DETECTION AND PHYLOGENETIC ANALYSIS OF PRUNUS NECROTIC RINGSPOT VIRUS ISOLATES FROM STONE FRUITS IN IRAN

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SUMMARY

Symptomatic stone fruit trees suspected to be infected with prunus necrotic ringspot virus (PNRSV) were sampled in the west and northwest parts of Iran and tested by enzyme-linked immunosorbent assay (ELISA). Then, total RNA from 103 samples from the infected orchards were tested by reverse transcription polymerase chain reaction (RT-PCR) with the ilarvirus universal primer set, Ilar1/ Ilar2. An expected 206 bp DNA fragment was amplified from 30 samples and when these positive samples were subjected to RT-PCR with a pair of specific primers, VP81/ VP103, the coat protein (CP) gene was amplified from 21 out of the 30 samples. Sixteen of the amplified CP fragments were purified, sequenced and the sequence data were compared among the newly sequenced isolates, and with the CP of previously reported isolates. The new isolates were 97-100% and 96-100% homologous at the nucleotide (nt) and deduced amino acid (aa) levels, respectively. However, there were 83-99% (nt) and 74-99% (aa) homologies between these and previously reported isolates. Neighbor-Joining phylogenetic trees were generated on the basis of the nt or aa data of the CP showing that the newly-sequenced isolates were placed within the previously defined PNRSV group known as PV-96-II, which comprises isolates from diverse geographical regions such as Poland, Uruguay, China, Chile, Montenegro, and the USA. This is the first report of detection and genetic analysis of PNRSV isolates from stone fruits in Iran. Also, PNRSV in nectarine is being reported for the first time from the country.

Keywords: Coat protein, ELISA, *Ilarvirus*, phylogenetic, PNRSV, RT-PCR.

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INTRODUCTION

Prunus species including peach, plum, apricot, sweet cherry and almond are vulnerable to a number of viruses among which prunus necrotic ringspot virus (PNRSV) is one of the most frequently occurring ones. This virus belongs to the genus *Ilarvirus* in the family *Bromoviridae*, and it is an economically important virus of many *Prunus* spp. and *Rosa* spp. (Pallas *et al.*, 2012). PNRSV is transmitted by pollen, seed and propagation material (Fiore *et al.*, 2008) and induces necrotic and chlorotic rings, mosaic and deformations in leaves, flowers and fruits. It delays maturation, reduces growth and decreases productivity, but may also be present in a latent form causing no symptoms.

PNRSV is a positive-sense single-stranded RNA virus with a tripartite genome. RNA-1 and RNA-2 encode proteins involved in virus replication whereas RNA-3 is dicistronic and codes for the movement protein (MP) and coat protein (CP) at its 5' and 3' proximities, respectively. The CP is expressed via a subgenomic RNA named sgRNA-4. Sequence comparisons and phylogenetic analyses based on RNA-3, RNA-4 and the CP amino acid (aa) sequences have revealed that all PNRSV variants are clustered into three groups represented by three sequenced PNRSV isolates known as PV32, PE5, and PV96. PE5-type group is distinct from the other two groups because its 5' untranslated region is clearly different. PV32-type group possesses an additional hexanucleotide sequence, a duplication of the six immediately preceding nucleotides (nt) (Aparicio *et al.*, 1999), whereas PV96-II group does not have the six nt repeat.

PNRSV has been detected from different hosts in several Middle East countries including Syria (Ismaeil *et al.*, 2002), Lebanon (Choueiri *et al.*, 2001), Palestine (Myrta *et al.*, 2003) and Egypt (Abdel-Salam *et al.*, 2008); and in the Mediterranean countries such as Cyprus, Greece, Italy, Malta, Tunisia, Turkey and Spain (Myrta *et al.*, 2003). It has also been isolated from a *Rosa* sp. in Iran (Rakhshandehroo *et al.*, 2006). This, however, is the first report of detection and genetic analysis of PNSRV isolates from stone fruits in the west and northwest regions of Iran. Nucleotide sequences of the CP gene of newly detected isolates were generated and compared with those of other PNRSV variants retrieved from GenBank in order to determine their phylogenetic positions.

Table 1. Abbreviations, accession numbers, and origin of PNRSV strains/isolates including Iranian isolates detected in this study.

Isolate	Host	Origin	Accession No.
–	*Prunus mahaleb*	Germany	S78312
AprIt.caf1	apricot, variety Cafona	Italy	AJ133199
PlmCl.mrb1	Plum cv. Mirabolan	Chile	EF565260
Beijing	Cherry	China	DQ300178
Rose-Br	*Rosa* sp.	Brazil	KJ958527
143	Montezuma	Poland	DQ983498
RM-5	*Rosa* sp.	India	AY948441
–	Peach	Argentina	AY007217
PV32	*Malus* sp.	Spain	Y07568
PchBr.unk2	Peach cv. unknown	Brazil	EF565265
Pch-b	Peach	China	HQ833199
–	*Prunus persica*	USA	L38823
FA48-esb1	Rosa × damascena	Iran	KJ599816
162/12	*Prunus persica* (peach)	Montenegro	KF420289
–	*Rosa* sp.	China	FJ610342
E260	*Rosa* sp.	India	AJ619958
ChrT54	*Prunus cerasus*	USA	FJ231734
P2	Peach	Iran	KY484014
PN1	Peach	Iran	KY484015
PN3	Almond	Iran	KY484016
PN10	Almond	Iran	KY484017
PN50	Cherry	Iran	KY484018
PN100	Sour Cherry	Iran	KY484019
SH3	Cherry	Iran	KY484020
SH4	Almond	Iran	KY484021
SH6	Peach	Iran	KY484022
SH12	Apricot	Iran	KY484023
ZK15	Apricot	Iran	KY484024
ZKN-52	Nectarine	Iran	KX353930
SHN-6	Nectarine	Iran	KX353932
SHN-12	Nectarine	Iran	KX353933
SHN-31	Nectarine	Iran	KX353934
SHN-40	Nectarine	Iran	KX353935

MATERIAL AND METHODS

Plant material. One-hundred and three samples (leaf and fruit) showing symptoms characteristic of PNRSV were collected from different stone fruit species including almond, apricot, sour cherry, sweet cherry, peach, plum and nectarine in the west and northwest part of Iran during spring through autumn 2014 and 2015. Generally, a portion of each sample was used as inoculum in glasshouse studies and the remainder was stored at 4°C for RNA extraction. However, for a few randomly-selected samples another portion was subjected to serological assays.

Greenhouse studies. The collected samples were examined for their host range and symptomatology. *Cucumus sativum* (cucumber) and *Nicotiana tabacum* cv. Samsun were inoculated with randomly selected representative samples and maintained in a greenhouse for symptom development. Extraction was done in STEP buffer [0.1 M Na_2HPO_4-NaH_2PO_4, pH 8.3, containing 0.02 M of Na_2SO_3 and ethylene diamine tetraacetate (EDTA), and 1.5% Triton X-100] and the sap was rubbed on carborundum-dusted leaves (Abdel-Salam *et al.*, 2008). After inoculation the plants were washed thoroughly to remove carborundum. Non-inoculated plants were left as healthy controls.

Serological analysis. Eight to twelve leaves with different symptoms randomly selected from each orchard were tested collectively. Leaf samples were analyzed using a 1:1000 dilution of anti-PNRSV polyclonal antiserum (Bioreba, Switzerland) following the established protocol for DAS-ELISA (Clark and Adams, 1977) with modification as described elsewhere (Bashir *et al.*, 2006). The light absorbance values, at 405 nm wavelength, of the ELISA plate wells were measured using an Anthos 2002 ELISA Plate Reader (Anthos, Austria). The threshold was set as twice the mean absorbance value of the healthy control, a previously certified non-infected plum seedling which had been supplied by the Seed and Plant Improvement Institute, Karaj, Iran.

RNA extraction. Total RNA extractions were performed using RNX-PLUS kit (SinaClon, Iran) from 100 mg of symptomatic leaves and fruits according to manufacturer's instructions and finally dissolved in 30 µl sterile distilled water. The purified RNA samples were kept at – 80°C until they were subjected to RT-PCR.

RT-PCR. Initially, the forward Ilar1 (5'-TTCTAGCAGGTCTTCATCGA-3') and reverse Ilar2 (5'-CAACCGAGAGGTTGGCA-3') primers corresponding to conserved regions at the 3' end of the CP-coding region on the RNA-3, which allow amplification from both PNRSV and another ilarvirus, apple mosaic virus (ApMV), were used (Moury *et al.*, 2000). Amplification by the use of Ilar1 and Ilar2 produced a 206 bp DNA fragment from PNRSV. Then, a pair of PNRSV CP-specific primers, VP81 (5'-AGTGGATCCATGGTTTGCCGAATTTGC-3') and VP103 (5'-ACATAAGCTTCTAGATCTCAAGCAGGTC-3') (Aparicio *et al.*, 2003) was used to amplify an expected DNA fragment of 694 bp, encompassing the entire CP gene (675 bp) plus 9 and 10 bp extensions including restriction sites engineered in the forward and reverse primers, respectively.

In a 10 µl reverse transcription reaction, 2 pmol (0.4 µl) of the reverse primer (Ilar2) was mixed with 1.1 µl RNase-free sterile distilled water and 1 µl aliquot of the purified total RNA sample. The mixture was incubated at 70°C for 5 min (to remove secondary structures from the ssRNA sample), then immediately placed on ice and 7.5 µl of Hyperscript master mix (Genall, South Korea) were added to each reaction before incubation at 42°C for 60 min in a thermal cycler model ASTEC PC-320 (Taiwan). Inactivation of the reverse transcriptase was carried out at 90°C for 5 min. Then, 5 µl of the reverse-transcribed mix were added to 12.5 µl PCR master mix which contained 1 pmol Ilar1 primer, 50 mM Tris-HCl, pH 8.5, 25 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM of each dNTP, 0.2

Units Ampliqon *Taq* DNA polymerase and adjusted to a final volume of 25 µl by adding double distilled H_2O. The reaction components for amplification of the full CP gene were similar with the exception that the reverse transcription was done with 1 pmol of VP89 primer and the PCR mix contained 1 pmol of the reverse primer, VP103.

When Ilar1/Ilar2 primers were used, the initial denaturing was performed at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 48°C for 45 s and extension at 72°C for 45 s. In the case of the PNRSV CP-specific primers, the initial denaturing was similarly done, but followed by 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 60 s. A final polymerization step at 72°C for 5 min was also applied in both cases. PCR products were run on a 1% agarose gel in 0.5 × TBE buffer, stained with ethidium bromide and photographed by a gel documentation apparatus (Kiagen, Iran).

Sequencing and phylogenetic analyses. Each of 16 out of 21 PCR products was purified and sequenced by the the use of both VP81 and VP103 primers (Bioneer, South Korea). For each fragment, sequencing data resulting from use of VP103 primer were reverse-complemented and aligned with the sequence data coming from the use of VP81. As a result, an overlapping region was established in the middle that facilitated determination of the whole CP sequence. The generated sequences were submitted to BLAST (Altschul *et al*., 1990) to identify if they belonged to PNRSV. Then, the generated sequences were aligned with those of previously reported PNRSV strains/isolates (Table 1) by the use of ClustalW (Larkin *et al*., 2007). Neighbor-joining phylogenetic trees were generated with MEGA6 (Tamura *et al*., 2013) and ApMV was designated as the outgroup species. Initially, the phylogenetic trees were drawn for 38 isolates including 16 PNRSV isolates whose CP sequences were generated in this study. Additional analysis for phylogenetic inference was carried out by omission of isolates having < 1% genetic distance from other isolates.

RESULTS

The most common symptoms on the collected samples were mosaic, necrotic ring spots and shot holes on leaves of all the hosts, but chlorotic ring spots on fruits were evident on the nectarine fruits only. Bud failure was seen on peach, apricot, sour cherry, plum and nectarine trees. Necrotic ring spots and shot holes were evident on infected leaves in the majority of the visited orchards. Infected trees had fewer blossoms and smaller fruits. Inoculated *C. sativum* and *N. tabacum* expressed symptoms, including common mosaic, mottling and stunting, 15-20 days post inoculation (dpi).

An expected DNA fragment 206 bp in length corresponding to a segment of PNRSV RNA-1 was amplified with the universal primers from 30 out of 103 symptomatic leaf samples, whereas there was no amplification from the healthy control. Thus, PNRSV was successfully detected by RT-PCR in apricot, peach, plum, sour cherry, and from fruit and leaves of nectarine. A full-length CP fragment, 694 bp in size, was amplified from 21 out of the 30 samples (Table 2). The amplified DNA fragments were in agreement with the expected size based on the positions of the primers on the published nucleotide (nt) sequence of PNRSV (Scott *et al*., 1998). There was no amplification from the healthy control.

When the CP nucleotide sequences of the newly characterized isolates were aligned with those of previously reported PNRSV isolates it appeared that the newly generated sequences corresponded to the CP gene of PNRSV. The identities between the new isolates were 97-100% and 96-100% at the nt and the deduced amino acid (aa) sequences, respectively. When the sequences were subjected to BLAST, high similarities (83% to 99%) were found between the newly-sequenced PNRSV isolates and the other isolates that were previously reported from Iran, Poland and India. Also, alignment of the aa sequences showed that the identities between the new isolates from Iran and the other isolates were 74-99%. The lower percentage of similarity was noted between an isolate from the USA (L38823) belonging to the PE5 group and two members of the other groups (PV-96 and PV-32) being approximately 83% and 74%, based on nt and aa data, respectively.

Phylogenetic analysis based on nt or aa sequences of the CP gene from 33 PNRSV isolates including those from Iran showed that all the newly-sequenced 16 isolates and a previously reported isolate from Iran (KJ599816) together with isolates from the USA (FJ231734), China (DQ300178), Montenegro (KF420289), Italy (AJ133199), Germany (S78312) and Chile (EF565260) were placed in the PV-96-II subclade (Fig. 1 and 2)

DISCUSSION

Virus diseases cause serious yield losses in orchards in the west and northwest regions of Iran. Detection of plant virus isolates and determination of their characteristics are effective steps in the control of such diseases. Therefore, a rapid and accurate identification method is essential for application of appropriate control measures. Various viral symptoms including necrotic ring spots and shot holes associated with virus infection that cause significant yield losses were observed in the orchards in the course of this study. Prior to this survey, PNRSV had been reported from Iran only in rose plants (Rakhshandehroo *et al*., 2006). In the present study several PNRSV isolates from Iranian stone fruits were characterized. A large number of PNRSV isolates have already been characterized in the USA, Europe and Mediterranean countries (Sala-Rejczak and Paduch-Cichal, 2013; Salem *et al*., 2003). Virus

Table 2. List of PNRSV isolates from Iran and results from serological and molecular analyses.

Isolate	location	Original host	RT-PCR[a] (Ilar1/Ilar2)	RT-PCR[b] (VP81/VP103)	DAS-ELISA[c]	Phylogenetic subgroup[d]
P2	Tarom	Peach	+	+	+	PV-96-II
PN1	Tabriz	Peach	+	+	NA	PV-96-II
PN3	Zanjan	Almond	+	+	+	PV-96-II
PN10	Mianeh	Almond	+	+	NA	PV-96-II
PN50	Sardroud	Cherry	+	+	NA	PV-96-II
PN100	Basmenj	Sour Cherry	+	+	+	PV-96-II
SH3	Shit	Cherry	+	+	+	PV-96-II
SH4	Shit	Almond	+	+	+	PV-96-II
SH6	Tarom	Peach	+	+	NA	PV-96-II
SH12	Tarom	Apricot	+	+	+	PV-96-II
ZK15	Zanjan	Apricot	+	+	+	PV-96-II
ZKN-52	Zanjan	Nectarine	+	+	+	PV-96-II
SHN-6	Abhar	Nectarine	+	+	NA	PV-96-II
SHN-12	Khodabandeh	Nectarine	+	+	+	PV-96-II
SHN-31	Abhar	Nectarine	+	+	NA	PV-96-II
SHN-40	Mahneshan	Nectarine	+	+	+	PV-96-II
SHN-30	Mahneshan	Peach	+	+	NA	NA
P5	Tarom	Cherry	+	+	NA	NA
P14	Tarom	Cherry	+	+	+	NA
Z4	Zanjan	Almond	+	+	+	NA
Qa1	Abhar	Apricot	+	+	+	NA
Ma1	Maragheh	Peach	+	–	NA	NA
Ma2	Maragheh	Cherry	+	–	NA	NA
AH30	Abbar	Cherry	+	–	NA	NA
Ha1	Haronabad	Apricot	+	–	NA	NA
Da1	Jizlan dasht	Apricot	+	–	NA	NA
ALT1	Zahir	Peach	+	–	NA	NA
ALS1	Zanjan	Almond	+	–	NA	NA
Alb1	Abbar	Almond	+	–	NA	NA
Ab	Abbar	Cherry	+	–	NA	NA

[a] PCR with the universal ilarvirus primers Ilar1 and Ilar2 corresponding to the part of virus RNA1.
[b] PCR with a pair of primers corresponding to the PNRSV CP gene, NO: not optimized.
[c] DAS-ELISA by specific antibodies of PNRSV, NA: DAS-ELISA was not performed.
[d] Subgrouping based on the phylogenetic analysis, NA: sequencing not done.

symptoms were observed in a majority of the visited orchards; however, chlorotic spots and mosaic on the leaves were most common in spring, whereas shot-holes and ring spots were observed in late summer and early autumn. Mosaic and ring spot are the typical symptoms caused by PNRSV (Choueiri *et al*., 2003). Fruit setting was dramatically reduced in infected plum and apricot trees due to cessation of flower development and flower dropping. Chlorotic spots were observed only on the nectarine fruits. PNRSV infection in nectarine trees is being reported for the first time from Iran.

In regard to glasshouse studies, although *C. sativus* has been recommended as an especially useful host to differentiate PNRSV isolates, no significant differences were found between pathogenicity of the presently studied PNRSV isolates. Similar results have been reported by others (Paduch-Cichal *et al*., 2003; Sala-Rejczak and Paduch-Cichal, 2013; Salem *et al*., 2003).

RT-PCR as a highly sensitive test for detection of PNRSV (Yang *et al*., 2007; Hou *et al*., 2006) was applied in this study to detect PNRSV isolates from the collected samples. A pair of primers that covered the full CP gene of PNRSV was applied to detect the virus using by RT-PCR. The CP fragment was amplified from 21 out of 30 tested samples (data not shown). However, PNRSV was not detected in some samples which showed typical symptoms of PNRSV infection. The PNRSV-specific primers are not degenerate at any position, and therefore display no heterogeneity; there may be variation between the unique primer sequences (VP81 and VP103) and the target sequences of particular isolates, resulting in a failure to amplify the anticipated product. The other possibility is that because the samples were kept at 4°C until processing, the RNA extracted from them may have been of poor quality. It is suggested RNA extraction be done immediately after sampling.

PNRSV isolates have been assigned to three phylogroups known as PV32-I, PV96-II, PE5-III according to their CP gene sequences (Hammond, 2003). A similar grouping was also proposed although with an alternative nomenclature for each phylogroup (I, II and III, respectively) (Vaskova *et al*., 2000). In the present study, all the PNRSV isolates from Iran were placed in the PV96-II group (Fig. 1 and 2) because two extra residues (N/S42 and R43) and Y81 that are typical characteristic features of PV-32 are absent in the sequences of the new PNRSV isolates from Iran. Alignment of the CP sequences also

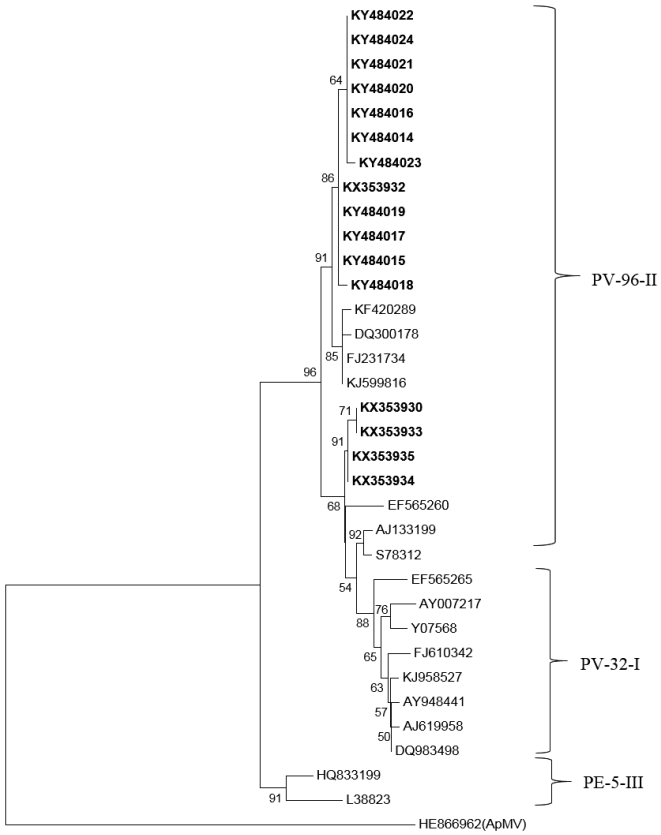

Fig 1. Neighbor joining phylogenetic trees generated by the use of Mega 6 program on the basis of amino acid sequences of coat protein of PNRSV isolates. Branch lengths are proportionate to the genetic distances and the scale bar represents 0.05 genetic distance. Branches with bootstrap value of <50% are unresolved. *Apple mosaic virus* (ApMV) is assigned as the outgroup species.

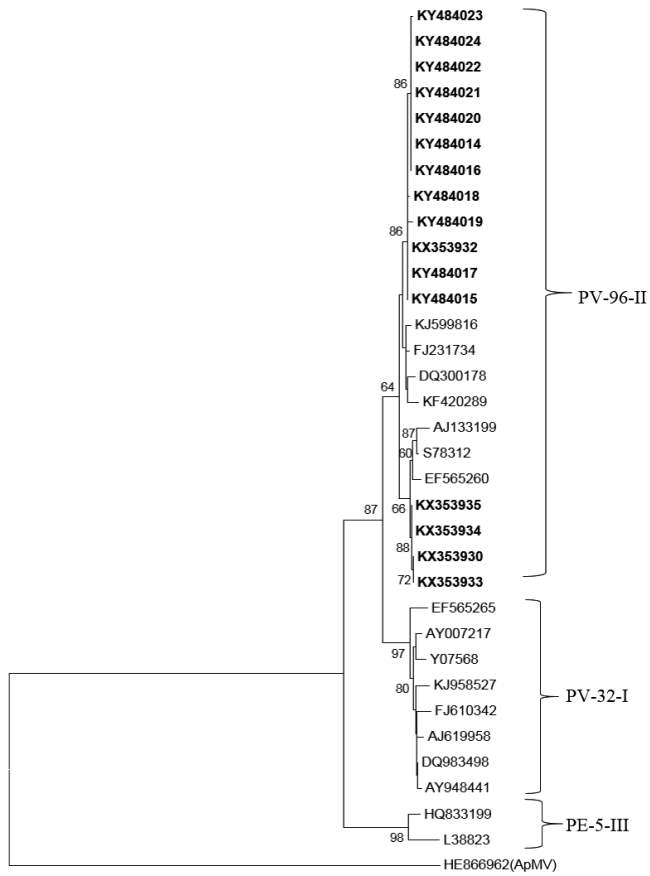

Fig 2. Neighbor joining phylogenetic trees based on nucleic acid sequences of CP gene of PNRSV isolates generated by Mega 6 program. Branch lengths are proportionate to genetic distances and the scale bar represents 0.05 genetic distance. Branches with <50% bootstrap values are unresolved. *Apple mosaic virus* (ApMV) is assigned as the outgroup.

revealed that the CP is highly conserved especially at the C-terminus, whereas most differences were found at the N-terminus. All the new isolates from Iran are conserved between aa residues 30 and 50. Three to five residues in this domain are important to differentiate PNRSV groups. Further, Arg residues at positions 30, 34, 41, and 47 were observed in the alignment of newly-generated sequences that are highly conserved in all PNRSV isolates, whereas there is an additional Arg residue at position 43 present only in the CP of the PV32-type group. This region could be defined as an Arg-rich region with a high tendency to adopt an α-helical secondary structure and, interestingly, is located downstream from a C2C2 putative zinc-finger motif (Fiore *et al*., 2008). Amino acid residues K59, N121, R139, N142, and I181 that are specific for the PE5-III group were not found in presently reported isolates. Also, we found a domain located between aa residues 9 and 27 relative to the C-terminal end that has been reported to be highly conserved for all members of *Ilarvirus* and critical for dimer stabilization by a putative intermolecular interaction which is mediated via aromatic residues (Aparicio *et al*., 2006).

Sequences of the CP gene in PV32-I group are 6 nt longer than that of isolates of the PV-96 group. The extra residues of the PV32-I group are located inside the RNA-binding domain, which is essential for the protein to bind PNRSV RNA *in vitro*. In a study by Hammond (2003), 68 PNRSV isolates were classified in PV32-I group based on sequences of the CP gene containing the additional 6 nt. In another study, several isolates from rose plants were characterized forming an independent phylogenetic line (Moury *et al*., 2001). Accordingly, these isolates could be categorized into the PV96-II or PV32-I group. Similarly, 25 PNRSV isolates from plums and sour cherries from Slovakia were classified into one group represented by the PV96-II isolate (Glasa *et al*., 2002). Many isolates of PNRSV from the USA, central Europe and the Mediterranean Basin have been grouped into PV96-II (Cui *et al*., 2012). PNRSV isolates from cherry trees in New York were assigned in the predominant group PV-96-II (Cui *et al*., 2012). The majority of the examined isolates were assigned to the PV96-II group based on phylogenetic and RFLP analysis, and one from peach to PV32-I, whereas none belonged to PE5-III (Ulubas and Ertunc, 2004). In

the present work, all new isolates from *Prunus* cultivars and also those previously reported from rose in Iran were placed into PV-96 group.

The length of the deduced aa sequences of the CP gene was in the range of 224-226 residues. The aa identity among the isolates from Iran was 96-100%. The frequency of distribution of different phylogroups (PV32-I, PV96-II and PE5-III) of PNRSV isolates may represent an equilibrium distribution in which the abundance of a given type depends on factors such as its fitness, growth (replication), and transmission rates under different environmental conditions and different host species. The distribution may also reflect the isolates present in different regions and, in particular, the isolates present in budwood sources. This is most likely the major determinant of which types of isolate are found in any region. However, it is noteworthy that a lower number of PNRSV isolates are assigned to the PE5-III phylogroup and previous observation has shown that PE5-III has an accelerated rate of molecular evolution for the CP gene (Sala-Rejczak and Paduch-Cichal, 2013). Collectively, these observations suggest that selective pressures acting on PE5-III isolates are stronger than that on the other phylogroups. Types of specific pressures acting on PE5-III, and particularly whether its actual frequency is stable or transiently changing toward higher levels may suggest a faster rate of molecular evolution which is still a question to be explored. Analysis of all the isolates sequenced so far shows the existence of certain phylogroup-specific residues for PV32-I and PE5-III but not for PV96-II.

This study revealed infections with PNRSV in stone fruits in Iran which necessitates close attention to the virus-free status of plant propagation material in order to prevent further spread of the virus. It was also determined that PNRSV isolates from Iran belong to the PV96-II group. In line with other studies on this virus, it appeared that placement of PNRSV isolates in one of the several main groups is not correlated with the geographical origin of a given isolate, although it cannot be ruled out that variation in the aa sequence of the CP can affect biological properties of the virus.

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REFERENCES

Abdel-Salam A.M., Ibrahim A., Abdelkader H.S., Aly A.M., El-Saghir S.M., 2008. Characterization of two isolates of *Prunus necrotic ringspot virus* (PNRSV) from peach and apricot in Egypt. *Arab Journal Biotechnology* **11**: 107-112.

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Aparicio F., Sanchez-Pina M.A., Sanchez-Navarro J.A., Pallas V., 1999. Location of *Prunus necrotic ringspot* ilarvirus within pollen grains of infected nectarine trees: evidence from RT-PCR, dot-blot and in situ hybridisation. *European Journal of Plant Pathology* **105**: 623-627.

Aparicio F., Vilar M., Perez-Paya E., Pallas V., 2003. The coat protein of *Prunus necrotic ringspot virus* specifically binds to and regulates the conformation of its genomic RNA. *Virology* **313**: 213-223.

Aparicio F., Sa´nchez-Navarro J.A., Pallas V., 2006. *In vitro* and *in vivo* mapping of the Prunus necrotic ringspot virus coat protein C-terminal dimerization domain by bimolecular fluorescence complementation. *Journal of General Virology* **87**:1745-1750.

Bashir N.S., Kalhor M.R., Zarghani S.N., 2006. Detection, differentiation and phylogenetic analysis of *Cucumber mosaic virus* isolates from cucurbits in the northwest region of Iran. *Virus Genes* **32**: 277-288.

Choueiri E., Haddad C., Abou Ghanem-Sabanadzovic N., Jreijiri F., Issa S., Saad A., Di Terlizzi B., Savino V., 2001. A survey of peach viruses in Lebanon. *Bulletin OEPP/EPPO Bulletin* **31**: 493-497.

Choueiri E., Abou Ghanem-Sabanadzovic N., El Zammar S., Jreijiri F., 2003. Viruses of stone fruit trees in Lebanon. Virus and virus-like diseases of stone fruits, with particular reference to the Mediterranean region. *Options Méditerranéennes: Série B. Etudes et Recherches* **45**: 25-27.

Clark M.F., Adams A., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**: 475-483.

Cui H., Hong N., Wang G., Wang A., 2012. Detection and genetic diversity of Prunus necrotic ringspot virus in the Niagara Fruit Belt, Canada. *Canadian Journal of Plant Pathology* **34**: 104-113.

Fiore N., Fajardo T.V., Prodan S., Herranz M.C., Aparicio F., Montealegre J., Elena S.F., Pallas V., Sanchez-Navarro J., 2008. Genetic diversity of the movement and coat protein genes of South American isolates of *Prunus necrotic ringspot virus*. *Archives of Virology* **153**: 909-919.

Glasa M., Betinova E., Kudela O., Subr Z., 2002. Biological and molecular characterisation of *Prunus necrotic ringspot virus* isolates and possible approaches to their phylogenetic typing. *Annals of Applied Biology* **140**: 279-283.

Hammond R., 2003. Phylogeny of isolates of *Prunus necrotic ringspot virus* from the Ilarvirus Ringtest and identification of group-specific features. *Archives of Virology* **148**: 1195-1210.

Hou Y., Zhang L., Yu Y., Yu D., 2006. Detection of PNRSV in sweet cherry variety Hong Deng' by RT-PCR. *Journal of Dalian University* **4**: 14.

Ismaeil F., Myrta A., Abou Ghanem-Sabanadzovic N., Al Chaabi S., Savino V., 2002. Viruses and viroids of stone fruits in Syria. *Bulletin OEPP/EPPO Bulletin* **32**: 485-488.

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., Higgins D.G., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Moury B., Cardin L., Onesto J.-P., Candresse T., Poupet A., 2000. Enzyme-linked immunosorbent assay testing of shoots grown *in vitro* and the use of immunocapture-reverse transcription-polymerase chain reaction improves the detection of *Prunus necrotic ringspot virus* in rose. *Phytopathology* **90**: 522-528.

Moury B., Cardin L., Onesto J.-P., Candresse T., Poupet A., 2001. Survey of *Prunus necrotic ringspot virus* in rose and its variability in *Rosa* and *Prunus* spp. *Phytopathology* **91**: 84-91.

Myrta A., Di Terlizzi B., Savino V., Martelli G., 2003. Virus diseases affecting the Mediterranean stone fruit industry: a decade of surveys. Virus and virus-like diseases of stone fruits, with particular reference to the Mediterranean region. *Options Méditerranéennes: Série B. Etudes et Recherches* **45**: 15-23.

Paduch-Cichal E., Szyndel M., Sala-Rejczak K., Skrzeczkowska S., 2003. Characterization of *Prunus necrotic ringspot virus* (PNRSV) isolates from plum trees. *Phytopathologia Polonica* **29**.

Pallas V., Aparicio F., Herranz M., Amari K., Sanchez-Pina M., Myrta A., Sanchez-Navarro J., 2012. Ilarviruses of *Prunus* spp.: a continued concern for fruit trees. *Phytopathology* **102**: 1108-1120.

Rakhshandehroo F., Zamani Zadeh H., Modarresi A., Hajmansoor S., 2006. Occurrence of *Prunus necrotic ringspot virus* and *Arabis mosaic virus* on rose in Iran. *Plant Disease* **90**: 975-975.

Sala-Rejczak K., Paduch-Cichal E., 2013. Molecular variability of the coat protein gene of *Prunus necrotic ringspot virus* isolates. *Acta Scientiarum Polonorum. Hortorum Cultus* **12**: 35-42.

Salem N., Mansour A., Al-Musa A., Al-Nsour A., 2003. Incidence of *Prunus necrotic ringspot virus* in Jordan. *Phytopathologia Mediterranea* **42**: 275-279.

Scott S., Zimmerman M., Ge X., MacKenzie D., 1998. The coat proteins and putative movement proteins of isolates of *Prunus necrotic ringspot virus* from different host species and geographic origins are extensively conserved. *European Journal of Plant Pathology* **104**: 155-161.

Tamura K., Stecher G., Peterson D., Filipski A., Kuma S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725-2729.

Ulubas C., Ertunc F., 2004. RT-PCR Detection and molecular characterization of *Prunus necrotic ringspot virus* isolates occurring in Turkey. *Journal of Phytopathology* **152**: 498-502.

Vaskova D., Petrzik K., Karesova R., 2000. Variability and molecular typing of the woody-tree infecting prunus necrotic ringspot ilarvirus. *Archives of Virology* **145**: 699-709.

Yang H., Wu Y., Yan Y., Fu H., Yang M., Wang Y., Zhou J., Yang S., 2007. Studies on PCR dete mination cherry of prunus necrotic ring-spot virus. *Journal of Shaanxi Normal University (Natural Science Edition)*: S1.

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