MOLECULAR CHARACTERIZATION OF VIRUSES INFECTING POTATO AND VEGETABLES IN IRAQ

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Dedication:-

To

My Wife, Parents

&

Children

For the love and support

Nawres

DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently submitted for any degree other than that of Doctor of Philosophy (PhD) being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

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ABSTRACT

Due to the lack of published molecular information concerning plant viruses from Iraq, this study was initiated to investigate the diversity of viruses infecting potato and vegetables in Iraq on a molecular basis. Based on the economic importance and incidence worldwide, eight virus genera were investigated in 175 potato and vegetable samples collected from fields in Baghdad, Anbar and Najaf provinces in Iraq. Using genus/family specific primers, published in the literature, polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) were performed to screen samples for potyviruses, begomoviruses, carlaviruses, tombusviruses, potexviruses, cucumoviruses, tobamoviruses and alfamoviruses. Circular DNA viruses were screened by rolling circle amplification (RCA). Products resulting from PCR/RT-PCR and RCA were cloned and sequenced and data obtained were used for sequence analyses. The above approach led to the first molecular characterisation of three potyviruses; *Potato virus Y* (PVY), *Bean yellow mosaic virus* (BYMV) and *Zucchini yellow mosaic virus* (ZYMV), one begomovirus; *Tomato yellow leaf curl virus*, two carlaviruses, *Potato virus S* (PVS) and *Cowpea mild mottle virus* (CPMMV) and one tombusvirus; *Grapevine Algerian latent virus* (GALV) in Iraqi potato and vegetable samples. Based on nucleotide (nt) sequence analyses, BYMV from broad bean and ZYMV from zucchini were 97% and 99% identical to equivalent sequences from the GenBank sequences, respectively. Two PVY strains were distinguished when sequences from potato and tomato showed 99% maximum nt identity to equivalent $PVY^{O: N}$ and PVY^{NTN} GenBank sequences, respectively. Full-length sequence from tomato amplified by RCA showed 99% maximum nt identity to equivalent TYLCV sequences from the GenBank. Sequence comparison of carlavirus sequences isolated from potato and cowpea were 99% and 96% identical to equivalents PVS and CPMMV sequences from the GenBank, respectively. All tombusvirus sequences amplified from tomato and eggplant showed 93% maximum nt identity to equivalent GALV GenBank sequences. The high similarities (93-99%) of virus sequences isolated suggest, viruses isolated may have been introduced into Iraq from other countries, through international trading of plant materials used for cultivation as Iraq import most of plant materials for agriculture.

TABLE OF CONTENTS

FIGURES

TABLES

APPENDICES

ABBREVIATIONS

µFD	Microfarad(s)
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromole(s)
µm	Micrometer(s)
6K1	Six kilo Dalton first protein
6K2	Six kilo Dalton second protein
aa	Amino acid
Acc	GenBank accession number
***Alu*I**	*Arthrobacter luteus* restriction enzyme
AMV	*Alfalfa mosaic virus*
ApMV	*Apple mosaic virus*
A-rich	Adenine rich region
ArMV	*Arabis mosaic virus*
***Bam*HI**	*Bacillus amyloliquefaciens* H restriction enzyme
BanMMV	*Banana mild mosaic virus*
BCMV	*Bean common mosaic virus*
BLAST	Basic alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
BStMV	*Brome streak mosaic virus*
BVY	*Blackberry virus Y*
BYMV	*Bean yellow mosaic virus*
^{o}C	Degree Celsius
CABI	Centre for agricultural bioscience international
CaMV	*Cauliflower mosaic virus*
cDNA	Complementary DNA
CI	Cytoplasmic inclusion
CIRSV	*Carnation Italian ring spot virus*
cm	Centimetre(s)
CMV	*Cucumber mosaic virus*
CNV	*Cucumber necrosis virus*
CP	Coat protein or capsid protein
CPMMV	*Cowpea mild mottle virus*
CPMoV	*Cowpea mottle virus*
CR	Common region
CTAB	Cetyltrimethylammonium bromide
CuVCV	*Cucumber vein-clearing virus*
CymMV	*Cymbidium mosaic virus*
dB	Data base
***Dde*I**	*Desulfovibrio desulfuricans* restriction enzyme
dI	Deoxyinosine
DI RNA	Defective interfering RNAs
DNA	Deoxyribonucleic acid
dNTP	Dideoxynucleotides
ds	Double stranded
E	Elite class
E. coli	*Escherichia coli*

EcoRI	*Escherichia coli* restriction enzyme
EDTA	Ethylenediaminetetracetic acid
eIF(iso) 4E	Eukaryotic translation initiation factor
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EMCV	*Eggplant mottled crinkle virus*
EPPO	European and Mediterranean Plant Protection
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization statistics
g	Gram(s)
g	Gravity
GALV	*Grapevine Algerian latent virus*
GFkV	*Grapevine fleck virus*
GFLV	*Grapevine fan leaf virus*
GVA	*Grapevine virus A*
GX	Gauge X
h	Hour
HC	High concentration
HC-Pro	Helper-component-proteinase
HEL	Helicase
HSP70	Heat shock protein 70 kDa
IC-RT-PCR	Immunocapture RT-PCR
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropylthiogalactoside
ISEM	Immunosorbent electron microscopy
kb	Kilo base
KCl	Potassium chloride
***Kpn*I**	*Siellkleba pneumoniae* restriction enzyme
KV	Kilo volte
lacZ	Lactose operon in *E. coli*
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani medium
LIR	Large intergenic region
LWSV	*Leek white stripe virus*
M	Molar(s)
mA	Milliampere
MEGA 5	Molecular Evolutionary Genetics Analysis version 5
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute(s)
ML	Maximum likelihood
ml	Millilitre(s)
mM	Millimole(s)
mm	Millimetre(s)
MP	Movement protein
***Mse*I**	*Micrococcus species* restriction enzyme
***Msp*I**	*Moraxella species* restriction enzyme
MSV	*Maize streak virus*
MT	metric tonnes

MTase	Methyltransferase
MYaV	*Melon yellowing-associated virus*
NaCl	Sodium chloride
NB	Nucleic acid binding protein
***Nco*I**	*Nocardia corallina* restriction enzyme
NCR	Non coding region
NIa-Pro	Nuclear inclusion a protein
NIb	Nuclear inclusion b protein
NJ	neighbor-joining
nm	Nanometre (s)
nt	Nucleotide(s)
oligo(dT)	Oligodeoxythymidine
ONMV	*Oat necrotic mottle virus*
ORF	Open reading frame
ori	Origin of replication region
ORSV	*Odontoglossum ringspot virus*
OTU	Operational taxonomic units
P1	First protein
P3	Third protein
PCR	Polymerase chain reaction
PDV	*Prune dwarf virus*
PLRV	*Potato leaf roll virus*
pmol	Picomole
PMTV	*Potato mop top virus*
PNRSV	*Prunus necrotic ringspot virus*
Poly A	Poly adenine or polyadenylic acid tail
PotLV	*Potato latent virus*
P-Pro	Protein like protease
PRDV	*Potato rough dwarf virus*
***Pst*I**	*Providencia stuartii* restriction enzyme
PSV	*Peanut stunt virus*
PVA	*Potato virus A*
PVM	*Potato virus M*
PVP	*Potato virus P*
PVS	*Potato virus S*
PVS^{A}	*Potato virus S* the Andean strain
PVS^{O}	*Potato virus S* the ordinary strain
PVX	*Potato virus X*
PVY	*Potato virus Y*
PVY^{c}	*Potato virus Y Capsicum* strain
PVY^{N}	*Potato virus Y* veinal necrosis strain
PVY^{NTN}	*Potato virus Y* necrotic tuber necrosis strain
PVY^{NW}	*Potato virus Y* necrotic Wilga recombinant strain
PVY^{O}	*Potato virus Y* ordinary strain
PVY$^{O: N}$	*Potato virus Y* ordinary: necrotic recombinant strain
RCA	Rolling circle amplification
RdRp	RNA dependent RNA polymerase
REn	Replication enhancer protein
REP	Replication initiation protein

RFLP	Restriction fragment length polymorphism
RJ	Recombination junction
RNA	Ribonucleic acid
RNAi	RNA interference
RRP	RNA replicase protein
***Rsa*I**	*Rhodopseudomonas sphaeroides* restriction enzyme
RT-LAMP	Reverse transcription loop mediated isothermal amplifiction
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
S RNA	Small RNA
***Sac*I**	*Streptomyces achromogenes* restriction enzyme
***Sal*I**	*Streptomyces albus* restriction enzyme
SDT	simple direct tube
SDW	Sterile distilled water
SE	Super elite class
SOC	Super optimal catabolise repression medium
***Sph*I**	*Streptomyces phaeochromogenes* restriction enzyme
ss	Single stranded
SVYV	*Squash vein yellowing virus*
***Taq*I**	*Thermophilus aquaticus* restriction enzyme
TBE	Tris-borate EDTA
TBSV	*Tomato bushy stunt virus*
TCTV	*Turnip curly top virus*
TE	Tris EDTA buffer
TEV	*Tobacco etch virus*
TGB	Triple gene block
TLCOV	*Tomato leaf curl Oman virus*
TLCV	*Tomato leaf curl virus*
TLCV-AU	*Tomato leaf curl virus- Australia*
ToLCD	Tomato leaf curl disease
TrAP	Transcription activator protein
Tris-HCl	Tris-hydrochloride
tRNA	Transfer RNA-like structure
TRV	*Tobacco rattle virus*
TSAP	Thermal stable alkaline phosphatase
TSWV	*Tomato spotted wilt virus*
TYLCAxV	*Tomato yellow leaf curl Axarquia virus*
TYLCCNV	*Tomato yellow leaf curl China virus*
TYLCD	Tomato yellow leaf curl disease
TYLCGuV	*Tomato yellow leaf curl Guangdong virus*
TYLCIDV	*Tomato yellow leaf curl Indonesia virus*
TYLCMalV	*Tomato yellow leaf curl Malaga virus*
TYLCMLV	*Tomato yellow leaf curl Mali virus*
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
TYLCTHV	*Tomato yellow leaf curl Thailand virus*
TYLCV	*Tomato yellow leaf curl virus*
TYLCVNV	*Tomato yellow leaf curl Vietnam virus*
TYLVKaV	*Tomato yellow leaf curl Kanchanaburi virus*
U	Unit

UTR	Untranslated region
v	Volume
v/v	Volume by volume
vac	Vacuum
VIGS	Virus-induced gene silencing
Vo	Volt
VPg	Virus protein genome-linked
w/v	Weight by volume
WMV	*Watermelon mosaic virus*
X-gal	5-bromo-4-chloro-3-indolyl- β-D-galctopyranoside
***Xho*I**	*Xanthomonas holcicola* restriction enzyme
YCNMV	*Yam chlorotic necrotic mosaic virus*
YEB	Yeast extract beef
ZYMV	*Zucchini yellow mosaic virus*
α	Alpha
β	Beta
Ø	Phi
Ω	Omega

CHAPTER 1: INTRODUCTION

1. General introduction

Plant viruses are one of the factors that limit the production of potato and vegetables worldwide, including in Iraq. Viral diseases can cause serious losses of up to 50% on potato (Loebenstein, 2009) and 3-5% on vegetables (Caciagli, 2009). Losses can be even higher on vegetables in developing countries due to lower emphasis on plant virus disease control (Caciagli, 2009) due to a lack of adequate use of diagnostic methods for plant viruses and less awareness of plant viruses' economic importance. There are over 50 different viruses reported to infect potato worldwide (Brunt *et al*., 1996; ICTVdB, 2012). Plant viruses have also been reported worldwide to attack a wide range of other crops in Iraq, e.g. commonly grown broad bean, cucumber, zucchini, bottle gourd, snake cucumber, cowpea and eggplant are reported to be infected by 107, 143, 62, 6, 45, 141 and 44 different viruses globally (Brunt *et al*., 1996; ICTVdB, 2012), many of which can cause serious economic losses.

Viruses limit potato production and their detection has therefore been included in seed testing and certification of potato tubers. Particular concern has been placed on potato viruses in Iraq, because virus spread through progeny is often in vegetatively propagated rather than seed propagated crops, which increases economic losses (Loebenstein, 2009). Despite the serious damage caused by plant viruses, no precise estimation of their diversity or effects on crop production and yields has been carried out in Iraq.

Most potato and vegetable seeds imported are submitted for seed certification under quarantine rules established by the State Board for Testing and Certificate and Plant Quarantine in the Ministry of Agriculture in Iraq. Imported vegetable seeds should pass through seed health testing before being released to local farmers. However, this seed health testing is restricted to fungal and bacterial infection only as seed testing for viruses has not been included within seed health testing in Iraq except for potato tubers. Potato tubers imported are tested by enzyme linked immunosorbent assay (ELISA) for viral indexing upon arrival. The tolerance limits used for virus laboratory testing in Iraq is less stringent than other bordering countries. As comparison, the total virus indexing including *Potato virus X*, *Potato virus Y*, *Potato virus M*, *Potato virus A*, *Potato virus S*, and *Potato leaf roll virus*) in Iraq are: class super elite (SE) 1%, class elite (E) 2% and class A 5%, whereas the total virus tolerance limits are 0.2% (nil for PLRV) in Kuwait, 2% (for PVY, PLRV and PVA combined) and 4%

(for PVX and PVS combined) in Jordan, 0.5% (for PVY, PVA, PVX, PVM and PLRV) for SE class and 2% for E class in Saudi Arabia (Anonymous, 2009). Many different plant viruses have been reported in local Iraqi publications to infect potato, tomato, cucurbits and broad bean in Iraq (e.g. Sadeq, 1999). These viruses have been identified based on biological properties, serology and electron microscopy (Table 1). However, molecular diagnostic and virus diversity studies have not been carried out on Iraqi viruses due to a lack of the required equipment and expertise to conduct such molecular studies in Iraq. Molecular techniques are often preferable to other diagnostic techniques as they can have much greater sensitivity than other techniques and can also differentiate among closely related viruses, strains or sometimes even isolates. Besides they can be used alongside other techniques to support the classification of plant viruses and identification of new species there of (Koenig *et al*, 2009).

This study was, therefore, initiated with the aim of obtaining preliminary information on the diversity of viruses infecting potato and selected vegetable crops in Iraq. The objectives of the current study were to:

1. Investigate selected groups of plant viruses in potato and vegetable samples collected from Iraqi fields by PCR/RT-PCR using genus/family specific degenerate primers.
2. Clone and sequence the amplified DNA fragments and compare their sequences to GenBank sequences to identify viruses infecting the tested samples.
3. Determine the relatedness of viruses isolated from Iraq to those from GenBank, based on sequence analyses and phylogeny, to identify whether the viruses isolated were originated in/ or have been introduced to Iraq.

Due to the large number of plant viruses that can infect potato and vegetables, the first step was to decide which virus groups/families should be investigated? The chosen option was to investigate the most significant and ubiquitous viral groups that cause serious losses over the world. The family *Potyviridae* is the most important and the largest family of plant viruses and it was selected as a first choice for detection. Due to the large number of viruses within the family *Potyviridae*, the next step was to select the best strategy to screen the 175 collected Iraqi potato and vegetable samples. The most applicable solution was to investigate potyviruses as a group using RT-PCR and universal primer sets instead of individual viruses. Then, sequences obtained were cloned, sequenced and compared to GenBank sequences to identify and differentiate among individual viruses, strains or isolates using phylogenetic analysis. This strategy enabled the detection of different viruses from the family *Potyviridae*

in one reaction. Besides, this strategy facilitated detection of potyviruses up to isolate level and indicated the possible origin of potyviruses infecting potato and vegetables in Iraq.

The second group of viruses selected for investigation were those belonging to the family *Geminiviridae.* These were targeted using genus specific primers as well as the rolling circle amplification (RCA) technique. The family *Geminiviridae* include over 200 definite and tentative species grouped into four genera *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* (ICTVdB, 2012). The RCA approach enables amplification of the full-length genomes of geminiviruses, including DNA (A), DNA (B) and DNA (β), which will be functional for sequence analyses and phylogeny and then constructing an infectious clone for infectivity studies in later steps. RCA also facilitates screening for sequences belonging to viruses of other families with circular DNA, e.g. the families *Caulimoviridae* and *Nanoviridae*.

Finally, investigations were performed to search for viruses belonging to other genera of economic importance, using specific primer sets to alfamo-, cucumo-, tobamo-, carla- and potexviruses.

The above approach confirmed the detection of three potyviruses; namely, *Potato virus Y* (PVY), *Bean yellow mosaic virus* (BYMV) and *Zucchini yellow mosaic virus* (ZYMV), one begomovirus; namely *Tomato yellow leaf curl virus*, two carlaviruses, namely, *Potato virus S* (PVS) and *Cowpea mild mottle virus* (CPMMV) and one tombusvirus; namely, *Grapevine Algerian latent virus* (GALV) in potato and vegetable samples collected from Iraq and revealing their relatedness to GenBank sequences.

Table 1: Plant viruses identified in Iraq based on biological, serological and electron microscopy (EM) techniques

Virus name	Group	Crop(s)	Location(s)	Detection method(s)	Reference(s)
Alfalfa mosaic virus	alfamoviruses	tomato, broad bean	Baghdad, Anbar, Diyala, Wasit	biology & serology	El-Muadhidi *et al.*, 2001; 2010
Bean common mosaic virus	potyviruses	common bean	Baghdad	biology & EM	Al-Fadhil, 1984
Bean yellow mosaic virus	potyviruses	broad bean, common bean	Baghdad, Anbar, Diyala, Tamim, Nineveh	biology, serology& EM	Salih, 1983; Zwein, 1985; El-Muadhidi *et al.*, 2001; Ramadan & Al-Sama'a, 2005; Al-Ani R., 2011
Broad bean mottle virus	bromoviruses	broad bean	Baghdad	biology	Zwein, 1985
Broad bean wilt virus	fabaviruses	broad bean	Nineveh, Anbar	serology	El-Muadhidi *et al.*, 2001
Cowpea severe mosaic virus	comoviruses	cowpea	Baghdad	Biology	Abd-Alghafor, 1988
Cucumber mosaic virus	cucumoviruses	eggplant, cucumber, tomato,	Baghdad, Anbar, Nineveh, Diyala	biology & serology	Shawkat& Fegla, 1979; Al-Zobaidy, 1988; El-Muadhidi *et al.*, 2001; Younis & Kassim, 2003; Kassim & Husain, 2006; Albayati *et al*., 2012
Chickpea chlorotic dwarf	mastreviruses	broad bean	Nineveh, Anbar	serology	El-Muadhidi *et al.*, 2001
Curly top virus	curtoviruses	tomato	Baghdad	biology	Abdul-Razak, 1988
Faba bean necrotic yellows	nanoviruses	broad bean	Nineveh	serology	El-Muadhidi *et al.*, 2001
Potato leaf roll virus	polerovirus	potato	Baghdad	biology & serology	Al-Ani *et al.*, 2010
Potato virus A	potyviruses	potato	Baghdad, Anbar	biology and serology	Sadeq, 1999
Potato virus X	potexviruses	potato, tomato	Baghdad, Nineveh, Basrah, Diyala, Wasit	biology, serology and EM	Khamass, 1983; El-Muadhidi *et al.*, 2010
Potato virus Y	potyviruses	potato, pepper	Baghdad, Nineveh	biology, serology & EM	Khamass, 1983; Younis & Kassim, 2003; Al-Ani *et al.*, 2011d

Table 1: continued.

Virus name	Group	Crop(s)	Location(s)	Detection method(s)	Reference(s)
Tobacco mosaic virus	tobamoviruses	potato, pepper, tomato	Baghdad, Nineveh, Basrah, Diyala, Wasit	biology, serology and EM	Khamass, 1983; Al-Molla, 1984; Younis & Kassim, 2003; El-Muadhidi *et al.*, 2010
Tomato aspermy virus	cucumoviruses	tomato	Nineveh	biology & serology	Al-Chero, 2010
Tomato bushy stunt virus	tombusviruses	tomato	Nineveh	biology & serology	Al-Chero, 2010
Tomato mosaic virus	tobamoviruses	tomato	Nineveh	Biology& serology	Al-Chero, 2010
Tomato spotted wilt virus	tospoviruses	tomato	Ninava	biology &, serology	Al-Chero, 2010
Tomato yellow leaf curl virus	begomoviruses	tomato, common bean	Baghdad, Najaf, Nineveh, Basrah	biology & serology	Al-Chero, 2010; Al-Ani *et al*., 2011b&c; Khalaf *et al.*, 2011; Al-Fadhal, 2012
Watermelon mosaic virus	potyviruses	vegetable marrow	Ninava	biology	Shawkat& Fegla, 1979
Zucchini yellow mosaic virus	potyviruses	zucchini, cucumber	Baghdad, Diyala, Babil, Wasit	biology &, serology	Al-Janabi & Abd El-Hadi, 2006; Al-Ani *et al.*, 2011a

CHAPTER 2: LITERATURE REVIEW

2. Literature review

2.1. The current status of potato and vegetables in Iraq

Potato and vegetables grown in Iraq have significant economic importance due to their wide consumption (Bishay, 2003). They provide a source of direct income as well as employment. Annually, potato is grown twice during the year in two seasons in Iraq, namely the spring and autumn seasons. The spring season starts from mid-February until late June and the autumn season starts from mid-August until end of February (Sadeq, 1999). The main growing regions for potato production in Iraq are Baghdad, Anbar and Nineveh provinces. Broad bean is cultivated in open fields once a year; mainly from mid-December till harvest in April. The main production regions for broad bean are Babylon (Babel) and Nineveh, although, this crop is grown throughout most regions of Iraq. Bottle gourd squash, snake cucumber and cowpea are cultivated to replace other vegetables in the summer season (mid-June-early September). Tomato, cucumber and zucchini squash are not grown in this season due to the high temperatures and a water shortage at this time of year. In contrast, bottle gourd, snake cucumber and cowpea are more tolerant to hot dry weathers as well as salinity and poor soil conditions (Padilla, *et al*, 2009; Bohnert, 2011).

Taxonomically, potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicon* L.) and eggplant (*Solanum melongena* L.) belong to the *Solanaceae* family. Cucumber (*Cucumis sativus* L.), zucchini squash (*Cucurbita pepo* L. subsp. *pepo*), bottle gourd (*Lagenaria cineraria* (Molina.) Standl.) and snake cucumber (*Cucumis melo* L. subsp. *melo* var. *flexuosus* (L.) Naudin) are members of the *Cucurbitaceae* family. Broad bean (*Vicia faba* L.) and cowpea (*Vigna unguiculata* (L.) Walp.) are members of the family *Fabaceae* (Anonymous, 2012). The estimated yield of the solanaceous crops in Iraq were about 557401, 1059540 and 452050 metric tonnes (MT) for potato, tomato and aubergine, respectively (Anonymous, 2011). Whereas the production of cucurbits (including cucumber, zucchini, bottle gourd, watermelon and snake cucumber) and leguminous vegetables (including broad bean and cowpea) were, 1162702 and 232129 MT, respectively (Anonymous, 2011) (Figure 2). Based on FAO statistics, Iraq ranked the 20th in tomato production among other countries, whereas it ranked the 12th, 7th, 4th in cucumber, aubergine and leguminous vegetables, respectively (Anonymous, 2011).

Figure 1: Map of Iraq

The map shows locations where potato and vegetables samples were collected during this study (red marks).

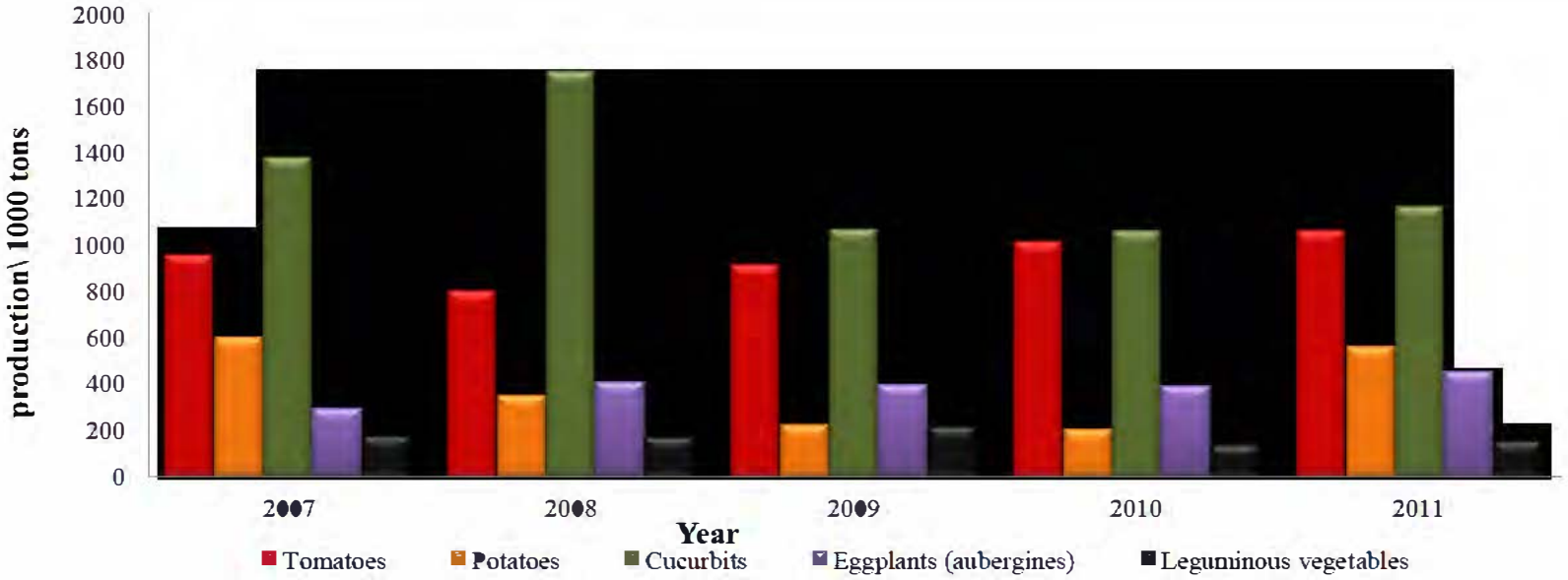


Figure 2: Production of potato and vegetables in Iraq

The illustration shows the estimated yields of potato and vegetables over the time period 2007-2011. Data adapted from FAO statistics (Anonymous, 2011).

In Iraq, potato growers usually cultivate imported potato tubers to grow potato in the spring season, but then store tubers produced from the spring season to cultivate these in the autumn season. They split single tubers using knives to be able to cultivate a greater area. Tomato and eggplant are grown by sowing the seeds into small pots to produce seedlings, and then transplanting into the field or greenhouse two months later. Cucurbit and legume seeds are sown directly into ground. Recently, farmers in Iraq have been using imported seeds extensively to grow potato, tomato, cucumber, zucchini squash, and eggplant (Bishay, 2003). Broad bean, snake cucumber, cowpea and bottle gourd are still produced from local seeds (Bishay, 2003).

2.2. Potato viruses

Virus diseases are one of the major limiting factors in potato production worldwide as they reduce yield by more than 50%, as well as resulting in additional costs to control such virus diseases or their vectors (Loebenstein, 2009). At least 37 viruses infect potato worldwide (Loebenstein, 2009); although 56 plant viruses have been reported to infect potato (Table 2), it is believed that several of them are only tentative species or possibly strains rather than different species (Kerlan, 2009). Only eight of the potato viruses cause serious losses on potato worldwide and, therefore, only these have been included in potato seed testing and certification. These are *Potato virus Y* (PVY), *Potato leaf roll virus* (PLRV), *Potato virus X* (PVX), *Potato Virus A* (PVA), *Potato virus S* (PVS), *Potato virus M* (PVM), *Tobacco rattle virus* (TRV) and *Potato mop top virus* (PMTV) (Kerlan, 2009, Loebenstein, 2009).

PVY is one of the most important of the above described eight important potato viruses. It infects most potato cultivars and causes significant yield losses (up to 80%) (Dabijev *et al.*, 2005; Kerlan, 2009; Kostiw, 2011). Tuber quality can be severely affected due to necrosis and a reduction in size (Kerlan, 2009, Kerlan & Moury, 2009; Loebenstein, 2009). Typical symptoms associated with PVY infections are based on strain, potato cultivar or environmental conditions (Jones *et al.,* 2009). Typical symptoms range from symptomless or mild mosaic to mosaic, crinkle, necrosis and stunting and leaf drops giving rise to a palm shape feature to the infected plant (Kerlan, 2009; Kerlan & Moury, 2009). The virus is aphid transmissible in a non-persistent manner that results in effective PVY spread in the fields (Jones *et al*., 2009; Kerlan, 2009; Kerlan & Moury, 2009).

Table 2: Number of viruses reported from different virus groups reported to infect potato and vegetables globally.

Group		potato	tomato	broad bean	cucumber	squash	bottle gourd	snake cucumber	cowpea	eggplant
1	alfamoviruses	1	1	1	1	1	-	1	1	1
2	alphacryptoviruses	-	-	1	-	-	-	-	1	-
3	begomoviruses	7	47	-	3	2	-	3	3	3
4	bromoviruses	-	-	4	4	2	-	-	2	-
5	capilloviruses	-	-	1	-	-	-	-	1	-
6	carlaviruses	4	4	7	6	3	-	3	7	4
7	carmoviruses	1	4	2	6	1	-	2	7	1
8	castreviruses	-	2	-	-	-	-	-	-	-
9	caulimoviruses	-	-	1	-	-	-	-	2	-
10	closteroviruses	1	-	3	3	1	-	2	1	-
11	comoviruses	1	1	7	2	1	-	1	7	-
12	criniviruses	-	1	-	-	-	-	-	-	-
13	cucumoviruses	1	3	2	3	2	1	2	3	2
14	curtoviruses	1	1	1	1	1	-	-	1	2
15	cytorhabdoviruses	-	1	1	-	-	-	1	1	-
16	dianthoviruses	-	1	3	3	-	-	-	3	-
17	enamoviruses	-	-	1	-	-	-	-	-	-
18	fabaviruses	-	-	2	1	-	-	-	1	-
19	furoviruses	1	2	1	2	1	-	-	1	-
20	ilarviruses	1	4	3	11	4	-	4	12	1
21	ipomoviruses	-	1	-	-	-	-	-	-	-
22	luteoviruses	4	6	4	2	1	-	-	3	-
23	macluravirus	-	-	-	-	-	-	-	1	-
24	nanoviruses	-	-	3	-	-	-	-	2	-
25	necroviruses	-	1	1	2	2	-	1	3	-
26	nepoviruses	11	15	10	29	11	-	5	20	3
27	nucleorhabdoviruses	1	4	2	-	-	-	-	-	3
28	ourmiaviruses	-	3	3	3	1	-	1	3	-
29	phytoreovirus	-	1	-	-	-	-	-	-	-
30	potexviruses	4	9	3	8	1	-	1	8	2
31	potyviruses	7	26	20	14	15	3	11	18	6
32	sequiviruses	-	1	-	2	-	-	-	-	-
33	sobemoviruses	-	-	1	2	1	-	-	3	1
34	tobamoviruses	2	9	5	10	2	2	2	3	6
35	tobraviruses	1	2	2	3	-	-	1	1	1
36	tombusviruses	1	3	-	6	4	-	1	5	2
37	topocuviruses	-	1	-	-	-	-	-	-	-
38	torradoviruses	-	3	-	-	-	-	-	-	-
39	tospoviruses	1	2	1	1	-	-	-	2	1
40	trichoviruses	1	-	1	1	-	-	-	2	1
41	tymoviruses	3	6	3	6	4	-	3	7	4
42	umbraviruses	-	2	1	-	-	-	-	-	-
43	varicosaviruses	1	1	-	1	-	-	-	-	-
44	satellite viruses	-	1	-	-	1	-	-	-	-
45	ungrouped	-	1	-	-	-	-	-	7	-
	Total	56	170	107	143	62	6	45	141	44

Data adapted from (Brunt *et al*., 1996) and (ICTVdB, 2012).

Over 70 aphid species have been reported to transmit PVY, however *Myzus persicae* is the most efficient vector in potato fields (Kerlan, 2009; Kerlan & Moury 2009, Loebenstein, 2009). Many outbreaks of PVY were reported on potato worldwide due to the introduction of new potato varieties or emergence of new virus strains (Dabijev *et al*., 2005; Kerlan, 2009; Kostiw, 2011). PVY is a high variable virus with many different strains having been reported worldwide (Kerlan & Moury, 2009). Initially, PVY isolates were grouped into three main strains; namely, PNY^{O}, PVY^{N} and PVY^{C}, based on their ability to cause a hypersensitive reaction on certain potato cultivars (Singh *et al*., 2008; Kerlan & Moury, 2009). However, more recently many other recombinant strains have been described, e.g. PVY^{NTN} , $PVY^{N:O}$ and PVY^{NW} , which show different or shared properties to the three main strains (Singh *et al*., 2008; Kerlan, 2009; Kerlan & Moury, 2009). Recent studies showed these new strains have been drived from the non-recombinant strains PNY^{O} and PVY^{N} due to the recombination event (Visser *et al*., 2012). However, a recombination between two recombinant strains PVY^{NTN} and PVY^{NW} has occurred resulted in emerge PVY^{NTN-NW} a new recombinant strain from Syria (Chikh Ali *et al*., 2010). Recombination event has been reported in P1, HC-Pro, P3, 6K2, NIaVPg, NIa and NIb/CP regions within potyvirus genome (Hu *et al*., 2009; Chikh Ali *et al*., 2010). Recombination is a common feature of PVY (Visser *et al*., 2012). As recombination has resulted in emerging of PVY recombinant strains that cause seriuos losses in potato and affect tuber quality, it has been required to design primer sets that diffrentiate among PVY strains (Nie & singh, 2003; Rigutti & Gugerli, 2007, Schubert *et al*., 2007a; Kogovšek *et al.* 2008). Those primer sets have been developed for rapid differntiation among strains which would be useful to use in potato seed testing and certification programmes and routine tests (Nie & singh, 2003; Rigutti & Gugerli, 2007, Schubert *et al*., 2007a; Kogovšek *et al.* 2008). Most primer sets used to diffrentiate between recombinant and non-recombinant strains/isolates of PVY have been designed to target three recombinant junctions (RJ) (Kogovšek *et al.* 2008; Chikh Ali *et al*., 2010). These RJs locate in HC-Pro, NaI and CP genes (Kogovšek *et al.* 2008; Chikh Ali *et al*., 2010). Various nucleic acid based approaches have successfully been used for rapid sensitive detction to diffrentiate the recombinant from non-recombinant PVY strains/isolates like multiplex RT-PCR (Nie & singh, 2003, Schubert *et al*., 2007a) one-step triplex RT-PCR (Rigutti & Gugerli, 2007) and single step RT/real-time PCR (Kogovšek *et al.* 2008). *Potato virus Y* is the type member of the genus *Potyvirus* the family of *Potyviridae* (ICTVdB, 2012).

PLRV is the second important virus that infects potatoes worldwide. It induces serious losses of about 33-50% (Jones *et al*., 2009; Loebenstein, 2009) and can reach infection levels of up to 90% (Jones *et al*., 2009; Kerlan, 2009). Typical symptoms induced by PLRV are pale yellowing of foliage, upward leaf rolling, phloem necrosis and plant stunting but giving rise to an erect shape (Jones *et al*., 2009; Kerlan, 2009). Tubers produced from PLRV infected plants are usually small to medium size with internal net necrosis that affects tuber quality. PLRV is aphid transmissible in a persistent manner and it is not transmitted mechanically (Kerlan, 2009). At least 10 different aphid species have been reported to transmit the virus, with *M. persicae* reported to be the most efficient vector of PLRV in potato fields (Kerlan, 2009). *Potato leaf roll virus* is the type member of the genus *Polerovirus*, family *Luteoviridae* (ICTVdB, 2012)

PVX is the next most important virus on potato (Kerlan, 2009). The virus is the type member of the genus *Potexvirus*, family *Flexiviridae* (Jones *et al*., 2009; ICTVdB, 2012). PVX induces variable symptoms on potato, based on the cultivar and the presence of mixed infections with other viruses (Kerlan, 2009).They range from a mild mosaic and mottle to severe mosaic, rugosity, crinkle and tuber necrosis (Jones *et al*., 2009; Kerlan, 2009). PVX generally causes a yield reduction of about 10-15%, however yield losses can be up to 50% in cases of mixed infection with PVY or *Potato virus A* (PVA) (Kerlan, 2009) or 40-70% with PLRV (Loebensstein, 2009). The virus is mechanically transmissible and could be transmitted by contact between two plants in potato fields or through shared agricultural equipments (Ryu & Hong, 2009).

PVA is another member of the genus *Potyvirus* that infects potato and has been reported to cause yield losses of up to 40% in certain susceptible cultivars such as Desiree (Kerlan, 2009; Wang *et al*., 2011). Virus infection is often symptomless, but symptoms when present range from mild mosaic, mottling to plant crinkling often due to mixed infection with PVX or PVY. PVA is an aphid-transmissible virus and it is transmitted by *M. persicae* and *Aphis* spp. in a non-persistent manner (Jones *et al*., 2009; Kerlan, 2009).

PVS and PVM are other important viruses that infect potato worldwide. They belong to the genus *Carlavirus*,family *Flexiviridae* (Jones *et al*., 2009; Ryu & Lee, 2009). Yield reduction due to PVS and PVM range 3-20% and 5-15%, respectively (Loebenstein, 2009) although higher losses have been reported in certain cultivars (Kerlan, 2009). PVS and PVM have been reported to cause reductions in potato yields of up to 30% in certain countries (e.g. Poland and Bulgary) (Dabijev *et al*., 2005; Kostiw, 2011). Mixed infections of PVS and PVM

can result in even higher tuber yield losses of about 40-75% (Loebenstein, 2009). Symptoms induced by PVS are rather mild, ranging from symptomless to leaf mottling, whereas PVM induces slight mottling and mild abaxial rolling of leaves (Jones *et al*., 2009; Kerlan, 2009). These viruses are aphid-transmissible in a non-persistent manner and transmitted by several aphid species like *M. persicae* and other *Aphis* species (Kerlan, 2009).

Tobacco rattle virus (TRV) and *Potato mop top virus* (PMTV) are the type members of the genera *Tobravirus* and *Pomovirus*, respectively, within the family *Virgaviridae*. They can cause serious losses in potato production (Kerlan, 2009). TRV causes necrotic corky arcing ('spraing' ringspot) in tuber flesh and stem mottling symptoms on infected plants (Jones *et al*., 2009; Kerlan, 2009). PMTV induces blotching or mottling on shoots and chlorotic V-shape markings in the leaflets as well as internal brown arcing ('spraing') seen as brown rings on the tuber surfaces (Jones *et al*., 2009; Kerlan, 2009). TRV is transmitted by at least 12 nematode species of the genera *Paratrichodorus* and *Trichodorus*, whereas PMTV is vectored by the fungus *Spongospora subterranea* (Jones *et al*., 2009; Kerlan, 2009).

2.3. Vegetable viruses

Significant losses due to plant virus diseases also affect vegetable crop production worldwide, with the prevalence and severity of viral diseases on vegetables varying from year to year (Celetti & Fraser, 2004; Caciagli, 2009). About 3-5% of total vegetable production is lost due to virus diseases according to different estimations; however yield losses could be higher based on host, pathogen and environment (Caciagli, 2009). At least 200 viruses belonging to 30 genera within 15 families have been reported to infect vegetables (Caciagli, 2009). Most of these viruses belong to two genera, the genus *Potyvirus* (family *Potyviridae*) and the genus *Begomovirus* (family *Geminiviridae*). About 40 potyviruses and 35 begomoviruses have been found to infect different vegetable crops (Caciagli, 2009). Most of the begomoviruses infect tomato (Caciagli, 2009). Other viruses commonly reported on vegetables are *Cucumber mosaic virus* (CMV) and *Alfalfa mosaic virus* (AMV), the type members of the genera *Cucumovirus* and *Alfamovirus* respectively within the family *Bromoviridae* (Celetti & Fraser, 2004; Caciagli, 2009).

Tomato is infected by 53 different viruses (Caciagli, 2009), but it is worth noting that a total of 170 viruses have been reported to infect tomato, naturally or experimentally, according to Brunt *et al*. in (1996) (Table 2). The difference between these two statistical data for virus incidence on vegetables is due to Caciagli (2009) excluding the cryptoviruses,

satellites and viroids within tomato viruses as well as excluding torradoviruses, a new virus group on tomato (ICTVdb, 2012). Besides, several of ~170 viruses are only tentative species or possibly strains rather than different species (Kerlan, 2009). Cucurbits have been found to be infected by at least 30 different plant viruses (Coutts *et al.*, 2011), wherease 59 different viruses, reported in data bases, have been characterized to infect cucrbits worldwide (Lecoq & Desbiez, 2012). About 50 viruses naturally infect broad bean worldwide (Makkouk *et al*., 2012), wherease, around107 viruses have been reported to infect broad bean naturally and experimantly, found in databases (Brunt *et al*., 1996; ICTVdb, 2012) (Table 2). Cowpeas are infected by 144 viruses naturally and experimentally (Brunt *et al*., 1996; Salem *et al*., 2010, ICTVdb, 2012) (Table 2).

Zucchini yellow mosaic virus (ZYMV) belongs to the genus *Potyvirus* (family *Potyviridae*). This virus is one of the most devastating pathogens that threaten cucurbits world-wide (Celetti & Fraser, 2004; Caciagli, 2009; Lecoq & Desbiez, 2009). The virus can cause severe symptoms leading to complete yield losses (Lecoq & Desbiez, 2009; Coutts *et al.*, 2011). Disease severity depends on the plant species and the growth stage of the host plant when infection occurs (Celetti & Fraser, 2004). ZYMV induces symptoms on cucurbits that range from vein clearing and mosaic of leaves to leaf blisters, knobby fruit, plant stunting and small leaves (Celetti & Fraser, 2004; Lecoq & Desbiez, 2009; Coutts *et al.*, 2011). ZYMV is transmitted by several aphid species in a non-persistent manner (Celetti & Fraser, 2004; Caciagli, 2009; Lecoq & Desbiez, 2009). Seed transmission may occur, but at a low level of 0.3-15 % (Tobias *et al*., 2008; Lecoq & Desbiez, 2009; Coutts *et al.*, 2011).

PVY has been reported to infect solanaceous vegetables, mainly tomato, pepper (Kerlan & Moury, 2009) and eggplant (Bhat *et al.*, 1999). PVY has been considered to be of secondary importance on tomato (Kerlan & Moury, 2009) as it induces only a mild mosaic. Since 1980, new PVY strains have emerged causing serious quantity and quality reductions in tomato fruit. Those strains induce necrotic lesions on leaves and necrotic streaks on stem in all tomato varieties in both protected agriculture and open fields. Higher losses in tomato production may occur due to mixed infection of PVY and other viruses like *Cucumber mosaic virus* (CMV) (Kerlan & Moury, 2009).

Bean yellow mosaic virus (BYMV) is one of the potyviruses infecting legumes worldwide including broad bean. It can reduce broad bean production by ~60% (Albrechtsen, 2006). BYMV causes vein chlorosis, leaf mottling and yellow or green mosaic symptoms on broad bean (Bos, 1970; El-Muadhidi *et al*., 2001). The virus is aphid-transmissable in a non-

persistent manner by at least 20 diffferent species, and seed transmission can also occur at a low level. In a serological based survey carried out in Iraq, the virus was shown to infect broad bean at a high incidence (80%) (El-Muadhidi *et al*., 2001).

Tomato yellow leaf curl disease (TYLCD) is considered as one of the most devastating ubiquitous virus diseases in the world (Duffy & Holms, 2007; Glick *et al.*, 2009; Díaz-Pendón *et al.*, 2010; Lefeuvre *et al.*, 2010; Kim *et al.*, 2011). This disease has been recorded to impact tomato crops by being capable of causing 100% yield losses in over 30 countries (Glick *et al.*, 2009; Díaz-Pendón *et al.*, 2010). TYLCD is caused by various members of the genus *Begomovirus* within the family *Geminiviridae*, including 11 virus species approved by the International Committee on Taxonomy of Viruses (ICTV) (Díaz-Pendón *et al.*, 2010): *Tomato yellow leaf curl Axarquia virus* (TYLCAxV), *Tomato yellow leaf curl China virus* (TYLCCNV), *Tomato yellow leaf curl Guangdong virus* (TYLCGuV), *Tomato yellow leaf curl Indonesia virus* (TYLCIDV), *Tomato yellow leaf curl Kanchanaburi virus* (TYLVKaV), *Tomato yellow leaf curl Malaga virus* (TYLCMalV), *Tomato yellow leaf curl Mali virus* (TYLCMLV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato yellow leaf curl Thailand virus (*TYLCTHV*)*, *Tomato yellow leaf curl Vietnam virus* (TYLCVNV) and *Tomato yellow leaf curl virus* (TYLCV) have been reported to induce TYLCVD (Díaz-Pendón *et al.*, 2010). TYLCD symptoms may vary, but the main features of diseased plant are yellowing and leaf curling (Anonymous 2000; Melzer *et al.*, 2009; Díaz-Pendón *et al.*, 2010). TYLCD has been reported to attack other solanaceous crops like tobacco, potato, eggplant and pepper and leguminous plants like bean but with fewer or no symptoms. Unlike most begomoviruses whose genomes are bipartite, viruses causing TYLCD have monopartite genomes except for TYLCTHV and TYLVKaV which have bipartite genomes. Viruses causing TYLCD are transmitted by the whitefly *Bemisia tabaci*, the only known vector for the genus *Begomovirus*, in a persistent manner (Seal *et al.*, 2006; Glick *et al.*, 2009; Díaz-Pendón *et al.*, 2010; Lefeuvre *et al.*, 2010; Ohnishi *et al.*, 2011).

Tomato leaf curl disease (ToLCD) is another devastating begomovirus disease that impacts tomato cultivation worldwide and is capable of causing complete yield losses (Chakraborty, 2009). Viruses causing ToLCD symptoms are characterized by severe plant stunting, upward and downwards rolling and crinkling of leaves, interveinal yellowing, vein clearing and puckering (Chakraborty, 2009). ToLCD is caused by a range of begomoviruses, which are transmitted by their whitefly vector in a persistent manner (Chakraborty, 2009).

Cucumber mosaic virus (CMV) is one of the most common viruses found in many vegetable crops. This virus is aphid-transmissible by about 70 aphid species in a non-persistent manner (Celetti & Fraser, 2004; Caciagli, 2009; Garcia-Arenal & Palukaitis, 2009). Seed transmission of CMV has been reported in many plant species, varying from 1% to 50% (Garcia-Arenal & Palukaitis, 2009). CMV has a wide range of host plants. Several strains of CMV have been reported to infect over 1300 different plant species in more than 500 genera within 100 families, including cucurbits, broad beans, cowpea, tomato and eggplant (Table 2) (Celetti & Fraser, 2004; Caciagli, 2009; Garcia-Arenal & Palukaitis, 2009). Symptoms induced by CMV may vary. Based on the plant species infected, symptoms range from yellow to green mottling on leaves and fruit, to severe stunting of plants. On infected tomato plants, CMV causes a reduction in leaf size which is known as fern leaf disease. On cucurbits, symptoms induced by CMV range from mild mosaic to leaf narrowing and distortion of plants (Celetti & Fraser, 2004; Caciagli, 2009; Garcia-Arenal & Palukaitis, 2009).

AMV is another common virus that infects vegetables. It occurs worldwide and infects about 600 species falling into 70 plant families (Bol, 2009). AMV is aphid-transmissible in a non-persistent manner, with at least 15 aphid species known to vector the virus (Bol, 2009). Seed transmission has been reported for AMV at up to 50% (Bol, 2009). AMV causes severe necrosis on tomato and yellow mosaic on cowpea, mild mottle and stem necrosis and death of plants for broadbean (Jaspars & Bos, 1980).

Tobamoviruses have been reported to infect vegetables worldwide including cucurbits, solanaceous vegetables (including tomato and eggplant) (Lewandowski, 2009) and legumenous vegetables (including broad bean and cowpea) (Table 2). Tobamoviruses are easily transmissible by mechanical inoculation. Tobamovirus members can be transmitted through contaminated seed coats but not through the seed embryos (Caciagli, 2009).

2.4. The family *Potyviridae*

The family *Potyviridae*, whose name was abbreviated after type member Potato virus Y (PVY: genus *Potyvirus*; family *Potyviridae*), is one of the largest virus families (the other being the family *Geminiviridae*). It contains the greatest number (~30%) of known plant viruses (Gibbs & Ohshima, 2010). About 172 members reported within this family have been grouped into eight (Table 3) with just two unassigned species (ICTVdB, 2012). These divisions were based on genome organization (mono- or bi-partite), genome sequencing and vector transmission. They infect a wide range of plants worldwide causing serious losses (Gibbs & Ohshima, 2010). Virus particles of the family *Potyviridae* consist of elongated

Table 3: Genera of the families *Potyviridae* and *Geminiviridae*

Potyvirus genus	**Type member**	**Genome type (ssRNA)**	**Vector**	**Particle length/nm**	**Species**
Brambyvirus	*Blackberry virus Y*	monopartite	unknown	800	1
Bymovirus	*Barley yellow mosaic virus*	bipartite	fungus (*Polymyxa) graminis*)	500-600 and 200-300	6
Ipomovirus	*Sweet potato mild mottle virus*	monopartite	whitefly (*Bemisia tabaci*)	750-950	5
Macluravirus	*Maclura mosaic virus*	monopartite	aphid (*Myzus persicae*)	650-660	6
Poacevirus	*Triticum mosaic virus*	monopartite	mite (*Aceria tosichella*)	680-750	2
Potyvirus	*Potato virus Y*	monopartite	aphid (*Myzus persicae*)	720-850	146
Rymovirus	*Ryegrass mosaic virus*	monopartite	mite (*Abacarus hystrix*)	690-720	3
Tritimovirus	*Wheat streak mosaic virus*	monopartite	mite (*Aceria tulipae*)	680-750	3
Geminivirus genus	**Type member**	**Genome type (ssDNA)**	**Vector**	**Host**	**Species**
Begomovirus	*Bean golden yellow mosaic virus*	monopartite or bipartite	whitefly (*Bemisia tabaci*)	dicotyledonous plants	192
Curtovirus	*Beet curly top virus*	monopartite	leafhopper	dicotyledonous plants	7
Mastrevirus	*Maize streak virus*	monopartite	leafhopper	monocotyledonous plants	14
Topocuvirus	*Tomato pseudo-curly top virus*	monopartite	treehopper	dicotyledonous plants	1

capsids of a filamentous flexuous nature that are about 12-15 nm wide and vary in length depending on whether they have monopartite (680-900 nm) or bipartite (500-600 nm and 200-300 nm) genomes (Table 3). The genome of members of the family *Potyviridae* is monopartite, linear, positive sense, single stranded RNA containing around 8,500-10,000 nucleotides (nt) (Adams et al., 2005; Fauquet et al., 2005; Gibbs & Ohshima, 2010). The genus *Potyvirus* is the largest within the family Potyviridae and the second largest (after the genus *Begomovirus* within the family *Geminiviridae*) amongst the 73 genera of other families (Gibbs et al., 2008b; ICTVdB, 2012). It includes at least 146 definite and tentative species. Its members infect a wide range of hosts which belong to different plant families including the *Solanaceae*, *Cucurbitaceae* and *Leguminosae* (Gibbs & Ohshima, 2010). The genus *Potyvirus* probably originated and diverged for the first time 7,250 years ago from a virus infecting a monocotyledonous plants (Gibbs & Ohshima, 2010). The genomes of potyviruses are monopartite, organized in a single open reading frame (ORF) (Adams et al., 2005) that comprises a genome-linked protein (VPg), a 5` untranslated region (5`UTR) and a 3` untranslated region (3`UTR) and a poly (A) tract at the 3`-end (Figure 3). The giant polyprotein encoded by this ORF is cleaved by three virus encoded proteinase enzymes to produce 10 mature proteins (Adams et al., 2005). The possible function of these proteins are as follows: P1 or the first protein function is a proteinase and required for genome amplification as well as being an accessory factor for suppression of RNA silencing. Then the HC-Pro (helper component protein) is a proteinase and involved in cell-to-cell and long-distance movement, genome amplification, aphid transmission as well as being a suppressor of RNA silencing induced by host plant (Adams et al., 2005). The P3 third protein is involved in genome amplification. The six kilo Dalton first protein (6K1) is required for RNA replication. Cytoplasmic inclusion (CI) functions are helicase and cell-to-cell movement. The six kilo Dalton second protein (6K2) is required for virus replication and long-distance movement. The viral genome protein (VPg) is involved in linked RNA replication, cell-to-cell and long distance movement and forms a complex with eukaryotic translation initiation factor eIF (iso) 4E. NIa-Pro or nuclear inclusion a protein function is a proteinase. The nuclear inclusion b protein (NIb) functions as a RNA-dependent RNA-polymerase. The coat protein (CP) is required for encapsidation of viral RNA, cell-to-cell and long-distance movement, genome amplification and aphid transmission (Adams et al., 2005). According to the ICTV, a potyvirus can be classified as a novel species when it shows less than 80% of the CP amino acid similarity or 85% of complete genome nucleotide identity to other potyviruses within

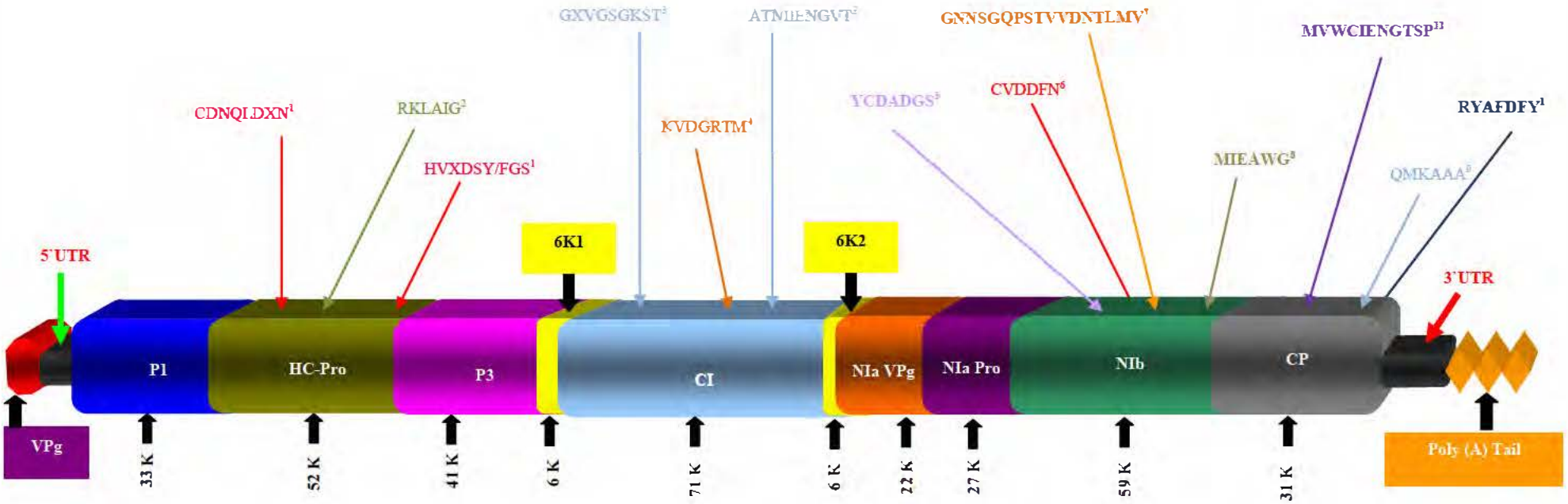


Figure 3: Genome map of the genus *Potyvirus*

Genome map of the genus *Potyvirus* showing the genome organization, conserved amino acid motifs (numbered above) to which primers have been designed. The names of typical potyvirus genes are abbreviated as follows: VPg: Viral genome protein linked, UTR: Untranslated region, P1: P1 protein, HC-Pro: Helper component protein, P3: Third protein, CI: Cytoplasmic inclusion, NIa: Nuclear inclusion a protein, NIb: Nuclear inclusion b protein, CP: Coat protein, Poly (A) tail: Poly adenine tail. Data adapted from Adams *et al.*, 2005. References for numbered motifs are as follows: (1& 3) Ha *et al.*, 2008a&b, (2) (Spetz & Valkonen, 2003; Spetz *et al.*, 2003, (4 & 6) Zheng *et al.*, 2008a; 2010, (5) Colinet *et al.*, 1994, (7) Gibbs and Mackenzie, 1997, (8) Pappu *et al.*, 1994, (9&11) Langeveld *et al.*, 1991 (10) Zheng *et al.*, 2008a; Yamamoto & Fuji, 2008.

the genus *Potyvirus* (Fauquet *et al*., 2005, Gibbs & Ohshima, 2010).

2.5. The family *Geminiviridae*

The family *Geminiviridae* is one of the largest plant virus families (Yadava *et al*., 2010). It was established for the first time in 1978 (Brown, 2009). The name is based on its members possessing geminate or twinned quasi-isometric particles (Seal *et al.*, 2006; Lefeuvre *et al*., 2007; Brown, 2009). The geminivirus genome is composed of a circular single stranded DNA makes this family unusual among other plant viruses that generally have RNA genomes (Brown, 2009; Yadava *et al*., 2010). To date, 214 definite geminivirus members have been classified within the family *Geminiviridae* (ICTVdB, 2012). Due to the recent and increasing high impact of geminiviruses on various crops worldwide, they are considered as emerging viral diseases (Seal *et al*., 2006). Yield losses in infected crops due to geminiviruses can also reach 100% depending on the virus-host combination and age of plant at infection (Brown, 2009). The family *Geminiviridae* are divided into four genera based on their genome organization and biological properties (Fauquet *et al*., 2008; ICTVdB, 2012(). The genus *Begomovirus* (type member *Bean golden yellow mosaic virus*) is the biggest genus within the family *Geminiviridae*, and includes the majority of geminiviruses as 192 out of 214 definite members are classified within this genus (ICTVdB, 2012). Begomoviruses are transmitted by the sweet potato whitefly *Bemisia tabaci* (Gennadius) complex to dicotyledonous plants (Brown, 2009). Begomovirus genomes can be either monopartite or bipartite (Table 3). The genome of bipartite members consists of two segments of circular DNA; namely DNA-A and DNA-B, and they are referred to as New World begomoviruses. Whereas, the genome of monopartite members consists of DNA-A only and they are referred to as Old World begomoviruses (Seal *et al.*, 2006; Brown, 2009). Certain old world monopartite begomoviruses are associated with a non-viral circular single stranded DNA of about 1.3 kb size termed a DNA-β satellite (Briddon *et al*., 2002; Seal *et al.*, 2006; Brown, 2009). The function of DNA-β satellites is thought to be for the viral pathogenicity and disease development by suppressing host-induced gene silencing (Seal *et al.*, 2006; Brown, 2009). More recently, assosiation of DNA-α satellite with monopartite begomoviruses and DNA-β satellite has been identified in China forming begomovirus complex (Xie *et al*., 2010). The DNA-β satellite ranges from 1360 to 1376 nucleotides and contains three conserved features: a single open reading frame (Rep), a conserved hairpin structure, and an adenine-rich (A-rich) region (Xie *et al*., 2010) (Figure 4). Begomoviruses represent the most destructive group of plant viruses in tropical and subtropical regions of the world, due to a combination of factors

including agricultural intensification, genome recombination and increases in populations of their whitefly vector *B. tabaci* (Seal *et al*., 2006). The genome of begomoviruses is about 2.6-2.8 kb for each of the DNA segments (DNA-A and DNA-B). DNA-A is organized into four to six open reading frames (ORF) (Figure 4) (Brown, 2009). The first ORF region is AV1 (or V1 in monopartite begomoviruses) which encodes the viral capsid protein CP. The CP region is the most highly conserved begomovirus gene. It is required for encapsidation of the viral genome, whitefly transmission and systemic spread for monopartite and some bipartite begomoviruses (Seal *et al.*, 2006; Brown, 2009). The AV2 ORF is required for virus accumulation and symptom development in Old World begomoviruses, but is lacking in the New World begomoviruses (Seal *et al.*, 2006). The AC1 ORF encodes the REP, a replication initiation protein (Seal *et al.*, 2006; Brown, 2009). The AC2 ORF encodes proteins for gene expression and suppression of gene silencing induced by the host plant (Brown, 2009). AC3 encodes the replication enhancer protein (*REn*) (Figure 4) (Seal *et al.*, 2006; Brown, 2009). ORFs BV1 and BC1 in DNA-B of bipartite begomoviruses are responsible for nuclear transport of viral DNA and cell to cell movement respectively (Seal *et al.*, 2006; Brown, 2009). DNA-A and DNA-B share a common region (CR) of 200 nt length. CR is a highly conserved sequence between the equivalent component in bi-partite begomovirus. It is termed the large intergenic region (LIR) in monopartite begomoviruses (Brown, 2009). The (LIR/CR) include modular *cis*-acting elements of the origion of replication (*ori*) and promoter elements which are required for regulating viral replication and transcription (Seal *et al.*, 2006; Brown, 2009). According to the ICTV, a geminivirus can be classified as a new species if pairwise sequence comparison shows less than 89% of complete nucleotide sequence to other species within the genus (ICTVdB, 2012).

2.6. The genus *Carlavirus* within the family *Betaflexiviridae*

The genus *Carlavirus*, whose name was abbreviated from the name of its type member *Carnation latent virus*, is one of seven genera within the family *Betaflexiviridae* (Ryu & Lee, 2009). The other six genera within the family are *Foveavirus* (4 species), *Capillovirus* (2 species), *Vitivirus* (6 species), *Tepovirus* (1 species), *Trichovirus* (5 species), *Citrivirus* (1 species) and five unassigned species (ICTVdB, 2012). The family *Betaflexiviridae* has been approved by the ICTV since 2004 and is so named due to the flexuous filamentous virion morphology of its members (Adams, 2009). The genus *Carlavirus* is the largest among other genera of the family *Betaflexiviridae* and includes at least 43 definite members and 25 tentative species (Ryu & Lee, 2009; ICTVdB, 2012).

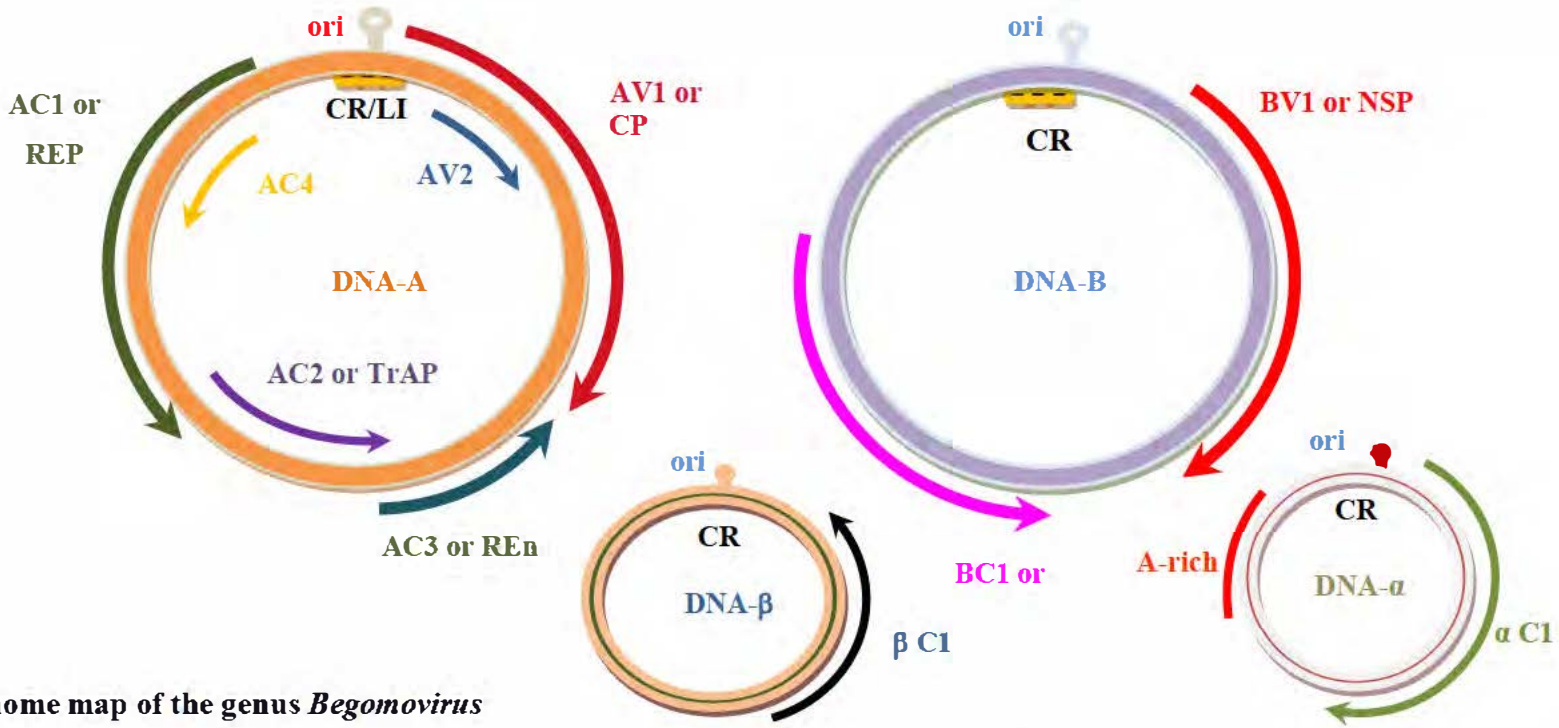


Figure 4: Genome map of the genus *Begomovirus*

Genome map illustrates the main genomic components that occur in begomoviruses. Arrows refer to the orientations of the viral open reading frames (ORFs), which are labelled according to their genomic component (A, B, α or β), and whether they are transcribed from the viral (V) or complementary (C) DNA strand, and their function. In monopartite viruses, gene AV1 is often referred to as V1. The common region (CR) refers to the most homologous part of the intergenic region containing the origin of replication (*ori*) bipartite begomoviruses from the New World lack the AV2 gene. AC3 or replication inhacner protein (*REn*), AC1, (βC1, αC1) or replication initiation protein (REP), AC2 or transcription activator protein (TrAP), BV1 or nuclear shuttle protein (NSP), BC1 or movement protein (MP), A-rich or poly adenine signal. Data adapted from Seal *et al.*, 2006 and Xi *et al.*, 2010.

Carlaviruses induce mild symptoms or symptomless infections on their host plants, and thus the name "latent" is associated with names of many species within the genus (Ryu & Lee, 2009). Most carlaviruses are aphid-transmissible in a non-persistent manner. However, three species, namely *Cowpea mild mottle virus* (CPMMV), *Cucumber vein-clearing virus* (CuVCV) and *Melon yellowing-associated virus* (MYaV), have been found to be transmitted by the whitefly *B. tabaci* in a non-persistent manner (Ryu & Lee, 2009). Carlaviruses have flexuous filamentous particles that are about 610-700 nm in length and 12-15 nm in diameter. The viral genome is monopartite, consisting of linear ssRNA ranging from 7.4-9.1 kb in length. The carlavirus genome is organized into six ORFs with a poly (A) tail at the 3` terminus, a methylated nucleotide cap at 5` terminus, 3`UTR and 5`UTR (Figure 5). The possible gene functions of ORFs are: ORF1: viral replicase (methyltransferase, papain like protease, helicase and RNA-dependent RNA polymerase), ORF2, ORF3 and ORF4 : these form the triple gene block which facilitates cell to cell movement, ORF5: coat protein and ORF6: vector transmission, viral transcription, viral pathogenicity and a supressor of gene silencing (Adams, 2009; Ryu & Lee, 2009).

2.7. The genus *Tombusvirus* (the family *Tombusviridae*)

The genus *Tombusvirus*, whose name was abbreviated from the name of its type member *Tomato bushy stunt virus* (TBSV), is the second largest genus (with 17 species) after the genus *Carmovirus* (20 species) within the family *Tombusviridae* (ICTVdB, 2012). In addition to these two genera, the family *Tombusviridae* includes the following genera; namely, *Aureusvirus* (4 species), *Avenavirus* (1 species), *Carmovirus* (20 species), *Dianthovirus* (3 species), *Machlomovirus* (1 species), *Necrovirus* (7 species), *Panicovirus* (2 species) and two unassinged viruses within the family *Tombusviridae* (Lommel & Sit, 2009). The family *Tombusviridae*, whose name was derived from the genus *Tombusvirus*, consists of more than 57 (ICTVdB, 2012) definite and 15 tentative species (Lommel & Sit, 2009), at least ten of them have been recorded to infect vegetables (Caciagli, 2009). Members of the genus *Tombusvirus* are transmitted in various ways, namely mechanical transmission, fungal transmission, seed and pollen transmission, vegetative propagation and grafting, and through infected soil (Lommel & Sit, 2009). The tombusvirus particles are icosahedral and ~30-38 nm diameter with T=3 symmetry. Their genome is a single molecule of positive-sense single stranded RNA (ssRNA) of about 4.8 kb in length organized in four ORFs (Figure 6) (Hull, 2009).

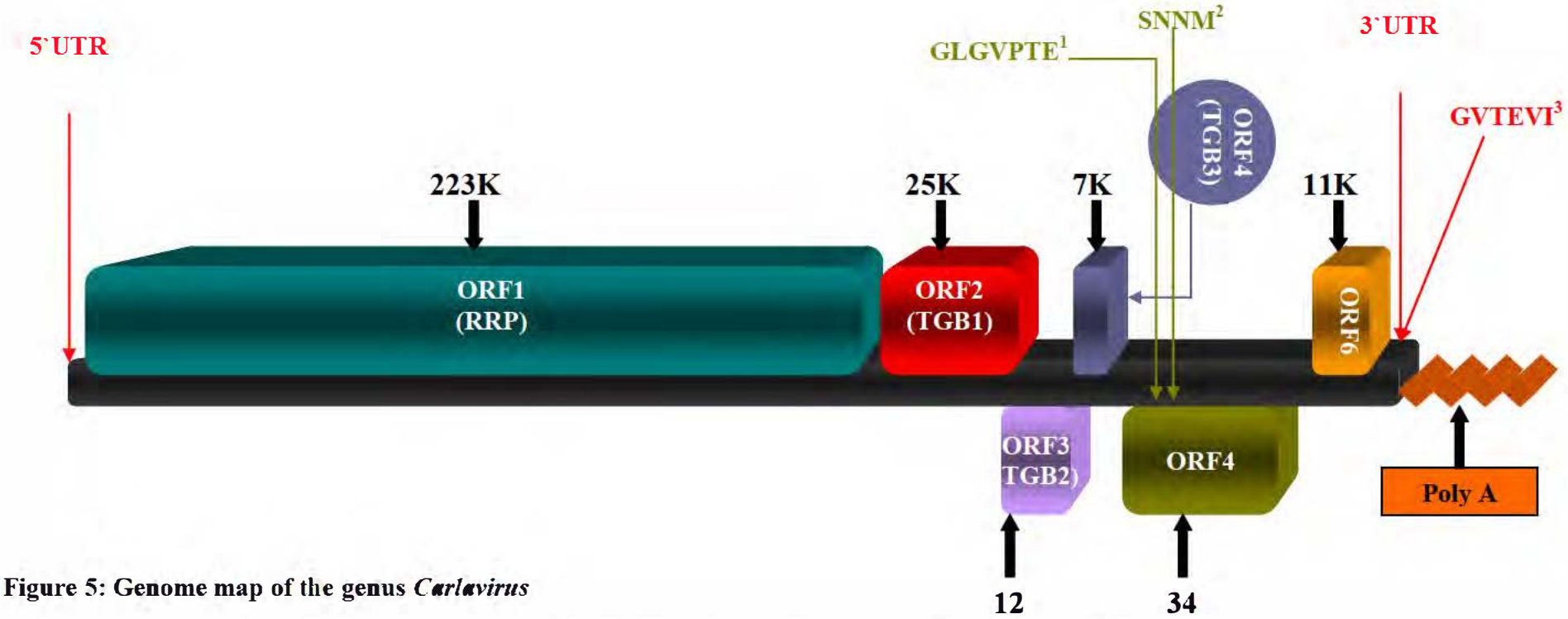


Figure 5: Genome map of the genus *Carlavirus*

Genomic map of the genus *Carlavirus* showing the genome organization and conserved motifs to which primers were designed. The names of typical genome structure of the genus *Carlavirus* family *Flexiviridae* genes are abbreviated as follows: The 5-proximal one large ORF encodes an RNA-dependent RNA polymerase, three overlapping ORFs encode the putative cell to cell movement proteins MPs or triple gene block (TGBs) and two ORFs encodes coat protein (CP) and nucleic acid binding protein (NB). Motifs in the RNA replicase protein (RRP) are methyltransferase (MT) protein like protease (P-Pro), helicase (HEL) and RNA dependent RNA polymerase. References for numbered motifs are: (1) Nie *et al*., 2008 (2) Gasper *et al*., 2008 (3) Badge *et al*., 1996. Data adapted from Ryu & Lee, 2009.

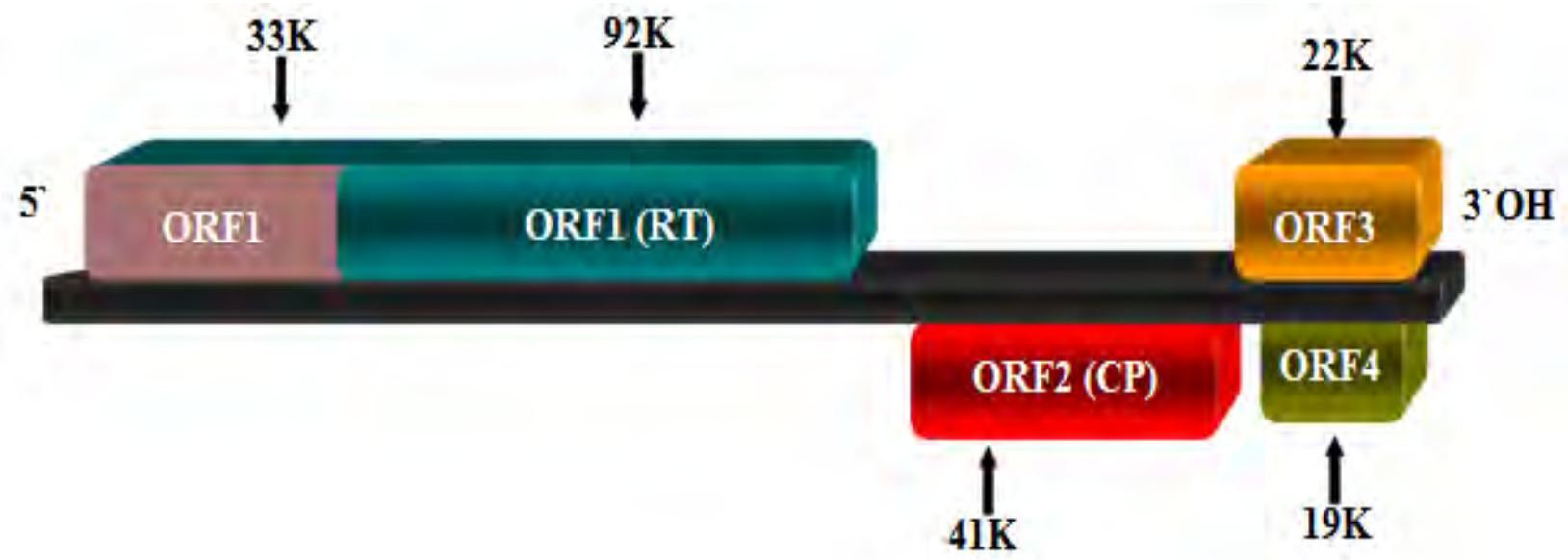


Figure 6: Genome map of the genus *Tombusvirus*

Genomic map of the genus *Tombusvirus* illustrating its genome organization. The names of typical genome structure of the genus *Tombusvirus* family *Tombusviridae* genes functions are as follows: ORF1: viral replicase with read-through of a weak stop codon, ORF1RT: RNA dependent RNA polymerase motif, ORF2: coat protein (CP), ORF3: cell to cell movement, ORF4: suppressor of RNA silencing. 3`OH: 3` hydroxyl group. Data adapted from Hull, 2009.

Species demarcation criteria within the genus *Tombusvirus* outlined in the ICTV guidelines are that a distinct tombusvirus species shows less than 87% amino acid similarity in their CP sequence to other members of this genus (Koenig *et al.*, 2004b).

2.8. Conventional techniques used for virus detection

Plant virus diseases are one of the factors that limit agriculture worldwide (Hull, 2009), it is important; therefore, to characterize, identify the source and the route of spread of the virus(s) causing the diseases to develop control measures (Dickinson, 2003; Hull, 2009). Viral diagnosis is important as well, with the increase in global trade of plant materials, used for cultivation, and associated risk of viruses and their vectors movement from one country to another (Dickinson, 2003). Detection of plant viruses in early stage of infection enables to prevent new outbreaks and the potential impact of virus diseases (Dickinson, 2003). Reliability, simplicity and rapidity, as well as low cost, are essential for technique used in plant virus diagnosis (Dickinson, 2003; Hull, 2009). Diagnostic techniques are necessary for several applications like virus identification and classification, routine detection of plant viruses in seed certification and quarantine programmes, epidemiological investigation for virus spread monitoring and to test virus resistant verities in plant breeding programmes (Hull, 2009; Koenig *et al*., 2009). Methods used for virus detection can be grouped into four categories based on virus properties into biological, physiochemical, serological and molecular tests (Hull, 2009). Biological assays are involved direct inspection of virus symptoms on plant, infectivity assay on indicator plant/hosts to achieve Koch`s postulates, *In vitro* virus properties, growing on tests for screening seed borne viruses and transmission assays to identify the vector and transmission manner (Hull, 2009). However, the biological assays are time and labour consuming, requiring space and facilities for assay plant growth, which may add extra test cost when used to test samples at a large scale (Albrechtsen, 2006; Hull, 2009). Physiochemical diagnosis approaches detect viruses based on measuring the composition and outside feature of virus particles (Hull, 2009). The physiochemical properties measured of the virus are density, sedimentation and diffusion coefficients and ultraviolet (UV) absorption spectrum (Hull, 2009). Size, shape and any surface features of the virus particles are essential for virus identification and classification (Hull, 2009; Koenig *et al*., 2009). Those features can be measured by electron microscopy (Hull, 2009; Koenig *et al*., 2009). Physiochemical approaches are reliable and highly sensitive techniques. However, these require expensive equipments (i.e. electron microscopes, ultracentrifuges and ultraviolet spectrophotometers) beside high skills; this limits use of these techniques in routine testing

for virus indexing (Hull, 2009; Koenig *et al*., 2009). Serological test approaches are one of the most important techniques used to detect plant viruses. They involve specific reaction between mono- and polyclonal antibodies prepared against viral protein (antigen) by immunizing an experimental animal (Albrechtsen, 2006; Hull, 2009). Enzyme linked immunosorbent assay (ELISA) is the most widely used among other serological test (Albrechtsen, 2006; Hull, 2009; Koenig *et al*., 2009). Double sandwich ELISA (DAS-ELISA) (Clark and Adams, 1977) is the most used ELISA among other approaches (Albrechtsen, 2006; Hull, 2009; Koenig *et al*., 2009). It involves trapping virus particles by specific antibodies, already immobilized on inner surfaces of multiwell polystyrene plates (Clark and Adams, 1977; Koenig *et al*., 2009). The trapped virus particles are detected by means of enzyme labelled antibodies (Clark and Adams, 1977; Koenig *et al*., 2009). DAS-ELISA reliability, high sensitivity, simplicity and low cost, have enabled use of this technique in routine diagnosis for virus routine testing in vegetable and potato seeds (Albrechtsen, 2006; Kerlan, 2009). The flexibility of serological approaches has enabled combining them with other approaches like Immunosorbent electron microscopy (ISEM) and Immunocapture polymerase chain reaction (IC-PCR) to improve virus detection (Hull, 2009; Koenig *et al*., 2009).

2.9. Molecular techniques used in plant virus detection

Nucleic acid based techniques are the most powerful and reliable characterisation approaches that have widespread application in plant virus identification (Dietzgen, 2002; Potter *et al.*, 2007; Coen, 2009; Koenig *et al.*, 2009). Such methods for the detection of plant pathogens have been used since the late 1970s, when the first nucleic acid hybridisation protocols were developed for the diagnosis and detection of viruses and viroids (Mumford *et al*., 2006). However, molecular methods failed to play a major role in routine diagnoses until the 1990s, when the development of the polymerase chain reaction (PCR) in the mid 1980s started to have an impact (Mullis *et al*., 1986; Saiki *et al*., 1988). In the last two decades, significant progress has been made in molecular (nucleic acid-based) diagnostic methods. The availability of commercial kits for nucleic acid extraction, PCR, restriction digestion and cloning, laboratory equipments like thermal cyclers and consumables, commercial sequencing and oligonucleotide synthesis services, software and data base for sequence analyses, together with high sensitivity, specificity and speed, have led to more extensive use of molecular techniques for the detection of plant pathogens (Mumford *et al*., 2006; Koenig *et al.*, 2009). Using molecular techniques such as PCR has expanded the range of targeted pathogens

detected and minimized the false diagnosis of plant pathogens. However, while the detection of pathogens in individual infected samples has become easier, there are still many challenges in the use of these techniques in plant pathogen diagnosis. Amongst the challenges are the detection of pathogens in soil due to find a reliable method for soil extraction and the low concentration of virus particles in soil samples (Mumford *et al*., 2006). Other challenges are detection of viruses in their vectors, high throughput testing and the development of generic methods, which enable simultaneously screening of samples for large numbers of pathogens (Mumford *et al*., 2006). The challenges in applying molecular techniques to test potato and vegetable samples in broad spectrum will be reducing the test cost as the molecular techniques require using expensive equipments (like thermocyclers) and high cost reagents (especially for RNA viruses that require reverse transcription step) (Tomlinson *et al*., 2012). Molecular techniques require using reliable extraction methods to obtain a quality nucleic acid (Thomson & Dietzgen, 1995; Seal & Coates, 1998; Fenby *et al*., 1998; Mumford *et al.*, 2006; Sipahioglu *et al*., 2007; Li *et al*., 2008), which will be time consuming and add an extra cost to the test beside the labour costs compared to ELISA (Abarshi *et al*., 2010; Tomlinson *et al*., 2012). The challenge, therefore, will be to optimize reliable, simple, low cost and rapid approaches to extract nucleic acids from potato and vegetables, suitable for molecular detection. Loop mediated isothermal amplification (LAMP) is a promising molecular approach, which may be an alternative to PCR (Notomi *et al*., 2000, Koenig *et al.*, 2009; Tomlinson *et al*., 2012). LAMP involves amplification of targeted DNA at isothermal temperature (65 ºC) using four sets of primer and DNA polymerase with high strand displacement activity (Notomi *et al*., 2000). The test is rapid as it can be performed in 1 h and the amplification product can be visual to sight (Notomi *et al*., 2000; Tomlinson *et al*., 2012). LAMP has been developed to include reverse transcription step, termed as (RT-LAMP), and shown to be a powerful tool to detect plant RNA viruses (e.g. Tomlinson *et al*., 2012) and viroids (Tsutsumi *et al*., 2010). LAMP/RT-LAMP are rapid, sensitive, specific and low in cost as it can be performed without using expensive equipment (i.e. thermocyclers) or further gel electrophoresis step especially when combined to lateral flow approaches (Tomlinson *et al*., 2012). LAMP/RT-LAMP can be alternatives to ELISA in developing countries, due to simplicity, sensitivity and low cost, for virus detection in potato and vegetables, especially when used to test samples in broad spectrum (Tomlinson *et al*., 2012).

2.10. Nucleic acid extraction

PCR/RT-PCR could be unreliable for the detection of plant viruses if the tissues from infected plants contain RT- or PCR-inhibitory substances (Thomson & Dietzgen, 1995; Seal & Coates, 1998; Fenby *et al*., 1998; Mumford *et al.*, 2006; Sipahioglu *et al*., 2007; Li *et al*., 2008). Many PCR applications devoted for plant virus diagnosis require rapid, easy-to-use, efficient, and reliable methods for nucleic acid isolation from purified virus preparations or plant tissue extracts, soil, or virus vectors (Dietzgen, 2002). Due to the high sensitivity of PCR, only pico- or femtogram amounts of target template are required. Total plant genomic and non-genomic nucleic acids as well as genomic viral nucleic acids could serve as a template for PCR or RT-PCR amplification (Dietzgen, 2002). Although many PCR applications do not require exceptionally high template purity, a removal or reduction of inhibitory components will be beneficial for efficient activity of the reverse transcriptase or DNA polymerase enzymes (Dietzgen, 2002). PCR amplification will be hampered due to the presence of inhibitory substances in plant extracts, such as polysaccharides and phenolic compounds (Seal & Coates, 1998). Type, location, and concentration of such inhibitors may vary depending on plant age and cultivar. Some plant leaves, roots, tubers, corms, and seeds, have been shown to contain potent PCR inhibitory compounds (Dietzgen, 2002).

Hence, different methodologies for nucleic acid extraction have been used for the diagnosis of plant viruses by PCR/RT-PCR or RCA. One common method based on total nucleic acid extraction by cetyltrimethylammonium bromide (CTAB) has been used successfully to extract viral total nucleic acids for both RNA viruses (e.g.; Abarshi *et al*., 2010) and DNA viruses (e.g. Pratap *et al.*, 2011). CTAB is a strong detergent used for separation of protein from nucleic acids. This method has been adapted from Doyle and Doyle method (1987) to isolate barley DNA and detect plant viruses (Doyle & Doyle, 1987; Li *et al*., 2008). The CTAB method was particularly developed to extract nucleic acids from plants due to its suitability for plants rich in polyphenolic and polysaccharide compounds and it is relatively inexpensive compared to commercial kits (Li *et al*., 2008; Abarshi *et al*., 2010). However, the CTAB method was not found suitable for extraction of RT- or PCR-amplifiable potyvirus RNA from sweet potato (Fenby *et al*., 1998), possibly due to the exceptionally high levels of polysaccharides in this plant species. For the latter, more success was achieved using commercial kits, such as the Plant RNeasy extraction kit (Qiagen Inc.) (e.g. Chen *et al*., 2001a; Zheng *et al*., 2010) and TRIZOL-based methods (e.g. Colinet *et al*., 1998 a&b; Souto *et al*., 2003). Conventional methods like guanidium thyocyanate combined with liquid nitrogen /phenol-chloroform method were used to extract nucleic acids as well (e.g. Chavi *et*

al., 1997; Colinet *et al*., 1997; Fenby *et al*., 1998). Methods have been developed for some potyviruses that allow RNA to be amplified directly by RT-PCR without RNA isolation (Mumford & Seal, 1997; Berger & Shiel 1998; Sharman *et al*., 2000). The methods are generally dependent on capturing the virus particles on tubes, either directly or using antibodies to selectively concentrate the viruses from the plant sap (Thomson & Dietzgen, 1995; Mumford & Seal, 1997; Suehiro *et al*., 2005). The latter technique is termed immunocapture-RT-PCR (Koenig *et al.*, 2009).

2.11. PCR/RT-PCR

Within nucleic acid based diagnostic tests, approaches based on PCR have become the most extensively used for plant virus detection (Dietzgen, 2002; James *et al.*, 2006; Potter *et al.*, 2007; Koenig *et al.*, 2009). PCR is a rapid and sensitive technique that can be used to amplify with high specificity a product from an extremely small amount of targeted DNA in biologically complex samples (Dietzgen, 2002; Potter *et al.*, 2007; Coen, 2009, Koenig *et al.*, 2009). The specificity of the PCR is determined predominantly through the design of oligonucleotide primers that are annealing to complementary regions flanking the fragment to be amplified (Dietzgen, 2002; Potter *et al.*, 2007, Coen, 2009). Resultant DNA fragments can be analyzed by size fractionation on agarose gels, restriction digestion, hybridization with probes or by DNA sequencing for further characterisation (Potter *et al.*, 2007; Coen, 2009). Due to their high sensitivity and potential specificity, PCR techniques have widespread application in plant pathogen routine diagnosis, identification and population studies (Dietzgen, 2002; Potter *et al.*, 2007; Hull, 2009).

Historically, the polymerase chain reaction (PCR) was proposed for the first time in 1971. Several elements required for PCR, particularly a thermostable DNA polymerase, had not yet been discovered at that time. In 1983, PCR was independently developed by Kary Mullis when a better understanding of how DNA synthesis worked had been achieved (Mullis *et al*., 1986). Mullis used *Escherichia coli* polymerase I rather than a thermal stable DNA polymerase. Hence, the enzyme had to be added after each cycle of the chain reaction. Later, in 1988, Saiki and colleagues had found that by using thermal stable DNA polymerase isolated from *Thermus aquaticus*, an archaea species isolated from a hot spring environment (50-85ºC) (Saiki *et al*., 1988). Then no additional polymerase was required to be added for each time after a PCR cycle (Potter *et al.*, 2007). Since then, PCR has become one of the most important tools for molecular biologists (Dietzgen, 2002; Potter *et al.*, 2007).

PCR is nowadays useful for routine detection of plant viruses, and it can be performed directly on plant viruses that have a DNA genome, whereas RNA-containing viruses require a reverse transcription (RT) step to generate complementary (c) DNA prior to PCR amplification (RT-PCR) (Dietzgen, 2002; Potter *et al.*, 2007; Hull, 2009; Koenig *et al.*, 2009). It facilitates simple, sensitive, rapid and accurate detection of plant viruses in broad spectrum samples (Seal & Coates, 1998; James *et al*., 2006; Tomassoli *et al*., 2007; Salem *et al*., 2010). Applications of PCR-based plant virus diagnosis include germplasm screening, field surveys to resolve virus incidence and geographic distribution, provision of virus-free planting material, domestic and international plant quarantine, detection of mixed virus infections, analyses of virus distribution in different plant tissues, identification of alternative host plants, evaluation of virus-resistant or -tolerant cultivars, analysis of virus transmission by insect, nematode, or fungal vectors (Dietzgen, 2002). Very small amounts of sample are needed for PCR, and sample tissues can be fresh, dried, or frozen, and even partially degraded (Dietzgen, 2002; Mumford *et al*., 2006; Hull, 2009; Koenig *et al.*, 2009).

2.12. Group specific primers

'Universal' primers refer to group-specific (at species, genus, or family level) primers designed to target conserved nucleotide or amino acid sequence motifs, shared by all or several members within the same taxonomic group (Dietzgen, 2002; James *et al.*, 2006; Rodoni, 2009). The extreme differences among plant viruses with RNA and DNA genomes limit the identification of conserved regions shared by all viruses, and hence designing "plant virus-specific" primers is likely to remain elusive (James *et al.*, 2006). However, by restricting the scope of the assay to lower-order taxa, such as genera or families, it is possible to identify short conserved amino acid motifs within the viral proteins (James *et al.*, 2006). Due to the genetic code degeneracy, it still requires the use of 'degenerate' primers to achieve such group-specific primer design. A 'degenerate' primer is a mixture of multi-copy oligonucleotides in which the nucleotides at one or more defined positions vary such that all variations complementary to the consensus sequence are achieved. Incorporation of deoxyinosine at nucleotide positions with more than two fold degeneracy reduces primer complexity and increases sensitivity over fully degenerate primers (Dietzgen, 2002; James *et al.*, 2006); hypoxanthine, the base present in the nucleosides I and deoxyinosine (dI), is able to base pair with all four normal nucleosides. Another potential problem associated with the use of such degenerate primers is amplification of non-targeted sequences due to low-

stringency of amplification conditions employed to enable primer annealing to targeted sequences (Dietzgen, 2002; James *et al.*, 2006; Rodoni, 2009).

Group-specific primer sets can be applied effectively in quarantine and seed certification programs. The aspects of application could be, for example, as a useful tool when new crops are imported and there is limited information on their pathogens, or alternatively when there are several known quarantinable viruses in the same plant genera or family a generic test can reduce costs (Rodoni, 2009). Specific primers have been used individually or in combination with degenerate primers for group detection (James *et al.*, 2006). For example, the use of a primer containing an oligodeoxythymidine that targets the polyadenylic acid (poly A) tail at the 3` end within the genome of a number of viruses, have been extensively used in combination with genus- or family-specific forward primers to detect potyviruses (Gibbs & Mackenzie 1997; Chen *et al*., 2001a) and carlaviruses (Badge *et al*., 1996; Chen *et al*., 2002a; Gasper *et al*., 2008; Nie *et al*., 2008). Most group-specific primers have been designed from conserved motifs within coat protein (CP) and RNA-dependent RNA polymerase (RdRp) regions (Table 4), as these two regions have some of the most highly conserved motifs among genomic regions. CP and RdRp genes, therefore, are approved by ICTV criteria for virus classification and demarcation within the many genera (Fauquet *et al*., 2005; ICTVdB, 2012). Similarly, a non-degenerate primer set has been designed to detect virus members within the genus *Tombusvirus*, and this also targets the highly conserved motifs within the CP region (Koenig *et al*., 2004a&b). RT-PCR was introduced for the first time to detect individual potyviruses in 1990 -1991 (Nicolas & Laliberte, 1992; Gibbs & Mackenzie, 1997). It was used successfully for potyvirus detection using group specific primers (Langeveld *et al*., 1991; Nicolas & Laliberte, 1992).

It was concluded that using RT-PCR based group specific degenerate primers was a rapid and sensitive approach to detect new potyviruses (Langeveld *et al*., 1991), discriminate strains (Pappu *et al*., 1994; 1998) as well as to determine the taxonomic status (Pappu *et al*., 1997; Colinet *et al*., 1998b). About 17 conserved sites have been discovered by sequence composition in the *Potyvirus* genome, particularly in HC-Pro, CIP, NIb and CP regions using sequence comparison (Korniichuk *et al*., 2007; Zheng *et al*., 2008a). However, most potyvirus specific primers have been designed to target six conserved motifs only within the whole potyvirus genome (James *et al*., 2006; Webster, 2008; Zheng *et al*., 2008a) (Figure 3).

Table 4: Group-specific primers designed for different plant virus genera/families

Specificity	Target region	Reference(s)
Allexvirus genus	3`UTR/Poly(A)	Chen *et al*., 2001b
Badnavirus genus	tRNA/RT/RNase H	Lockhart & Olszewski, 1993
Badnavirus genus	RT/ RNase H	Thompson *et al*., 1996
Begomovirus genus	CP	Reddy *et al*., 2005
Begomovirus genus	AC1 / AV1 (DNA-	Rojas *et al*., 1993
Begomovirus genus	BC1/ ori (DNA-B)	Rojas *et al*., 1993
Begomovirus genus	ori / AR1	Deng *et al*., 1994
Begomovirus genus	capsid protein "core"	Wyatt & Brown, 1996
Begomovirus genus (containing DNA B)	DNA B	Reddy *et al*., 2005
Begomovirus genus (containing DNA β)	DNA β	Briddon *et al*., 2002
Bromoviridae family	helicase (RNA1)	Untiveros *et al*., 2010
Carlavirus genus	NBP /poly(A)	Badg *et al*., 1996
Carlavirus genus	CP / poly(A)	Gasper *et al*., 2008
Carlavirus genus	CP / poly(A)	Nie *et al*., 2008
Carlavirus genus	TGB2 / poly(A)	Chen *et al*., 2002a
Capillovirus genus	putative RNA	Marinho *et al.,* 1998
Carmovirus, *Dianthovirus* & *Tombusvirus*	RdRp	Morozov *et al*., 1995
Closterovirus genus	HSP70	Karasev *et al.*, 1994
Closteroviridae family	HSP70 homolog	Dovas & Katis, 2003a
Closterovirus & *Crinivirus* genera	HSP70 homolog	Tian *et al.*, 1996
Comoviridae family	RdRp	Maliogka *et al*., 2004
Cucumovirus genus (CMV, PSV&TAV)	(3′NCR) /CP	Choi *et al.*, 1999
Ilarvirus genus	RdRp (RNA2)	Untiveros *et al*., 2010
Ilarvirus genus (PNRSV plus ApMV)	CP	Candresse *et al.*, 1998
Ilarvirus genus (PNRSV, PDV & ApMV)	3′NCR / CP	Saade *et al.*, 2000
Luteoviridae family	CP	Chomicˇ *et al*., 2010
Luteovirus & *Polerovirus* genera	CP/17K	Robertson *et al*., 1991
Mastrevirus genus	C2	Rybicki & Hughes, 1990
Nepovirus genus (ArMV plus GFLV)	Movement protein	Wetzel *et al.*, 2001
Nepovirus genus (subgroups a&b)	RdRp	Wei & Clover, 2008
Ophiovirus genus	RNA polymerase	Vaira *et al*., 2003
Polerovirus genus	RdRp/NCR /CP	Shang *et al*., 2009
Pospiviroid genus	Central conserved	Bostan *et al.*, 2004
Potexvirus genus	RdRp	Gibbs *et al*., 1998
Potexvirus genus	RdRp	Vlugt & Berendsen, 2002
Potyviridae family	NIb /poly(A)	Gibbs & Mackenzie, 1997
Potyviridae family	NIb / poly(A)	Chen *et al.*, 2001a
Potyvirus genus	NIb / CP gene	Hsu *et al.*, 2005
Potyvirus genus	NIb / CP gene	Colinet *et al.*, 1994

Table 4 continued

Specificity	Target region	Reference(s)
Potyvirus genus	NIb /CP gene	Langeveld *et al.*, 1991
Potyvirus genus	CP / poly(A)	Pappu *et al.*, 1994
Potyvirus genus	NIb / poly(A)	Pappu *et al.*, 1994
Potyvirus genus	CP / PolyA	Vlugt *et al.*, 1999
Potyvirus genus	HC-Pro	Ha *et al.*, 2008a
Potyvirus genus	CI	Ha *et al.*, 2008a
Potyvirus genus	NIb	Zheng *et al.*, 2008a
Potyvirus genus	NIa or CP	Janzac *et al.*, 2009
Tobamovirus (ORSV) & *Potexvirus*	RdRp	Seoh *et al.*, 1998
Tobamovirus genus	(nested PCR) RdRp	Dovas *et al.*, 2004
Tobamovirus genus	RdRP	Gibbs *et al.*, 1998; 2004
Tobamovirus genus (subgroup I)	3′NCR	Letschert *et al.*, 2002
Tombusvirus genus	CP	Koenig *et al.*, 2004a&b
Tospovirus genus	nucleocapsid/3' SRNA	Mumford *et al.*, 1996; Dewey *et al.*, 1996
Tospovirus genus	N gene / 3′NCR (S RNA)	Okuda & Hanada, 2001
Trichovirus, *Capillovirus* & *Foveavirus* plus BanMMV	RdRp	Foissac *et al.*, 2005
Tymovirus & *Marafivirus* genera and GFkV	RdRp (MT or polymerase)	Sabanadzovic *et al.*, 2000
Vitivirus & *Foveavirus* genera	RdRp	Dovas & Katis, 2003b
Vitivirus genus	RdRp	Saldarelli *et al.*, 1998

C2: complementary, CI: cytoplasmic inclusion, CP: capsid protein, HC-Pro: Helper component-protease, HSP70: heat shock protein 70 kDa, MT: methyltransferase, NBP: nuclear binding protein, 3′NCR: 3′ noncoding region, NCR: intergenic noncoding region, NIa: nuclear inclusion a, NIb: nuclear inclusion b, oligo(dT): oligodeoxythymidine, *ori*: origin of replication, polyA: polyadenylic acid tail, RdRp: RNA dependent RNA polymerase, S RNA: small RNA, tRNA: tRNA-like structure, TGB2: triple gene block2, 3`UTR: 3` untranslated region.

Abbreviations for virus species: ApMV: *Apple mosaic virus,* ArMV: *Arabis mosaic virus*, BanMMV: *Banana mild mosaic virus*, CMV: *Cucumber mosaic virus*, CymMV: *Cymbidium mosaic virus*, GFkV: *Grapevine fleck virus*, GFLV: *Grapevine fanleaf virus*, ORSV: *Odontoglossum ringspot virus*, PDV: *Prune dwarf virus*, PNRSV: *Prunus necrotic ringspot virus*, PSV: *Peanut stunt virus*, TAV: *Tomato aspermy virus*, TSWV: *Tomato spotted wilt virus.*

Data in this table were adapted from Dietzgen, 2002 and James *et al.*, 2006.

Potyvirus primer sets have frequently been designed to target conserved motifs in the CP region because it is less variable than other regions and has been approved by ICTV for species demarcation and identification of potyviruses (Zheng *et al*., 2008a; 2010). According to the ICTV, a potyvirus can be classified as a novel species when it shows less than 80% of the CP aa similarity to other potyviruses within the genus *Potyvirus* (Fauquet *et al*., 2005). A universal degenerate primer set was designed to detect most members of the family *Potyviridae*, the forward primer 'Potyvirid primer 2' was designed to target GNNSGQP, a family conserved motif in the NIb gene sequence, and the reverse primer 'Potyvirid primer 1' contained oligodeoxythymidine and was derived from the poly-A tail at 3` end (Gibbs & Mackenzie, 1997). Thus, primer sets derived from this motif have been used to detect RNA sequences of over 300 members of the family *Potyviridae* in a wide range of plants from variable geographical locations (Gibbs *et al*., 2003; James *et al*., 2006). Incorporating inosine, a nucleotide derivative, within the forward primer has reduced the nucleotide degeneracy in potyvirus family-specific primer set (Mackenzie *et al*., 1998). Genus/family specific primers have also been successfully used to screen for seed-borne viruses in cowpea for the genera *Cucumovirus*, *Carmovirus* and *Carlavirus* in and for the families *Potyviridae* and *Comoviridae* (Salem *et al*., 2010). Bulb crops have been screened for potyviruses, carlaviruses, potexviruses and allexviruses using genus/family specific primers (Chen *et al.*, 2001b; 2002a&b). Begomovirus-specific primers enabled the detection of five different begomoviruses able to cause ToLCD when tomato and weed samples were screened for mixed begomovirus infections (Reddy *et al*., 2005).

It is common that a gel electrophoresis approach is used to visualize DNA fragments of the expected size amplified by PCR using group specific primers. However, this approach often does not provide information on the virus (es) present in the starting plant material. Restriction fragment length polymorphism (RFLP) analysis of the amplified fragments (Marie-Jeanne *et al*., 2000; Okuda & Hanada 2001; Reddy *et al*., 2005), or direct amplicon sequencing (Foissac *et al*., 2005), can be used to provide such information. However, the interpretation of the results obtained may be significantly complicated in the case of mixed infections (James *et al.*, 2006). Cloning of DNA fragments amplified by group specific primers is an alternative strategy to RFLP or direct sequencing (James *et al.*, 2006). Other possibilities include the use of ELISA-PCR approaches using capture or detection probes (Hsu *et al.*, 2005).

2.13. Rolling circle amplification (RCA)

Techniques involve amplification of whole genomes in a single step are powerful tools for phylogenetic analyses, epidemiological studies and studies on genome organization (Johne *et al*., 2009). Rolling-circle amplification (RCA) approach is among the new available techniques for whole genome amplification, using the DNA polymerase isolated from bacteriophage Phi 29 (Ø 29) (Demidov, 2002; Johne *et al*., 2009). The Ø 29 DNA polymerase possesses several cleaving functions, such as strand displacement activity, proof-reading activity and amplification of very long synthesis products, which enable for the efficient amplification of circular DNA molecules from complex of molecules within biological samples (Johne *et al*., 2009). Small circular DNA molecules are rare in higher organisms; but they are abundant in several microorganisms. Thus, Ø 29 DNA polymerase-dependent RCA, therefore, has been applied to the study of several virus families of circular DNA genomes and has been of great value. As specific primers are not required for this technique, many novel viruses have been sequenced and infectious genomic clones generated (Johne *et al*., 2009).

The basics of RCA are illustrated in Figure 7. After primer (random hexamers) annealing to the single-stranded template molecule, the Ø29 DNA polymerase incorporates nucleotides in a 5'-3' direction complementary to the template strand. When, the polymerase reaches the primer binding site again, it displaces the newly synthesized strand and proceeds DNA synthesis for several rounds dependent on the template length. Meanwhile, amplification continues as the random primers anneal to the displaced linear DNA and Ø29 DNA polymerase starts to synthesize a second strand of DNA using the displaced linear DNA as a template. As a result, a large concatemeric of double stranded (ds) DNA molecule is produced consisting of repeated copies of the template sequence (Demidov, 2002; Johne *et al*., 2009). The full genome length DNA fragments can be released by applying a single cutting restriction enzyme to RCA products, and then fragments can subsequently be cloned and sequenced. The 3'-5' DNA exonuclease activity of the Ø29 polymerase enables the enzyme to perform proofreading, which makes it even more suitable for genetic studies due to possessing high fidelity characteristics (Johne *et al*., 2009). RCA has been applied for circular DNA amplification since 1989 (Johne *et al*., 2009).

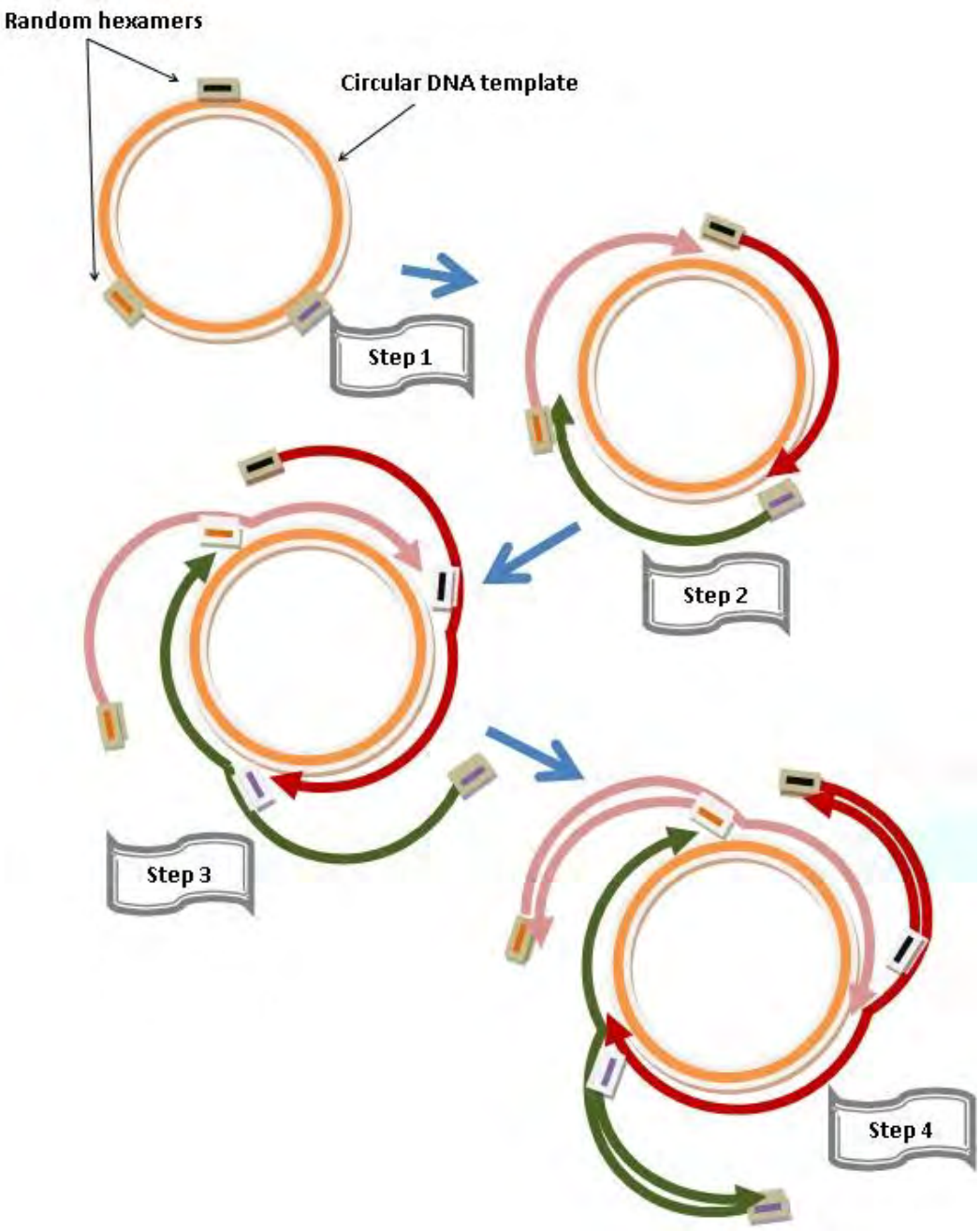


Figure 7: Rolling circle amplification

An illustration showing the mechanism of RCA for circular DNA amplification. Step1: Primer annealing of random hexamers to circular DNA template. Step 2: Extension and displacing of linear DNA amplified by Phi 29 DNA polymerase. Step 3: Primer annealing of random hexamers to displaced linear DNA. Step4: Extension of second strand of linear DNA while Phi 29 DNA polymerase extension continues amplifying the circular DNA template and displacing the multi-copy DNA. The diagram was adapted from Johne *et al*., 2009.

However, it has been introduced into plant viruses for the first time in 2004 when multiply-primed RCA was first applied to the bipartite geminiviruses infecting tomatoes to both DNA-A and DNA B component (Inoue-Nagata *et al.*, 2004). The advantages of RCA are the suitability of the RCA product for direct sequencing, restriction fragment length polymorphism analyses as well as cloning of the entire genome segment being made easily possible. Furthermore, the RCA technique requires no expensive equipments or particularly costly consumables, and it is possible to detect any circular DNA component without any *a priori* knowledge of sequence information (Schubert *et al.*, 2007b; Haible *et al.*, 2006). Besides, using the random hexamers enables the amplification of distinct variants or novel sequences and quasispecies, which provides comprehensive information about the sequence pool in mixed populations (Johne *et al*., 2009). RCA has been applied to several monopartite and bipartite geminiviruses (Johne *et al*., 2009). It has been used to amplify full genomes of monopartite begomoviruses that cause leaf curl diseases in tomato (Pandey *et al*., 2010; Pratap *et al.*, 2011), tomato yellow leaf curl bipartite begomoviruses (Knierim & Maiss, 2007), or monopartite begomoviruses (Al-Abdallat *et al*., 2011) or monopartite with a β satellite DNA genome (Khan *et al*., 2008). RCA has successfully been used to amplify other circular DNA viruses such as mastreviruses (Owor *et al.*, 2007a&b; Briddon *et al*., 2010b; Mumtaz *et al.*, 2011), curtoviruses (Briddon *et al*., 2010a), nanoviruses (Grigoras *et al*., 2010) and caulimoviruses (James *et al*., 2011). Nevertheless false reactions can occur when using multiply-primed RCA in diagnostic applications on plant viral diseases, with sequence analyses of restriction fragments amplified by RCA having revealed mitochondrial sequences for some of the fragments (Homs *et al.*, 2008). Therefore, to minimize the false positive results, it is advisable to use additional precautions such as sequencing of RCA products to identify such problems (Homs *et al.*, 2008; Johne *et al*., 2009).

2.14. Gel electrophoresis and restriction fragment length polymorphism (RFLP)

DNA fragments amplified using PCR can be visualized by agarose gel electrophoresis followed by staining with ethidium bromide. Gel electrophoresis separates the PCR products according to size (Sambrook *et al*., 2006) targeted DNA fragments can then be extracted from gels for cloning and sequencing (e.g. Odedara *et al*., 2007). Agarose gel electrophoresis has been used frequently to analyse DNA fragments resulted from RT-PCR amplification of potyvirus genome using universal primers (Chavi *et al*., 1997; Raj *et al*., 2009).

PCR-based RFLP approaches have been used to differentiate among potyviruses (Dekker *et al*., 1993; Yang & Mirkov, 1997). DNA fragments resulting from RT-PCR using

potyvirus specific primers could be digested with certain restriction enzymes and then analyzed by gel electrophoresis to discriminate among these fragments. Certain restriction enzymes, like *Rsa*I, *Msp*I, *Alu*I, *Mse*I or *Dde*I, have been used to cleave PCR products generated by universal primer sets designed from MVWCIEN and QMKAAA motifs in CP region and poly-A tail. The resulting fragments have been used to discriminate different species and isolates from the family *Potyviridae* (Vlugt *et al*., 1999; Marie-Jeanne *et al*., 2000; Lebas, 2002; Sertkaya *et al*., 2003). However *in silico* sequence analyses approaches are still superior in discriminating among potyviruses, as they have been based on sequence comparison using computer based analyses to discriminate among different potyviruses and they give more information for comparing closely related sequences (Gibbs & Ohshima, 2010).

RFLP have also been used to differentiate various geminiviruses and to study genomic components of begomoviruses since the 1980s (Bisaro *et al*., 1982; Rojas *et al*., 1993; Reddy *et al*., 2005). The RFLP technique has frequently been used to analyze the product amplified by RCA (Johne *et al*., 2009). It enables differentiation among circular DNA viruses, viral genome components and to obtain the whole genome of geminiviruses and other circular DNA viruses (Inoue-Nagata *et al*., 2004; Haible *et al*., 2006; Schubert *et al*., 2007b). RFLP has been applied to generate infectious clones from both RCA and group specific primers for many geminviruses (Knierim & Maiss, 2007; Ito *et al*., 2009; Mumtaz *et al*., 2011).

2.15. Cloning

Cloning has been adapted to amplify and sequence plant DNA and RNA (Domier *et al.*, 1986; Rybicki & Hughes, 1990) some years before the PCR/ RT-PCR techniques were used first to amplify plant virus sequences (Langeveld *et al*., 1991). Different cloning systems have been used to clone PCR/RT-PCR amplified plant virus DNA fragments for sequencing. The pGEM-T cloning system from Promega has been used to increase the ligation efficiency of PCR-amplified DNA fragments. Accordingly, pGEM-T is a linearized plasmid vector with T-overhangs at both 3`-ends which hybridise to the 3' adenine overhang added to amplified DNA fragments through the terminal transferase activity of *Taq* polymerase. Due to this design, ligations can be performed efficiently without restriction enzyme cleavage as both DNA fragments and the vectors have sticky ends (Brown, 2010). The recombinant plasmid resulted from ligation is then transformed into *E. coli* in order to multiply the recombinant plasmid. As a result, large numbers of a single copy of the targeted sequence are obtained (Brown, 2010). To identify the recombination, the pGEM-T vector includes the ampicillin

resistance and the *lacZ* genes within the insertion site. The *lacZ* encodes part of the enzyme β-galactosidase which catalyzes the lactose into glucose and galactose. The selective growth medium (i.e. broth-agar), used for growing the desired *E. coli* competent cells, includes the ampicillin antibiotic, the X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside) a lactose analogue and the enzyme inducer IPTG (isopropylthiogalactoside). The non-recombinant colonies that synthesise the β-galactosidase and catalyze the X-gal will generate a blue colour, whereas the recombinant colonies with a disrupted *lacZ* gene, are unable to synthesise the β-galactosidase and so will be white on the selective medium (Brown, 2010). In addition, the pGEM-T vector includes a T7/SP6 promoter located on either side of its polylinker, which enables amplification of the inserted fragment for further confirmation of recombination (Brown, 2010).

Other systems that have been used for cloning potyvirus cDNA fragments include cloning vectors such as the pCRII plasmid cloning vector (Invitrogen), which also allows cloning without the need for restriction enzyme digestion (Yang & Mirkov, 1997; Colinet *et al*., 1998a&b; Chen *et al*., 2006a&b; Monger *et al*., 2001; Guaragna *et al*., 2006b; Wang *et al*., 2007). In addition, the pDrive cloning vector (Qiagen), which includes a U-overhang instead of a T-overhang at its 3`-terminal, has been used for cloning DNA fragments obtained from RT-PCR using potyvirus-family specific primer (Odedara *et al*., 2007) or carlavirus-specific primers (Nie *et al*., 2008; Nie, 2009). In contrast, DNA fragments of ~ 1-3 kb have been cloned successfully using Bluescript plasmid systems. Cloning using this system does require restriction enzyme cleavage of both the vector and the DNA fragment for ligation (Desbiez & Lecoq, 2004). Other vectors like M13 (Dekker *et al*., 1993), pUC 18 (Benscher *et al*., 1996) and pCR-Script SK(+) (Colinet *et al*., 1997; Gambley *et al*., 2004), all of which require restriction enzyme cleavage, have been used to clone DNA fragments obtained from PCR product using potyvirus-specific primer sets.

Alternatively, RT-PCR products obtained from potyvirus genomes have been sequenced directly (i.e. without cloning), by incorporating either T7 or SP6 promoters into the 5`-end of the potyvirus-specific primers (Gibbs *et al*., 1998; Mackenzie *et al*., 1998; Marie-Jeanne *et al*., 2000; Moran *et al*., 2002; Spetz & Valkonen, 2003; Spetz *et al*., 2003; Grisoni *et al*., 2006; Yamamoto & Fuji, 2008). In addition, pGEM-T vector was used to clone DNA viruses. Full-length fragments of DNA-A and DNA-B components, amplified by begomovirus-specific primers, were successfully cloned into 'pGEM-T Easy' vector and then sequenced to identify begomoviruses in legumes (Rouhibakhsh *et al*., 2008). The pGEM-T

Easy vector was used to clone DNA fragments amplified by begomovirus specific primers isolated from tomato and sweet pepper to identify *Tomato yellow leaf curl virus* by sequence analyses or constructing infectious clones (Khan *et al*., 2007; Ito *et al*., 2009).

Full-length geminivirus genomes amplified by RCA have been successfully cloned into pGEM vectors and used later for sequncing and sequence analyses (Schubert *et al*., 2007b; Al-Musa *et al*., 2008; Owor *et al*., 2007a&b; Shepherd *et al*., 2008; Briddon *et al.*, 2010a&b). Alternatively, RCA products were sequenced directly to obtain the full-length sequence for geminiviruses (Haible *et al*., 2006; Schubert *et al*., 2007b).

2.16. Sequencing

Nucleotide sequencing is a powerful tool to distinguish plant viruses (Koenig *et al*., 2009; Gibbs & Ohshima, 2010). Plant DNA viruses were first sequenced in 1982 (Rybicki & Hughes, 1990) whereas plant RNA viruses were first completely sequenced in the mid 1980s (Riechmann *et al*, 1992). There are several approaches for DNA sequencing, among them is the chain termination method innovated by Fred Sanger and colleagues in the mid-1970. This approach is the most used method due to simplicity, the method can be automated for commercial use and can sequence longer DNA fragment (up to 1500 nucleotide) (Sanger *et al*., 1977; Brown, 2010). Chain termination sequencing (or referred to as the Sanger method) is based on the single stranded DNA molecules that differ in length by a single nucleotide only. DNA molecules of 10-1500 nt size can be separted from one another by polyacrylmide gel electrophoresis, into series of bands in a slab or capillary gel (Brown, 2010). The approach inolves preparing identical single stranded DNA molecules. A short oligonucleotide is used to anneal to each molecule at the same position. Subsequently, this oligonucleotide acts as a primer to synthesize a new complementary DNA strand from the template. The reaction requires adding small quantities of modified, differentely fluoresent labelled dideoxynucleotide triphosphates (ddNTPs) together with the normal four deoxynucleotide triphosphate (dNTPs) set and DNA polymerase enzyme as well. Once the polymerase incorporates the dideoxynucleotide, DNA extension stops and no further elongation of DNA molecule occurs due to lack of 3`-hydroxyl group needed to form connection to the next nucleotide (Brown, 2010). But, the reaction would not be terminated at the same position of all DNA molecules present in the reaction as the normal dNTPs occur within the reaction. Thus, the resulted DNA fragment represent a set of different length molecules that end at the equivalent position in the template DNA (Brown, 2010). Polyacrylamide gel electrophoresis separation is applied to discriminate each chain terminated fragment according to their

length. Then, fluorescent detector is used to enable distinguishing the different fluorescent dyes, tag each terminated molecule at the end, whether they are A, T, C or G (Brown, 2010).

Due to small size genome and circular feature of most plant DNA viruses, full genome sequences can directly be obtained using the RCA approach without previous sequence information as random primers are used. RNA viruses require cDNA synthesis step before applying PCR (Koenig *et al*., 2009). Complete cDNA synthesis could be obtained using rapid amplification of cDNA ends (RACE) approaches (Koenig *et al*., 2009). cDNA fragments from RT-PCR product of universal primers could be sequenced and compared to other sequences in GenBank to identify potyviruses. This approach has enabled to identify many genera, species and strains in different geographical regions (Gibbs & Mackenzie, 1997; Gibbs *et al*., 2003; Koenig *et al*., 2009).

Currently, 90 out of 111 definite and 11 out of 86 tentative potyvirus members have been sequenced partially or in full and included within GenBank database (Zheng *et al*., 2008a). They are represented in more than 6000 sequences (Gibbs & Ohshima, 2010) (approximately 11383 potyvirus sequences have been included in the GenBank Database (data retrieved from GenBank website http://www.ncbi.nlm.nih.gov/ in the 17th of April 2013). Potyvirus specific primers have been used alongside sequence analyses to confirm the identification of virus isolates as a new potyvirus in bulbs (Langeveld *et al*., 1991; Dekker *et al*., 1993; Chen *et al*., 2006a; Raj *et al*., 2009), cereal (Seifers *et al*., 2000), peanut, orchids (Mackenzie *et al*., 1998; Guaragna *et al*., 2006b; Zheng *et al*., 2008b), apiaceous crops (Moran *et al*., 2002; Ori'lio *et al*., 2009) and sweet potato (Souto *et al*., 2003). Besides, sequencing of RT-PCR products amplified by potyvirus specific primers has also been used to distinguish among different potyvirus species or strains in single or mixed infection samples of sweet potato (Colinet *et al*., 1994; 1997; 1998a; Souto *et al*., 2003; Wang *et al*., 2007), peanut and sesame (Pappu *et al*., 1997; 1998), sugarcane (Yang & Mirkov, 1997; Gambley *et al*., 2004), cruciferous crops, bulbs, legumes, cereal, celery and lettuce (Chen *et al*., 2001a&b; Ha *et al*., 2008a&b) solanaceous crops (Spetz *et al*., 2003) and vanilla (Grisoni *et al*., 2006). Partial nucleotide sequence and similarity analyses of DNA fragments amplified by potyvirus specific primer have been used to identify potyviruses/strains in non-original hosts (Benscher *et al*., 1996; Chen *et al*., 2001a; 2002a&b; 2006b). In addition; a recombination between two different potyviruses has been recognized depending on complete nucleotide sequence and using potyvirus family specific primer sets (Desbiez & Lecoq, 2004). Information from

nucleotide sequence of potyviruses had a major role in designing universal primers (Langeveld *et al*., 1991; Zheng *et al*.; 2008a; 2010; Janzac *et al*., 2009).

Group specific primers have been used to obtain the full-length sequence of new carlavirus species or strains when a fragment, amplyfied by carlaviruses specific primers. was sequenced to design species specific primers to obtain the remains genomic sequence (Chen *et al*., 2001b; Nisbet *et al*., 2006; Zheng *et al*., 2006; Nie, 2009). New carlaviruses have been identified based on sequencing of DNA fragments obtained from carlavirus-specific primers (Belintani *et al*., 2002; Mollov *et al*., 2007). Sequences obtained from DNA fragments amplified by tombusvirus specific primers were used to detect known tombusviruses or identify new species and isolates (Koenig *et al*., 2004 a&b; Fujinaga *et al*., 2009). About 9112 geminivirus sequences have been included in the GenBank Database (data retrieved from GenBank website http://www.ncbi.nlm.nih.gov/ on 17 April 2013) represent over 1500 species, isolates and strains (Fauquet *et al*., 2008). Many of the sequenced geminviruses have been amplified using the RCA or group specific primers approach which provided important sequence data for geminivirus identification (Reddy *et al*., 2005; Knierim & Maiss, 2007; Johne *et al*., 2009; Briddon *et al*., 2010 a&b; Pandey *et al*., 2010).

2.17. Phylogeny and sequence analyses

Nucleotide sequences provide valuable information about the extent of relationships between plant viruses (Hull, 2004). It is the most reliable approach to identify plant viruses up to isolate level. The rapid accession to data bases, the exponential increase of sequence data and the computer programme development enable simplifying the use of sequence analyses in plant virology (Hull, 2004; Koenig *et al*., 2009). Short and full-length sequences can be compared to study the relatedness between two virus isolates (Hull, 2004). Nucleotide as well as amino acid sequences can be compared using various alignment computer programs. Deduced amino acid can be generated based on the conserved motifs and data obtained from the GenBank data base and used to study the relationship among plant viruses (Hull, 2004).

Reconstruction of ancestral relationships from contemporary data has extensively been used to provide both evolutionary and functional information into biological systems. The exponential rise in available DNA sequence data has increased the interest in phylogenetic analyses (Pearson *et al*., 1999). Three general classes of phylogenetic reconstruction methods are commonly used to analyze sequence data sets: parsimony methods distance based methods, and maximum likelihood methods (Pearson *et al*., 1999). Parsimony- and distance-based methods are most often used, because they are computationally speed and allow a larger

number of potential phylogenetic trees to be evaluated (Pearson *et al*., 1999). The neighbor-joining (NJ) method is widely used in molecular biology due to its efficiency and simplicity in reconstructing large phylogenies. Besides, it has computational speed and the high accuracy in phylogenetic inference as revealed in computer simulation studies (Pearson *et al*., 1999; Kumar & Gadagkar, 2000). The NJ is a heuristic method, joins at each step, the two closest sub-trees that are not already joined and it is based on the minimum evolution principle (Pearson *et al*., 1999). One of the important concepts in the NJ method is ***neighbours***, which are defined as two taxa that are connected by a single node in an enrooted tree. Neighbor-joining method is proposed for reconstructing phylogenetic trees from evolutionary distance data. The method is based on finding pairs of operational taxonomic units (OTUs [=neighbours]) that minimize the total branch length at each stage of clustering of OTUs starting with a star like tree. The branch lengths as well as the topology of a parsimonious tree can quickly be obtained by using this method (Saitou & Nei, 1987).

The use of comparative analyses of molecular sequence data enables reconstructing the evolutionary history of species, besides inferring the nature and extent of selective forces shaping the evolution of genes and species (Tamura *et al*., 2011). Molecular Evolutionary Genetics Analysis version 5 (MEGA5) is a user-friendly software for mining online databases, building sequence alignments and phylogenetic trees (Tamura *et al*., 2011). It enables using methods of evolutionary bioinformatics in basic biology, biomedicine, and evolution (Tamura *et al*., 2011). The MEGA5 software package is a collection of maximum likelihood (ML) analyses for inferring evolutionary trees, selecting best-fit substitution models, whether they are nucleotide or amino acid, inferring ancestral states and sequences (alongside probabilities) and estimating evolutionary rates site-by-site. In computer simulation analyses, the ML tree inference algorithms in MEGA5 compared to other software packages in terms of computational efficiency and the accuracy of the estimates of phylogenetic trees, substitution parameters and rate variation among sites (Tamura *et al*., 2011).

The bootstrap is a computer-based technique for assessing the accuracy of almost any statistical estimate. It is particularly useful in complicated nonparametric estimation problems, where analytic methods are impractical. The bootstrap has been introduced in the estimation of phylogenetic trees. The technique provides assessments of ‘‘confidence’’ for each clade of an observed tree, based on the proportion of bootstrap trees showing that same clade (Efron *et al*., 1996; Soltis & Soltis, 2003). Due to the increasing emphasis in biology on reconstruction

of phylogenetic trees, concerns about how confident one should be in a given phylogenetic tree and how support for phylogenetic trees should be measured have arisen (Soltis & Soltis, 2003). It has been suggested that bootstrapping be applied across characters of a taxon-by-character data matrix to produce replicate "bootstrap data sets," each of which is then analyzed phylogenetically, with a consensus tree constructed to summarize the results of all replicates. The proportion of trees/replicates, in which a grouping is recovered, is presented as a measure of support for that group (Soltis & Soltis, 2003). Thus, bootstrapping has become a common feature of phylogenetic analysis. The advantages of phylogenetic bootstrapping is potentially limited by a number of features, such as the size of the data matrix and the underlying assumptions of the phylogeny reconstruction program (Soltis & Soltis, 2003).

Phylogeny and nucleotide sequence analyses have provided critical information regarding the extent of relationship among genera, species and strains, resolved many taxonomic themes, recognized distinction among geographical isolates and enabled understanding the origin and evolution of plant viruses (Hall *et al*., 1998; Hull, 2004; Chare & Holmes, 2006; Gibbs *et al*., 2008a&b; Gibbs & Ohshima, 2010). Both full-length and partial sequences can be used in phylogenetic analyses to elucidate relationships of plant viruses from different hosts, samples and geographical locations using various sequence analysis software. Small sequences of ~350 bp were compared to GenBank sequences to give evidence that they belong to a member within the genus *Potyvirus*. These sequences were amplified from CP gene core using potyvirus specific primer set targeting MVWCIEND and QMKAAA motifs (Raj *et al*, 2009). A full-length sequence of 10,113 nucleotides of a virus isolated from lupine has been compared to sequences from GenBank to reveal that this virus is a new member of potyviruses. Full-length genome of the new potyvirus has been obtained by a PCR based primer-walking approach using degenerate potyvirus primers designed from conserved motifs in NIb, CI and HC-Pro genes (Sarkisova & Petrzik, 2011).

According to ICTV criterion, a virus can be described as a new species when it shows less than 80% or 85% similarity of full-length CP amino acid or complete genome sequences, respectively to other potyviruses (Fauquet *et al.*, 2005). Species demarcation point for potyviruses could be decreased to 42.2-77.6% nucleotide identity when full-length sequence of NIb is used (Zheng *et al.*, 2010). Using potyvirus family/genus specific primers, RT-PCR, cloning, sequencing and phylogenetic analysis have enabled rapid determination of taxonomical status of potyviruses as distinct viruses, strains or isolates of the same virus in different crop plants. In addition, deduced amino acid sequences have been used in

phylogenetic analyses to compare coat proteins of different potyviruses, their strains and isolates in single or mixed infection of infected bulbs (Chen *et al*., 2001b; 2006b), cruciferous crops (Chen *et al*., 2002b), cucurbits (Desbiez & Lecoq, 2004) cereal (Seifers *et al*., 2000) solanecious crops (Monger *et al*., 2001; Spetz *et al*., 2003; Spetz & Valkonen, 2003) legumes and pulses (Benscher *et al*., 1996; Berger *et al*., 1997; Guaragna *et al*., 2006a), and sweet potato (Souto *et al*., 2003; Wang *et al*., 2007).

Phylogenetic analyses based on full-length genome of geminiviruses amplified by RCA have extensively been used to reveal virus relatedness and the evolutionary relationship of highly diverged geminiviruses (Khan *et al*., 2008; Varsani *et al*., 2009; Briddon *et al*., 2010a&b; Mumtaz *et al*., 2011) beside study molecular diversity of many geminiviruses (Schubert *et al*., 2007b; Ilyas *et al*., 2009; Briddon *et al*., 2010c). Phylogenetic analyses based on core region and complete sequence of CP amplified by begomovirus specific primers have been used to study molecular diversity of begomoviruses isolated from tomato (Reddy *et al*., 2005; Maruthi *et al*., 2007). Phylogentic analyses have resolved the taxonomic status of distinct species within carlavirus (Ruy & Lee, 2009; Menzel *et al*., 2011) and tombusvirus goups (Koenig *et al*., 2004a&b).

2.18. Infectious clones and agro-inoculation

Agro-inoculation is a technique that enables the inoculation of cloned viral genomes into a wide variety of host plants using the bacteria *Agrobacterium tumefaciens* (Saeed, 2008). Genome-sized viral DNA, therefore, is produced and systemically spreads throughout the plant and induces disease symptoms (Grimsley *et al*., 1987). The potential of using *Agrobacterium* as a vector to generate transgenic plants with genes of interest has been known since the early 1980s. The first successful attempt to construct an infectious clone of plant virus has been achieved in 1986 (Grimsley *et al*., 1987; Vaghchhipawala & Mysore, 2008). They were able to clone the genomes of *Cauliflower mosaic virus* (CaMV) and the *Maize streak virus* (MSV) into *Agrobacterium* binary vectors and induced disease symptoms upon "agro-infection" (Vaghchhipawala & Mysore, 2008). Since then the agro-infection approach (commonly known as agro-inoculation) has extensively been used to deliver viruses inside plants for the following applications: to validate both mono- and bipartite viruses as causal agents of disease, characterize novel viruses and their genomes via mutagenesis, recombination analyses between related viruses, and for transient RNA interference (RNAi), and virus-induced gene silencing (VIGS) studies (Vaghchhipawala & Mysore, 2008). Agro-inoculation has also been invaluable in identification of plant lines resistant to virus infection

(Vaghchhipawala & Mysore, 2008). As most geminiviruses are naturally transmitted only by specific insect vectors, agro-inoculation has greatly simplified the study of this group of viruses. Agro-inoculation has been used to conduct infectivity analyses and to determine the host range of geminiviruses and to confirm the role of DNA-β in severity of symptom on plant infected by monopartite begomovirus (Seal *et al*., 2006; Saeed, 2008). However, the disadvantages of agroinoculation are it requires time-consuming sub-cloning procedures to introduce the viral DNA, which is more than one unit in length, into the binary vector. Another pathogen (*A. tumefaciens*) is introduced into the host plant as well. In some cases, agroinoculation of cloned begomovirus DNA does not mimic whitefly transmission, probably due to the difficulties encountered with *A. tumefaciens* in the infection of some hosts (Guenoune-Gelbart *et al*., 2010). A one-step cloning approach has been applied to agro-inoculation using partial restriction digestion of RCA product to obtain repeated tandems of begomovirus DNA (Ferreira *et al*., 2008). This approach enabled to construct infectious clone for both DNA-A and DNA-B from bipartite begomoviruses with reduced cloning steps (Ferreira *et al*., 2008). Biolistic approach has extensively been used to inoculate infectious clones into plants (Guenoune-Gelbart *et al*., 2010). A modified biolistic approach has been applied to infectious clone inoculation using RCA product (Guenoune-Gelbart *et al*., 2010). The approach is relatively simple and rapid, enables the creation of begomovirus cultures without the use of cloning or whiteflies transmission. Biolistic inoculation can be used to construct an infectious DNA to recover viruses from old collections, separate begomoviruses from other viruses in mixed infection, test Koch's postulates, preserve pure virus cultures and to screen hosts for resistance to viruses (Guenoune-Gelbart *et al*., 2010). However, agro-inoculation is widely used in infectious clone inoculation, as it is cost-effective compared to biolistic approach (Saeed, 2008).

CHAPTER 3: MATERIALS AND METHODS

3. Materials and methods

3.1. Plant sampling and handling

All potato and vegetable samples collected from Iraq, sample information, key description and abbreviations were listed in (Appendix 1). The following strategy was used for plant sampling: to test viruses already spread in fields, leaf samples were collected separately from symptomatic potato, tomato, eggplant, cucumber, squash, snake cucumber and bottle gourd plants (Figure 8) from fields in the Abu Ghraib district of Baghdad province in November 2008, March 2009 and April 2011 The original source for potato samples collected from fields in Baghdad was from Syria (Appendix 1).

To test seed/tuber transmitted and high incident viruses, potato tubers from Anbar Province were planted and grown in pots in open fields at the College of Agriculture/University of Baghdad-Abu Ghraib in February 2009. Tomato seedlings from seed of a local variety were grown in the glasshouse, and then transplanted into the field at the College of Agriculture/University of Baghdad-Abu Ghraib in December 2008. Leaf samples from grown potato and tomato were collected from each plant individually in April and June 2009, respectively. Cowpea seed obtained from local varieties in Najaf were sown in the glasshouse at the College of Agriculture/University of Baghdad-Abu Ghraib in November 2008 and leaf samples were collected in April 2009 (Appendix 1).

Leaf samples were dried by calcium chloride in plastic bags at 4 ºC for two weeks, and sent to NRI at Greenwich University, UK and kept in the laboratory at room temperature. Samples of leaf tissues were crushed, transferred into screw-cap plastic tubes containing silica and also kept in the laboratory at room temperature. A certain scheme was followed to screen selected virus groups in 175 potato and vegetable samples (Figure 9).

3.2. Nucleic acid extraction

Total nucleic acids were extracted from dried leaf samples using an adapted CTAB protocol (Lodhi *et al*., 1994; Maruthi *et al*., 2002; Abarshi *et al*., 2010) as follows: Around 30-40 mg of dried plant tissue was placed into a thick gauged plastic bag and ground using a hand-held ball bearing sample grinder (Bioreba AG, Switzerland).

Figure 8: Pictures illustrating leaf symptoms of naturally infected plants collected from fields in Baghdad-Iraq

Description as follows: A. Cucumber Leaves: mottle (L.), healthy (R.), B. Bottle gourd leaf (mosaic), C. Zucchini Leaf (yellow mosaic), D. Zucchini leaf (mosaic), E. Snake cucumber leaf (green vein banding). F. Tomato leaves: leaf curling and yellowing.

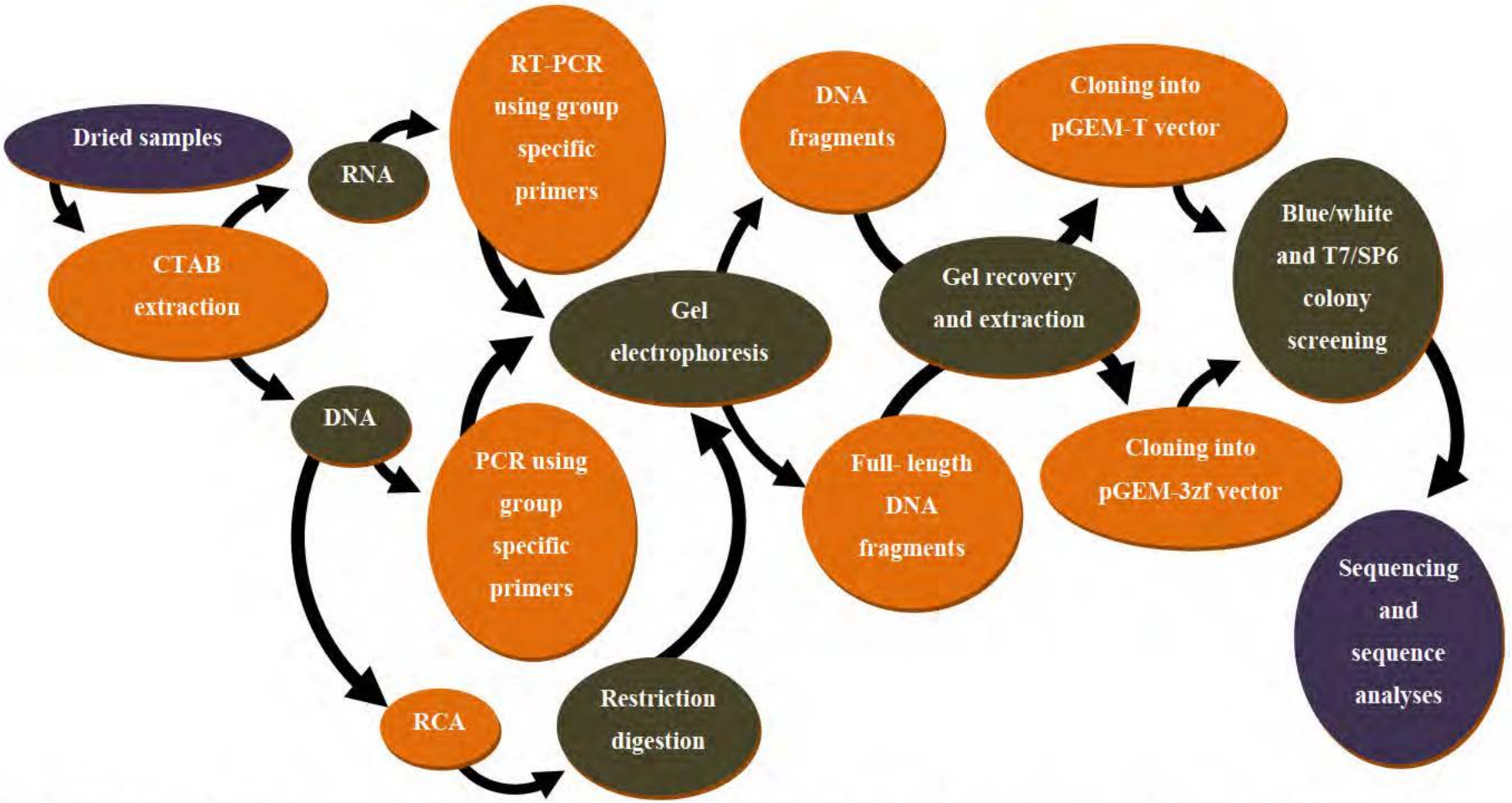


Figure 9: Scheme used for group screening and plant virus detection in potato and vegetable samples collected from Iraq.

About 30 volumes (1ml) of CTAB extraction buffer (2 % (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl) contain 0.2 % (v/v) 2-mercaptoethanol were added to the crushed tissue and homogenised using a wallpaper seam roller (Wickes, UK). Approximately 750 µl of each mixture was transferred into a 1.5 ml tube and incubated in a water bath for 30 min at 60º C. Then, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) was added to the extract, mixed vigorously and centrifuged at 13,000 *g* for 10 min. The supernatant was collected carefully, avoiding any interphase material, and transferred into a new 1.5 ml tube. Nucleic acids were precipitated by adding 0.6 volumes (300/µl) of ice cold (-20 ºC) isopropanol, the mixture was mixed gently and incubated overnight at -20 ºC. Precipitated nucleic acids were collected by centrifuging the mixture at 15,000 *g* for 10 min at 4 ºC. The supernatant was discarded and the resulting pellet was washed by adding 0.5 ml 70% ethanol, mixing and re-centrifuging (15,000 *g*, 5 min). The supernatant was discarded and the ethanol residue removed from the pellet by vacuum drying for 5 min in a Spin vac (Model Savant DNA 120 speed vac concentrator, Thermo Scientific, USA). Finally, the pellet was re-suspended in 1 ml of 1x TE buffer and stored at -20 ºC till further analysis.

3.3. **Group specific primer sets**

Primer sets used for detection of various plant virus families/genera are shown in (Table 5). Selected samples from potato, cucurbits and broad bean, which showed typical virus symptoms, were used for primer testing and screening. In contrast, information on genus/family specific primers, reaction conditions and their sequences were obtained from previous publications (Table 5) synthesized by Invitrogen, UK. The remaining samples were then tested using the primer sets that detected viruses in the highest number of selected samples tested.

3.4. **RT-PCR for RNA viruses**

3.4.1. **cDNA synthesis**

Initially, cDNA synthesis was performed using ImProm-II™ Reverse transcription system (Promega, UK) and Oligo-dT primer (Invitrogen, UK) by a modification of their recommended procedure as follows: 5 µl of extracted nucleic acids of each sample were mixed with 5 µl of primer solution (0.5 µg Oligo-dT and 4 µl nuclease-free sterile distilled deionised water (SDW)) in a PCR tube. For nucleic acid denaturation, the mixture was incubated for 5 min in a 75º C water bath and then immediately chilled on ice.

Table 5: Primer sets tested for detection of plant virus genomes from Iraqi samples

Primer	Virus/group	Sequence	Motif /region	References
PNIbF1(F)	Potyviruses	5'-GGBAAYAATAGTGGNCAACC-3'	GNNSGQP/NIb	Hsu *et al*., 2005
PNIbF5(F)		5'-GCCAGCCCTCCACCGTNGTNGAYAA-3'	GQPSTVVDN/NIb	
PCPR1(R)		5'-GGGGAGGTGCCGTTCTCDATRCACCA-3'	WCIENGTSP/CP	
POT1(R)		3'-ACCACRTADCTBTTACCTAGGTCAG-5'	WCIEN/CP	Colinet *et al*., 1994
POT2(F)		5'-GACGAATTCTGYGAYGCBGATGGYTC-3'	YCDADGS/NIb	
U335(F)		5'-GAATTCATGRTNTGGTGYATHGANAAYGG-3'	MVWCIENG/CP	Langeveld *et al*., 1991
D335(R)		5'-GAGCTCGCNGYYTTCATYTGNRHDWKNGC-3'	QMKAAA/CP	
U341(F)		5'-CCGGAATTCATGRTITGGTGYATIGAIAAYGG-3'	MVWCIENG/CP	
D341(R)		5'-CGCGGATCCGCIGYYTTCATYTGIRIIWKIGC-3'	QMKAAA/CP	
U1000(F)		5'-ACIGTIGTIGAYAAYWSIYATGG-3'	TVVDNTLMV/NIb	
DI000(R)		5'-GTICCRTTITCIATRCACCAIAYCAT-3'	MVWCIENG/CP	
Sprimer(F)		5'-GGNAAYAAYAGYGGNCARCC-3'	GNNSGQP/NIb	Chen *et al*., 2001a
M4T(R)		5'-GTT TTCCCAGTCACGACT(15)-3'	Oligo dT /Poly A Tail	
PV2I(F)		5'-GGIAAYAAYAGYGGICARCC-3'	GNNSGQP/NIb	Mackenzie *et al*., 1998
PV1(R)		5'-CACGGATCCCGGG(T)17V-3'	Oligo_dT /Poly_A	
Carla-Uni(F)	Carlaviruses	GGAGTAACYGAGGTGATACC	11k /3`UTR	Badge *et al*., 1996
Carla-CP(F)		5'-GGBYTNGGBGTNCCNACNGA-3'	GLGVPTE/CP	Gasper *et al*., 2008
Car-F1(F)		5'_CNRTBTCNAAYAAYATGGC-3'	SNNMA/CP	Nie *et al*., 2008
Potex1(F)	Potexviruses	5'-CAYCARCARGCNAARGAYSA-3'	HQQAKD/RdRp	Gibbs *et al*., 1998
Potex2(R)		5'-TCDGTRTTDGCRTCRAADGT-3'	TFDANT/Pol	
CPTALL5(F)	Cucumoviruses	5'-YASYTTTORGGTTCAATTCC-3'	HLLGSI/CP	Choi *et al*., 1999
CPTALL3(R)		5'-GACTGACCATTTTAGCCG-3'	RLKWSV/CP	
Tobamo3(F)	Tobamoviruses	5'-CARACNATWGTBTAYCA-3'	RdRp	Gibbs *et al*., 1998 & 2004
Tobamo2(R)		5'-TTBGCYTCRAARTTCCA-3'		
AMVF2(F)	Alfamoviruses	5'-ATCATGAGTTCTTCACAAAAGAA-3'	IMSSSQK/CP	Xu & Nie, 2006
AMVR2(R)		5'-TCAATGACGATCAAGATCGTC-3'	DDLDRH/CP	
CIR1(F)	Tombusviruses	5'-GACTCCGCCGTAGCTTGACC-3'	DSAVA /CP	Koenig *et al*., 2004a&b
CIR2(R)		5'-GGTTTATTGACTTGTTCGTATTCAG-3'	LNTNKSIN /CP	
Deng A(F)	Begomoviruses	5'-TAATATTACCKGWKGVCCSC-3'	ORi	Deng *et al*., 1994
Deng B(R)		5'-TGGACYTTRCAWGGBCCTTCACA-3'	AV1 CP	
TYLCV-F	TYLCV	5'-GGATAAGCACATGGAGATGTG-3'	C4	This study
TYLCV-R		5'-CAAGATAACAGAACACAGCCA-3'	C3	

Different primer sets, their sequences and targeted motifs used to detect plant viruses in samples collected from Iraq. Key: A= Adenine; C= Cytosine; T= Thymine; G= Guanine; I= Inosine; N= A, G, C, or T; R= A or G; Y= C or T; W= A or T; K= G or T; V= A, C or G; D= A, G or T; H= A, C or T; B= C, G or T; F= forward primer, and R= reverse primer.

Then, reaction solution (1x ImProm buffer, 5 mM $MgCl_2$, 0.25 mM dNTP, and 5 U of ImProm reverse transcriptase) was added and the final volume of reaction mixture was adjusted to 20 µl using nuclease-free SDW. Using a Gene Amp PCR System thermo cycler machine (Model 2720, a&b\ Applied Biosystems, UK), cDNA was synthesized using the following regime: mixture from above was incubated for 5 min at 25 ºC, for 60 min at 40 ºC, for 15 min at 70 ºC for (annealing), (extension) and (reverse transcriptase inactivation) respectively. The resultant cDNA was used directly for PCR or stored at -20 ºC.

3.4.2. RT-PCR for potyvirus sequence detection

The resultant cDNA was amplified using Red Hot *Taq* DNA polymerase (Thermo Scientific Inc., UK) following the manufacturer's instructions with slight modifications: 23 µl of reaction solution (containing 0.625 U of Red Hot DNA polymerase, 1 x reaction buffer, mixed dNTPs (0.2 mM of each nucleotide), 2.5 mM $MgCl_2$, 20 pmol/µl each of forward and reverse primers. This was mixed with 2 µl of cDNA in PCR tube and the final volume was adjusted to 25 µl using SDW. For cDNA amplification, PCR tubes containing reactants were loaded in a PCR machine with cycling programmed as follows: a cycling regime was applied to each set of primers using one pre-denaturation cycle for 2 min at 94 ºC, 35 amplification cycles including denaturation for 1 min at 94 ºC, annealing for 1 min at 50 ºC and extension for 2 min at 72 ºC. A final extension cycle was used for 10 min at 72 ºC. The cycle for the Sprimer/M4T set of primers was the same except annealing was performed by incubation for 1 min at 47 ºC.

In contrast, the following program was used for Pot2/Pot1 primer set (Colinet *et al*., 1994): a pre-denaturation cycle for 1 min at 94 ºC, followed by 5 cycles of pre-amplification using denaturation for 30 s at 94 ºC, annealing for 30 s at 45 ºC and extension for 2 min at 72 ºC. Then, 35 amplification cycles in which there was denaturation for 30 s at 94 ºC, annealing for 30 s at 50 ºC and extension for 2 min at 72 ºC were performed. A final extension step for 10 min at 72 ºC was used for this set. PCR products were used for cloning and sequencing later on.

3.4.3. RT-PCR for carlavirus sequence detection

cDNA was synthesised as described in (3.4.1). PCR reactions, prepared as in (3.4.2), were used for Carla-CP/M4T and Car-F1/M4T primer sets. For amplification with the 0.2 µl (4 pmol/µl Carla-Uni/M4T primers set was used and the reaction was adjusted to 23 µl using SDW. The following PCR cycle was used for Carla-CP/M4T and Car-F1/M4T primer sets (Gasper *et al*, 2008; Nie *et al*., 2008) respectively:

A pre-denaturation cycle for 3 min at 94 ºC, 35 amplification cycles, where denaturation was 30s at 94 ºC, annealing 30s at 50 ºC and extension 2 min at 72 ºC, were applied. A final extension cycle was used for 10 min at 72 ºC. The same PCR cycle was used for Carla-Uni/M4T except the annealing temperature was 56 ºC and extension was 30s at 72 ºC.

3.4.4. **RT-PCR for tombusviruses**

The reverse primer CIR2 was used for cDNA synthesis using the same protocol described in (3.4.1). PCR reactions were prepared as described in (3.4.2) and 0.5 µl (10 pmol/µl) of each of CIR1/CIR2 primers were used. The following PCR cycle for CIR1/CIR2 primer set was used (Koenig *et al*., 2004a&b): A pre-denaturation cycle for 3 min at 94 ºC, 35 amplification cycles (denaturation for 30s at 94 ºC, annealing for 45s at 60 ºC and extension for 2 min at 72 ºC), with a final extension cycle of 10 min at 72 ºC.

3.4.5. **RT-PCR for other groups**

The reverse primers Potex2, CPTALL3, AMVR2 and Tobamo2 for potexviruses, cucumoviruses, alfamoviruses and tobamoviruses respectively were used for cDNA synthesis as described in (3.4.1). PCR reactions prepared in (3.4.2) were applied to Potex1/Potex2, CPTALL5/CPTALL3, AMVR1/AMVR2 and Tobamo3/Tobamo2 primer sets. The following PCR cycle was used for the Potex1/Potex2 primer set (Gibbs *et al*., 1998): A pre-denaturation cycle for 3 min at 94 ºC, 35 amplification cycles (denaturation 30s/ 94 ºC, annealing 30s/56 ºC, extension 2 min /72 ºC) with a final extension cycle of 10 min at 72 ºC. The same protocol was used to detect tobamoviruses except the annealing temperature used was 50 ºC (Gibbs *et al*., 1998). To screen samples for cucumoviruses, the following cycling regime was used for PCR (Choi *et al*., 1999): one pre-denaturation cycle for 5 min at 94 ºC, 40 amplification cycles (denaturation 30s /94 ºC, annealing 30s/ 48 ºC, extension 1 min /72 ºC) with a final extension cycle of 10 min at 72 ºC. The following PCR cycle was used for AMVR1/AMVR2 primer set (Xu & Nie, 2006): one initial denaturation cycle for 5 min at 94ºC, followed by 35 cycles of 95 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 30 seconds. The final extension was at 72 ºC for 10 min.

3.5. **Agarose gel electrophoresis**

PCR products were analysed using agarose gel electrophoresis according to standard protocol (Sambrook & Russell, 2006). Agarose gels (1% w/v) were prepared using 0.5X TBE buffer (4.5 mM Tris-borate, 0.1 mM EDTA). PCR products (25 µl) were mixed with 5 µl Orange-G loading dye (2 ml 50X TAE, 0.15 g Orange G, 60 ml glycerol adjusted to 100 ml

with SDW) and loaded onto gels. Electrophoresis was performed for 1.5 h at 8 Vo/cm (21 mA). The gel was stained in 0.5 µg/ml ethidium bromide solution, visualized and photo captured using SYNGENE G: Box photo gel image and analysis system (Synoptics group, UK).

3.6. Cloning and sequencing

3.6.1. DNA recovery and purification

DNA was recovered from the gel slices using a QIAquick gel extraction kit (Qiagen, UK) according to the manufacturer's protocol with slight modification. Gel slices were weighed individually and ~3 volumes of QG buffer were added to each slice and dissolved by incubating in a 50 ºC dry block for 10 min with vortex mixing every 2-3 min throughout to increase dissolving of the gel slice(s).

Approximately, 1 gel volume of isopropanol was added; the mixture was transferred into QIAquick column and spun to bind the DNA. All centrifugation steps were performed for 1 min at 12,000 *g*. The flow-through was discarded, 500 µl QG buffer were added, centrifuged as before and the flow-through was discarded. The bound DNA was washed with 750 µl PE buffer and the column left to stand for 2-5 min and then centrifuged. The flow-through was discarded and columns spun again to remove the residual ethanol. The column was moved into a clean 1.5 ml tube and DNA was eluted by adding 30 µl nuclease-free SDW; the column was left to stand for 5 min and centrifuged. The column was discarded and purified DNA was prepared for ligation.

3.6.2. Ligation

Purified DNA was ligated into pGEM®-T easy using the vector system produced by Promega, UK, according to the manufacturer's protocol with slight adaption as follows: approximately, 2 µl of DNA were mixed with ligation reaction (1X rapid ligation buffer, 0.25 ng pGEM®-T easy vector and 0.3 U T4 DNA ligase). The final volume was adjusted to 10 µl using nuclease-free SDW. A positive control containing 2 µl of control insert DNA in the ligation reaction, and a background control including only ligation reaction (no DNA) were included. The ligation mixtures were incubated overnight at 4 ºC.

3.6.3. *E. coli* transformation

The standard bacterial transformation protocol was followed according to the manufacturer's instructions (Promega, UK) with slight modifications as follows: About 2 µl of each ligation reaction mixture including the recombinant plasmids from the previous step was added to a 1.5 ml tube on ice. In the meantime, JM109 high efficiency competent cells

(Promega, UK) were placed on ice bath until just thawed (~ 5 min). Then, cells were mixed by flicking the tube gently and 30-50 µl of the competent cells were transferred into each tube carefully. Then tubes were flicked gently and re-placed onto ice for 20 min. The cells were heat-shocked by placing tubes in a 42 ºC water bath for 45-50 s. Tubes were immediately returned to the ice bath for 2 min.

About 500 µl of 4ºC SOC medium (2% w/v Bacto® -tryptone, 0.5% w/v Bacto® -yeast extract, 0.01 M NaCl, 2.5 mM KCl, 0.1 M $MgCl_2$, 0.1 M $MgSO_4$, 0.2 M glucose) was added into the transformed cell tubes and incubated for 90 min at 37 ºC with shaking at approximately 150 rpm. Agar plates were prepared by autoclaving the agar medium (1.5% w/v agar, 1% w/v Bacto® -tryptone, 0.5% w/v Bacto® -yeast extract and 0.2 M NaCl) for 20 min at 121 ºC. The medium was allowed to cool to 50 ºC and mixed with the following ingredients: 100 µg/ml ampicillin, 0.5 mM IPTG, and 80 µg/ml X-Gal. Around 20 ml of medium was poured into 85 mm Petri dishes and left to solidify in a sterile environment at room temperature. Transformation culture (250 µl) was plated onto LB/Ampicillin/IPTG/X-Gal plates, spread by L-shaped glass rod and allowed to dry. Plates were incubated overnight at 37 ºC. Successful cloning of recombinant colonies was identified visually by blue/white colour screening followed by PCR screening.

3.6.4. Colony screening and sequencing

The recombinant colonies were confirmed by PCR amplification using the T7/SP6 primer set. Selected recombinant colonies were picked by a micropipette tip and suspended in 25 µl PCR reaction mixture and loaded into a PCR machine, programmed as follows: an initial denaturation cycle for 3 min at 94 ºC (to lyse bacterial cells), 35 cycles of 94 ºC 30 s, 52º C 30 s, 72 ºC for 2 min, and one single final extension cycle for 10 min at 72 ºC. PCR products were analyzed by agarose gel electrophoresis. The selected recombinant colonies were re-streaked onto LB/Ampicillin/IPTG/X-Gal plates before sending for sequencing (Source Bioscience Gene Service, Source Bioscience UK Limited, UK) using either T7 or SP6 forward and reverse primers in both directions.

3.7. Recombinant plasmid extraction and RFLP for potyviruses

3.7.1. Recombinant plasmid extraction

Plasmids containing recombinant DNA were purified from *E. coli* colonies using QIAprep miniprep kit (Qiagen, the Netherlands & Germany) before sending for sequencing. The miniprep was performed following the manufacturer's (Qiagen) protocol. Colonies were picked using a micropipette tip and grown in a culture of 5 ml LB medium (100 µg/ml

ampicillin, 1% w/v Bacto® -tryptone, 0.5% w/v Bacto® -yeast extract and 0.2 M NaCl) for 12-16 h at 37 ºC with shaking at 150 rpm. Fifteen ml tubes were used for growing cultures according to the manufacturer. Bacterial clumps were harvested by centrifugation (500 *g*, 10 min/4 ºC). The supernatant was discarded and the pellet was re-suspended in 250 µl buffer P1 and the tube was shaken vigorously until no cell clumps were visible. The suspension was transferred into 1.5 ml tubes, 250 µl of buffer P2 was added and mixed gently by inverting the tubes 4-6 times. Then, 350 µl of buffer N was added, mixed as before and centrifuged (12,000 *g*, 10 min). The supernatant was applied to a QIAprep spin column and centrifuged (30-60 s at 12,000 *g*) and then the flow-through was discarded. The column was washed using 0.5 ml buffer PB and centrifuged as previously. Then, the columns were washed again by adding 0.75 ml buffer PE. An additional centrifugation step (1 min, 12,000 *g*) was carried out to remove residual washing buffer. For DNA elution, the spin column was placed into a clean 1.5 ml tube, 50 µl of nuclease free SDW were added to the column, allowed to stand for 1 min and centrifuged (1 min, 12,000 *g*). The spin column was removed and the quality and quantity of nucleic acids were estimated using a spectrophotometer (Biophotometer, Eppendorf, UK).

3.7.2. **RFLP for potyviruses**

Recombinant plasmids were digested using *Eco*RI (Promega, UK) according to manufacturer's guidelines with slight adaptation. About 5 µl of plasmid DNA were mixed with reaction solution including 5 U *Eco*RI, 2 µl buffer H and the final volume was adjusted to 20 µl using nuclease-free SDW in a 0.5 ml tube. Tubes were incubated for 90 min at 37 ºC. Then, the restriction enzyme was inactivated by incubating for 15 min at 65 ºC and tubes were placed on ice. PCR products from each clone, which showed the expected fragment size, were compared by RFLP approach using *Alu*I and *Taq*I (Promega, UK) according to manufacturer's guidelines with slight adaptation. Restriction digestion was applied (1X *Alu*I buffer or *Taq*I buffer, 5 U *Alu*I or *Taq*I restriction enzymes, the final volumes adjusted to 20 µl using nuclease-free SDW) for each clone in 0.5 ml tubes. The reactions were incubated at 37 ºC and 65 ºC for 60 min, for *Alu*I and *Taq*I respectively. Then, enzymes were inactivated by heating for 5 min at 80 ºC and placed on ice subsequently. Around 4 µl of Orange-G loading dye were added to 20 ul reactions and the digested DNA analyzed using agarose gel electrophoresis.

3.8. Sequence comparison and phylogenetic analyses

The CP sequences obtained from Source Bioscience Gene Service were analyzed using the MEGA5 software system (Tamura *et al*., 2011). Sequences were compared to corresponding

sequences in GenBank for virus characterization using MEGA BLAST search. Both amplified and GenBank sequences were aligned and their amino acid sequences deduced. Neighbour-Joining phylogenetic trees were constructed using both obtained and GenBank sequences that showed the maximum identity with the sequences resulted from each sample. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were calculated based on scores above 70% of cut-off value only to support tree topology and give high confidence values for bootstrap test whereas relatedness with bootstrap values below 70% were collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site (Tamura *et al*., 2011).

3.9. **Detection of DNA viruses:**

3.9.1. **PCR for begomoviruses detection**

PCR amplification was performed using Red Hot *Taq* DNA polymerase kit (Thermo Scientific Inc., USA) and Deng A/Deng B begomovirus specific primers according to Reddy *et al*. (2005) with the following modification: DNA template (1 µl) obtained from CTAB extraction was mixed with 24 µl of PCR reaction (containing 0.625 U Red Hot DNA polymerase, 1 x reaction buffer, 1.5 mM $MgCl_2$ and mixed dNTPs (0.25 mM of each nucleotide) and the reaction was adjusted to 24 µl using SDW. A PCR routine of one cycle of pre-denaturation cycle for 2 min at 94 ºC, 35 cycles for 45 s, 30 s and 2 min at 94 ºC, 53 ºC and 72 ºC respectively, and a final extension cycle for 10 min at 72 ºC was used for amplification. PCR products were analysed by ethidium bromide gel electrophoresis, cloned and sequenced as described in (3.5) and (3.6) steps.

3.9.2. **Rolling circle amplification (RCA)**

RCA approach was performed using an Illustra Templiphi 100 Amplification Kit (GE Healthcare Limited, UK) according to manufacturer's protocol: DNA template (1 µl) obtained from CTAB extraction was mixed with 5 µl sample buffer and the mixture was heated for 3 min at 95 ºC then cooled to room temperature. About 0.2 µl of enzyme mixture (containing bacteriophage ø29 DNA polymerase, a mixture of random hexamers in 50% glycerol) was mixed with 5 µl of reaction buffer then added to DNA/sample buffer mixture. The reactants were incubated for 18-24 h at 30 ºC.

3.9.3. **RFLP of RCA product**

The following restriction enzymes were tested to cleave the repeated tandem of concatemeric molecules resulting from RCA*: Nco*I, *Sph*I, *Sac*I, *Bam*HI, *Kpn*I, *Pst*I, *Xho*I, *Eco*RI and *Sal*I (Promega, UK). About 2 µl of RCA product was added to restriction digestion mixture (containing 2 µl reaction buffer, 0.5 µl restriction enzyme, 0.2 bovine serum albumin (BSA) the final volume was adjusted to 20 µl using SDW). The reactants were incubated for 2 h at 37 ºC, then, enzyme was inactivated by incubating for 15 min at 65 ºC and tubes were placed on ice. The digested DNA fragments were analysed by gel electrophoresis as mentioned in step 4.5 using 0.6% (w/v) agarose.

3.9.4. **Cloning**

DNA fragments obtained from RFLP using *Nco*I restriction enzyme were purified from gels as described in step (3.6.1) and cloned into pGEM-3zf plasmid (Promega, UK) as follows: pGEM-3zf plasmid vector was linearized and dephosphorylated using *Nco*I endonuclease and thermal sensitive Alkaline Phosphatase (TSAP) enzymes (Promega, UK) following the manufacturer's protocol by adding about 1 µl pGEM-3zf to dephosphorylation /restriction digestion reaction (containing 2 µl multi-core reaction buffer, 1.5 *Nco*I, 2 µl TSAP and 0.2 µl BSA), the final volume was adjusted to 20 µl using SDW. The reactants were incubated for 2 h at 37 ºC, then heat inactivated for 15 min at 80 ºC. Ligation was performed using T4 (10-20 U/ µl) ligase enzyme (Promega, UK) according to manufacturer's instruction with a slight modification. To construct the recombinant plasmid, 10 µl of ligation reaction was prepared, 3 µl of purified DNA was mixed with 1 µl of digested dephosphorylated pGEM-3zf vector then 6 µl of ligation reaction (containing 1 µl 10x ligase buffer, 1 µl T4 ligase HC (10-20 U/ µl) and 4 µl SDW), then incubated overnight at 4 ºC. Transformation and clone screening was performed as in (3.6.3) and (3.6.4) with slight modification by changing the extension step into 3 min rather than 2 min.

3.9.5. **Sequencing and sequence analyses**

Recombinant colonies were processed and sent for sequencing as described in (3.6. 4). Sequencing data obtained were used to design sequencing primers referred as (TYLCV-F/TYLCV-R) in (Table 5) to sequence the gap region from DNA fragments to obtain the complete nucleotide sequence of recombinant DNA fragments. Motifs selected from forward and reverse sequences obtained from T7/SP6 sequencing were tested for self annealing, hair pin formation, and 3` complimentarity using Oligo Calc: Oligonucleotide Properties Calculator online software from Northwestern University (USA) (http://www.basic.northwestern.edu/biotools/oligocalc.html), compared to GenBank

sequences and then oligonucleotides were synthesized by Invitrogen (UK). Recombinant colonies were re-sent to Source Bioscience UK Limited (UK) for sequencing based on the synthesized oligonucleotides. Complete nucleotide sequences were analysed as described in step 3.9. Further sequence analyses were performed by comparing restriction sites for both partial and full-length sequences obtained with those sequences from the GenBank sequence database showed the highest sequence identity. The newly designed TYLCV-F/TYLCV-R primers were used later on to detect TYLCV infected samples and recombinant colonies, using the following PCR routine: one pre denaturation cycle for 2 min at 94 ºC, 35 cycles of denaturation step for 30 sec at 94 ºC, annealing step for 30 sec at 52 ºC and extension step for 2 min at 72 ºC, to test whether they are able to detect TYLCV.

3.9.6. **Construction of an infectious clone**

To obtain an infectious clone of the begomovirus, the approach previously described by Ferreira *et al*. in (2008) was used. This approach involves generating a repeated tandem fragment of begomovirus DNA to obtain the full length begomovirus genome of the right orientation, gene arrangement and appropriate starting point as follows: RCA products were partially digested by adding about 4 µl of RCA product to 18 µl restriction digestion mixture (containing 2 U *Sac*I, 2 µl reaction buffer D and volume adjusted using SDW) on ice. Restriction digestion reactants were incubated for 30 min at 37 ºC followed by inactivation step for 15 min at 80 ºC. Restriction digestion pattern was visualized by ethidium bromide gel electrophoresis using 0.6% (w/v) agarose gel. The two tandem repeated DNA fragments were recovered from the gel as described in (3.6.1). The purified DNA fragments were cloned directly into pCAMBIA-1301 binary vector (Cambia, Australia) as follows: about 3 µl pCAMBIA was cleaved by *Sac*I endonuclease mixture (containing 2 µl 10x multicore buffer, 5 U *Sac*I, 0.2 µl BSA and the final volume was adjusted to 20 µl with SDW). The reactants were incubated for 2 h at 37 ºC and then inactivated for 15 min at 80 ºC. The ratio 1:1 (insert: vector) was used for cloning by mixing 1 µl of purified DNA fragment with 1 µl of linearized pCAMBIA vector then T4 ligation was performed by mixing the ligation mixture (containing 1 µl T4 HC (10-20 U/ µl), 1 µl 10x reaction buffer and the final volume was adjusted to 10 µl using SDW). The reactants were incubated overnight at 4 ºC. Transformation into *E. coli* competent cells was performed by heat shock treatment as described in 4.5.3 using 100 µg/ ml kanamycin rather than ampicillin. Recombinant colonies were indicated by white/blue colour screening. Recombinant pCAMBIA plasmids were purified as mentioned in 4.6.1, then restriction digestion using *Sac*I was perform to confirm recombination as follows: about 2 µl

of purified recombinant pCAMBIA plasmid was mixed with restriction digestion mixture (containing 2 µl 10x multi-core buffer, 5 U *Sac*I, 0.2 µl BSA and the final volume was adjusted to 20 µl using SDW) The reactants were incubated for 2 h at 37 ºC then inactivated for 15 min at 80 ºC. Restriction digestion pattern was visualized by agarose gel (1% w/v) electrophoresis and ethidium bromide staining. Further confirmation of recombinant plasmids was performed by PCR using DengA / DengB primer set as described in (3.9.1) using 5 min pre-denaturation step at 94 ºC.

3.9.7. **Agroinoculation**

Competent cells of *Agrobacterium tumefaciens* strain LBA 4404 were prepared according to adapted protocol of Kim Schlegel (personal communication) as follows: a single colony of *Agrobacterium tumefaciens* strain LBA 4404 was grown in 10 ml liquid YEB medium (containing 0.1% w/v yeast extract, 0.5% w/v beef extract, 0.5% w/v sucrose, 2 mM $MgSO_4$, 25 µg/ ml rifampicin, 50 µg/ ml streptomycin and the final volume was adjusted to 100 ml using SDW) for 48 h at 28 ºC. The cultures were centrifuged for 20 min at 3000 *g* at 4 ºC and then the supernatant was decanted. The pellet was washed with 10 ml of glycerol (10 ml glycerol adjusted to 100 ml with SDW) (sterilized by filtration using 0.45 µm Millipore filter) then centrifuged (20 min, 3000 *g*, 4 ºC). The supernatant was decanted and the washing step was repeated. The resultant cells were re-suspended in 200 µl glycerol and transferred into 1.5 ml tubes. Cell transformation was performed using electroporation approach as follows: the bacterial cultures, the recombinant pCAMBIA plasmids and 0.1 cm electroporation cuvettes (Invitrogen, UK) were chilled on ice then 1 µl of recombinant plasmid was mixed with 200 µl of competent bacterial cells and transferred into 0.1 cm electroporation cuvettes then kept on ice for 2 min. Electric shock was performed using Gene Pulser X cell model (Bio RAD, UK) as follows: The Gene Pulser was set at 2.5 KV, 200Ω and 25 µFD and the cuvettes were placed in the Gene Pulser and an electric shock was applied to each cuvette. Then the cuvettes were placed immediately on ice and 800 µl of YEB medium (including the antibiotics) was added immediately to each cuvette and mixed using the micropipette. The mixture was transferred into a 1.5 ml tube then incubated for 3 h at 28 ºC. Liquid cultures were spread on YEB solid medium plates (including YEB liquid medium, 1.5 g agar, 25µg/ ml rifampicin, 50 µg/ ml streptomycin and 50 µg/ ml kanamycin) and incubated for 48 h at 28 ºC. *Agrobacterium* colonies were screened for recombinant plasmids by PCR using DengA / DengB primer set and re-streaked on YEB solid plates and incubated overnight at 28 ºC. Colonies harbouring recombinant pCAMBIA binary vector were re-suspended in 100 µl

SDW and inoculated into *Nicotiana benthamiana* plants at 5-6 leaf (3-4weeks) by injecting stem three times using a disposable syringe (U-100, 29 GX ½, 033X1.2mm) and needle and kept in glasshouse. Mock inoculation was performed using bacteria harbouring pCAMBIA vector only. Leaves from symptomatic plants were extracted using CTAB method as described in step (3.2). PCR amplification using DengA / DengB was performed to detect infectious clones in extracted samples. PCR products were analysed by gel electrophoresis as described in section (3.5).

CHAPTER 4: THE POTYVIRUSES

Summary

This study confirmed the occurrence of potyviruses in Iraq using molecular techniques. RT-PCR amplification using various potyvirus genus- and family-specific primer sets showed that the primer set Sprimer/M4T was the best amongst the tested sets in detecting potyvirus sequences in 175 potato and vegetable samples from Iraq. Sequence analyses revealed the occurrence of three potyviruses, namely *Bean yellow mosaic virus* (BYMV) in broad bean, *Potato virus Y* (PVY) in potato and tomato and *Zucchini yellow mosaic virus* (ZYMV) in zucchini squash. The BYMV sequences showed maximum nucleotide identity of 97% to corresponding sequences of BYMV from Japan (Acc. AB439731). Eight out of 14 PVY sequences showed 99% maximum identity to $PVY^{O: N}$ from USA (Acc. FJ643479, EF026074, DQ008213, DQ157178, EF026076 and DQ157179), Poland (Acc. EF558545), Germany (Acc. AJ890350) and Canada (Acc. AY745491 & AY745492). Other PVY sequences showed 99% maximum nucleotide identity to PVY^{NTN} GenBank sequences from the UK (Acc. EF016294), USA (Acc. FJ204166, AY884982 & EF026075), Syria (Acc. AB185833) and Vietnam (Acc. DQ925437). The ZYMV sequence showed 98% maximum nucleotide identity to the corresponding sequences from Slovakia (Acc. DQ124239) and Israel (Acc. EF062583, EF062582 & AY188994). The results obtained indicated that the potyviruses detected are likely to have been introduced to Iraq through imported planting materials.

4. Detection of potyviruses

4.1. Introduction

The genus *Potyvirus* is named after the type member PVY and is the largest genus within the family *Potyviridae.* It also represents the second largest plant virus genus, after the genus *Begomovirus* within the family *Geminiviridae*, amongst the 73 genera within other families (Gibbs *et al.*, 2008a&b). The genus currently includes 143 definitive and tentative potyvirus species. The genus *Potyvirus* is also one of the most important plant virus genera, as its members cause serious yield losses worldwide on a wide range of hosts, belonging to different plant families including the *Solanaceae*, *Cucurbitaceae* and *Leguminosae*. Members of this genus are transmitted mechanically, by aphids in a non-persistent manner and through seed (Gibbs *et al.*, 2008b; Gibbs & Ohshima, 2010).

Virions of the genus *Potyvirus* members are filamentous particles (~680-900 nm long, ~11-13 nm wide) with a helical symmetry of about 3.4 nm (Fauquet *et al*., 2005). The virus genome of this genus is monopartite and consists of one linear positive sense ssRNA molecule of about 9.7 kb size and encapsidated within a coat protein (CP) of ~30-47 kDa and 267 amino acids (aa) in length. The genome of potyviruses is organized in a single open reading frame (ORF) with a genome-linked protein (VPg), a 5` untranslated region (5`UTR) and a 3` untranslated region (3`UTR) and poly (A) tract at the 3`-end (Figure 3) (Adams *et al*., 2005).

Potato virus Y (PVY) is considered as one of the most economically important viruses that causes serious losses on the potato crop as well as other solanaceous crops such as pepper, tomato and tobacco (Singh *et al.*, 2008). PVY is highly variable and includes many strains and isolates; however three main strains, namely PVY^{O}, PVY^{N} and PVY^{C} have been identified based on their biological properties (Singh *et al.*, 2008). New strains of PVY emerged recently such as PVY^{NTN} and PVY^{NTN-NW} due to recombination (Dullemans *et al.*, 2011; Chikh Ali *et al.*, 2010; 2011; Hosseini *et al.*, 2011). In Iraq, the two strains PVY^{O} and PVY^{N} were distinguished based on their varying serological and biological properties in potato fields (Al-Ani *et al.*, 2011c).

Bean yellow mosaic virus (BYMV) is another important potyvirus and infects legumes worldwide causing serious losses (El-Muadhidi *et al.*, 2001; Mali *et al*., 2003; Makkouk *et al*., 2012). A serology-based survey conducted in 37 broad bean fields in Iraq revealed that BYMV was infecting about 80% of broad bean plants (El-Muadhidi *et al.*, 2001). BYMV has been reported to be transmitted through broad bean seed at levels of up to 17% (Mali *et al*., 2003).

Zucchini yellow mosaic virus (ZYMV) was isolated from zucchini squash for the first time in Italy in the 1980s (Brunt *et al*., 1996; Lecoq & Desbiez, 2009). Soon after, it was identified in different cucurbit-growing areas worldwide. It was suggested that ZYMV spread so rapidly due to a combination of long distance distribution via infected seed (as it was found to be seed-transmissible up to ~15%) combined with short distance transmission by aphids (Tobias *et al.*, 2008). A field study in Iraq to estimate yield losses in zucchini due to ZYMV showed that this virus caused 90-100% yield reduction in naturally infected zucchini plants (Al-Ani *et al*., 2011a). Other members of the genus *Potyvirus* considered to be amongst the most damaging viruses globally are *Turnip mosaic virus* on crucifer crops, *Lettuce mosaic virus* on lettuce, *Papaya ring spot virus* on papaya and *Plum pox virus* on stone fruit (Ha, 2007).

In this chapter, the objectives were to investigate potyviruses in potato and vegetable samples collected from fields in Iraq using molecular techniques and to obtain the first molecular sequence data such that the relatedness of these potyviruses could be compared to equivalent sequences in GenBank to gain an insight into the presence and origin of these viruses.

4.2. Results

4.2.1. Detection of potyviruses by RT-PCR

Primer screening results, using a limited number of between 10 and 20 samples, suggested that the Sprimer/M4T primer pair was the best set for screening these samples for potyvirus infection, as it detected potyviruses from the highest number of the Iraqi samples selected (Table 6); the Sprimer/M4T set detected 11 out of 20 positives, followed by the second best primer pair Pot2/Pot1 which detected 10 positives out of 20 samples. When the whole collection of potato, tomato and broad bean samples was tested with Sprimer/M4T, 54 out of 138 samples were scored as 'potyvirus-positive' showing the expected 1.6-1.8 kb RT-PCR amplified DNA fragment with this primer set (Figure 10 and Appendix 1). It is interesting to note that Pot2/Pot1 when also used on the full range of samples, actually detected potyvirus sequences in 44 out of 138 samples (Appendix 1). DNA fragments amplified by the Pot2/Pot1 set varied from 1.3-1.45 kb in size (Figure 11). Some samples showed false positives when screened by RT-PCR using both primer sets (referred to as ±) as sequencing results later on, showed that fragment amplified from those samples belong to plant genome (Table 6 and Appendix 1) . Forward primers, (namely PNIbF1, PV2I and PV2) which target the same GNNS motif as Sprimer/M4T, detected potyviruses in fewer of the tested samples, when used either with Oligo-dT or other reverse primers such as D335 and PCPR1 (Table 6). Based on positive results obtained and a detection of a wide range of potyviruses within the whole family, the Sprimer/M4T primer set was chosen to screen future batches of samples for potyvirus infection (Appendix 1).

4.2.2. Cloning

Successful cloning of the potyvirus cDNA was confirmed by visual inspection for white-coloured colonies on the selective medium and subsequent screening by PCR using T7/SP6 primers to confirm that these were indeed recombinant colonies containing the correct insert size .

Table 6: RT-PCR screening results of various genus-/family-specific potyvirus primer sets for their ability to detect potyvirus sequences in Iraqi plant samples

Primer set	product size (kb)	BB1	Cu1	Cu2	CM	LM	Cr1	Cr2	PMO	PC	PM	To23	P8	BB7	To31	To1	To32	To21	P53	P28	BB9
Pot1/Pot2	1.3-1.4	+	±	±	±	±	+	±	+	+	+	+	+	+	+	+	±	±	±	-	NS
U341/D341	0.341	NS	-	-	-	-	-	-	-	NS	-	NT	+	NS	±	-	-	+	NS	-	-
U1000/D1000	1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+	+	-	-	-	-	-	-	-
PV2I/D335	1.3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NT	-	-	-	-	-	-	-	-	-
PNIbF1/PCPR1	1-1.2	+	NS	NS	NS	NS	+	-	-	+	+	NT	NS	+	+	+	-	-	+	-	-
PNIbF5/PCPR1	1	-	-	-	-	-	-	-	-	-	-	NT	+	+	+	+	-	-	-	+	-
U335/D335	0.335	+	+	+	-	-	NS	NS	+	+	+	NT	-	-	-	-	-	-	-	-	-
PV2I/PCPR1	1-1.1*	+	NS	NS	NS	NS	+	+	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT
PV2/Oligo-dT	1.6-2.1	+	NS	NS	NS	NS	+	NS	NS	NS	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
PV2/D335	1.3	NS	NS	NS	NS	NS	+	NS	NS	NS	NS	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
PV2I/Oligo-dT	1.6-2.1	+	NS	NS	NS	NS	+	NS	NS	NS	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT
Pot2/Oigo-dT	1.6-	+	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
U1000/Oligo-	1.2*	NS	NS	NS	NS	NS	NS	NS	-	-	NS	NS	NT	NT	NT	NT	NT	NT	NT	NT	NT
PNIbF1/Oligo-	1.6-	+	-	+	+	-	+	-	+	+	-	+	NT	NT	NT	NT	NT	NT	NT	NT	NT
Sprimer/M4T	1.6-2.1	+	±	±	±	±	+	±	+	+	+	+	+	+	+	+	-	-	-	+	±

Key: NT: not tested, NS: non-specific bands, +: positive.-: negative, ±: false positive.*: expected product size. Samples; BB1, BB7 & BB9: broad bean, Cu1& Cu2: cucumber, CM: snake cucumber, LM: bottle gourd, Cr1& Cr2: zucchini, PMO: potato mosaic, PC: potato crinkle, PM: potato mottle, P8, P28 & P53: potato, To1, To23, To31&To32: tomato.

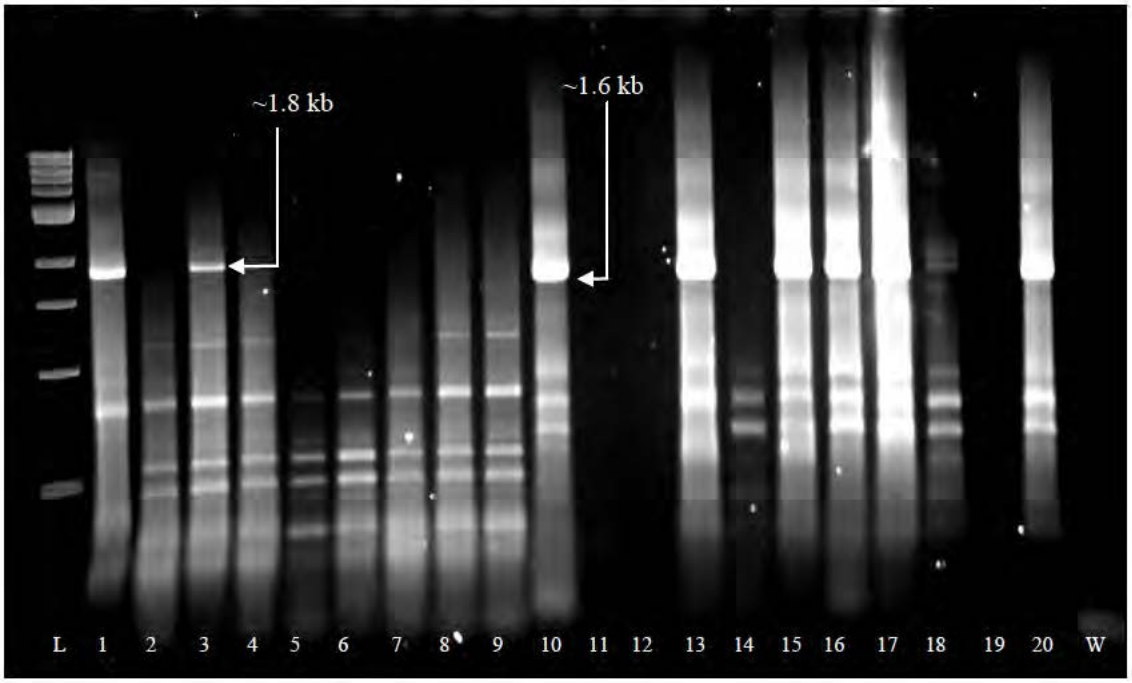


Figure 10: Screening of plant samples by RT-PCR for potyvirus sequences using the Sprimer/M4T primer set

Electrophoretic analysis of PCR products amplified from potato, tomato and broad bean samples by RT-PCR using the Sprimer/M4T primer set. The 1.6-1.8 kb bands indicate amplification of cDNA fragments representing the NIb/CP/3`UTR /poly A tail regions of the potyvirus genomes. All tested samples that contain potyviruses show the presence of a 1.6-1.8 kb sized fragment in this figure. Key to lanes: 1: P54A, 2: To36, 3: To5, 4: P40, 5: To26, 6: To6, 7: To40, 8: To34, 9: To20, 10: BB15, 11: BB16, 12: BB3, 13: BB4, 14: BB21, 15: BB22, 16:BB12, 17: BB13, 18: BB23, 19: BB24, 20: BB5, W: water control. L: 1 kb DNA ladder marker (New England Biolabs, UK)

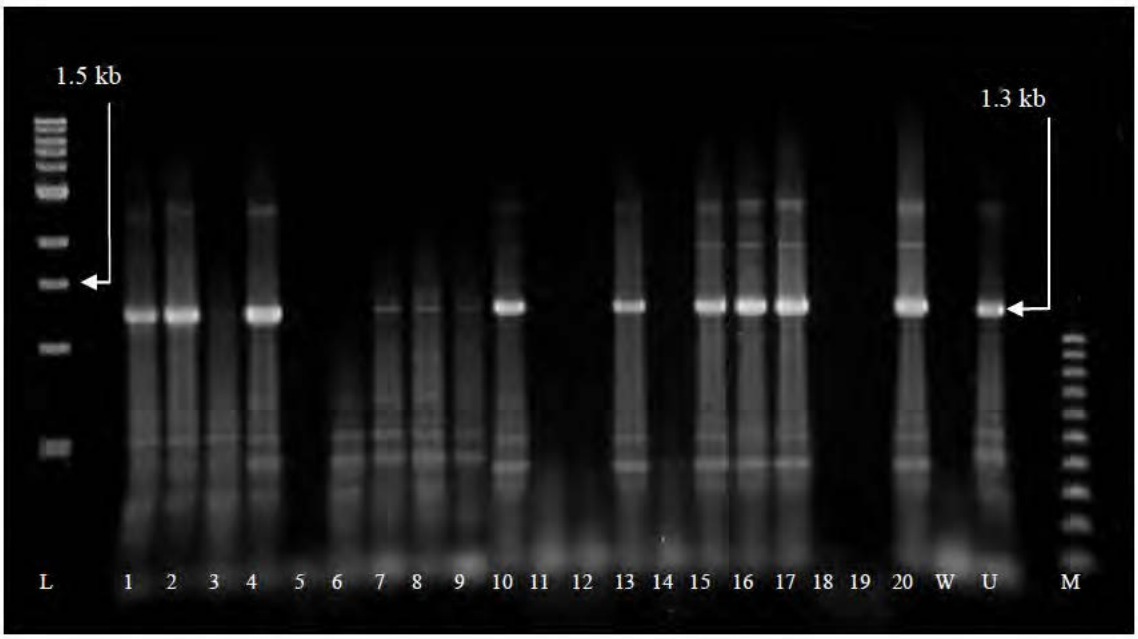


Figure 11: Screening of plant samples by RT-PCR for potyvirus sequences using the Pot2/Pot1 primer set.
Gel electrophoresis of RT-PCR products from broad bean, tomato and potato using Pot2/Pot1 primers shows ~1.3 kb amplified fragments indicating amplification of the Nlb/CP region of potyviruses. No bands are shown in 3, 5, 6, 11, 12, 14, 18 and 19 lanes which are recorded as potyvirus-free samples. Lane 7, 8 and 9 show faint bands which may indicates either weak titres of potyviruses or the presence of RT-PCR inhibitory compounds. 1: P54A, 2: P8, 3: P28, 4: To1, 5: To26, 6: To6, 7: To32, 8: To21, 9: To20, 10: BB15, 11: BB16, 12: BB3, 13: BB4, 4: BB21, 15: BB22, 16: BB12, 17: BB13, 18: BB23, 19: BB24, 20: BB5, W: water control, u: P11 positive control, L & M: 1 kb & 100 bp DNA ladder markers (New England Biolabs, UK).

Recombinant DNA fragments of ~1.8 kb were obtained and cloned from broad bean sample BB1, zucchini sample Cr1, potato samples PC and PM, and tomato samples To23 and To24 (see Appendix 1 for sample details) (Figure 12). In RFLP experiments, clones obtained from BB1, Cr1, PC, PM, To23 and To24 samples showed different patterns when digested by *Alu*I or *Taq*I restriction enzymes. These results indicated the possible occurrence of different potyviruses in these samples (Figure 13 & Figure 14A&B). Some clones isolated from the same sample showed multiple digestion patterns within the same sample (Figure 14B), and these multiple clones from the same sample which were sequenced to identify whether they represented mixed potyvirus infections or they were due to non-specific products amplified from the host plant genomes.

4.2.3. **Detection of potato and vegetable potyviruses**

Sequence comparison to the GenBank database sequences confirmed the prevalence of potyviruses in potato and vegetable samples collected from fields in Baghdad and Anbar provinces in Iraq. The potyviruses detected are described in more detail below.

4.2.3.1. **Detection of *Bean yellow mosaic virus* in broad bean**

RT-PCR results showed that the family specific primer set Sprimer/M4T amplified 1.6-1.8 kb fragments in 17 out of 23 broad bean samples (Appendix 1). Sequences of 1629-1631 bp were obtained when cloned DNA fragments amplified from broad bean samples were sequenced in both directions (Table 7). These sequences included partial NIb/CP/3`UTR of BYMV genomic regions, and showed 96-97% nucleotide identity (except for BYMV-IRQ2 sequence) to the corresponding genomic region of *Bean yellow mosaic virus* (BYMV: genus *Potyvirus;* family *Potyviridae*) sequences in the GenBank (Table 8 & Appendix 2). Analyses of the potyvirus sequences of broad bean samples confirmed that all sequenced clones represented BYMV sequences, showing the highest (90-97%) identity to a NIb/CP/3`UTR nucleotide sequence of BYMV- 90-2 from Japan (Acc. AB439731) isolated from *Vicia faba*, and lowest nt identity of 86% with other BYMVs (MB4, G1 and M11) from Japan (Acc. D28819, AB439730 & AB079886 respectively) and other isolates (BYMV-S, Masdevallia, Lisianthus, Pullman and Gladules), (Acc. U47033, AF185961, AM884180, EU144223 and X63358 respectively) from Australia, Germany, Taiwan, USA and The Netherlands respectively (Table 8). Based on aa and nt sequence alignments (Appendix 2 & Appendix 3), comparison of CP aa also showed that BYMV sequences were most similar (98%) to GenBank isolate 90-2 from Japan (Acc. AB439731) (Table 9).

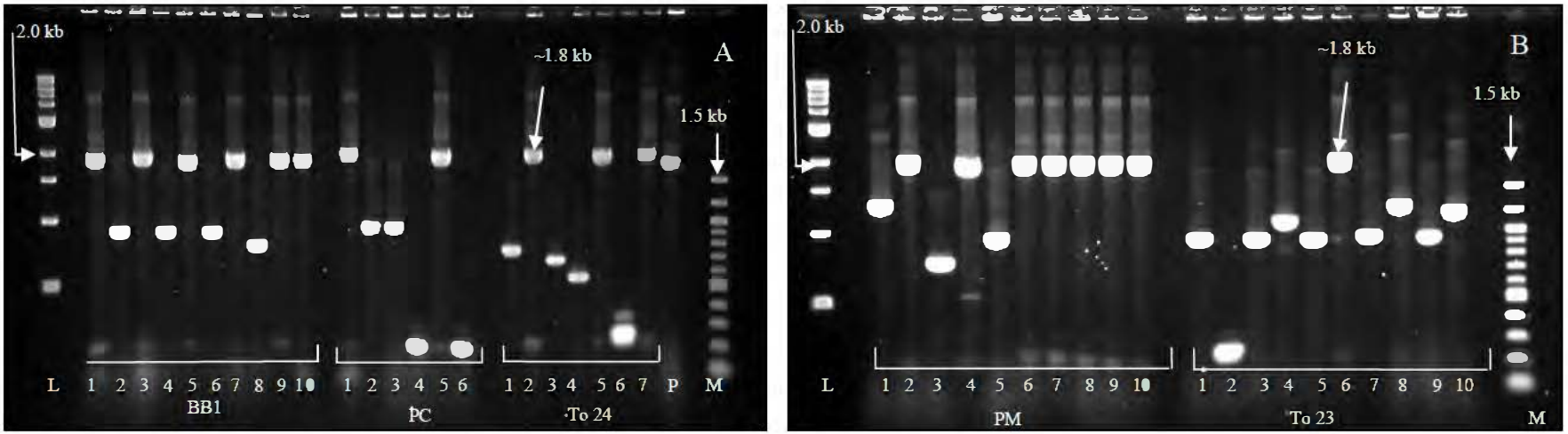


Figure 12: Screening recombinant clones for their insert size by PCR using T7/SP6 primers

Gel electrophoretic analysis of PCR products where 1.8 kb bands indicate successful cloning of ~1.6 kb NIb/CP/UTR sequences. Clones generated from plant samples: (A) broad bean BB1, potato crinkle PC, and tomato To24, (B), potato mottle PM and tomato To23 using Sprimer/M4T primers are shown. L & M refer to 1.0 kb and 100 bp DNA ladder markers, respectively (New England Biolabs, UK). P: positive control clone.

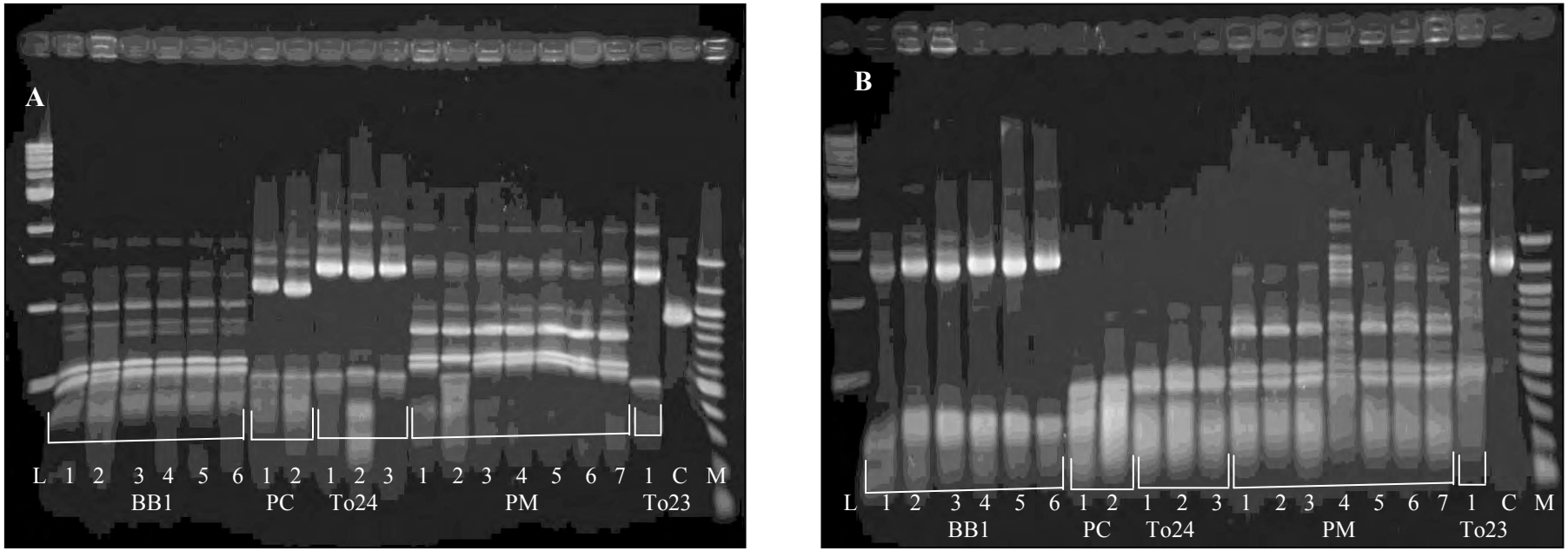


Figure 13: Restriction fragment length polymorphism analysis of recombinant clone insert DNA using *Alu*I & *Taq*I

Restriction digestion pattern using (A) *Alu*I, (B) *Taq*I of PCR products amplified from clones by T7/SP6 primers to differentiate DNA inserted. L & M are 1 kb & 100 bp DNA ladder markers (New England Biolabs, UK) respectively. The labelling in the lanes shows numbers in the upper row representing individual clones for each of the plant samples indicated in the lower row, where BB1: broad bean infected by BYMV, PC: potato infected by PVY^{NTN}, and To23 and To24 are tomato samples infected by PVY $^{NTN-NW}$, PM: potato sample infected by PVY $^{N:O}$, C: recombinant positive control clone with non viral fragment.

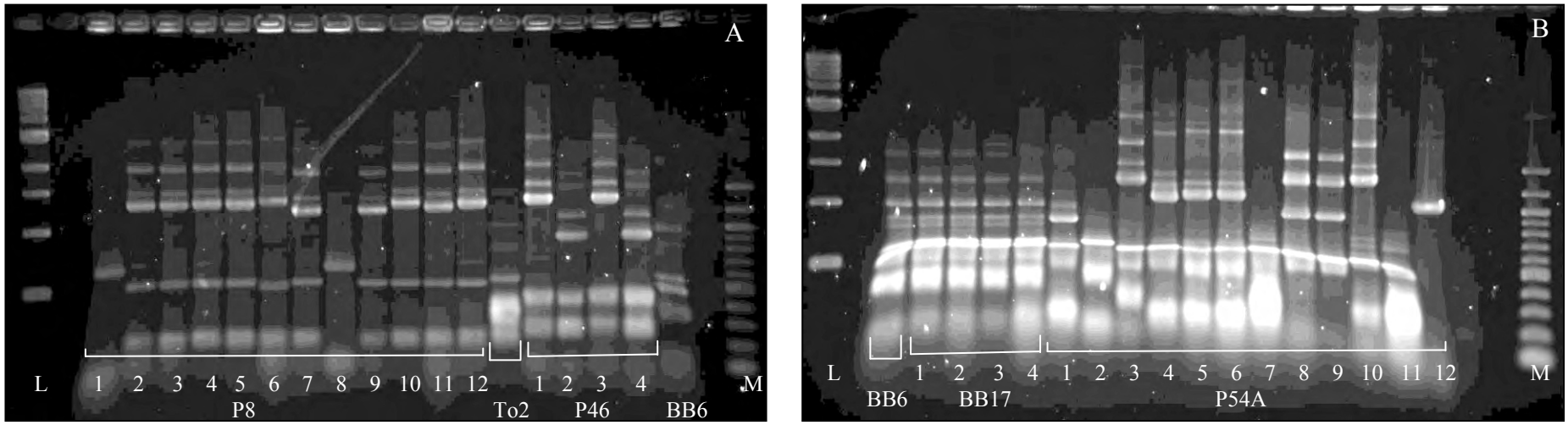


Figure 14 (A&B): Restriction fragment length polymorphism analysis of recombinant clone insert DNA using *Alu*I

Restriction digestion patterns of insert DNAs from recombinant clones amplified by T7/SP6 PCR and then cleaved with *Alu*I, where A: P8 & P46 clones represent PVY $^{N:O}$ infected potato samples, BB6 clone represents BYMV infected broad bean sample. Lanes P8-1, -8 and To2 show pattern from recombinant clones containing fragments amplified from potato, broad bean and tomato genome. B: Clones represent samples BB6 and BB17 from BYMV-infected broad bean samples and P54A from PVY-infected potato sample. Clones from sample P54A show variable band patterns due to the occurrence of more than one isolate (lanes 1, 3 and 5) or fragment from plant origin (lanes 2, 7, 8 and 11). L&M are 1 kb & 100 bp DNA ladder markers (New England Biolabs, UK) respectively.

Table 7: Geographical and host origin details of potyvirus sequence clones

Details are given for sequences amplified and cloned from selected samples, including their source details, GenBank accession numbers, length in base pairs after sequence editing, and the virus in GenBank they are most similar to.

Acc. code	Clone	Sample	Length bp	Crop	Location	Virus detected
JQ025996	BYMV-IRQ1	BB1	1629	broad bean	Baghdad	BYMV
JQ026000	BB1-1 IRQ	BB1	1629	broad bean	Baghdad	BYMV
JQ025998	BB3 IRQ	BB1	1629	broad bean	Baghdad	BYMV
JQ025997	BYMV-IRQ2	BB2	1631	broad bean	Baghdad	BYMV
JQ026021	BB3-1 IRQ	BB3	1629	broad bean	Baghdad	BYMV
JQ026002	BB3-2 IRQ	BB3	1629	broad bean	Baghdad	BYMV
JQ026001	BB5 IRQ	BB5	1630	broad bean	Baghdad	BYMV
JQ026004	BB6 IRQ	BB6	1629	broad bean	Baghdad	BYMV
JQ026003	BB2 IRQ	BB15	1629	broad bean	Baghdad	BYMV
JQ025999	BB15 IRQ	BB15	1629	broad bean	Baghdad	BYMV
JQ026005	BB17 IRQ	BB17	1629	broad bean	Baghdad	BYMV
JQ026007	PVY-O:N IRQ	PM	1771	potato	Baghdad	PVY
JQ026016	PM6 IRQ	PM	1771	potato	Baghdad	PVY
JQ026017	PM9 IRQ	PM	1770	potato	Baghdad	PVY
JQ026018	PM8 IRQ	PM	1771	potato	Baghdad	PVY
JQ026019	PM2 IRQ	PM	1771	potato	Baghdad	PVY
JQ026009	P55 IRQ	P55	1770	potato	Anbar	PVY
JQ026010	P8-2 IRQ	P8	1771	potato	Anbar	PVY
JQ026011	P8-6 IRQ	P8	1771	potato	Anbar	PVY
JQ026012	P46-1 IRQ	P46	1771	potato	Anbar	PVY
JQ026013	P54A-5 IRQ	P54A	1771	potato	Anbar	PVY
JQ026014	P54A-1 IRQ	P54A	1558	potato	Anbar	PVY
JQ026015	P54A-3 IRQ	P54A	1558	potato	Anbar	PVY
JQ026008	PVY-To IRQ	To1	1771	tomato	Baghdad	PVY
JQ026006	PVY-NTN IRQ	PC	1771	potato	Baghdad	PVY
JQ026020	ZYMV IRQ	Cr1	1038	zucchini	Baghdad	ZYMV

PMO: potato mosaic, PC: potato crinkle, PM: potato mottle

Table 8: Identity percentages of *Bean yellow mosaic virus* sequences

Nucleotide identities of BYMV sequences obtained from the GenBank database (accession number given in the first column) to sequences detected in broad bean samples from Baghdad-Iraq (sequences listed in the middle of top row). Information on the GenBank accession sequences, such as the isolate name, country of origin and crops infected are also given where known.

GenBank acc. code	Isolate name	Identity %											Location	Crop
		BB17 IRQ	BB3-1 IRQ	BB3-2 IRQ	BB1-1 IRQ	BB5 IRQ	BB2 IRQ	BB6 IRQ	BB3-IRQ	BYMV-IRQ1	BYMV-IRQ2	BB15-IRQ		
AB439731	90-2	97	94	96	97	96	97	94	96	96	90	97	Japan	broad bean
D28819	MB4	85	83	85	85	85	85	85	85	85	83	85	Japan	------
AB439730	G1	85	83	85	85	85	85	85	85	85	83	85	Japan	Gladiolus
U47033	BYMV-S	86	84	85	86	86	86	86	85	86	87	86	Australia	------
AF185961	Masdevallia	85	83	84	85	85	85	85	84	85	82	85	Germany	Masdevallia
AM884180	Lisianthus	85	84	85	86	85	86	86	85	85	84	86	Taiwan	*Eustoma russellianum*
AB079886	M11	86	84	85	86	85	86	86	85	85	84	86	Japan	------
EU144223	Pullman	86	84	86	86	85	86	84	86	86	87	86	USA	*Lupinus luteus*
X63358	Gladiolus	85	83	84	85	84	85	83	85	85	83	85	Netherlands	gladiolus

Table 9: Comparison of Iraqi BYMV sequences to each other and GenBank sequences

Nucleotide identity of partial NIb/CP/3`UTR sequences (upper right of table) and the similarity of their deduced amino acids (partial NIb/CP) (lower left of table) of Iraqi BYMV sequences from broad bean (bold lettering) and GenBank sequences. Evolutionary Divergence conducted by pairwise comparison and calculated by p-Distance method from (MEGA5) (Tamura *et al*., 2011).

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	**BYMV-IRQ1**		**87**	**97**	**98**	**99**	**98**	**94**	**97**	**98**	**94**	**98**	**84**	**83**	**96**	**84**	**84**	**84**	**83**	**84**	**84**	**83**
2	**BYMV-IRQ2**	77		**87**	**88**	**88**	**87**	**84**	**87**	**88**	**92**	**88**	**73**	**74**	**87**	**74**	**74**	**73**	**74**	**73**	**73**	**72**
3	**BB3 IRQ**	97	78		**98**	**98**	**98**	**94**	**99**	**98**	**94**	**99**	**84**	**83**	**97**	**83**	**84**	**84**	**83**	**83**	**83**	**82**
4	**BB15 IRQ**	97	78	98		**99**	**98**	**96**	**98**	**100**	**96**	**99**	**84**	**84**	**97**	**85**	**85**	**84**	**84**	**84**	**84**	**83**
5	**BB1-1 IRQ**	97	77	98	98		**99**	**95**	**98**	**99**	**95**	**99**	**85**	**84**	**97**	**84**	**84**	**85**	**84**	**84**	**84**	**83**
6	**BB5 IRQ**	96	76	96	97	97		**95**	**98**	**98**	**95**	**99**	**84**	**84**	**97**	**84**	**84**	**84**	**83**	**83**	**83**	**82**
7	**BB3-1 IRQ**	86	67	87	88	87	86		**95**	**96**	**92**	**95**	**80**	**80**	**93**	**81**	**81**	**80**	**80**	**80**	**80**	**80**
8	**BB3-2 IRQ**	96	77	99	98	97	96	87		**98**	**95**	**98**	**84**	**83**	**96**	**84**	**84**	**84**	**83**	**83**	**83**	**82**
9	**BB2 IRQ**	96	76	97	98	99	97	86	96		**96**	**99**	**84**	**84**	**97**	**85**	**85**	**84**	**84**	**84**	**84**	**83**
10	**BB6 IRQ**	89	83	90	91	90	89	79	90	89		**96**	**80**	**80**	**94**	**81**	**81**	**80**	**80**	**80**	**80**	**79**
11	**BB17 IRQ**	98	79	99	99	99	98	88	99	98	91		**84**	**84**	**98**	**84**	**84**	**84**	**84**	**84**	**84**	**83**
12	BYMV-S	94	76	94	94	93	92	83	94	93	87	95		**88**	**85**	**89**	**88**	**100**	**88**	**85**	**84**	**82**
13	VM-23	91	74	92	93	92	91	82	92	91	85	93	92		**83**	**98**	**87**	**88**	**99**	**88**	**88**	**83**
14	90-2	95	77	96	97	96	95	86	96	95	89	97	93	92		**83**	**84**	**85**	**83**	**83**	**83**	**83**
15	M11	92	74	93	93	93	92	82	92	92	86	94	92	97	92		**88**	**89**	**98**	**89**	**89**	**84**
16	Pullman	94	76	94	94	94	93	83	94	93	86	95	95	94	94	94		**88**	**87**	**84**	**84**	**83**
17	Masdevallia	94	76	94	94	93	92	83	94	93	87	95	100	92	93	92	95		**88**	**85**	**84**	**82**
18	Lisianthus	92	74	93	94	93	92	82	93	92	86	94	93	98	92	99	94	93		**89**	**88**	**83**
19	MB4	92	73	92	93	92	91	82	92	92	85	93	91	93	92	94	92	91	94		**99**	**91**
20	G1	92	74	92	93	92	92	82	92	92	85	93	91	93	92	94	92	91	94	100		**91**
21	Gladiolus	91	73	92	92	92	91	82	92	91	84	93	91	91	91	92	92	91	92	95	95	

4.2.3.2. **Detection of *Potato virus Y* in potato and tomato**

RT-PCR results revealed that the Sprimer/M4T primer set detected potyviruses in 28/ 60 potato and 7/45 tomato samples (Appendix 1). Two different sequences (1558 bp and 1771 bp excluding the poly-A tail) were obtained from the potato samples. These sequences, as well as those from the tomato samples, showed the highest nucleotide identity to the PVY sequences present in GenBank (Table 7). PVY-NTN IRQ, a sequence obtained from potato sample PC (Baghdad), showing severe mosaic and crinkle symptoms, showed maximum identity (98%) to GenBank PVY ^{NTN} -(PVY-12 (Acc. AB185833), v94249 (Acc. EF016294), PVY-VNP2 (Acc. DQ925437), HR1 (Acc. FJ204166) and 423-3 (Acc. AY884982)) sequences from Syria, UK, Vietnam and USA, respectively. Two sequences obtained from potato sample P54A (Anbar), namely P54A-5 IRQ (1777 bp) and P54A-1 IRQ (1558), also showed highest identity (96-99%) to PVY^{NTN} GenBank sequences (Table 10). In contrast, most other sequences from potato samples showed 94-99% similarity to PVY $^{N: O}$ which is a recombinant strain of PVY (Singh *et al.*, 2008). PVY N: O-IRQ (Baghdad). A sequence from another potato sample showing leaf mottling, was 97-98% identical to all PVY $^{N: O}$ from GenBank (Table 10). Two additional sequences, P55-IRQ and P8-6 IRQ, isolated from potato samples from Anbar, showed maximum identity (99%) to PVY $^{N: O}$ -(ID-1(Acc. DQ157178), PB209 (Acc. EF026076) & OR-1(Acc. DQ157179)) and PVY $^{N: O}$ -(N:O-Mb112 (Acc. AY745491) and N:O-L56(Acc. AY745492)) from USA and Canada, respectively. A comparison of the deduced aa of the partial NIb/CP region revealed that the PVY sequences isolated from potato and tomato samples also showed highest (97-99%) similarity to PVY GenBank sequences (Table 11).

The three sequences from P54A, a sample collected from potato fields in Anbar, showed the maximum amount of sequence variation. Surprisingly, based on sequence analyses, two of the sequences, P54A-1 IRQ (1558 bp) and P54A-5 IRQ (1771bp) were 99% identical to PVY^{NTN} strain (Table 11), whereas a third sequence P54A-3 (1558 bp) was 97% identical to PVY^{NTN-NW}, a novel recombinant strain from Syria (Acc. AB461453). Phylogenetic analysis confirmed the relatedness between the P54A-1 IRQ (1558 bp) and P54A-5 IRQ (1771bp) afterwards. PVY-To IRQ sequence isolated from tomato sample from Baghdad, and P46-1 IRQ sequence isolated from potato sample P46 from Anbar, showed 98% and 97% identity to $^{PVYNTN-NW}$, respectively. Results obtained from RFLP digestion of P54A clones with *Alu*I (Figure 14A&B) also revealed the presence of more than one potyvirus isolate/strain in P54A.

Table 10: Comparison of *Potato virus Y* sequences to each other and GenBank sequences

Identity percentages of PVY isolate sequences (infecting potato) obtained from GenBank database to sequences detected in potato samples from Iraq. Information on the GenBank accession sequences, such as the isolate name and country of origin are also given where known.

GenBank acc. code	GenBank PVY name	Identity % of Iraqi sequences to GenBank sequence														Location
		PVY-NTN IRQ	PVY-O:N IRQ	PVY-To IRQ	P55-1 IRQ	P8-2 IRQ	P8-6 IRQ	P46-1 IRQ	P54A-5 IRQ	P54A-1 IRQ	P54A-3 IRQ	PM2 IRQ	PM6 IRQ	PM8 IRQ	PM9 IRQ	
EF016294	V942490	98	89	93	90	89	90	92	98	98	93	89	88	89	89	UK
FJ204166	HR1	98	89	93	90	89	90	92	98	98	92	89	88	89	89	USA
AY884982	423-3	98	89	93	89	89	89	92	98	98	92	89	88	89	89	USA
AJ889866	Dec-94	98	89	93	89	89	90	92	98	98	92	89	88	89	89	Poland
EF026075	PB312	98	89	93	90	90	90	92	98	98	92	89	88	89	89	USA
AB185833	PVY-12	98	88	93	88	89	90	92	98	98	92	89	88	89	89	Syria
DQ925437	PVY-VNP2	98	89	93	90	90	90	92	99	98	92	89	88	89	89	Vietnam
FJ666337	N Nysa	96	90	93	90	90	90	92	97	96	92	90	89	90	90	Poland
GQ200836	HN2	94	93	96	93	93	93	96	95	94	95	93	91	93	93	China
FJ643479	ME173	89	98	94	98	98	98	93	90	89	94	98	96	98	98	USA
EF026074	PVY-Oz	89	98	94	98	98	98	93	90	89	94	98	96	99	99	USA
DQ008213	PN10A	89	98	94	99	98	98	94	90	89	94	99	97	99	99	USA
AJ890350	Wilga	89	98	94	98	98	98	94	90	89	94	98	96	99	99	Germany
EF558545	Wilga(N)	88	97	94	98	97	98	93	89	88	93	98	96	99	98	Poland
DQ157178	ID-1	89	98	95	99	98	99	94	90	89	94	98	96	99	99	USA
EF026076	PB209	89	98	95	99	98	99	94	90	88	94	98	96	99	99	USA
AY745491	N:O-Mb112	89	98	95	99	98	99	94	90	88	94	98	96	99	99	Canada
DQ157179	OR-1	89	98	95	99	98	99	94	90	89	94	98	96	99	99	USA
AY745492	N:O-L56	89	98	95	99	98	99	94	90	88	94	98	96	99	99	Canada

Table 11: Sequence comparison among PVY sequences

Nucleotide identity (partial NIb/CP/3`UTR)(upper right) and deduced amino acid similarity (partial NIb/CP)(Lower left) percentages comparison among PVY sequences isolated from potato and tomato samples (bold letters) and GenBank isolates. Evolutionary Divergence conducted by pairwise comparison and calculated by p-Distance method from (MEGA5) (Tamura *et al*., 2011).

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	**PVY-NTN IRQ**		**98**	**98**	**85**	**98**	**98**	**98**	**98**	**98**	**96**	**85**	**86**	**86**	**86**	**86**	**86**	**86**	**86**	**86**	**86**	**90**	**85**	**86**	**86**	**86**	**90**	**98**	**98**	**91**	**84**	**86**	**86**	**85**	**93**	**91**
2	PVY-12	97		**99**	**87**	**99**	**99**	**99**	**99**	**99**	**97**	**87**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**92**	**87**	**88**	**87**	**88**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**87**	**95**	**92**
3	HR1	97	99		**87**	**100**	**100**	**100**	**99**	**100**	**97**	**87**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**92**	**87**	**88**	**87**	**88**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**87**	**95**	**92**
4	Poland	90	93	93		**87**	**87**	**87**	**87**	**87**	**88**	**98**	**99**	**98**	**99**	**98**	**98**	**98**	**98**	**98**	**99**	**93**	**97**	**99**	**98**	**98**	**93**	**87**	**87**	**94**	**96**	**98**	**98**	**98**	**92**	**95**
5	PVY-VN/P2	96	99	99	93		**100**	**100**	**99**	**100**	**97**	**87**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**92**	**87**	**88**	**87**	**88**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**87**	**95**	**92**
6	v942490	97	99	100	93	99		**100**	**99**	**100**	**97**	**87**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**92**	**87**	**88**	**87**	**88**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**87**	**95**	**92**
7	PB312	97	99	100	93	99	99		**99**	**99**	**97**	**87**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**88**	**92**	**87**	**88**	**87**	**88**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**87**	**94**	**92**
8	12-94	96	99	99	92	99	99	99		**99**	**97**	**87**	**87**	**87**	**87**	**87**	**87**	**88**	**88**	**88**	**88**	**92**	**86**	**88**	**87**	**87**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**87**	**94**	**92**
9	423-3	96	99	100	93	99	99	99	99		**97**	**87**	**87**	**87**	**87**	**87**	**87**	**88**	**88**	**88**	**88**	**92**	**86**	**87**	**87**	**87**	**92**	**99**	**99**	**92**	**86**	**87**	**87**	**86**	**94**	**92**
10	N Nysa	96	98	98	93	97	98	98	97	97		**88**	**89**	**89**	**89**	**89**	**89**	**89**	**89**	**89**	**89**	**92**	**88**	**89**	**88**	**89**	**92**	**97**	**97**	**92**	**87**	**88**	**88**	**88**	**95**	**92**
11	**PVYO:N IRQ**	90	93	92	98	92	92	92	92	92	92		**99**	**99**	**99**	**99**	**99**	**99**	**99**	**98**	**99**	**94**	**98**	**99**	**98**	**98**	**94**	**87**	**87**	**94**	**98**	**100**	**100**	**100**	**92**	**95**
12	PN10A	91	93	93	99	93	93	93	93	93	93	99		**99**	**99**	**99**	**99**	**99**	**99**	**99**	**99**	**94**	**98**	**100**	**99**	**99**	**94**	**88**	**88**	**94**	**98**	**99**	**99**	**99**	**93**	**96**
13	PB209	90	93	93	99	93	93	93	93	93	93	98	100		**99**	**100**	**100**	**100**	**100**	**99**	**99**	**94**	**98**	**99**	**99**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**96**
14	Wilga	90	93	93	99	92	93	92	92	92	93	98	100	99		**99**	**99**	**99**	**99**	**99**	**99**	**94**	**98**	**99**	**98**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**95**
15	N:O-L56	90	93	93	99	93	93	93	93	93	93	98	100	100	99		**99**	**100**	**100**	**99**	**99**	**94**	**98**	**99**	**99**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**96**
16	N:O-Mb112	91	94	93	99	93	93	93	93	93	94	98	100	100	99	100		**100**	**100**	**99**	**99**	**94**	**98**	**99**	**99**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**96**
17	OR-1	90	93	93	99	93	93	93	93	93	93	98	100	100	99	100	100		**100**	**99**	**99**	**94**	**98**	**99**	**99**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**96**
18	ID-1	91	94	93	99	93	93	93	93	93	94	98	100	100	99	100	100	100		**99**	**99**	**94**	**98**	**99**	**99**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**96**
19	ME173	91	94	94	99	93	93	93	93	93	94	98	100	99	99	99	99	99	99		**100**	**94**	**98**	**99**	**98**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**95**
20	PVY-Oz	91	94	93	99	93	93	93	93	93	94	98	100	99	99	99	100	99	100	100		**94**	**98**	**99**	**98**	**99**	**94**	**88**	**88**	**94**	**97**	**99**	**99**	**98**	**93**	**96**
21	**PVY-To IRQ**	91	94	94	96	93	93	93	93	93	94	96	96	96	96	96	97	96	97	96	96		**93**	**94**	**93**	**94**	**99**	**92**	**92**	**98**	**92**	**94**	**94**	**93**	**96**	**98**
22	**P55 IRQ**	88	91	91	96	90	91	90	90	90	91	96	97	97	97	97	97	97	97	97	97	94		**98**	**98**	**98**	**93**	**87**	**87**	**93**	**96**	**98**	**98**	**97**	**92**	**94**
23	**PVY-Wi**	91	93	93	99	93	93	93	93	93	93	99	100	100	100	100	100	100	100	100	100	96	97		**99**	**99**	**94**	**88**	**88**	**95**	**98**	**99**	**99**	**99**	**93**	**96**
24	**P8-2 IRQ**	90	93	93	98	92	92	92	92	92	93	97	99	99	98	99	99	99	99	98	99	96	96	99		**99**	**94**	**87**	**87**	**94**	**96**	**98**	**98**	**98**	**92**	**95**
25	**P8-6 IRQ**	90	93	93	99	93	93	93	92	93	93	98	99	99	99	99	100	99	100	99	99	96	97	99	99		**94**	**88**	**88**	**94**	**97**	**99**	**98**	**98**	**93**	**95**
26	**P46-1 IRQ**	91	94	94	96	93	94	93	93	93	94	96	97	97	96	97	97	97	97	96	96	98	94	97	96	96		**92**	**92**	**98**	**92**	**94**	**94**	**94**	**97**	**99**
27	**P54A-5 IRQ**	96	99	99	94	99	99	99	99	99	98	93	94	94	93	94	94	94	94	94	94	94	91	94	93	94	94		**100**	**92**	**86**	**87**	**87**	**87**	**94**	**92**
28	**P54A-1 IRQ**	96	99	99	94	99	99	99	99	99	98	93	94	94	93	94	94	94	94	94	94	94	91	94	93	94	94	100		**92**	**86**	**87**	**87**	**87**	**94**	**92**
29	**P54A-3 IRQ**	89	91	91	93	91	91	91	90	91	91	93	94	94	94	94	94	94	94	94	94	94	91	94	93	94	94	92	92		**92**	**94**	**94**	**94**	**96**	**99**
30	**PM6 IRQ**	82	84	84	89	84	84	84	84	84	84	89	89	89	89	89	89	89	89	89	89	87	86	89	88	88	87	85	85	84		**98**	**98**	**98**	**91**	**94**
31	**PM9 IRQ**	90	93	93	98	92	92	92	92	92	93	99	99	98	98	98	99	98	99	98	99	96	96	99	97	98	96	93	93	94	89		**100**	**99**	**92**	**95**
32	**PM8 IRQ**	90	93	93	98	92	92	92	92	92	93	99	99	98	98	98	99	98	99	98	99	96	96	99	97	98	96	93	93	94	90	100		**99**	**92**	**95**
33	**PM2 IRQ**	89	92	92	97	91	92	91	91	91	92	99	98	98	98	98	98	98	98	98	98	95	95	98	97	97	96	93	93	93	89	99	99		**92**	**95**
34	HN2	93	96	96	96	96	96	96	96	96	96	96	97	97	96	97	97	97	97	97	97	97	94	97	96	96	97	96	96	94	86	96	96	95		**97**
35	SYR-II-DrH	92	94	94	97	94	94	94	94	94	94	98	98	98	98	98	98	98	98	98	98	98	95	98	97	98	98	95	95	96	88	98	98	97	97	

4.2.3.1. **Detection of *Zucchini yellow mosaic* virus in squash**

The Sprimer/M4T primer set detected potyviruses in all cucurbit samples, but only one RT-PCR product was cloned successfully from the zucchini sample Cr1 (Table 7). Sequence analyses showed that the cloned sequence (1038 bp) referred to as ZYMV IRQ shared 99% nucleotide identity to *Zucchini yellow mosaic virus* (ZYMV) isolates obtained from GenBank (Table 12). ZYMV IRQ showed the maximum identity (~ 99%) to sequences ZYMV-AG (Acc. EF062583), NAT (Acc. EF062582) and B (Acc. AY188994) from Israel and ZYMV-Kuchyna (Acc. DQ124239) isolate from Slovakia (isolated from *Cucurbita pepo*), whereas ZYMV IRQ showed less identity to other GenBank accessions (Table 13) based on nt sequence alignment comparison (Appendix 3). Based on aa and nt sequence alignment analyses (Appendix 2 & Appendix 3), comparison of deduced aa sequences of ZYMV IRQ partial NIb/CP region showed maximum similarity (99%) to ZYMV GenBank sequences from Israel and Mali (Table 13).

4.2.4. **Sequence analyses**

The Neighbor-Joining phylogenetic tree constructed from NIb/ CP/3`UTR nucleotide sequences from Iraqi and allied sequences from GenBank clustered in three groups related to their corresponding virus species (BYMV, PVY and ZYMV) within the genus *Potyvirus* (Figure 15A-D). Comparison of deduced aa sequences of partial NIb/CP regions confirmed that all sequences obtained belonged to the genus *Potyvirus* due to the presence of potyvirus conserved motifs in their genomes (Appendix 2), in addition to a good nucleotide sequence alignment. The aa alignment of partial NIb/CP regions revealed slight differences among BYMV sequences isolated from broad bean from Iraq and GenBank sequences except for the sequence BYMV-IRQ2 which showed 87% maximum aa similarity to partial NIb/CP regions of GenBank sequence 90-2 (Acc. BAA22880) from Japan (Table 9 & Appendix 2). In contrast, isolates of PVY ^{NTN} (99% maximum aa similarity to GenBank sequences SYR-111-L4 (Acc. BAJ79037) and ID155 (Acc. AEI52912) from Syria and the USA respectively, and PVY $^{N:O}$ (99% maximum aa similarity to GenBank isolate Wigla MV175 (Acc. CCE 46024) from Germany) were highly similar (Table 11 & Appendix 2). P46-1 IRQ, P54A-3 IRQ and PVY-To IRQ sequences showed variation in aa sequences among others because they are more likely to be isolates of another strain, PVY^{NTN-NW} from Syria. The ZYMV IRQ sequence (99% maximum aa similarity to GenBank sequences AG (Acc. ABL01531), B (Acc. AY188994) and NAT (ABL01532) from Israel) showed slight divergence in aa sequence with other GenBank sequences in its CP region (Appendix 2).

Table 12: Identity percentages among *Zucchini yellow mosaic virus* sequences

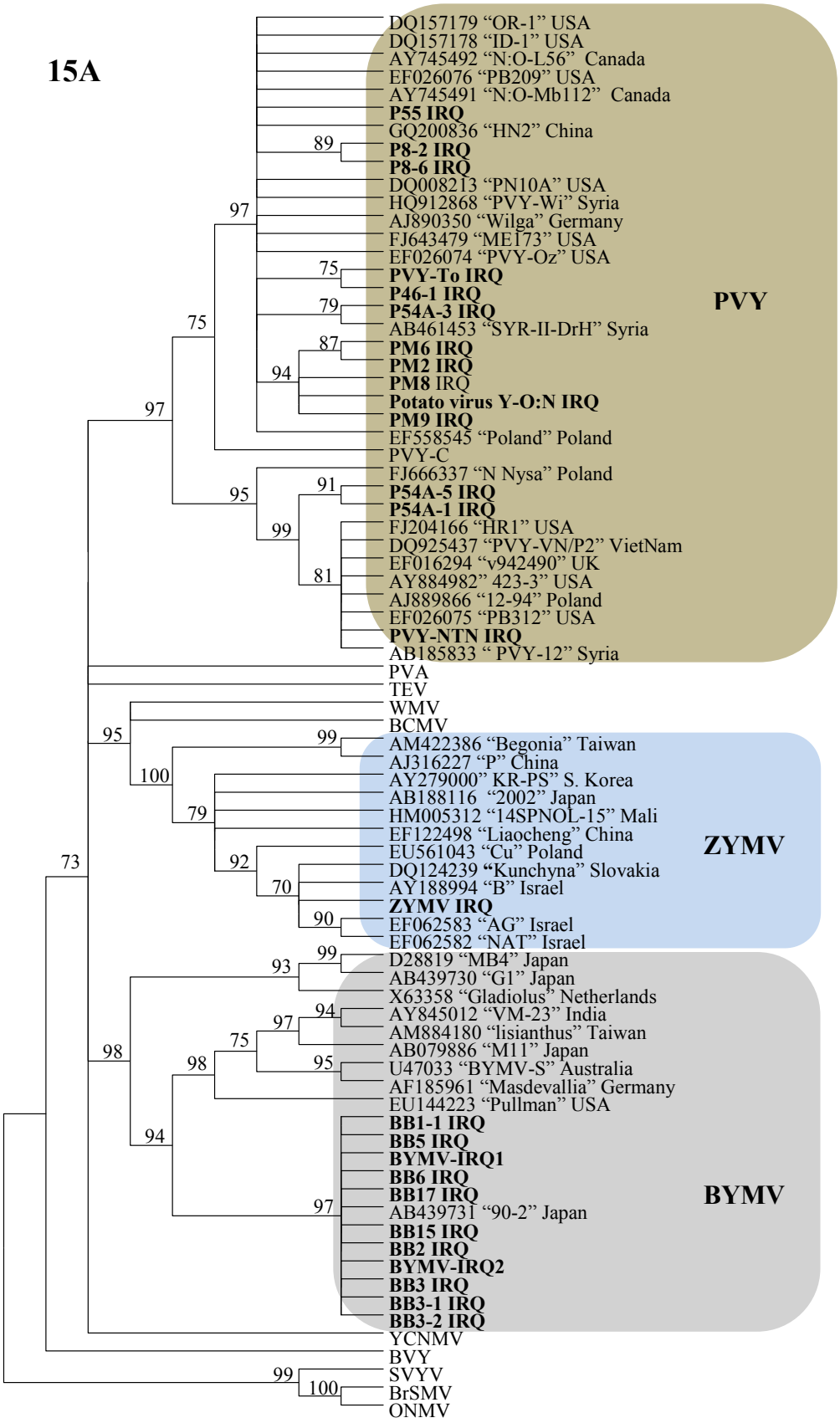
Identity percentages of ZYMV isolates obtained from the GenBank database to the ZYMV IRQ sequence detected from a cucurbit sample from Iraq. Details of the GenBank sequence such as accession code, country and crop of origin are also given.

GenBank acc. code	Isolate name	Identity%	Location	Crop
ZYMV IRQ				
EF062583	AG	99	Israel	-
EF062582	NAT	99	Israel	-
AY188994	B	99	Israel	-
DQ124239	Kunchyna	98	Slovakia	*Cucurbita pepo*
EU561043	Cu	97	Poland	cucumber
EF122498	Liaocheng	95	China	pumpkin
AM422386	begonia	95	Taiwan	*Begonia*
HM005312	14spno1-5	95	Mali	watermelon
AB188116	2002	95	Japan	cucumber
AJ316227	P	95	China	pumpkin
AY279000	KR-PS	93	South Korea	*Cucurbita moschata*

Table 13: Sequence comparison percentages among ZYMV isolates

Nucleotide identity (partial NIb/CP/3`UTR) (upper right) and deduced amino acid similarity (partial NIb/CP) (lower left) percentages comparison between ZYMV IRQ sequence isolated from Cr1 (bold letter), a zucchini sample and GenBank isolates. Evolutionary Divergence conducted by pairwise comparison and calculated by p-Distance method from (MEGA5) (Tamura *et al*., 2011).

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	**ZYMV IRQ**		**99**	**99**	**99**	**99**	**97**	**94**	**94**	**92**	**94**	**94**	**94**	**52**	**53**
2	Kunchyna	98		**99**	**99**	**99**	**97**	**94**	**94**	**92**	**94**	**94**	**94**	**52**	**53**
3	AG	99	98		**100**	**99**	**97**	**95**	**95**	**92**	**94**	**94**	**95**	**52**	**53**
4	NAT	99	98	100		**100**	**97**	**95**	**95**	**92**	**95**	**95**	**95**	**52**	**53**
5	B strain	99	98	100	100		**97**	**95**	**95**	**92**	**95**	**95**	**95**	**52**	**52**
6	Cu	98	98	98	98	98		**94**	**95**	**92**	**94**	**94**	**94**	**51**	**52**
7	Begonia	97	97	98	98	98	97		**93**	**91**	**93**	**93**	**99**	**53**	**52**
8	Liaocheng	98	98	98	98	98	98	97		**93**	**97**	**97**	**93**	**52**	**53**
9	KR-PS	97	97	97	97	97	96	97	97		**93**	**93**	**91**	**53**	**52**
10	2002	98	98	98	98	98	98	97	98	97		**96**	**94**	**53**	**53**
11	14SPNOL 15	99	99	99	99	99	99	98	99	98	99		**94**	**53**	**52**
12	P	97	97	98	98	98	97	100	97	97	97	98		**53**	**53**
13	*Bean common mosaic virus*	33	33	33	33	33	33	33	33	33	33	33	33		**61**
14	*Watermelon mosaic virus*	34	34	34	34	34	34	35	34	34	34	34	35	34	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P54A-3 IRQ
AB461453 "SYR-II-DrH" Syria
PM6 IRQ
PM2 IRQ
PM8 IRQ
Potato virus Y-O:N IRQ
PM9 IRQ
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PVY-C
FJ666337 "N Nysa" Poland
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PVY-NTN IRQ
AB185833 " PVY-12" Syria
PVY
PVA
TEV
WMV
BCMV
AM422386 "Begonia" Taiwan
AJ316227 "P" China
AY279000" KR-PS" S. Korea
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HM005312 "14SPNOL-15" Mali
EF122498 "Liaocheng" China
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AY188994 "B" Israel
ZYMV IRQ
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ZYMV
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U47033 "BYMV-S" Australia
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BVY
SVYV
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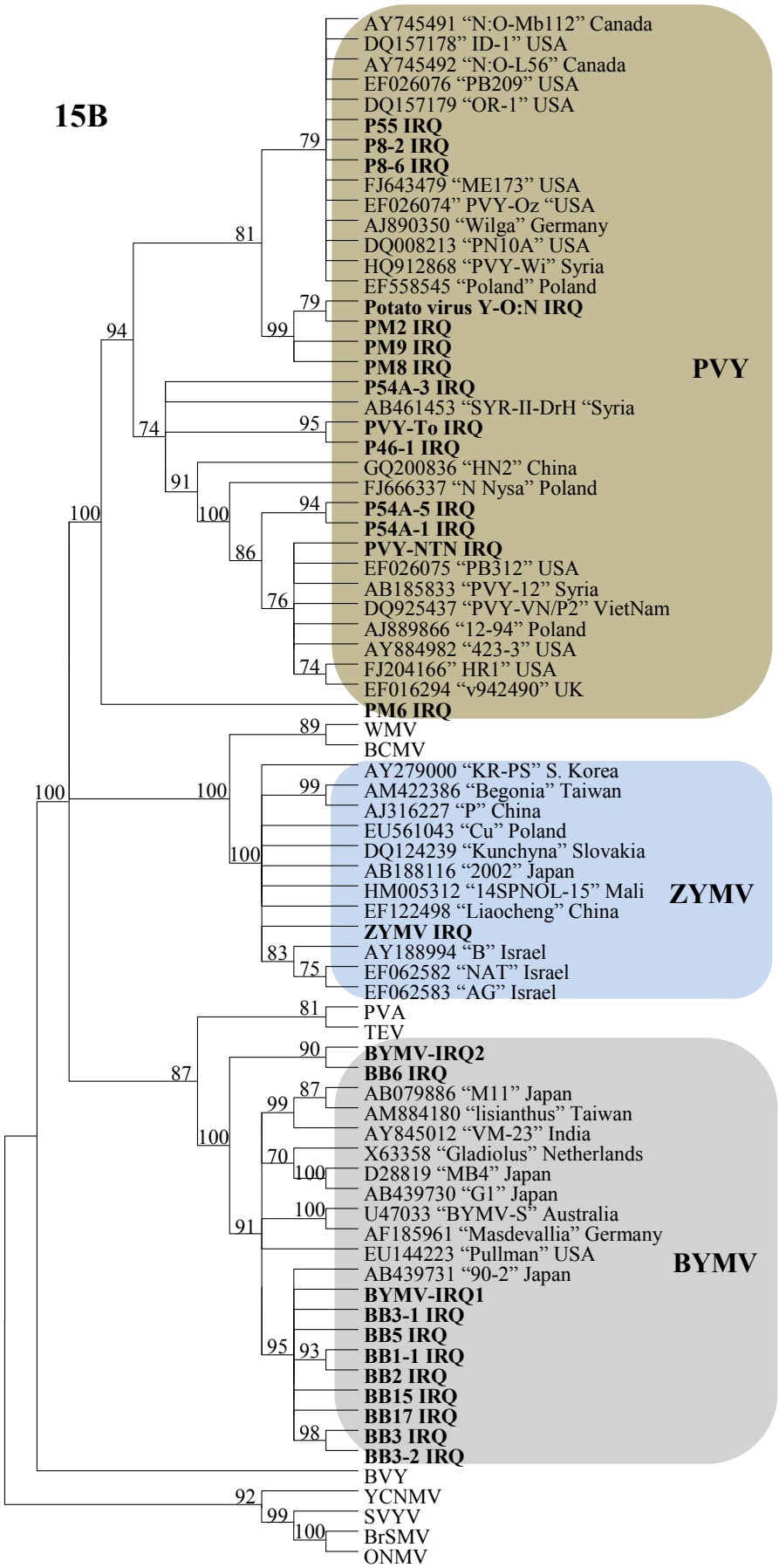
ONMV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WMV
BCMV
AY279000 "KR-PS" S. Korea
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EF122498 "Liaocheng" China
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PVA
TEV
BYMV-IRQ2
BB6 IRQ
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X63358 "Gladiolus" Netherlands
D28819 "MB4" Japan
AB439730 "G1" Japan
U47033 "BYMV-S" Australia
AF185961 "Masdevallia" Germany
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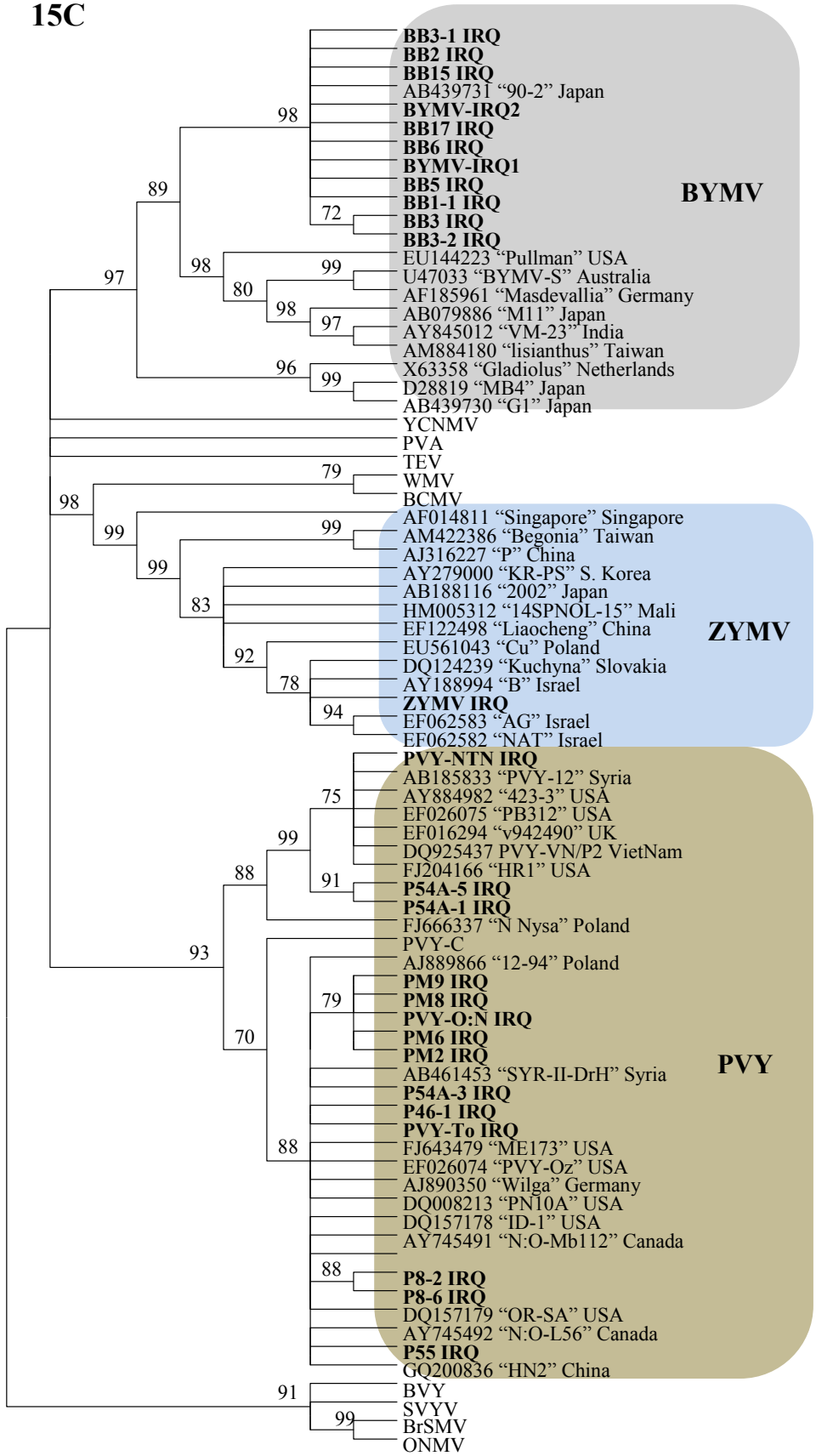
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TEV
WMV
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DQ925437 PVY-VN/P2 VietNam
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P54A-1 IRQ
FJ666337 "N Nysa" Poland
PVY-C
AJ889866 "12-94" Poland
PM9 IRQ
PM8 IRQ
PVY-O:N IRQ
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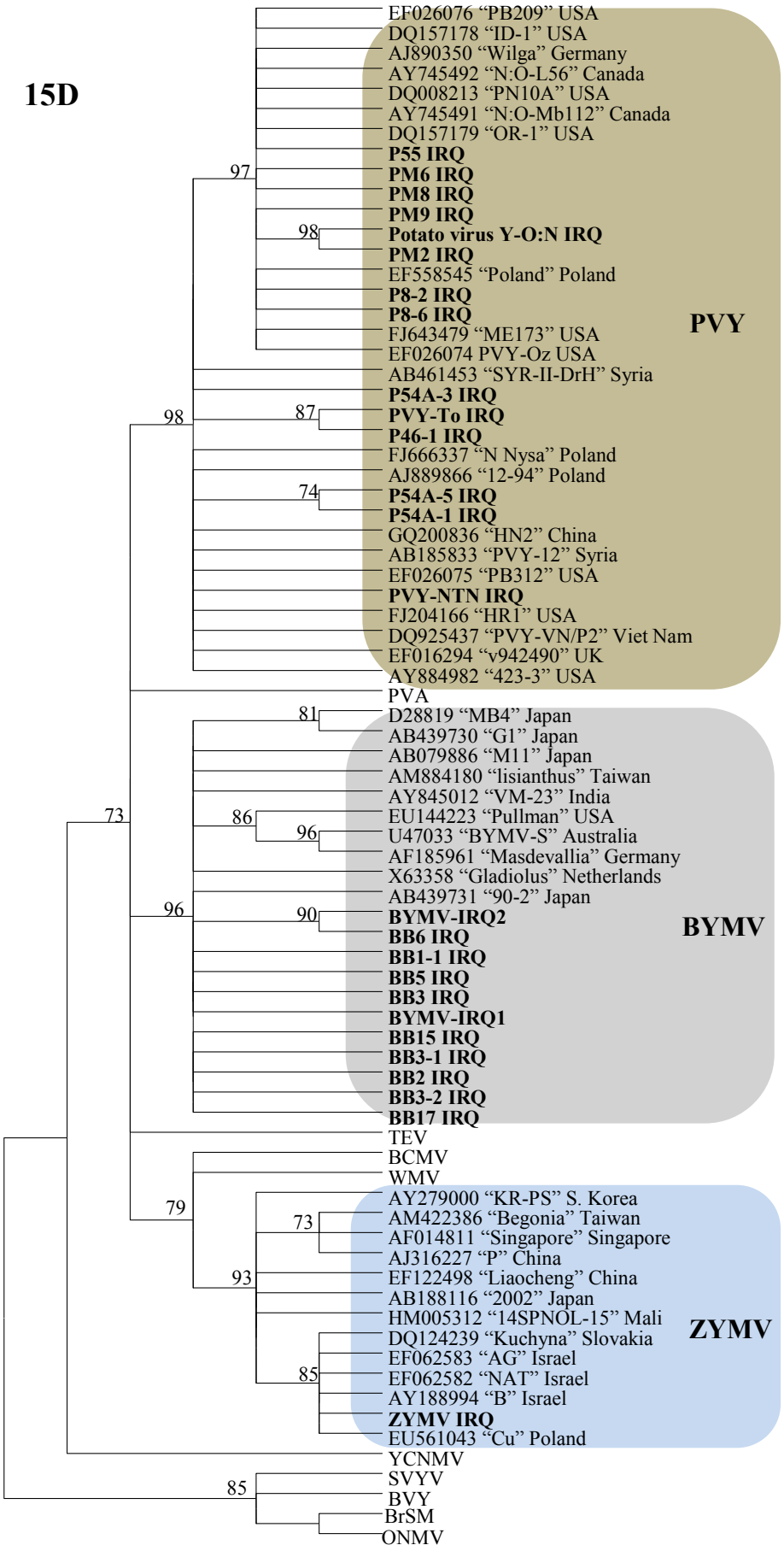
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Potato virus Y-O:N IRQ
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BYMV-IRQ1
BB15 IRQ
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EF062582 "NAT" Israel
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ZYMV IRQ
EU561043 "Cu" Poland
ZYMV
YCNMV
SVYV
BVY
BrSM
ONMV
97
98
87
74
81
86
96
90
73
96
79
73
93
85
98
85

Figure 15(A-D): Evolutionary relationships of potyviruses sequences found in potato and vegetables from Iraqi samples

Phylogenetic topology shows Neighbor-Joining tree based on A: partial NIb (GNNS motif) /CP/3`UTR regions nucleotide sequences, B: partial NIb (GNNS motif) /CP amino acid sequences, C: CP region nucleotide sequences and D: partial NIb (GNNS motif) region nucleotide sequences of four groups of Iraqi potyvirus isolates (bold letters) and related sequences of potyvirus sequences from GenBank referred as (accession number "isolate name" geographical location) for each isolate. Other selected sequences from the family *Potyviridae* with corresponding genera were included as a comparison. *Potato virus A* (PVA), *Watermelon mosaic virus* (WMV), *Tobacco etch virus* (TEV), *Bean common mosaic virus* (BCMV) are members of the genus *Potyvirus*. *Oat necrotic mottle virus* (ONMV), *Brome streak mosaic virus* (BStMV), *Blackberry virus Y* (BVY), *Yam chlorotic necrotic mosaic virus* (YCNMV) the genus and *Squash vein yellowing virus* (SVYV) are members of the genera *Rymovirus*, *Tritimovirus*, *Brambyvirus*, *Macluravirus* and *Ipomovirus*, respectively.

The Neighbor-Joining phylogenetic tree based on nucleotide sequences of NIb/CP/3`UTR grouped virus sequences into three clusters according to virus species. Sequences within the BYMV node clustered into four clades which indicated that sequences obtained from Iraqi broad bean samples were isolates of BYMV (Figure 15 A-D). All sequences isolated from Iraqi broad bean samples were clustered into the same clade with BYMV-90-2 from Japan, at 97% maximum identity of NIb/CP/3`UTR nucleotide sequences. Whereas other related BYMV isolates from GenBank, were clustered into different clades (Figure 15A & C).However, trees constructed from deduced aa sequences as well as those from the NIb region did not show this relationship between the Iraqi and Japanese sequences (Figure 15B & D). Sequences obtained from Iraqi potato and tomato samples diverged into two different clades within PVY node according to their strains, supported by a 97% bootstrap value. Most sequences were clustered within PVY $^{N:\ O}$, including sequences that showed similarity to PVY^{NTN-NW} (Figure 15A-C) and phylogenetic topology even when NIb/CP sequences and CP aa sequences were compared separately.

ZYMV IRQ sequence from zucchini clustered into the same clade with other GenBank sequences of ZYMV; namely, B, NAT, AG isolates from Israel and Kuchyna isolate from Slovakia, respectively. Isolate Cu from Poland diverged from ZYMV IRQ supported by low bootstrap value at 70%. Other GenBank sequences from South Korea, Mali, Japan and China were supported by high bootstrap score of 92% (Figure 15A). Similar results were obtained when Neighbor-Joining phylogenetic tree based on NIb/CP region were constructed (Figure 15C & D). The lack of distinct divergence among ZYMV IRQ and other GenBank sequences (Figure 15B) may indicate that the changes in nucleotides did not affect aa composition (Appendix 2).

4.3. **Discussion**

A total of 175 samples collected from Iraqi fields in Baghdad and Anbar provinces were tested for potyviruses using RT-PCR amplification followed by sequence analyses of selected products. Results obtained confirmed the occurrence of potyviruses in Iraqi potato, tomato, cucurbits and broad bean. This study provided the first molecular characterization of potyviruses in Iraq.

In RT-PCR experiments, different genus- and family-specific primer sets were screened to detect potyviruses in Iraqi samples. Among the primers tested, the Sprimer/ M4T

primer set was the most efficient when detected potyvirus infection in the highest number (54 out of the 138) of samples tested. The Sprimer/M4T was chosen based on sequencing data, the number of samples detected to be positive for potyviruses and that it amplified the full-length CP region making it more useful for phylogenetic comparisons (Gibbs *et al.*, 2003). In addition, this primer set detects a wider range of members within the family *Potyviridae* in a single reaction (Gibbs *et al.*, 2003; James *et al.*, 2006). This primer set, therefore, was chosen to screen other tomato, eggplant and cowpea samples for potyvirus indexing.

The primer set Sprimer/M4T was designed to be specific by targeting the GNNSGQP amino acid sequence in the GNNSGQPSTVVDN highly conserved motif in the NIb coding region. Hence the Sprimer/M4T is suitable for detection of viruses within the family *Potyviridae*, due to the presence of conserved motif GNNS and poly-A tail at 3`end in their genomes (Gibbs *et al.*, 2003; James *et al.*, 2006; Ha *et al.*, 2008b).

The Pot2/Pot1 set was less efficient in detecting potyviruses in the Iraqi samples and this primer set was designed to target the conserved motifs YCDADGS and WCIEN in the NIb and CP encoding regions of genomes of the genus *Potyvirus* (Colinet *et al*., 1994). The difference between the two sets in detecting potyviruses is likely to be due to differences in the targeted motifs and their frequency in the potyvirus genomes. Zheng *et al*. (2008a) indicated that GNNSGQ was ranked the seventh most conserved motif in the genus *Potyvirus*, when a comparison of consensus sequences was made using GenBank sequences. YCDADGS was not included in the analyses because of its much higher variability in sequence. The WCIEN motif, which is targeted by the reverse primer Pot1, was ranked 12th based on its frequency in the CP region of potyviruses (Zheng *et al*., 2008a). The Pot2/Pot1 primer set was therefore less efficient than the Sprimer/M4T set due to a low frequency of these motifs in genomes of potyviruses from Iraq and it remains possible that the lower detection was due to false negative reactions. However, the former explanation seems more probable as the PNIbF1/PCPR1 primer set was also less efficient at detecting potyviruses in Iraqi samples, and within this set the reverse primer PCPR1 also targets the WCIEN motif.

Besides, these degenerate primers may not cover all nucleotide variation in the targeted conserved motifs which may cause primer mismatch and hence false negative results, i.e. Sprimer, the forward family-specific potyvirus primer includes more degenerate nucleotides than PNIbF1 and PV2I (for example replacing B in the third position from 5' terminal with N may give an advantage in annealing more variable nucleotides in this position) (Table 5) (Zheng *et al.*, 2008a; 2010). Similarly, that could be applied to PV2I

forward primer, by replacing the Inosine, a nucleotide derivative that has a high affinity to Adenine but it bonds poorly to Guanine and Thymine, in the third and 15th positions from 5' terminal with N in Sprimer (Mackenzie *et al.*, 1998; Chen *et al*., 2001a; Gibbs *et al*., 2003). The GQPSTVVDN motif in NIb encoding region was ranked 9th among other conserved motifs (Zheng *et al*., 2008a), and hence the PNIbF5/ PCPR1primer set which targets this motif may be less efficient at amplifying potyvirus sequences due to the low frequency of these motifs and primer mismatch as mentioned previously. In addition, primers designed to target WCIENG motif have been reported to show non-specific reactions and false positive results (Zheng et al., 2010). In this study, two samples (Ca1 and Cr1), which generated 1.3 kb bands using the Pot2/Pot1 primer set, were in fact false positive results, as the sequences cloned turned out to share most identity (97-98%) to plant genome sequences. Thus, non-specific reaction may result in false detection of potyviruses in non-infected samples using the Pot2/Pot1primer set or primers derived from WCIENG or YCDADGS motifs (Zheng et al., 2010). Degenerate primers could also hybridize non targeted sites due to the low annealing temperature. Thus, PCR products could be contaminated by non-specific PCR product of different molecular weight when using degenerate primers (Chen et al., 2001a). Some potyviruses include A-rich sites in their genomes which may result in amplifying lower molecular weight fragments of about 0.6-1.0 kb (Gibbs & Mackenzie, 1997; Gibbs et al., 2003) as unspecific bands were easily detectable when the PCR products were visualized by ethidium bromide electrophoresis (Figure 10. The 0.6-1.0 kb and low molecular weight non-specific fragments could be highly competitive for PCR reagents and hence lead to false negative results. Confirmation of the PCR products by sequencing was therefore essential for diagnosing potyviruses accurately, for differentiating specific and non-specific amplified fragments.

RFLP was used on cloned PCR products for discriminating viruses (Figure 13 &Figure 14). RFLP approach was used to differentiate among the clones of the same and different samples, which enabled to select the desired clones for sequencing as well as to identify mixed infections (Marie-Jeanne *et al.*, 2000; Gibbs *et al.*, 2003).

A high rate of mutation is reported to occur in RNA viruses due to the low fidelity of RNA replication enzymes, insertion and deletion of RNA fragments, in addition to recombination, which are all common in members of the family *Potyviridae* (Malinowski, 2005; Gibbs & Ohshima, 2010). Mutation could also happen during cDNA synthesis as the reverse transcriptase enzyme shows no proof-reading activity (Kolb *et al.*, 2008). All the above

factors will make mutation of the conserved motifs in potyviruses more likely, and presumably these motifs persist as they are essential for functional virus replication. Carrying out RT-PCR screening with more than one primer set, e.g. a genus-specific primer set in addition to a family-specific *Potyviridae* primer sets would assist the identification of false negatives.

RT-PCR and sequence analyses revealed the occurrence of three potyviruses on potato and vegetable samples collected from fields in Baghdad and Anbar provinces. BYMV was detected in broad bean. BYMV sequences isolated showed 100% and 99% maximum nt identity and aa similarity when compared to each other (Table 9). All sequences obtained from broad bean samples showed 99% and 97% maximum nt identity and aa similarity respectively, to 90-2 Japanese BYMV isolate from broad bean. Phylogenetic and sequence analysis confirmed high similarity when all sequences from Iraq clustered alongside 90-2 isolate, suggesting that BYMV from Iraq and Japan have a common origin. BYMV may have been introduced to Iraq within legume seeds, as BYMV is seed-transmissible through broad bean by up to 3% (El-Muadhidi *et al*., 2001; Makkouk *et al*., 2012) or in certain broad bean cultivars up to 17% (Mali *et al.*, 2003). BYMV-IRQ2, a sequence obtained from BB2 broad bean sample which reacted negatively when screened by Pot2/Pot1, but was positive using the Sprimer/M4T set, showed 96% similarity in the MEGA BLAST analyses of the CP nucleotide sequences. These results indicated that this is not a new strain of BYMV as the % CP nucleotide identities are greater than 92% required by the ICTV for classifying new potyvirus strains (Fauquet *et al.*, 2005).

PVY was another potyvirus detected in potato and tomato samples collected from fields in Baghdad and Anbar provinces. PVY sequences isolated showed 100% nt identity and aa similarity when compared to each other (Table 11). Amongst PVY, three recombinant strains, namely, PVY ^{NTN}, PVY $^{NTN-NW}$ and PVY $^{N:O}$ were identified by phylogenetic and sequence analyses. Phylogenetic analyses grouped most PVY sequences with PVY $^{N:O}$ from GenBank, which indicates that this recombinant strain is more frequent in Iraq than PVY ^{NTN}, the necrotic tuber strain, in potato and tomato samples. The Neighbor-Joining phylogenetic tree based on NIb/CP aa sequences revealed PM6 IRQ diverged in distinct clade from other PVY sequences, supported by 100% bootstrap value (Figure 15 B), while other Neighbor-Joining trees based on nt sequences did not show PM6 IRQ divergence. This sequence showed 89% maximum aa similarity to PVY sequences compared to 99% maximum nt identity. Based on

sequence analyses and phylogeny, unexpected results were obtained from the three sequences isolated from the potato sample from Anbar P54A. Both P54A-1 IRQ and P54A-3 IRQ were 1558 bp in length but shown they belong to different strains when showed 99% and 97% maximum nt identities to PVY^{NTN} and PVY^{NTN-NW}, respectively and were 93% identical to each others. Whereas P54A-1 IRQ shown 98% similarity to P54A-5 IRQ (1771bp) the third P54A IRQ sequence. NJ phylogenetic analyses confirmed the relatedness when grouped P54A-1 IRQ and P54A-5 IRQ together within PVY^{NTN} clade and diverged P54A-3 IRQ separately. Regardless of sequence length, it is possible that both P54A-1 (1558) and P54A-5 (1771) were originated in Iraq, whereas P54A-3 was from Syrian origin when grouped to SYR-II-DrH (Acc No. AB46145) from Syria, based on NJ phylogenetic tree (Figure 15A).

The high identity percent to GenBank sequences alongside the phylogenetic analyses suggest that PVY isolated have been introduced to Iraq from other countries in the recent past. The possible route is through infected potato tubers imported from neighbouring countries. The tuber origin of some PVY infected potato samples was from tubers imported from Syria, as local potato growers prefer to cultivate low-priced potato seed. In addition, PVY $^{NTN-NW}$ a regional strain from Syria was detected in some potato (P46 and P54A-3 from Anbar) and tomato (To23 from Baghdad) samples. PVY $^{NTN-NW}$ a newly described recombinant strain is a high prevalence and locally emerged in Syria between 2002 and 2007 (Chikh Ali *et al.*, 2010). PVY $^{NTN-NW}$ detection in samples collected from Iraq, high identity percent of PVY sequences to GenBank sequences and the origin of potato tubers grown in Iraq, therefore, support that PVY has been introduced to Iraq. To confirm the origin of PVY in Iraq, further survey studies are required to investigate PVY in potato and other crops (including weeds), using molecular based approaches, as serological approaches (ELISA) cannot differentiate between recombinant and non-recombinant strains/isolates (Nie and Singh, 2003; Rigotti & Gugerli, 2007). The survey should be expanded to include more potato growing fields in Iraq, a large number of samples and different potato growing seasons.

The third characterized potyvirus was ZYMV. The ZYMV IRQ sequence obtained from the Cr1 cucurbit sample from Baghdad showed (99%) maximum nt identity and aa similarity (99%) to ZYMV GenBank sequences from Israel. Phylogenetic analyses grouped the ZYMV IRQ with ZYMV GenBank sequences from Israel, in addition to sequences from Poland and Slovakia in the same clade which indicated that they are closely related (Figure 15 A-D). ZYMV may have been introduced into Iraq through seed, as it is seed-transmitted up to ~ 15% (Tobias *et al.*, 2008). Translation of the nucleotide sequences into aa enabled the

identification of potyvirus-conserved motifs, namely GNNS in the NIb region and MVWCIEN and QMKAAA in the CP region. Presence of these motifs in all sequences amplified by Sprimer/M4T confirmed that these sequences belong to the genus *Potyvirus* (Zheng *et al.*, 2008a).

It is likely that these viruses were introduced into Iraq through infected planting materials from other countries through international trading (Mali *et al.*, 2003; Gibbs *et al*. 2008; Tobias *et al*. 2008). So, it is critical to use rapid sensitive techniques to test for potyviruses in imported planting material. Promising techniques for this would be nucleic acid based (like RT-PCR) as these have been used successfully to screen samples on a large scale, e.g. vegetables in Korea (Lee *et al*., 2011), for similar quarantine purposes. However, a number of limitations should be addressed first before molecular based approaches can be applied in Iraqi quarantine laboratories. Due to the lack of adequate expertise, long term training programmes are required to train staff to perform molecular techniques. The expensive reagents and equipments will add extra costs to the testing process (Tomlinson *et al.*, 2012) which may add unacceptably high extra costs to imported plant material desired for cultivation purposes. The high value of planting material imported, therefore, may impose farmers of low income to use uncertified plant material for cultivation, which will be a primary source for virus spread. Loop-mediated isothermal amplification (LAMP) is also a promising nucleic acid based approach that has been applied successfully for the detection of potyviruses (Nie, 2005, Tomlinson *et al.*, 2012). LAMP involves specific amplification of target nucleic acid sequences without thermal cycling (Notomi *et al*., 2000; Tomlinson *et al.*, 2012). The advantage of this approach is that it does not require expensive equipments such as a thermal cycling device and it is rapid, sensitive and specific (Tomlinson *et al.*, 2012). Due to its simplicity and flexibility, it is possible the LAMP technique could be optimized to test imported plant material in Iraq. By combining various group specific primers, LAMP can be improved to detect a wide range of potyviruses in a single reaction. It will enable large scale screening of samples for potyvirus indexing in a short time of period and thus lead to reduced costs.

CHAPTER 5: THE BEGOMOVIRUSES

Summary

Begomoviruses were indicated to be present in potato and vegetable samples collected from fields in Iraq by PCR using genus-specific primers as well as rolling circle amplification (RCA). Sequencing of PCR and RCA products revealed the occurrence of *Tomato yellow leaf curl virus* (TYLCV) in tomato samples collected from Baghdad, Iraq. Analysis of the full-length sequence of this TYLCV sequence from Iraq (TYLCV IRQ) showed that it shared its maximum identity (99%) to GenBank TYLCV sequences from the Reunion Islands (Acc. AM409201), Spain (Acc. AJ489258) and Mauritius (Acc. HM448447). Phylogenetic analyses grouped TYLCV IRQ separately from other GenBank sequences within TYLCV group, which may indicate a distinct origin for TYLCV IRQ. Analyses of deduced amino acid sequences indicated the presence of six open reading frames (ORFs) in TYLCV IRQ, which is identical to known TYLCV isolates. A pair of primers (TYLCV-F/TYLCV-R) designed to detect TYLCV IRQ, successfully amplified a 1.7 kb fragment containing the C4, V2, V1 and C2 regions. Trials to construct an infectious clone from TYLCV IRQ using partial digestion of the viral genome did not succeed unfortunately. This was thought to be due to multiple insertion of the repeated tandem, which may have affected transformation of the recombinant binary vector into *Agrobacterium*. The high sequence identity of the Iraqi sequences to each other and sequences from neighbouring countries, suggests that the virus may have been introduced to Iraq in the recent past.

5. Detection of begomoviruses

5.1. Introduction

Tomato *Solanum lycopersicon* is one of the most important solanaceous crops grown in Iraq. Due to its high economic importance, tomato is grown throughout the year in both protected culture and open fields. The estimated annual yield of the tomato crop in Iraq is around one million metric tonnes (MT) (Anonymous, 2011). But yields are clearly decreased by tomato being susceptible to a wide range of pathogens including many viruses; 167 viruses from different groups have been reported to infect tomato worldwide naturally and experimentally (Brunt *et al*., 1996; ICTVdB, 2012).

Tomato yellow leaf curl disease (TYLCD) is considered as one of the devastating diseases worldwide (Duffy & Holms, 2007; Glick *et al.*, 2009; Díaz-Pendón *et al.*, 2010; Lefeuvre *et*

al., 2010; Kim *et al.*, 2011). TYLCD has been recorded to impact tomato by causing serious losses in yield in over 30 countries (Glick *et al.*, 2009). The disease is caused by various members of the genus *Begomovirus* within the family *Geminiviridae* including various strains of *Tomato yellow leaf curl virus* (TYLCV) (Díaz-Pendón *et al.*, 2010). The symptoms caused by TYLCV on tomato are stunting of the plant, leaf wrinkling, reduction of leaf size, interveinal yellowing and upward curling of leaf margins giving them a cup-shape appearance. Flowers may form but usually will be aborted before fruit set based on the susceptibility of the plant. No symptoms appear on fruit (Anonymous, 2000; Melzer *et al.*, 2009; Díaz-Pendón *et al.*, 2010). TYLCV has also been reported to attack other solanaceous crops including tobacco, potato, eggplant and pepper and leguminous plants like bean, but in the latter infection appears symptomless.

TYLCV is a circular single stranded DNA virus with a monopartite genome and transmitted by the whitefly *Bemisia tabaci* in a persistent manner (Seal *et al.*, 2006; Glick *et al.*, 2009; Lefeuvre *et al.*, 2010; Ohnishi *et al.*, 2011). Tomato yellow leaf curl disease (TYLCD) is one of the factors that severely limits tomato production in Iraq, as it attacks tomato cultivated in both protected and open fields (Al-Fadhal, 2012). In Iraq**,** TYLCD incidence may reach up to 100% and can cause economic losses between 50-90% especially when the virus infects tomato plants in the early growing stages (Al-Ani *et al*., 2011b). TYLCV was reported for the first time in Iraq in 1978 (Glick *et al.*, 2009), and recent studies were carried out to characterize the virus based on biological and serological methods (Al-Ani & Hamad 2002; Al-Ani *et al.*, 2010; 2011d; Al-Fadhal, 2012). However, these studies did not investigate the genetic diversity of TYLCV to determine the nature of the virus causing disease on molecular bases (Al-Kuwaiti *et al*., 2013).

Due to the economic importance and the lack of molecular studies concerning TYLCV in Iraq, this study was initiated with the aim to obtain baseline information on begomoviruses infecting vegetables in Iraq, to characterize these viruses, to determine their genetic diversity and for establishing the genetic relationship between begomoviruses found in Iraq with those present in the GenBank global sequence database.

5.2. Results

5.2.1. Begomovirus detection by degenerate primers and virus characterization

Results obtained from the 175 samples screened for the presence of begomovirus DNA sequences using the begomovirus-specific primer set Deng A/Deng B , showed that ~540 bp fragments were amplified from six tomato samples only; namely ToIq1, ToIq2, ToIq5, ToIq6, ToIq7 and ToIq9 (Figure 16). No bands, or non-specific products, were obtained from other tomato, potato, eggplant, cucurbits and legume samples (Appendix 1). Cloning and sequencing of the 540 bp fragment and subsequent sequence analyses confirmed that all sequences obtained were highly similar to the partial coat protein region of TYLCV as expected. The maximum nucleotide sequence identities, of partial V2/CP gene sequences, ranged between 97- 98% to sequences of TYLCV from Mauritius, Jordan, The Netherlands, Spain and Iran, except for the sequence obtained from ToIq5 which showed only a maximum of 90-91% identity to TYLCV GenBank sequences. For other viruses in the genus *Begomovirus*, sequences obtained in this study showed <74% nucleotide identities (Table 14). Analyses of sequences by Neighbor-Joining method revealed that all TYLCV sequences obtained in this study clustered together with the GenBank sequences of TYLCV. The clade was supported by high bootstrap value (97%) and diverged from other species within the genus *Begomovirus* (Figure 17).

5.2.2. Rolling circle amplification and restriction digestion

Restriction digestion of RCA products indicated the presence of single DNA fragments of ~2.7 kb size in ToIq1, ToIq2, ToIq5, ToIq6, ToIq7 and ToIq9 tomato samples when cleaved by *Nco*I, *Sph*I and *Sac*I (Figure 18). Two fragments of 2.1 and 0.8 kb were obtained from *Eco*RI restriction digestion of RCA products (Figure 18). No fragments were obtained when *Bam*HI, *Kpn*I, *Pst*I, *Xho*I and *Sal*I were used. Successful recombination of *Nco*I digested products into vector pGEM-3Zf (+) was confirmed when 2.7 kb fragments were amplified by PCR using the T7/SP6 (Figure 19).

5.2.3. Full-length sequencing and sequence analysis of TYLCV

The complete sequence of TYLCV (referred to as TYLCV IRQ) was obtained by sequencing (Appendix 4). The full-length sequences were 2780 bp long, and contained the begomovirus conserved motif TAATATTAC within the origin of DNA replication (*ori*) region.

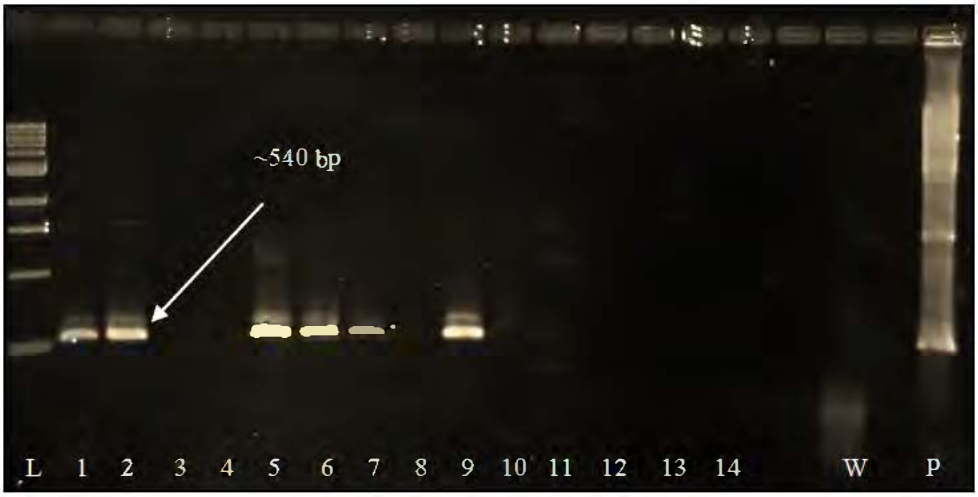


Figure 16: Screening of plant samples by RT-PCR for begomovirus sequences using the 'DengA/B' primer set

Gel electrophoresis pattern shows DNA fragments resulted from PCR amplification of tomato samples using Deng A /Deng B primer set. Lanes 1: ToIq1, 2: ToIq2, 3: ToIq3, 4: ToIq4, 5: ToIq5, 6: ToIq6, 7: ToIq7, 8: ToIq8, 9: ToIq9 from tomato samples. Lanes 10 from a weed sample, 11, 12, 13 and 14 represent eggplant samples. Lane P positive control obtained from a control positive begomovirus-infected DNA. W: water control. L: 100 bp DNA ladder marker (New England Biolabs, UK). Samples ToIq3, ToIq4 and IqTo8 were obtained from tomato plants showing typical fern leaf symptoms.

Table 14: **Identity percentage of TYLCV sequences**

Nucleotide identity percentage comparison of V2/CP among TYLCV sequences isolated from tomato samples collected from Iraq (bold letters) and those sequences obtained from GenBank. GenBank accession codes for Iraqi sequences are: ToIq1: JQ025990, ToIq2: JQ025991, ToIq5: JQ025992, ToIq6: JQ025993, ToIq7: JQ025994 & ToIq9: JQ025995.

GenBank acc. code	Isolate/virus name	Identity percentage						Location
		ToIq1	**ToIq2**	**ToIq5**	**ToIq6**	**ToIq7**	**ToIq9**	
HM448447	TYLCV-Mauritius	98	97	91	98	98	98	Mauritius
EU143754	TYLCV-Squash	98	97	91	97	97	98	Jordan
FJ439569	TYLCV-3181291	97	97	91	97	97	97	Netherlands
AJ519441	TYLCV-CB1/99	98	97	91	98	98	98	Spain
AB110217	TYLCV-Ng	97	97	90	96	97	98	Israel
GU076444	TYLCV-IL	97	97	90	97	97	97	Iran
S53251	*Tomato leaf curl virus*-AU	69	67	63	67	67	67	Australia
D88773	*Tomato yellow leaf curl China virus*	67	67	61	67	67	67	China
AF511528	*Tomato yellow leaf curl Kanchanaburi virus*	40	40	39	40	40	41	Thailand
AF271234	*Tomato yellow leaf curl Malaga virus*	74	74	68	74	74	74	Spain
X61153	*Tomato yellow leaf curl Sardinia virus*	74	73	67	74	74	74	Italy
X63015	*Tomato yellow leaf curl Thailand virus*-1	68	68	63	68	68	68	Thailand
AY998122	*Indian cassava mosaic virus*	21	22	20	21	21	22	India
HQ141673	*Tomato leaf curl New Delhi virus*	61	60	57	61	61	61	India

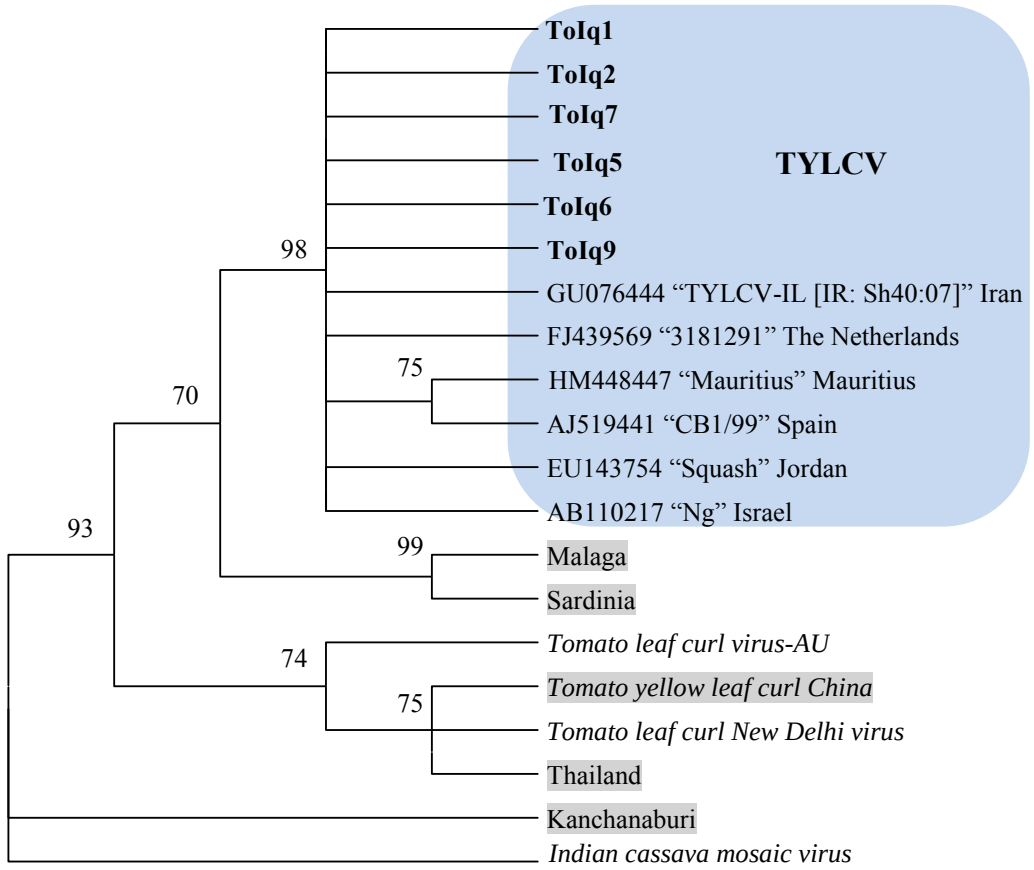


Figure 17: Genetic relationships of *Tomato yellow leaf curl virus* isolated from tomato in Iraq

Neighbor-joining phylogenetic tree constructed based on 540 bp sequences amplified by Deng primers. The topology shows the relatedness of sequences isolated from infected tomato from Iraq (bold letters) to GenBank sequences of TYLCV referred to as (GenBank acc number "isolate name" geographical region) begomoviruses causing TYLCVD (highlighted) and other begomoviruses. Begomoviruses GenBank accession numbers: Malaga: *Tomato yellow leaf curl Malaga virus* (Acc. AF271234), Sardinia: *Tomato yellow leaf curl Sardinia virus* (Acc. X61153), Thailand: *Tomato yellow leaf curl Thailand virus*-1(Acc. X63015), Kanchanaburi: *Tomato yellow leaf curl Kanchanaburi virus* (Acc. AF511528), *Tomato leaf curl virus*-AU (Acc. S53251), *Tomato yellow leaf curl China virus* (Acc. D88773), *Indian cassava mosaic virus* (Acc. AY998122) and *Tomato leaf curl New Delhi virus* (Acc. HQ141673).

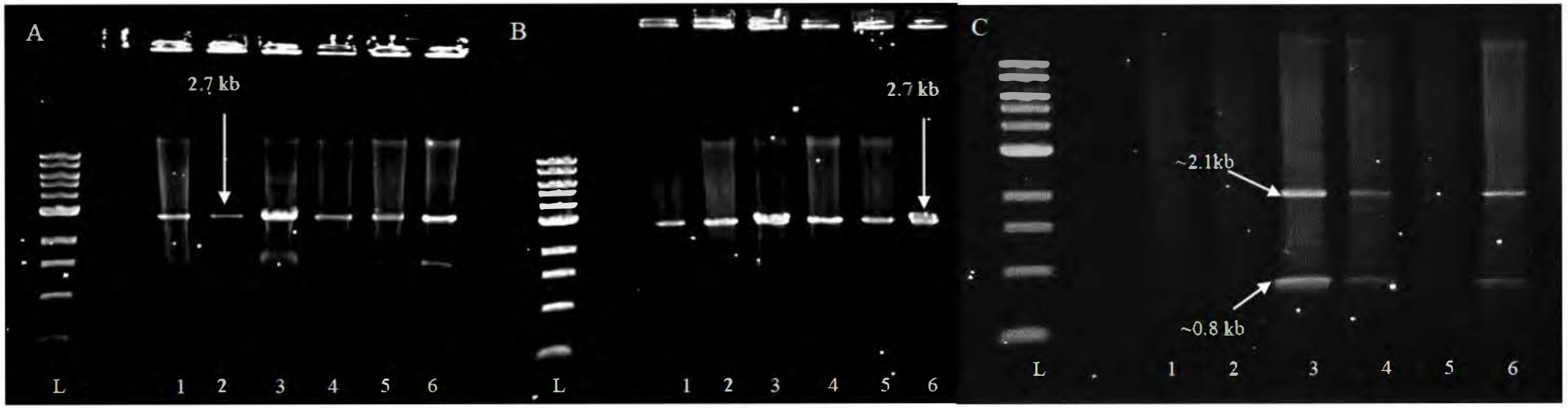


Figure 18: Restriction digestion pattern of RCA products

Restriction digestion patterns of RCA products cleaved by (A): *Nco*I & (B): *Sph*I showing fragments of ~2.7 kb in size representing multiple copies of TYLCV DNA amplified from tomato samples. C: shows restriction digestion patterns of RCA products cleaved by *Eco*RI. Lanes 1-6 are ToIq1, ToIq2, ToIq5, ToIq6, ToIq7 & ToIq9 tomato samples respectively. L: 1 kb DNA marker (New England Biolabs, UK).

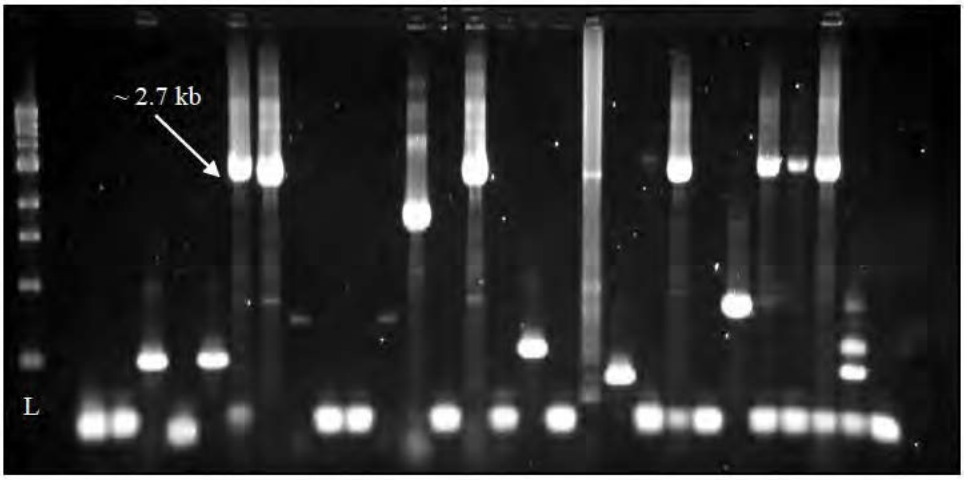


Figure 19: Screening for recombinant plasmid for TYLCV DNA

Ethidium bromide stained gel shows 2.7 kb DNA fragment amplified by T7/SP6 primer set to detect recombinant pGEM-3Zf (+) vector. DNA fragments were obtained from *Nco*I restriction digestion of RCA products and ligated to *Nco*- linearized dephosphorylated pGEM-3Zf (+) vector. L: 1 kb DNA marker (New England Biolabs, UK).

The virus consisted of six open reading frames (ORFs) and was 99% identical to GenBank sequences of Almeria Spain (Acc. AJ489258), Reunion Island (Acc. AM409201) and Mauritius (Acc. HM448447) (Table 15). Comparison of deduced amino acid sequences of TYLCV IRQ ORFs showed up to 100% identity to the equivalent ORFs of GenBank sequences (Table 16 & Appendix 4). Phylogenetic analyses of full sequence of TYLCV IRQ and those sequences from GenBank grouped TYLCV IRQ within the TYLCV cluster but diverged into a separate clade from TYLCV isolates from Spain, Reunion Island, Morocco, New Caledonia, and The Netherlands (Figure 20).

5.2.1. Detecting TYLCV IRQ using virus-specific primers

Non-degenerate primers, TYLCV-F/TYLCV-R, designed for sequencing the remaining fragment of full-length TYLCV IRQ, were able to amplify 1.7 kb fragments (Figure 21) when used to test the TYLCV infected tomato samples. The 1.7 kb fragment includes partial C2, V2, CP and partial C3 TYLCV genomic regions.

5.2.2. Developing a TYLCV IRQ infectious clone

Ethidium bromide gel electrophoresis revealed the partial digestion of RCA products using *Sac*I to produce the required 5.4 kb repeated tandem fragment that includes a full-length copy of TYLCV IRQ for subsequent cloning (Figure 22). The 5.4 kb fragment was cloned and the RFLP analyses of extracted recombinant pCAMBIA binary vectors using *Sac*I confirmed the presence of the 5.4 kb fragment. Recombinant *E. coli* colonies screening by PCR amplification using TYLCVF/TYLCVR primers confirmed successful recombination of the 5.4 kb TYLCV repeated tandem DNA fragments into the binary vector pCAMBIA when 1.7 kb fragments were amplified (Figure 24). In agroinoculation tests on *Nicotiana benthamiana* plants using *Agrobacterium tumefaciens* bacterial colonies harbouring the recombinant pCAMBIA binary vectors, one out of twenty clones was shown to be infectious when leaf curl and plant stunting symptoms developed on *N. benthamiana* 10-12 days after inoculation. PCR using TYLCV-F/TYLCV-R primers did not amplify the TYLCV fragment, neither in agro-inoculated plants nor in recombinant *A. tumefaciens* colonies harbouring pCAMBIA-TYLCV IRQ recombinant binary vector.

Table 15: Identity percentages of full-length sequences of *Tomato yellow leaf curl virus* isolates

Comparison of full-length sequences of TYLCV IRQ (Acc No. JQ354991) isolated from tomato in Iraq to equivalent TYLCV sequences in GenBank

GenBank acc. code	Isolate/virus name	Identity	Location
		TYLCV IRQ	
AJ489258	Almeria	99	Spain
AM409201	RE4	99	Reunion Islands
HM448447	Mauritius	99	Mauritius
FJ439569	3181291	98	The Netherlands
HE603243	New Caledonia:Noumea 3:2010	98	New Caledonia
EF060196	Moroccan	98	Morocco
AJ223505	Cuba	98	Cuba
EF054893	Jordan	98	Jordan
AF024715	Dominican	98	The Dominican Republic
GU325634	Bos	98	S. Korea
AB116631	Japan:Misumi:Stellaria	98	Japan
AB636409	TYLCV-GJ	97	S. Korea
GU076444	Shiraz:Iran	97	Iran
JF451352	KISR	95	Kuwait
FJ956700	*Tomato leaf curl Oman virus*	85	Oman
S53251	**Tomato leaf curl virus-[AU]*	74	Australia
D88773	**Tomato yellow leaf curl China*	70	China
GU456685	**Turnip curly top virus*	50	Iran

* Pairwise comparison

Table 16: Amino acid similarity percentages of genome region among TYLCV isolates

Comparison of deduced amino acid similarity percentages for V2 (pre coat protein), CP (coat protein), C3 or replication enhancer protein (*REn*), C2 transcription activator protein (TrAP), C1 replication initiation protein (REP) and C4 genes and complete nucleotide sequence (nt) between TYLCV IRQ and GenBank isolates.

GenBank acc.	Isolate	V2	CP	C3	C2	C1	C4	nt%	location
JQ354991	TYLCV IRQ								
AJ489258	Almeria	100	99	99	99	99	99	99	Spain
HM448447	Mauritius	100	99	99	99	98	99	99	Mauritius
AM409201	RE4	99	99	99	99	99	99	99	Reunion Islands
FJ439569	3181291	99	99	98	99	98	99	99	The Netherlands
EF060196	Moroccan	99	98	97	97	97	95	98	Morocco
AJ223505	Cuba	100	99	97	98	98	96	98	Cuba
AF024715	Dominican	100	99	97	98	98	96	98	The Dominican Republic
AB116631	Japan-Misumi	100	99	99	98	98	97	98	Japan
EF054893	Jordan	100	99	99	97	97	98	98	Jordan
GU325634	Bos Korea	99	99	97	97	97	97	98	S. Korea
AB636409	TYLCV-GJ	100	99	98	97	97	97	97	S. Korea
GU076444	Iran	100	99	97	97	97	89	97	Iran
JF451352	KISR	96	96	93	96	81	88	95	Kuwait
FJ956700	*Oman	96	99	91	81	73	27	85	Oman

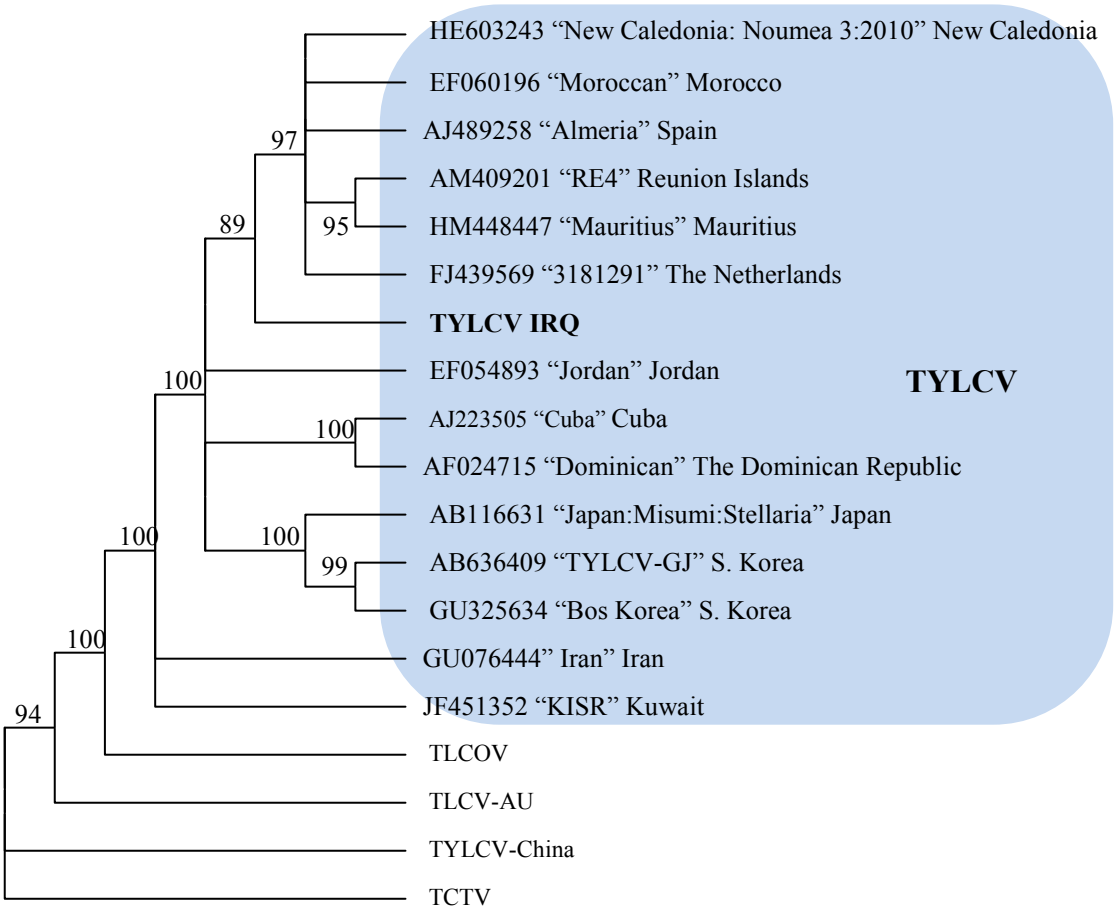


Figure 20: Genetic relationships of *Tomato yellow leaf curl virus* isolated from tomato in Iraq

Neighbor-joining phylogenetic tree constructed based on full-length sequences of TYLCV IRQ isolated from tomato samples in Iraq (bold lettering), compared to sequences of TYLCV isolates and other members of the family *Geminiviridae* from GenBank referred to as 'GenBank acc. number "isolate name" geographical region'. TLCOV: *Tomato leaf curl Oman virus* (TLCOV), TLCV-AU: *Tomato leaf curl virus-[AU]* (TLCV, TYLCV-China: *Tomato yellow leaf curl China virus* (TYLCCV) are members of the genus *Begomovirus*. TCTV *Turnip curly top virus* (TCTV) belongs to the genus *Curtovirus*.

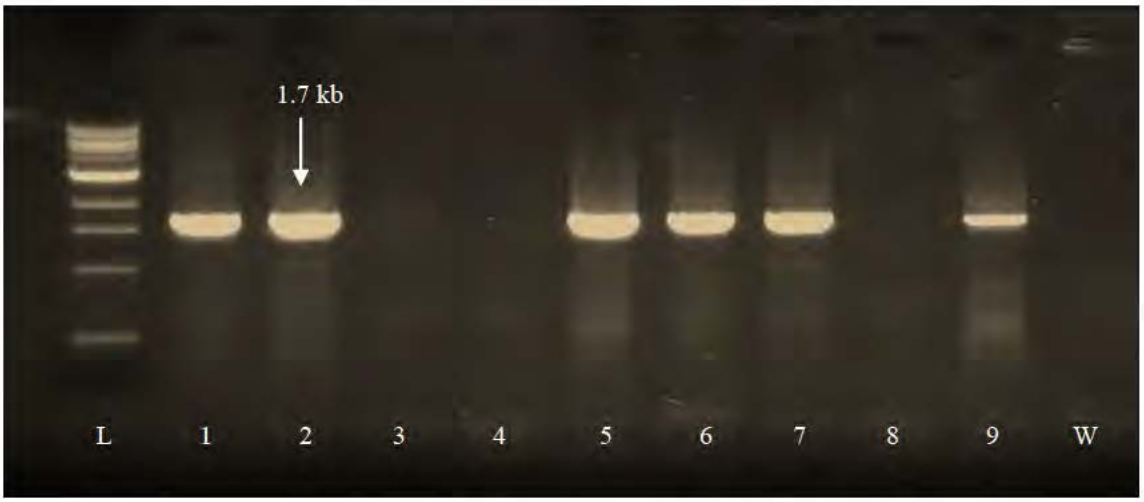


Figure 21: PCR using TYLCV-F/TYLCV-R primers set

Gel electrophoresis analysis showing 1.7 kb DNA fragments amplified by TYLCV-F/TYLCV-R primers designed from TYLCV IRQ. Lane 1: ToIq1, 2: ToIq2, 3: ToIq3, 4: ToIq4, 5: ToIq5, 6: ToIq6, 7: ToIq7, 8: ToIq8, 9: ToIq9, W: water control and L: 1 kb DNA marker (New England Biolabs, UK).

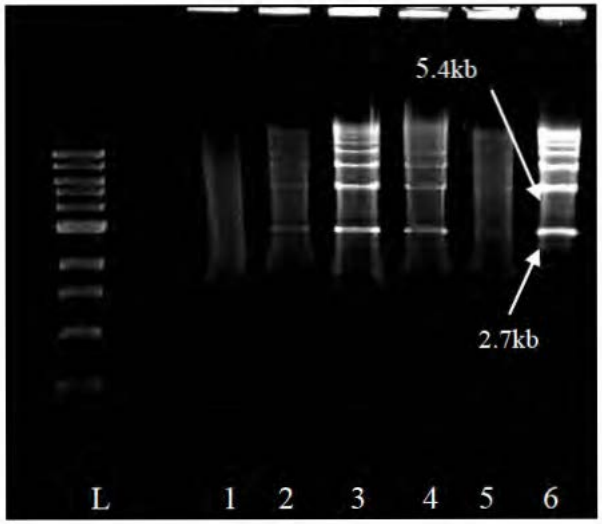


Figure 22: Partial digestion of RCA products

Ethidium bromide stained gel pattern shows partially digested TYLCV IRO RCA product using *Sac*I restriction enzyme to generate 5.4 kb fragment for cloning and subsequent agroinoculation. Lanes 1-6 are ToIq1, ToIq2, ToIq5, ToIq6, ToIq7 and ToIq9 tomato samples respectively. L: 1 kb DNA marker (New England Biolabs, UK).

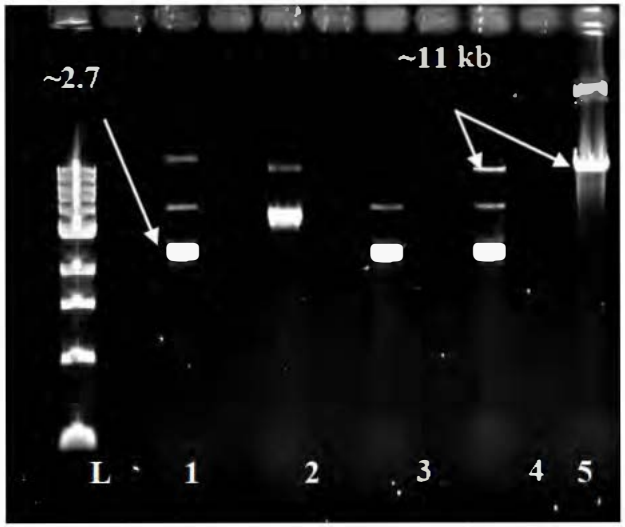


Figure 23: Restriction digestion of recombinant pCAMBIA-TYLCV IRQ

Restriction digestion pattern shows recombinant pCAMBIA 3001 binary vector cleaved by *Sac*I restriction enzyme. The released 2.7 kb fragments represent TYLCV IRQ DNA isolated from ToIq9 tomato sample and the 11 kb fragments represent linearized pCAMBIA 3001 plasmid, confirming successful recombination of pCAMBIA-TYLCV IRQ 5.4 kb. Lanes 1-4: represent recombinant plasmids. Lane 5: a non-recombinant plasmid. Lane L: 1 kb DNA marker (New England Biolabs, UK).

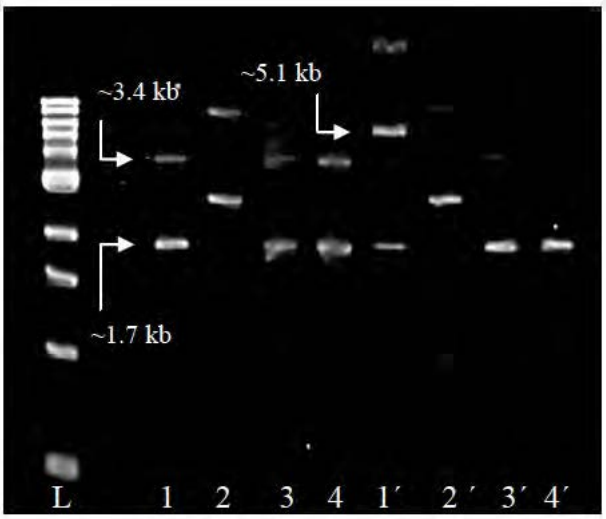


Figure 24: Screening recombinant clones using TYLCV-F/TYLCV-R primers

Gel electrophoresis pattern showing 1.7 kb DNA fragments amplified by TYLCV-F/TYLCV-R primer set to confirm pCAMBIA-TYLCV IRQ recombination. Lanes 1-4: represent recombinant clones obtained from ToIq9 sample. Lanes 1′- 4′are duplicates of lanes 1- 4. The 3.4 & 5.1 kb fragments are double and triple copies of the 1.7 kb fragment. L: 1 kb DNA marker (New England Biolabs, UK).

5.1. Discussion

The occurrence of TYLCD in Iraq has been reported before based on symptoms seen on tomato and other assay hosts, and by the detection of the virus by serology (Al-Ani & Hamad 2002; Al-Ani *et al.*, 2010; 2011b). These studies; however, did not obtain sequence and hence species information, and the prevalence of TYLCV was thus not confirmed. Degenerate primer set Deng A/ Deng B has been used to amplify ~ 540 bp from the coat protein gene of the DNA-A component of many begomoviruses and the sequences of this fragment are commonly used to identify begomoviruses (Reddy *et al*., 2005; Maruthi *et al*., 2007). Virus detection using these 'Deng' primers together with cloning, sequencing and comparison of sequences to GenBank enabled identification of the causal agent of tomato yellow leaf curl disease at the isolate level in this study. The molecular diagnosis using Deng A/ Deng B primers suggested that the diseased tomato samples were infected by a single infection of TYLCV as no other begomovirus sequences were amplified from the samples, and these primers are known to amplify a wide range of begomoviruses (Reddy *et al*., 2005). Sequence comparison of ~540 bp DNA fragments amplified from tomato samples by Deng primers showed 97- 98% maximum identity to GenBank sequences from Mauritius (Acc. HM448447), Jordan (Acc. EU143754), The Netherlands (Acc. FJ439569), Spain (Acc. AJ519441), Iran (Acc. GU076444) and Israel (Acc. AB110217) except for ToIq5 sample that showed only 91% maximum identity to the same TYLCV GenBank sequences. The sequences showed less than 75% identity to other begomoviruses reported to cause TYLCVD on tomato such as *Tomato yellow leaf curl Sardinia virus* or other begomoviruses from GenBank (Table 14). All sequences obtained were clustered with isolates from Mauritius (Acc. HM448447), Jordan (Acc. EU143754), The Netherlands (Acc. FJ439569), Spain (Acc. AJ519441), Iran (Acc. GU076444) and Israel (Acc. AB110217) within one clade of TYLCV node, this relation was supported by 98% bootstrap value. The Neighbor-Joining phylogenetic tree did not show divergence among sequences obtained and GenBank sequences, especially with ToIq5 that showed lower sequence similarity to GenBank sequences. Neighbor-Joining phylogenetic tree not only showed the relationship of TYLCV sequences, but also illustrated the divergence of TYLCV sequences from other begomoviruses sequences.

When combined with RFLP, RCA was shown to be reliable for TYLCV detection in tomato samples collected from Iraq. As Ø29 polymerase amplifies from circular DNA templates, RCA/RFLP approaches confirmed the single infection of TYLCV in tomato

samples from Iraq when restriction digestion of concatemeric molecules amplified from all nine samples showed the same pattern on the gel when cleaved by several restriction enzymes; i.e. *Nco*I, *Sph*I, *Sac*I, *Bam*HI, *Kpn*I, *Pst*I, *Xho*I, *Sal*I and *Eco*RI. Use of restriction enzymes previously reported to have unique recognition site on TYLCV genome has enabled the release of a full-length DNA fragment of TYLCV from concatemeric molecules which was later used for cloning and sequencing. The RCA/RFLP approaches not only enabled amplifying the full-length DNA fragment but they were useful to obtain identical copies of TYLCV DNA genome due to the proof reading ability of Ø29 polymerase (Johne *et al*., 2009), which minimizes the errors that occur due to mutation and nucleotides displacement and provides accurate data for sequence analyses.

Comparison of full-length TYLCV IRQ sequences showed similar results when MEGA BLAST search was performed against GenBank sequences. Three TYLCV isolates; namely Mauritius (Acc. HM448447), RE4 (Acc. AM409201) and Almeria (Acc. AJ489258) from Mauritius, Reunion Islands and Spain respectively, were 99% identical to TYLCV IRQ; whereas, TYLCV GenBank sequences from The Netherlands (Acc. FJ439569), New Caledonia (Acc. HE603243), Morocco (Acc. EF060196), Cuba (Acc. AJ223505), Jordan (Acc. EF054893), The Dominican Republic (Acc. AF024715), South Korea (Acc. GU325634) and Japan (Acc. AB116631) showed slightly lower identity of 98%. The slight variation in sequence identities (98% maximum nt identity for sequences amplified by PCR compared to 99% nt identity for the sequence amplified by RCA) between Iraqi TYLCV sequences resulted using Deng primers and RCA could be due to low fidelity of *Taq* polymerase used in PCR, compared to Ø29 polymerase that has proof reading activity (Johne *et al*., 2009). The low fidelity may result in producing less identical copies of targeted DNA fragments with nucleotide errors that differ from the original template of TYLCV (Brown, 2010). The full TYLCV sequence showed higher sequence identity than partial sequences obtained from the highly conserved motifs within TYLCV genome. Despite the high nt identity percentage (99%), Neighbor-Joining phylogenetic tree, constructed from full-length sequences of TYLCV, diverged TYLCV IRQ from equivalent GenBank sequences supported by 97% bootstrap value (Figure 20), which may indicate that TYLCV IRQ is a different strain. Phylogenetic tree based on full-length sequences of begomoviruses is a powerful tool for strain and variant demarcation (Fauquet *et al*., 2008), however, further biological and serological studies, are required to support that TYLCV IRQ is a distinct TYLCV strain (Fauquet *et al*., 2008).

The two virus-specific primers; TYLCV-F/TYLCV-R, were designed to sequence gaps between the overlapping fragments that could not be sequenced using T7/SP6 promoter primers. Coincidentally, the TYLCV-F/TYLCV-R primers were found to target the conserved region of consensus sequences. The forward primer TYLCV-F anneals to TYLCV genomic region at 2443-2463 nucleotide position within C1 or REP region, and the reverse primer TYLCV-R anneals at 1374-1394 nucleotide positions within C3 or REn region which enables amplification of a 1.7 kb fragment including partial C1, the origin of replication (*ori*), V2, CP, and REn regions (Figure 25A). The TYLCV-F/TYLCV-R primer set showed high specificity when used to screen individual *E coli* colonies harbouring pCAMBIA recombinant plasmid vectors with full-length fragments of TYLCV which suggested that this primer set could be used to screen recombinant colonies for successful pCAMBIA-TYLCV recombination as well as detecting samples for TYLCV infection. Furthermore, DNA fragments amplified from the pCAMBIA-TYLCV recombinant plasmid showed varied fragment sizes (1.7, 3.4 and 5.1 kb) when screened using TYLCV-F/TYLCV-R primer set (Figure 24). The ~3.4 and 5.1 kb fragments amplified may represent double and triple size of the 1.7 kb fragment (Figure 24) and they were possibly produced from repeated tandem copies of TYLCV IRQ (Figure 25B). However, further sequencing of the fragment 3.4-5.1 kb is required to confirm that they are repeated tandems of 1.7 kb.

Deduced amino acid analyses obtained from full-length sequences revealed the occurrence of six ORFs within TYLCV IRQ, which is identical to the typical TYLCV genome. The putative V2 region exhibited the highest identity compared to other regions with 100% identity to some other TYLCV isolates. The putative CP region also showed high sequence identity within the diverged isolates from Oman (Acc.FJ956700) with 99% amino acid similarity to TYLCV IRQ and other GenBank sequences. The V2 and CP regions are reported as reliable for sequence comparison and strain or species classification and demarcation according to the ICTV criteria (Fauquet *et al*., 2003). An infectious clone of TYLCV IRQ was constructed from the partial digestion of RCA products to obtain a repeated tandem of TYLCV IRQ of ~5.4 kb including the full- length genome of TYLCV IRQ in the right orientation starting from nucleotide no.1 and ending at nucleotide no. 2780. The approach involved cloning the resulting fragments directly into pCAMBIA binary vector and then transformation into *A. tumefaciens* for subsequent agroinoculation. The technique required dephosphorylation of the pCAMBIA plasmid to prevent self-ligation of the vector as a single restriction enzyme had been used for vector digestion (Ferreira *et al*., 2008; Brown, 2010).

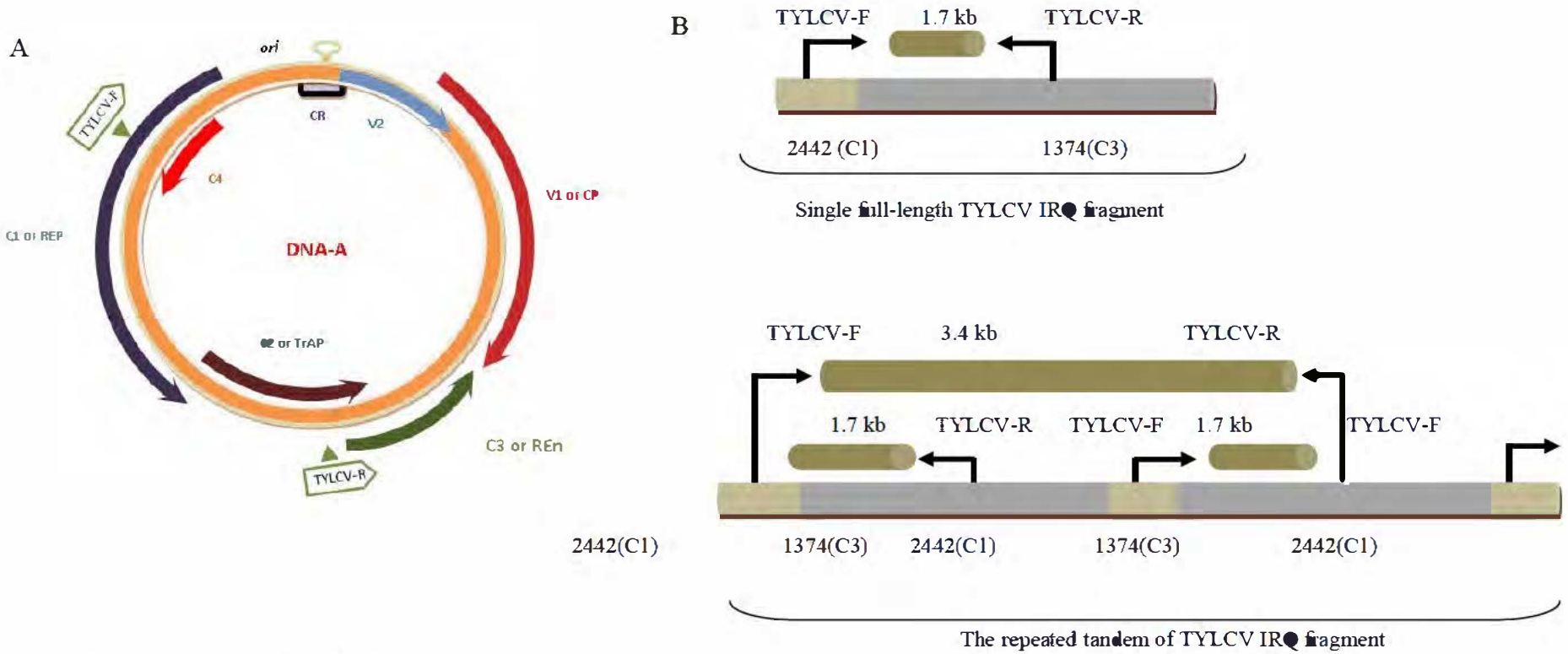


Figure 25: Genome map of TYLCV IRQ

A map illustrates the genomic region and annealing sites of TYLCV-F/TYLCV-R primer set that amplifies the 1.7 kb fragment between 2442 and 1374 nucleotide amplified positions (A). (B) A scheme illustrates amplification of 1.7 and 3.4 kb fragments by TYLCV-F/TYLCV-R from the 2.7 kb TYLCV fragment released from RCA product by *Sac*I and a repeated tandem fragment constructed by *Sac*I partial digestion of TYLCV amplified by RCA.

However, multiple insertions of 5.4 kb occurred throughout ligation caused in a recombinant pCAMBIA plasmid with a double or triple 5.4 kb TYLCV fragment. That multiple insertion fragments seemed to affect transformation of recombinant plasmid into *A. tumefaciens* as recombinant pCAMBIA binary vector did not transform successfully into the bacteria. Cloning large fragments of TYLCV IRQ DNA proved difficult as viral DNA was not detected in *A. tumefaciens* using the TYLCV-F/TYLCV-R primer set. Alternative approaches may be suggested to construct an infectious clone of TYLCV IRQ, such as using subsequent restriction digestion steps to construct 1.4-1.8 mer infectious clones (Ito *et al.*, 2009; Al-Abdallat *et al*., 2011) or by using PCR with appropriate adapters to amplify the genome from an appropriate start point (Lapidot *et al*., 2007), rather than using partial restriction digestion of clones.

The current study provided the first evidence of molecular data for TYLCV in Iraq, which is concurrent with reports from neighbouring countries of Iran, Jordon, Saudi Arabia and Turkey (Glick *et al.*, 2009), Syria (Cohen and Lapidot, 2007) and Kuwait (Fauquet *et al*., 2003). The low diversity and high sequence similarity to GenBank sequences suggested TYLCV IRQ might be introduced to Iraq from other countries. The way that TYLCV was introduced to Iraq is not known, however one possible route could have been from neighbouring countries through infected ornamental plants (Caciagli, 2007). The virus may have been transmitted into tomato subsequently by whitefly, as ornamental plants imported from neighbouring countries are grown alongside tomato seedlings in nurseries used for commercial production. Confirmation of the nature of begomovirus causing TYLCD in Iraq will help design sustainable disease control strategies by identifying tomato varieties resistant to TYLCV IRQ.

CHAPTER 6: OTHER VIRUSES

Summary

Research was initiated to investigate carlavirus, tombusviruses, alfamoviruses, potexviruses, tobamoviruses and cucumoviruses infection of potato and vegetable samples collected from fields in Iraq by RT-PCR using genus specific primer sets. Eleven out of 23 cowpea, 10 out of 61 potato and three out of four eggplant samples were infected with carlaviruses when tested with Car-F1/M4T carlavirus-specific primer set. Sequence analyses revealed the occurrence of two diverged carlaviruses in detected samples. *Potato virus S* (PVS) was identified in potato samples collected from Baghdad and Anbar. Sequences isolated showed 99% identity with isolates from Syria (Acc. AB364945), Iran (Acc. HQ875140 & HQ875132) and slightly lower levels of 95-97% similarity with sequences of other isolates in GenBank. Comparison of deduced amino acid sequences of CP region showed maximum identity (100%) to PVS isolates from Syria (Acc. AB364945), Iran (Acc. HQ875140 & HQ875132), USA (Acc. FJ813514 & FJ813513) and China (Acc. AJ889246). Another carlavirus found in the samples included *Cowpea mild mottle virus* (CPMMV) which was identified in cowpea samples collected from Najaf. CPMMV showed 80% maximum nucleotide identity to CPMMV isolate from Venezuela (Acc. JX310549) and Ghana (Acc. HQ184471) and 96% maximum aa similarity to CPMMV CP from Brazil (Acc. DQ885940). High nucleotide identity percent suggested that PVS may have been introduced into Iraq from two neighbouring countries, Syria and Iran. However, for CPMMV as there is no sequence information in GenBank regarding CPMMV from countries bordering Iraq and hence introduction cannot be deduced. This study is the first of its kind with regards to the diversity of carlaviruses found in Iraq. Results revealed the infection of 9 tomato and 2 eggplant samples from Baghdad with tombusvirus. Sequence analyses for the 10 sequences isolated, showed maximum (93%) nucleotide identity to the CP region *Grapevine Algerian latent virus* (GALV) isolates from Japan (Acc. AY830918). The maximum amino acid similarity was (98%) to an isolate from Italy (Acc. AF540885). The other two sequences showed similarity to plant genome. All 10 Iraqi GALV sequences showed 98-100% nt identity and aa similarity when compared to each other. Neighbor-Joining phylogenetic tree grouped the 10 sequences isolated into a single group. Although the tombusvirus sequences from Iraq are clearly representative of the species GALV, their distinct properties (infection of previously

unreported hosts, and phylogenetic position) suggest that GALV from Iraq could be a distinct strain. No potex-, tobamo, alfamo- and cucumoviruses were detected in potato and vegetable samples when screened using group specific primers.

6. Detection of other virus groups

6.1. Introduction

Five recognised species of carlaviruses have been found to infect potato worldwide *Potato virus S* (PVS), *Potato virus M* (PVM), *Potato latent virus* (PotLV), *Potato rough dwarf virus* (PRDV) and *Potato virus P* (PVP) while the taxonomic status of one additional species (Southern potato latent virus) remain to be determined (Ryu & Lee, 2009; ICTVdb, 2012). *Potato virus S* (PVS: genus *Carlavirus*; family *Betaflexiviridae*) was first characterized in 1952 and this is the most economically important member within the genus *Carlavirus*. It infects potato worldwide (Jones *et al*., 2009) causing up to 20% yield losses (Loebenstein, 2009). PVS is spread mechanically by contact between plants and is readily transmitted by the aphid vector *Myzus persicae* in a non-persistent manner (Jones *et al*., 2009; Kerlan, 2009). PVS was reported to cause symptomless infection on potato, but careful examination of infected plants can reveal slight rugosity of leaves and deepening of veins on the under surface of leaves. Some cultivars react with faint mottling, vein-banding, and waving of leaf margins (Jones *et al*., 2009; Kerlan, 2009). Two strain groups, namely PVS^{O} (the ordinary strain) and PVS^{A} (the Andean strain) have been recognized for this virus based on symptoms on experimental hosts such as *Chenopodium* spp. Symptoms caused by PVS vary with the virus strain, potato cultivar and environmental conditions with PVS^{A} tending to cause more severe symptoms than PVS^{O} (Kerlan, 2009).

Cowpea mild mottle virus (CPMMV: genus *Carlavirus*; family *Betaflexiviridae*) was first isolated from cowpea *Vigna unguiculata* in Ghana in 1973 (Brunt *et al.* 1996). Amongst the carlaviruses, CPMMV, *Cucumber vein-clearing virus* (CuVCV) and *Melon yellowing-associated virus* (MYaV) are the only members of the genus *Carlavirus* transmitted by whiteflies (Ryu & Lee, 2009, Menzel *et al.*, 2011, ICTVdB 2012). CPMMV is transmitted by the sweet potato whitefly *Bemisia tabaci* in a non-persistent manner and through seeds of cowpea, soybean and French bean (Naidu *et al*., 1998). The natural host range of CPMMV is narrow and restricted to leguminous and solanaceous crops. In addition to cowpea, the virus was reported to infect the following natural hosts; *Canavalia ensiformis*, *Arachis hypogaea*, *Phaseolus lunatus*, *P. vulgaris*, *Psophocarpus tetragonolobus*, *Glycine max*, *Solanum lycopersicon*, *Solanum melongena*, *Vicia faba*, cowpeas cv. Blackeye (*Vigna unguiculata*),

Vigna subterranean and *Vigna mungo*; besides, the virus occurs in various weeds (*Fabaceae*), namely *Stylosanthes* and *Tephrosia* spp. (EPPO/CABI, 1997; Mansour *et al*., 1998; Naidu *et al*., 1998). CPMMV is widely distributed in different geographical regions in African countries of Côte d'Ivoire, Egypt, Ghana, Kenya, Malawi, Mozambique, Nigeria, Sudan, Tanzania, Togo, Uganda and Zambia. In Asia, it has been reported from India, Indonesia, Iran, Israel, Malaysia, Thailand and Yemen. The virus has also been found in the South American countries Brazil, Argentina and Puerto Rico, but CPMMV has not been found in Europe to date (EPPO/CABI, 1997; Naidu *et al*., 1998; Tavasoli *et al*., 2009). Symptoms induced by CPMMV may vary according to host (Tavasoli *et al*., 2009). On cowpea the virus causes diffused chlorotic blotches on the primary leaves, as well as systemic mottling and leaf distortion (EPPO/CABI, 1997; Naidu *et al*., 1998; Tavasoli *et al*., 2009). Two strains, namely CPMMV-H and CPMMV-M have been distinguished based on symptoms on different indicator plants (Naidu *et al*., 1998) beside a unique strain that causes pale chlorosis disease on tomato described from Israel (EPPO/CABI, 1997).

GALV is a member of the genus *Tombusvirus* within the family *Tombusviridae*. It was described for the first time on naturally infected grapevine plants in Italy in 1989 (Fujinaga *et al*., 2009). The natural host range of this virus was recorded to be narrow infecting plant species belonging to three families, namely *Chenopodiaceae*, *Solanaceae* and *Vitidaceae.* The virus was shown to infect a wider host range experimentally, namely eggplant, cucumber, common bean and broad bean and cowpea causing local lesion infection (Koenig *et al*., 2004b; Ohki *et al*., 2006; Fujinaga *et al*., 2009). GALV was reported on a naturally infected nipple-fruit *Solanum mammosum*, an ornamental solanaceous plant in Japan and a range of other ornamental plants in different geographical regions (Koenig *et al*., 2004b; Fujinaga *et al*., 2009) but the virus has not been reported to infect vegetables or potato naturally. GALV is mechanically transmissible and transmitted through contaminated soils and has also been detected in river waters (Koenig *et al*., 2004b; Ohki *et al*., 2006; Fujinaga *et al*., 2009). Most tombusviruses are transmitted by fungi and through seed and pollen, although the transmission of GALV through seed or by a fungal vector has to date not been confirmed (Fujinaga *et al*., 2009).

6.2. **Results**

6.2.1. **Detection of carlaviruses**

6.2.1.1. **Primer screening and RT-PCR**

Four published carlavirus specific primer sets were used to screen a limited number of samples to choose the best primer set for carlavirus detection. Carla-uni/M4T was found to detect carlaviruses in the highest number of samples compared to results from the other three primer sets. Amongst 11 tested samples, Carla-uni/M4T amplified the expected 120 bp fragment size of 3`UTR region from three samples (PMo, PC and CP1) whereas Carla-Cp/M4T and Car-F1/M4T detected two samples to be carlavirus positives while Pcar/M4T detected only one positive sample (Table 19). However, primer set Carla-uni/M4T was not selected to screen the remaining samples because this set amplifies only a short fragment of ~ 120 bp (Figure 26), the sequences obtained from such a small fragment is insufficient to carry out subsequent phylogenetic analyses. In terms of the efficacy for virus detection, Car-F1/M4T was the second best primer set; it detected carlaviruses in two out of 12 Iraqi samples. This primer set also amplified a bigger DNA fragment size of about 950 bp which included the full-length CP region and therefore was successful for sequencing and subsequent phylogenetic analyses. RT-PCR results showed that Car-F1/M4T, the chosen primer set used to screen the remaining samples for carlaviruses, detected carlaviruses in 21 out of 175 tested samples (Appendix 1) by amplifying the expected ~950 bp DNA fragment size from cowpea and potato samples (Figure 27 & Figure 28). In addition, DNA fragments amplified by (Appendix 1) and other two primer sets, namely Carla-uni/M4T and Carla-Cp/M4T, were successfully cloned and sequenced for further confirmation of the presence of carlavirus in samples. Clone screening revealed T7/SP6 could amplify the expected size fragments from 14 recombinant colonies (Figure 29). Three out of four eggplant samples were detected false positives for carlaviruses (referred to as ±) when screened using Car-F1/M4T (Appendix 1), however sequencing results revealed that fragment amplified from eggplant samples belong to plant genome.

6.2.1.2. **Detection and sequence analyses of *Potato virus S* in potato**

RT-PCR amplification showed that 10 out of 61 potato samples were indicated to be infected by carlaviruses when using Car-F1/M4T primers (Appendix 1). DNA fragments amplified by Car-F1/M4T primers and Carla-Cp/M4T were about 883-898 bp and 770-896 bp (excluding the poly A tail), respectively when sequenced (Table 18).

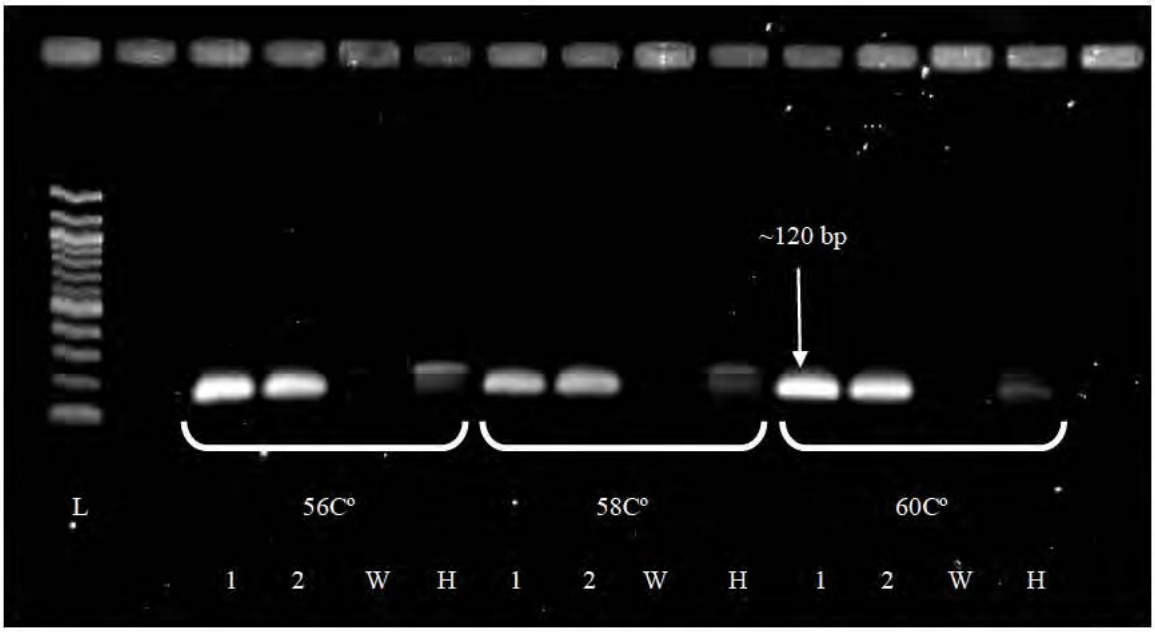


Figure 26: Optimizing annealing temperature for the Carla-uni/M5T primer set to detect carlaviruses.

Gel electrophoresis pattern of PCR products showing target ~120 bp DNA fragment amplified by Carla-uni/M4T carlavirus specific primers at annealing temperatures ranging from 56-60 ^{0}C. Lanes 1 and 2 represent PVS samples, W: water control, H: low positive control sample, L: 100 bp DNA ladder marker (New England Biolabs, UK).

Table 17: Screening of carlavirus specific primer set

Carlavirus primer screening to detect potato and vegetable samples collected from Iraqi fields and the expected product size of each primer set.

Primer set	product size (bp)	BB1	Cu1	Cu2	CF	LM	Cr1	Cr2	PMO	PC	PM	To23	CP1
Carla-uni/M4T	120	ns	ns	ns	ns	ns	ns	ns	++	++	-	-	++
Pcar/M4T	900	-	-	-	-	-	-	-	-	+	-	-	-
Car-F1/M4T	950	-	-	-	-	-	-	-	++	++	-	-	-
Carla-CP/M4T	800	-	-	-	-	-	-	-	++	++	-	-	-

++: strong positive, +: low positive, -: negative & ns: non-specific products.

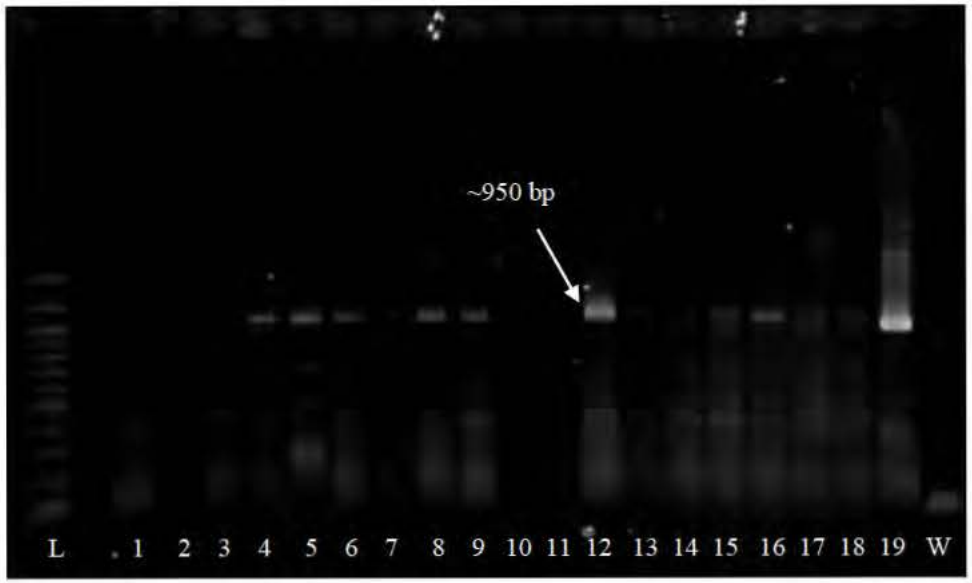


Figure 27: RT-PCR on cowpea samples using Car-F1/M4T primer set to screen for carlaviruses

Gel electrophoresis pattern of PCR products showing ~950 bp DNA fragment amplified by Car-F1/M4T carlavirus-specific primer set from cowpea samples. Lanes 1:Cp6, 2: CP7, 3: CP8, 4:CP9, 5: CP10, 6:CP11, 7:CP12, 8: CP15, 9: CP16, 10:CP17, 11: Cp18, 12:Cp19, 13: CP20, 14: CP21, 15: CP22, 16: CP23, 17: CP24 and 18: CP25, 19: positive control, W: water control. L & M: 100 bp & 1 kb DNA ladder markers respectively (New England Biolabs, UK).

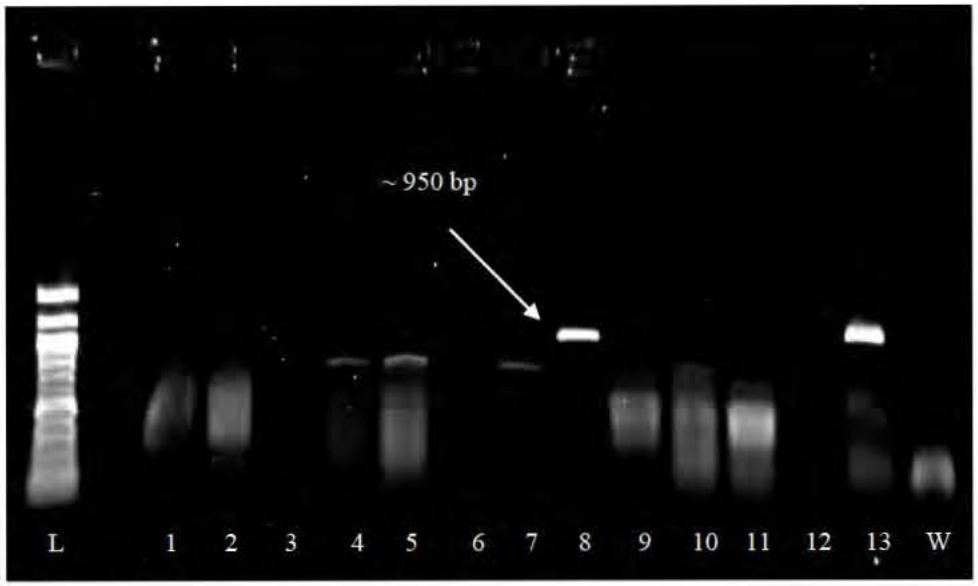


Figure 28: RT-PCR on potato samples using Car-F1/M4T primer set to screen for carlaviruses

Gel electrophoresis pattern of PCR products showing ~950 bp DNA fragment amplified by Car-F1/M4T carlavirus specific primers from potato samples Lanes 1: P54, 2: P40, 3: P11, 4: P23, 5: P17, 6: P24, 7: P12, 8: P4, 9: P54A, 10: P52, 11: P27 & 12: P53. 13: positive control. W: water control. L: 100 bp DNA ladder marker (New England Biolabs, UK).

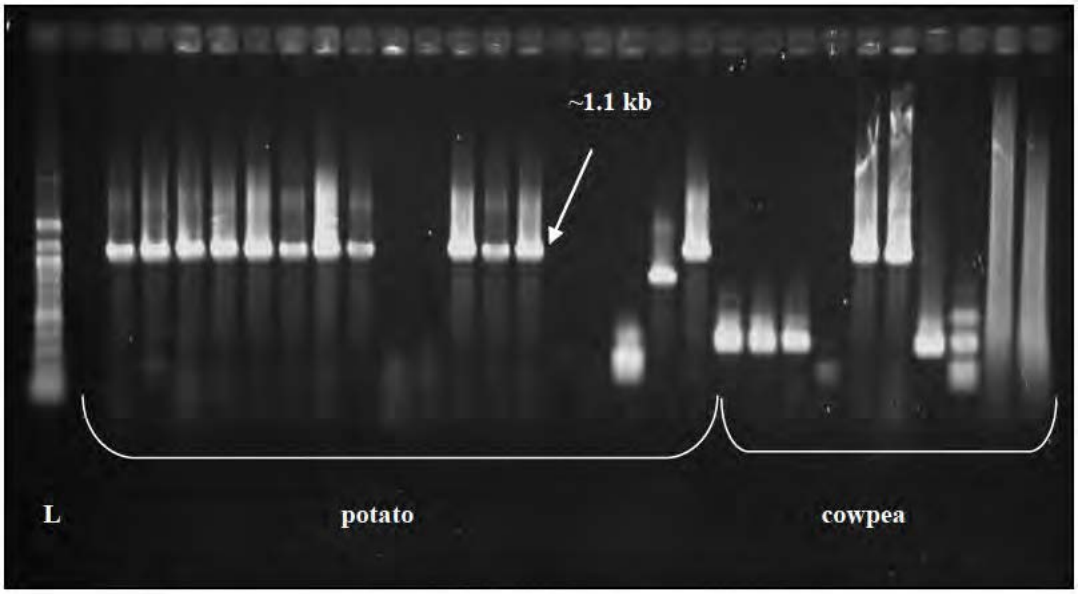


Figure 29: Screening for desired insert size of 950 bp in recombinant *E. coli*

Gel electrophoresis pattern of PCR products showing ~1100 bp DNA fragment amplified by T7/SP6 primers representative of 950 bp (+ ~150 bp vector sequences) insert successfully ligated into pGEM-T cloning vector. The 950 bp DNA fragments amplified from potato and cowpea samples using Car-F1/M4T carlavirus specific primer set. L: 100 bp DNA ladder marker (New England Biolabs, UK).

Table 18: Profiles of carlavirus clones sequenced

Clones isolated from selected samples used for sequencing and their lengths in base pair after sequencing and editing using MEGA 5 software. This table also illustrates the crop from which clones were isolated and the sample location and the carlavirus detected in each sample in this study.

GenBank acc. Code	Sequence	Sample	Length bp	Crop	Location	Virus detected	Primer set
KC677756	PMO2-CP	PMo	889	potato	Baghdad	PVS	Carla-CP/M4T
KC677757	PC11-CP	PC	896	potato	Baghdad	PVS	Carla-CP/M4T
KC677758	P19-18-CP	P19	893	potato	Al-Anbar	PVS	Carla-CP/M4T
KC677759	P19-23-CP	P19	770	potato	Al-Anbar	PVS	Carla-CP/M4T
KC677760	P19-24-CP	P19	770	potato	Al-Anbar	PVS	Carla-CP/M4T
KC677761	PC6	PC	887	potato	Baghdad	PVS	Car-F1/M4T
KC677767	P3-1	P3	895	potato	Al-Anbar	PVS	Car-F1/M4T
KC677768	P3-2	P3	894	potato	Al-Anbar	PVS	Car-F1/M4T
KC677769	P3-3	P3	894	potato	Al-Anbar	PVS	Car-F1/M4T
KC677770	P3-4	P3	891	potato	Al-Anbar	PVS	Car-F1/M4T
KC677762	P3-11	P3	878	potato	Al-Anbar	PVS	Car-F1/M4T
KC677763	P3-12	P3	898	potato	Al-Anbar	PVS	Car-F1/M4T
KC677764	P4-23	P4	898	potato	Al-Anbar	PVS	Car-F1/M4T
KC677765	P18-33	P18	898	potato	Al-Anbar	PVS	Car-F1/M4T
KC677766	P18-34	P18	883	potato	Al-Anbar	PVS	Car-F1/M4T
KC677771	CP2-8	CP2	854	cowpea	Al-Najaf	CPMMV	Car-F1/M4T
KC677772	CP2-9	CP2	854	cowpea	Al-Najaf	CPMMV	Car-F1/M4T

Sequence comparison revealed that all sequences isolated from potato samples collected from Iraq belong to PVS (Table 18) and that most of these sequences showed 99% maximum identity to partial CP/NB (nucleic acid binding protein)/3`UTR (3` untranslated region) regions of isolates from Syria (Acc. AB364945) and Iran (Acc.HQ875140 & HQ875132) (Table 19) and slightly lower similarity (95-97%) was shown to other isolates from, e.g. China (Acc. AY512653 & AJ889246), the USA (Acc. FJ813514 & FJ813513) and Germany (Acc. Y15612) (Table 19) (Appendix 6).Deduced aa sequences represented two putative viral proteins, namely the coat protein (CP) and nucleic acid binding protein (NB) and comparisons to GenBank sequences confirmed this (Appendix 7). Comparison of CP aa sequences showed maximum identity (100%) to PVS sequences from Syria (Acc. AB364945), Iran (Acc. HQ875140 & HQ875132), USA (Acc. FJ813514 & FJ813513) and China (Acc. AJ889246) whereas they showed less identity percentages to other GenBank sequences (Table 20). Phylogenetic analyses based on nucleotide sequence of partial CP/NB/3`UTR regions showed that all clones obtained from potato samples were grouped with other GenBank sequences of PVS which confirmed the occurrence of PVS in potato samples (Figure 30A). Similar results were obtained when Neighbor-Joining phylogenetic tree was constructed from deduced amino acid sequences of CP region (Figure 30B).

6.2.1.3. **Detection and sequence analyses of *Cowpea mild mottle virus* in cowpea**

Two clones were sequenced from cowpea samples CP2-8 and CP2-9 (Table 18). Comparison of sequences revealed that the highest identity was shown by the CP2-9 sequence, which was 80% identical to CP/NB/3`UTR sequences of *Cowpea mild mottle virus* (CPMMV: genus *Carlavirus*; family: *Betaflexiviridae*) from Venezuela (Acc. JX310549) and Ghana (Acc. HQ184471 The sequence CP2-8 also showed maximum identity to those Venezuelan and Ghanaian CPMMV sequences but at lower identity (77%) than CP2-9 (Table 21). Both CP2-8 and CP2-9 sequences showed less similarity (68-72% for CP2-8, and 70-73% for CP2-9) to isolates from other countries (Table 22& Appendix 6). Deduced aa sequences constructed from CPMMV showed that both CP2-8 and CP2-9 include two putative proteins from the carlavirus genome, namely the CP and NB protein when compared to GenBank sequences (Appendix 7). In addition, deduced aa comparison of the CP region revealed that CP2-8 and CP2-9 showed maximum identities to GenBank CP aa sequences (96 and 94% respectively) to an isolate from Brazil (Acc. DQ885940) (Table 22).

Table 19: PVS identity percentage of CP, NB and 3`UTR nucleotide sequence

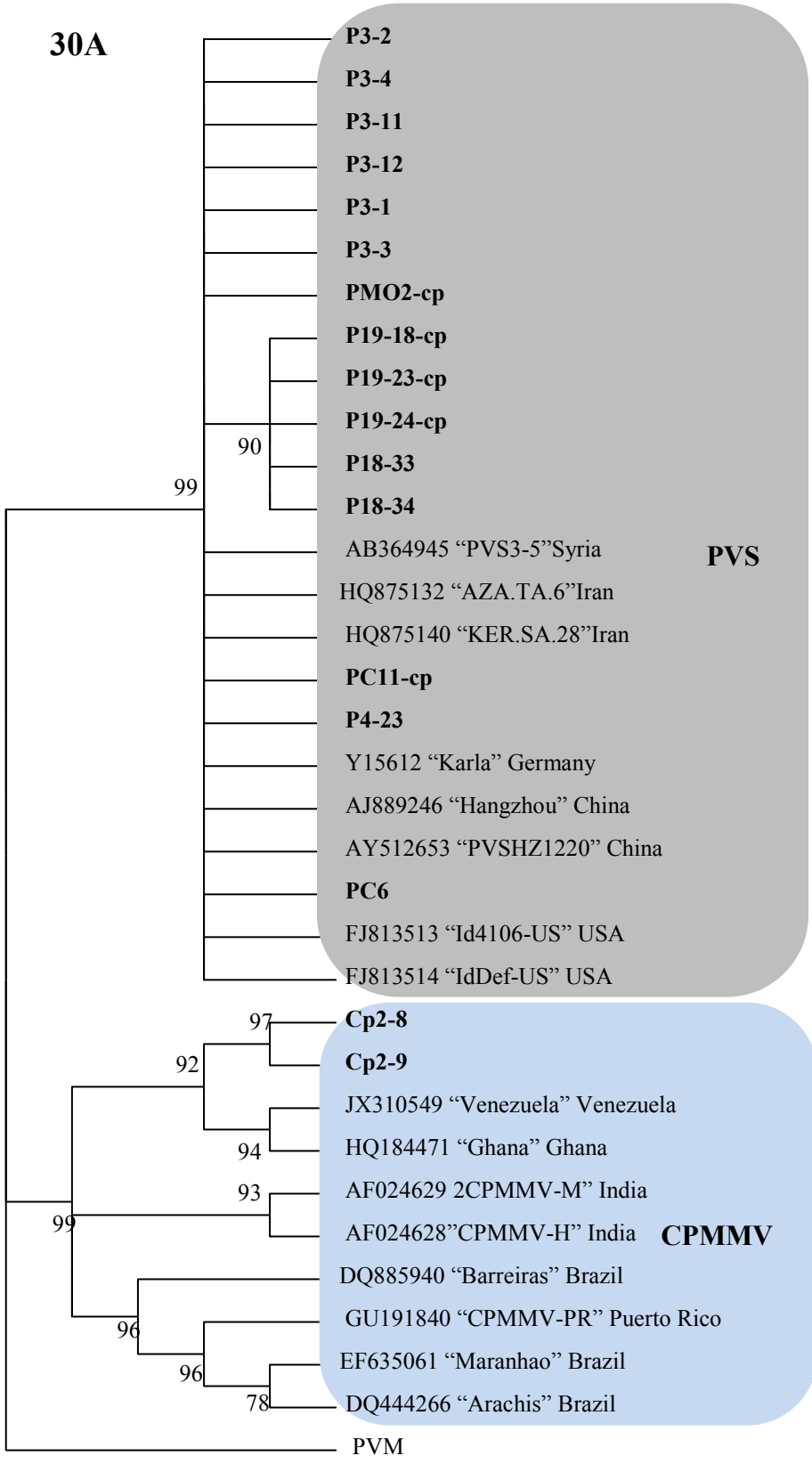
Identity percentage comparison between nucleotide sequences of (*Potato virus S*) isolated from samples in Iraq (bold letters) to those sequences found in GenBank

GenBank acc. code	Isolate/virus name	Identity percent (%)																
		PMo2-cp	**PC11-cp**	**P19-18-cp**	**P19-23-cp**	**P19-24-cp**	**PC6**	**P3-11**	**P3-12**	**P4-23**	**P18-33**	**P18-34**	**P18-34**	**P3-1**	**P3-2**	**P3-3**	**P3-4**	**Location**
AY512653	PVS-PVSHZ1220	96	95	96	96	96	95	96	95	96	96	96	96	96	96	96	96	China
FJ813514	PVS-IdDef-US	96	95	96	96	96	96	96	96	96	96	96	97	96	96	96	96	USA
FJ813513	PVS-Id4106-US	96	95	96	96	96	97	96	95	96	96	96	96	96	96	96	96	USA
AJ889246	PVS-Hangzhou	96	96	96	96	96	95	96	96	97	96	96	96	96	96	96	96	China
AB364945	PVS-PVS3-5	99	97	99	99	99	96	99	98	98	99	99	99	99	99	99	99	Syria
HQ875140	PVS-KER.SA.28	99	98	99	99	99	96	99	98	98	99	99	99	99	99	99	99	Iran
HQ875132	PVS-AZA.TA.6	98	97	98	99	99	95	98	98	98	98	98	99	99	99	98	98	Iran
Y15612	PVS-Karla	95	95	96	96	96	95	95	95	96	96	96	96	95	96	95	96	Germany

Table 20: Sequence comparison of PVS

Nucleotide identity (partial CP/NB/3`UTR) (upper right) and deduced amino acid similarity (partial CP) (lower left) of PVS isolated from Iraqi potato samples (bold letters) and GenBank sequences. Sequences 24 and 25 are CPMMV isolated from cowpea in this study. Evolutionary divergence conducted by pairwise comparison and calculated by p-Distance method from (MEGA5) (Tamura *et al*., 2011).

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	**PMO2-cp**		**98**	**100**	**100**	**100**	**96**	**100**	**99**	**99**	**100**	**99**	**99**	**100**	**99**	**100**	**96**	**96**	**96**	**97**	**100**	**100**	**99**	**96**	**57**	**58**
2	**PC11-cp**	100		**98**	**98**	**98**	**94**	**98**	**98**	**98**	**98**	**98**	**98**	**98**	**98**	**98**	**95**	**95**	**96**	**96**	**98**	**98**	**98**	**95**	**57**	**58**
3	**P19-18-cp**	100	100		**100**	**100**	**96**	**99**	**99**	**99**	**100**	**100**	**99**	**99**	**99**	**99**	**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
4	**P19-23-cp**	100	100	100		**100**	**96**	**99**	**99**	**99**	**100**	**100**	**99**	**99**	**99**	**99**	**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
5	**p1924-cp**	100	100	100	100		**96**	**99**	**99**	**99**	**100**	**100**	**99**	**99**	**99**	**99**	**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
6	**PC6**	98	98	98	98	98		**96**	**96**	**95**	**96**	**96**	**96**	**96**	**96**	**96**	**98**	**97**	**96**	**95**	**96**	**96**	**96**	**95**	**57**	**58**
7	**P3-11**	100	100	100	100	100	98		**100**	**99**	**99**	**99**	**100**	**100**	**100**	**100**	**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
8	**P3-12**	99	99	99	99	99	97	99		**99**	**99**	**99**	**100**	**100**	**99**	**100**	**96**	**96**	**96**	**96**	**99**	**99**	**99**	**96**	**57**	**58**
9	**P4-23**	97	97	97	97	97	95	97	96		**99**	**99**	**99**	**99**	**99**	**99**	**96**	**96**	**96**	**96**	**99**	**99**	**99**	**96**	**58**	**59**
10	**P18-33**	100	100	100	100	100	98	100	99	97		**100**	**99**	**99**	**99**	**99**	**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
11	**p18-34**	100	100	100	100	100	98	100	99	97	100		**99**	**99**	**99**	**99**	**96**	**96**	**96**	**96**	**99**	**99**	**99**	**96**	**57**	**58**
12	**P3-1**	100	100	100	100	100	98	100	99	97	100	100		**100**	**100**	**100**	**96**	**96**	**96**	**96**	**99**	**99**	**99**	**96**	**57**	**58**
13	**P3-2**	100	100	100	100	100	98	100	99	97	100	100	100		**100**	**100**	**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
14	**P3-3**	99	99	99	99	99	97	99	98	96	99	99	99	99		**100**	**96**	**96**	**96**	**96**	**99**	**99**	**99**	**96**	**57**	**58**
15	**P3-4**	100	100	100	100	100	98	100	99	97	100	100	100	100	99		**96**	**96**	**96**	**96**	**100**	**99**	**99**	**96**	**57**	**58**
16	Id4106-US	100	100	100	100	100	98	100	99	97	100	100	100	100	99	100		**98**	**97**	**96**	**96**	**96**	**96**	**96**	**58**	**58**
17	IdDef-US	100	100	100	100	100	98	100	99	97	100	100	100	100	99	100	100		**96**	**96**	**96**	**96**	**96**	**95**	**57**	**58**
18	Hangzhou China	100	100	100	100	100	98	100	99	97	100	100	100	100	99	100	100	100		**97**	**96**	**96**	**97**	**95**	**58**	**59**
19	China	98	98	98	98	98	96	98	97	95	98	98	98	98	97	98	98	98	98		**96**	**97**	**96**	**96**	**59**	**59**
20	PVS3-5 Syria	100	100	100	100	100	98	100	99	97	100	100	100	100	99	100	100	100	100	98		**100**	**99**	**96**	**58**	**59**
21	KER.SA.28 Iran	99	99	99	99	99	97	99	98	96	99	99	99	99	98	99	99	99	99	97	99		**100**	**96**	**58**	**59**
22	AZA.TA.6 Iran	100	100	100	100	100	98	100	99	97	100	100	100	100	99	100	100	100	100	98	100	99		**96**	**58**	**59**
23	Karla	98	98	98	98	98	96	98	97	95	98	98	98	98	97	98	98	98	98	98	98	97	98		**57**	**58**
24	**Cp2-8**	50	50	50	50	50	48	50	50	49	50	50	50	50	50	50	50	50	50	48	50	50	50	48		**93**
25	**Cp2-9**	52	52	52	52	52	50	52	52	51	52	52	52	52	52	52	52	52	52	50	52	52	52	50	95	


30A
P3-2
P3-4
P3-11
P3-12
P3-1
P3-3
PMO2-cp
P19-18-cp
P19-23-cp
P19-24-cp
90
P18-33
99
P18-34
AB364945 "PVS3-5"Syria
PVS
HQ875132 "AZA.TA.6"Iran
HQ875140 "KER.SA.28"Iran
PC11-cp
P4-23
Y15612 "Karla" Germany
AJ889246 "Hangzhou" China
AY512653 "PVSHZ1220" China
PC6
FJ813513 "Id4106-US" USA
FJ813514 "IdDef-US" USA
97
Cp2-8
92
Cp2-9
JX310549 "Venezuela" Venezuela
94
HQ184471 "Ghana" Ghana
93
AF024629 2CPMMV-M" India
99
AF024628"CPMMV-H" India
CPMMV
DQ885940 "Barreiras" Brazil
GU191840 "CPMMV-PR" Puerto Rico
96
96
EF635061 "Maranhao" Brazil
78
DQ444266 "Arachis" Brazil
PVM

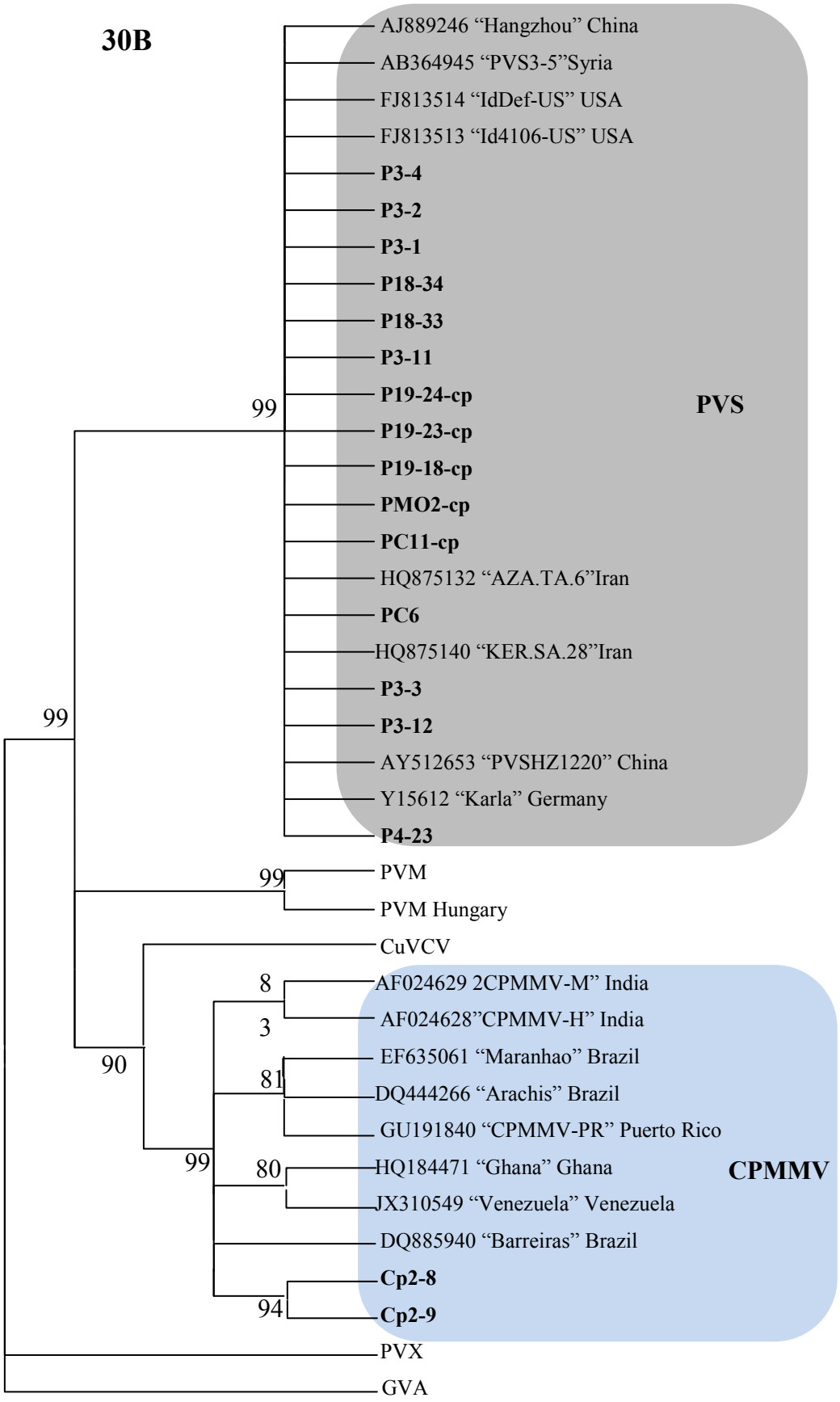

30B
AJ889246 “Hangzhou” China
AB364945 “PVS3-5”Syria
FJ813514 “IdDef-US” USA
FJ813513 “Id4106-US” USA
P3-4
P3-2
P3-1
P18-34
P18-33
P3-11
P19-24-cp
P19-23-cp
P19-18-cp
PMO2-cp
PC11-cp
HQ875132 “AZA.TA.6”Iran
PC6
HQ875140 “KER.SA.28”Iran
P3-3
P3-12
AY512653 “PVSHZ1220” China
Y15612 “Karla” Germany
P4-23
PVS
PVM
PVM Hungary
CuVCV
AF024629 2CPMMV-M” India
AF024628”CPMMV-H” India
EF635061 “Maranhao” Brazil
DQ444266 “Arachis” Brazil
GU191840 “CPMMV-PR” Puerto Rico
HQ184471 “Ghana” Ghana
JX310549 “Venezuela” Venezuela
DQ885940 “Barreiras” Brazil
Cp2-8
Cp2-9
CPMMV
PVX
GVA
99
99
90
99
8
3
81
80
94

Figure 30: Neighbor-Joining phylogenetic tree of carlaviruses

Neighbor-Joining phylogenetic topology based on nucleotide sequences of CP/3`UTR regions (A) and deduced amino acid sequences of CP region (B) of *Potato virus S* (PVS: genus *Carlavirus*; family: *Betaflexiviridae*) and *Cowpea mild mottle virus* (CPMMV: genus *Carlavirus*; family: *Betaflexiviridae*) sequences isolated from potato and cowpea respectively (bold letters) and GenBank sequences referred to as (GenBank acc. No. "isolate name" geographical location). PVM: *Potato virus M* (PVM: genus *Carlavirus*; family: *Betaflexiviridae*) (Acc. EF397747) and Hungary isolate (Acc. GQ923785), PVX: *Potato virus* X (PVX: genus *Potexvirus*; family: *Alphaflexiviridae*) (Acc. AF260640), GVA: *Grapevine virus A* (GVA: genus *Vitivirus*, family: *Betaflexiviridae*) (Acc. HQ676999) and CuVCV: *Cucumber vein-clearing virus* (CuVCV: genus *Carlavirus*; family: *Betaflexiviridae*) (Acc. JN591720).

Table 21: CPMMV identity percentage of CP, NB and 3`UTR nucleotide sequence

Identity percentage comparison between nucleotide sequences of *Cowpea mild mottle virus* (CPMMV) isolated from cowpea samples in Iraq (bold letters) to those sequences found in GenBank. *Identity percentage calculated using pairwise alignment EMBOSS Needle from www.ebi.ac.uk

GenBank Accession code	Isolate/virus name	Percentage nucleotide identity		
		Cp2-8	Cp2-9	Geographical Origin
JX310549	*CPMMV -Venezuela	76	80	Venezuela
HQ184471	*CPMMV-Ghana	75	80	Ghana
EF635061	*CPMMV- Maranhao	64	68	Brazil
DQ444266	*CPMMV-Arachis	64	68	Brazil
DQ885940	*CPMMV-Barreiras	66	67	Brazil
GU191840	*CPMMV-PR	64	65	Puerto Rico
AF024629	*CPMMV-M	67	69	India
AF024628	*CPMMV-S	69	70	India

Table 22: Sequence comparison of CPMMV

Nucleotide identity (partial CP/NB/3`UTR) (upper right) and deduced amino acid similarity (partial CP) (lower left) percentages (approximated) of *Cowpea mild mottle virus* isolated from Iraqi cowpea samples (bold letters) and GenBank sequences. Sequences 11 and 12 are PVS isolated from potato in this study. Evolutionary divergence was conducted by pairwise comparison and calculated by p-Distance method from MEGA5 (Tamura *et al*., 2011).

	Isolate	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	**Cp2-8**		**93**	**77**	**77**	**71**	**72**	**69**	**68**	**69**	**69**	**56**	**55**
2	**Cp2-9**	95		**80**	**80**	**73**	**73**	**70**	**70**	**70**	**70**	**57**	**56**
3	Ghana	91	93		**84**	**74**	**72**	**73**	**73**	**73**	**73**	**57**	**56**
4	Venezuela	93	95	98		**75**	**74**	**74**	**74**	**74**	**74**	**57**	**57**
5	CPMMV-M India	89	90	91	93		**80**	**74**	**74**	**74**	**75**	**57**	**56**
6	CPMMV-H India	89	91	92	94	97		**75**	**74**	**75**	**75**	**57**	**57**
7	Maranhao Brazil	91	94	95	96	92	95		**87**	**100**	**99**	**57**	**57**
8	Barreiras Brazil	94	96	96	98	93	94	98		**87**	**86**	**58**	**58**
9	Arachis Brazil	91	93	94	95	91	94	99	97		**99**	**57**	**57**
10	CpMMV-PR Puerto Rico	92	94	94	96	93	96	99	99	99		**57**	**57**
11	**PMO2-cp**	51	54	55	56	56	56	56	56	56	57		**98**
12	**PC11-cp**	51	54	55	56	56	56	56	56	56	57	99	

A phylogenetic tree generated by the Neighbor-Joining analysis of partial CP/NB/3`UTR nucleotide sequences confirmed that CP2-8 and CP2-9 belong to CPMMV as these sequences grouped within the CPMMV branch (Figure 30A). Results were supported through Neighbor-Joining analysis which generated a similar phylogenetic tree from deduced aa sequences of the CP region (Figure 30B).

6.2.2. **Detection of tombusviruses**

6.2.2.1. **Detection of *Grapevine Algerian latent virus***

Sample screening for tombusviruses by RT-PCR, using CIR1/CIR2 tombusvirus-specific primers (Koenig *et al*., 2004a&b), revealed the occurrence of tombusviruses in 11 out of 175 samples (Appendix 1). A diagnostic ~1.2 kb PCR product was amplified from RNA extracted from 2 eggplant (namely Eg1 and Eg3) and 9 tomato samples (ToIq1 - ToIq9) (Figure 31 and Figure 32). Sequencing of 10 cloned fragments produced 1119-1244 bp when sequenced in both directions using primers targeting the T7 and SP6 promoter regions (Table 23). Sequence analyses confirmed that 10 sequences obtained (Appendix 8) were 92-93% identical to sequences in GenBank for the CP region of *Grapevine Algerian latent virus* (GALV: genus *Tombusvirus*; family: *Tombusviridae*), infecting nipple fruit *Solanum mammosum* in Japan (Acc. AY830918) and *Gypsophila paniculata* L., in The Netherlands (Acc. AY500880) (Table 24). Deduced amino acid sequences of putative CP region of some of the sequenced clones (Appendix 9) showed 98% maximum identity to CP region of Apulia from Italy (Acc. AF540885) (Table 25). The remaining two sequences showed similarity to plant genome. Phylogenetic analyses based on nucleotide and amino acid sequences grouped all sequences in a separate clade within the GALV branch and diverged from other viruses from the same or other genera (Figure 15A&B). Mechanical inoculation trials, using dried leaf materials from both tomato and eggplant to *N. benthamiana*, tomato and eggplant did not succeed, and RT-PCR using CIR1/CIR2 primers did not generate GALV or any tombusvirus-expected PCR products from inoculated plants. Sequence comparison confirmed that the eggplant samples Eg2 and Eg4 were false positives (refered to as ±) (Appendix 1), as the 1, 2 kb fragments amplified from these two samples were similar to plant genome in the GenBank.

6.2.3. **Sample screening using alfamo-, cucumo-, potex- and tobamovirus specific primer sets**

RT-PCR using AMVF2/AMVR2 alfamovirus specific primer set could not detect

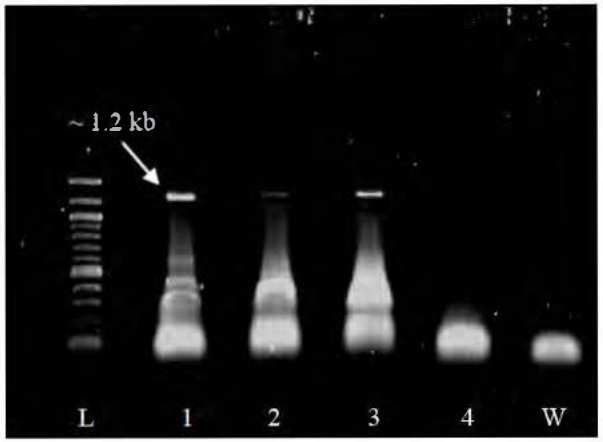


Figure 31: Amplification of tombusviruses genome by RT-PCR using CIR1/CIR2 primers from eggplant samples

Gel electrophoresis profile shows ~1.2 kb DNA fragment amplified by CIR1/CIR2 tombusvirus specific primers from eggplant samples. Lanes were as follows: 1: Eg1, 2: Eg2, 3: Eg3, 4: Eg4, W: water control. L: 100 bp DNA marker (New England Biolabs, UK). Eg2 sample (lane 2) shows false positive based on sequencing data.

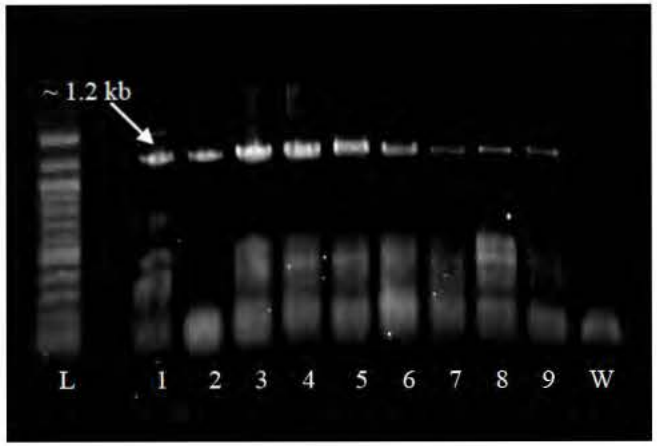


Figure 32: Amplification of tombusviruses genome by RT-PCR using CIR1/CIR2 primers

Gel electrophoresis profile shows ~1.2 kb DNA fragment amplified by CIR1/CIR2 tombusvirus specific primers obtained from collection of samples detected to be positive when tested in previous experiments. Lanes 1-8: tomato samples, 9: eggplant sample, W: water control and L: 100 bp DNA marker (New England Biolabs, UK).

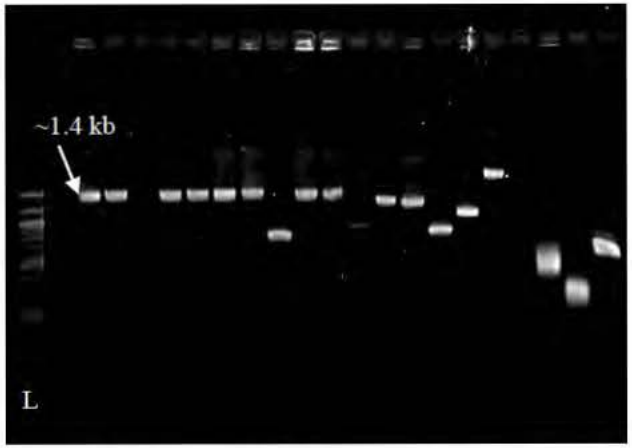


Figure 33: Screening of clones using T7/SP6 primers

Gel electrophoresis profile shows DNA fragment amplified by T7/SP6 primer set to screen *E. coli* colonies for recombinant plasmids. The ~ 1.4 kb fragments represent the DNA fragment amplified by CIR1/CIR2 primer set and confirm successful recombination. L: 100 bp DNA marker (New England Biolabs, UK).

Table 23: *Grapevine Algerian latent virus* clones profile

This table shows GALV clones isolated from eggplant and tomato samples from Baghdad, Iraq, and their accession numbers in GenBank and fragment length after cloning and sequencing.

GenBank accession code	clone	sample	crop	Fragment size/bp
JQ042281	Tom1	Egg1	eggplant	1244
JQ042290	Tom5	Egg3	eggplant	1243
JQ042282	Tom6	ToIq1	tomato	1243
JQ042283	Tom7	ToIq2	tomato	1244
JQ042284	Tom8	ToIq3	tomato	1244
JQ042285	Tom9	ToIq4	tomato	1244
JQ042286	Tom10	ToIq5	tomato	1244
JQ042287	Tom11	ToIq6	tomato	1242
JQ042288	Tom12	ToIq7	tomato	1244
JQ042289	Tom13	ToIq9	tomato	1119

Table 24: *Grapevine Algerian latent virus* identity percentage of coat protein nucleotide sequences

Identity percentage comparison of CP nucleotide sequences of GALV isolated from eggplant and tomato in Iraq (bold letters) and GenBank sequences. Tom1 and Tom5 were isolated from eggplant and Tom6-Tom13 were isolated from tomato plants.

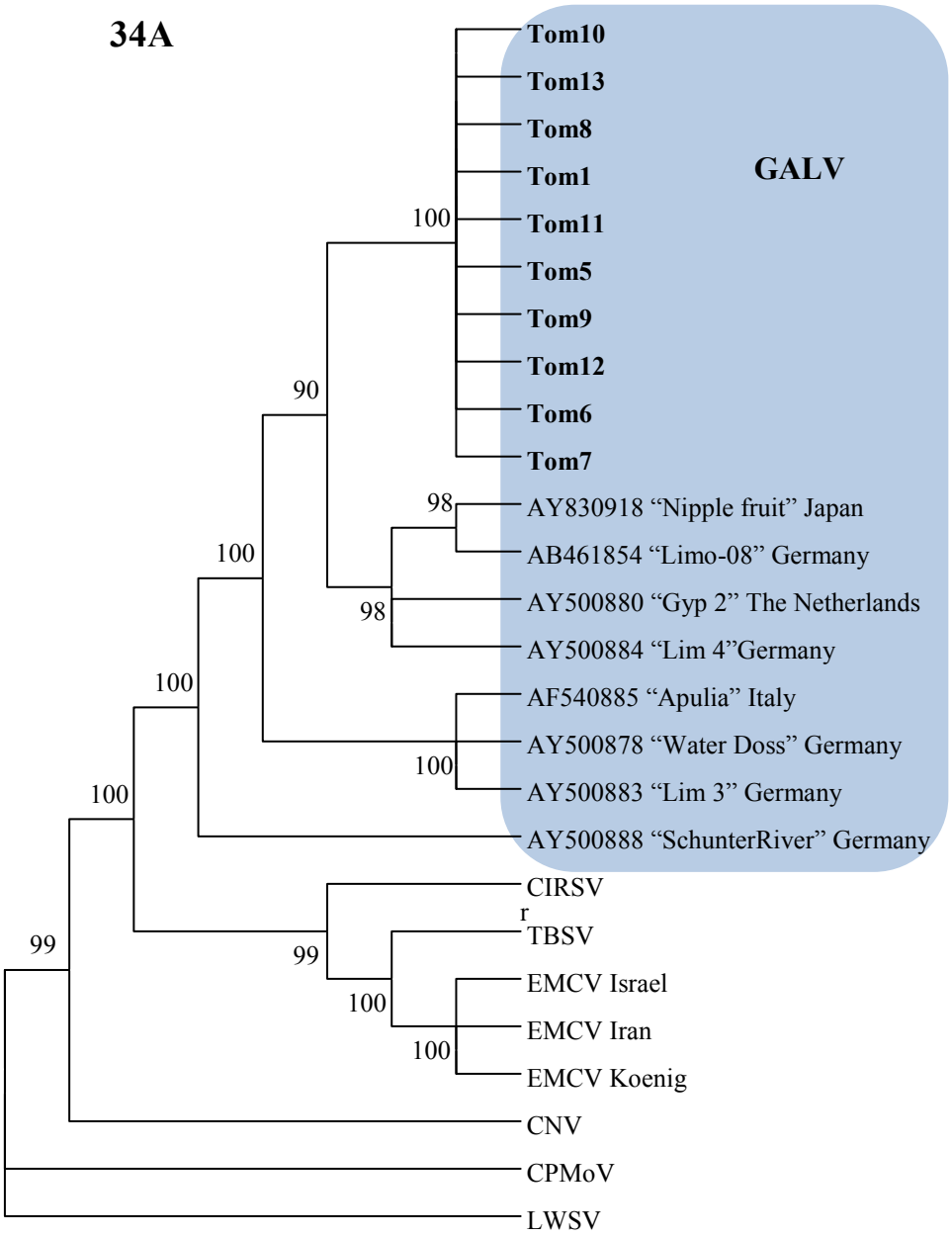
GenBank acc. Code	Isolate/virus name	Nucleotide identity percent%										Location
		Tom1	**Tom5**	**Tom6**	**Tom7**	**Tom8**	**Tom9**	**Tom10**	**Tom11**	**Tom12**	**Tom13**	
AY830918	Nipple fruit	93	93	93	93	93	92	93	93	93	93	Japan
AY500880	Gyp 2	93	93	93	93	93	93	93	93	93	93	The Netherlands
AB461854	Limo-08	92	92	92	92	92	92	92	92	92	92	Germany
AY500884	Lim 4	91	91	91	91	91	91	91	91	91	91	Germany
AY500878	Water Doss	90	90	90	90	90	90	90	90	90	91	Germany
AY500883	Lim 3	90	90	90	90	90	90	90	90	90	91	Germany
AF540885	Apulia	90	90	90	90	90	90	90	90	90	91	Italy
AY500888	Schunter River	84	84	84	84	84	84	84	84	84	84	Germany
AY579432	**Tomato bushy stunt virus*	43	43	42	43	42	42	43	43	42	37	Japan
X85215	**Carnation Italian ring spot virus*	55	53	55	54	55	54	55	55	55	53	Italy
M25270	**Cucumber necrosis virus*	49	50	49	49	50	49	49	49	49	47	-
U20976	**Cowpea mottle virus*	42	42	44	42	42	42	42	42	43	42	-
X94560	**Leek white stripe virus* genome	37	37	37	38	37	37	37	37	37	38	Italy
EU327529	**Eggplant mottled crinkle virus*	51	51	51	52	51	51	51	51	51	52	Iran
AY500890	**Eggplant mottled crinkle virus*	52	52	52	52	51	52	52	52	52	50	Germany
FJ872112	**Eggplant mottled crinkle virus*	53	53	53	53	53	53	53	53	53	51	Israel

*Pairwise alignment

Table 25: Comparison of GALV CP sequences

Nucleotide identity (upper right) and deduced amino acid similarity (lower left) CP sequences of *Grapevine Algerian latent virus* sequences isolated from Iraqi samples (bold letters) with corresponding sequences from GenBank compared to nt sequences identity percent (in bold numbers). Evolutionary divergence conducted by pairwise comparison and calculated by p-Distance method from (MEGA5) (Tamura *et al*., 2011).

	Isolate/virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	**Tom1**		**100**	**100**	**99**	**100**	**99**	**100**	**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
2	**Tom5**	100		**99**	**99**	**100**	**99**	**100**	**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**15**	**36**	**37**	**35**
3	**Tom6**	100	99		**99**	**100**	**99**	**100**	**100**	**100**	**100**	**94**	**94**	**92**	**93**	**91**	**91**	**91**	**85**	**38**	**37**	**14**	**36**	**37**	**35**
4	**Tom7**	100	100	100		**99**	**98**	**99**	**99**	**99**	**99**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**86**	**38**	**39**	**15**	**37**	**38**	**36**
5	**Tom8**	100	100	100	100		**99**	**100**	**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
6	**Tom9**	98	98	98	98	98		**99**	**99**	**99**	**99**	**93**	**92**	**91**	**92**	**90**	**90**	**90**	**84**	**36**	**35**	**14**	**35**	**36**	**34**
7	**Tom10**	100	100	100	100	100	98		**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
8	**Tom11**	100	99	99	100	100	98	100		**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
9	**Tom12**	100	99	100	100	100	98	100	99		**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**15**	**36**	**37**	**35**
10	**Tom13**	100	100	100	100	100	98	100	100	100		**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
11	Japan	96	96	96	96	96	95	96	96	96	96		**97**	**95**	**99**	**93**	**93**	**93**	**86**	**37**	**37**	**14**	**35**	**36**	**33**
12	GYP2	97	97	97	97	97	95	97	97	97	97	98		**96**	**96**	**93**	**92**	**93**	**86**	**37**	**37**	**14**	**36**	**37**	**35**
13	Lim 4	96	96	96	96	96	95	96	96	96	96	96	97		**95**	**91**	**91**	**91**	**85**	**37**	**36**	**14**	**36**	**37**	**35**
14	Limo 08	97	96	96	97	97	95	97	97	96	97	100	99	97		**92**	**92**	**92**	**86**	**38**	**38**	**14**	**34**	**36**	**33**
15	Water Doss	97	97	97	97	97	95	97	97	97	97	96	97	95	97		**100**	**100**	**86**	**40**	**38**	**14**	**37**	**38**	**36**
16	Lim 3	97	96	96	97	97	95	97	96	96	97	96	97	95	96	100		**100**	**86**	**39**	**37**	**14**	**37**	**38**	**36**
17	Apulia	98	97	97	98	98	96	98	97	97	98	97	98	96	97	100	99		**86**	**40**	**37**	**14**	**37**	**38**	**36**
18	Schunter River	95	94	94	95	95	93	95	95	94	95	95	95	94	95	95	95	96		**37**	**38**	**15**	**39**	**41**	**39**
19	TBSV	57	57	57	57	57	56	57	57	57	57	57	57	57	58	58	58	58	57		**52**	**7**	**66**	**67**	**66**
20	CIRSV	64	64	63	64	64	62	64	64	63	64	63	63	63	63	64	64	64	64	69		**10**	**47**	**48**	**47**
21	CNV	43	44	43	43	43	43	43	43	43	43	45	44	44	44	43	43	43	46	44	45		**7**	**8**	**7**
22	EMCV Iran	60	60	60	60	60	59	60	60	60	60	60	60	59	59	60	60	60	60	75	68	42		**99**	**97**
23	EMCV Koenig	60	60	60	60	60	59	60	60	60	60	60	60	59	59	60	60	60	60	77	69	42	98		**98**
24	EMCV Israel	59	59	59	59	59	58	59	59	59	59	58	58	58	58	59	59	59	59	75	69	42	96	97	


34A
Tom10
Tom13
Tom8
Tom1
Tom11
Tom5
Tom9
Tom12
Tom6
Tom7
GALV
AY830918 “Nipple fruit” Japan
AB461854 “Limo-08” Germany
AY500880 “Gyp 2” The Netherlands
AY500884 “Lim 4”Germany
AF540885 “Apulia” Italy
AY500878 “Water Doss” Germany
AY500883 “Lim 3” Germany
AY500888 “SchunterRiver” Germany
CIRSV
TBSV
EMCV Israel
EMCV Iran
EMCV Koenig
CNV
CPMoV
LWSV
100
90
98
100
98
100
100
100
99
99
100
100

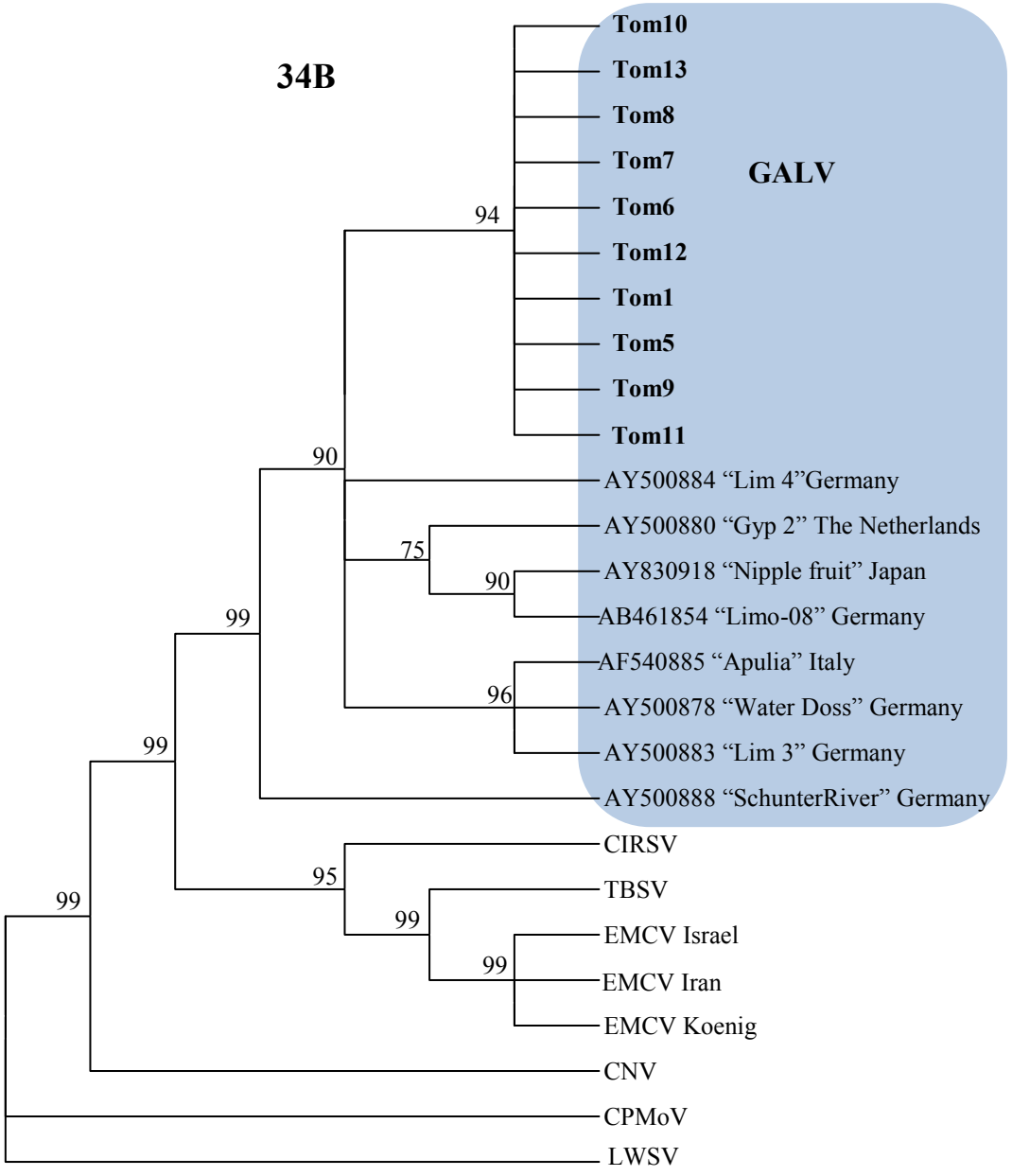

34B
GALV
Tom10
Tom13
Tom8
Tom7
Tom6
Tom12
Tom1
Tom5
Tom9
Tom11
AY500884 “Lim 4”Germany
AY500880 “Gyp 2” The Netherlands
AY830918 “Nipple fruit” Japan
AB461854 “Limo-08” Germany
AF540885 “Apulia” Italy
AY500878 “Water Doss” Germany
AY500883 “Lim 3” Germany
AY500888 “SchunterRiver” Germany
CIRSV
TBSV
EMCV Israel
EMCV Iran
EMCV Koenig
CNV
CPMoV
LWSV
94
90
75
90
99
96
99
99
95
99
99

Figure 34: Phylogenetic tree of *Grapevine Algerian latent virus*

Neighbor-Joining phylogenetic analysis of CP nucleotide sequences (A) and CP amino acid sequences (B) of GALV isolated from eggplant and tomato (bold letters) and GALV GenBank sequences referred as (GenBank acc. No. "isolate name" geographical location). TBSV: *Tomato bushy stunt virus* (TBSV: genus *Tombusvirus*; family: *Tombusviridae*), CIRSV: *Carnation Italian ring spot virus* (CIRSV: genus *Tombusvirus*; family: *Tombusviridae*), EMCV: *Eggplant mottled crinkle virus* (EMCV: genus *Tombusvirus*; family: *Tombusviridae*), CNV: *Cucumber necrosis virus* (CNV: genus *Tombusvirus*; family: *Tombusviridae*), CPMoV: *Cowpea mottle virus* (CPMoV: genus *Carmovirus*; family: *Tombusviridae*) & LWSV: *Leek white stripe virus* (LWSV: genus *Necrovirus*; family: *Tombusviridae*).

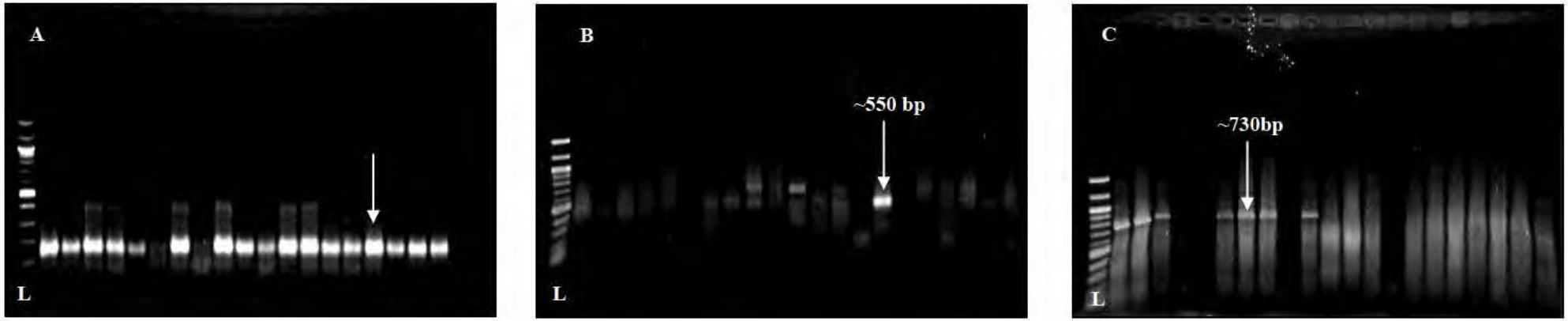


Figure 35: Screening potato and vegetable samples for different virus groups using group specific primers

Gel electrophoresis profile shows non-specific fragments amplified by CPTALL5/CPTALL3 a cucumovirus specific primer set from tomato (A) and false positive fragments amplified by Tobamo3/Tobamo2 a tobamovirus specific primer set from cucumber(B) and Potex1'Potex2 a potexvirus specific primer set from broad bean(C). The expected size fragments in (B) and (C) were false positives based on sequencing results when sequence comparison against GenBank sequences revealed that fragment amplified by tobamo- and potexvirus specific primer sets belong to plant genome. L: 1 kb and 100 bp DNA markers respectively (New England Biolabs, UK).

alfamoviruses in all potato and vegetable samples tested when no fragments were amplified from the tested samples. The expected fragment size for AMVF2/AMVR2 primer set is 669 bp (Xu & Nie, 2006). Non-specific fragments only were amplified from potato and vegetable samples when screened using CAPALL5/CAPALL3 cucumovirus specific primer set, as this primer set could not amplify the expected fragment size (938-966 bp) (Choi *et al.*,1999) from tested samples (Figure 35). Both Potex1/Potex2 potexvirus primer set and Tobamo3/Tobamo2 tobamovirus specific primer set could amplify the expected size fragments ~550 bp and ~730 bp, respectively (Gibbs *et al.*, 1998; 2004) (Figure 35), however, sequencing results and analyses revealed that these fragments were amplified from plant genome. No positive control samples were included in the analyses with these other primers.

6.3. **Discussion**

Due to a lack of molecular information regarding carlaviruses in Iraq, this study was carried out to investigate the occurrence of carlaviruses in potato and vegetable samples collected from Baghdad, Anbar and Najaf provinces in Iraq. Occurrence of carlaviruses in Iraq was confirmed by sequence analyses when all 15 sequences isolated from potato samples collected from fields in Baghdad and Anbar Provinces in Iraq grouped within PVS clade. PVS sequences isolated showed 100% maximum CP/NB/3`UTR nt sequence identity and CP aa sequence similarity when compared to each other (Table 20). All sequences obtained from potato showed high nucleotide sequence identities to isolates from Syria (Acc. AB364945), and Iran (Acc. HQ875140 & HQ875132) except for PC6 that showed high sequence identity to an isolate from the USA (Acc. FJ813513), which may indicate that sequences obtained from Iraq are closely related to Syrian and Iranian isolates. Detection of PVS was further supported when analyses of CP aa sequences showed 100% similarity to those PVS isolates from Syria, Iran, China and USA. Neighbor-Joining phylogenetic tree constructed from CP aa sequences showed high relatedness among PVS sequences from Iraq to those from the GenBank at isolate level. Sequence comparison and NJ phylogenetic relatedness of PVS were highly supported, as CP is highly conserved region and hence recommended by ICTV for carlavirus species and strain demarcation, (Adams *et al*., 2004; Ryu & Lee, 2009; ICTVdb 2012). High similarities among Iraqi sequences and to the PVS sequences from the GenBank may suggest that PVS has been introduced into Iraq from Iran and/ or Syria through potato tubers as Iraqi farmers import potato tuber seeds from these two countries from eastern and western sides of Iraq in the recent years. We need to test more potato samples from different locations, especially from potato growing area approaching to Iraqi boarders, to confirm that

PVS was introduced into Iraq from neighbouring countries. CPMMV was the second carlavirus species detected in Iraqi samples. Two sequences were isolated from cowpea samples collected from Najaf Provence in Iraq. CP2-8 and CP2-9 nt sequences were 93% identical in partial CP/NB/3`UTR regions and 95% similar in partial CP aa sequences (Table 21).CP2-9 showed maximum (80%) nucleotide sequence homology to CPMMV from Venezuela (Acc. JX310549) and Ghana (Acc. HQ184471), whereas CP2-8 showed 75-76% maximum identity (Table 21) and 77% nt identity (Table 22) to the same isolates. Comparison of amino acid sequences of the CP region showed 96% and 94% identity for the CP2-8 and CP2-9, respectively, to a Brazilian isolate (DQ885940). Based on these identities CP2-9 and CP2-8 can be considered as strains of CPMMV as these sequences were higher than 72% nucleotide identity and 80% aa CP homology to the GenBank sequences of CPPMV according to ICTV criteria for carlavirus species and strain demarcation (Adams *et al*., 2004; Ryu & Lee, 2009; ICTVdB 2012). Neighbor-Joining phylogenetic tree based on nt nucleotide sequences and CP aa sequence diverged CP2-9 and CP2-8 in separated clade from other CPMMV GenBank sequences. This divergence was supported by 97% and 94 % bootstrap values for nucleotide and aa sequences based trees respectively (Figure 30a&b). This divergence suggests CP2-8 and CP-9 sequences could be distinct Iraqi isolates. Further comparison was performed when *Cucumber vein-clearing virus* (CuVCV), a whitefly transmitted carlavirus, was included within NJ phylogenetic tree to support that CP2-8 and CP2-9 are closely related to CPMMV GenBank sequences, as CuVCV is the closest among other carlaviruses to CPMMV (63% CP sequence identity) (Menzel *et al*., 2011). Thus, the Neighbor-Joining phylogenetic topology of CP aa sequences grouped CP2-8 and CP2-9 within CPMMV and diverged from the CuVCV clade, which was supported by 90% bootstrap value.

This study is the first record of CPMMV in Iraq as no previous studies have been carried out regarding detection of CPMMV in Iraq besides providing new molecular information regarding CPMMV in Iraq. CPMMV is likely to have been introduced into Iraq through infected cowpea seeds as this virus can be seed-borne by 2-90% in leguminous host (EPPO/CABI, 1997). The possible origin of the virus in Iraq is still unknown as sequences isolated did not show high diversity and shown a relatively high CP homology of 96% to GenBank sequences. However, CPMMV could be introduced to Iraq from Jordan (Mansour *et al*., 1998) and Iran (Tavasoli *et al*., 2009) as this virus has been reported in these two countries bordering Iraq. In addition, CPMMV sequences isolated showed the same

maximum identity percent of 80% to sequences from Venezuela (Acc. JX310549) (76-80%), Ghana (75-80%) and low sequence identity less than 72% to other GenBank sequences from India, Brazil and Puerto Rico. Phylogenetic analyses showed that CPMMV sequences isolated were distinct isolates may indicate that Iraqi and GenBank sequences are not closely related. Further analyses; however, are required to confirm the relatedness among sequences isolated and the Iranian and Jordanian CPMMV isolates/strains as sequence data for these are currently unavailable. False carlavirus infection was detected in eggplant samples when Car-F1/M4T primers amplified the 950 bp fragments from three samples, however sequence comparison comfirmed that the amplified fragments were plant genome. In this study, tombusviruses were investigated in potato and vegetable samples collected from Iraq. Symptoms observed in the fields of Baghdad on eggplant resembled those induced by *Tomato bushy stunt virus* (TBSV) or *Eggplant mottled crinkle virus* (EMCV) (leaf mottling and malformation and fruit blisters and malformation) (Makkouk *et al*., 1981; Martelli *et al*., 2001). As both TBSV and EMCV are members of the genus *Tombusvirus*, CIR1/CIR2 primer set (Koenig *et al*., 2004a&b) was used to screen the four symptomatic eggplant samples collected. The remaining potato and vegetable samples were screened by CIR1/CIR2 later on. This non-degenerate primer set has been designed from the highly conserved motifs in CP gene. It has been used to amplify coat protein region for many tombusvirus members (Koenig *et al*., 2004a&b). Sequence comparison showed unexpected results when sequences obtained from eggplant samples showed homology to GALV rather than TBSV or EMCV as these two viruses have been reported to infect eggplant naturally. Unlike other two tombusviruses, GALV has been reported not only to infect eggplant experimentally but also to induce local lesion infection on inoculated leaves only and not to induce systemic infection on eggplant (Ohki *et al*., 2006; Fujinaga *et al*., 2009). In addition, another unexpected result was obtained when sequences isolated from tomato samples showed homology to GALV when compared to the GenBank database as GALV was not shown to infect tomato naturally, only experimentally (Ohki *et al*., 2006; Fujinaga *et al*., 2009). All eggplant and tomato samples shown to be infected by GALV were collected from the same greenhouse in Baghdad. Eggplant and tomato could potentially be infected by GALV through contaminated soils in greenhouse as all tombusviruses are efficiently transmitted in the soil, even without biological vectors, due to high concentration of tombusvirus particles in root system as this virus has been isolated from infected plants (Lommel & Sit, 2009). Another possible source for GALV infection is the water as intact particles of this virus has been isolated from rivers and ground

water and used for mechanical inoculation (Koenig et al., 2004a&b) which suggests that eggplant and tomato might be infected through irrigation water. All sequences obtained from eggplant and tomato showed minimum aa similarity of up to 93% to GALV CP region and they could belong to a new strain of existing GALV species (Table 25). Based on ICTV criteria of species demarcation and classification, members within the genus *Tombusvirus* are separated as species at less than 85% nucleotide identity in CP region (ICTVdB, 2012). Besides, all sequences showed the highest homology (97-98%) to CP aa region of GALV isolate Apulia (Acc. AF540885) from Italy, whereas they showed maximum homology of 93% to CP nucleotide sequence of Nipple fruit and Gyp2 isolates of GALV from Japan (Acc. AY830918) and the Netherlands (AY500880) respectively. Despite the high homology percentages, both the Neighbor-Joining phylogenetic trees constructed from aa and nucleotide sequences of CP showed that all GALV sequences isolated diverged from other GenBank sequences within the GALV group supported by 99% and 94% bootstrap values for nt and aa based trees respectively (Figure 34 a&b). Based on new host range, sequence data and phylogenetic divergence, GALV sequences may belong to separate strain. However, further biological and serological studies are required to confirm that GALV isolated is a unique strain. Infectivity tests using fresh infected plant material (leaves or roots) or contaminated soil are required to confirm the infectivity of GALV isolate obtained to tomato and eggplant as mechanical inoculation trials using dried leaf materials from both tomato and eggplant to *N. benthamiana*, tomato and eggplant did not succeed. Thus, the means of GALV infectivity to eggplant and tomato is still unknown. The infectivity of the GALV isolated might be slightly affected in dried plant materials, as the GALV is highly stable in vitro and can survive in dried samples (Mehle & Ravnikar, 2012). However, low GALV titration in infected samples used for inoculation might result in infectivity failure. The infected plant materials used for mechanical inoculation trials were leaves, whereas GALV titre is higher in plant roots rather than the leaves (Lommel & Sit, 2009). Another possible reason for low titration is that tombusviruses generated defective interfering RNAs (DI RNAs) during virus replication in infected tomato and eggplant samples. DI RNAs are sub-viral replicons, not packaged into virions. They are deletion mutants derived from the viral genome, due to errors in replication through rearrangement or recombination (Lommel & Sit, 2009). DI RNAs replicate to very higher titre and lead to interference with the viral genome replication (Lommel & Sit, 2009). They have been found to affect the tombusviruses pathogenicity alongside the titration causing attenuation of symptoms and suppress the infection (Lommel & Sit, 2009). Thus, it is

useful to include serological techniques (like ELISA) together with nucleic acid based techniques to quantify the virus titration. Serological techniques that target the virus coat protein will enable to measure GALV titration based on the intact virions rather than the DI RNAs.

The number of GALV sequences available in the GenBank is quite low (≤ 10 sequences). Further investigations are required to resolve whether tomato and eggplant GALV isolates were originated in Iraq or in other countries. The virus could be introduced to Iraq through infected plant materials or through river water as a possible long distance transmission. GALV and other tombusviruses were isolated from river and underground water (Mehle & Ravnikar, 2012) while some tombusviruses were shown to be seed transmissible in certain plant species (Lommel & Sit, 2009). No seed transmission has been reported for GALV. The possible route of GALV infection to eggplant and tomato plants could be through roots by irrigation water, as GALV and other tombusviruses are highly stable and can survive in both water and soil and easily transmitted through root system (Lommel & Sit, 2009; Mehle & Ravnikar, 2012). Study of the full-length of GALV detected in tomato and eggplant samples is required to identify whether the virus is a recombinant tombusvirus. Recombination occurs in tombusviruses, between both homologous and heterologous sequences, to repair the damaged or deleted 3`end (Lommel & Sit, 2009). Tombusviruses suppress gene silencing defence mechanism in plant by the gene ORF4 (P19), which locates at the 3`end of the virus genome (Hull, 2009). A recombination in P19 gene between GALV detected and other tombusviruses infecting tomato and eggplant may enable GALV to infect tomato and eggplant. The losses on tomato and eggplant due to GALV infection is unknown and should be addressed as the virus may impact tomato and eggplant production in Iraq in future.

However, we could not detect potexviruses, tobamoviruses, alfamoviruses and cucumoviruses in all samples and reasons for this are unknown. One reason may be that the failure to detect these viruses in the tested samples might be genuine due to these samples being free of the respective viruses, or infected by members from other groups rather than the viruses targeted by the primers used. Other possible reasons are that the primer sets could not detect Iraqi viruses or isolates due to these showing variability outside that detected by the primers (Zheng *et al.*, 2008a). Alternatively, the viruses could not be recovered from the samples due to low quality of the preserved samples as these had been dried either by air or calcium chloride; the resulting viral RNA after CTAB extraction is likely to be of lower

quality and quantity than if these samples had been used fresh (Seal & Coates, 1998). These group specific primer sets could not be optimized using a positive control as no positive control could be obtained from virus infected samples from Iraq. It was unreliable to use a positive control from other sources could not be used as these primer sets were designed from conserved motifs in sequences available in GenBank, not from Iraqi isolates. It may result in primer mismatch, as conserved motifs for these groups may not occur in Iraqi isolates. These group specific primer sets, therefore, should be optimized using true positive samples isolated from Iraqi potato and vegetables to obtain reliable results.

CHAPTER 7: GENERAL DISCUSSION

7. General discussion and conclusions

7.1. Detection of potato and vegetable viruses occurred in Iraq using molecular techniques

This study was carried out to investigate the molecular diversity of plant viruses in potato and vegetables in Iraq; as such molecular information concerning plant viruses from Iraq is absent. Based on the economic importance and incidence of plant viruses on potato and vegetables worldwide, eight virus genera were investigated in 175 samples from symptomatic and asymptomatic potato and vegetable plants collected from fields located in three governorates in Iraq (Baghdad, Anbar and Najaf). PCR/RT-PCR approaches, using genus-/family-specific degenerate primer sets published in the scientific literature, were used to screen the 175 samples for the wide diversity of potyviruses, begomoviruses, carlaviruses, tombusviruses, potexviruses, cucumoviruses, tobamoviruses and alfamoviruses reported to occur in these host plants worldwide. In addition, the RCA approach was used to screen samples for DNA viruses which have a circular genome. Products obtained from PCR/RT-PCR and RCA were cloned and sequenced and data obtained were used for phylogenetic analyses. When combined with cloning and sequencing, PCR/RT-PCR using group-specific primers and RCA techniques were shown to be reliable approaches to detect plant viruses in Iraqi samples. Using these techniques, seven different viruses belonging to four virus groups within four families were identified up to isolate level in potato and vegetable samples. In addition, the approaches enabled the study of the genetic relationships of these viruses isolated from Iraqi potato and vegetable samples to viruses from different geographical regions by comparing their sequences to those available in GenBank. Using group specific primers and RCA enabled the screening of several viruses within the same genera or families in one reaction, which helped to reduce the time, consumables and reagents required to test large numbers of samples rather than using specific primers designed to detect individual viruses (James *et al.*, 2006). Another advantage of using group-specific primers and RCA was to enable the detection of new viruses which may occur in potato and vegetable samples collected from Iraq, as group-specific primers have been designed to target conserved motifs within genera or families (James *et al.*, 2006; Webster, 2008) and RCA for amplification of any circular DNA.

7.2. Molecular epidemiology of potato and vegetable viruses isolated from Iraq

Molecular techniques are being used increasingly to study plant virus epidemiology (Thresh *et al.*, 2003) and biodiversity (Roossinck, 2011; Paga´n *et al*., 2012). The approach, molecular epidemiology, has been introduced for the first time in 2000 to study virus populations, virus/vector relationships and the interactions between virus strains, in order to resolve the origin of plant viruses and state epidemiological effects (Thresh *et al*., 2003). Among the tested samples from Iraq, the number of viruses that were found to infect tomato samples was the highest as samples from zucchini squash, broad bean and cowpea were infected by ZYMV, BYMV and CoMMV, respectively. Mixed infections were detected in some tomato and potato samples; some tomato samples showed mixed infection of TYLCV and GALV; while some of the potato samples tested were mixed infected with PVY and PVS.

Amongst the other virus groups screened for, potyviruses showed the highest incidence of 30.8% (54 out of 174) infection in potato and vegetable samples based on RT-PCR using family specific primers Sprimer/M4T (Gibbs & Mackenzie, 1997; Chen *et al.*, 2001a; Gibbs *et al*., 2003). The maximum incidence of 66.6 % (16 out of 24 samples) was detected in broad bean samples were detected to be infected by potyviruses (BYMV) and this was followed potato with 49.1% incidence (30 out of 61). Carlaviruses were the next in incidence with 12% (21 out of 175) detection in potato and vegetable samples based on the genus specific primer set Car-F1/M4T (Nie *et al*., 2008). The maximum incidence of 47.8% (11 out of 23 samples) was detected in cowpea samples and the next highest incidence of 16.4% (10 out of 61 samples) was in potato. In eggplant, products of the expected size were detected for carlaviruses in three out of four samples tested; however, sequence analyses showed that these fragments were amplified from the plant genome. Hence, these were considered negative for virus infection.

Based on CIR1/CIR2 (Koenig *et al*., 2004a&b) the genus-specific primers, tombusviruses ranked the third amongst other groups in terms of virus incidence of 7.4% in potato and vegetable samples. Eggplant samples showed the maximum incidence of 100% for tombusvirus infection when tombusviruses (GALV) were detected in all four tested samples. Tomato samples were the next highest for tombusvirus incidence with 16.67% (nine out of 54 tomato samples) positive when tested.

Using the Deng primer set (Deng *et al.*, 1994), begomovirus incidence was the lowest at 3.43% among the virus groups screened in potato and vegetable samples. Begomoviruses incidence of 11.1% was in tomato samples only as no begomoviruses were detected in other

tested samples. No virus incidence was detected for tobamovirus, potexvirus, alfamovirus and cucumovirus groups in any of potato and vegetable samples when screened using genus-specific primers.

Based on comparison of CP regions to sequences found in GenBank, data obtained from molecular screening of potato and vegetable samples showed that most sequences isolated belong to insect-transmissible viruses, as CP involvement in aphid transmission for PVY, ZYMV, BYMV and PVS (Adams *et al*., 2005; Adams, 2009; Ruy & Lee, 2009). ZYMV IRQ isolate showed the same 99% identity to ZYMV-(NAT (Acc. EF062582), AG (Acc. EF062583) & B (Acc. AY188994)) GenBank sequences from Israel as NAT (a severe field strain), AG, and B (laboratory constructed mutants) sequences are non-aphid transmissible (Gal-On & Raccah 2000; Desbiez *et al*., 2003). Deduced aa comparisons showed that ZYMV IRQ CP sequence include DAG domain required for aphid transmission, whereas, NAT, B and AG isolates have a DTG domain within the CP region (Appendix 2) occurred in non-aphid transmissible isolates (Lecoq & Desbiez, 2009). DAG domain interaction with PTK domain in HC-Pro within potyvirus genome causes the aphid transmission in potyviruses (Ng & Falk 2006; Hull, 2009; Lecoq & Desbiez, 2009). The DAG domain is replaced by NAG domain in BYMV CP in aphid transmissible isolates (Larsen *et al.*, 2008) as all PVY and BYMV sequences isolated shown to have DAG and NAG domains in CP region; respectively (Appendix 2).

This highlights the important role that insect vectors may play with regard to virus transmission in Iraq for short distance transmission. This role involves the spread of plant viruses in Iraqi potato and vegetables in short distance transmission, especially for those of low incidence seed transmission like ZYMV and BYMV (Albrechtsen, 2006; Tobias *et al.*, 2008). Four of the viruses detected in potato and vegetable samples were aphid-transmissible in non-persistent manner, when PVY, BYMV, ZYMV and PVS sequences isolated showed high similarity in CP region to aphid-transmissible isolates from GenBank. CP (together with HC-Pro and NB genes for potyviruses and carlaviruses respectively) is required for aphid transmission for these viruses (Adams *et al*., 2005; Adams, 2009; Ryu & Lee, 2009). Two viruses have been reported to be whitefly transmissible, i.e. CPMMV and TYLCV, in semi-persistent and persistent manners, respectively (Ryu & Lee, 2009; Díaz-Pendón *et al.*, 2010). All TYLCV sequences isolated from tomato showed high identities to GenBank TYLCV isolates which are whitefly transmissible begomoviruses (Díaz-Pendón *et al.*, 2010); whereas, CPMMV isolated from cowpea samples has been reported as a whitefly-transmissible

carlavirus (Naidu *et al*., 1998; Ryu & Lee, 2009). Aphid-borne viruses showed a higher incidence than whitefly-borne viruses in potato and vegetable samples, which may indicate that aphids have a more major role than whiteflies in virus spread as an insect vector in Iraq. The data obtained may reflect the data previously reported for aphid borne viruses worldwide, as the number of viruses reported to be aphid-transmissible is higher than of those transmitted by whiteflies worldwide (28% of viruses are aphid-transmissible compared to 18% are whitefly-transmissible) (Ng & Falk 2006; Hogenhout *et al.*, 2008). Besides, the numbers of aphid species able to transmit plant viruses are quite high (192 out of 4000 aphid species) compared to whitefly species (3 out of 1200) that can transmit viruses (Hull, 2009). The specificity of transmission may affect the virus incidence as most viruses reported are stylet-borne viruses and could be transmitted by more than one aphid species in non-persistent manner. Hence, the virus could easily spread compared to those which have specific relatedness to their vector and cannot be spread in the absence of the specific vector (Hull, 2009). In Iraq, many aphid-borne viruses have been reported in local publications (e.g. El-Muadhidi *et al.*, 2001; Al-Ani *et al.*, 2010; 2011a) compared to whitefly and other insect borne viruses, may demonstrate the major role of aphids, in short distance transmission, for virus epidemiology in potato and vegetable diseases in Iraq. Recently, many aphid species were shown to be associated with the potato and vegetable viruses and tested for their ability of virus transmission in Iraq. Among other aphid species, *Myzus persicae* is the most efficient in virus transmission to potato and vegetables in Iraq. It was reported to transmit CMV, PVY, ZYMV, BYMV, and *Watermelon mosaic virus* (WMV) in Iraqi potato and vegetable fields (Ali *et al.*, 2006; Kassim & Husain, 2006; Al-Ani, 2011; Albayati *et al.*, 2012). Other aphid species reported to transmit plant viruses are *Aphis craccivora* which transmit CMV, WMV and ZYMV on cucurbits (Kassim & Husain, 2006) and common bean (Al-Ani, 2011). The following aphid species *Acyrthosiphum pisum*, *Aphis faba*, *A. craccivora*, *A. neri*, *Myzus persicae*, and *Rhopalosiphum padi* have been reported to transmit BYMV on common bean (Al-Ani, 2011). In contrast, the whitefly *B. tabaci* was reported to transmit one virus only, the TYLCV, on tomato in Iraq (Al-Ani *et al*., 2011b). Due to high incidence of BYMV (up to 100%) on broad bean in Iraqi fields, it has been suggested that the aphid *A. faba* was responsible for spreading the BYMV causing the high incidence of the virus, but not the seed, as this virus is seed-transmissible by less than 1% (El-Muadhidi *et al.*, 2001).

The high similarity (93-99%) of most virus sequences isolated from potato and vegetables to those GenBank sequences indicated that plant viruses in Iraq might have been

introduced from other countries through international trading of plant materials used for culture as Iraq import most of plant materials for agriculture. Besides, no new viruses were detected in crops grown neither from imported plant material nor from local seeds produced by farmers in Iraq, like broad bean and cowpea for the eight screened virus groups in this study. Conceivably, the underestimate of potato and vegetable viruses in Iraq resulted in virus spread and epidemiology. The wrong culture practicing currently used in Iraq for virus monitoring and control like; the low stringency criteria of tolerance limits applied in seed testing and certification and plant quarantine for potato viruses. Based on ELISA test and regardless of virus strain/species, virus indexing of 10% is an acceptable level for imported potato tuber seed and classified as a class B potato seeds. Potato seeds with high virus indexing, therefore, will be released to potato growers and farmers for growing in potato fields in Iraq (Anonymous, 2009). The use of less accurate techniques based on viral protein detection such as ELISA; rather than nucleic acid based approaches in testing samples, and the lack of seed testing for vegetable viruses especially for devastating viruses like ZYMV, alongside the agriculture intensification may result in virus spread and epidemiology in Iraq (Hull, 2004; Albrechtsen, 2006; Seal *et al.*, 2006).

About 11 out of 175 potato and tomato samples showed mixed infections of viruses from different groups. Mixed infections have been reported to increase the damage caused by viruses on potato and vegetables (Kerlan, 2009; Kerlan & Moury, 2009; Loebenstein, 2009). Single and mixed infections of PVY and PVS only were detected in some potato samples, collected from Baghdad and Anbar, which may indicate these two viruses are dominant in potato fields in Iraq. No infection for other common potato viruses screened, namely PVM, PVX and PVA was detected in potato samples based on group specific primers. Mixed infection of TYLCV and GALV was detected in some tomato samples; however, symptomatology and the effect occurred on tomato due to the mixed infection with these two viruses has not been investigated in this study. For some viruses, like potato viruses (PVA, PVM, PVS, PVX and PVY), epidemic impact due to mixed infection may happen, because mixed infection can cause serious economic losses and could be more significant than single infection (Kerlan, 2009; Loebenstein, 2009). Mixed infection can modify virus characters such as host range, insect transmission and titration (Martı´n & Elena, 2009; Lecoq & Desbiez, 2009). Severe damage due to mixed infection may occur due to synergetic exacerbation caused by double or multiple virus infection (Hull, 2009, Martı´n & Elena, 2009) which may lead to express severe symptoms on infected plant and reduce the yield.

Members of potyviruses can interfere with and suppress the gene silencing process in plant by HC-Pro gene, which increase the plant sensitivity to be infected by other viruses (Martı´n & Elena, 2009). In addition, mixed infection was shown to change aphid behaviour and biology when aphid fecundity and preference were higher in PVY/PLRV mixed infected plants than single or non-infected plants (Srinivasan & Alvarez, 2007). Insect vectors may play an important role in epidemic impact of plant viruses in mixed infection through Co-transmission. Non aphid transmitted isolates co-transmitted with aphid transmissible viruses is common in potyviruses, as a non aphid transmitted isolate ZYMV-NAT could be transmitted by aphid together with *Papaya ring spot virus* aphid -transmitted isolates (Lecoq & Desbiez, 2009). Whiteflies show the ability to co-transmit two different TYLCV strains from mixed infection fields or even single plants (Ohnishi *et al*., 2011). Thus, mixed infection may be at the same spread, through co-transmission to healthy plants or to reservoir hosts, which may expand the host range of the transmitted viruses or lead to emergence of new virus strain/species due to recombination between different strains or species and increase the virus diversity (Seal *et al*., 2006; Martı´n & Elena, 2009; Navas-Castillo *et al*., 2011).

7.3. Sequence variability and molecular diversity of potato and vegetable viruses isolated from Iraq

Sixty-one different virus sequences, belong to potyviruses, begomoviruses, carlaviruses and tombusviruses, were obtained from the molecular screening of samples collected. They provided primary molecular information about plant viruses infecting potato and vegetables in Iraq. Sequence data obtained have given information to study the variability and the diversity of plant viruses occurred in Iraq. Among other viruses detected, PVY was the highest in variability when sequences isolated showed similarities to three different recombinant strains, the PVYO: N, PVYNTN and PVYNTN-NW. PVY was detected in both potato and tomato samples collected from two location in Baghdad and Anbar. The high variability of PVY sequences was evident when Neighbor-Joining phylogenetic tree constructed from NIb/ CP/3`UTR showed that PVY sequence isolated grouped into two distinct clades according to strain. Sequence variation of PVY may lead to false detection of PVY isolates using molecular techniques (Schubert et al., 2007a). High rate of PVY recombination has resulted in the emergence of new virus strains worldwide (Kerlan & Moury, 2009). Other virus sequences obtained did not show distinct variation when compared to GenBank sequences. BYMV sequences isolated showed slight differences in aa sequences of partial NIb/CP regions, but the Neighbor-Joining phylogenetic tree did not show unique divergence in

BYMV sequences isolated as all sequences grouped within the same clade (see Figure 14B). Variability of ZYMV isolated could not be resolved as one sequence only was obtained from zucchini samples despite the fact that ZYMV is highly variable virus worldwide (Lecoq & Desbiez, 2009). One TYLCV sequence only; namely ToIq5, was shown to be highly variable among other TYLCV sequences isolated when compared to GenBank sequences. ToIq5 showed identity percent of 91% in partial V2 and CP sequences some differences in restriction sites profile when compared to other sequences isolated and GenBank sequences. However, Neighbor-Joining phylogenetic dendogram (Figure 24) did not show distinct relation of ToIq15 to other sequences. PVS and GALV isolated from potato and tomato were less variable when aa CP sequences were compared to GenBank sequences and the Neighbor-Joining phylogenetic tree constructed from CP sequences showed that all sequences were grouped in one clade. The two CPMMV sequences showed high variability when compared against each other using pair wise sequence alignment. The similarity in CP aa sequences was 77-80%, whereas the maximum identity percent of CP aa sequences was 96% calculated by pairwise alignment (Table 22) (Tamura et al., 2011). However, the low similarity percent did not indicate whether the two sequences belong to different strains, as they did not show distinct relatedness when compared using Neighbor-Joining approach. The number of sequences available in GenBank for CPMMV isolates is quite low and this makes it difficult to resolve the level of sequence divergence obtained. However, sequence comparison alone may not resolve the strain demarcation level as it requires further biological and serological test to identify the two sequences are belonging to two different strains (Adams et al., 2004). Molecular screening and sequence analyses revealed that virus diversity was highest in potyviruses, as three different potyviruses, namely PVY, BYMV and ZYMV were identified in potato and vegetable samples. The next divergent virus group was the carlaviruses with two different viruses, namely PVS and CPMMV. Begomoviruses and tombusviruses showed less diversity with one virus identified for each group namely, TYLCV and GALV, respectively. Most plant materials used for growing are imported from other countries, which may result in long distance transmission of plant viruses in Iraq. The wide host range and the type of crop grown, growing season may result in the diversity of potyviruses in Iraqi samples compared to the other detected groups. Thus, molecular approaches involving group detection may resolve the variability and diversity of plant viruses occurring in samples. Study of the molecular diversity, therefore, can be a useful tool to predict and monitor the emergence of

virus diseases infecting potato and vegetables in Iraq for the near future (Roossinck, 2011; Paga´n et al., 2012).

7.4. **Application of molecular based techniques to plant virus control in Iraq**

Molecular techniques have been shown to be reliable and could be used in routine diagnosis of plant viruses (Koenig *et al.*, 2009). The high sensitivity, specificity and rapidity make it suitable for use to control plant viruses in Iraq. The correct identification of the virus diseases is essential for effective control measures to be applied (Hull, 2009). Molecular techniques, therefore, could be included to improve conventional methods for virus control, especially for those which are based on avoiding infection such as use of virus free plant material used for grown and quarantine regulation (Albrechtsen, 2006; Hull, 2009; Rodoni, 2009). The low stringency criteria applied to seed testing and certificate and quarantine in Iraq (Anonymous, 2009), using serological based test (ELISA), the low range of virus tested (virus indexing test is restricted to test 6 potato viruses only) (Anonymous, 2009), may allow significant level of virus infected plant material to pass through. Release of such infected plant materials for cultivation may increase the virus source of infection and result in high virus incidence. Molecular techniques should be applied to virus indexing tests to improve simultaneous seed testing and certification and quarantine programmes in Iraq to minimize the risk of infected plant material to escape detection through the existing approach. High sensitivity and the reliability of molecular based tests will minimize the source of virus infection by excluding virus infected plant materials to be released for cultivation (Albrechtsen, 2006; Hull, 2009; Rodoni, 2009). Molecular techniques could be included into local Iraqi programmes for virus control such as tissue culture and integrated pest management programmes used for sustainable agriculture (Bishay, 2003, Anonymous, 2008) to produce virus free plant materials, as those techniques could be used for the routine examination to test virus infection in the plant material produced. In addition, those techniques could be used to evaluate the new promising approaches, used in Iraq for virus control, such as application of plant extracts to control virus infection on potato and vegetables (Al-Ani *et al*., 2010; 2011a, b&c) which result in effective control of virus diseases in Iraq. The problem should be addressed before applying molecular techniques to seed testing and certification and quarantine is that some virus infected plant materials may pass through seed testing, as primers used may fail to detect some viruses due to high variability of virus sequences (Malinowski, 2005). The source of variation may be caused by high mutation rates in RNA viruses due to the low fidelity of RNA replication enzyme, insertion and

deletion of RNA fragments, in addition to recombination, which are common in the families *Potyviridae* and *Geminiviridae*, among species, strain and isolates (Malinowski, 2005; Seal *et al*., 2006; Martı´n & Elena, 2009; Gibbs & Ohshima, 2010; Navas-Castillo *et al*., 2011). For RNA viruses, mutation could also happen during cDNA synthesis as proof reading ability is absent in reverse transcriptase enzyme (Kolb *et al.*, 2008). Thus, the conserved motif could be changed or removed (Zheng *et al*., 2008a), and may result in the false negative detection of infected seed samples when tested.

7.5. General conclusions

This study revealed the occurrence of three potyviruses, one begomovirus, two carlaviruses and one tombusvirus in Iraq, when PCR/RT-PCR were successfully used to screen 175 potato and vegetable samples collected from Iraq. The reliability of PCR/RT-PCR using group-specific primers alone, however, could be affected by false positive and negative reaction so it is vital to use more than one group specific primer set to test samples for virus groups. PCR/ RT-PCR and RCA approaches could be combined with other techniques like RFLP, cloning and sequencing and *in silico* analyses to identify individual viruses by sequence comparison using GenBank database. Sprimer/M4T primer set was the best among other sets in detecting potyviruses in Iraqi samples, due to its ability to detect viruses in all genera within the family *Potyviridae*, which is important for group and broad-spectrum detection. This set also amplifies full-length CP, which enables the characterization of potyviruses by sequence analyses. Sprimer/M4T primers, showed their specificity to detect viruses from most samples, without false positive, comparing to other screened primer sets when tested for potyviruses induction. Neighbor-Joining tree topology proved that all Iraqi sequences belonged to potyviruses, to which they showed the maximum identity, and did not show any relatedness to other genera in the family *Potyviridae*.

Molecular techniques were used to detect begomoviruses in potato and vegetable samples collected from Iraq and they provided the first useful information about begomoviruses that occur in Iraq. This study confirmed the occurrence of *Tomato yellow leaf curl virus* (TYLCV) (genus: *Begomovirus*; family: *Geminiviridae*) based on sequence information obtained by PCR amplification using genus specific primers; namely Deng A/ Deng B primers which amplify core protein region of begomoviruses. RCA technique was used successfully to obtain full-length sequence of TYLCV genome from Iraqi samples without the previous knowledge of sequence information. Phylogenetic and *in silico* analyses of restriction mapping enabled to differentiate TYLCV IRQ as a distinct isolate when

compared to GenBank TYLCV isolates. The study confirmed the occurrence of two carlaviruses, provided molecular information regarding carlaviruses in Iraq and showed that molecular techniques can be used to detect carlaviruses in potato and vegetable samples from Iraq as two carlaviruses, namely PVS and CPMMV were detected in potato and cowpea, respectively. The study revealed a new record of CPMMV on cowpea in Iraq and showed a new geographical location for CPMMV which gives useful information regarding the virus epidemiology. The study showed successful use of molecular techniques to detect tombusviruses in potato and vegetable samples collected from Iraq. The study also provided molecular information about the occurrence of tombusviruses in Iraq and it is the first report of GALV on tomato and eggplant in Iraq. Phylogenetic analyses based on nucleotide and deduced amino acid sequence of CP region showed that GALV sequences isolated could be a unique Iraqi isolate. The high identity of sequences obtained to isolates from GenBank suggests that plant viruses detected may have been introduced into Iraq within imported planting material.

7.6. **Future work**

Use molecular techniques to investigate other viruses starting from virus groups of economic importance on potato and vegetables, like comoviruses, poleroviruses, nepoviruses and other groups, to resolve the virus status and to provide molecular information about plant viruses occurred in Iraq. The molecular data obtained from detected samples will be included in GenBank data base and provide source of molecular information, which can be used in sequence analyses and phylogeny by other workers. Sequence information accumulated will enable to resolve the virus status and the origin of plant viruses that infect potato and vegetables in Iraq, based on identity percent comparison of different virus group sequences obtained to those in GenBank databases. Screening potato and vegetable samples for other virus groups, occurring in Iraq, based on group specific primers will enable the detection of more than one virus at the same time, increase the possibility for new virus strain or species characterisations, as those types of primers were designed to target conserved motifs occurred in most virus species within the group. Based on accumulative molecular information obtained, full understanding of potato and vegetable viruses in Iraq will make it easier to address whether plant viruses infecting potato and vegetables in Iraq have been originated or introduced from other countries.

Expanding sampling to include potato and vegetables from other geographical region in Iraq to investigate the spread of potyviruses, geminiviruses, tombusviruses and carlaviruses

within Iraqi Governorates beside other groups could not be detected or investigated in the current study. Our current molecular screening, alongside recent local studies based on biological and serological tests in local publications, show the incidence of plant viruses belong to these groups. Besides, current and previous local publications have been reported members from these groups on potato and vegetables in other Iraqi Governorates. Programme sampling to include local varieties of vegetables and weeds from different geographical regions in Iraq as well, will enable the virus epidemiological status to be resolved and whether local varieties can be infected by members from these groups. In addition, use routine sampling approach, by collecting potato and vegetable and insect vector samples every season from random fields, to screen them for potyviruses, geminiviruses, tombusviruses and carlaviruses using molecular techniques. Data collected will support virus monitoring and control in Iraq and provide details of the spread of these virus groups (short distance transmission) in Iraqi potato and vegetable fields.

A study to include molecular based analysis techniques, alongside ELISA will enhance seed testing and certification and quarantine programmes in Iraq. Plant viruses detected could be introduced into Iraq from other countries through plant materials by international trading, as shown in this study, and the tolerance limits for seed tests and certification for virus indexing is less stringent. These factors could be involved in virus spread in Iraq, therefore, by using molecular techniques, to seed certification and quarantine programme in Iraq will enable to improve virus detecting in Iraq and eliminate most of virus infection sources. It should be noted that molecular technique should be optimised for testing samples in broad spectrum, as molecular techniques include using highly toxic and harmful chemicals, i.e. CTAB extraction method used in this study involves using phenol and 2-mercapto ethanol, for total nucleic acid extraction and using ethidium bromide staining in gel electrophoresis to visualize PCR products. The usage of such toxic chemicals should be avoided to minimize the risk of exposure to these chemicals and to reduce the costs due to dispose the hazardous waste resulted after each test. Alternative extraction approaches such as simple direct tube (SDT) methods should be considered. SDT can be performed without using hazardous chemicals (Suehiro *et al.*, 2005) and the time required for this approach is quite short (about 15 min) compared to the CTAB method (Suehiro *et al.*, 2005). Another advantage of SDT is that only viral nucleic acid could be obtained with less contamination of plant nucleic acid, which makes it suitable for routine diagnosis and sample test at a large scale in Iraq. For ethidium bromide staining, alternative environment friendly dyes or

alternative approach, like PCR-ELISA can be used to minimize the risk of using mutagenic dyes for testing plant viruses in Iraq.

Test the reliability of group specific primers to differentiate viruses belong to the same group in mixed infection samples. Mixed infection of plant viruses belong to same group, like potyviruses or carlaviruses on potato and begomoviruses on tomato, can be occurred in the same sample increasing yield losses on potato and vegetables. In this approach, mixed infected samples with viruses belong to the same group can be screened using group specific primers, then the resulted fragments could be cloned and sequenced to test whether the group specific primers are able to amplify DNA fragment from all viruses belong to the same group in the mixed infection sample tested. The approach could be useful for testing samples in large scale, because it will enable to reduce time, reagents and consumables required to perform the test, through decreasing the number of tests required to screen many samples for mixed infection, as more than one virus belong to the same group will be detected at the same time in one PCR tube. Using cloning and sequencing will raise the cost of tests higher than using virus specific primers screening. The cost of tests, therefore, should be estimated for both group and virus specific primer screening to identify the economical and reliability to screen samples at a large scale for mixed infection in Iraqi potato and vegetables.

PCR/RT-PCR approaches using group specific primers need to be compared to ELISA approaches using group specific antibodies, commercially available; in order to state the best at detection of plant viruses occurred in potato and vegetables in Iraq. Among other serological techniques, ELISA is familiar approach in Iraq and widely used to test samples at a large scale for plant viruses. It has been included to test potato viruses in seed testing and certification and quarantine programme in Iraq. The group specific antibodies as well as molecular techniques have not been applied to detect plant viruses in Iraq so far, therefore it is worthwhile to compare those approaches to test potato and vegetable samples for virus indexing to find out which is the best at detection. Basically, ELISA is less in sensitivity and specificity than PCR/RT-PCR (Lebas, 2002; Hull, 2004; Koenig *et al.*, 2009). In contrast, the possibility of contamination in the PCR/RT-PCR is higher; besides, primers are available on request only compared to already prepared antibodies for plant viruses. However both approaches are required to be investigated in terms of producing false negative and positive reactions, especially when the use for legislation and regulation purposes in seed testing and certification and quarantine programme in Iraq. It is possible to use both molecular and

serological approaches, using group-specific primers/antibodies, when required or in combination to screen samples for plant viruses at a large scale in Iraq.

Test cost may be one of the factors that limit the molecular based approaches to replace the serological approaches in Iraq. Nucleic acid based approaches, which do not involve using expensive instruments, like loop mediated isothermal amplification (LAMP), could be optimized to test virus indexing in plant materials (Tomlinson *et al*., 2012). LAMP/RT-LAMP approaches may reduce the test cost and simplify the virus detection due to overcome using expensive instruments to perform the test, espcially when used to test samples in large scales. Besides, it does not require further confirmation steps to visualize the amplified products (Tomlinson *et al*., 2012). LAMP /RT-LAMP could be optimized to screen wide range of viruses in single reaction using group specific primer sets for simultaneous detection. It may enable to reduce the test cost and time required to screen several viruses, when test different samples in large scale, for seed certification and quarantine. Accordingly, test cost can be reduced due to minimize use of chemicals reagents and consumable required to perform the test and the extra cost occurred due to waste dispose that result.

Molecular based techniques shown to be a promising approach to detect plant viruses isolated from Iraq. These techniques should be innovated to support virus research studies to improve and advance research in different aspects of plant virology including diagnosis, epidemiology and control of virus diseases in Iraq. Altering the current conventional studies carried out on plant viruses in Iraq to molecular based research will enable better understanding to plant viruses and add a new perspective to both academic and applied virology in Iraq. Investigating plant viruses as molecular entities rather than biological factors only will innovate new topics and methodology to plant virus research conducted in Iraq. Intensifying and expanding virus research toward molecular based studies and applying molecular technique to characterize and study plant viruses alongside preparing and familiarizing staff and technicians through training programmes to gain adequate expletive in molecular techniques, will enable for better monitoring and management of virus disease infecting crops in Iraq. Thus, progress in plant virus studies will enable to minimize losses in crop production due to virus diseases and further the sustainable agriculture sector in Iraq.

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Appendix 1: Detection of different virus groups in Iraqi samples using genus/family specific primer sets

Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
1	BB1	Broad bean	Baghdad	March 2009	+	+	nt	-	-	-	-	mosaic
2	BB2	Broad bean	Baghdad	March 2009	+	-	nt	-	±	-	-	mosaic
3	BB3	Broad bean	Baghdad	March 2009	+	-	nt	-	-	-	-	mosaic
4	BB4	Broad bean	Baghdad	March 2009	-	+	nt	-	-	-	-	mosaic
5	BB5	Broad bean	Baghdad	March 2009	+	+	nt	-	±	-	-	mosaic
6	BB6	Broad bean	Baghdad	March 2009	+	+	nt	-	±	-	-	mosaic
7	BB7	Broad bean	Baghdad	March 2009	+	+	nt	-	±	-	-	mosaic
8	BB8	Broad bean	Baghdad	March 2009	+	+	nt	-	+	-	-	mosaic
9	BB9	Broad bean	Baghdad	March 2009	±	-	nt	-	+	-	-	mosaic
10	BB10	Broad bean	Baghdad	March 2009	+	+	nt	-	+	-	-	mosaic
11	BB11	Broad bean	Baghdad	March 2009	-	-	nt	-	+	-	-	mosaic
12	BB12	Broad bean	Baghdad	March 2009	+	+	nt	-	nt	-	-	mosaic
13	BB13	Broad bean	Baghdad	March 2009	-	+	nt	-	-	-	-	mosaic
14	BB14	Broad bean	Baghdad	March 2009	+	-	nt	-	-	-	-	mosaic
15	BB15	Broad bean	Baghdad	March 2009	-	+	nt	-	±	-	-	mosaic
16	BB16	Broad bean	Baghdad	March 2009	-	-	nt	-	-	-	-	mosaic
17	BB17	Broad bean	Baghdad	March 2009	+	-	nt	-	+	-	-	mosaic
18	BB18	Broad bean	Baghdad	March 2009	-	-	nt	-	-	-	-	mosaic
19	BB19	Broad bean	Baghdad	March 2009	+	+	nt	-	-	-	-	mosaic
20	BB20	Broad bean	Baghdad	March 2009	+	+	nt	-	±	-	-	mosaic
21	BB21	Broad bean	Baghdad	March 2009	+	-	nt	-	±	-	-	mosaic
22	BB22	Broad bean	Baghdad	March 2009	+	+	nt	-	-	-	-	mosaic
23	BB23	Broad bean	Baghdad	March 2009	-	-	nt	-	-	-	-	mosaic

Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
24	BB24	Broad bean	Baghd	March 2009	+	-	nt	-	-	-	-	mosaic
25	DYM	Datura	Baghd	November 2008	-	-	nt	-	n	-	-	mosaic, chlorosis
26	Ca1	Ornamental cabbage	Baghdad	November 2008	-	±	-	-	nt	-	-	mosaic
27	Cp1	Cowpea	Najaf	April 2009	-	nt	nt	-	+	-	-	nr
28	Cp2	Cowpea	Najaf	April 2009	-	nt	nt	+	+	-	-	nr
29	Cp3	Cowpea	Najaf	April 2009	-	nt	nt	-	+	-	-	nr
30	Cp4	Cowpea	Najaf	April 2009	-	nt	nt	-	+	-	-	nr
31	Cp5	Cowpea	Najaf	April 2009	-	nt	nt	-	+	-	-	nr
32	Cp6	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
33	Cp7	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
34	Cp8	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
35	Cp9	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
36	Cp10	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
37	Cp11	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
38	Cp12	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
39	Cp15	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
40	Cp16	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
41	Cp17	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
42	Cp18	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
43	Cp19	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
44	Cp20	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
45	Cp21	Cowpea	Najaf	April 2009	-	nt	-	-	nt	-	-	nr
46	Cp22	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
47	Cp23	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
48	Cp24	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
49	Cp25	Cowpea	Najaf	April 2009	-	nt	-	+	nt	-	-	nr
50	LM	Bottle gourd	Baghdad	November 2008	±	+	-	-	-	-	-	mosaic

Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
51	CF	Snake cucumber	Baghdad	November 2008	-	+	-	-	-	-	-	mosaic
52	Cr1	Zucchini	Baghdad	November 2008	+	+	-	-	-	-	-	mosaic
53	Cr2	Zucchini	Baghdad	November 2008	±	±	-	-	-	-	-	yellow mosaic
54	Cu1	Cucumber	Baghdad	November 2008	±	-	nt	-	-	-	-	mottle
55	Cu2	Cucumber	Baghdad	November 2008	-	+	-	-	-	-	-	leaf curl
56	Eg1	Eggplant	Baghdad	April 2011	-	nt	nt	±	±	-	+	mosaic, leaf deformation
57	Eg2	Eggplant	Baghdad	April 2011	-	nt	nt	±	+	-	±	mosaic, leaf deformation
58	Eg3	Eggplant	Baghdad	April 2011	-	nt	nt	±	+	-	+	mosaic, leaf deformation
59	Eg4	Eggplant	Baghdad	April 2011	-	nt	nt	-	±	-	±	mosaic, leaf deformation
60	PC	Potato	Baghdad	November 2008	+	+	+	+	+	-	-	severe mosaic, crinkle
61	PM	Potato	Baghdad	November 2008	+	+	-	-	-	-	-	mottle
62	PMTV	Potato	Baghdad	November 2008	-	-	-	-	nt	-	-	V shape chlorosis
63	PMo	Potato	Baghdad	November 2008	+	+	+	+	+	-	-	severe mosaic
64	P1	Potato	Anbar	April 2009	-	+	-	-	nt	-	-	tuber deformation
65	P2	Potato	Anbar	April 2009	-	-	-	+	nt	-	-	tuber deformation
66	P3	Potato	Anbar	April 2009	+	+	±	+	-	-	-	tuber deformation
67	P4	Potato	Anbar	April 2009	+	-	nt	+	nt	nt	-	tuber deformation
68	P5	Potato	Anbar	April 2009	+	-	-	+	nt	-	-	tuber deformation
69	P6	Potato	Anbar	April 2009	+	-	nt	nt	nt	-	-	tuber deformation
70	P7	Potato	Anbar	April 2009	+	-	nt	-	-	-	-	tuber deformation
71	P8	Potato	Anbar	April 2009	+	+	nt	nt	nt	nt	-	tuber deformation
72	P9	Potato	Anbar	April 2009	+	+	nt	-	-	nt	-	tuber deformation
73	P10	Potato	Anbar	April 2009	+	-	-	-	nt	-	-	tuber deformation
74	P11	Potato	Anbar	April 2009	+	+	-	-	nt	nt	-	tuber deformation
75	P12	Potato	Anbar	April 2009	-	-	-	-	nt	nt	-	tuber deformation

Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
76	P13	Potato	Anbar	April 2009	+	+	nt	nt	nt	-	-	tuber deformation
77	P14	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
78	P15	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
79	P16	Potato	Anbar	April 2009	-	+	-	-	nt	-	-	tuber deformation
80	P17	Potato	Anbar	April 2009	+	-	-	-	nt	nt	-	tuber deformation
81	P18	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
82	P19	Potato	Anbar	April 2009	+	-	+	+	nt	-	-	tuber deformation
83	P20	Potato	Anbar	April 2009	+	+	-	-	nt	-	-	tuber deformation
84	P21	Potato	Anbar	April 2009	+	-	nt	-	-	nt	-	tuber deformation
85	P22	Potato	Anbar	April 2009	-	+	nt	-	nt	-	-	tuber deformation
86	P23	Potato	Anbar	April 2009	-	-	-	-	nt	nt	-	tuber deformation
87	P24	Potato	Anbar	April 2009	+	+	-	-	nt	nt	-	tuber deformation
88	P25	Potato	Anbar	April 2009	+	-	-	-	nt	-	-	tuber deformation
89	P26	Potato	Anbar	April 2009	-	-	nt	-	-	nt	-	tuber deformation
90	P27	Potato	Anbar	April 2009	+	-	nt	-	nt	nt	-	tuber deformation
91	P28	Potato	Anbar	April 2009	+	-	nt	-	-	nt	-	tuber deformation
92	P29	Potato	Anbar	April 2009	+	+	nt	-	-	nt	-	tuber deformation
93	P30	Potato	Anbar	April 2009	-	-	-	-	nt	-	+	tuber deformation
94	P31	Potato	Anbar	April 2009	+	-	-	-	nt	-	-	tuber deformation
95	P32	Potato	Anbar	April 2009	+	-	nt	-	-	nt	-	tuber deformation
96	P33	Potato	Anbar	April 2009	-	-	nt	-	-	nt	-	tuber deformation
97	P34	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
98	P35	Potato	Anbar	April 2009	+	+	nt	nt	nt	-	-	tuber deformation
99	P36	Potato	Anbar	April 2009	-	-	nt	-	-	-	-	tuber deformation
100	P37	Potato	Anbar	April 2009	-	-	nt	-	nt	-	-	tuber deformation

Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
101	P38	Potato	Anbar	April 2009	-	-	nt	-	nt	-	-	tuber deformation
102	P39	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
103	P40	Potato	Anbar	April 2009	-	+	-	-	nt	-	-	tuber deformation
104	P41	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
105	P42	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
106	P43	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
107	P44	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
108	P45	Potato	Anbar	April 2009	-	+	-	+	nt	-	-	tuber deformation
109	P46	Potato	Anbar	April 2009	+	+	-	-	nt	-	-	tuber deformation
110	P47	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
111	P48	Potato	Anbar	April 2009	-	-	nt	-	nt	-	-	tuber deformation
112	P49	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
113	P50	Potato	Anbar	April 2009	+	-	-	+	nt	-	-	tuber deformation
114	P51	Potato	Anbar	April 2009	-	-	nt	nt	nt	-	-	tuber deformation
115	P52	Potato	Anbar	April 2009	-	-	nt	-	nt	-	-	tuber deformation
116	P53	Potato	Anbar	April 2009	-	+	nt	-	nt	-	-	tuber deformation
117	P54	Potato	Anbar	April 2009	+	+	-	+	-	-	-	tuber deformation
118	P55	Potato	Anbar	April 2009	+	-	-	-	nt	-	-	tuber deformation
119	P56	Potato	Anbar	April 2009	-	-	-	-	nt	-	-	tuber deformation
120	P54A	Potato	Anbar	April 2009	+	+	nt	-	-	nt	-	tuber deformation
121	To1	Tomato	Baghdad	June 2009	-	+	nt	-	nt	-	-	nr
122	To2	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
123	To3	Tomato	Baghdad	June 2009	-	-	nt	-	-	-	-	nr
124	To4	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
125	To5	Tomato	Baghdad	June 2009	+	+	nt	-	nt	-	-	nr
126	To6	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
127	To7	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr

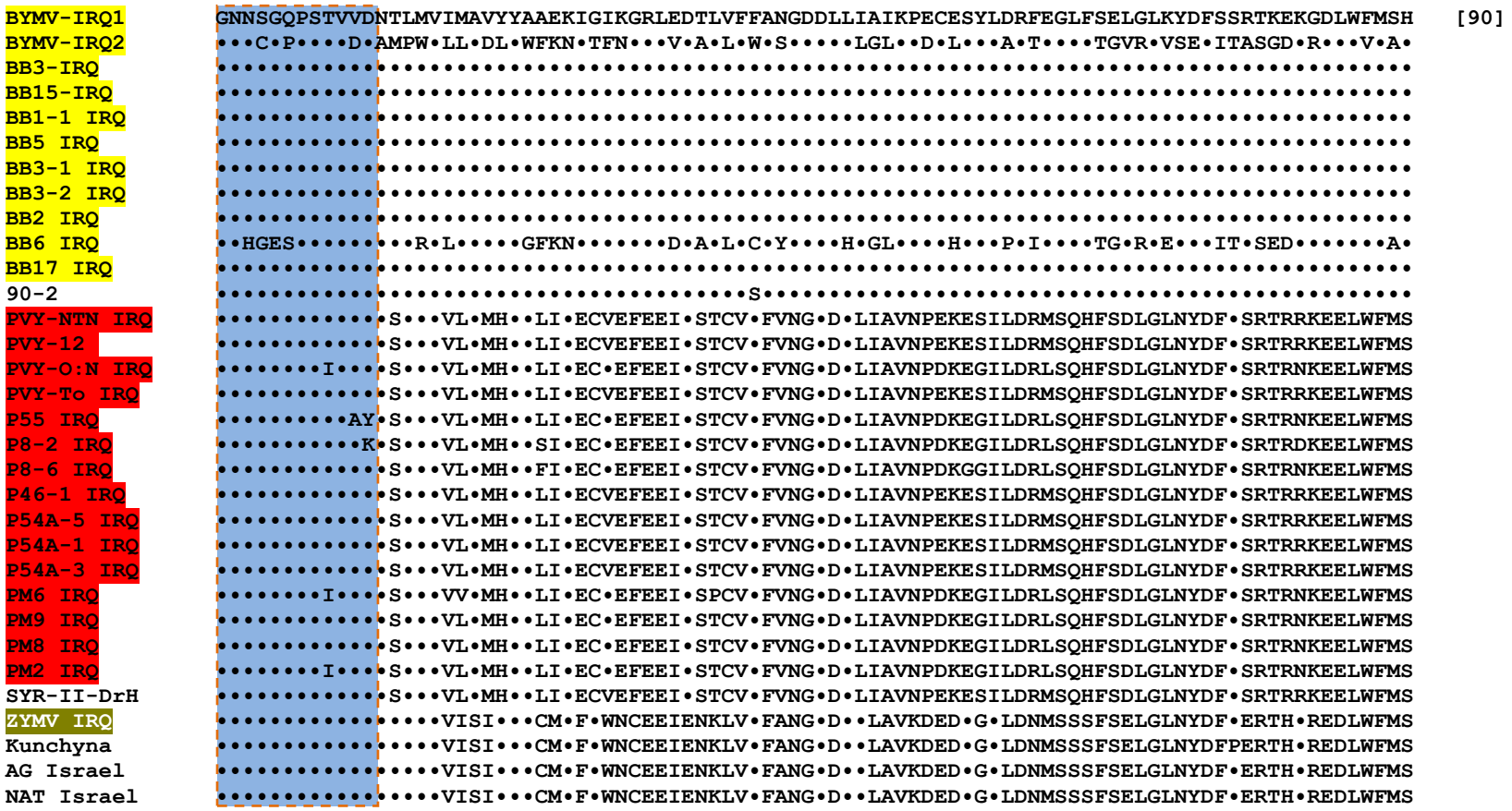
Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
128	To8	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
129	To9	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
130	To10	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
131	To11	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
132	To12	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
133	To14	Tomato	Baghdad	June 2009	+	+	nt	-	nt	-	-	nr
134	To15	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
135	To18	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
136	To19	Tomato	Baghdad	June 2009	-	-	nt	-	-	-	-	nr
137	To20	Tomato	Baghdad	June 2009	+	±	nt	-	nt	-	-	nr
138	To21	Tomato	Baghdad	June 2009	-	±	nt	-	nt	-	-	nr
139	To22	Tomato	Baghdad	June 2009	+	±	nt	-	nt	-	-	nr
140	To23	Tomato	Baghdad	June 2009	+	+	-	-	nt	-	-	nr
141	To24	Tomato	Baghdad	June 2009	+	+	nt	-	-	-	-	nr
142	To25	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
143	To26	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
144	To27	Tomato	Baghdad	June 2009	-	-	nt	-	-	-	-	nr
145	To28	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
146	To29	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
147	To30	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
148	To31	Tomato	Baghdad	June 2009	-	+	nt	-	nt	-	-	nr
149	To31A	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
150	To32	Tomato	Baghdad	June 2009	-	±	nt	-	nt	-	-	nr
151	To33	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
152	To34	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
153	To35	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
154	To36	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
155	To37	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr

Number	Sample	Host	Location	Collection date	Potyviruses		Carlaviruses			Begomoviruses	Tombusviruses	Symptoms
					Sprimer/M4T	Pot1/Pot2	Carla-CP/M4T	Car-F1/M4T	Carla-uni/M4T	DengA / DengB	CIR1/CIR2	
156	To38	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
157	To39	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
158	To40	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
159	To41	Tomato	Baghdad	June 2009	+	+	nt	-	nt	-	-	nr
160	To42	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
161	To43	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
162	To44	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
163	To45	Tomato	Baghdad	June 2009	-	-	nt	-	nt	-	-	nr
164	To19A	Tomato	Baghdad	June 2009	-	-	nt	-	-	-	-	nr
165	To46	Tomato	Baghdad	June 2009	-	+	nt	-	±	-	-	nr
166	ToIq1	Tomato	Baghdad	April 2011	-	nt	nt	-	+	+	+	yellow leaf curl
167	ToIq2	Tomato	Baghdad	April 2011	-	nt	nt	-	+	+	+	yellow leaf curl
168	ToIq3	Tomato	Baghdad	April 2011	-	nt	nt	-	+	-	+	fern leaf
169	ToIq4	Tomato	Baghdad	April 2011	-	nt	nt	-	+	-	+	fern leaf
170	ToIq5	Tomato	Baghdad	April 2011	-	nt	nt	-	+	+	+	yellow leaf curl
171	ToIq6	Tomato	Baghdad	April 2011	-	nt	nt	-	+	+	+	yellow leaf curl
172	ToIq7	Tomato	Baghdad	April 2011	-	nt	nt	-	+	+	+	yellow leaf curl
173	ToIq8	Tomato	Baghdad	April 2011	-	nt	nt	-	±	-	+	fern leaf
174	ToIq9	Tomato	Baghdad	April 2011	-	nt	nt	-	+	+	+	yellow leaf curl
175	W1	*Rumex*	Baghdad	April 2011	-	nt	nt	-	+	-	-	nr

Results obtained from screening potyviruses in Iraqi samples using different primer sets. Key description: +: positive, ±: false positive, -: negative, nr: not reported, nt: not tested, P: potato, W weed, Ca: cabbage, To: tomato, ToIq: tomato Iraq (next batch samples), Eg: eggplant, PMo: potato with mosaic symptoms, PC: potato with crinkle symptoms, PM: potato with mottle symptoms, CF: cucumber variety *flexuosus*, LM: *Lagenaria* with mosaic symptoms, Cu: cucumber, Cr: cucurbit, DYM: *Datura* with yellowing and mosaic symptoms, BB: broad bean, PMTV: potato with mop top symptoms, Cp: cow pea.

Appendix 2: Amino acid sequence alignment of potyvirus sequences

Deduced amino acid sequence alignment of partial NIB starting from GNNS motif and complete CP regions in genomes of BYMV (yellow highlighted), PVY(red highlighted) and ZYMV sequences obtained from Iraqi samples, to compare sequences isolated from Iraqi samples (tagged with (IRQ) and different closely related isolates obtained from GenBank data base. The assented, deleted amino acids and stop condones were marked with •, - and *. Discontinuous rectangles refer to conserved motifs of potyviruses. This alignment performed using MEGA5 software (Tamura *et al*., 2011).


BYMV-IRQ1 GNNSGQPSTVVDNTLMVIMAVYYAAEKIGIKGRLEDTLVFFANGDDLLIAIKPECESYLDRFEGLFSELGLKYDFSSRTKEKGDLWFMSH [90]
BYMV-IRQ2 •••C•P••••D•AMPW•LL•DL•WFKN•TFN•••V•A•L•W•S•••••LGL••D•L•••A•T••••TGVR•VSE•ITASGD•R•••V•A•
BB3-IRQ
BB15-IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ ••HGES•••••••••R•L•••••GFKN•••••••D•A•L•C•Y••••H•GL••••H•••P•I••••TG•R•E•••IT•SED•••••••A•
BB17 IRQ
90-2 ••••••••••••••••••••••••••••••S••
PVY-NTN IRQ ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
PVY-12 ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
PVY-O:N IRQ ••••••••I•••S•••VL•MH••LI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
PVY-To IRQ ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
P55 IRQ ••••••••••AY•S•••VL•MH••LI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
P8-2 IRQ ••••••••••••K•S•••VL•MH••SI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRDKEELWFMS
P8-6 IRQ ••••••••••••S•••VL•MH••FI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKGGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
P46-1 IRQ ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
P54A-5 IRQ ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
P54A-1 IRQ ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
P54A-3 IRQ ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
PM6 IRQ ••••••••I•••S•••VV•MH••LI•EC•EFEEI•SPCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
PM9 IRQ ••••••••••••S•••VL•MH••LI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
PM8 IRQ ••••••••••••S•••VL•MH••LI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
PM2 IRQ ••••••••I•••S•••VL•MH••LI•EC•EFEEI•STCV•FVNG•D•LIAVNPDKEGILDRLSQHFSDLGLNYDF•SRTRNKEELWFMS
SYR-II-DrH ••••••••••••S•••VL•MH••LI•ECVEFEEI•STCV•FVNG•D•LIAVNPEKESILDRMSQHFSDLGLNYDF•SRTRRKEELWFMS
ZYMV IRQ ••••••••••••••••VISI•••CM•F•WNCEEIENKLV•FANG•D••LAVKDED•G•LDNMSSSFSELGLNYDF•ERTH•REDLWFMS
Kunchyna ••••••••••••••••VISI•••CM•F•WNCEEIENKLV•FANG•D••LAVKDED•G•LDNMSSSFSELGLNYDFPERTH•REDLWFMS
AG Israel ••••••••••••••••VISI•••CM•F•WNCEEIENKLV•FANG•D••LAVKDED•G•LDNMSSSFSELGLNYDF•ERTH•REDLWFMS
NAT Israel ••••••••••••••••VISI•••CM•F•WNCEEIENKLV•FANG•D••LAVKDED•G•LDNMSSSFSELGLNYDF•ERTH•REDLWFMS

```
BYMV-IRQ1        KGVQIDGMWIPKLEEERIVSILEWDRALQPEHRLEAVCAAMIEAWGYPILLNHIRKFYLWVLGQAPYSQLSAEGKAPYISEVALKHLYTE   [180]
BYMV-IRQ2        GR•L•H•LR••Q••••S•••••G••K••L•K•S•STW•••L••F•••LT••••TNS••••D••RSSGGG•TG••••••V•D•••R•••••
BB3-IRQ          ••••••••••••••••••••••••••••••••••••••••••••••••••T••••••••••••••••••••••••••••••••••••••••••
BB15-IRQ         ••••••••••••••••••••••••••••••••••••••••••••••••••T••••••••••••••••••••••••••••••••••••••••••
BB1-1 IRQ        ••••••••••••••••••••••••••••••••••••••••••••••••••T••••••••••••••••••••••••••••••••••••••••••
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BB2 IRQ          ••••••••••••••••••••••••••••••••••••••••••••••••••T••••••••••••••••••••••••••••••••••••••••••
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BB17 IRQ         ••••••••••••••••••••••••••••••••••••••••••••••••••T••••••••••••••••••••••••••••••••••••••••••
90-2             R•••••••••••••••••••••••••••••••••I••••••••••T••••••••••••••••P••••••T••••••••••••••••••
PVY-NTN IRQ      HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFSTI•QEGKAPYLASMALKKLYV
PVY-12           HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFSTI•QEGKAPYIASMALKKLYM
PVY-O:N IRQ      HRGLLIEGMYVPKL••ERIVSILQWDRADLAEHRLEAIFFAMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
PVY-To IRQ       HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFSTI•HEGKAPYIASMALRKLYM
P55 IRQ          HRGLLIEGMYVPKL••ERIVSILQWDRADLAEHRLEAIC•AMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
P8-2 IRQ         HRGLLIEGMYVPKL••ERIVSILQWDGADLAEHRLEAIC•AMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
P8-6 IRQ         HRGLLIEGMYVPKL••ERIVSILQWDRADLAEHRLEAIC•AMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
P46-1 IRQ        HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFSTI•QEGKAPYIASMALRKLYM
P54A-5 IRQ       HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFSTI•QEGKAPYIASMALRKLYM
P54A-1 IRQ       HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFSTI•QEGKAPYIASMALRKLYM
P54A-3 IRQ       HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYFE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
PM6 IRQ          HRGLLIEGMYVPKL••ERIVSFLQWDRADLAEHRLEAIC•AMIESWGYSE•T•PIRRFYSW•L•QQPFVTI•HEGKAPYIASMALRKLYM
PM9 IRQ          HRGLLIEGMYVPKL••ERIVSILQWDRADLAEHRLEAIC•AMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
PM8 IRQ          HRGLLIEGMYVPKL••ERIVSILQWDRADLAEHRLEAIC•AMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
PM2 IRQ          HRGLLIEGMYVPKL••ERIVSILQWDRADLAEHRLEAIFFFMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
SYR-II-DrH       HRGLLIEGMYVPKL••ERIVSILQWDRADLPEHRLEAIC•AMIESWGYSE•T•QIRRFYSW•L•QQPFATI•QEGKAPYIASMALRKLYM
ZYMV IRQ         HQAMLVDGMYIPKL•KERIVSILEWDRSKEIMHRTEAIC•AMIEAWGHTE•LQEIRKFYLWFVEKEEVREL•ALGRAPYIAETALRKLYT
Kunchyna         HQAMLVDGMYIPKL•KERIVSILEWDRSKEIMHRTEAIC•AMIEAWGHTE•LQEIRKFYLWFVEKEEVREL•ALGKAPYIAETALRKLYT
AG Israel        HQAMLVDGMYIPKL•KERIVSILEWDRSKEIMHRTEAIC•AMIEAWGHTE•LQEIRKFYLWFVEKEEVREL•ALGKAPYIAETALRKLYT
NAT Israel       HQAMLVDGMYIPKL•KERIVSILEWDRSKEIMHRTEAIC•AMIEAWGHTE•LQEIRKFYLWFVEKEEVREL•ALGKAPYIAETALRKLYT
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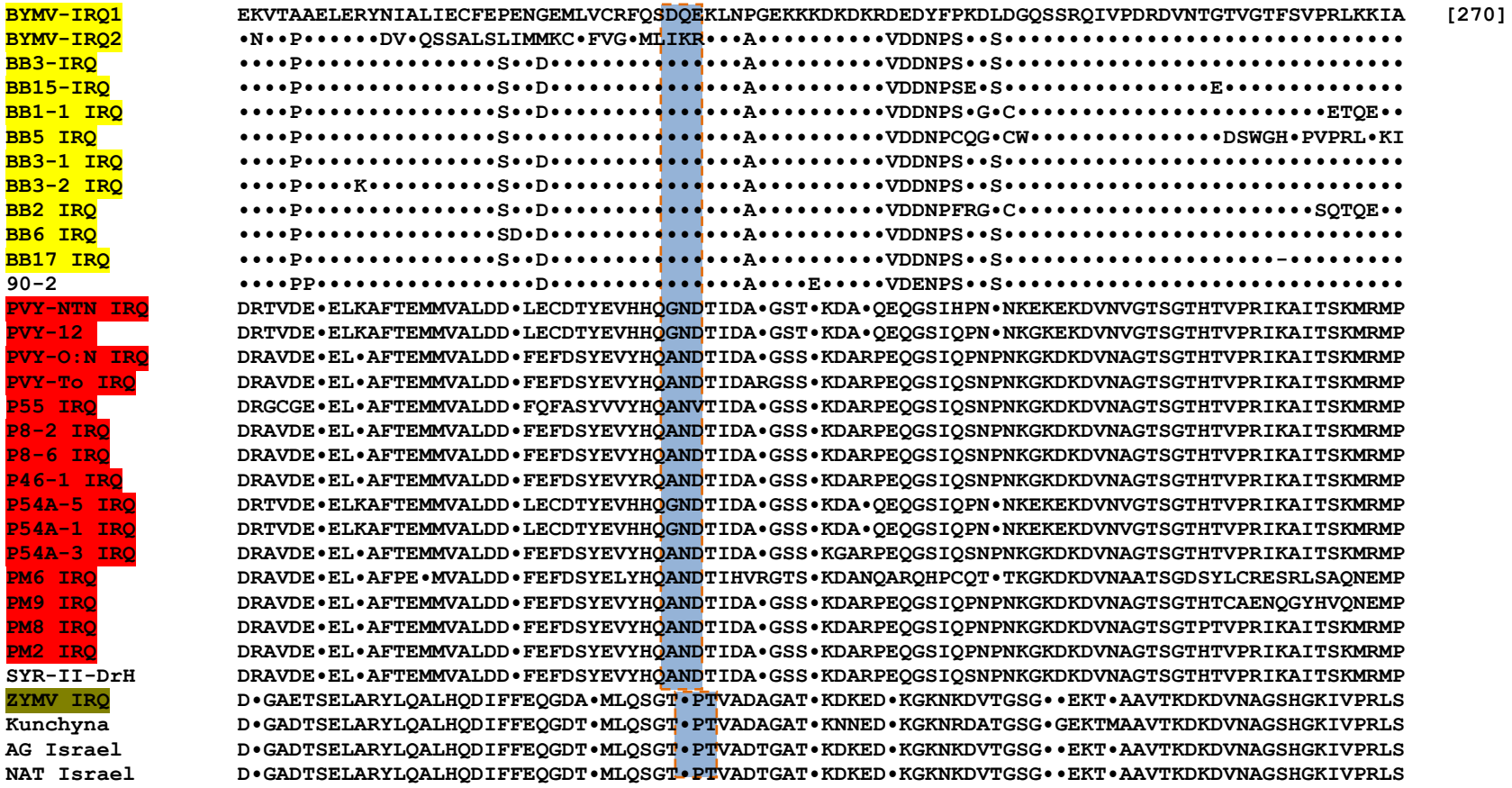
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BB5 IRQ ••••P••••••••••••••S••••••••••••••••A••••••••••VDDNPCQG•CW••••••••••••••••DSWGH•PVPRL•KI
BB3-1 IRQ ••••P••••••••••••••S••D••••••••••••••A••••••••••VDDNPS••S••••••••••••••••••••••••••••••
BB3-2 IRQ ••••P••••K••••••••••S••D••••••••••••••A••••••••••VDDNPS••S••••••••••••••••••••••••••••••
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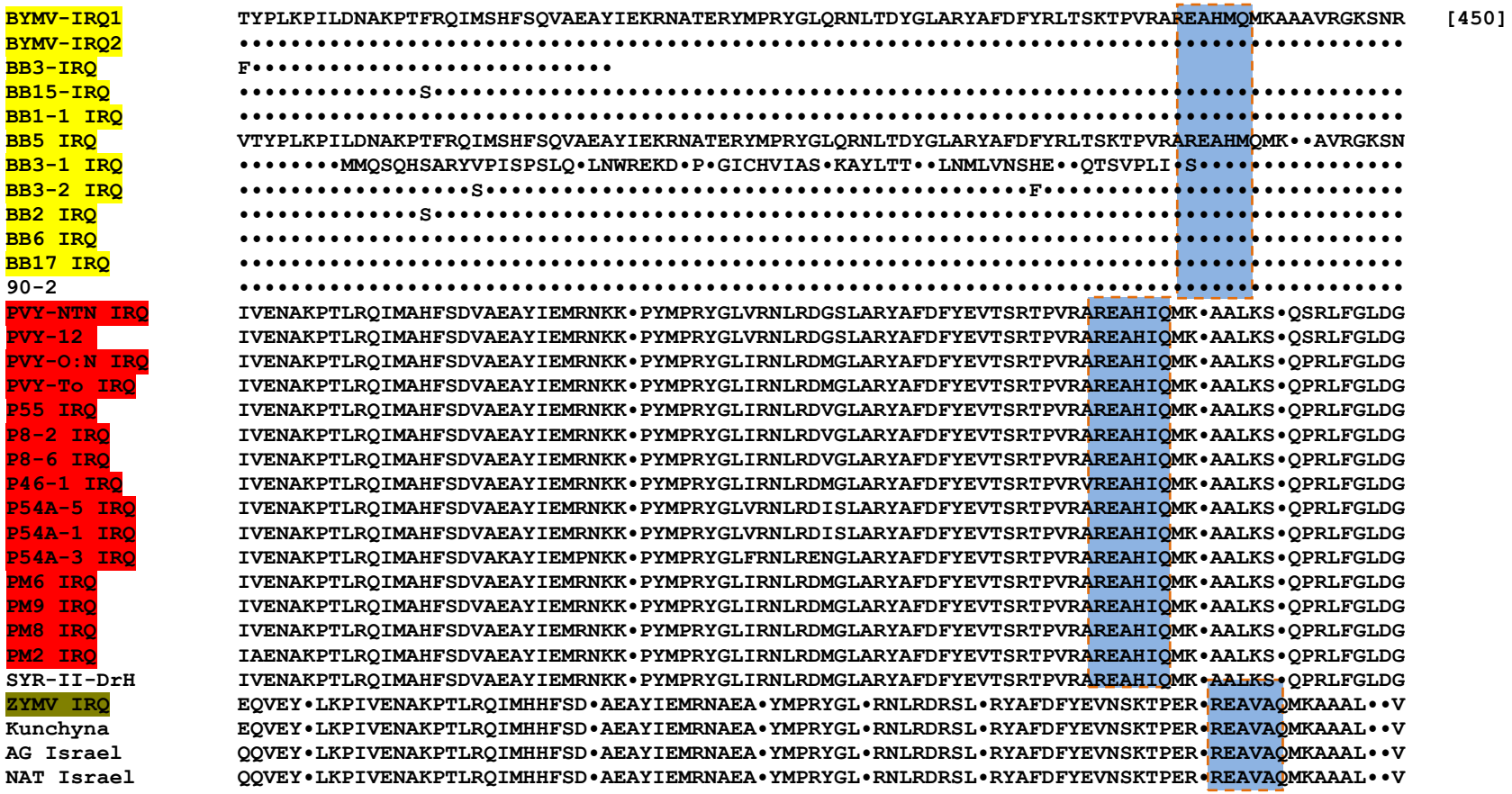
BB6 IRQ ••••P••••••••••••••SD•D••••••••••••••A••••••••••VDDNPS••S••••••••••••••••••••••••••••••
BB17 IRQ ••••P••••••••••••••S••D••••••••••••••A••••••••••VDDNPS••S••••••••••••••••••••-••••••••••
90-2 ••••PP••••••••••••••••D••••••••••••••A••••E•••••VDENPS••S••••••••••••••••••••••••••••••
PVY-NTN IRQ DRTVDE•ELKAFTEMMVALDD•LECDTYEVHHQGNDTIDA•GST•KDA•QEQGSIHPN•NKEKEKDVNVGTSGTHTVPRIKAITSKMRMP
PVY-12 DRTVDE•ELKAFTEMMVALDD•LECDTYEVHHQGNDTIDA•GST•KDA•QEQGSIQPN•NKGKEKDVNVGTSGTHTVPRIKAITSKMRMP
PVY-O:N IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQPNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
PVY-To IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDARGSS•KDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
P55 IRQ DRGCGE•EL•AFTEMMVALDD•FQFASYVVYHQANVTIDA•GSS•KDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
P8-2 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
P8-6 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
P46-1 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYRQANDTIDA•GSS•KDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
P54A-5 IRQ DRTVDE•ELKAFTEMMVALDD•LECDTYEVHHQGNDTIDA•GSS•KDA•QEQGSIQPN•NKEKEKDVNVGTSGTHTVPRIKAITSKMRMP
P54A-1 IRQ DRTVDE•ELKAFTEMMVALDD•LECDTYEVHHQGNDTIDA•GSS•KDA•QEQGSIQPN•NKEKEKDVNVGTSGTHTVPRIKAITSKMRMP
P54A-3 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KGARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
PM6 IRQ DRAVDE•EL•AFPE•MVALDD•FEFDSYELYHQANDTIHVRGTS•KDANQARQHPCQT•TKGKDKDVNAATSGDSYLCRESRLSAQNEMP
PM9 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQPNPNKGKDKDVNAGTSGTHTCAENQGYHVQNEMP
PM8 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQPNPNKGKDKDVNAGTSGTPTVPRIKAITSKMRMP
PM2 IRQ DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQPNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
SYR-II-DrH DRAVDE•EL•AFTEMMVALDD•FEFDSYEVYHQANDTIDA•GSS•KDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAITSKMRMP
ZYMV IRQ D•GAETSELARYLQALHQDIFFEQGDA•MLQSGT•PTVADAGAT•KDKED•KGKNKDVTGSG••EKT•AAVTKDKDVNAGSHGKIVPRLS
Kunchyna D•GADTSELARYLQALHQDIFFEQGDT•MLQSGT•PTVADAGAT•KNNED•KGKNRDATGSG•GEKTMAAVTKDKDVNAGSHGKIVPRLS
AG Israel D•GADTSELARYLQALHQDIFFEQGDT•MLQSGT•PTVADTGAT•KDKED•KGKNKDVTGSG••EKT•AAVTKDKDVNAGSHGKIVPRLS
NAT Israel D•GADTSELARYLQALHQDIFFEQGDT•MLQSGT•PTVADTGAT•KDKED•KGKNKDVTGSG••EKT•AAVTKDKDVNAGSHGKIVPRLS

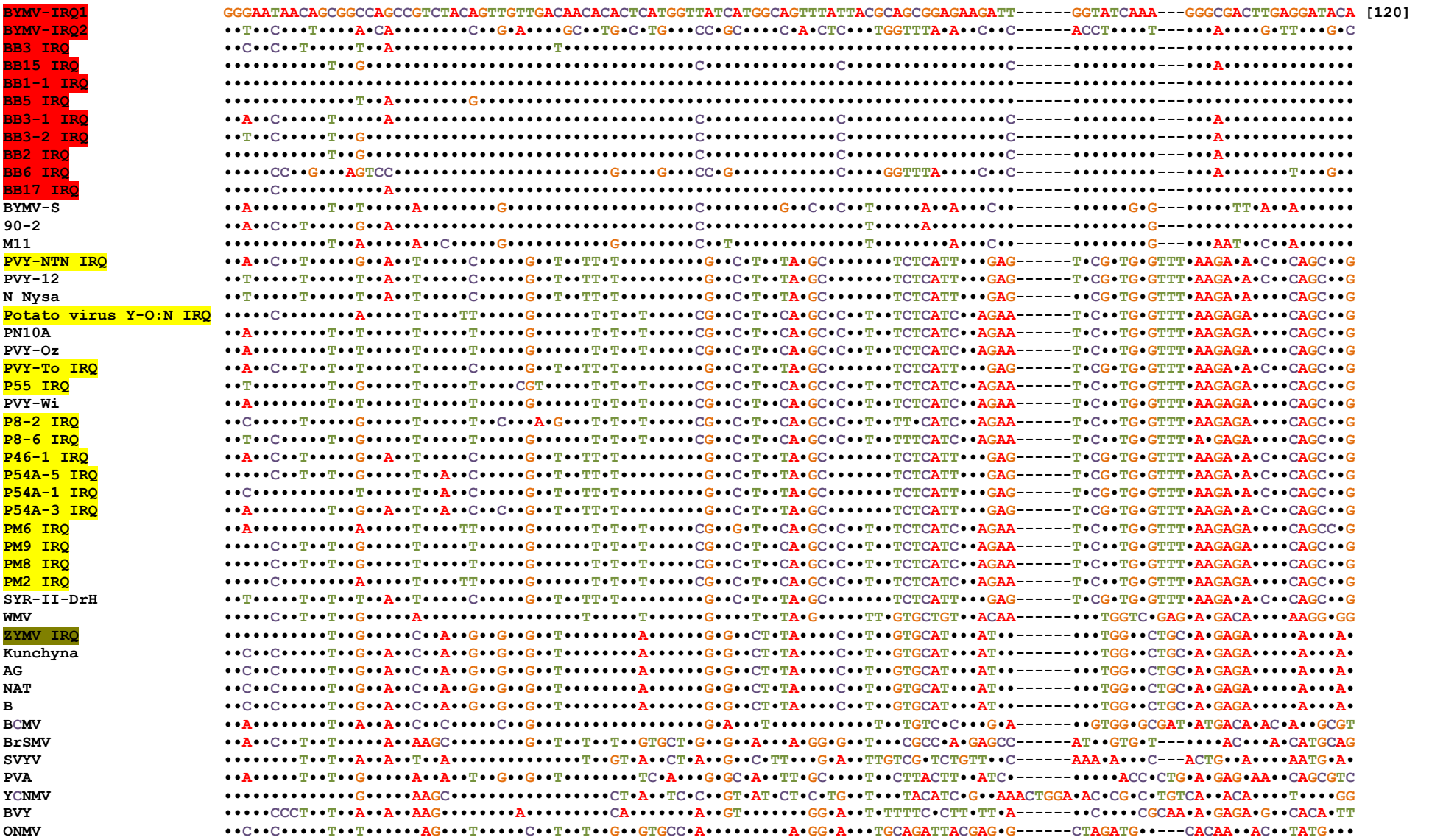
BYMV-IRQ1	GKLNIPKIGGKIVLNLDHLLDYNPPQDDISNTIATQAQFEAWYNGVKQAYEVEDSQMGTILNGLMVWCIENGTSGDLQGEWTMMDGEEQV	[360]
BYMV-IRQ2	••I••••••••••••A••••••••••••••••••	
BB3-IRQ	•••••••••••••••••••V••••••••••••••••••••••••••••••••••••••I•••••••••••••••••••••••L•••••••	
BB15-IRQ	••I•••••••••••••••••••••••••••••••	
BB1-1 IRQ	••I•••••••••••••••••••••••••••••••	
BB5 IRQ	AGKLNIPKI•GKIVLNLDH•LDYN•PQ•DISNTIATQAQFEAWYNGVKQAYEVEDSQMG•ILNGLMVWCIENGTSGDLQGEWT•MDG•EQ	
BB3-1 IRQ	••I•••••••••••••••••••••••••••••••	
BB3-2 IRQ	••I•••••••••••••••••••••••L•••••••	
BB2 IRQ	••I•••••••••••••••••••••••••••••••	
BB6 IRQ	••I•••••••••••••••••••••••••••••••	
BB17 IRQ	••I•••••••••••••••••••••••••••••••	
90-2	••••••••••••••••N••••••••••••••••••••••••••••••••••••••P••I••••••••••K••••••••••••••••••••	
PVY-NTN IRQ	KSKGATVLNLGNL•EYAPQQIDISNTRATQSQFD•CYEAVQLAFDIGETEIPSVMNGLLVWCIENGTPPNINGVWVMMDGYEQVEYPLNP	
PVY-12	KSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVQLAYDIGETEMPTVMNGLMVWCIENGTSPNINGVWVMMDGDEQVEYPLKP	
PVY-O:N IRQ	TSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTAMNGLMVWCIENGTSPNVNGVWVMMD•NEQVEYPLKP	
PVY-To IRQ	TSKGATVLNLEHL•EYAPQQIDISNTGATQSQFD•WYEAVRMAYDIGKLKMPTVMNGLMVWCIENGTSPNVNGVWVMMDGDEQVEYPLKP	
P55 IRQ	KSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYAAVRMAYDIGETEMPTGINGLMVWCIENGTSPNVNGVWVMMDGDEQVEYPLKP	
P8-2 IRQ	KSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTVMNGLLVWCIENGTSPNVNGVWVMMDGDEQVEYPLKP	
P8-6 IRQ	KSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMDGDEQVEYPLKP	
P46-1 IRQ	TSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMDGDEQVEYPLKP	
P54A-5 IRQ	KSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVQLAYDIGETEMPTVMNGLMVWCIENGTSPNINGVWVMMDGDEQVEYPLKP	
P54A-1 IRQ	KSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVQLAYDIGETEMPTVMNGLMVWCIENGTSPNINGVWVMMDGDEQVEYPLKP	
P54A-3 IRQ	TSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTVMNGLMVWCIE•EPRQMSTEF•LWMDGNEQVEYPLKP	
PM6 IRQ	TSKGATVLNYEHL•DML•NKIDISNTRATQSQFD•WYEAVRMAFDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMD•NEQVEYPLKP	
PM9 IRQ	TSKGATVLNLEHL•EYAPQQIDISNTRATHSQFD•WYEAVRMAYDIGETEMPTAMNGLMVWCIENGTSPNVNGVWVMMD•NEQVEYPLKP	
PM8 IRQ	TSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTAMNGLMVWCIENGTSPNVNGVWVMMD•NEQVEYPLKP	
PM2 IRQ	TSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMD•NEQVEYPLKP	
SYR-II-DrH	TSKGATVLNLEHL•EYAPQQIDISNTRATQSQFD•WYEAVRMAYDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMDGNEQVEYPLKP	
ZYMV IRQ	KITKKMSLPRVKGNVILDIDHLLEYKP•QIELYN•R•SHQQFASWFS•VKTEY•LNEQQMGVVMNGFMVWCIEN•TSPDINGVWVMMDGN	
Kunchyna	KITKKMSLPRVKGNVILDIDHLLEYKP•QIELYN•R•SHQQFASWFN•VKTEY•LNEQQMGVVMNGFMVWCIEN•TSPDINGVWVMMDGN	
AG Israel	KITKKMSLPRVKGNVILDIDHLLEYKP•QIELYN•R•SHQQFASWFN•VKTEY•LNEQQMGVVMNGFMVWCIEN•TSPDINGVWVMMDGN	
NAT Israel	KITKKMSLPRVKGNVILDIDHLLEYKP•QIELYN•R•SHQQFASWFN•VKTEY•LNEQQMGVVMNGFMVWCIEN•TSPDINGVWVMMDGN	

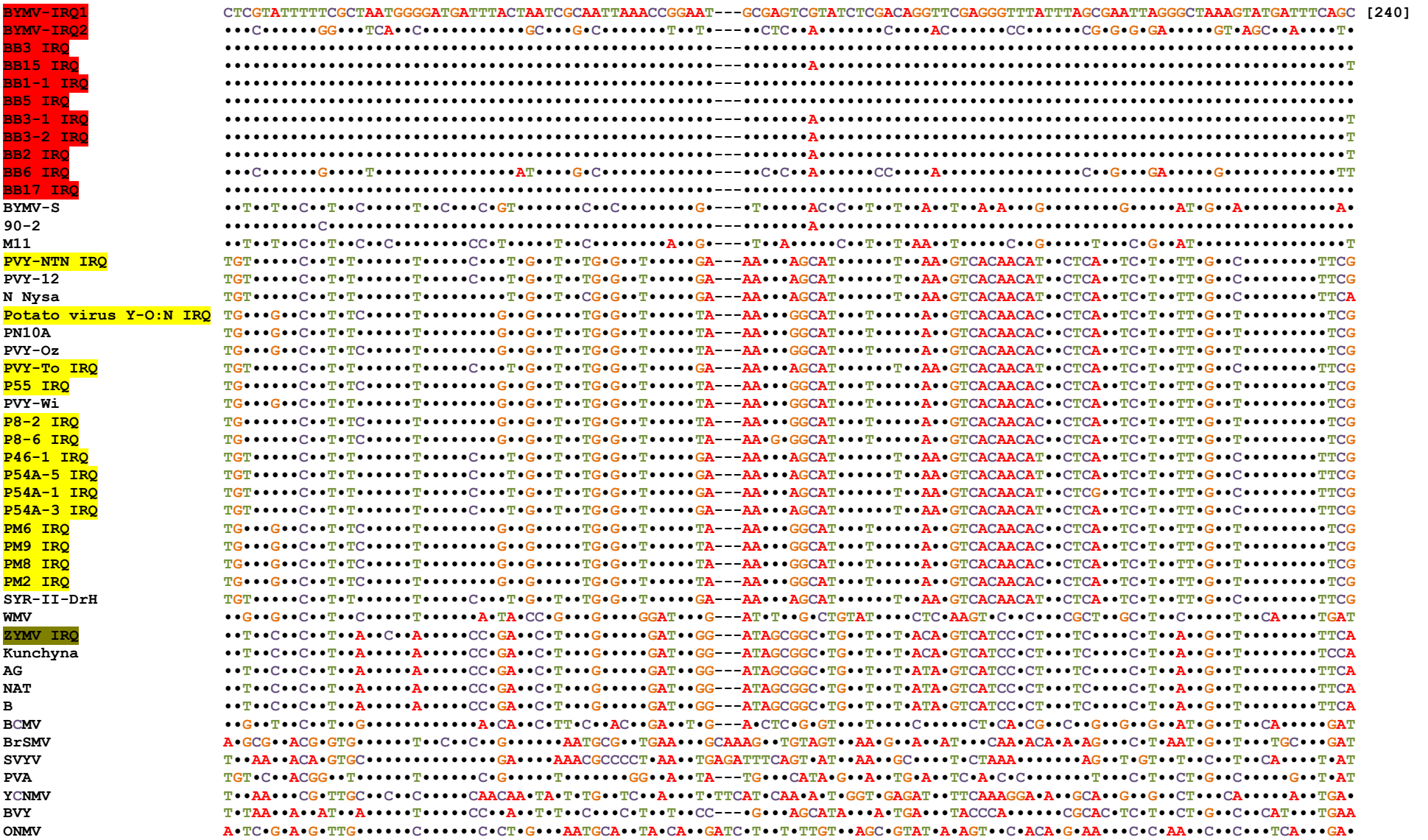

BYMV-IRQ1 TYPLKPILDNAKPTFRQIMSHFSQVAEAYIEKRNATERYMPRYGLQRNLTDYGLARYAFDFYRLTSKTPVRAREAHMQMKAAAVRGKSNR [450]
BYMV-IRQ2
BB3-IRQ F
BB15-IRQ S
BB1-1 IRQ
BB5 IRQ VTYPLKPILDNAKPTFRQIMSHFSQVAEAYIEKRNATERYMPRYGLQRNLTDYGLARYAFDFYRLTSKTPVRAREAHMQMK··AVRGKSN
BB3-1 IRQ ••••••••MMQSQHSARYVPISPSLQ•LNWREKD•P•GICHVIAS•KAYLTT••LNMLVNSHE••QTSVPLI•S
BB3-2 IRQ S F
BB2 IRQ S
BB6 IRQ
BB17 IRQ
90-2
PVY-NTN IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLVRNLRDGSLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QSRLFGLDG
PVY-12 IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLVRNLRDGSLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QSRLFGLDG
PVY-O:N IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
PVY-To IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
P55 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDVGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
P8-2 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDVGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
P8-6 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDVGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
P46-1 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRVREAHIQMK•AALKS•QPRLFGLDG
P54A-5 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLVRNLRDISLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
P54A-1 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLVRNLRDISLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
P54A-3 IRQ IVENAKPTLRQIMAHFSDVAKAYIEMPNKK•PYMPRYGLFRNLRENGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
PM6 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
PM9 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
PM8 IRQ IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
PM2 IRQ IAENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
SYR-II-DrH IVENAKPTLRQIMAHFSDVAEAYIEMRNKK•PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK•AALKS•QPRLFGLDG
ZYMV IRQ EQVEY•LKPIVENAKPTLRQIMHHFSD•AEAYIEMRNAEA•YMPRYGL•RNLRDRSL•RYAFDFYEVNSKTPER•REAVAQMKAAAL••V
Kunchyna EQVEY•LKPIVENAKPTLRQIMHHFSD•AEAYIEMRNAEA•YMPRYGL•RNLRDRSL•RYAFDFYEVNSKTPER•REAVAQMKAAAL••V
AG Israel QQVEY•LKPIVENAKPTLRQIMHHFSD•AEAYIEMRNAEA•YMPRYGL•RNLRDRSL•RYAFDFYEVNSKTPER•REAVAQMKAAAL••V
NAT Israel QQVEY•LKPIVENAKPTLRQIMHHFSD•AEAYIEMRNAEA•YMPRYGL•RNLRDRSL•RYAFDFYEVNSKTPER•REAVAQMKAAAL••V

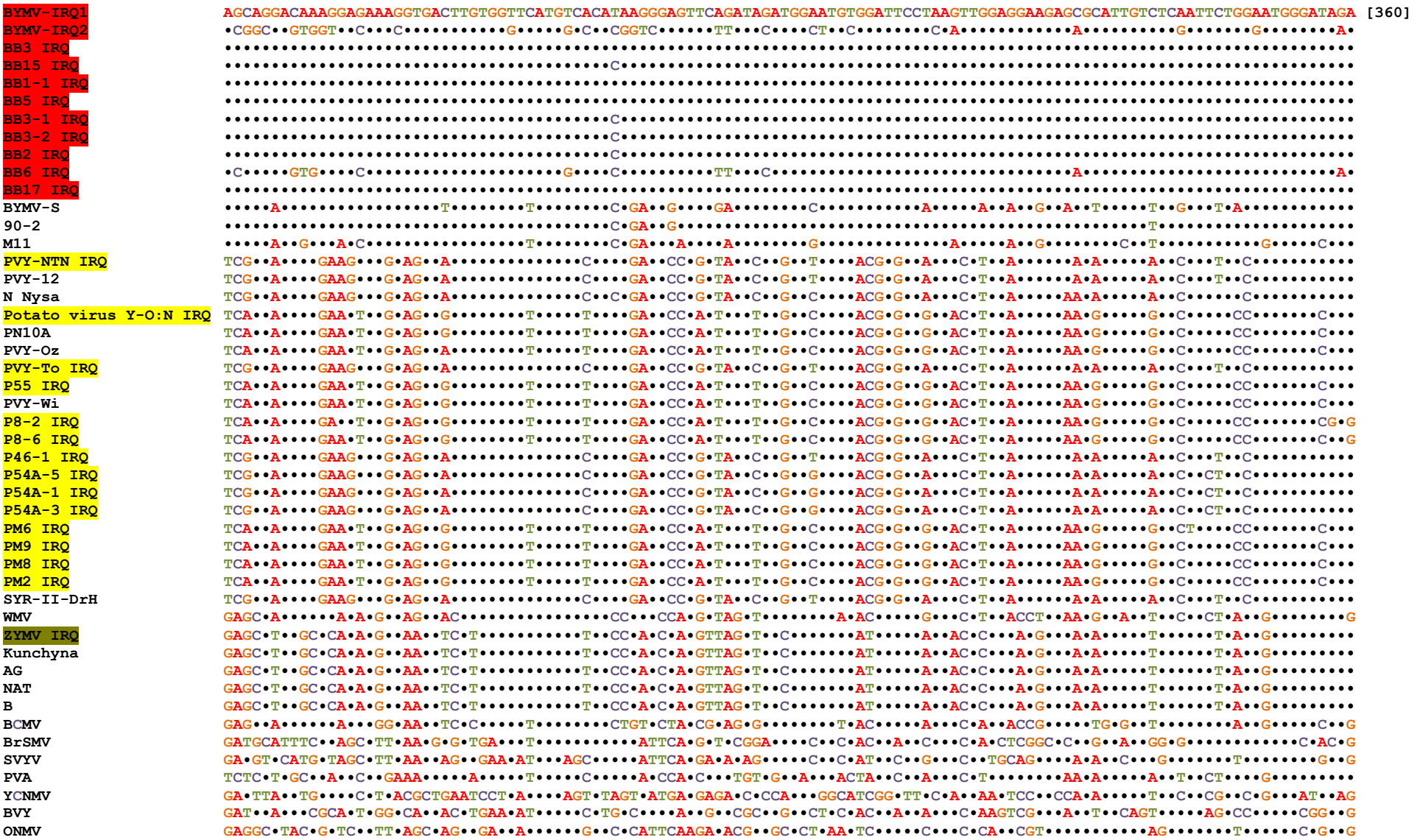
```
BYMV-IRQ1          LFGLDGNVGTDEENTERHTAGDVNRDMHTMLGVRV*-3`UTR [486]
BYMV-IRQ2          ••••••••••••••••••••••••••••••••••-3`UTR
BB3-IRQ            •••F••••••••••P•••••••••••••••••••-3`UTR
BB15-IRQ           ••••••••••••••••••••••••••••••••••-3`UTR
BB1-1 IRQ          ••••••••••••••••••••••••••••••••••-3`UTR
BB5 IRQ            RLFGLDGNVGTD•ENTERHTAGDVNRDMHTMLGVRV*-3`UTR
BB3-1 IRQ          ••••••••••••••••••••••••••••••••••-3`UTR
BB3-2 IRQ          •••F••••••••••P•••••••••••••••••••-3`UTR
BB2 IRQ            ••••••••••••••••••••••••••••••••••-3`UTR
BB6 IRQ            ••••••••••••••••••••••••••••••••••-3`UTR
BB17 IRQ           ••••••••••••••••••••••••••••••••••-3`UTR
90-2               ••••••••••••••••••••••••••••••••I•-3`UTR
PVY-NTN IRQ        GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR      [481]
PVY-12             GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
PVY-O:N IRQ        GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
PVY-To IRQ         GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
P55 IRQ            GISTQEENTERHPPEDVSPSMHTLLGVKN•*-3`UTR
P8-2 IRQ           GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
P8-6 IRQ           GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
P46-1 IRQ          GISTQEENPERHPTEDVFPSMHTLLGVKN•*-3`UTR
P54A-5 IRQ         GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
P54A-1 IRQ         GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
P54A-3 IRQ         GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
PM6 IRQ            GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
PM9 IRQ            GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
PM8 IRQ            GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
PM2 IRQ            GISTQEENTERHTTEDVSPSMHTLLGVKD•*-3`UTR
SYR-II-DrH         GISTQEENTERHTTEDVSPSMHTLLGVKN•*-3`UTR
ZYMV IRQ           SSR•F•LD•NVATTS•DTERHTARDVNRN•HTLLGVNTMQ*-3`UTR [492]
Kunchyna           SSR•F•LD•NVATTS•DTERHTARDVNRN•HTLLGVNTMQ*-3`UTR
AG Israel          SSR•F•LD•NVATTS•DTERHTARDVNRN•HTLLGVNTMQ*-3`UTR
NAT Israel         SSR•F•LD•NVATTS•DTERHTARDVNRN•HTLLGVNTMQ*-3`UTR
```

Appendix 3: Nucleotide sequence alignment of potyvirus sequences

Nucleotide sequence alignment of partial NIb /CP/UTR region of *Bean yellow mosaic virus* (BYMV: genus *Potyvirus*; family *Potyviridae*) (red highlighted), *Potato virus Y* (PVY: genus *Potyvirus*; family *Potyviridae*) (yellow highlighted) and *Zucchini yellow mosaic virus* (BYMV: genus *Potyvirus*; family *Potyviridae*) (grey highlighted) sequences obtained from Iraqi potato and vegetable samples and isolates obtained from GenBank data base. The assented and deleted nucleotides were marked with (.) and (-) respectively. This alignment performed using MEGA5 software. (Tamura *et.al.*, 2011).


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[120]


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[240]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BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[480]


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[600]


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
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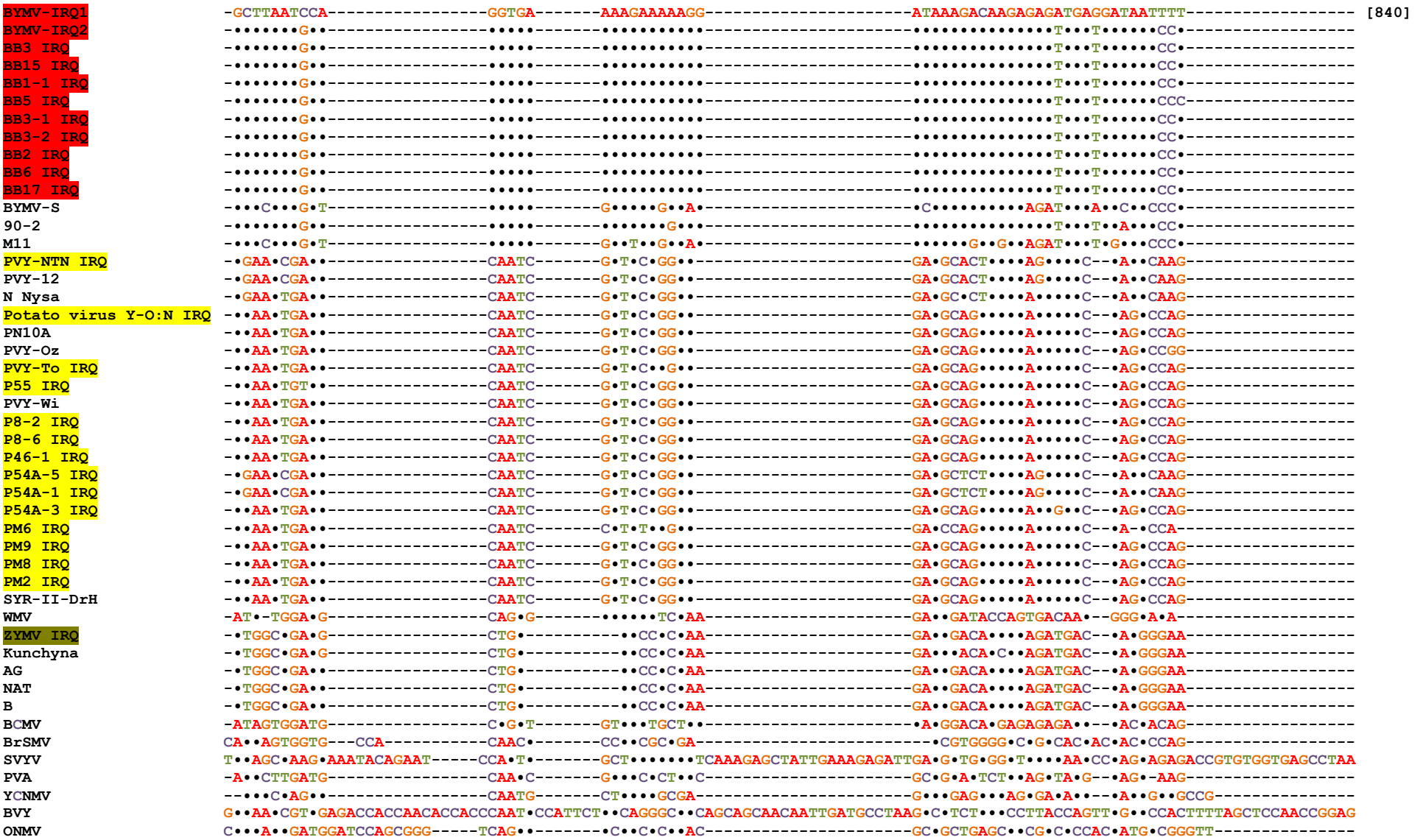
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[720]


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[840]


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
B
BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
[960]


BYMV-IRQ1
BYMV-IRQ2
BB3 IRQ
BB15 IRQ
BB1-1 IRQ
BB5 IRQ
BB3-1 IRQ
BB3-2 IRQ
BB2 IRQ
BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
P54A-1 IRQ
P54A-3 IRQ
PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
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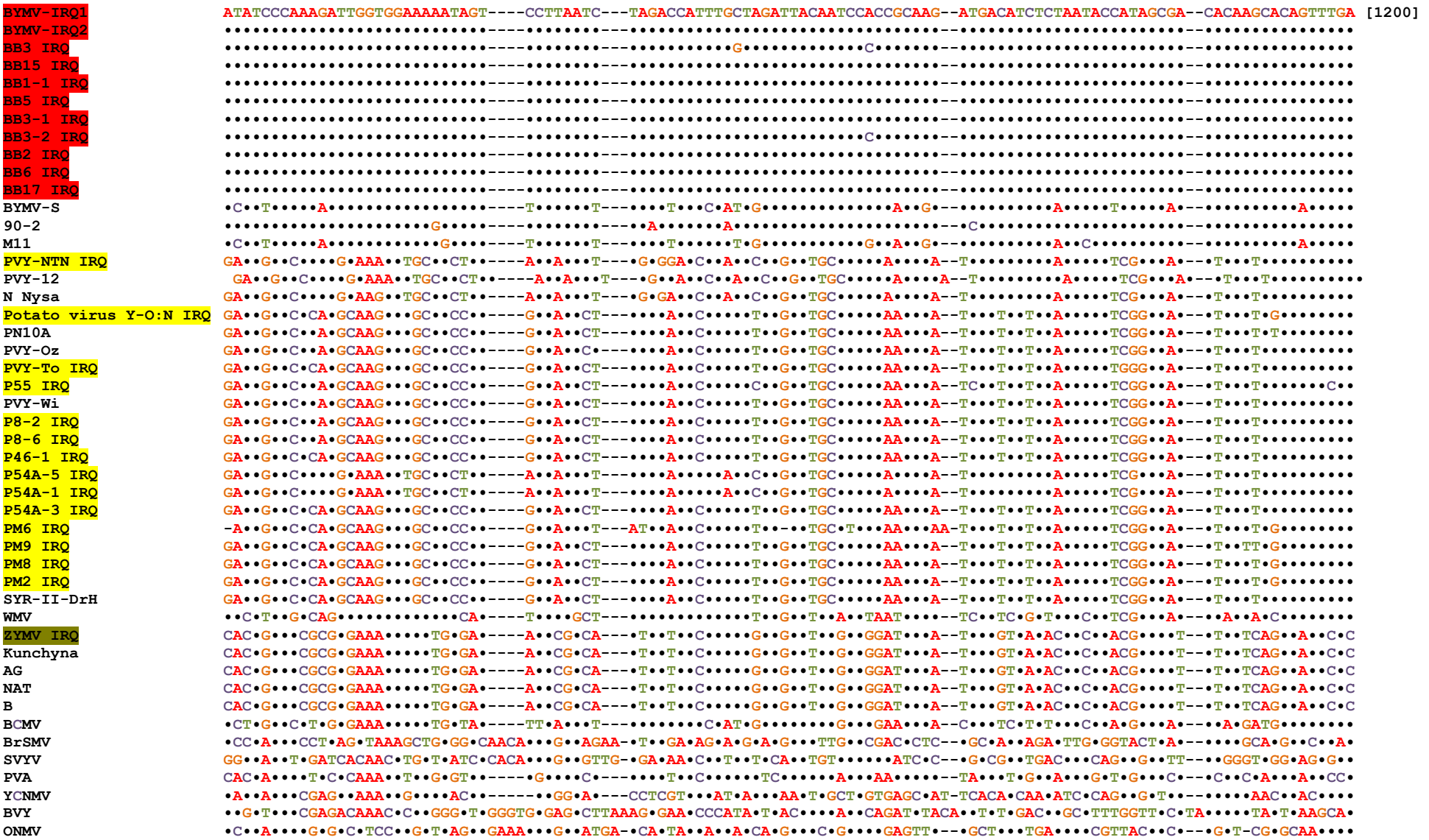
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BB5 IRQ
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PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
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P55 IRQ
PVY-Wi
P8-2 IRQ
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P54A-5 IRQ
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PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
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BrSMV
SVYV
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BVY
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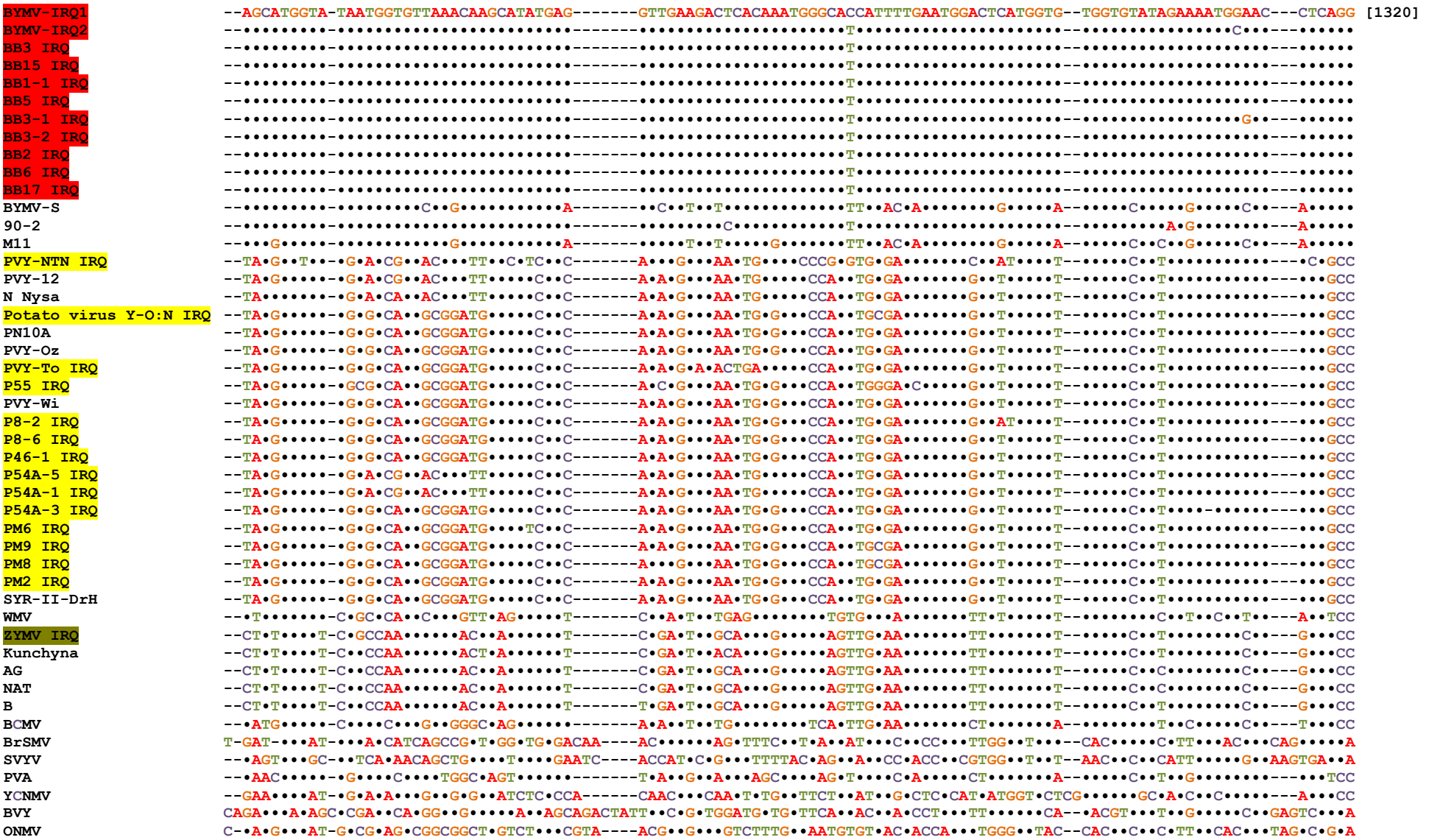
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PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
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PVY-Wi
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PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
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PVY-Wi
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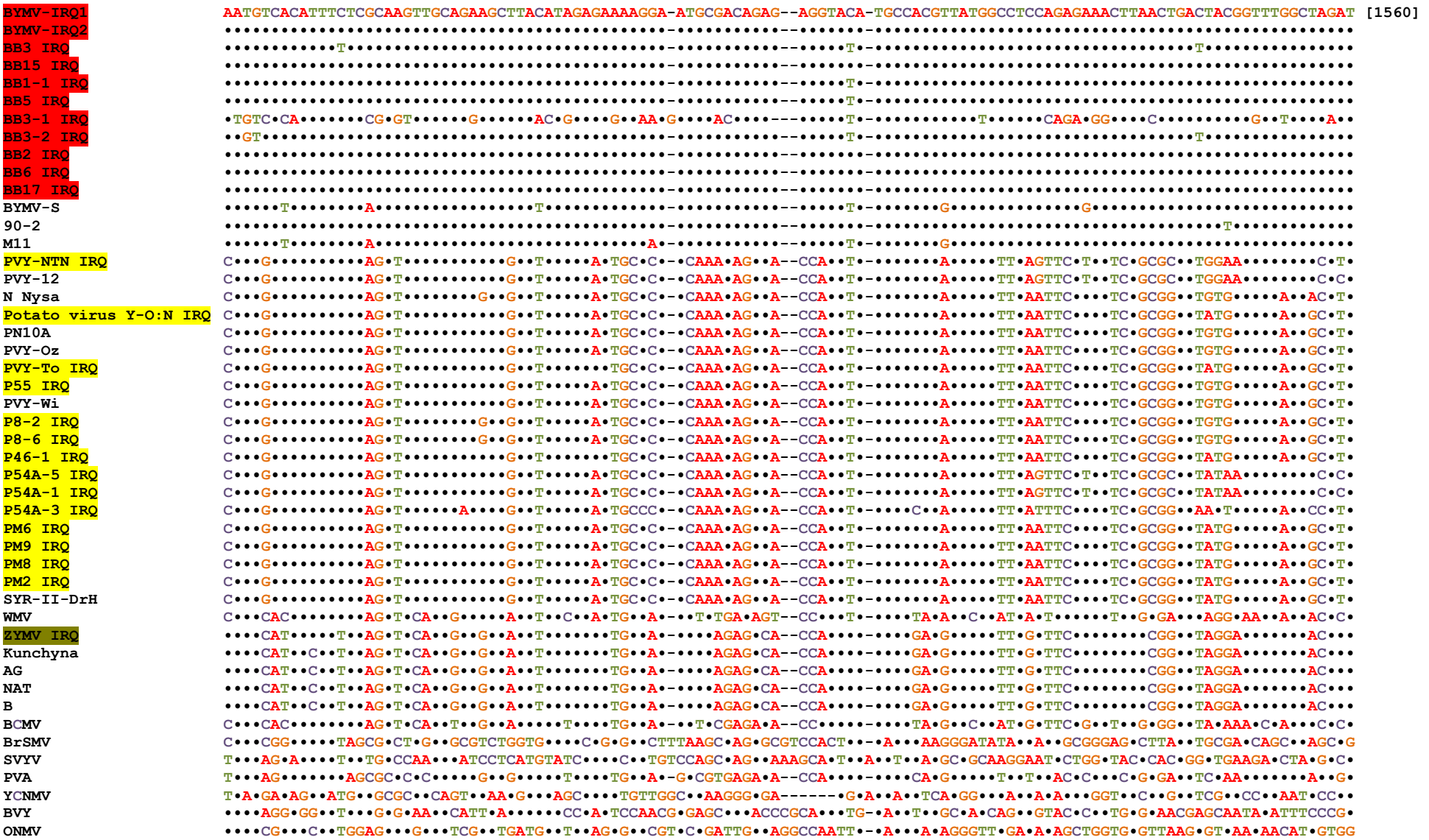
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BB17 IRQ
BYMV-S
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PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
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SYR-II-DrH
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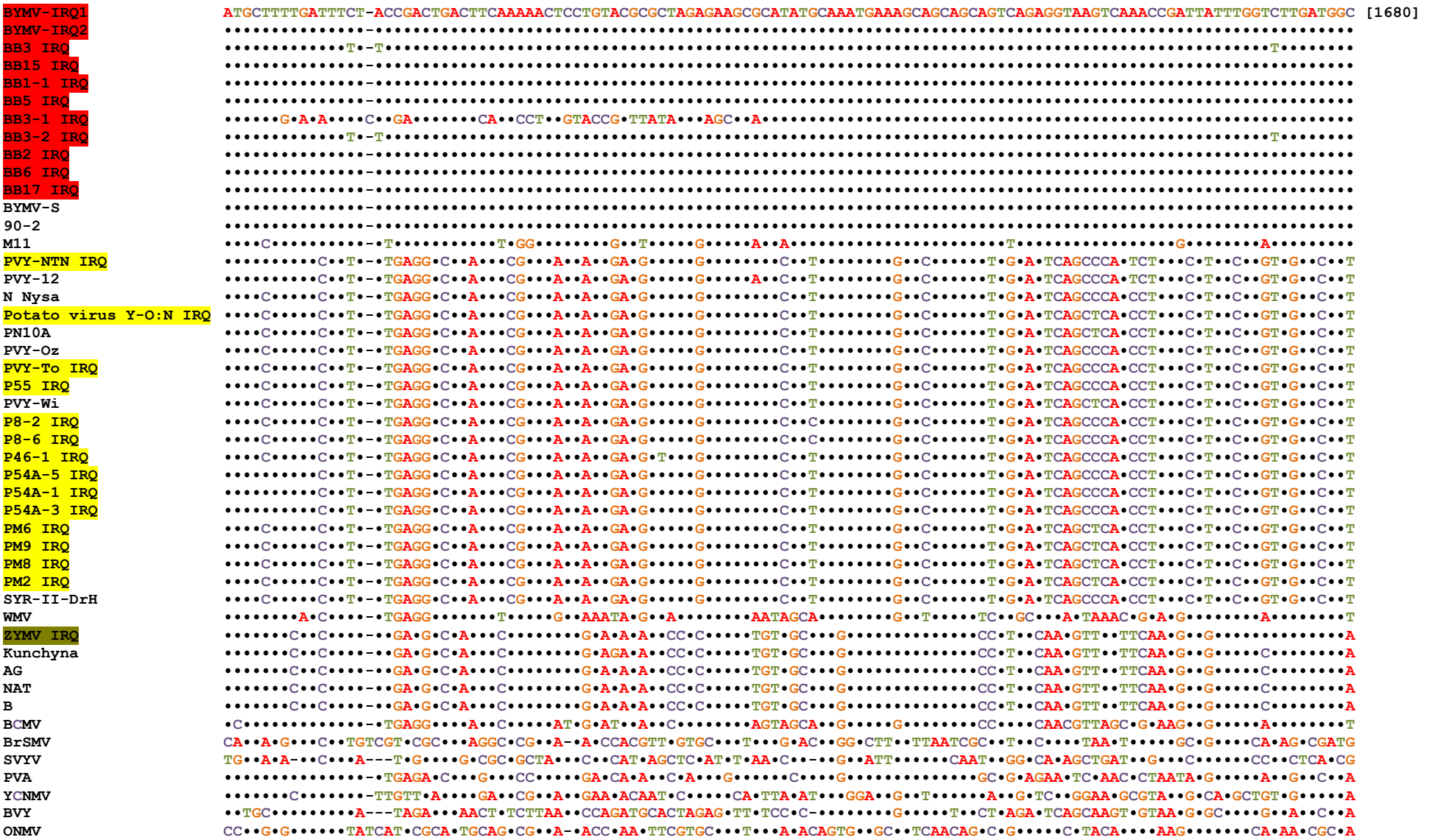
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BB6 IRQ
BB17 IRQ
BYMV-S
90-2
M11
PVY-NTN IRQ
PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
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P54A-1 IRQ
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PM6 IRQ
PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
WMV
ZYMV IRQ
Kunchyna
AG
NAT
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BCMV
BrSMV
SVYV
PVA
YCNMV
BVY
ONMV
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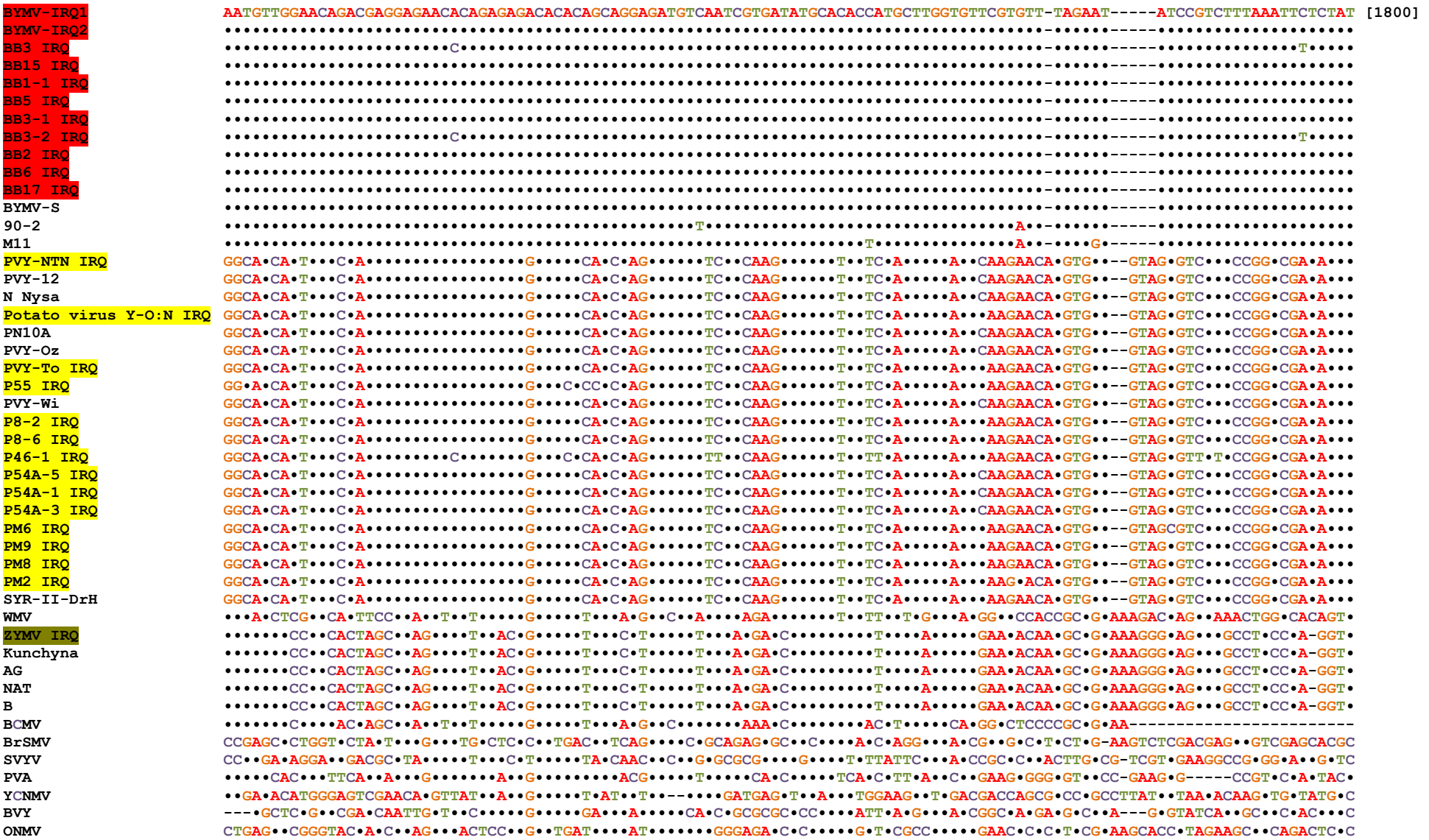
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PVY-12
N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
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WMV
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[1800]


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Potato virus Y-O:N IRQ
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PM8 IRQ
PM2 IRQ
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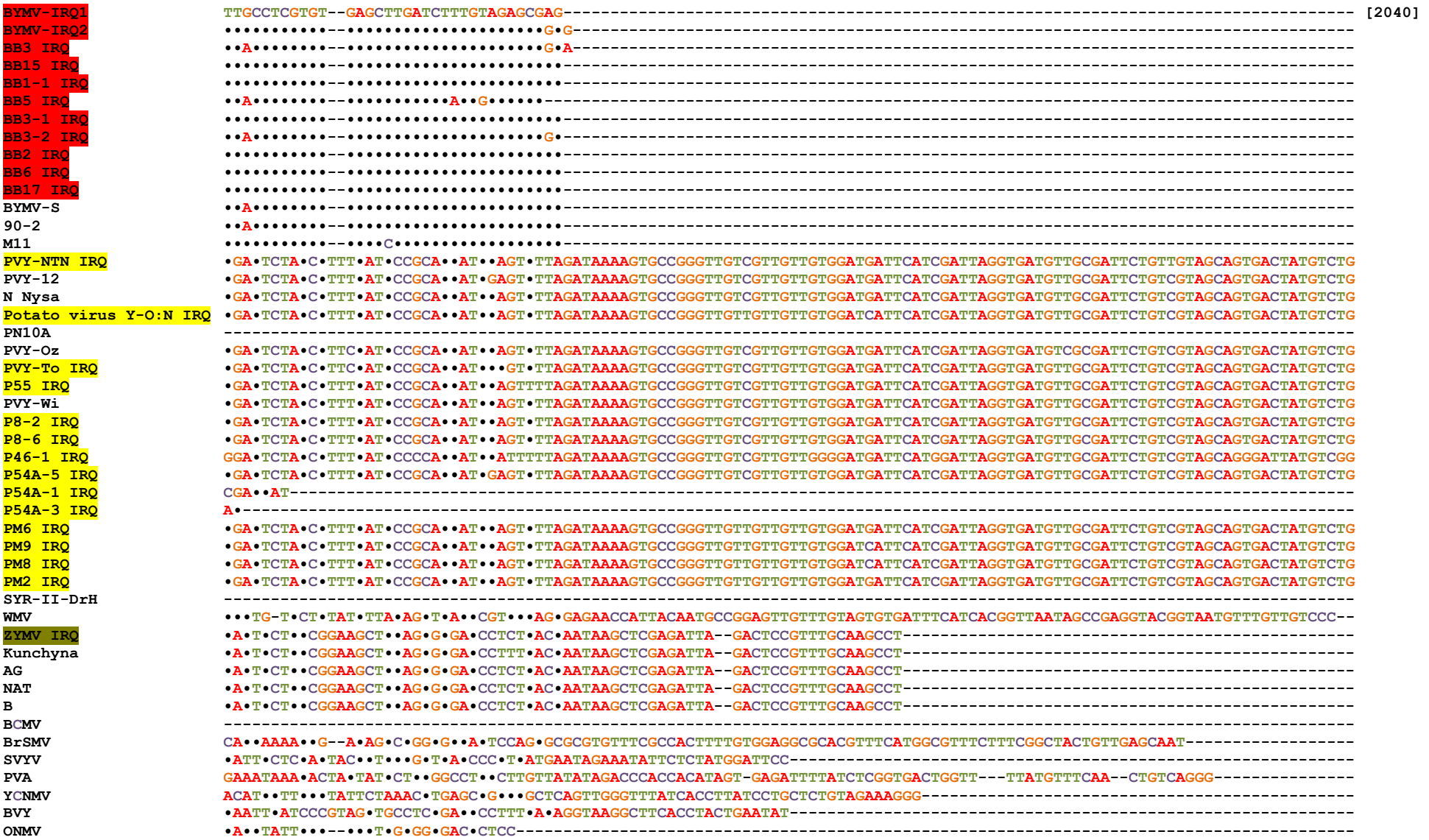
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N Nysa
Potato virus Y-O:N IRQ
PN10A
PVY-Oz
PVY-To IRQ
P55 IRQ
PVY-Wi
P8-2 IRQ
P8-6 IRQ
P46-1 IRQ
P54A-5 IRQ
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PM9 IRQ
PM8 IRQ
PM2 IRQ
SYR-II-DrH
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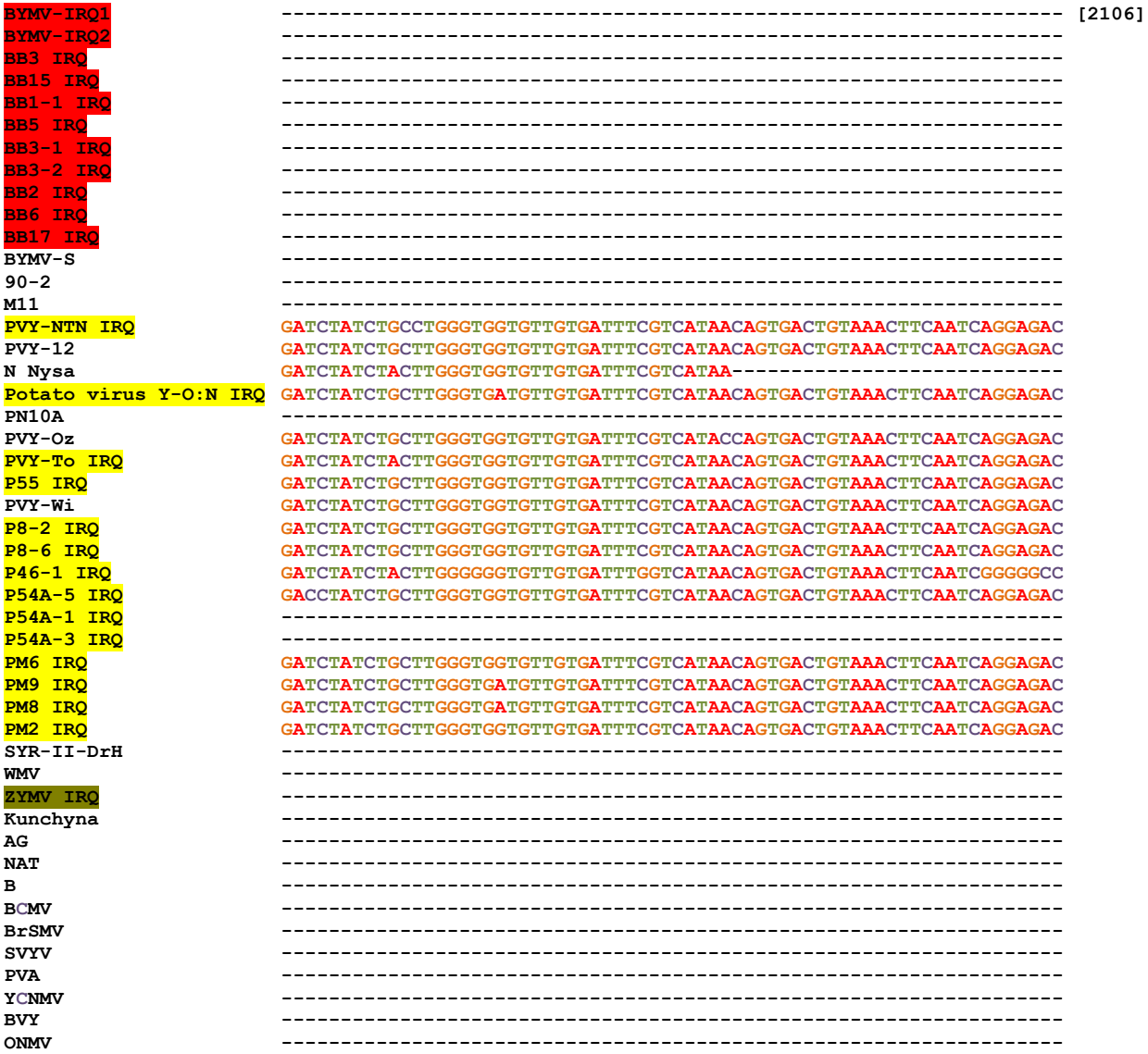
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BB2 IRQ ----------
BB6 IRQ ----------
BB17 IRQ ----------
BYMV-S ----------
90-2 ----------
M11 ----------
PVY-NTN IRQ GATCTATCTGCCTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
PVY-12 GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
N Nysa GATCTATCTACTTGGGTGGTGTTGTGATTTCGTCATAA----------
Potato virus Y-O:N IRQ GATCTATCTGCTTGGGTGATGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
PN10A ----------
PVY-Oz GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATACCAGTGACTGTAAACTTCAATCAGGAGAC
PVY-To IRQ GATCTATCTACTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
P55 IRQ GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
PVY-Wi GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
P8-2 IRQ GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
P8-6 IRQ GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
P46-1 IRQ GATCTATCTACTTGGGGGGTGTTGTGATTTGGTCATAACAGTGACTGTAAACTTCAATCGGGGGCC
P54A-5 IRQ GACCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
P54A-1 IRQ ----------
P54A-3 IRQ ----------
PM6 IRQ GATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
PM9 IRQ GATCTATCTGCTTGGGTGATGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
PM8 IRQ GATCTATCTGCTTGGGTGATGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC
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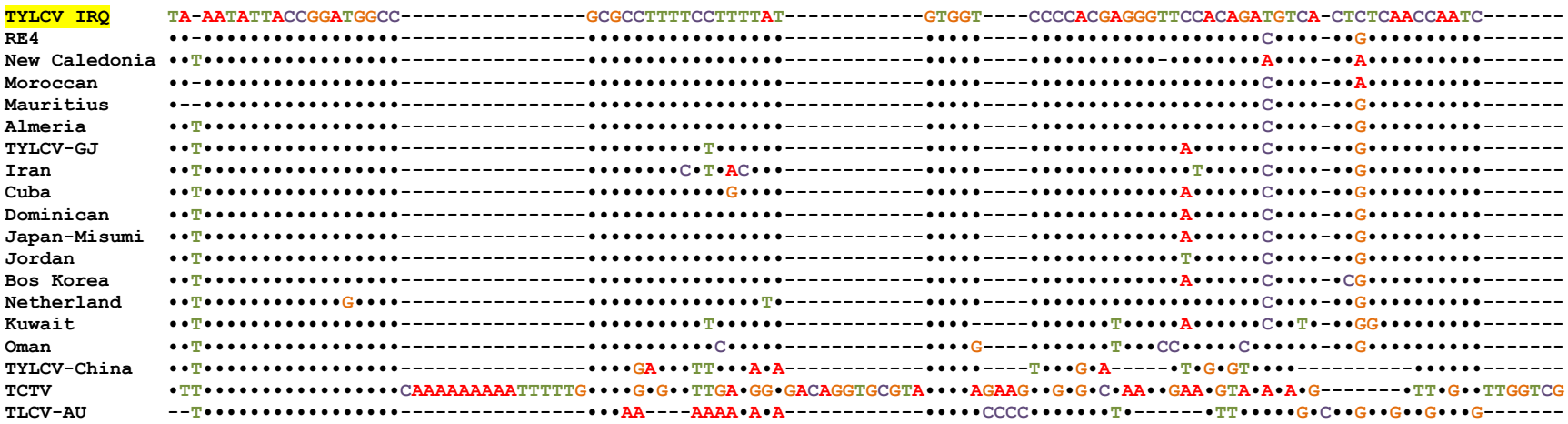
SYR-II-DrH ----------
WMV ----------
ZYMV IRQ ----------
Kunchyna ----------
AG ----------
NAT ----------
B ----------
BCMV ----------
BrSMV ----------
SVYV ----------
PVA ----------
YCNMV ----------
BVY ----------
ONMV ----------

Appendix 4: Nucleotide sequence comparison of TYLCV

Full-length nucleotide sequence alignment of *Tomato yellow leaf curl virus* (TYLCV: genus *Begomovirus*; family *Geminiviridae*) (yellow highlighted) isolated from tomato samples collected from Iraq and isolates obtained from GenBank database. The assented and deleted nucleotides were marked with (•) and (-) respectively. This alignment performed using MEGA5 software. (Tamura *et al.*, 2011)



```
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RE4           ----------•••••••••A•••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
New Caledonia ----------•••••••••AA••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
Moroccan      ----------•••••••••A•••••••••••A•••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
Mauritius     ----------•••••••••A•••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
Almeria       ----------•••••••••A•••••••••••A•••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
TYLCV-GJ      ----------•••••••••••••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•T•--------••••••••••••••
Iran          ----------•C•••A•••••••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
Cuba          ----------•••••••••••••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
Dominican     ----------•••••••••••••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•••--------••••••••••••••
Japan-Misumi  ----------•••••••••••••••••••••••••---••••----••••••••••••-•••••-•••••••••••--•••--------------•T•--------••••••••••••••
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Kuwait        ----------•••••C•••••••••••••••••••---••••----•C••••••••••-•••••-•••••••••••CA•GT--------------•••--------•••••A••••••••
Oman          ----------•••••TT•G•••GG•••••••••••---•C••----•C•TG•••••••-•••••-•••••••••••--•••--------------••G--------••••••••C•••A•
TYLCV-China   ----------•TGGCCGC••••••••GC•••AT••-----------••A•A•G•••CCC•••••A••••A•G••••--•••--------------••---------•••ACG••A-••C•
TCTV          TGTGGTCCCA••GG•AA•GTA•T•••GA••G•T••TTG••••CGAT•A••CG•••A•C-•G••CT•T•••TCT••G--•••CTAAGCTTCGGCTA•••AATTAGCA•CG••A••AGG•A•
TLCV-AU       ----------•G•ACCGCG•G•••TCGC•••TT••---•••T----••---•••••G•A•••••-••••••G•••T--•••--------------•••--------••A•G•CCAT••A•

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RE4           •••••••••••••T••••••--------••••••••••••••••••••••••••••••••••••••••••••••••••-------••••••••••••••••••••••••••••••••---
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Moroccan      •••••••••••••T••••••--------••••••••••••••••••••••••••••••••••••C•••••••••••••-------••••••••••••••••••••••••••••••••---
Mauritius     •••••••••••••T••••••--------••••••••••••••••••••••••••••••••••••••••••••••••••-------••••••••••••••••••••••••••••••••---
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TYLCV-GJ      •••••••••••••T••T•••--------••G•••••••••••••••••••••••••••••••••••••••••••••••-------••••••••••••••••••••••••••••••••---
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Bos Korea     •••••••••••••T••T•••--------••G•••••••••••••••••••••••••••••••••••••••••••••••-------••••••••••••••••••••••••••••••••---
Netherland    •••••••••••••T••••••--------••••••••••••••••••••••••••••••••••••••••••••••••••-------••••••••••••••••••••••••••••••••---
Kuwait        •••••••T•••••TG•T•••--------••••••••••••••C•••••••••••••••••••••••••••••••••••-------•••••••G•••••••••••••G••T•••••••---
Oman          ••••••••••T••T••G•••--------••••••••G•••••••••••••••••••••••••••C••••C••A•••••-------•••••••G••••••••C••••A••••••••••---
TYLCV-China   •••••••T••TT••••C••C--------••G••C••G••TA•C••A••T••G••CA•G••••••C•T••A••A•••••-------CC•••••CTT••A••AA•T••G••TTCT•••G---
TCTV          ••••---TAT•T••AA••••GATTTGGA••••CG•AAA•GA•GA•AA••••G••AG•GC••---••••G•••A•••A•AGAAGGT•••A•TC•TTA•C•GA•-T•T•••TTG••••CTTT
TLCV-AU       •••••••T••TT••G•CC•C--------••••••••••••A•C••••••••T••C•••••••••C•C••C•AC•••••-------••••TT•G•T•••••ATC••AA••••CT••GG---
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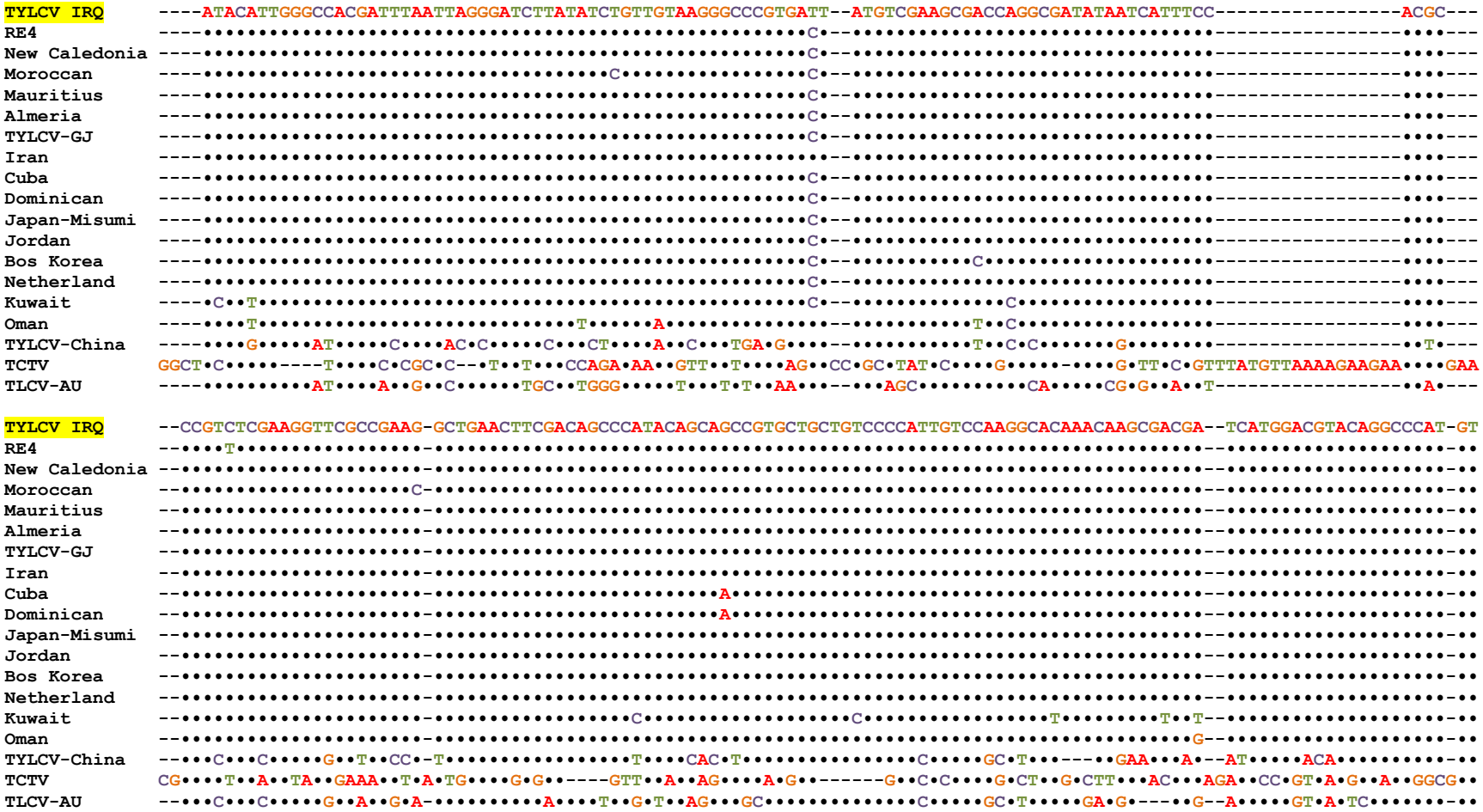
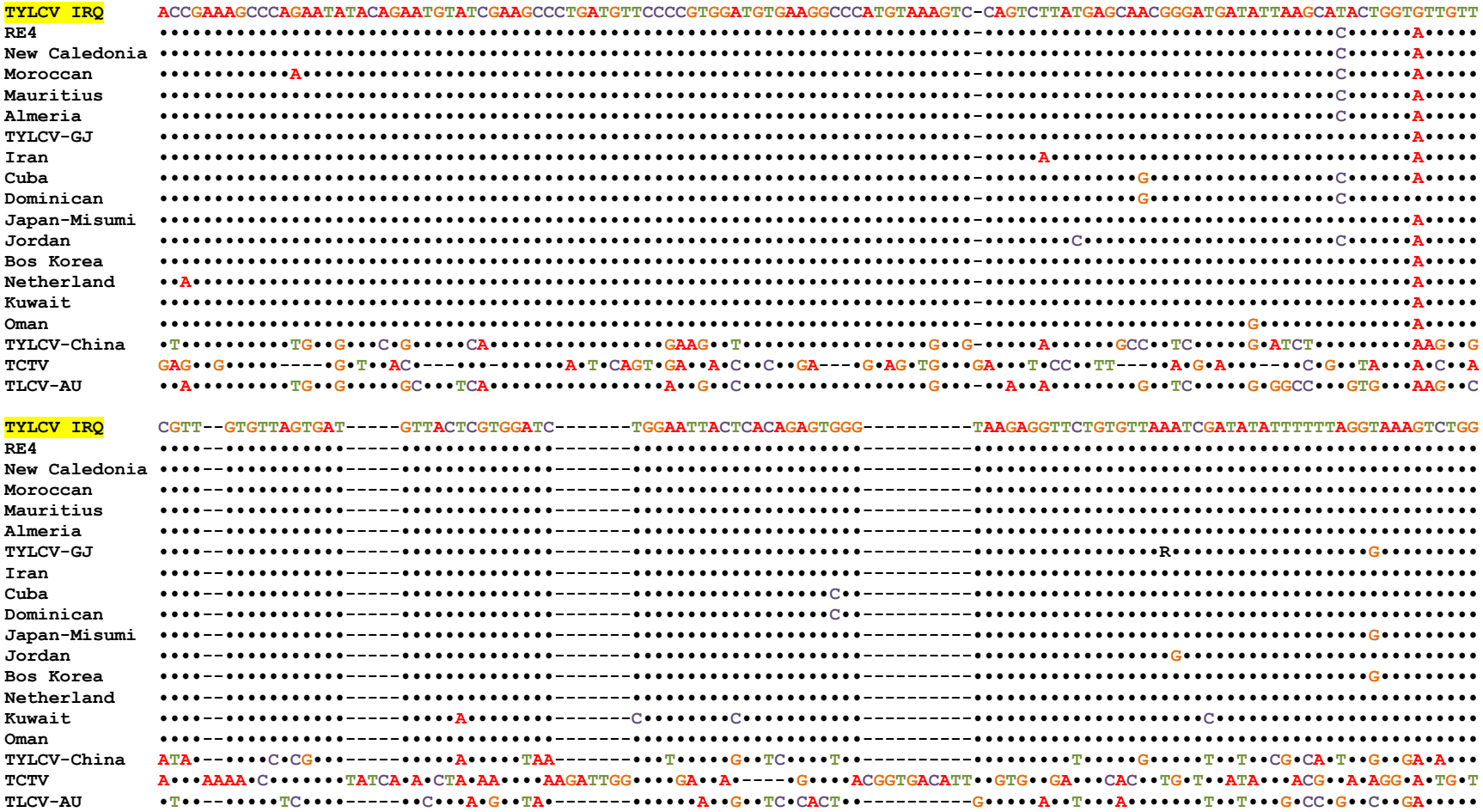
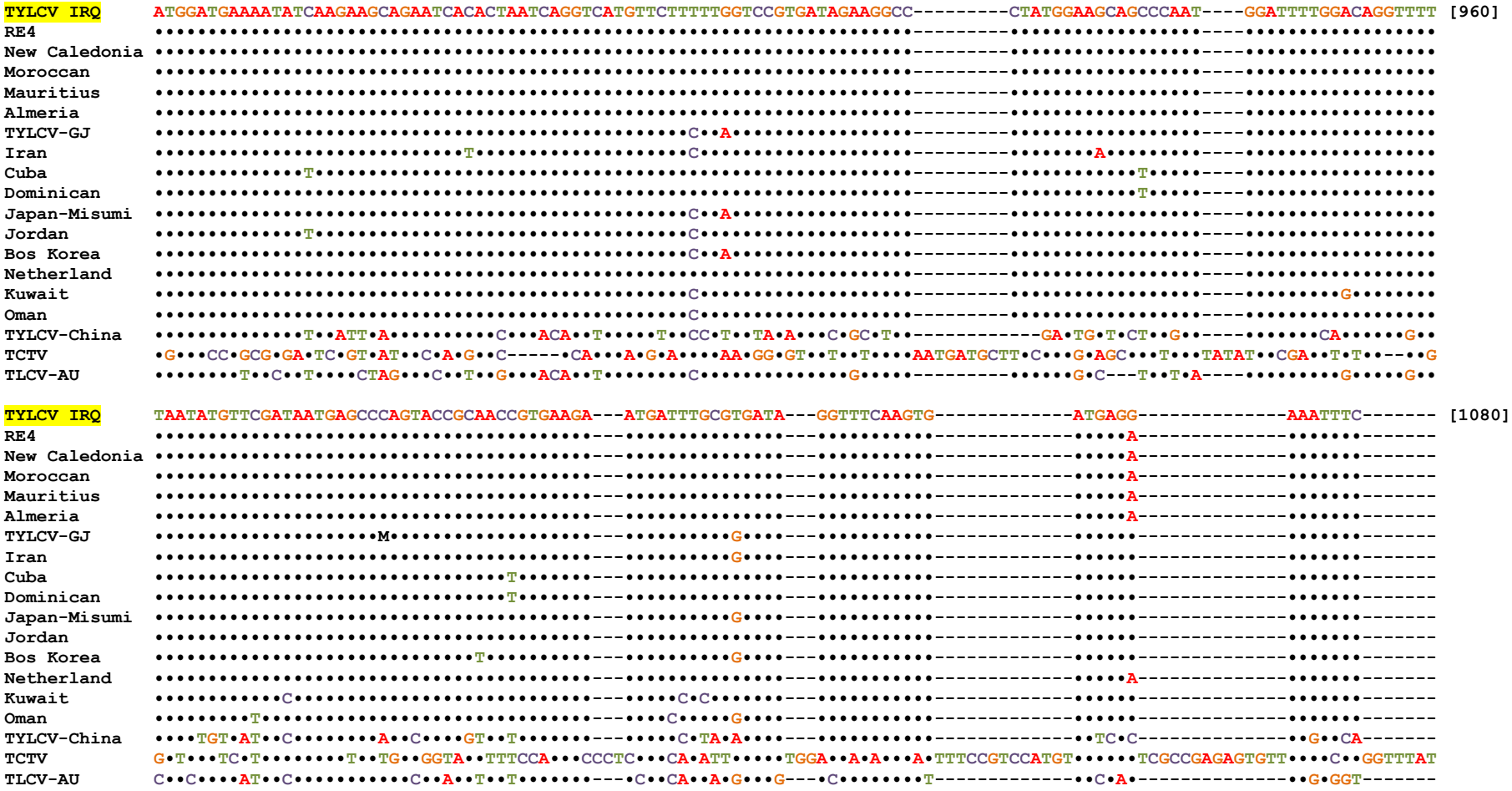
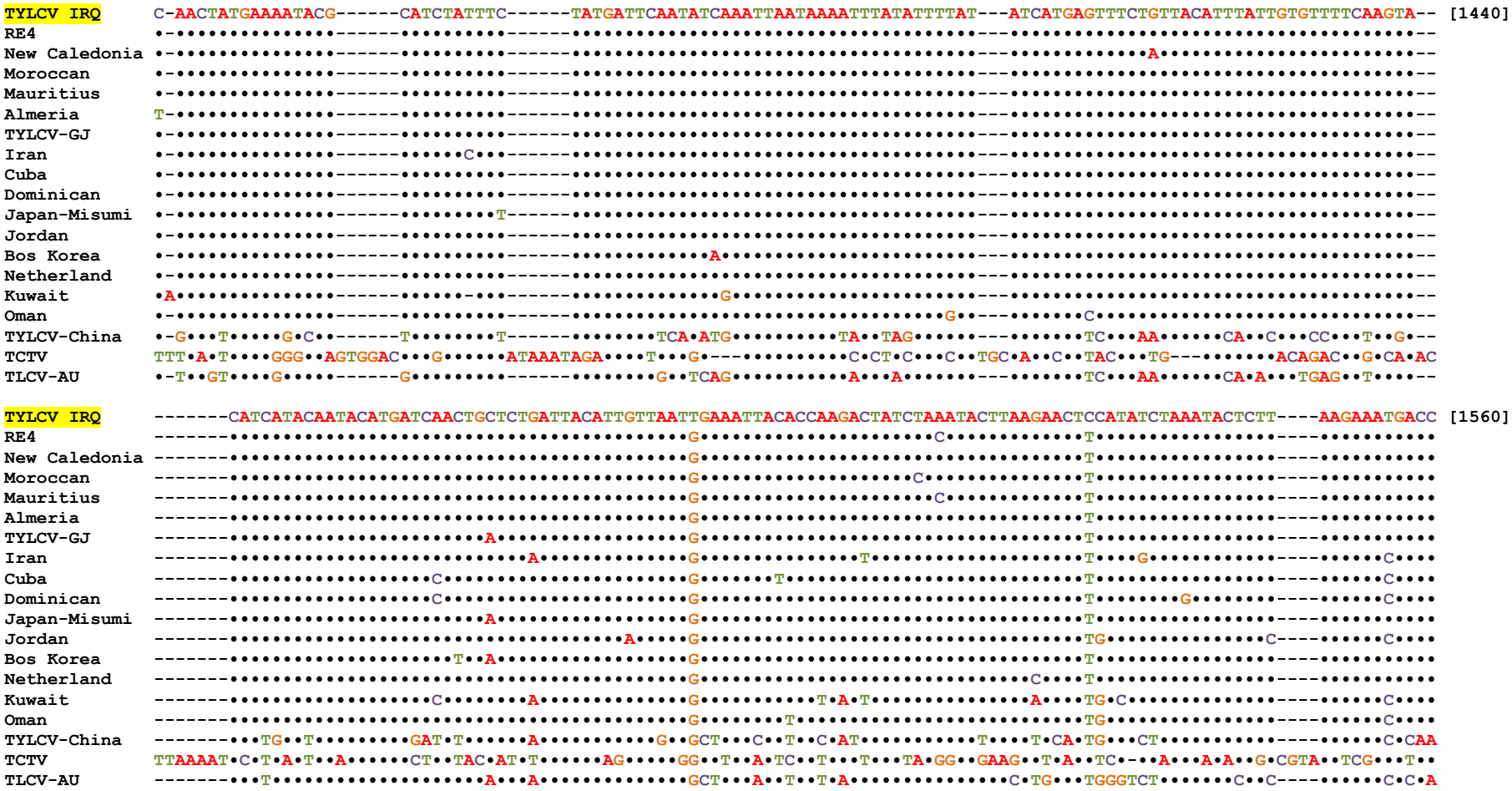

TYLCV IRQ ----ATACATTGGGCCACGATTTAATTAGGGATCTTATATCTGTTGTAAGGGCCCGTGATT--ATGTCGAAGCGACCAGGCGATATAATCATTTCC----------ACGC--- [480]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV GGCT•C•••••----T••••C•CGC•C---•T••T•••CCAGA•AA••GTT••T••••AG••CC•GC•TAT•C••••G•••••-••••G•TT•C•GTTTATGTTAAAAGAAGAA••••GAA
TLCV-AU
TYLCV IRQ --CCGTCTCGAAGGTTCGCCGAAG-GCTGAACTTCGACAGCCCATACAGCAGCCGTGCTGCTGTCCCCATTGTCCAAGGCACAAACAAGCGACGA--TCATGGACGTACAGGCCCAT-GT [600]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV CG••••T••A••TA••GAAA••T•A•TG••••G•G•-----GTT••A••AG••••A•G••------G••C•C••••G•CT••G•CTT•••AC•••AGA••CC•GT•A•G••A••GGCG••
TLCV-AU

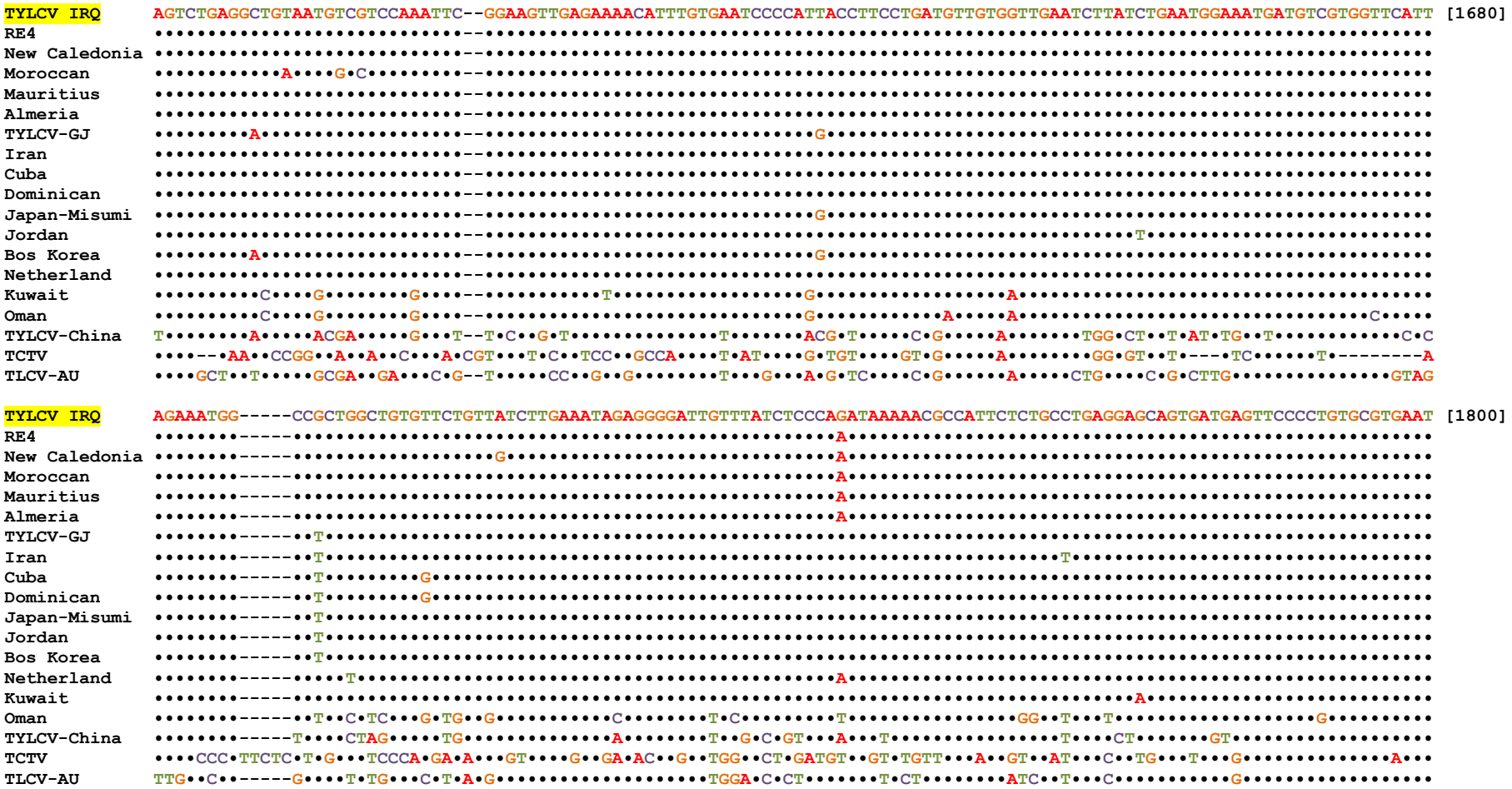

TYLCV IRQ ACCGAAAGCCCAGAATATACAGAATGTATCGAAGCCCTGATGTTCCCCGTGGATGTGAAGGCCCATGTAAAGTC-CAGTCTTATGAGCAACGGGATGATATTAAGCATACTGGTGTTGTT [720]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ CGTT--GTGTTAGTGAT-----GTTACTCGTGGATC-------TGGAATTACTCACAGAGTGGG----------TAAGAGGTTCTGTGTTAAATCGATATATTTTTTAGGTAAAGTCTGG [840]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU

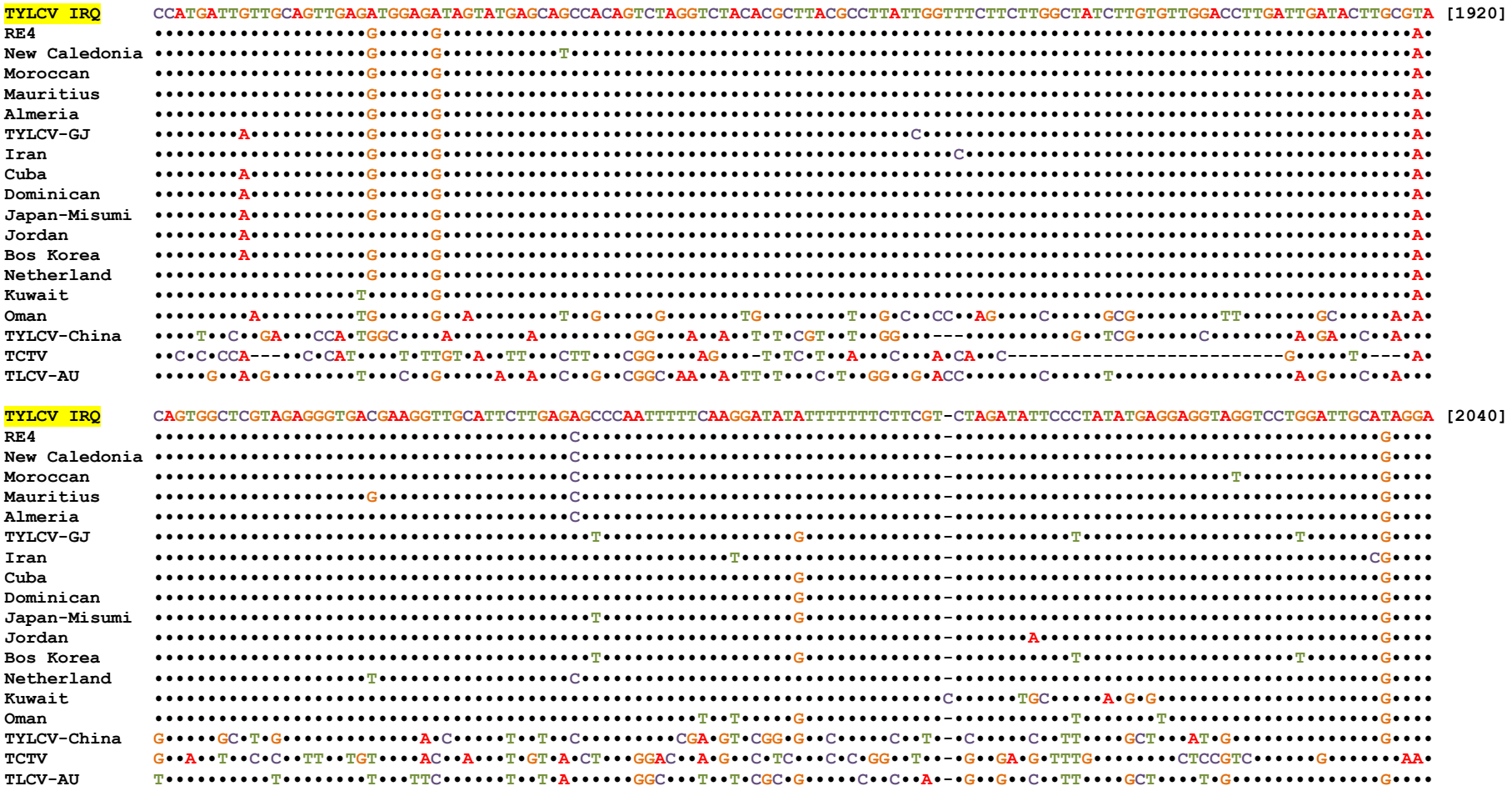

TYLCV IRQ ATGGATGAAAATATCAAGAAGCAGAATCACACTAATCAGGTCATGTTCTTTTTGGTCCGTGATAGAAGGCC---------CTATGGAAGCAGCCCAAT----GGATTTTGGACAGGTTTT [960]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ TAATATGTTCGATAATGAGCCCAGTACCGCAACCGTGAAGA---ATGATTTGCGTGATA---GGTTTCAAGTG-------------ATGAGG--------------AAATTTC-------- [1080]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU

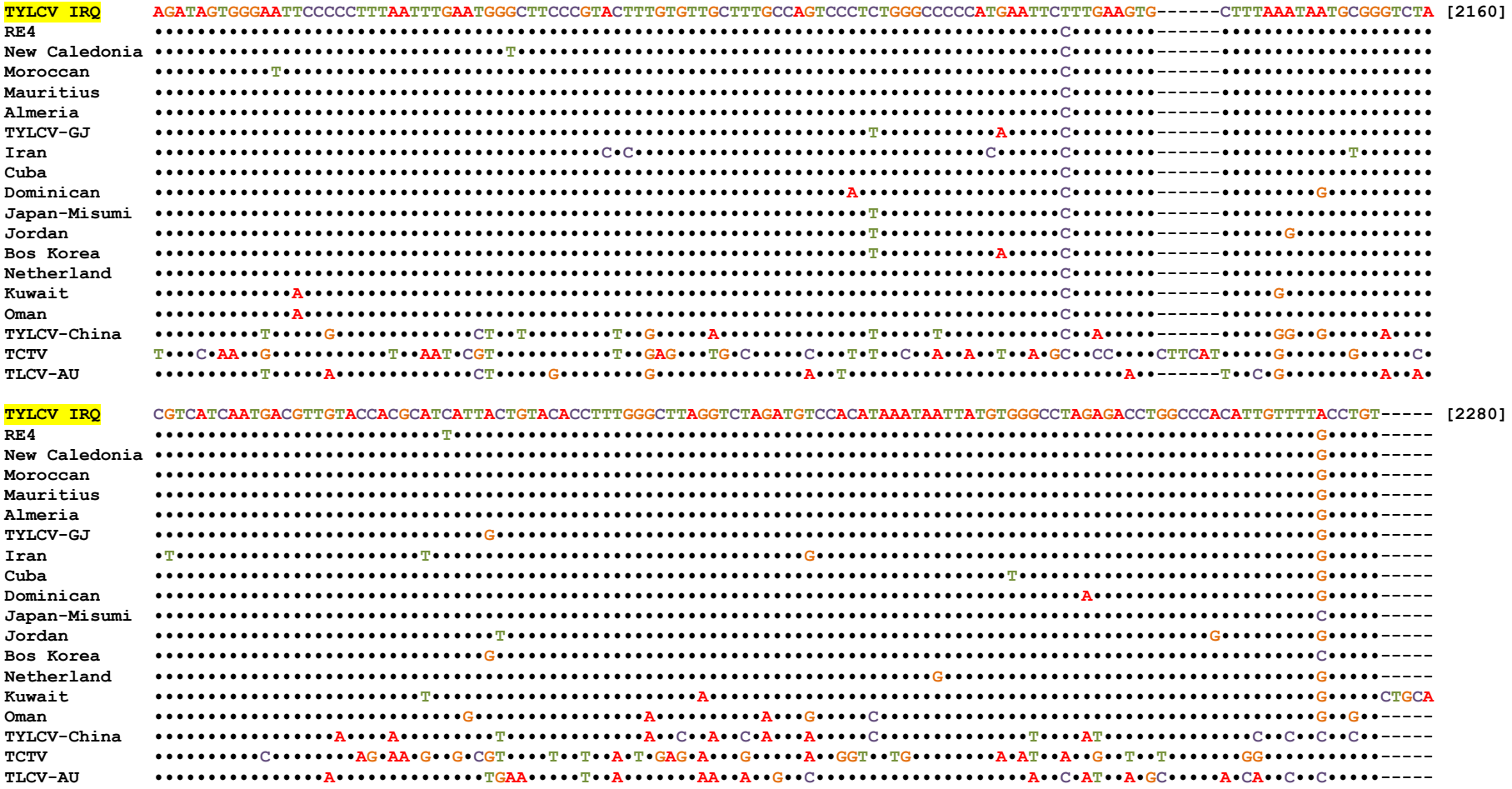
```
TYLCV IRQ      -------------ATGCAACAGT----------TATTGGTGGGCCCTCTGGAATGAAGGAA------CAGGCA------------TTAGTTAAGAGATTTTTTAAAATTAACAGTCATGT [1200]
RE4            -------------••••••••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
New Caledonia  -------------••••••••••----------••••••••••••••••••••••••A•••------••••••------------•••••••••••••••••••••••••••••••••••
Moroccan       -------------••••••••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Mauritius      -------------••••••••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Almeria        -------------••••••••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
TYLCV-GJ       -------------••••T•••••----------•••••••••A••••••••••••••••••------••••••------------•••••••••••••••••••W•••••••••••••••
Iran           -------------••••T•••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Cuba           -------------••••T•••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Dominican      -------------••••T•••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Japan-Misumi   -------------••••T•••••----------•••••••••A••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Jordan         -------------••••T•••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Bos Korea      -------------••••T•••••----------•••••••••A••••••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Netherland     -------------••••••••••----------••••••••••••T•••••••••••••••------••••••------------•••••••••••••••••••••••••••••••••••
Kuwait         -------------••••T•••••----------C••••••••A••A•••••••••••••••------•••••T------------•••••••••••••••••••••••••••••••••••
Oman           -------------••••T•••••----------••••••••••••••••••••••••••••------••••••------------•••••••••••••••••••G•••••••••••••••
TYLCV-China    -------------••TAT•TG••----------••C••••••••AA•A••CGTCC••A•••------••A•••------------G•G••G••••AG••C••GCGGG••••T•CC•••••
TCTV           TGTTAAGAGAAAG•C•G•G••T•ATTTACGGTC••CC••••TTG•T•T•••GGA•••AC••AATTATA••••GCCTAATGTTGGTCCG•••••A•AGCC•A•ACGT••G•--•••TTC••
TLCV-AU        -------------CC•••••T••----------C•C••••••••AA•A••CGTCT•••••G------•••••T------------A•TA•C••T••G••••A••••••AT•T•AC•••TG

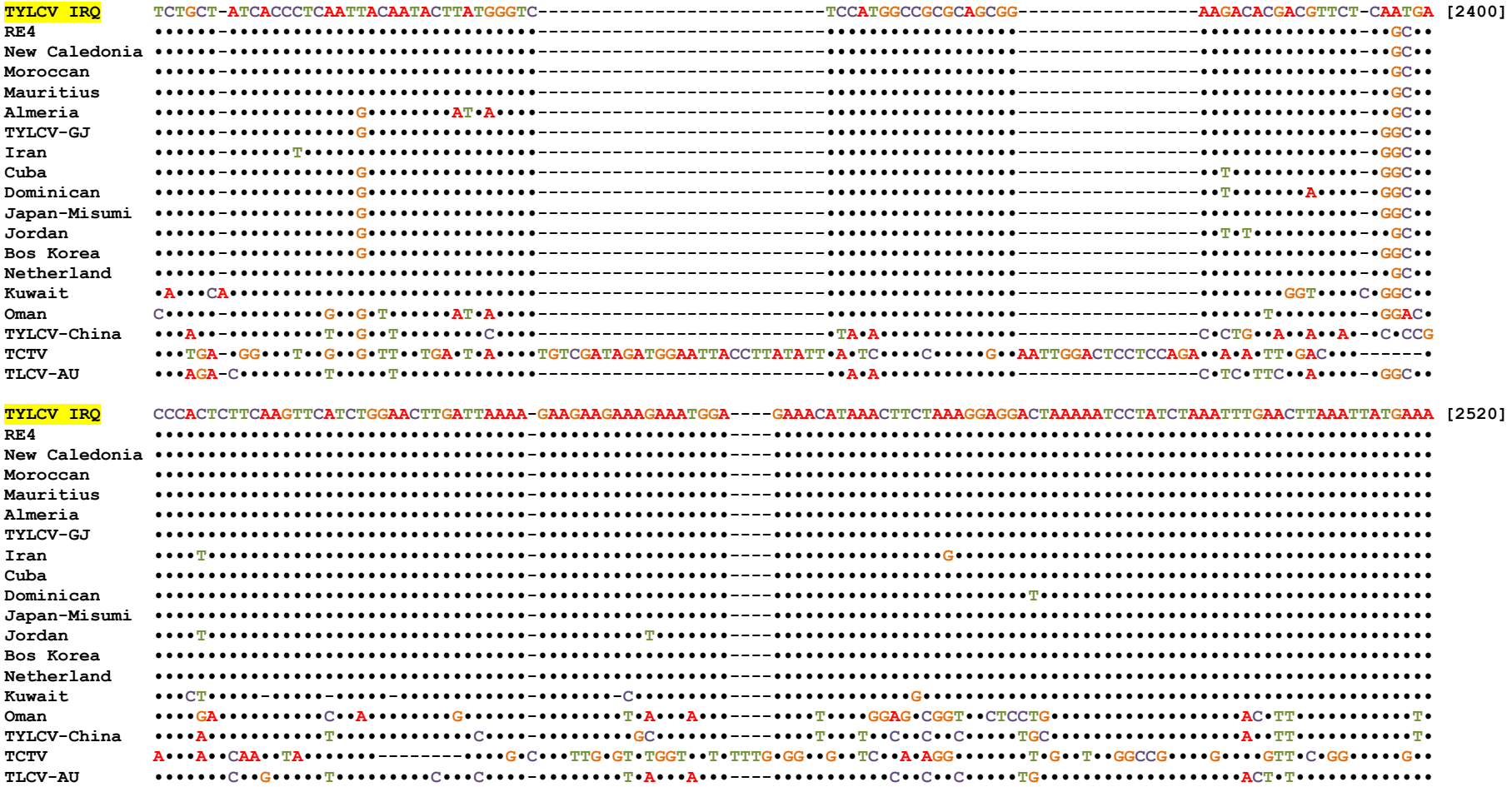
TYLCV IRQ      AACTTATAATCATC-----------AGGAGGCAGCC------AAGTACGAGAACCATACTGAAAACGCCTTGTTAT-----TGTATATGGCATGTACGCATGCCTCTAATCCAGTGTATG [1320]
RE4            ••••••••••••••-----------•••••••••••------•••••••••••••••••••••••••••••A••••-----•••••••••••••••••••••••••••••••••••••••
New Caledonia  ••••••••••••••-----------•••••••••••------••••••••A•••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Moroccan       ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Mauritius      ••••••••••••••-----------•••••••••••------•••••••••••••••••••••••••••••A••••-----•••••••••••••••••••••••••••••••••••••••
Almeria        ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
TYLCV-GJ       ••••••••••••••-----------•••••••••••------•••••••••••••••••••••••••••••••••C-----•••••••••••••••••••••••••G•••••••••••••
Iran           ••••••••••••••-----------•A•••••••••------••••••••••••••••••••••••••T•••••••-----•••••••••••••••••••••••••••••••••••••••
Cuba           ••••••••••••C•-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Dominican      ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Japan-Misumi   ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Jordan         ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Bos Korea      ••••••••••••••-----------•••••••••••------•••••••••••••••••••••••••••••••••C-----•••••••••••••••••••••••••G•••••••••••••
Netherland     ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----•••••••••••••••••••••••••••••••••••••••
Kuwait         ••••••••••••••-----------••••A••••••------•••••••••••••••••G••••••••••••C•••-----•••••••••••••••••••••••••••••••••G•••••
Oman           ••••••••••••••-----------•••••••••••------••••••••••••••••••••••••••••••••••-----••••••••••C•••••T••••••••••••••••••••••
TYLCV-China    TGTG••••••••••-----------•A••ACAG••G------•••••T••A••T••••••••G••T••G•••••G•-----•••••••••TG•••••T•••••TAG••••••T••A••••
TCTV           ••TA•••GGCAGC•TTCCGAGTGGA••••TA•G••TGGAGGA••A••••••G•TTTG•AGA••GGG••T•••••••ACGTA•••••T••TG••A---A••A•G••---•CT••A•T•TC•
TLCV-AU        T•••••C•••••••-----------••••A••T••A------•••••T•••••T••••••••G••T••A••A••••-----••••••••••••••••A•••••••••••C••••••••••
```

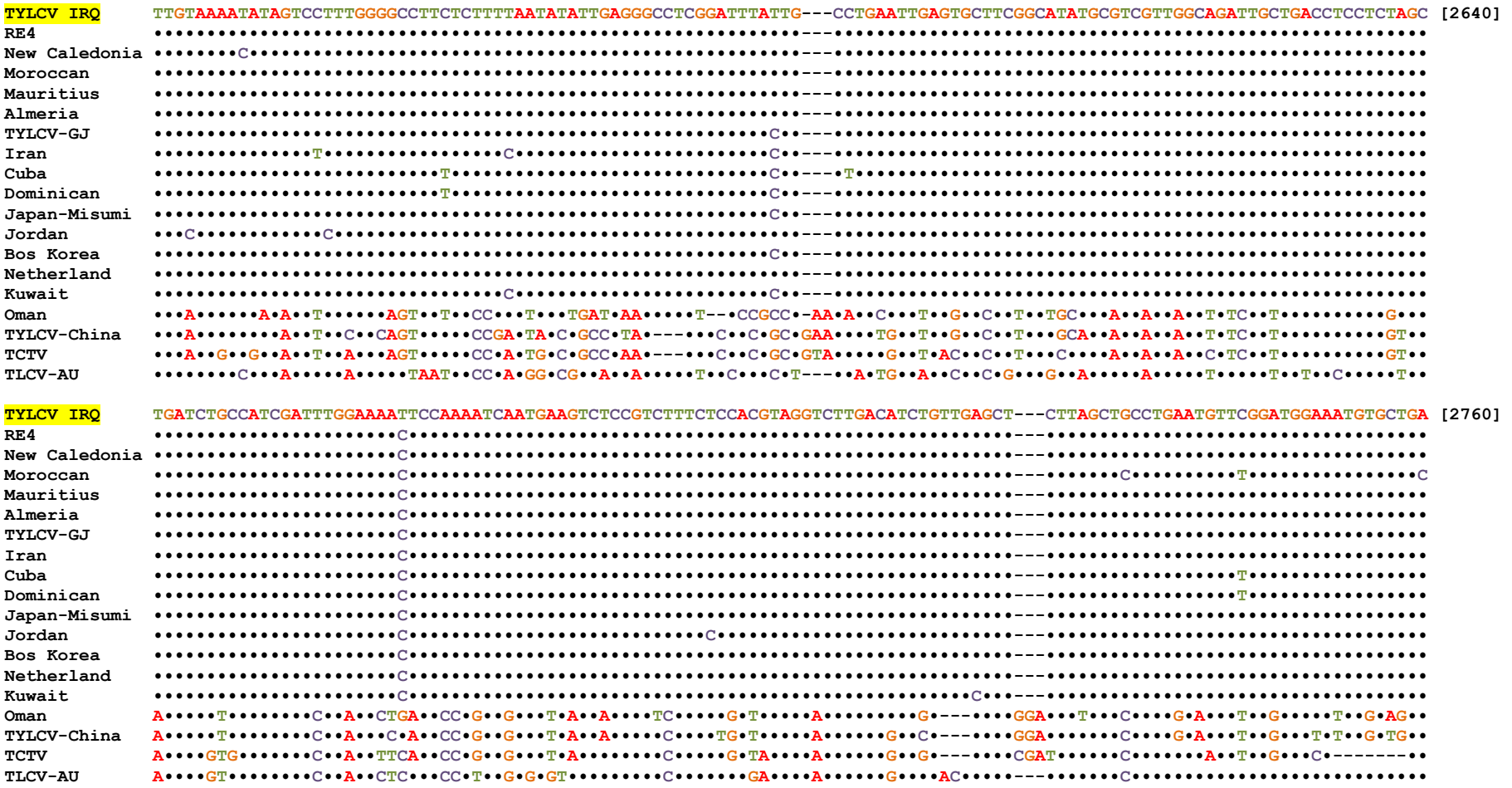

TYLCV IRQ C-AACTATGAAAATACG------CATCTATTTC------TATGATTCAATATCAAATTAATAAAATTTATATTTTAT---ATCATGAGTTTCTGTTACATTTATTGTGTTTTCAAGTA-- [1440]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ -------CATCATACAATACATGATCAACTGCTCTGATTACATTGTTAATTGAAATTACACCAAGACTATCTAAATACTTAAGAACTCCATATCTAAATACTCTT----AAGAAATGACC [1560]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU

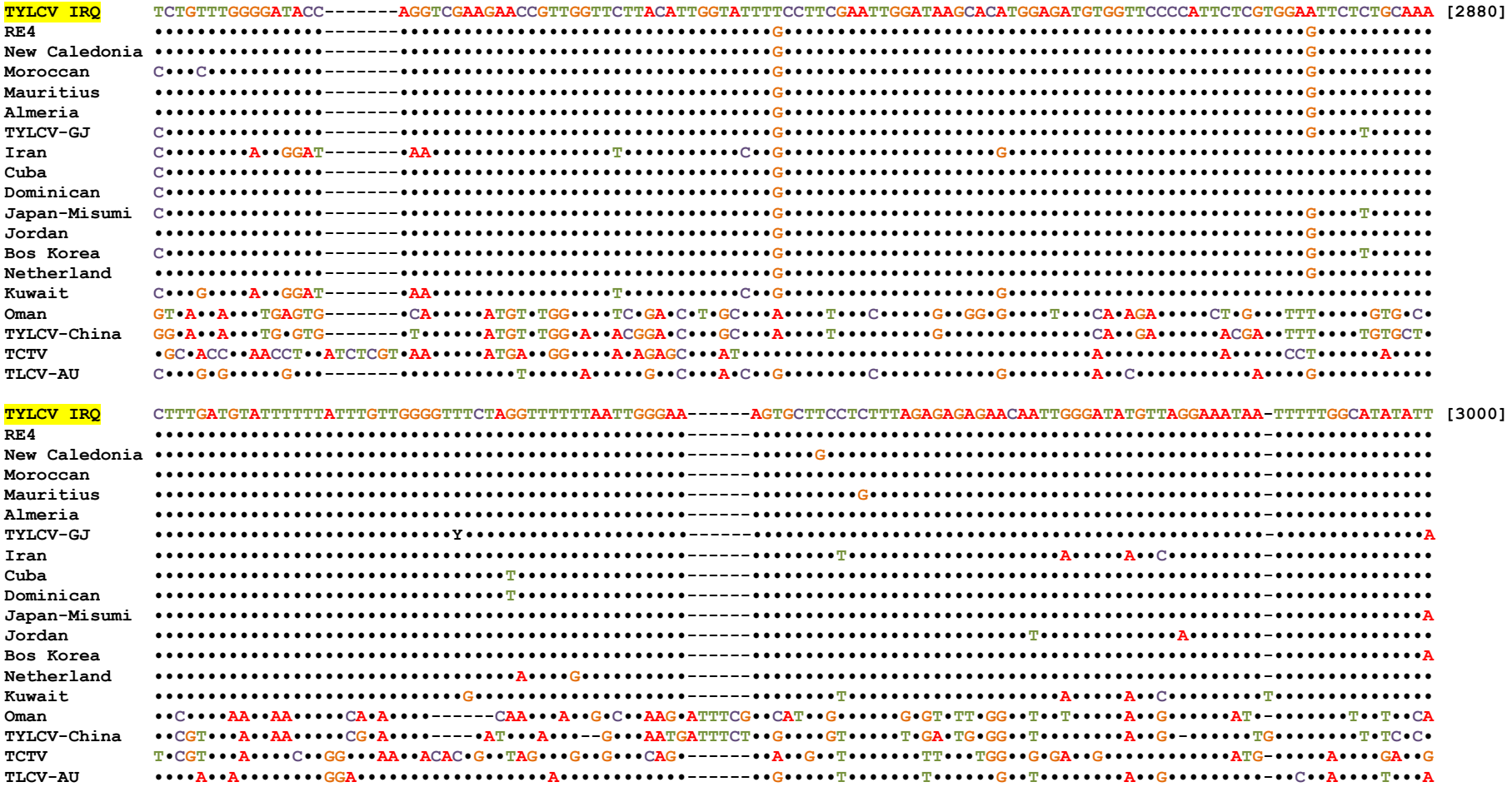

TYLCV IRQ AGTCTGAGGCTGTAATGTCGTCCAAATTC--GGAAGTTGAGAAAACATTTGTGAATCCCCATTACCTTCCTGATGTTGTGGTTGAATCTTATCTGAATGGAAATGATGTCGTGGTTCATT [1680]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ AGAAATGG-----CCGCTGGCTGTGTTCTGTTATCTTGAAATAGAGGGGATTGTTTATCTCCCAGATAAAAACGCCATTCTCTGCCTGAGGAGCAGTGATGAGTTCCCCTGTGCGTGAAT [1800]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU

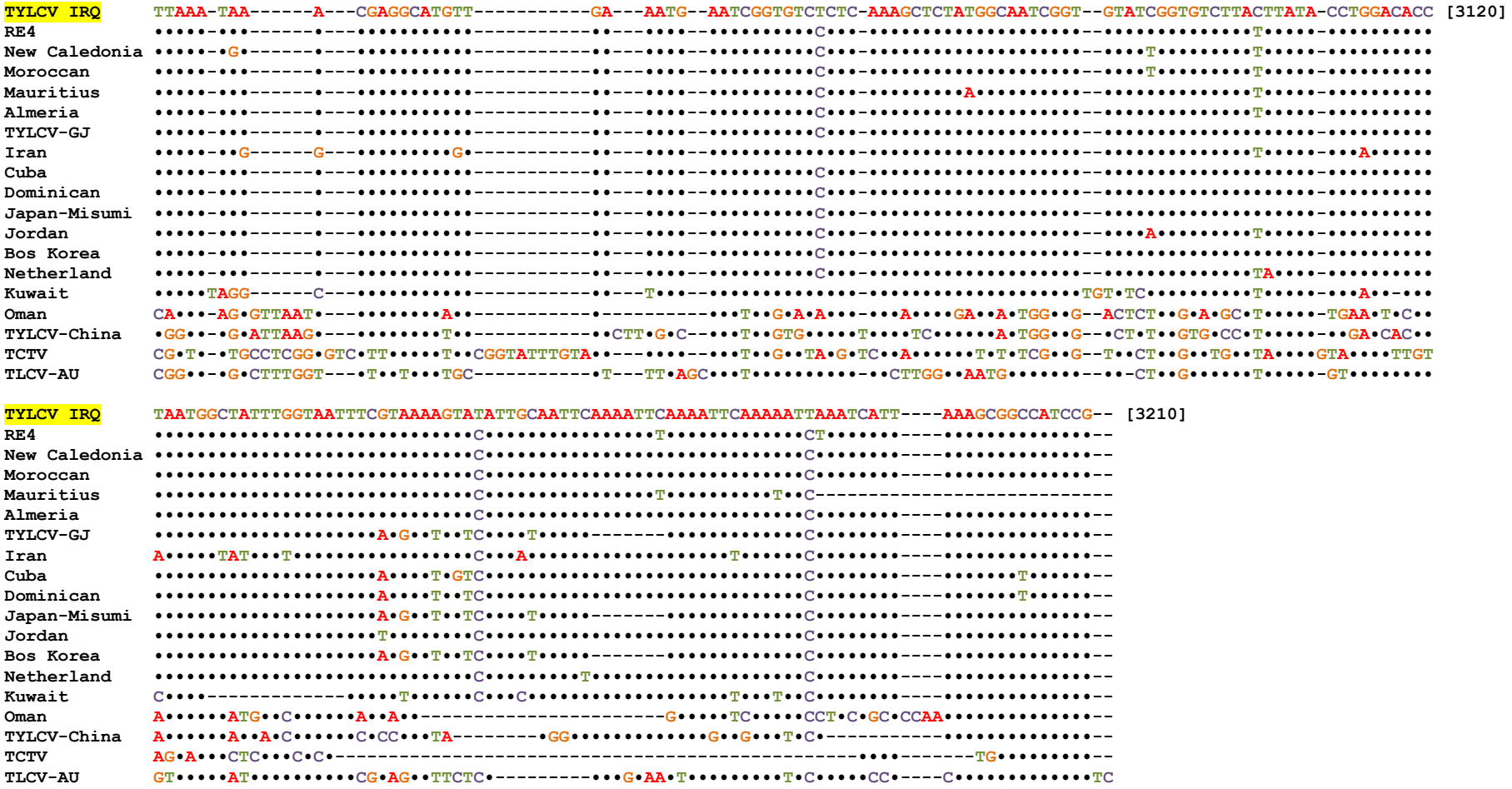

TYLCV IRQ CCATGATTGTTGCAGTTGAGATGGAGATAGTATGAGCAGCCACAGTCTAGGTCTACACGCTTACGCCTTATTGGTTTCTTCTTGGCTATCTTGTGTTGGACCTTGATTGATACTTGCGTA [1920]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ CAGTGGCTCGTAGAGGGTGACGAAGGTTGCATTCTTGAGAGCCCAATTTTTCAAGGATATATTTTTTTCTTCGT-CTAGATATTCCCTATATGAGGAGGTAGGTCCTGGATTGCATAGGA [2040]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU


TYLCV IRQ AGATAGTGGGAATTCCCCCTTTAATTTGAATGGGCTTCCCGTACTTTGTGTTGCTTTGCCAGTCCCTCTGGGCCCCCATGAATTCTTTGAAGTG------CTTTAAATAATGCGGGTCTA [2160]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ CGTCATCAATGACGTTGTACCACGCATCATTACTGTACACCTTTGGGCTTAGGTCTAGATGTCCACATAAATAATTATGTGGGCCTAGAGACCTGGCCCACATTGTTTTACCTGT----- [2280]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU


TYLCV IRQ TCTGCT-ATCACCCTCAATTACAATACTTATGGGTC----------TCCATGGCCGCGCAGCGG----------AAGACACGACGTTCT-CAATGA [2400]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ CCCACTCTTCAAGTTCATCTGGAACTTGATTAAAA-GAAGAAGAAAGAAATGGA----GAAACATAAACTTCTAAAGGAGGACTAAAAATCCTATCTAAATTTGAACTTAAATTATGAAA [2520]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU


TYLCV IRQ TTGTAAAATATAGTCCTTTGGGGCCTTCTCTTTTAATATATTGAGGGCCTCGGATTTATTG---CCTGAATTGAGTGCTTCGGCATATGCGTCGTTGGCAGATTGCTGACCTCCTCTAGC [2640]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ TGATCTGCCATCGATTTGGAAAATTCCAAAATCAATGAAGTCTCCGTCTTTCTCCACGTAGGTCTTGACATCTGTTGAGCT---CTTAGCTGCCTGAATGTTCGGATGGAAATGTGCTGA [2760]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU


TYLCV IRQ TCTGTTTGGGGATACC-------AGGTCGAAGAACCGTTGGTTCTTACATTGGTATTTTCCTTCGAATTGGATAAGCACATGGAGATGTGGTTCCCCATTCTCGTGGAATTCTCTGCAAA [2880]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
TYLCV IRQ CTTTGATGTATTTTTTATTTGTTGGGGTTTCTAGGTTTTTTAATTGGGAA------AGTGCTTCCTCTTTAGAGAGAGAACAATTGGGATATGTTAGGAAATAA-TTTTTGGCATATATT [3000]
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU


TYLCV IRQ
RE4
New Caledonia
Moroccan
Mauritius
Almeria
TYLCV-GJ
Iran
Cuba
Dominican
Japan-Misumi
Jordan
Bos Korea
Netherland
Kuwait
Oman
TYLCV-China
TCTV
TLCV-AU
[3120]
[3210]

Appendix 5: Amino acid comparison of TYLCV IRQ and GenBank sequences

Amino acid alignment of genome region of *Tomato yellow leaf curl virus* Iraqi isolate (TYLCV IRQ) and GenBank TYLCV isolates.

1. V1

```
TYLCV IRQ        MWDPLLNEFPESVHGFRCMLAIKYLQSVEETYEPNTLGHDLIRDLISVVRARDYVEATRRYNHFHARLEGSPKAELRQPIQQPCCCPHCPRHKQATIMDVQAHVPKAQNIQNVSKP [116]
RE4              ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Moroccan         ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Mauritius        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Almeria          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
TYLCV-GJ)        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Iran             ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Cuba             ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Dominican        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Japan-Misumi     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Jordan           ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Bos Korea        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Netherland       ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Oman             ••••••••••••••••••••••••••A••Q•••••••••••••••••I••••••••S•••••••••••••••••••••••••••••••••••V••••••••••••••••••
```

2. V2 or CP

```
TYLCV IRQ        MSKRPGDIIISTPVSKVRRRLNFDSPYSSRAAVPIVQGTNKRRSWTYRPMYRKPRIYRMYRSPDVPRGCEGPCKVQSYEQRDDIKHTGVVRCVSDVTRGSGITHRVGKRFCVKSIYFLGK [120]
RE4              ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Moroccan         ••••••••••••••••••T•••••••••••••••••••••••••••••••••K•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Mauritius        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Almeria          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
TYLCV-GJ         ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Iran             ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Cuba             ••••••••••••••••••••••••••••N••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Dominican        ••••••••••••••••••••••••••••N••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Japan-Misumi     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Jordan           ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Bos Korea        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Netherland       ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Oman             ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V••••I••••••••••••••••••••
```

```
TYLCV IRQ     VWMDENIKKQNHTNQVMFFLVRDRRPYGSSPMDFGQVFNMFDNEPSTATVKNDLRDRFQVMRKFHATVIGGPSGMKEQALVKRFFKINSHVTYNHQEAAKYENHTENALLLYMACTHASN [240]
RE4           ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Moroccan      ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Mauritius     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Almeria       ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
TYLCV-GJ      ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Iran          ••••••••••••••••••••••••••••N••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Cuba          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Dominican     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Japan-Misumi  ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Jordan        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Bos Korea     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Netherland    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Oman          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••R••••••••••••••••••••••••••••••••

TYLCV IRQ     PVYATMKIRIYFYDSISN [258]
RE4           ••••••••••••••••••
Moroccan      ••••••••••••••••••
Mauritius     ••••••••••••••••••
Almeria       •••V••••••••••••••
TYLCV-GJ      ••••••••••••••••••
Iran          ••••••••••••••••••
Cuba          ••••••••••••••••••
Dominican     ••••••••••••••••••
Japan-Misumi  ••••••••••••••••••
Jordan        ••••••••••••••••••
Bos Korea     ••••••••••••••••*•
Netherland    ••••••••••••••••••
Oman          ••••••••••••••••••
```

3. C3

```
TYLCV IRQ       MDSRTGELITAPQAENGVFIWEINNPLYFKITEHSQRPFLMNHDIISIQIRFNHNIRKVMGIHKCFLNFRIWTTLQPQTGHFLRVFRYGVLKYLDSLGVISINNVIRAVDHVLYDVLENT [120]
RE4             ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E••••V••••••••••••••••••••••••••
Moroccan        ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••RPF•••••••••••••E•••••••••••••••••••••••••••••••
Mauritius       ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E••••V••••••••••••••••••••••••••
Almeria         ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E•••••••••••••••••••••••••••••••
TYLCV-GJ        •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••H••••••••••••E••••••••••••••••••S••••••••••••
Iran            ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••R•••••S•E••••••N••••••••••••••••••••••••
Cuba            ••••••••••••••••••••••••••••••••D•••••••••••••••••••••••••••••••••••••••••••••••R•••••••E•••••••••••••••••••••••••••••••
Dominican       ••••••••••••••••••••••••••••••••D•••••••••••••••••••••••••••••••••••••••••••••••R••••S••E•••••••••••••••••••••••••••••••
Japan-Misumi    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E••••••••••••••••••S••••••••••••
Jordan          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••R•••••••Q•••••••••••••••••••••••••••••••
Bos Korea       •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••H••••••••••••E••••••••••••••••••SI•••••••••••
Netherland      •••••••••••••••••••••••••••••••••••K••••••••••••••••••••••••••••••••••••••••••••••••••••E•V•••••••••••••••••••••••••••••
Oman            •••••••••••H••Q••••••••E••••••••N•DE•••••S•••••••••••••••••L•••••••••••••••R••••R•••••••Q•••••••••••••••••••••••••••••••
Kuwait          ••••••••••V••••••••••••••••••••••••••••••••••••••••••••••••L•••••Y•••••••••R••••R••••••CQ•F••••N••••••••••••••••••••••••
```

```
TYLCV IRQ       INVTETHDIKYKFY [134]
RE4             ••••••••••••••
Moroccan        ••••••••••••••
Mauritius       ••••••••••••••
Almeria         ••••••••••••••
TYLCV-GJ        ••••••••••••••
Iran            ••••••••••••••
Cuba            ••••••••••••••
Dominican       ••••••••••••••
Japan-Misumi    ••••••••••••••
Jordan          ••••••••••••••
Bos Korea       ••••••••••••••
Netherland      ••••••••••••••
Oman            •••••S••••••••
Kuwait          ••••••••••••••
```

4. C2

```
TYLCV IRQ     MQPSSPSTSHCTQVSIKVQHKIAKKKPIRRKRVDLDCGCSYYLHLNCNNHGFTHRGTHHCSSGREWRFYLGDKQSPLFQDNRTQPAAISNEPRHHFHSDKIQPQHQEGNGDSQMFSQLPN [120]
RE4           ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Moroccan      ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Mauritius     ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Almeria       ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
TYLCV-GJ      ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E•••••••••••••••••••••H••••••••••
Iran          ••••••••••S••••••••••••R•••••••••••••••••••••••••••••••••••S••••••••••••••••••••E••••••••••••••••••••••••••••••••••
Cuba          ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••P••E••••••••••••••••••••
Dominican     ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••P••E••••••••••••••••••••
Japan-Misumi  ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E••••••••••••••••••••H••••••••••
Jordan        ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E••••••••••••N••••••••••••••••••••
Bos Korea     ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••E••••••••••••••••••••H••••••••••
Netherland    ••••••••••S•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••Q••••••••••••••••••••••••••••••••••
Oman          ••••••••••S••P••I••R•••T•AV••••I••••••••••••I••I••••••••••••••SP•••••M••RK••V•••HQPRRE•••••••••••••••••S•Y•••T••••••••••
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```
TYLCV IRQ     LDDITASDWSFLKSI [135]
RE4           ••••••••••••••••
Moroccan      •EA•S••••••••••••
Mauritius     ••••••••••••••••
Almeria       ••••••••••••••••
TYLCV-GJ      •••••S•••••••••••
Iran          ••••••••••••••••
Cuba          ••••••••••••••••
Dominican     ••••••••••••••••
Japan-Misumi  ••••••••••••••••
Jordan        ••••••••••••E••••
Bos Korea     •••••S•••••••••••
Netherland    ••••••••••••••••
Oman          •••L••••••••••••
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5. C1 or rep region

```
TYLCV IRQ     MPRLFKIYAKNYFLTYPNCSLSKEEALSQLKNLETPTNKKYIKVCREFHENGEPHLHVLIQFEGKYQCKNQRFFDLVSPNRSAHFHPNIQAAKSSTDVKTYVEKDGDFIDFGIFQIDGRS [120]
RE4           •••••••••••••••••••••••••••••••••••••••••••••••L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Moroccan      •••••••••••••••••••••••••••••••••••••••••••••••L•••••••••••••••••••••••••••••••S•A••••••••G•••••••••••••••••••••V•••••••
Mauritius     •••••••••••••••••••••••Q•••••••••••••••••••••••L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Almeria       •••••••••••••••••••••••••••••••••••••••••••••••L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
TYLCV-GJ      •••••N•••••••••••••••••••••••••••••••••••••••K•L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Iran          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••S•••••••••••••••••••••••••••••••••••V•••••••
Cuba          ••••••••••••••••••••••••••••••••I•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Dominican     ••••••••••••••••••••••••••••••••I•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Japan-Misumi  •••••N•••••••••••••••••••••••••••••••••••••••K•L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Jordan        •••••••••••••••••K•••••••••••••••••••••••••••••L••••••••••••••••••••••••••••••••••••••••••••••••••••••G•••••••••V•••••••
Bos Korea     •••••N•••••••••••••••••••••••••••••••••••••••K•L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Netherland    ••••••••••••••••••••••••••••••Q••••••••••••••••L••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Oman          •••INSFCVNTKNIFLTYPKCPIPKEQMLEILQSINCPSDKLFIRVSREKHQDGS••IHALIQF•GKA•FRNPRHFDVTHPNNSSQFHPNFQGAKSSSDVKSYIEKNGDYIDWG•FQIDG

TYLCV IRQ     ARGGQQSANDAYAEALNSGNKSEALNILKEKAPKDYILQFHNLSSNLDRIFSPPLEVYVSPFLSSSFNQVPDELEEWVIENVVSSAARPWRPISIVIEGDSRTGKTMWARSLGPHNYLCG [360]
RE4           ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Moroccan      ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Mauritius     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Almeria       ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••N•••••••••••••••••••••••••••
TYLCV-GJ      •••••••••••••••••••S••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Iran          •••••••••••••••••••S••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Cuba          ••••••••••••••••••SS••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••••Y•••••••••••••••••••••••••••••Q••••••
Dominican     •••••••••••••••••••S•••••••••••••••••••••••••••••••N••••••••••••••••••••••••••A••••Y••••••••••••••••••••••••••••••••••••
Japan-Misumi  •••••••••••••••••••S••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Jordan        •••••••••••••••••••••••••••••••••R••••••••••••••••••••••••••••I•••••••••••••••A••••Y•••••••••••••••••••••••R••••••••••••
Bos Korea     •••••••••••••••••••S••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Netherland    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••
Oman          RSARGGQQTAND•A•EALNAC•AEEALSIIREKLPKDFI•QYHNLKCNLDRIFQEPPAPYISPFL•SSFNQVPE•LE•WVSENVMSSAARPWRPNS•IIEGDSRTGKTMWARSLGPHNYL

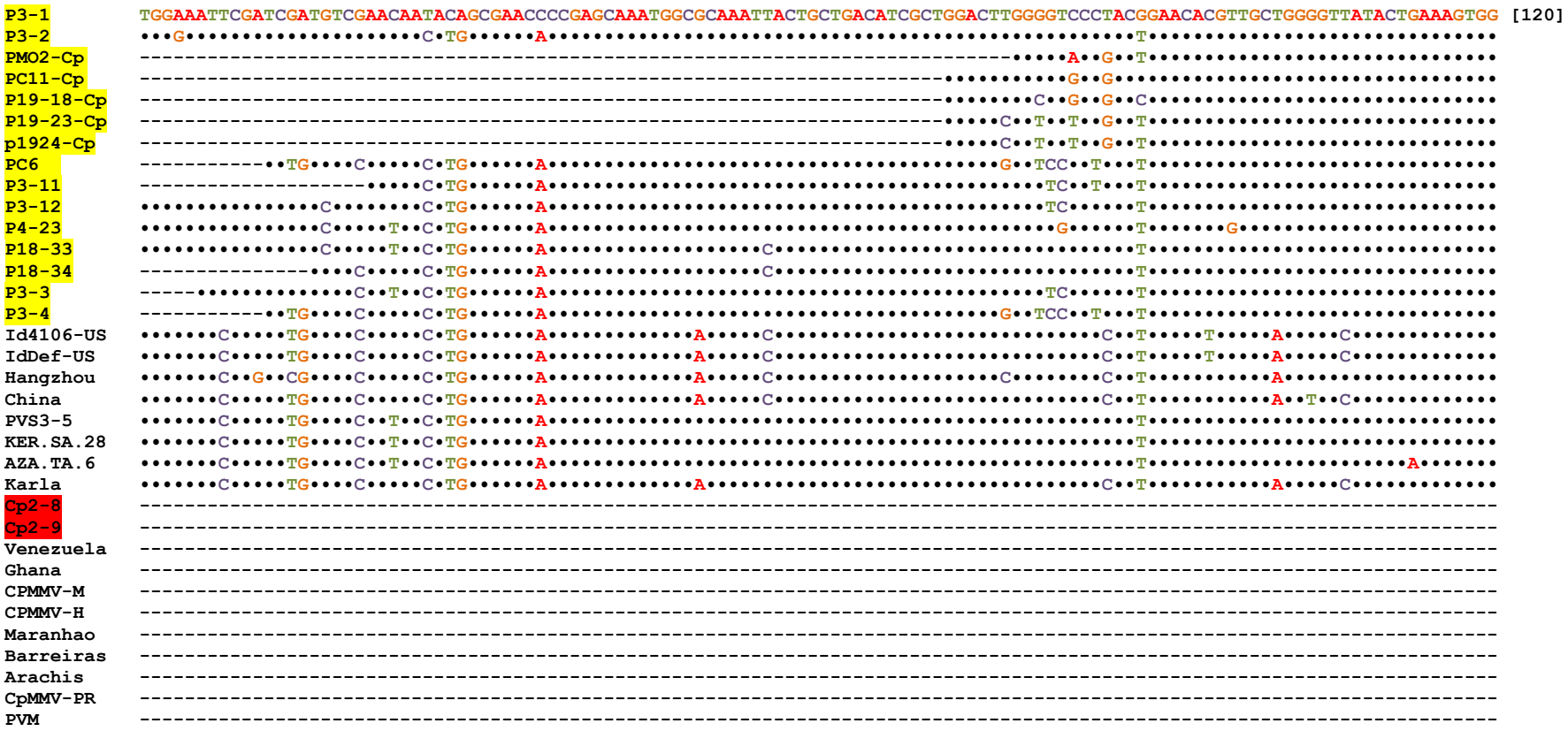
TYLCV IRQ     HLDLSPKVYSNDAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPIQIKGGIPTIFLCNPGPTSSYREYLDEEKNISLKNWALKNATFVTLYEPLYASINQGPTQDSQEETNKA [358]
RE4           •••••••••••N••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••F••••••••••••••••••
Moroccan      ••••••••••••••••••••••••••••••••••••••••••••••••••••••N•••••••••H•••••••••••••••••••••••••••••••••F••••••••••••••••••
Mauritius     •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••L••••••F••••••••••••••••••
Almeria       ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••F••••••••••••••••••
TYLCV-GJ      ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••Q••••••K••••••••••••••T•••••••••••••F•••••••••••••••D••
Iran          •••••••••••E•••••••E••••••••••••V••••••••••RD•••••••••••••••G•••••••••••••••••••••••••••••••••••••F••••••••••••••••••
Cuba          ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••F••••••••••••••••••
Dominican     ••••••••••••••••••••••••••••••••••••M•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••F••••••••••••••••••
Japan-Misumi  ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••F••••••••••••••••••
Jordan        •••••••••N••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••D•••••••••••••••••••••••••••F••••••••••••••••••
Bos Korea     ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••Q••••••K••••••••••••••T•••••••••••••F••••••••••••••••••
Netherland    •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••I••••••F••••••••••••••••••
Oman          CGH•DLSPKVYSTDAWYNID•VDPHYLKHFKEFMGAQRDWQSNTKYGKP•QIKGGIPTIFLCN•GPTSSYKEYLDEEKNISLKNWALKNATFVTLYEPLFSSANQNPTPHS•D*GSQ
```

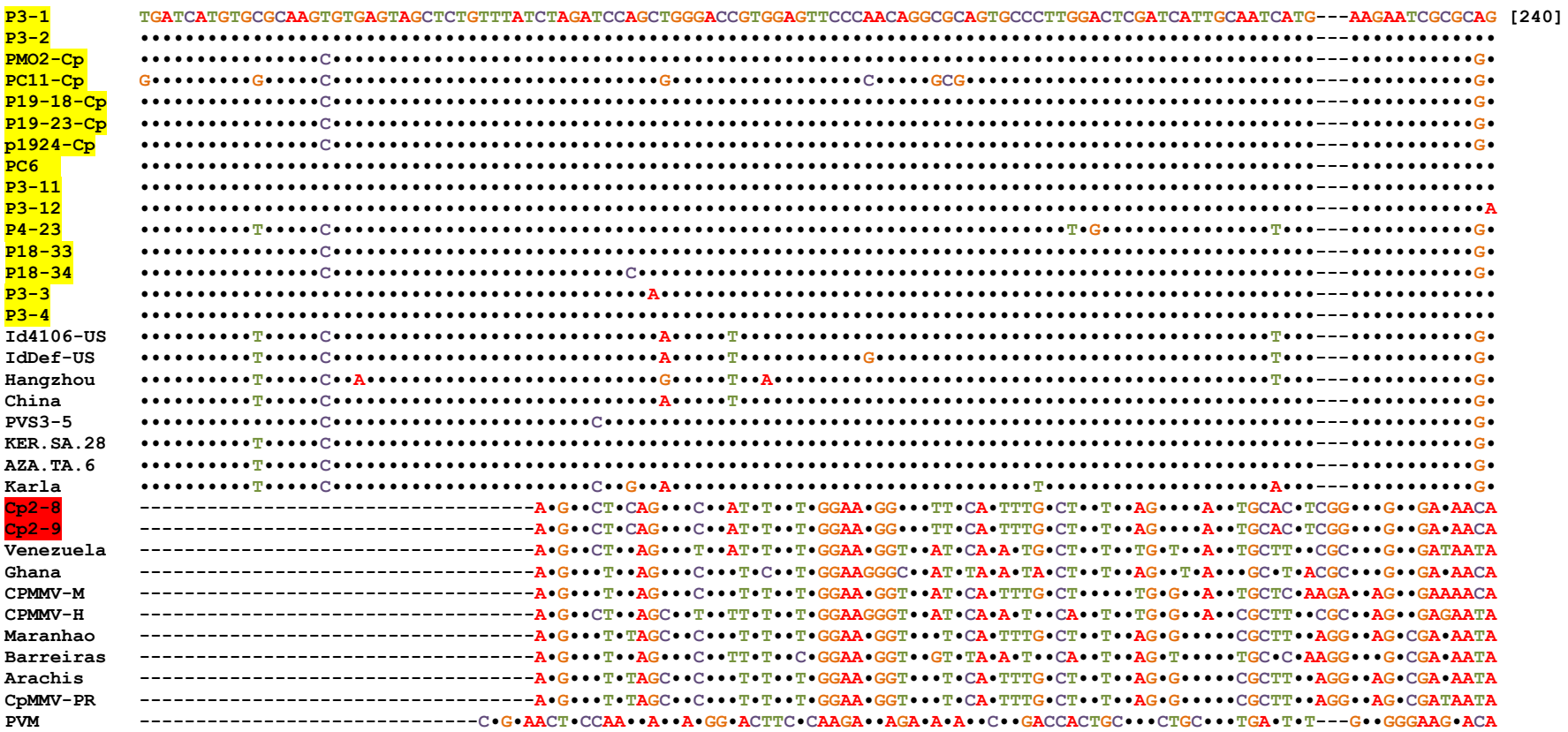
6. C4

```
TYLCV IRQ     MGNHISMCLSNSKENTNVRTNGSSTWYPQTDQHISIRTFRQLRAQQMSRPTWRKTETSLILEFSKSMADQLEEVSNLPTTHMPKHSIQAINPRPSIY [97]
RE4           ••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Moroccan      ••••••••••••A••••••••••••••AG•••••Q•••E•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Mauritius     ••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Almeria       ••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
TYLCV-GJ      ••••••••••••A••••••••••••••••G•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Iran          ••••••T••••••AS•••K•••••IYPL••G•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Cuba          ••••••••••••A••••••••••••••••G•••••Q•••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Dominican     ••••••••••••A••••••••••••••••G•••••Q•••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Japan-Misumi  ••••••••••••A••••••••••••••••G•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Jordan        ••••••••••••A•••••••••••••••••••••••••••••••••••G•••••••••••••••••••••••••••••••••••••••••••••••••••
Bos Korea     ••••••••••••A••••••••••••••••G•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••V•••••••
Netherland    ••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Oman          ••LC••TPS•S••VKPSSE•PDI•MSLTLITPPN•TQ•S•EQSPAP••S••S•R•VITSTGVSFR••E•L••••NRQLMMQQQRP*MHVRRK•LYQ*
```

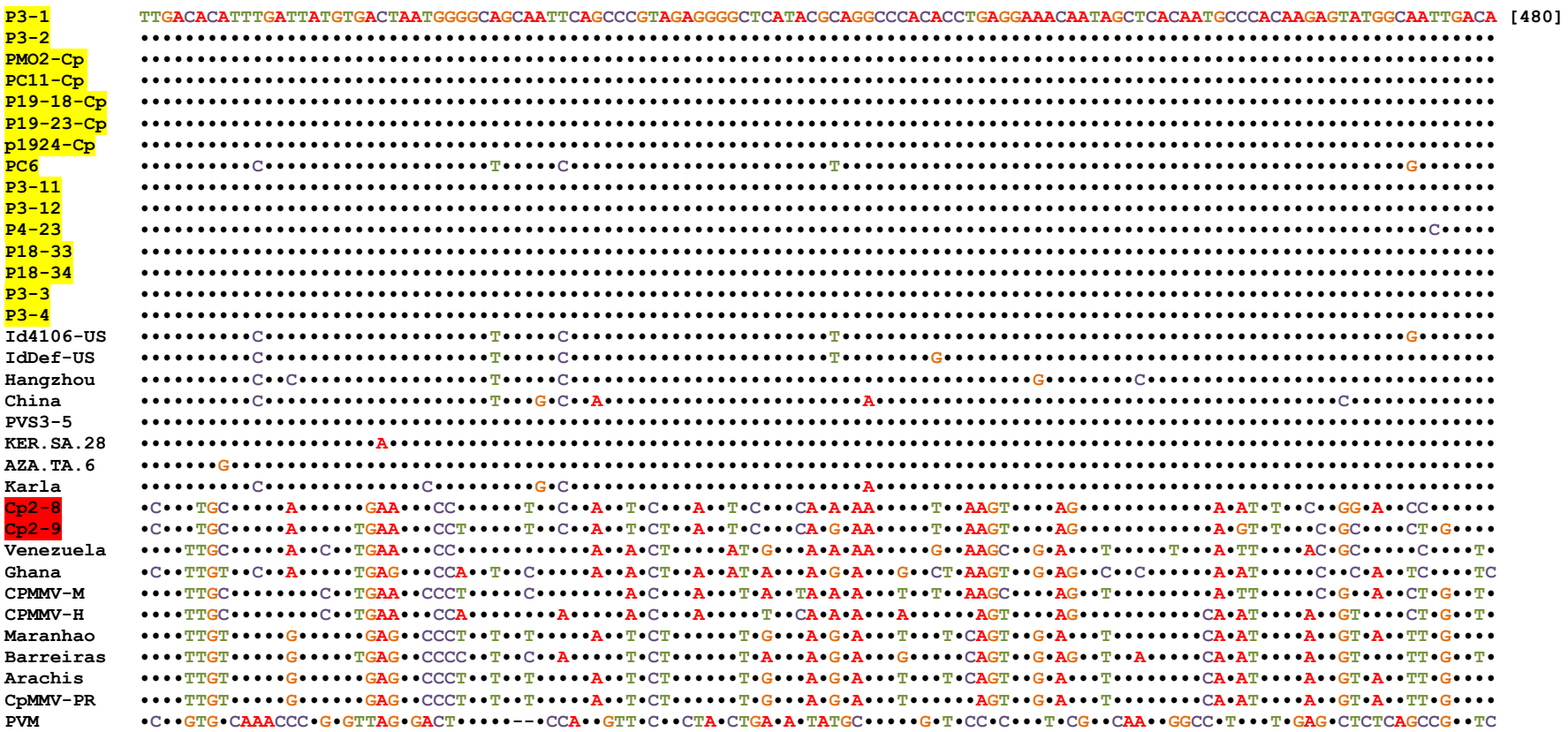
Appendix 6: Nucleotide sequence alignment of carlavirus sequences

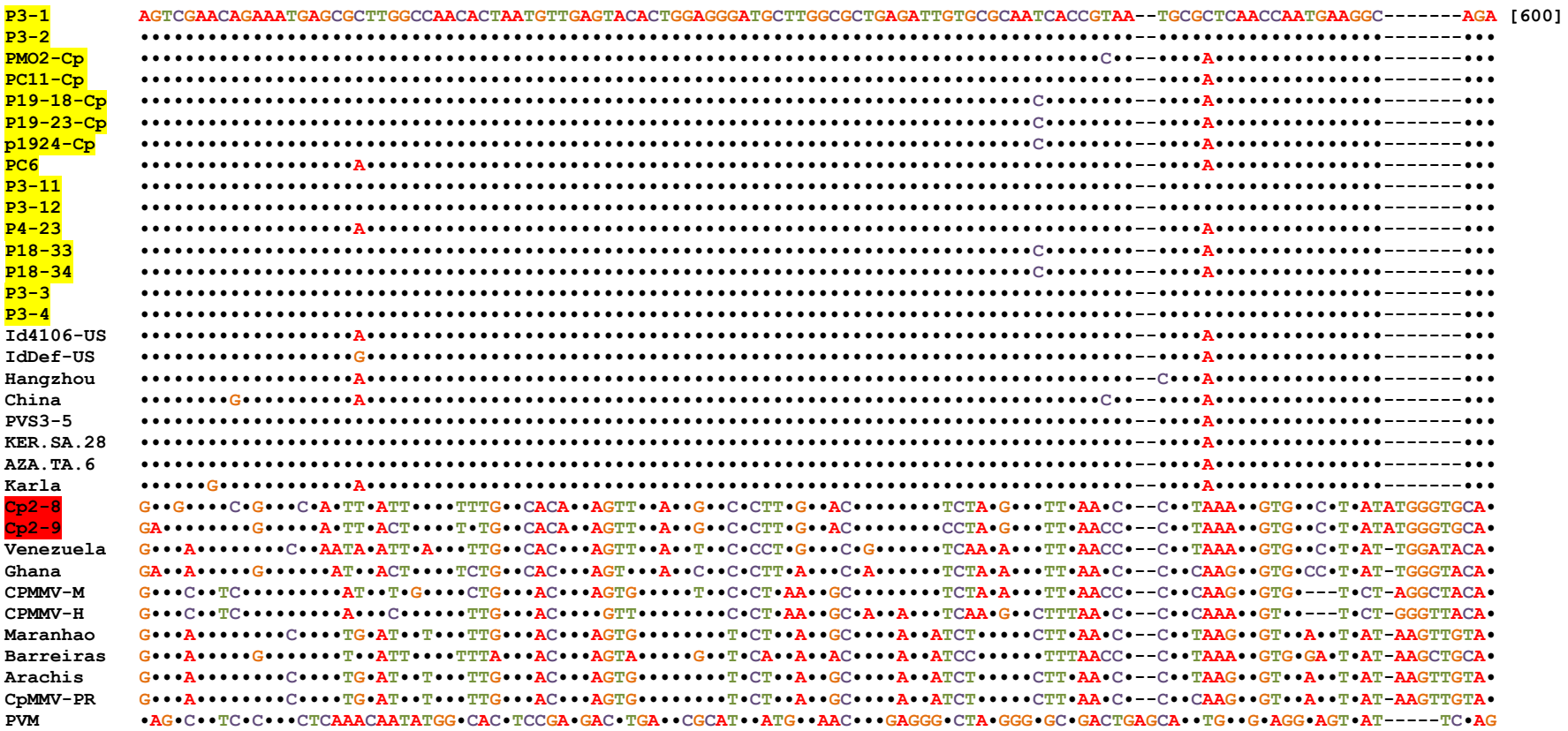
Nucleotide sequence alignment of partial CP/NB/UTR regions of *Potato virus S* (PVS: genus *Carlavirus*; family *Betaflexiviridae*) (yellow highlighted), *Cowpea mild mottle virus* (CPMMV: genus *Carlavirus*; family *Betaflexiviridae*) (red highlighted) sequences obtained from Iraqi potato and cowpea samples respectively and isolates obtained from GenBank database. The assented and deleted nucleotides and stop condones were marked with (•) and (-) respectively. This alignment performed using MEGA5 software (Tamura *et al*., 2011)

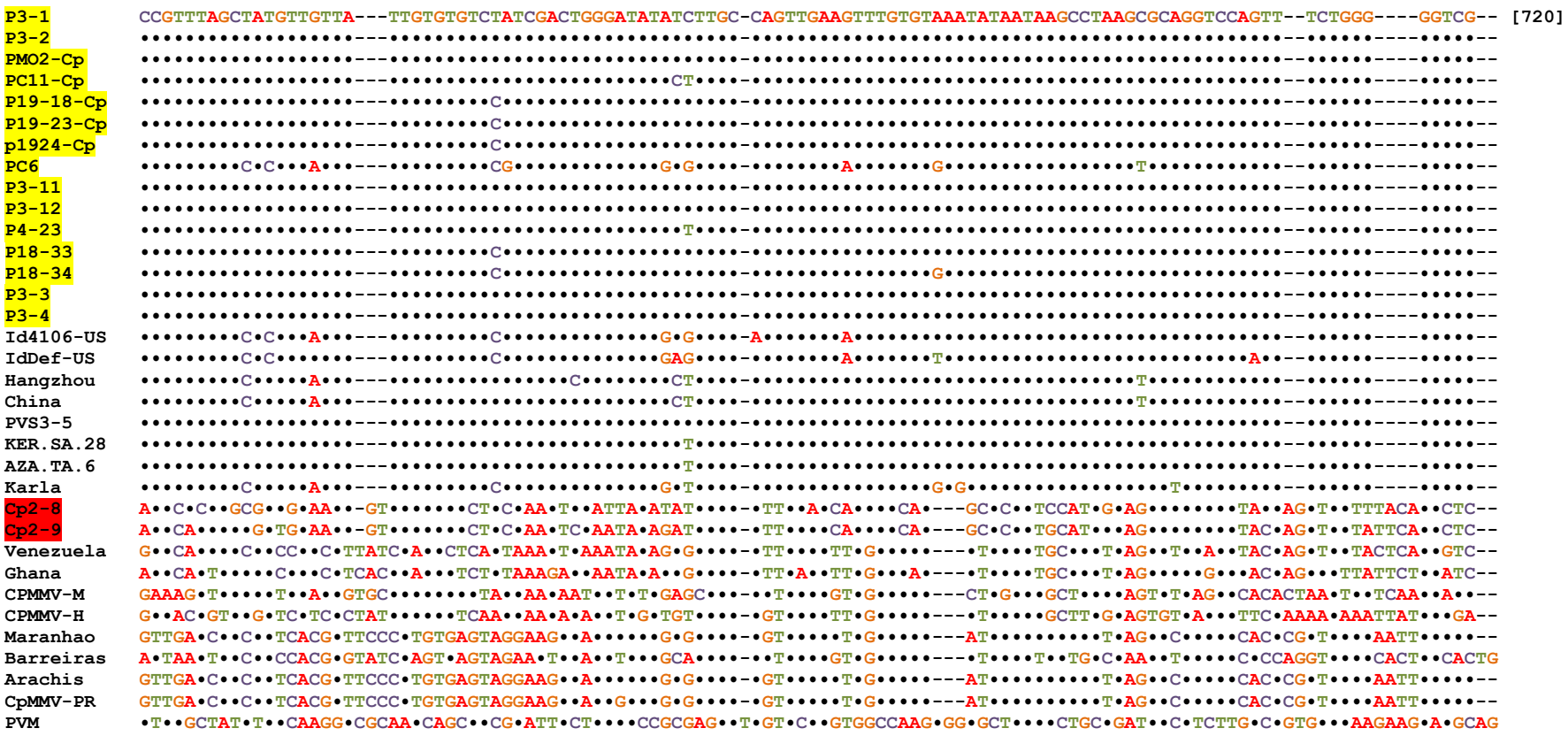


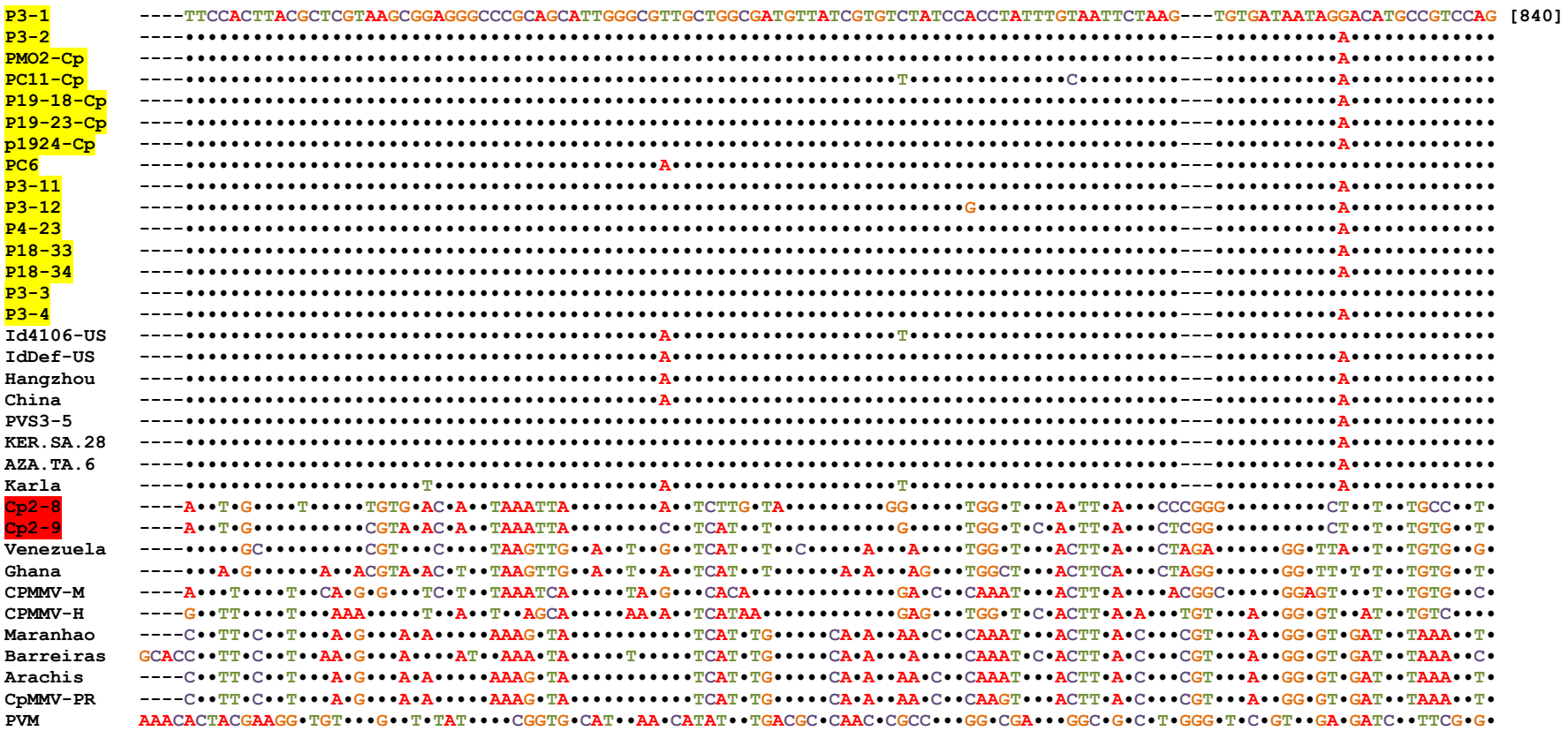

P3-1 TGATCATGTGCGCAAGTGTGAGTAGCTCTGTTTATCTAGATCCAGCTGGGACCGTGGAGTTCCCAACAGGCGCAGTGCCCTTGGACTCGATCATTGCAATCATG---AAGAATCGCGCAG [240]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM

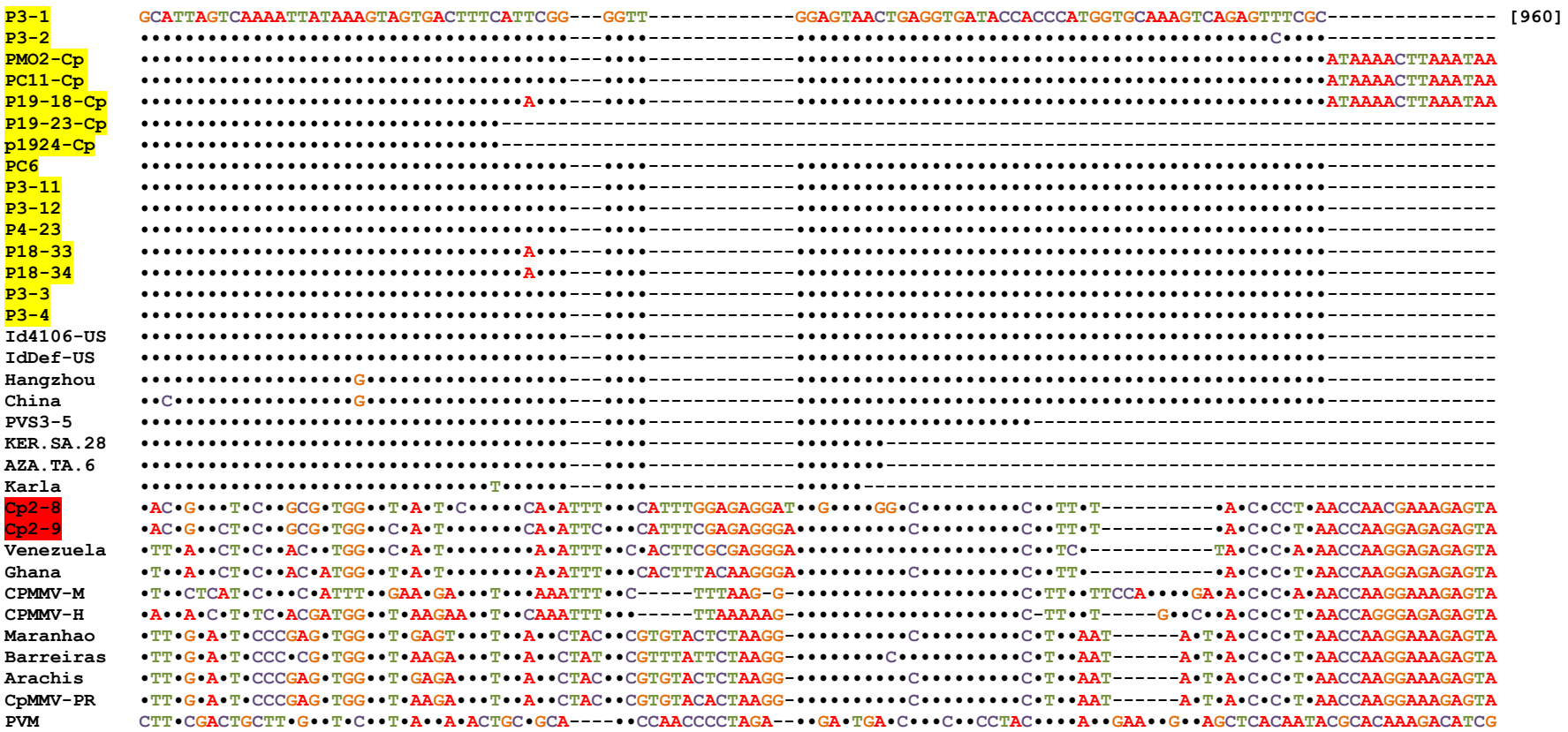

P3-1 GATTGAGAAAAGTGTGCAGGCTGTATGCTCCAGTCGTGTGGAATTACATGCTAGTTCAGAATAGGCCACCTTCGGATTGGCAGGCCATGGGATTTCAATGGAATGCACGCTTCGCCGCAT [360]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM

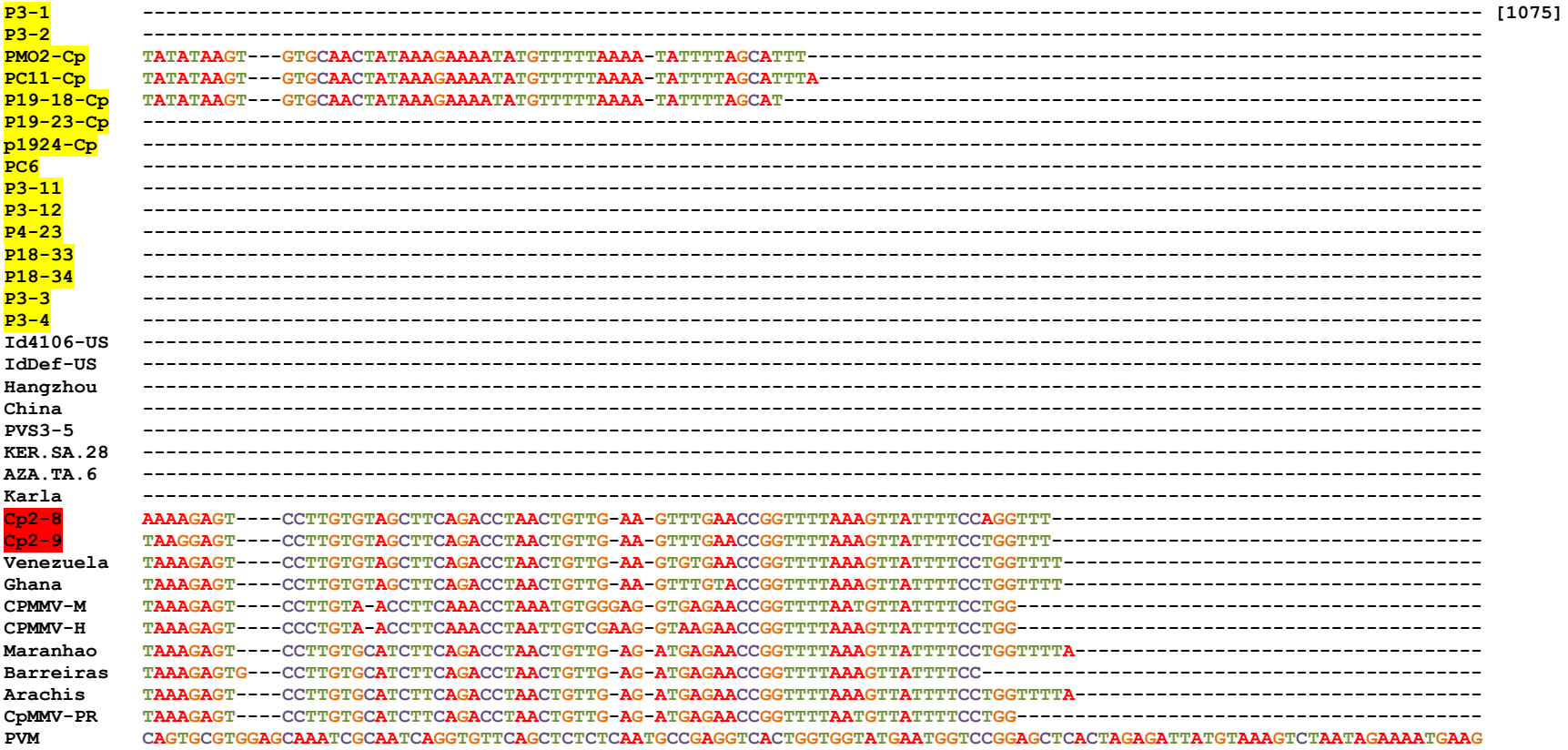

P3-1 TTGACACATTTGATTATGTGACTAATGGGGCAGCAATTCAGCCCGTAGAGGGGCTCATACGCAGGCCCACACCTGAGGAAACAATAGCTCACAATGCCCACAAGAGTATGGCAATTGACA [480]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM


P3-1 AGTCGAACAGAAATGAGCGCTTGGCCAACACTAATGTTGAGTACACTGGAGGGATGCTTGGCGCTGAGATTGTGCGCAATCACCGTAA--TGCGCTCAACCAATGAAGGC-------AGA [600]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM


P3-1 CCGTTTAGCTATGTTGTTA---TTGTGTGTCTATCGACTGGGATATATCTTGC-CAGTTGAAGTTTGTGTAAATATAATAAGCCTAAGCGCAGGTCCAGTT--TCTGGG----GGTCG-- [720]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM


P3-1 ----TTCCACTTACGCTCGTAAGCGGAGGGCCCGCAGCATTGGGCGTTGCTGGCGATGTTATCGTGTCTATCCACCTATTTGTAATTCTAAG---TGTGATAATAGGACATGCCGTCCAG [840]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM


P3-1 GCATTAGTCAAAATTATAAAGTAGTGACTTTCATTCGG---GGTT----------GGAGTAACTGAGGTGATACCACCCATGGTGCAAAGTCAGAGTTTCGC---------- [960]
P3-2
PMO2-Cp ATAAAACTTAAATAA
PC11-Cp ATAAAACTTAAATAA
P19-18-Cp ATAAAACTTAAATAA
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM


P3-1 [1075]
P3-2
PMO2-Cp
PC11-Cp
P19-18-Cp
P19-23-Cp
p1924-Cp
PC6
P3-11
P3-12
P4-23
P18-33
P18-34
P3-3
P3-4
Id4106-US
IdDef-US
Hangzhou
China
PVS3-5
KER.SA.28
AZA.TA.6
Karla
Cp2-8
Cp2-9
Venezuela
Ghana
CPMMV-M
CPMMV-H
Maranhao
Barreiras
Arachis
CpMMV-PR
PVM

Appendix 7: Amino acid comparison of carlaviruses isolates

Amino acid alignment of partial CP (1) and NB (2) regions of *potato virus S* (yellow highlighted) and *Cowpea mild mottle virus* (red highlighted) isolated from Iraqi samples and GenBank isolates. The assented, deleted amino acids and stop condones were marked with •, - respectively. This alignment performed using MEGA5 software (Tamura *et al*., 2011).

1. Carlavirus coat protein

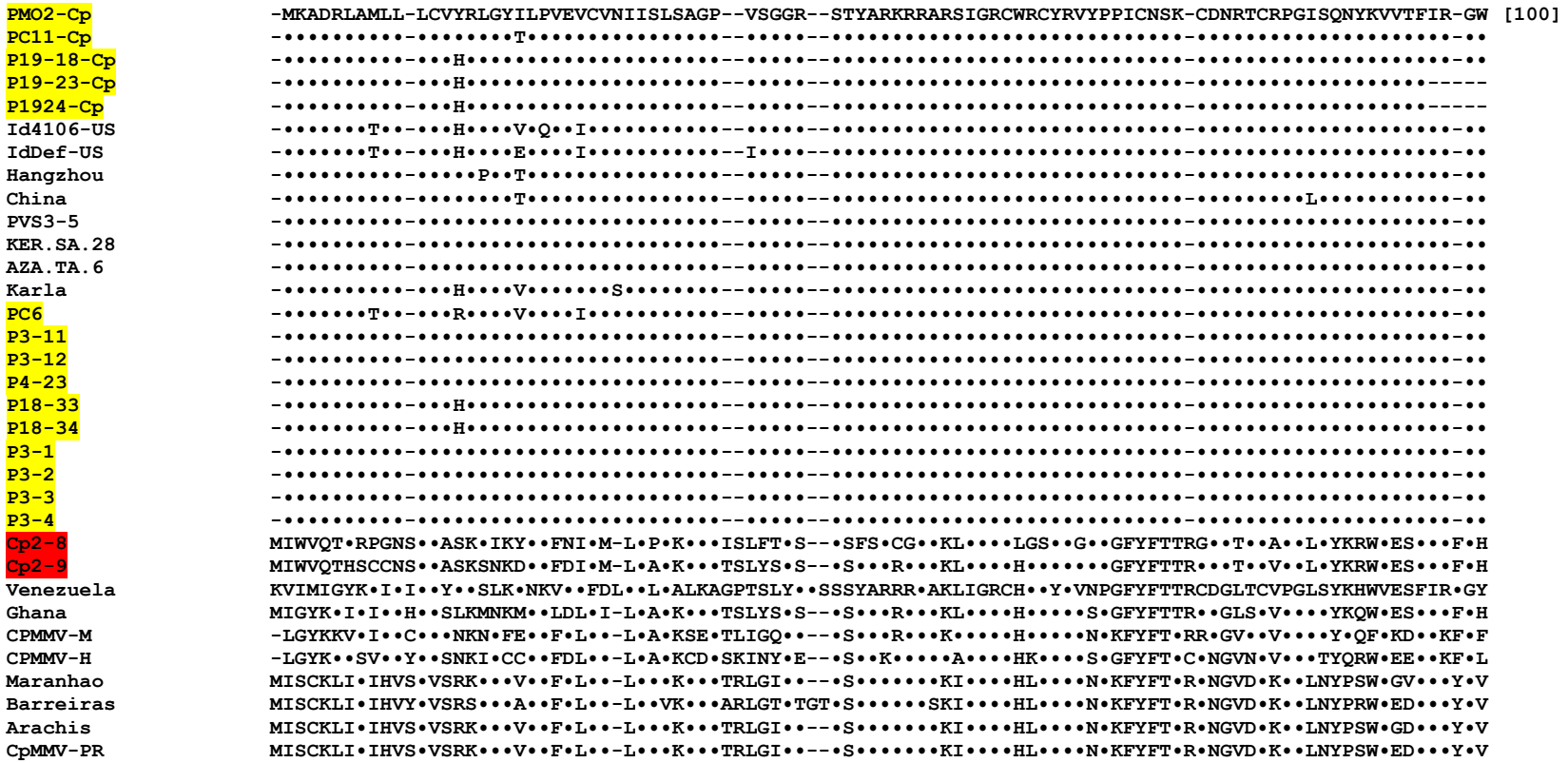
```
P3-1                ----------------------------------------------------------------------------------------------------EIRSMSNNT [120]
P3-2                ----------------------------------------------------------------------------------------------------G•••••••M
PMO2-cp             ----------------------------------------------------------------------------------------------------------------
PC11-cp             ----------------------------------------------------------------------------------------------------------------
P19-18-cp           ----------------------------------------------------------------------------------------------------------------
P19-23-cp           ----------------------------------------------------------------------------------------------------------------
P1924-cp            ----------------------------------------------------------------------------------------------------------------
PC6                 ----------------------------------------------------------------------------------------------------•V•••M
P3-11               ----------------------------------------------------------------------------------------------------••M
P3-12               ----------------------------------------------------------------------------------------------------••••I•••M
P4-23               ----------------------------------------------------------------------------------------------------••••I•••M
P18-33              ----------------------------------------------------------------------------------------------------••••I•••M
P18-34              ----------------------------------------------------------------------------------------------------•••M
P3-3                ----------------------------------------------------------------------------------------------------•••••••M
P3-4                ----------------------------------------------------------------------------------------------------•V•••M
Id4106-US           ----------------------------------------------------------------------------------------------------••••V•••M
IdDef-US            ----------------------------------------------------------------------------------------------------••••V•••M
Hangzhou China      ----------------------------------------------------------------------------------------------------••••V•••M
China               ----------------------------------------------------------------------------------------------------••••V•••M
PVS3-5 Syria        ----------------------------------------------------------------------------------------------------••••V•••M
KER.SA.28 Iran      ----------------------------------------------------------------------------------------------------••••V•••M
AZA.TA.6 Iran       ----------------------------------------------------------------------------------------------------••••V•••M
Karla               ----------------------------------------------------------------------------------------------------••••V•••M
Cp2-8               ----------------------------------------------------------------------------------------------------------------
Cp2-9               ----------------------------------------------------------------------------------------------------------------
Ghana               ----------------------------------------------------------------------------------------------------------------
Venezuela           ----------------------------------------------------------------------------------------------------------------
CPMMV-M India       ----------------------------------------------------------------------------------------------------------------
CPMMV-H India       ----------------------------------------------------------------------------------------------------------------
Maranhao Brazil     ----------------------------------------------------------------------------------------------------------------
Barreiras Brazil    ----------------------------------------------------------------------------------------------------------------
Arachis Brazil      ----------------------------------------------------------------------------------------------------------------
CpMMV-PR Puerto Rico ----------------------------------------------------------------------------------------------------------------
PVM                 --------ETAKDEGTSQEKREARPLPTAADFEGKDTSENTDGRAADADGEMSLERRLDSLREFLRERRGAIRVTNPGLETGRPRLQLAENMRPDPTNPYNRPSIEALSRIKPIAI•••M
PVM Hungary         MGEPTEKTEATKGAGTSKAVKGARPLPTAADFEERDEQAGQTVRGGEDDEEASLERRLDSLREFLRDRRGAIRVTNPGLETGRPKLILAEDMRPDPTNPYNRPSIEALSRIKPIAV•••M
CVV                 ----------------------MGDAKQAANTAEEKPKSASGVPIDGIEADLQKRLDALHEFWLKMQTQSVVTNPGLELGRPIPKAPAHLQKKKSSIYNKWTIDELSLLVSKPI••SM
PVX                 ------------------------------------------------------------MSAPASTTQATGSTTSTTTKTAGATPATASGLFTIPDGDFFSTARAIVA••AV
Grapevine virus A   ----------------------------------------------------------------------------------------------------------------
```

Carlavirus coat protein continued

Carlavirus coat protein continued

```
P3-1                 FAAFDTFDYVTNGAAIQPVEGLIRRPTPE---ETIAHNAHKSMAIDKS-NRNERLANTNVEYTGGML-GAEIVRNHRNALNQ [322]
P3-2                 ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••••
PMO2-cp              ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
PC11-cp              ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
P19-18-cp            ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
P19-23-cp            ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
P1924-cp             ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
PC6                  ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
P3-11                ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••••
P3-12                ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••••
P4-23                ••••••••••••••••••••••••••••••---••••••••••••T•••-••••••••••••••••-••••••••••I••
P18-33               ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
P18-34               ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
P3-3                 ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••••
P3-4                 ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••••
Id4106-US            ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
IdDef-US             ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
Hangzhou China       ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
China                ••••••••••••••••V•••••••••••••---••••••••••••••••-G•••••••••••••••-••••••••••I••
PVS3-5 Syria         ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
KER.SA.28 Iran       ••••••••••N•••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
AZA.TA.6 Iran        ••••••••••••••••••••••••••••••---••••••••••••••••-••••••••••••••••-••••••••••I••
Karla                ••••••••••••••••V•••••••••••••---••••••••••••••••-S••••••••••••••••-••••••••••I••
Cp2-8                Y••••C•E••E•P•••••A••P••K•••S----K••••TYNRV•L•RW-•••QLYS•L•T•V•••T•-•P••S••FNH•KK-
Cp2-9                Y••••C•E••E•P•••••A••P••K•••S----K••••TY•RL•L•R-•••••LYS•M•T•V•••T•-•P••P••FNH•KK-
Ghana                Y••••C•E••E•P•••••A•••••K••SS----K••••TY•RL•L•R-•••••IYS•L•T•V•••T•-•P••S••FNH•KK-
Venezuela            Y••••C•E••E•P•••••A•••••K•••S----K••••TY•RL•L•R-•••••IYS•L•T•V•••T•-•P••S••FNH•KK-
CPMMV-M India        Y••••C••••E•P•••••A•••••K•••S----K••••TY•RI•L•R-•••••HFG•L•T•V•••TQ-•P••S••FNH•KK-
CPMMV-H India        Y••••C••••E•P•••••A•••••K•••S----K••••TY•RL•L•R-•••••HF••L•T•V•••TQ-•P••S••FNH•KK-
Maranhao Brazil      •••••C•E••E•P•••••A•••••K••SS----K••••TY•RL•L•R-•••••LY••L•T•V•••T•-•P••S••FNH•KK-
Barreiras Brazil     •••••C•E••E•P•••••A•••••K•••S----K••••TY•RL•L•R-•••••LYS•L•T•V•••T•-•P••S••FNH•KK-
Arachis Brazil       •••••C•E••E•P•••••A•••••K••SS----K••••TY•RL•L•R-•••••LY••L•T•V•••T•-•P••S••FNH•KK-
CpMMV-PR Puerto Rico •••••C•E••E•P•••••A•••••K•••S----K••••TY•RL•L•R-•••••LY••L•T•V•••T•-•P••S••FNH•KK-
PVM                  •••••C••••E•T••V••L•••••••••R----KV•••T••DI•VRGA-•••QVFSSL•A•V••••N-•P•LT•DYVKSNRK
PVM Hungary          •••••C••••E•T••V••L•••••••••R----KV•••T••DI•LRGA-•••QVYGSL•A•V••••N-•P•LT•DY•K•K•-
CVV                  •••••C••F•E•P••V••F•••••K•••A----K••NE•••RI•L•R-•••••TY••LGT•I•••R•-•P••N•DFN••N•H
PVX                  •••••F•NG•••P•••M•K•••••P•SEA----•MN•AQTAAFVK•T•ARAQSNDF•SLDAAV•R•RIT•TTTAEAVVTLPPP
Grapevine virus A    LVVSELV?RMRT-LSVAV S••PVKGA•LRQMC•PF•Q••YDFLILM---AEMGTYSQLATKM•--------------------
```

2. Carlavirus NB (nucleic acid binding protein)

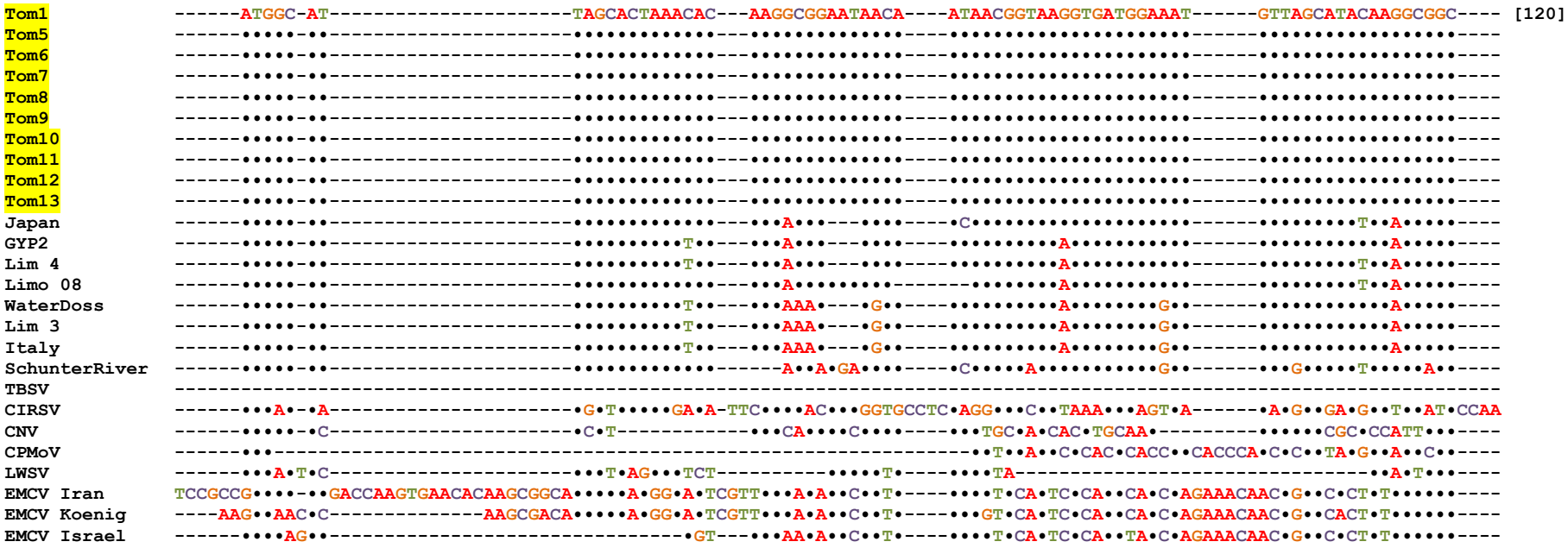


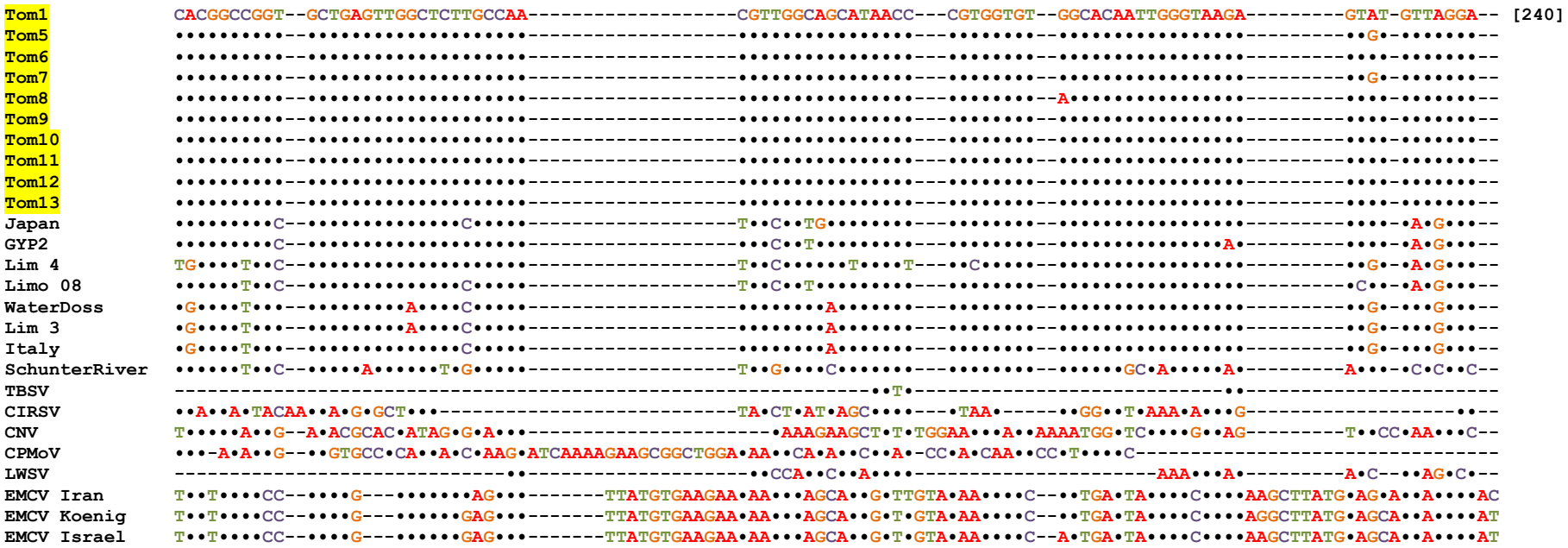
NB (nucleic acid binding protein) continued

```
PMO2-Cp             SN-------------- [116]
PC11-Cp             ••--------------
P19-18-Cp           ••--------------
P19-23-Cp           ----------------
P1924-Cp            ----------------
Id4106-US           ••--------------
IdDef-US            ••--------------
Hangzhou            ••--------------
China               ••--------------
PVS3-5              ••--------------
KER.SA.28           ••--------------
AZA.TA.6            ••--------------
Karla               ••--------------
PC6                 ••--------------
P3-11               ••--------------
P3-12               ••--------------
P4-23               ••--------------
P18-33              ••--------------
P18-34              ••--------------
P3-1                ••--------------
P3-2                ••--------------
P3-3                ••--------------
P3-4                ••--------------
Cp2-8               LERMG-----------
Cp2-9               FEREE-----------
Venezuela           FAREE-----------
Ghana               FTREE-----------
CPMMV-M             KGSN------------
CPMMV-H             KRE-------------
Maranhao            YSKGSNCGDTPQILNS
Barreiras           YSKGSNRGDTPQILNS
Arachis             YSKGSNCGDTPQILNS
CpMMV-PR            YTKGSNCGDTPQILNS
```

Appendix 8: Nucleotide sequence alignment of tombusvirus CP region

Nucleotide sequence alignment of CP region of *Grapevine Algerian latent virus* (GALV: genus *Tombusvirus*; family *Tombusviridae*) (yellow highlighted sequences obtained from Iraqi tomato and eggplant samples and isolates obtained from GenBank database. The assented and deleted nucleotides and stop condones were marked with (•) and (-) respectively. This alignment performed using MEGA5 software (Tamura *et al.*, 2011). TBSV: *Tomato bushy stunt virus* (TBSV: genus *Tombusvirus*; family: *Tombusviridae*), CIRSV: *Carnation Italian ring spot virus* (CIRSV: genus *Tombusvirus*; family: *Tombusviridae*),, EMCV: *Eggplant mottled crinkle virus* (EMCV: genus *Tombusvirus*; family: *Tombusviridae*), CNV: *Cucumber necrosis virus* (CNV: genus *Tombusvirus*; family: *Tombusviridae*), CPMoV: *Cowpea mottle virus* (CPMoV: genus *Carmovirus*; family: *Tombusviridae*) & LWSV: *Leek white stripe virus* LWSV: genus *Necrovirus*; family: *Tombusviridae*).


Tom1
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
Italy
SchunterRiver
TBSV
CIRSV
CNV
CPMoV
LWSV
EMCV Iran
EMCV Koenig
EMCV Israel
[120]


Tom1
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
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Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
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LWSV
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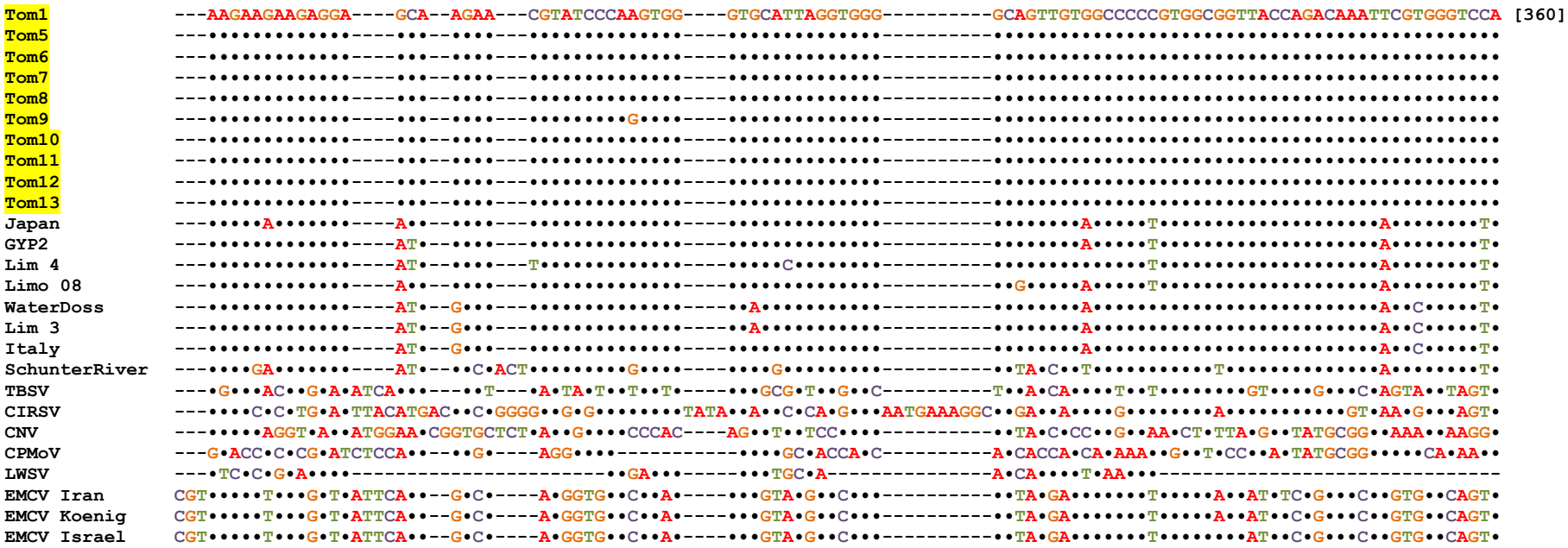
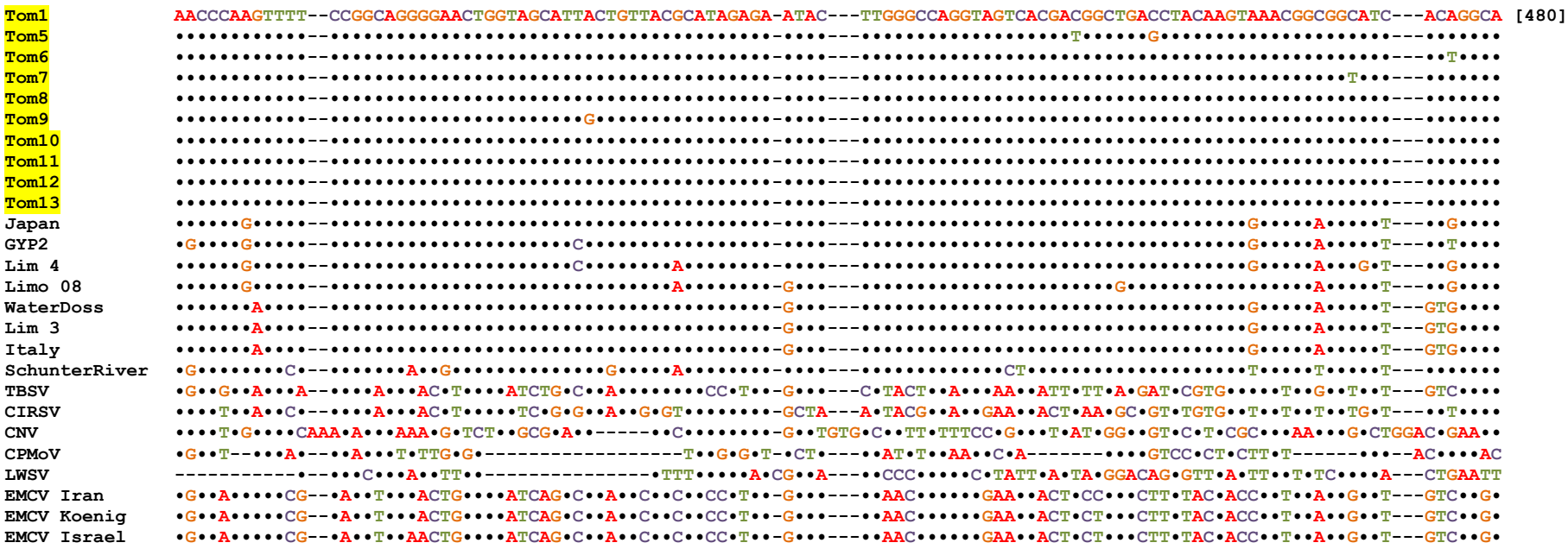
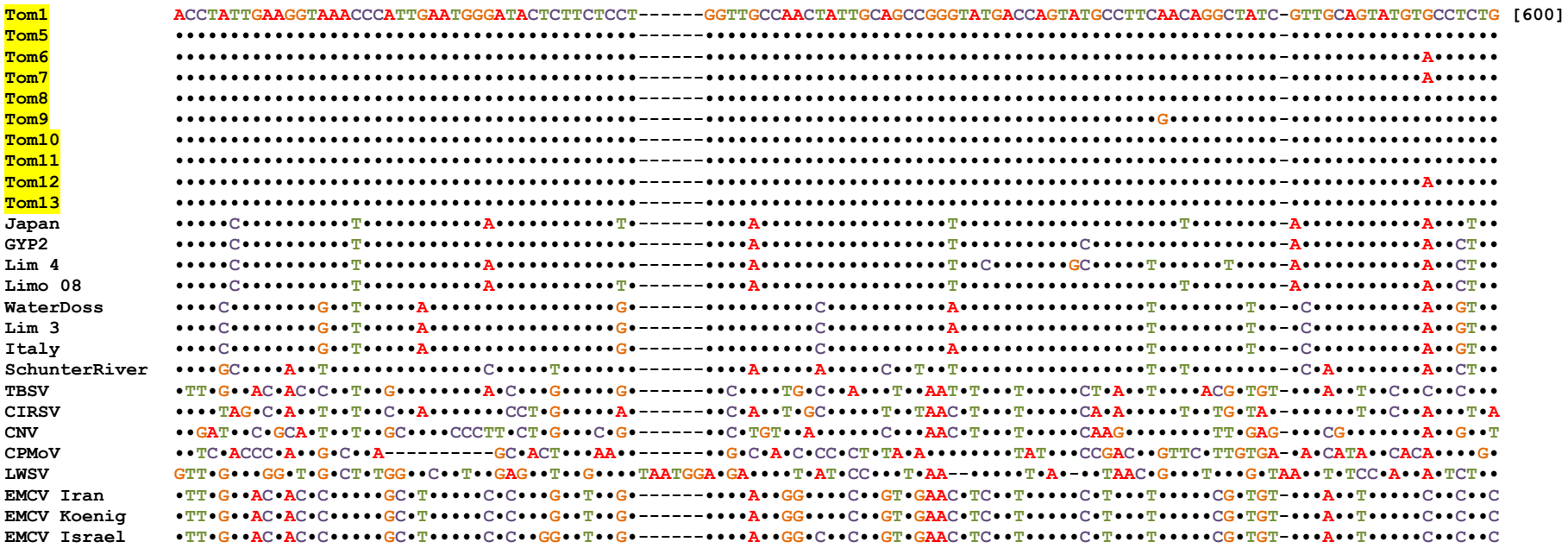
EMCV Koenig
EMCV Israel
[240]


Tom1 ---AAGAAGAAGAGGA----GCA--AGAA---CGTATCCCAAGTGG----GTGCATTAGGTGGG----------GCAGTTGTGGCCCCCGTGGCGGTTACCAGACAAATTCGTGGGTCCA [360]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
Italy
SchunterRiver
TBSV
CIRSV
CNV
CPMoV
LWSV
EMCV Iran
EMCV Koenig
EMCV Israel


Tom1 AACCCAAGTTTT--CCGGCAGGGGAACTGGTAGCATTACTGTTACGCATAGAGA-ATAC---TTGGGCCAGGTAGTCACGACGGCTGACCTACAAGTAAACGGCGGCATC---ACAGGCA [480]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
Italy
SchunterRiver
TBSV
CIRSV
CNV
CPMoV
LWSV
EMCV Iran
EMCV Koenig
EMCV Israel


Tom1 ACCTATTGAAGGTAAACCCATTGAATGGGATACTCTTCTCCT------GGTTGCCAACTATTGCAGCCGGGTATGACCAGTATGCCTTCAACAGGCTATC-GTTGCAGTATGTGCCTCTG [600]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
Italy
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EMCV Iran
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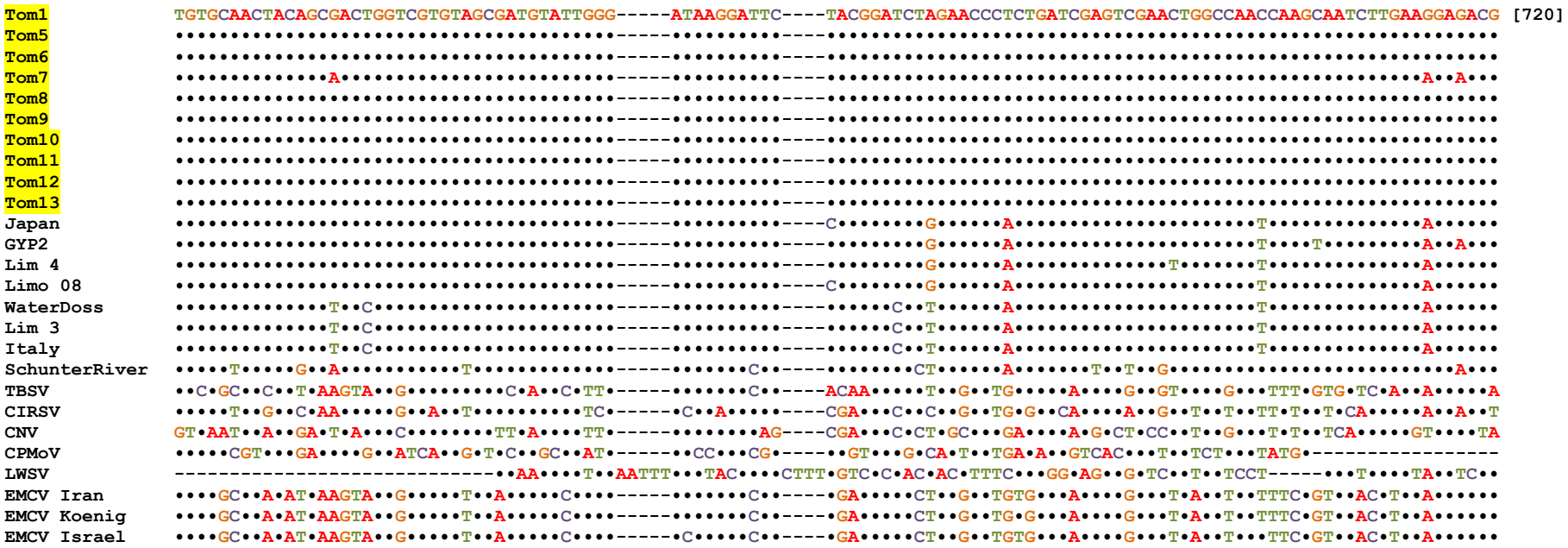
EMCV Israel


Tom1 TGTGCAACTACAGCGACTGGTCGTGTAGCGATGTATTGGG-----ATAAGGATTC----TACGGATCTAGAACCCTCTGATCGAGTCGAACTGGCCAACCAAGCAATCTTGAAGGAGACG [720]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
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Italy
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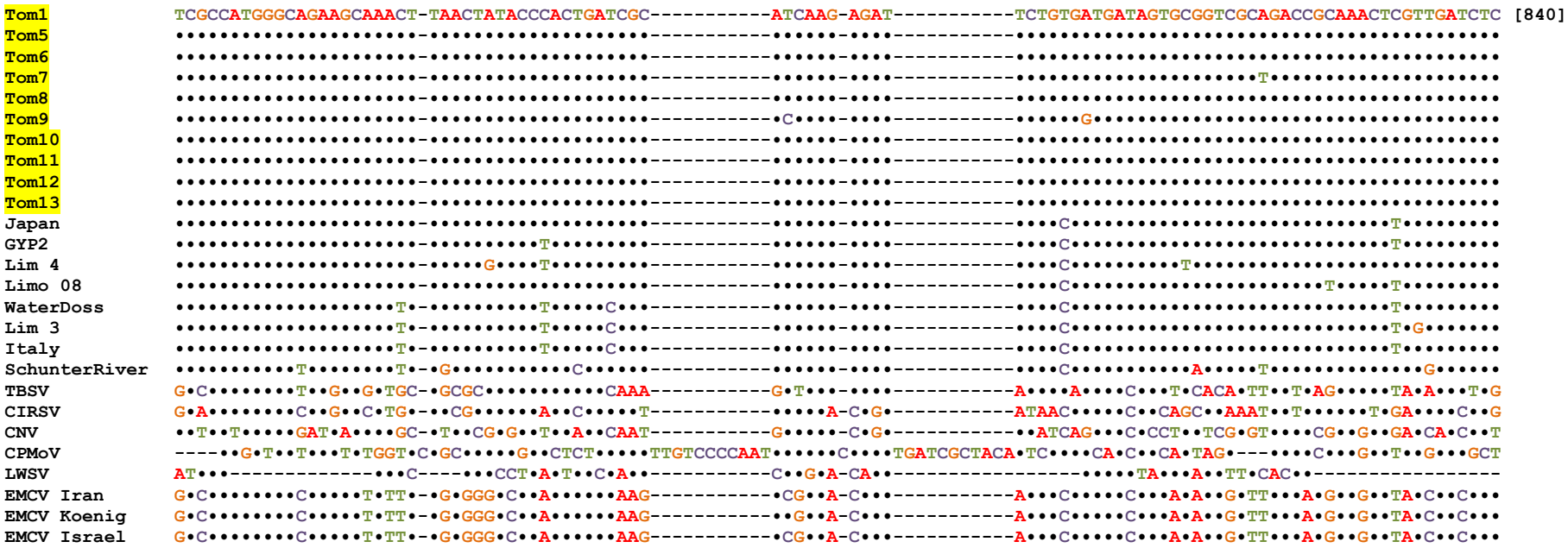
Tom1 TCGCCATGGGCAGAAGCAAACT-TAACTATACCCACTGATCGC-----------ATCAAG-AGAT-----------TCTGTGATGATAGTGCGGTCGCAGACCGCAAACTCGTTGATCTC [840]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
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Tom13
Japan
GYP2
Lim 4
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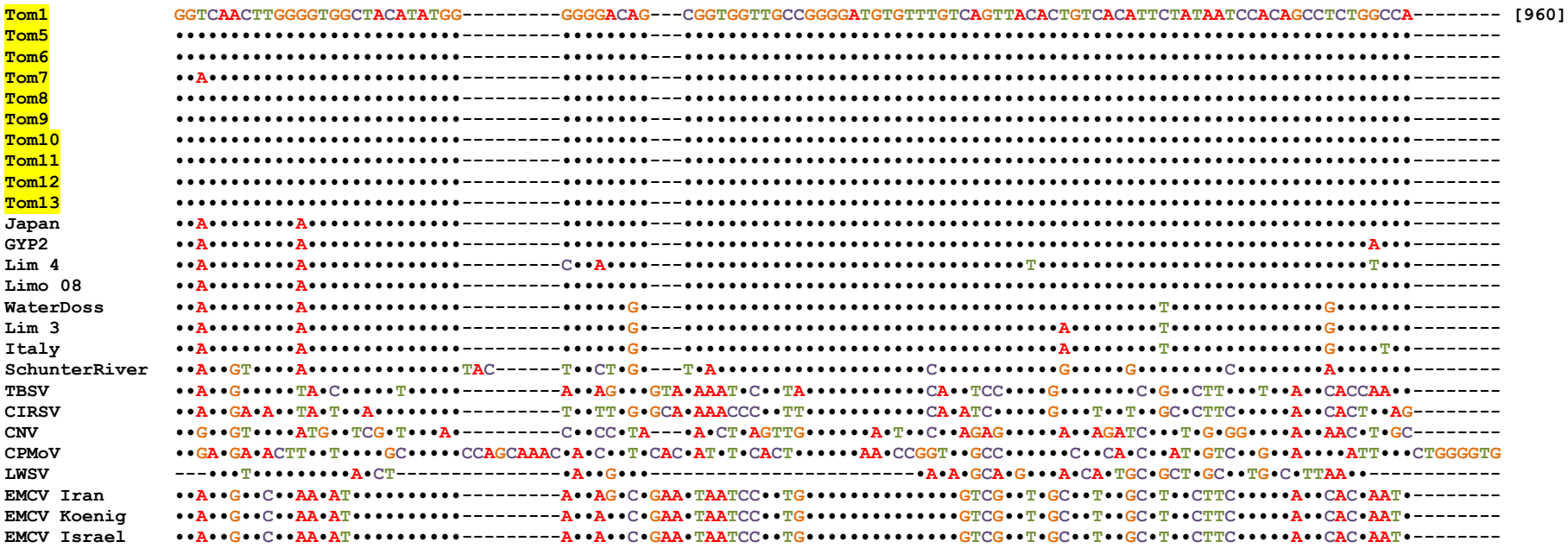
Tom1 GGTCAACTTGGGGTGGCTACATATGG---------GGGGACAG---CGGTGGTTGCCGGGGATGTGTTTGTCAGTTACACTGTCACATTCTATAATCCACAGCCTCTGGCCA-------- [960]
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Tom6
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Tom10
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Tom12
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GYP2
Lim 4
Limo 08
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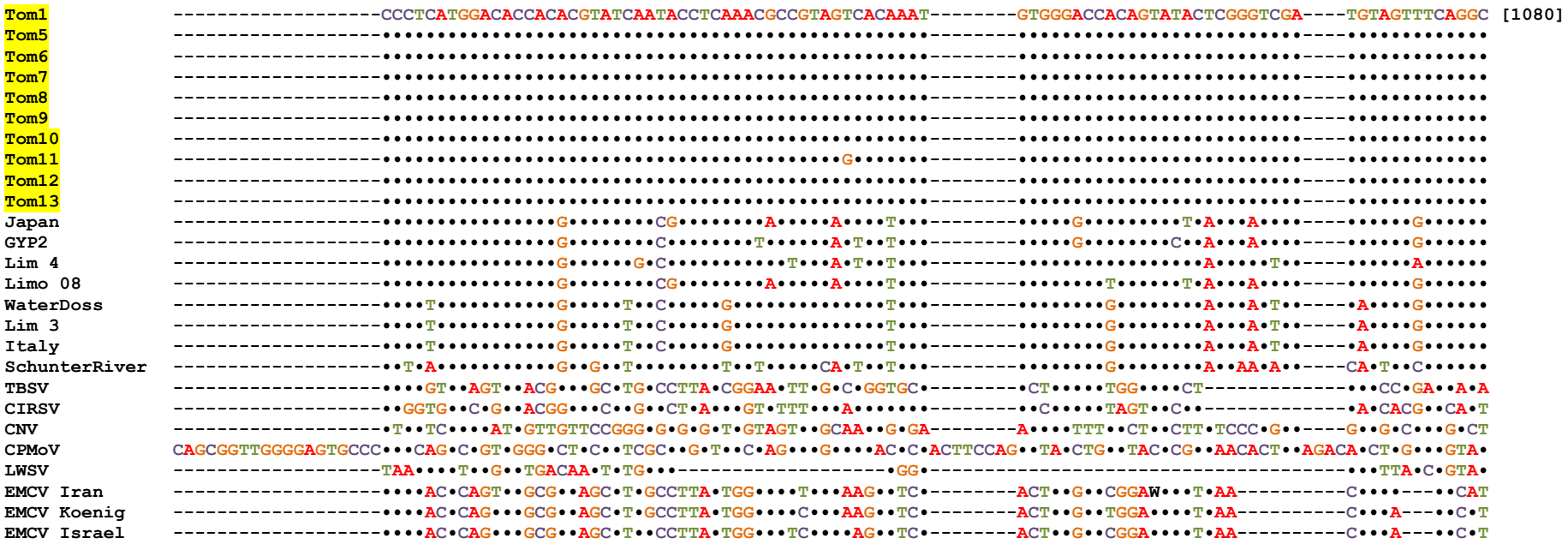
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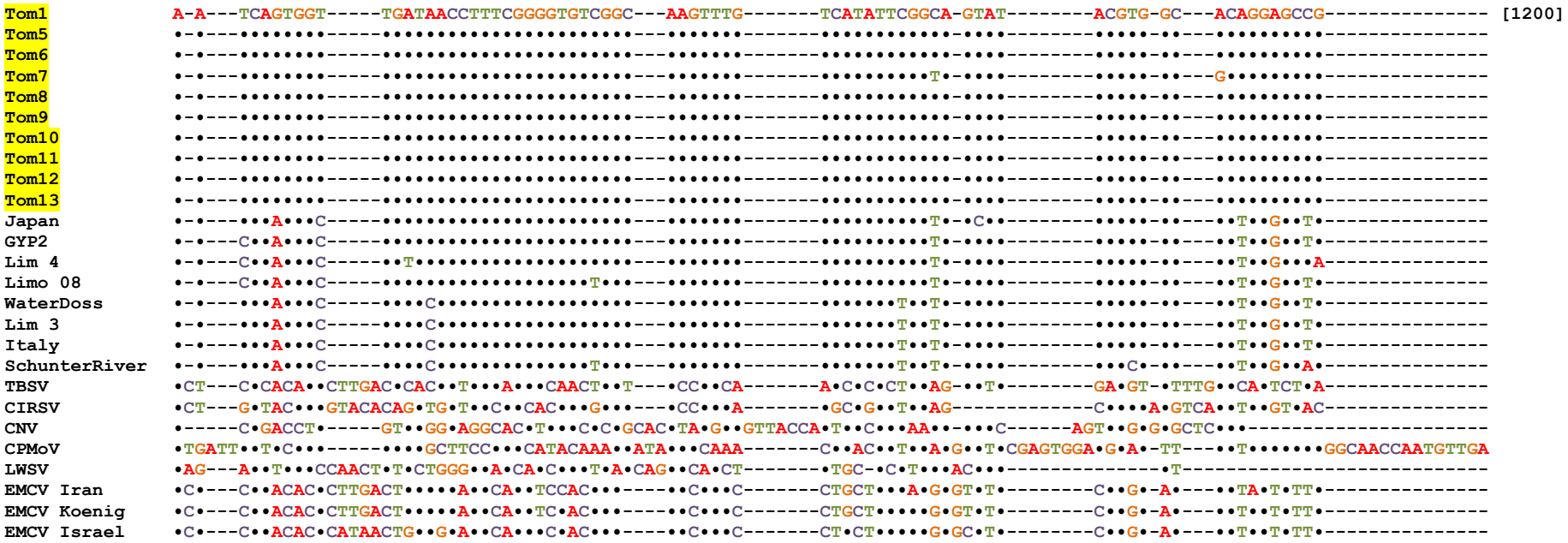
Tom1
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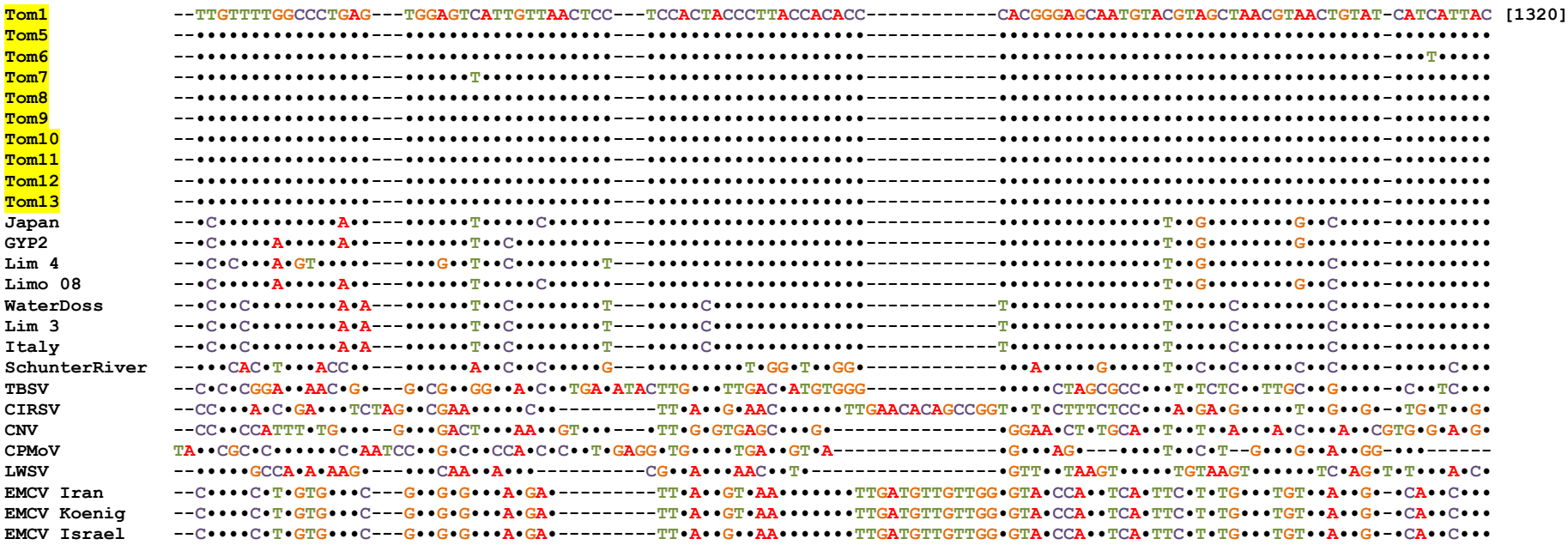
Tom1 --TTGTTTTGGCCCTGAG---TGGAGTCATTGTTAACTCC---TCCACTACCCTTACCACACC----------CACGGGAGCAATGTACGTAGCTAACGTAACTGTAT-CATCATTAC [1320]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
Italy
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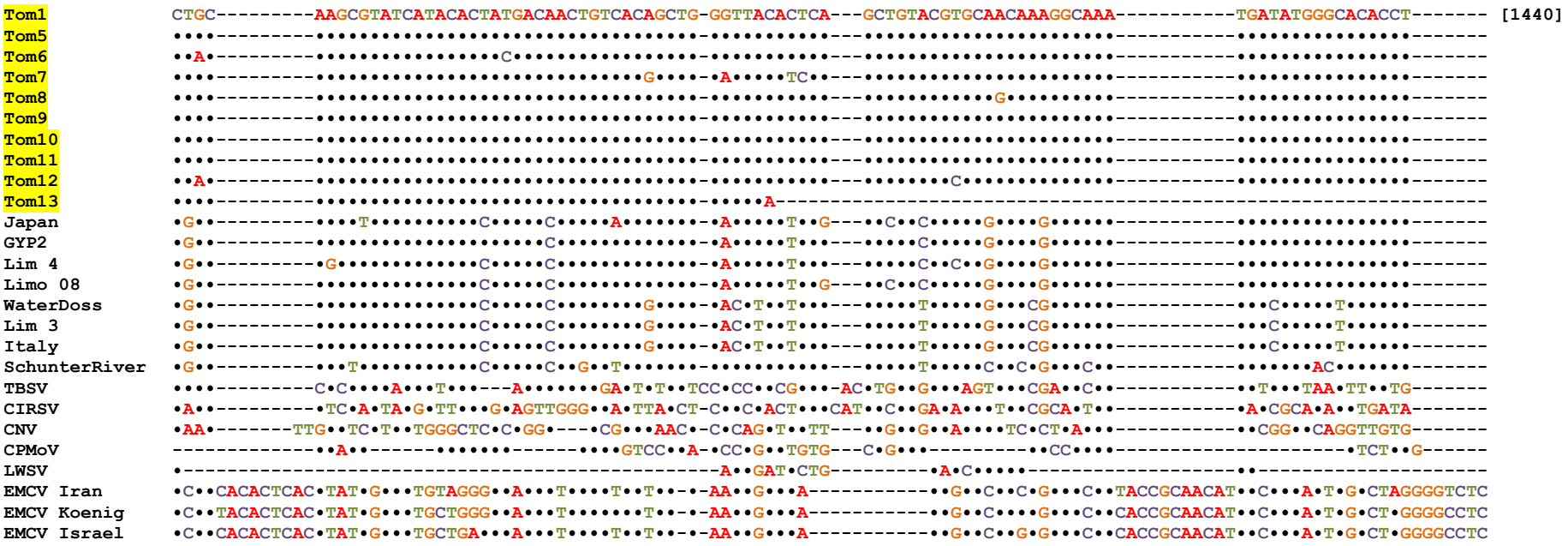
TBSV
CIRSV
CNV
CPMoV
LWSV
EMCV Iran
EMCV Koenig
EMCV Israel


Tom1 CTGC---------AAGCGTATCATACACTATGACAACTGTCACAGCTG-GGTTACACTCA---GCTGTACGTGCAACAAAGGCAAA-----------TGATATGGGCACACCT------- [1440]
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Tom6
Tom7
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Tom9
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Tom11
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Tom13
Japan
GYP2
Lim 4
Limo 08
WaterDoss
Lim 3
Italy
SchunterRiver
TBSV
CIRSV
CNV
CPMoV
LWSV
EMCV Iran
EMCV Koenig
EMCV Israel

Appendix 9: Amino acid comparison of tombusvirus isolates

Amino acid alignment of CP region of *Grapevine Algerian latent virus* (highlighted) isolated from tomato and eggplant in Iraq and GenBank isolates. The assented, deleted amino acids and stop condones were marked with •, - respectively. This alignment performed using MEGA5 software (Tamura *et al*., 2011).

```
Tom1           -------------MALALNTRR---NNNNGKVMEMLAYKAATAGAELALANVGSITRGVAQLGKSMLG-----KKKRSK-NVSQVGALGGAVVAPVAVTRQIRGSKPK-FSGRGTGSITV [120]
Tom5           -------------•••••••••--·••••••••••••••••••••••••••••••••••••••••V••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom6           -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom7           -------------•••••••••---••••••••••••••••••••••••••••••••••••••••V••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom8           -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom9           -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••A•
Tom10          -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom11          -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom12          -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Tom13          -------------•••••••••---•••••••••••••••••••••••••••••••••••••••••••-----••••••-••••••••••••••••••••••••••••-•••••••••••
Japan          -------------•••••••••---•••-••••••••••••••••••••••••G••••••••••••M•------•••N•-•••••••••••••••••••••••••••R-•••••••••••
GYP2           -------------•••••••••---•••-•••••••••••••••••••••••••••••••••••••M•------•••N•-•••••••••••••••••••••••••••R-•••••••••••
Lim 4          -------------•••••••••---•••-••••••••••••A••••••••••••L••••••••••VM•------•••N•-•••••••••••••••••••••••••••R-•••••••••••
Limo 08        -------------•••••••••---•••-•••••••••••••••••••••••••••••••••••••M•------•••N•-•••••••••••••••••••••••••••R-•••••••••••
Water Doss     -------------••••••••K---S••-••••••••••••A•••••T•••••N•••••••••••V••------•••NR-••••••T•••••••••••••••••••••-•••••••••••
Lim 3          -------------••••••••K---S••-••••••••••••A•••••T•••••N•••••••••••V••------•••NR-••••••T•••••••••••••••••••••-•••••••••••
Italy          -------------••••••••K---S••-••••••••••••A•••••••••••N•••••••••••V••------•••NR-••••••••••••••••••••••••••••-•••••••••••
Schunter River -------------•••••••••---•••-••••••••••••••••••••••••T••••••••••N••-------R••N•QL••••••••••I••••••••••••••••-•••••••••••
TBSV           -----------------------------------------------------------------------NRR•QGNQQIITH••GV••SIM•••••S••LV•••••-•T••TS••V••
CIRSV          -------------•TMV•RNSGTMVPQGGN•MSK•AKNASQ----QGLQ•GAAYLMSNPKGAL•KGKDAWNYMTAGGVPSGIVHS•GMK••IM••••••••L••••••-••••TS••V••
CNV            -------------•••VSRNNN-------MRTLAK••APL••••TRTIVD•KEA•WN••KWIWGKLPKG--KKG•NGNGALIAHPQ•FP••IA••ISYAYAVK•R••R-•QT-AK••VRI
CPMoV          -----------------M•GN--------ALTITQ••K••••Q•RASLSK•QKKRLDN----------------ANTNPIKSVTRT•RISTRKV•A•TSTT•TNGV•N-YAV•QNKPVIQ
LWSV           ------------------------------MTTSKKSNNNNKS•KSTPKP•K-------------------------NRRQSRKGQMVLQTI•E•S•RGSIY•NVI•PGIFN•DSVNLLS
EMCV Iran      PPWHDQVNTSGIA•EIVRRNNNNAIAI•KKQLAS•A•TA••G•LSNYVKN•GAM•VQ•AVNM••KAY•VVRNR•N•GNSQAMVH••GM•••MM•••••S••LV•••••-•A••TA••V••
EMCV Koenig    ------VNTSDIA•EIVRRNNNSAIAI•KKQLAT•A•TA••G•LSNYVKN•GAM•VQ•AVNM••KAY•AVRNR•N•GNSQAMVH••GM•••MM•••••S••LV•••••-•A••TA••V••
EMCV Israel    -------------•EIVRRNNNNAIAI•KKQLAS•A•TA••G•LSNYVKN•GAM•VQ•AVNM••KAY•AVRNR•N•GNSQAMVH••GM•••MM•••••S••LV•••••-•A••TA••V••
```

```
Tom1            THREYLGQVV-TTADLQVNGGITG-NLLKVNPLNGILFSWLPTIAAGYDQYAFNRLSLQYVPLCATTATGRVAMYWDKDSTDLEPSDRVELANQAILKETSPWAEANLTIPTDRIKRFCD [240]
Tom5            ••••••••••-M•E•••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Tom6            ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Tom7            ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Tom8            ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Tom9            ••••••••••-••••••••••••-••••••••••••••••••••••••••D•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T••••G
Tom10           ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Tom11           ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
Tom12           ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
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Japan           ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••••••••
GYP2            ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T••••••••V•••••••••••••••••••••••••
Lim 4           ••••••••••-••••••••••V••-••••••••••••••••••••••••••••C•••••••••••••••••••••••••••••T•••••••••••••••••••••••V•••••••••
Limo 08         ••••••••••-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••••••••
Water Doss      ••••••••••-•••••••••••V•-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••••••••
Lim 3           ••••••••••-•••••••••••V•-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••••••••
Italy           ••••••••••-•••••••••••V•-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••••••••
Schunter River  •••••••••A-••••••••••••-••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••P••T•••••••••••••••••••••••••••••••••••••
TBSV            ••••••T••N-NSSGFV•••••V•-•••QL•••••T••••••A••SNF•••S••NVV•H•••••G••EV••••L•F••••Q••••A•••••••FGV••••A•••••M•R••••KV••Y•N
CIRSV           S•••LIT••N-NSTQFV••••V••-••RQ••••••T••••••S•••NF•••T••SVT•H•••••••E•••••••F••••E••••A•••••••Y•T••••A•••••M•R•••••••YN•
CNV             •••••VSVLSG•NGEFLR•N•TGPN•DFSI••••PF••P••VN•••NF•••K••S•RFE••••VN••TN••••L•F••••E•PG•D••AA•••Y•H•S•I••••ITK••V•••NV•••IS
CPMoV           HVELWGTLMS---NTTESPAY••R----TL••SDPAT•N•VQPLST•••M•RLV•CEII•T•R••V•T••S•VLAY•P•AS•VN•DNVTD•L•M•GAESG•AYSPLS•VPNIKQ•D•YIR
LWSV            HTELFV•VDSGALGAFVL••----------GAIIPSQMTNCNRH•VNFSK•SWKY•EFI•I•FV•••FP•Q•VLAPNF•RS•AN•TSIAS•EQYDYAVS•PI•GGS-----EGSRRMHSN
EMCV Iran       ••••••T••N-NSPAFTT••••V•-•••QL•••••T••••••A••SNF•••V••SVV•H•••••G•NEV••••••••••E•P••V•••••••FG•••••A•••••I•RV•••KT••Y••
EMCV Koenig     ••••••T••N-NSSAFTT••••V•-•••QL•••••T••••••A••SNF•••V••SVV•H•••••G•NEV••••••••••E•P••A•••••••FG•••••A•••••I•RV•••KM••Y••
EMCV Israel     ••••••T••N-NSSAFTT••••V•-•••QL•••••TR•••••A••SNF•••V••SVV•H•••••G•NEV••••••••••E•P••V•••••••FG•••••A•••••I•RV•••KT••Y••
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Tom1 DSAVADRKLVDLGQLGVATYG----GTAVVAGDVFVSYTVTFYNPQPLATLMDTTRINTSNAVVTNVGPQYTRVDVVSGNQWLITFRGVGKFVIFGSIRG-----TGAVVLALSGV---- [360]
Tom5
Tom6
Tom7
Tom8
Tom9
Tom10
Tom11
Tom12
Tom13
Japan
GYP2
Lim 4
Limo 08
Water Doss
Lim 3
Italy
Schunter River
TBSV
CIRSV
CNV
CPMoV
LWSV
EMCV Iran
EMCV Koenig
EMCV Israel

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Tom1            IVNSSTTLTTP-TGAMYVANVTVSSLPASVSYTMTT-VTAGLHSAVRATKANDMGTP [417]
Tom5            ••••••••••-••••••••••••••••••••••••-••••••••••••••••••
Tom6            ••••••••••-••••••••••••L••T•••••T••-••••••••••••••••••
Tom7            ••••••••••-•••••••••••••••••••••••••••••••P••••••••••••
Tom8            ••••••••••-••••••••••••••••••••••••••••••••••••A••••••••
Tom9            ••••••••••-••••••••••••••••••••••••••••••••••••••••••••
Tom10           ••••••••••-••••••••••••••••••••••••••••••••••••••••••••
Tom11           ••••••••••-••••••••••••••••••••••••••••••••••••••••••••
Tom12           ••••••••••-•••••••••••••••T••••••••-••••••••••••••••••
Tom13           ••••••••••-•••••••••••••••••••••••••••••-----------------
Japan           ••••••••••-••••••••••••••••••L••••••-••••••••••••R•••••••
GYP2            ••••••••••-•••••••••••••••••••••••••••••••••••••R•••••••
Lim 4           ••••••••••-••••••••••••••••••G•••••••-••••••••••••R•••••••
Limo 08         ••••••••••-••••••••••••••••••••••••••••••••••••••R•••••••
Water Doss      ••••••••••-••••••••••••••••••••••••••••••••••••••R•••••••
Lim 3           ••••••••••-••••••••••••••••••••••••••••••••••••••R•••••••
Italy           ••••••••••-••••••••••••••••••••••••••••••••••••••R•••••••
Schunter River  ••••••••A•A-••••••••••••••••••••••••••••••••••••••R••••T••
TBSV            VI•DILAIDNVG•ASA•FL•C•••••••T•TF•T•G-ISSATVNV••S•R••VINLL
CIRSV           AI••N•••N•AG•AFS•MT•••••A••••ITF•VVGVI•SSTSH••••SRT•NADMI
CNV             TI•••FSVS•A-GSSA••••I•IR-VN•NL•LSGL•GA•NAQLF••••ITE•AVQVV
CPMoV           SRATNVDIRSALKSGATITFEAATDSN•E•YVVRDSGINDTIVVPSLHVPV•PKSL-
LWSV            -N•VAIQ•SVGKLYCK••IEFFNPID•••NM--------------------------
EMCV Iran       GI••Q•••DVVGVATSFLV•C•••T•••TLT•GSVG-I•SVTANCT•••R••TATLL
EMCV Koenig     GI••Q•••DVVGVATSFLV•C•••T•••TLT•GSAG-I•SVTANCT•••R••TATLL
EMCV Israel     GI••Q•••DVVGVATSFLV•C•••T•••TLT•GSAD-I•SVTANCT•••R••TATLL
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