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e-Xtra*

Insights Into the Etiology of Polerovirus-Induced Pepper Yellows Disease

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ABSTRACT

The study of an emerging yellows disease of pepper crops (pepper yellows disease [PYD]) in Greece led to the identification of a polerovirus closely related to *Pepper vein yellows virus* (PeVYV). Recovery of its full genome sequence by next-generation sequencing of small interfering RNAs allowed its characterization as a new poleroviruses, which was provisionally named Pepper yellows virus (PeYV). Transmission experiments revealed its association with the disease. Sequence similarity and phylogenetic analysis highlighted the common ancestry of the three poleroviruses (PeVYV, PeYV, and Pepper yellow leaf curl virus [PYLCV]) currently reported to be associated with PYD, even though significant genetic differences were identified among them, especially in the C-terminal region of P5 and the 3′ noncoding region. Most of the differences observed can be attributed to a modular type of evolution, which produces mosaic-like variants giving rise to these different poleroviruses Overall, similar to other polerovirus-related diseases, PYD is caused by at least three species (PeVYV, PeYV, and PYLCV) belonging to this group of closely related pepper-infecting viruses.

Additional keywords: etiology, evolution, Pepper yellows disease, *Polerovirus*, recombination.

Poleroviruses have only recently been shown to be important pathogens of pepper crops. It was not until the mid-1980s, when a *Beet western yellows virus* (BWYV) isolate was reported infecting pepper crops in the United States (Timmerman et al. 1985), followed by an Australian *Potato leafroll virus* (PLRV)-like isolate (Gunn and Pares 1990) and, potentially, a new polerovirus from Japan (Yonaha et al. 1995). This new virus was named *Pepper vein yellows virus* (PeVYV) and was associated with symptoms of interveinal yellowing, vein yellowing, upward leafroll, and fruit malformation, herein named pepper yellows disease (PYD). The complete sequence of PeVYV was obtained 16 years later (Murakami et al. 2011). Another virus associated with PYD was reported around the same time in Israel (Dombrovsky et al. 2010) and was named Pepper yellow leaf curl virus (PYLCV). Sequencing of PYLCV's genome revealed similarities to and substantial differences from PeVYV, which resulted in their classification as two different viral species within the genus *Polerovirus* (Dombrovsky et al. 2013). Both PeVYV and PYLCV have the typical genome organization of members in the genus *Polerovirus*, encoding seven open reading frames (ORF) (ORF0 to 5 and 3a) from a positive sense, single-stranded RNA which is packaged inside an icosahedral shell and is persistently transmitted by aphids (Domier 2011; Katis et al. 2007). PeVYV and PYLCV are closely related in the largest part of their genomes but exhibit significantly high variability in the C-terminal part of P5, after the recombination break point of the *Cucurbit aphid-borne yellows virus* (CABYV)-like sequence present in the N-terminal part (Dombrovsky et al. 2013). *Tobacco vein distorting virus* (TVDV) is phylogenetically the closest species in the genus to PeVYV and PYLCV, with which both viruses share highly similar RNA-dependent RNA polymerase (RdRp) and coat protein (CP) amino acid sequences. Thus far, natural infections of pepper plants by PeVYV or PYLCV have been reported from different regions around the globe, (Alabi et al. 2015; Alfaro-Fernández et al. 2014; Buzkan et al. 2013; Knierim et al. 2013; Tan et al. 2015; Villanueva et al. 2013), thus making the polerovirus-induced PYD a worldwide spread disease.

In Greece, during the last decade, pepper plants exhibiting symptoms of PYD were sporadically collected from commercial growers but, with the exception of BWYV (Lotos et al. 2014), no other viral species of the genus *Polerovirus* were identified infecting pepper in the country. Moreover, the disease had low incidence without a constant presence through the years and it was not a limiting factor in the cultivation of pepper in Greece. However, during spring 2013, a major outbreak emerged on the island of Crete. Pepper plants exhibiting typical symptoms of PYD (Fig. 1) were observed in different greenhouses throughout the Lasithi regional unit, with incidences of 40 to 60%. To identify the etiological agent of the disease, a generic polerovirus detection method (Lotos et al. 2014) was used and revealed a putative PeVYV/PYLCV-like isolate in the infected samples. It suggested that the causal agent of the disease in Greece was a divergent isolate of the *Polerovirus* genus. In order to characterize this newly detected isolate, viral-derived small interfering RNAs (siRNA) purified from a symptomatic plant were analyzed by next-generation sequencing (NGS) using the Ion Torrent platform. NGS technology makes it conceptually feasible to detect any viral agent without prior knowledge of the nucleotide sequence by high-throughput sequencing of nucleic acids from an infected host. It has already been used to recover other polerovirus genomes such as PYLCV, Strawberry polerovirus 1, and *Carrot red leaf virus* (Adams et al. 2014; Dombrovsky et al. 2013; Xiang et al. 2015). Our study revealed the presence of a novel polerovirus related to PeVYV and PYLCV in diseased pepper from Greece that was given the provisional name Pepper yellows virus (PeYV). Complete genome sequencing, phylogenetic analysis, and aphid transmission assays of this new virus

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were performed. These closely related poleroviruses (PeVYV, PYLCV, and PeYV) are associated with PYD symptoms and form a very distinct group, herein named "PYD virus group" (PYD group). The possible mechanisms driving the diversification and evolution of the poleroviruses causing PYD are discussed.

MATERIALS AND METHODS

Screening of plant materials. Samples from 35 pepper plants exhibiting PYD symptoms, typical of the infection by members of the genus *Polerovirus*, were collected during three consecutive years (2013 to 2015) from different greenhouses. The severity as well as the symptoms present varied among the tested plants, with the most common one being interveinal yellowing of variable intensity. The geographical origin of the majority ($n = 24$) of the samples was the island of Crete, where the outbreak of PYD occurred, and a few ($n = 7$) were collected from other locations in Greece. In addition, samples from four symptomatic pepper plants from the laboratory repository, collected in previous years, were also included in the analysis (Supplementary Table S1). Total RNA was purified according to Chatzinasiou et al. (2010) (method A), with the modifications described by Maliogka et al. (2015). All samples were tested for the presence of poleroviruses by genus-specific reverse-transcriptase polymerase chain reaction (RT-PCR) (Lotos et al. 2014), for the presence of BWYV by a species-specific assay (Lotos et al. 2014), and for the presence of a PYD group member using a new RT-PCR assay developed herein. This new PCR was designed to amplify a 240-bp part of the RdRp coding sequences of PeVYV, PYLCV, and TVDV. Briefly, the PYD group RT-PCR was performed using 2 µl of cDNA (from the polerovirus generic RT) in a 20-µl final volume reaction mixture containing 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM PYDGroupUp (5′-CAG AAC AAG CGA GAA ATC GCT C-3′) and 0.4 µM PYDGroupDown (5′-TGT GAG TTT GTT GCG GAC GAC C-3′) primers, and 1.5 U of Qiagen HotStarTaq DNA Polymerase. Cycling conditions consisted of one initial denaturation step at 95°C for 15 min; 40 cycles of 94°C for 30 s, 60°C for 20 s, and 72°C for 20 s; followed by a final extension step at 72°C for 5 min.

Characterization of pepper-infecting polerovirus isolates. NGS and analysis of siRNA reads. Isolate PX3, collected in 2014 from a PYD-symptomatic pepper plant from Chania (Crete), was selected for the NGS analysis. siRNAs were isolated from a pool of lyophilized pepper leaves using the mirPremier microRNA Isolation Kit (Sigma-Aldrich). Quality of the extracted RNA was verified by checking the values for optical density ratio at 260/280 nm using NanoDrop (Thermo Scientific). Subsequently, siRNA library construction and sequencing were performed by Life Sequencing S.L. NGS was performed in an Ion-Torrent platform using the Ion 318 chip (Life Technologies, Inc.). De novo assembly was performed using the Velvet plug-in in Geneious software (Biomatters Ltd.) and the resulting contigs were blasted (Blastn and Blastx) against GenBank databases. Contigs that showed similarity, in the previous step, with viral sequences were subsequently used in an iterative mapping and contig extension process using Geneious. Profiling of the siRNA coverage over the length of the genome was performed using MISIS (Seguin et al. 2014).

Determination of full genome and partial sequences using Sanger sequencing. The genome of isolate Pi21 collected in 2013 from Lasithi (Crete) was fully sequenced by Sanger sequencing. Overlapping amplicons covering the complete viral genome were generated using 26 primers in 15 RT-PCR (Fig. 2A; Supplementary Table S2). Primer design was based on the alignment of the genomic sequence that was reconstructed from the NGS and the sequences

Fig. 1. Symptoms of **A,** interveinal yellowing; **B,** severe upward leafroll; and **C,** stunted growth appearing on infected Sammy RZ F_1 hybrid (A and B) and Zafiro RZ F_1 hybrid (C) pepper plants in greenhouses located in the municipality of Ierapetra of the Lasithi regional unit. All photos were taken during March 2013, when the plants were approximately 7 months old.

from the two already characterized pepper poleroviruses (PeVYV and PYLCV).

For 12 of the amplifications, RT-PCR were performed according to the following protocol. For the reverse transcription of the RNA, the downstream primer of each subsequent PCR was used. A 20-µl final volume reaction mixture (containing 2 µl of the total RNA extraction, 50 mM Tris-HCl [pH 8.3 at 25°C], 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM each dNTP, 1 µM downstream primer, and 50 U of M-MLV reverse transcriptase) (Invitrogen) was incubated at 45°C for 1 h followed by 15 min at 70°C for enzyme deactivation.

PCR was performed using 2 µl of cDNA in a 20-µl final volume reaction mixture containing 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM upstream primer, 0.4 µM downstream primer, and 1.5 U of Qiagen HotStarTaq DNA Polymerase. Cycling conditions consisted of one initial denaturation step at 95°C for 15 min; 40 cycles of 94°C for 20 s, *X*°C for 20 s, and 72°C for *Y* s; followed by a final extension step at 72°C for 5 min, where *X* and *Y* were adjusted for each amplicon according to the manufacturer's specifications.

For the remaining three amplicons, number 8 (Fig. 2A) was amplified using the generic detection assay (Lotos et al. 2014) and numbers 9 and 10 were amplified as described by Lotos et al. (2016). For the amplification of amplicon number 10, the primer 1498Up (5′-ACG CCC ACG ACA GGT TCG-3′) was used.

Eight additional isolates—seven from Greece, including two collected in 2013 (Pi24 and Pi226), one in 2014 (PX1), and four in 2015 (PiL1, PiE1, PiE6, and PiE13); and one from Turkey (PY), collected in 2007—were selected for further characterization. ORF0 and the generic detection amplicons were sequenced from all eight isolates, the RdRp-CP region from isolates Pi24 and PY, and a part of the genome near the 3′ end from isolates Pi226, PX1, PiL1, PiE1, PiE6, and PiE13.

Amplification of ORF0 of the PYD group isolates was performed using primer P-892Down (5′-GAG AGT TGT CTT CAT GTT GC-3′) and primer P-5′Up (5′-ACA AAA TAT ACG AAG AGA GAG AG-3′) in the RT-PCR assay described above, modifying only the cycling conditions, as follows: one initial denaturation step at 95°C for 15 min; 40 cycles of 94°C for 20 s, 53°C for 20 s, and 72°C for 20 s; followed by a final extension step at 72°C for 5 min.

The portion of the genome which includes the last 719 nucleotides (nt) of ORF5 and the first 112 nt of the 3′ noncoding region (NCR) was amplified using primers P-5777Down for the RT and P-5102Up and P-5777Down for the PCR using the same RT-PCR assay described for the amplification of ORF0.

The amplicon DNA was purified from agarose gel using the NucleoTrap purification kit (Macherey-Nagel) according to the manufacturer's instructions and was directly sequenced at VBC-Biotech.

Sequence similarity and phylogenetic analysis. Putative ORFs were predicted using the annotation and prediction algorithm implemented in Geneious (Biomatters Ltd., Auckland, New Zealand) and ORF-finder (https://www.ncbi.nlm.nih.gov/gorf/gorf.html). The −1 frameshift which results in the expression of ORF2 was predicted

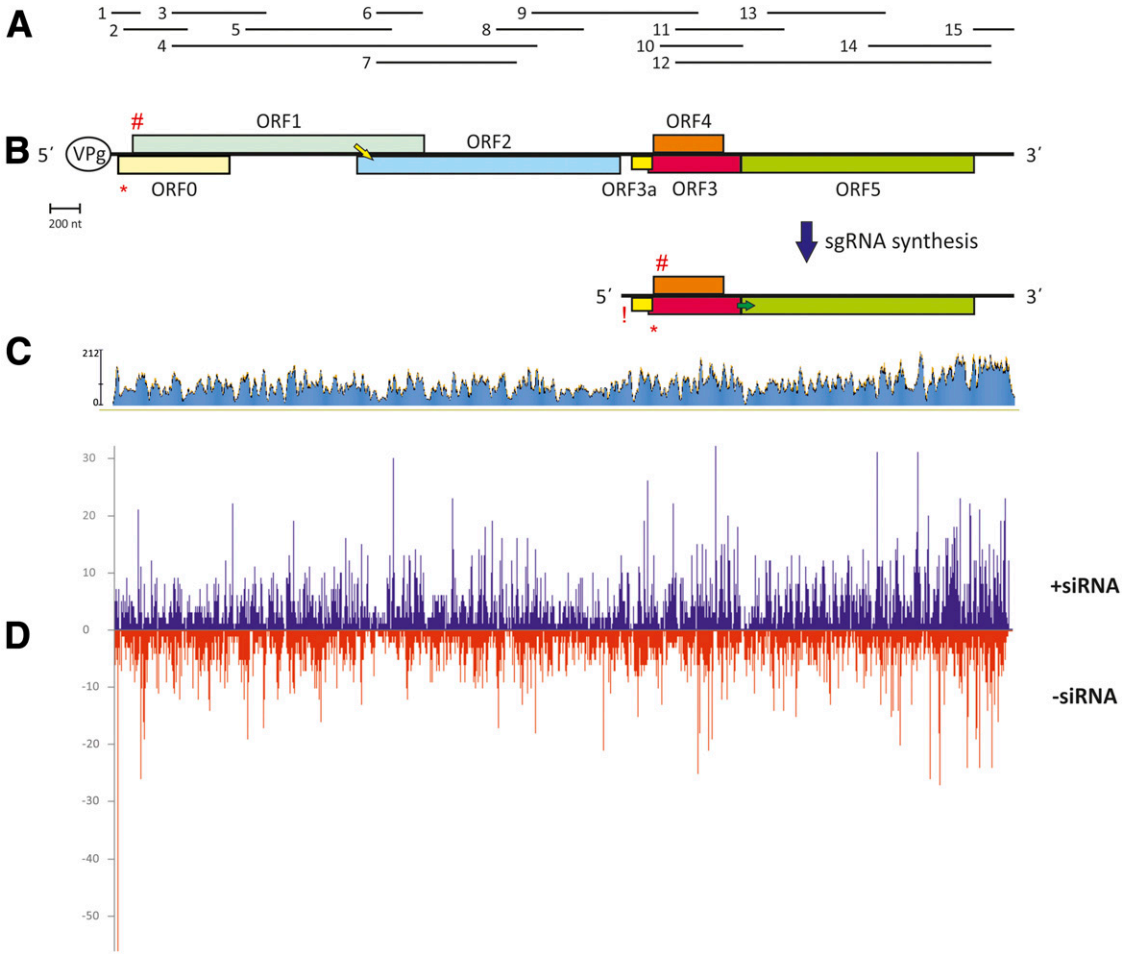

Fig. 2. A, The 15 overlapping amplicons used for sequencing of the Pi21 isolate relative to the genome. **B,** Genome organization, and predicted expression strategies of the Pi21 and PX3 isolates. Expression of proteins by first AUG codon (*), leaky scanning (#), non-AUG codon (!), frameshift (light arrow), and readthrough (dark arrow) are indicated next to each open reading frame (ORF)'s start. sgRNA = subgenomic RNA. **C,** Coverage and **D,** distribution relative to polarity of the small interfering RNA (siRNA) mapped to the genome of isolate PX3.

using the FSFinder software (Moon et al. 2004). Homology comparison of the resulting ORF was performed using the Blast(n/p) algorithms. Identities were calculated with the percent identity criterion implemented in Geneious. ProtTest, version 3.4 (Abascal et al. 2005) and JModelTest, version 2.1.6 (Darriba et al. 2012) were used for selecting the best evolutionary model needed for the construction of maximum-likelihood (ML) phylogenetic trees by PhyML, version 3.1 (Guindon and Gascuel 2003). The Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) for branch support was used. Possible recombination events were detected by RDP4 (Martin et al. 2010). All alignments were performed by MAFFT, version 7 (Katoh and Standley 2013).

Aphid transmission. Two pepper-colonizing aphid species were evaluated as possible vectors of the new pepper polerovirus. *Aphis gossypii* Glover. was collected from pepper plants and grown on *Cucurbita maxima* Duchesne and *Myzus persicae* Sulz. was grown on Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis* (Lour.) Hanelt). Both species were reared at 18 to 20°C under a photocycle of 16 h of light and 8 h of darkness. Isolate Pi21 was used in the transmission assays.

Adult apterous females from each species were transferred onto infected pepper (*Capsicum annuum* L., 'Sammy RZ' F_1 hybrid) leaves and were given an acquisition access period of 2 days, after which 8 to 10 individuals were transferred to each of the test plants (Sammy RZ F_1 hybrid) and given an inoculation access period of 3 days. Then, the aphids were removed with a brush and the plants were sprayed with an insecticide and grown in a growth chamber (photocyle of 16 h of light and 8 h of darkness, 22 to 25°C) for 6 weeks before testing the newly expanded leaves for the presence of the virus with the polerovirus generic and PYD group specific RT-PCR assays. Five pepper plants were used in the experiment with *A. gossypii* and seven in the one with *M. persicae*.

RESULTS

A putative PYD group polerovirus was detected in the majority of the symptomatic pepper samples. From the 39 samples screened, 28 tested positive for the presence of poleroviruses, the majority of which (27 samples) were found to be infected with a PYD-group-like isolate, and only one (PiFD1) was infected with BWYV. Of the pepper plants infected with the PYD-group-like isolates, 25 were collected from the island of Crete where the PYD outbreak occurred and 2 were older samples collected from Turkey (Antalya). The rest of the samples originated from the other locations, although symptomatic, tested negative for poleroviruses.

The full genome sequences of two PYD group polerovirus isolates were obtained by NGS and conventional sequencing. In total, 4,524,216 reads were obtained after the NGS run of the PX3 sample, from which 2,872,964 (62.5%) corresponded to the 21- to 24-nt siRNAs. De novo assembly using the velvet algorithm produced 2,030 contigs (k-mer 17) ranging from 50 to 489 nt, with a mean of 76.3 nt.

Blast analysis of the resulting contigs returned hits with sequences in the online databases for approximately 1,400 of them. Only 37 contigs were similar to PeVYV or PYLCV sequences whereas the majority of the rest were aligned with sequences from solanaceous plants representing either plant genes or endogenous virus-like sequences. A number of the contigs were related to viruses belonging to the family *Caulimoviridae* but exhibited low similarities to the already-characterized viral species. No other known viruses were identified in the sample.

Contigs related to poleroviral sequences were selected for the subsequent extension by iterative mapping, after which the reconstruction of a 6,096-nt-long polerovirus genome was possible. In total, 22,598 siRNAs, mainly of 21 to 22 nt in length, were mapped to the assembled sequence (Supplementary Fig. S1), with a coverage ranging from 2- to 212-fold (Fig. 2C) and a mean of coverage of 80.1-fold. siRNAs from both polarities were mapped to the sequence (54% forward and 46% reverse) with no apparent dicer "hotspot" because siRNAs were distributed almost evenly throughout the whole genome (Fig. 2D).

Using primers designed according to the polerovirus sequence reconstructed from the NGS, it was also possible to obtain the complete genome of isolate Pi21. Sequence and assembly of the 15 overlapping amplicons (Fig. 2A) resulted in a 6,091-nt-long genome, with the only unconfirmed sequence being the first 23 nt of the 5′ end and the last 25 nt of the 3′ end, which correspond to the P-5′Up and P-3′Down2 primer annealing sites, respectively.

Pi21 and PX3 isolates belong to a putative new polerovirus that differs significantly from PeVYV and PYLCV. The full genomes of Pi21 (LT559484) and PX3 (LT559483) (6,091 and 6,096 nt long, respectively) have the typical genome organization of poleroviruses (Fig. 2B). They encode all seven proteins (P0 to P5 and P3a) from the respective number of ORF (0 to 5 and 3a) and have the 5′, 3′, and intergenic NCR. ORF 0 and 3 (CP) are expressed from the first AUG found in the genomic and subgenomic RNA, respectively. ORF1 and 4 (movement protein [MP]) are expressed via leaky scanning and ORF2 (RdRp) is predicted to be translated

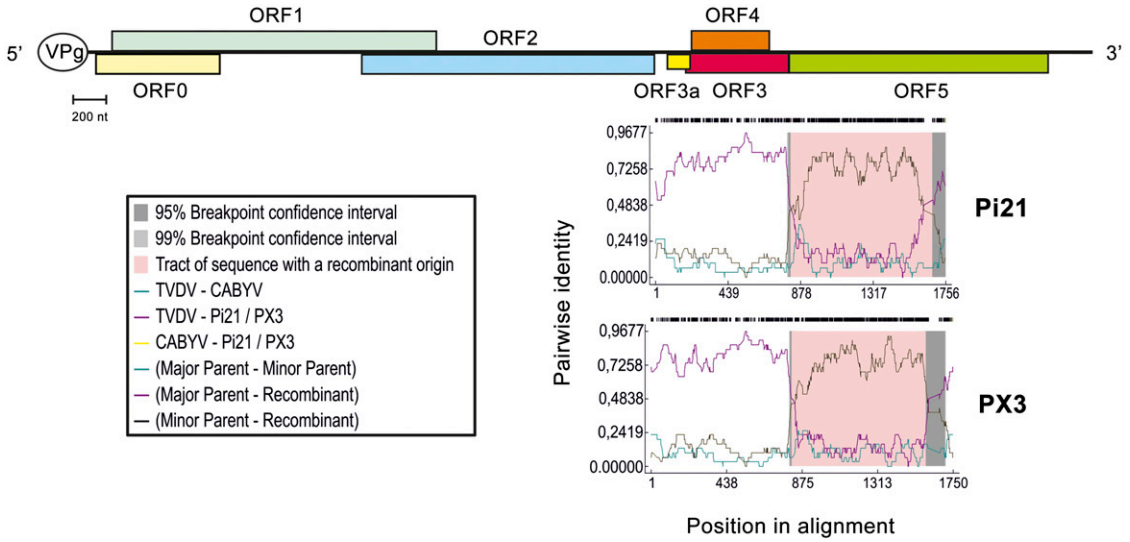

Fig. 3. Recombination events predicted by RDP in the N-terminal half of the P5 (open reading frame [ORF]5) of isolates Pi21 and PX3 plotted below the genome. Each line gives the pairwise identity for each position of the alignment. The sequence tract with a recombinant origin is marked by a light frame and the predicted breakpoints by a darker frame.

from a −1 frameshift during the translation of ORF1 at the slippery heptanucleotide GGGAAAC located in bases 1,653 to 1,659 for both isolates. ORF5 results from the translational readthrough of ORF3 and the newly characterized ORF3a is expressed by the initiation at an AUA codon located at positions 3,512 and 3,513 (Pi21 and PX3) in the formerly intergenic NCR (iNCR). Both isolates have a 50-nt-long 5′ NCR that starts with the poleroviral typical 5′-ACAAAA-3′ sequence and a 268-nt-long 3′ NCR. The distance between ORF2 and ORF3 is approximately 200 nt, which is common for poleroviruses, even though the addition of ORF3a reduced the iNCR to 81 and 80 nt for Pi21 and PX3, respectively.

Recombination analysis identified one recombination starting at the end of ORF3 (approximately 4,240 nt) and ending near the end of the conserved part of ORF5 (approximately 5,030 nt) (Brault et al. 2011), between a TVDV major parent and a CABYV minor one (Fig. 3). This recombination was present in both Greek isolates and was supported from six of seven programs used for the analysis (RDP, GENECONV, BootScan, MaxChi, Chimaera, and 3Seq) (Boni et al. 2007; Martin et al. 2005; Padidam et al. 1999; Posada and Crandall 2001; Smith 1992).

Even though the 3′ NCR among the Greek isolates, PeVYV, PYLCV, and TVDV differed substantially in length and appeared to be similar only in their 3′ end proximal region, they exhibit a very interesting sequence pattern. PeVYV, which has the longest NCR, has a unique starting sequence of 36 nt, followed by three replications of the first 69 nt of TVDV's NCR (5,816 to 5,884 nt; TVDV, EF529624), and ending with a 163-nt-long unique region. Using that as reference, it becomes apparent that PYLCV's 3′NCR lacks the starting sequence and the first replication, has only minor differences in the 163-nt region, and has a unique 15-nt tract at the end. The two Greek isolates lack the starting sequence and the first replication as well, exhibit variation and have major deletions in the second replication, but continue almost identical to PeVYV until their genomes' end (Fig. 4).

Although Pi21 and PX3 showed 95.2% nucleotide sequence identity, sequence variation was observed in all ORFs (Table 1), with the highest being in ORF0 (90.8% identical in amino acids) and the lowest in the CP/MP region (98.1% identical in amino acids). Moreover, the nucleotide sequences of the 5′NCR, 3′NCR, and iNCR were 98.0, 98.9, and 91.4% identical, respectively. The closest species in the genus *Polerovirus* were the two pepper-infecting viruses PeVYV (accession number AB594828) and PYLCV (accession number HM439608). However, the closest one, in terms of sequence identity, varied according to the ORF examined, with a specific pattern existing across the genomes of both Greek isolates. More specifically, Pi21 and PX3 exhibited higher amino acid sequence identity with PYLCV in ORF0, 1, 2, and the recombinant (5′) part of ORF5; and with PeVYV in ORF3a, 3, and 4. However, when taking into consideration a recently released sequence of PeVYV (PeVYVHN; accession number KP326573), PX3 shows higher similarity to this isolate in ORF0 and 1 than to Pi21 or PYLCV. The 3′ half of ORF5 is highly variable because it had less than 60% amino acid sequence identity with every known polerovirus. More specifically, the recombination break point was followed by a very short region with high amino acid sequence identity between all these isolates, after which the amino acid sequence varies in each one. PYLCV has a TVDV-like C-terminal part of P5 (starting at the approximately 290th amino acid of P5 [after the readthrough]) (Dombrovsky et al. 2013), whereas PeVYV and Pi21 or PX3 showed moderate identity to each other (approximately 60%) and very low to PLRV (approximately 35%) and to PYLCV or TVDV (17 to 23%). Differences in this part of the genome were present even between the two PeVYV isolates because they exhibited approximately 77% identities. Overall identity percentages are given in Table 1.

Sequence variability exists among isolates of the new virus species. Among the eight isolates which were sequenced to acquire additional information, Pi24 and PX1 were identical to Pi21 and

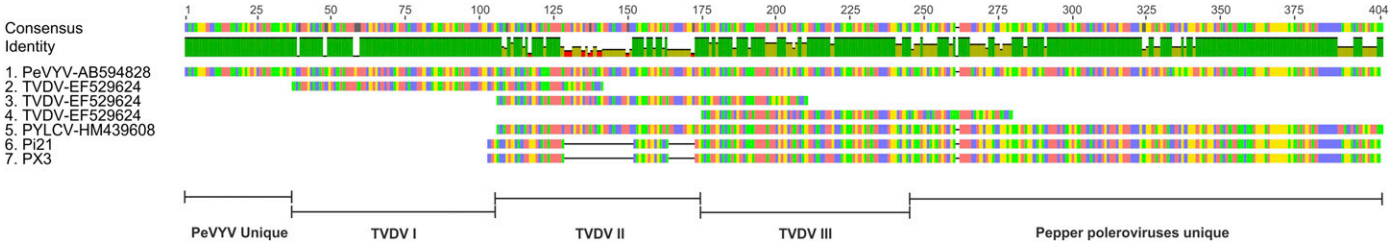

Fig. 4. Alignment of the 3′ noncoding regions (NCR) of *Pepper vein yellows virus* (PeVYV), Pepper yellows virus (PeYV), Pepper yellow leaf curl virus (PYLCV), and *Tobacco vein distorting virus* TVDV, indicating the three repetitions of the first 69 nucleotides of TVDV's 3′NCR as well as the unique regions of PeVYV and the other pepper-infecting poleroviruses.

TABLE 1. Identity percentages between the genomes of Pi21 and PX3 isolates and with those from the other species in the pepper yellows disease group[a]

	5′NCR	ORF0		ORF1		ORF2		iNCR	ORF3a		ORF3		ORF4		ORF5		3′NCR	Full genome
	nt	aa	nt	aa	nt	aa	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	nt	nt
Pi21/PX3[b]	98.0	90.8	94.0	96.2	95.4	97.8	96.3	91.4	91.1	89.1	98.1	97.7	98.1	98.9	97.3	94.3	98.9	95.2
Pi21																		
PeVYV	94.0	84.3	88.0	90.5	92.1	94.9	93.9	92.6	93.3	91.3	**97.1**	**96.6**	93.6	**97.2**	75.7	74.1	82.0	86.0
PeVYV-HN	94.0	90.0	92.9	95.4	94.7	95.4	93.6	**96.3**	**95.6**	92.8	**97.1**	96.3	**94.2**	**97.2**	**77.2**	**75.5**	80.0	87.1
PYLCV-Is	**100***	**98.4**	**98.7**	**98.0**	**97.6**	**98.3**	**97.1**	95.1	77.8	**97.5**	94.7	92.3	88.5	94.1	60.6	65.0	**82.5**	87.1
TVDV	81.0	75.9	81.7	77.5	82.9	90.2	88.9	84.1	**95.6**	88.4	87.9	89.5	81.4	90.4	26.1	40.1	51.3	70.1
PX3																		
PeVYV	92.0	85.9	90.4	91.6	93.3	94.7	95.4	92.6	93.3	93.5	**96.6**	**97.1**	94.2	**97.5**	76.1	73.8	81.0	86.9
PeVYV-HN	92.0	**94.8**	**96.4**	**97.1**	**96.4**	94.2	93.8	92.6	**95.6**	**94.9**	**96.6**	97.4	**94.9**	**97.5**	**78.3**	**78.0**	79.0	88.5
PYLCV-Is	**98.0**	90.8	94.1	96.5	95.8	**97.6**	**96.0**	**96.3**	68.9	86.7	94.7	92.3	89.1	94.3	60.0	61.5	**81.5**	85.1
TVDV	83.0	76.7	82.5	76.9	83.3	89.9	88.7	87.5	**95.6**	87.7	86.9	89.2	82.7	90.4	26.5	40.1	51.3	70.2

[a] NCR = noncoding region, ORF = open reading frame, iNCR = intergenic NCR, aa = amino acid, and nt = nucleotide. * = Highest percentages are underlined and in bold.

[b] Viruses *Pepper vein yellows virus* (PeVYV), PeVYV from China (PeVYV-HN), Pepper yellow leaf curl virus isolate IS (PYLCV-Is), and *Tobacco vein distorting virus* (TVDV).

PX3, respectively. However, sequence variation was present in these eight isolates, even between those collected during the same cultivating period. In the ORF0 gene, sequence identities ranged from 93.6 to 99.7% in nucleotides and 90.4 to 99.6% in amino acids. The generic PCR amplicon sequences (559 bp, without the primer annealing sites) exhibited 93.7 to 100% and 97.3 to 100% identities in nucleotides and amino acids, respectively. The variable region of ORF5 and the 3′NCR were similar to Pi21 and PX3, in all isolates sequenced, with nucleotide identities ranging from 96.5 to 100%. The RdRp-CP region of isolate PY from Turkey exhibited over 94%

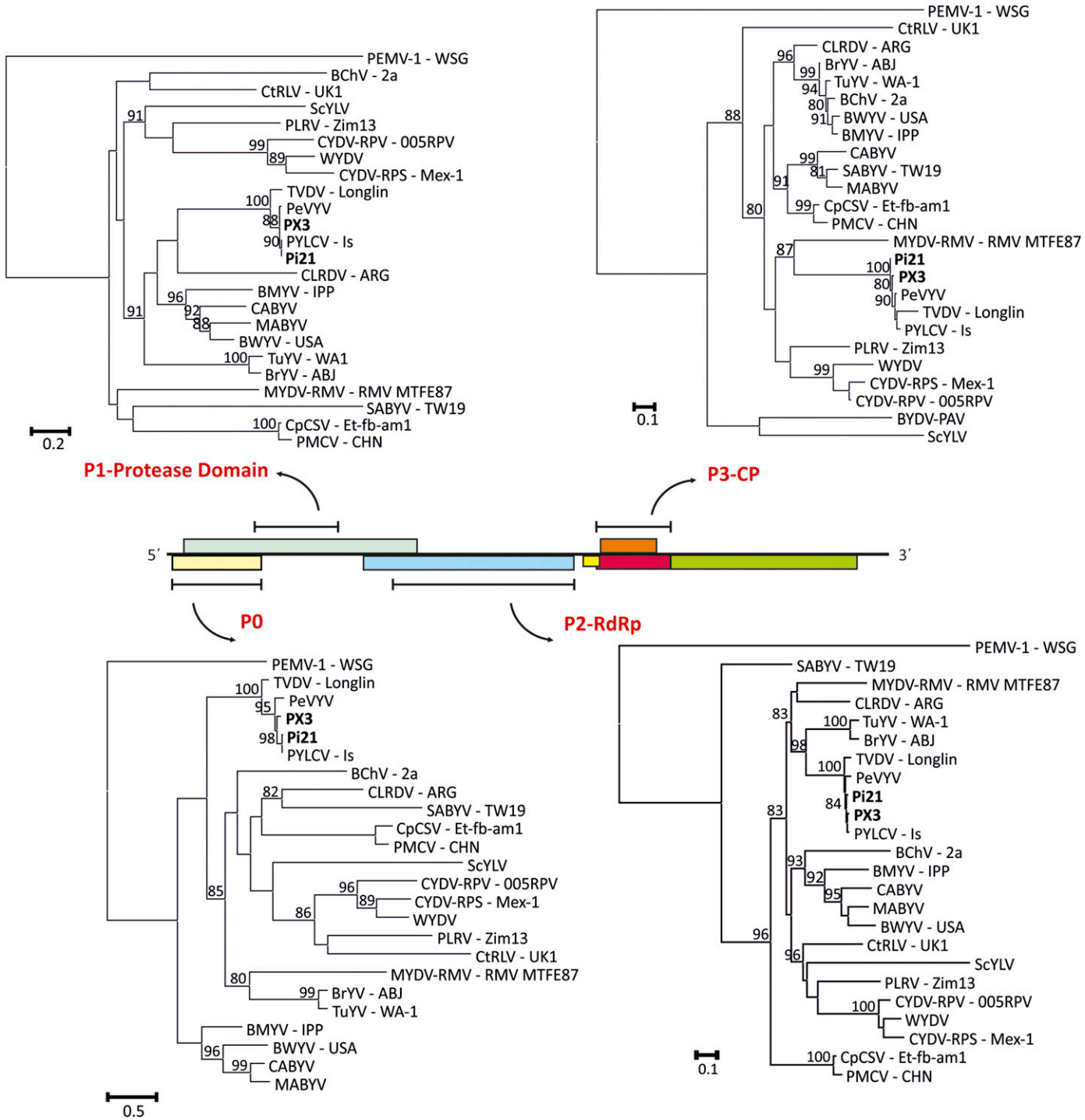

Fig. 5. Maximum-likelihood phylogenetic trees constructed using the amino acid sequence of the region indicated by each respective bar above or below the genome. Shimodaira-Hasegawa approximate likelihood ratio test scores >80 are given above each node and the bars on the left of each tree indicate substitutions per site. Abbreviations and accession numbers of the isolates used are BChV (*Beet chlorosis virus*, AF352024), BMYV (*Beet mild yellowing virus*, DQ132996), BrYV (Brassica yellows virus, KF015269), BWYV (*Beet western yellows virus*, AF473561), CABYV (*Cucurbit aphid-borne yellows virus*, JQ700305), CLRDV (*Cotton leafroll dwarf virus*, NC_014545), CpCSV (*Chickpea chlorotic stunt virus*, AY956384), CtRLV (*Carrot red leaf virus*, AY695933), CYDV-RPS (*Cereal yellow dwarf virus-RPS*, AF235168), CYDV-RPV (*Cereal yellow dwarf virus-RPV*, EF521827), MABYV (*Melon aphid-borne yellows virus*, JQ700307), MYDV-RMV (*Maize yellow dwarf virus-RMV*, NC_021484), PEMV-1 (*Pea enation mosaic virus 1*, NC_003629), PeVYV (*Pepper vein yellows virus*, AB594828), PLRV (*Potato leafroll virus*, AF453390), PMCV (Pea mild chlorosis virus, JF507725), PYLCV (Pepper yellow leaf curl virus, HM439608), SABYV (*Suakwa aphid-borne yellows virus*, JQ700308), ScYLV (*Sugarcane yellow leaf virus*, JF925154), TuYV (*Turnip yellows virus*, X13063), TVDV (*Tobacco vein distorting virus*, EF529624), and WYDV (Wheat yellow dwarf virus, FM865413).

identities in nucleotides and 98% in amino acids in the RdRp, P3a, CP, and MP protein sequences with isolates Pi21 and PX3. (Supplementary Tables S3, S4, S5, and S6).

The evolutionary relationships among the pepper-infecting poleroviruses cannot be accurately resolved. To evaluate the relationships of isolates Pi21 and PX3 within the genus *Polerovirus*, four ML phylogenetic trees from different genomic regions were constructed. The trees inferred were based on the amino acid sequences of the complete P0 (model VT+I+G+F), the protease domain of P1 (LG+G), a part of P2 (starting from the end of the ORF1/2 overlap until the end of P2) (LG+I+G+F) and the complete P3 sequence (JTT+G+F) (Fig. 5). With the exception of the CP tree, isolates Pi21 and PX3 were grouped together with PYLCV and PeVYV in a clade that evolved from a TVDV-like ancestor, with minor differences in this cluster's topology. However, the phylogenetic relationships of this cluster with the other species in the genus *Polerovirus* were variable, depending on the ORF examined.

To better resolve the evolutionary relationships between the Greek isolates and the other pepper-infecting poleroviruses, three additional phylogenetic trees were constructed (Fig. 6). The first one was inferred from the ORF0 nucleotide sequences (TPM2+G), the region exhibiting the highest diversity among poleroviruses; the second was inferred from the generic detection amplicon's nucleotide sequence (TrNef+G); and the last from the nucleotide sequence of the ORF2-ORF3 (RdRp-CP) region (TIM2ef+I+G) commonly used for the classification of PYD group's isolates. The ORF0 tree displayed a well-resolved phylogeny with high support, possibly representing an accurate reconstruction, indicating that multiple diversification events within the PYD clade are responsible for the variability detected in pepper poleroviruses at this point in time. However, the tree inferred from the generic detection amplicon exhibits a trichotomy, grouping similar isolates together but probably without an accurate reconstruction (data not shown), whereas the RdRp-CP tree didn't discriminate the three viral species.

The new polerovirus is aphid transmissible by *A. gossypii* and *M. persicae*. *A. gossypii* transmitted the new polerovirus to 5 of 5 pepper plants (100%) whereas *M. persicae* only to 2 of 7 plants (28.5%). The infected plants exhibited the typical PYD symptoms, similar to those appearing on the field, approximately 5 weeks post inoculation.

DISCUSSION

A new polerovirus associated with PYD symptoms was identified in Greece. Analysis of full genome sequences obtained from two isolates (Pi21 and PX3) showed that they had the typical genomic organization of poleroviruses. Moreover, the phylogenetic trees constructed from the predicted amino acid sequences of ORF0, 1, 2, and 3 placed both Pi21 and PX3 in an internal clade together with PeVYV and PYLCV, suggesting a close relationship between these pepper-infecting poleroviruses. However, sequence comparison revealed pronounced differences between the Greek isolates and the already characterized pepper poleroviruses (Table 1). PeVYV is the most distant one from Pi21 and PX3, even though isolate PeVYV-HN is closer to PX3 in ORF0 and 1. PYLCV is almost identical (in amino acids) to Pi21 in the 5′ part of the genome but differs significantly after the iNCR. Finally, the sequence of ORF5 after the

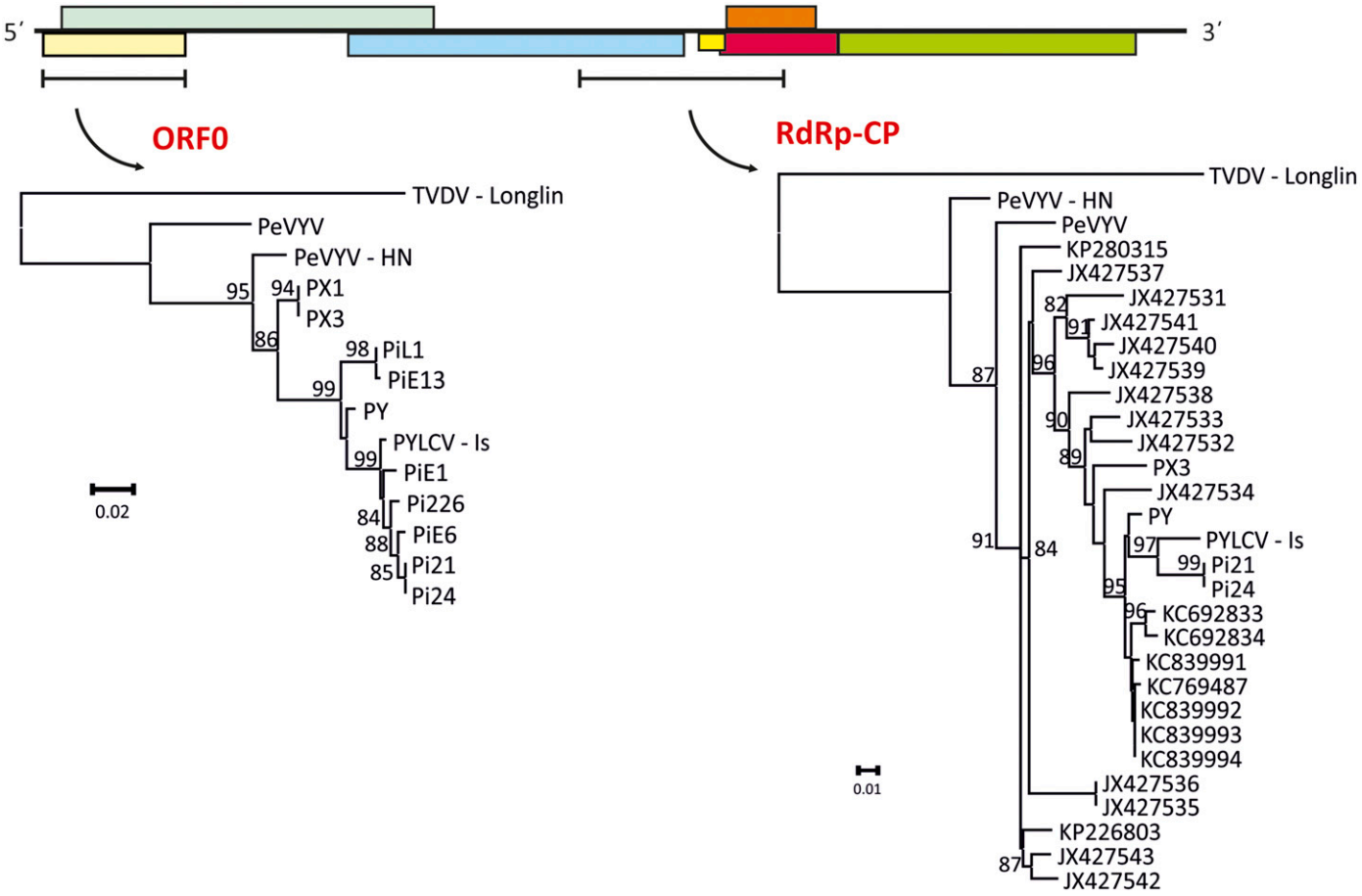

Fig. 6. Maximum-likelihood phylogenetic trees constructed using the nucleotide sequence of the region indicated by each respective bar below the genome. Shimodaira-Hasegawa approximate likelihood ratio test scores >80 are given above each node and the bars on the left of each tree indicate substitutions per site. Abbreviations and accession numbers of the isolates used are PeVYV (*Pepper vein yellows virus*, AB594828), PeVYV-HN (KP326573), and PYLCV (Pepper yellow leaf curl virus, HM439608). Accession numbers of each PeVYV-like isolate used for the construction of the tree are indicated on each respective clade.

CABYV-like recombination and the 5′ end of the 3′NCR are clearly the most diverse regions between PeVYV, PYLCV, and Pi21 or PX3, with each virus exhibiting unique features. Even though some genetic variability exists between Pi21 and PX3 (Table 1), they should be considered as diversified isolates of the same viral species, given their overall similarity, the almost identical genome lengths, the proximity of the collection sites, and the similarity of the symptoms they induce in the field. This new species differs from PeVYV and PYLCV for the following reasons: (i) the differences in amino acid percentages are above the 10% limit used as a demarcation criterion (Domier 2011) for ORF0 and 5 with PeVYV and ORF4 and 5 with PYLCV, (ii) neither Pi21 nor PX3 exhibit a global (genome-wise) similarity with either of the two viruses, and (iii) they both have a highly diversified P5 C-terminal end. The substantial differences in P5 cannot be considered insignificant because this protein plays a crucial role in the biology of poleroviruses, being related to symptom expression, virus accumulation, and movement (Boissinot et al. 2014; Bruyère et al. 1997; Rodriguez-Medina et al. 2015). A close association of the putatively new virus presence with the disease observed in the field was confirmed using the PYD group assay for all but one of the polerovirus-infected samples. Therefore, it is proposed that the causal agent of the PYD in Greece should be considered a new species in the genus *Polerovirus*, with the provisional name Pepper yellows virus (PeYV).

The "genus-wide" phylogenetic trees constructed using the amino acid sequences (Fig. 5) were not able to clearly elucidate the relationships between PeVYV, PYLCV, and PeYV because the topology differed in each tree. ORF5 was not included in this analysis because the recombination in the 5′ half renders the complete sequence unsuitable for phylogenetic reconstructions. However, when more isolates of the PYD group were included in the construction of the nucleotide sequence tree of ORF0 (Fig. 6), a more coherent phylogeny was inferred, producing a tree that has a "comb-like" topology, which indicates that this ORF could be under continuous selective pressure. Recent studies have shown that closely related viruses or their isolates have P0 RNA-silencing suppressors of variable efficiencies, up to the extent of being unable to suppress the RNA silencing mechanism (Almasi et al. 2015; Kozlowska-Makulska et al. 2010). Therefore, the ORF0 tree's topology could depict the ongoing selection for a protein exhibiting better RNA silencing suppression capacity after a recent host jump. Episodic selection has been proposed for viral suppressors of RNA silencing after a change in host range or during the adaptation to local host genotypes (Murray et al. 2013), which is consistent with this hypothesis. The topology could also be linked to the geographical distribution of the isolates used, given that PeVYV originated from Japan, PeVYV-HN from China, and the rest of the isolates from the Mediterranean basin. However, more data are needed in order to verify these hypotheses.

The generic amplicon's (559 bp) phylogenetic tree (data not shown), although supported by high SH-aLRT scores, cannot be used to accurately represent the phylogenetic history, probably because of the high similarity of the RdRp region sequences in combination with the relatively small length of the amplicon. The RdRp-CP phylogenetic tree (Fig. 6), which was constructed using the sequences from previously characterized isolates originating from three continents, doesn't have a clear isolate grouping. In this tree, PeVYV sequences (from the two fully sequenced isolates) appear to have diverged early, after the speciation of the common ancestor, whereas PYLCV and PeYV are intermixed with the rest of the PeVYV partially sequenced isolates. Possible recombination events, which are common in this region (Domier et al. 2002; Lim et al. 2015; Smith et al. 2000), in addition to the highly conserved RdRp and CP coding sequences could hinder the reconstruction of an accurate phylogeny. However, considering the fact that these isolates were characterized as PeVYV using only a small portion of the genome, it is quite possible that the incoherent phylogeny could, in fact, be the result of these isolates being erroneously characterized as PeVYV.

The inconsistencies of the observed phylogenies and the sequence variability present between the viral isolates of the PYD group can be attributed to a modular type of evolution with multiple recombination events among isolates that constantly produce mosaic-like variants. A modular type of evolution can explain (i) the unrelated sequences found in the C-terminal part of P5, which are unlikely to be the product of an evolution based on point mutations and selection only; (ii) the recombination with CABYV; and (iii) the sudden change in similarities of PYLCV and Pi21 after the iNCR. According to the pepper-infecting poleroviruses, at least four different modules must be acknowledged: the first is related to replication spans from the 5′NCR until the iNCR; the second includes ORF3a, 3, and 4 and is related to the structure and movement of the virus; and the last two represent the 5′ and 3′ parts of ORF5, which mainly control aphid transmission and movement, respectively. Recombination events in the region that encodes the CP and MP are well defined in the literature between either polero- or luteovirus parental sequences in different viral species (Domier et al. 2002; Lim et al. 2015; Smith et al. 2000), and break points in the rest of the genome that coincide with some of the putative modules also have been reported (Pagán and Holmes 2010).

Even though phylogenetic reconstruction could not produce a consensus evolutionary scenario, it clearly demonstrates that PeVYV, PYLCV, and PeYV descend from the same common TVDV-like ancestor. This hypothesis is also supported by their highly similar RdRp sequence, their common recombination in the CP-ORF5 region, and the almost identical 148-nt-long tract of the 3′NCR, which does not resemble any other known virus. Moreover, with a spatial distribution from Eastern Asia to Europe, it is rather improbable for all these traits to have been acquired independently or to have convergently evolved. Hence, we must consider that these viruses belong to the same pepper-infecting poleroviral lineage in which PeVYV diverged early from the group's common ancestor. Isolate PX3 has an "intermediate" genome which, although similar to the two PeVYV isolates (especially with the Chinese isolate PeVYV-HN), belongs to a different viral species. The genomes of isolates Pi21 and PYLCV have nearly identical 5′ ends, and if it weren't for the differences observed after the iNCR, they could be considered as isolates of the same viral species. The 3′ part of PYLCV's genome could be the product of either a single recombination event after the iNCR with a yet-uncharacterized virus of the same lineage still harboring the parental TVDV-like C-terminal part of P5 or two recombination events, the first in the CP/MP region and the second in the C-terminal part of P5.

The recombination present in the 5′ end of ORF5 of Pi21 and PX3 was identified previously for PeVYV and PYLCV (Dombrovsky et al. 2013). It is probable that this recombination gave the members of the PYD group the ability to be transmitted by both *A. gossypii* and *M. persicae*, like the parental CABYV, in contrast to TVDV, which is transmitted only by the latter, hence broadening their potential host range. Moreover, the unique sequences of the 3′ end of ORF5 imply the existence of two more unknown poleroviruses from which they were acquired through recombination. The repetitions observed in the 3′NCR could be indicative of multiple recombination events whereas the retention of at least one repetition of TVDV's 3′NCR and the pepper poleroviruses' unique region at the end of the genome could imply the existence of determinants for the capability to infect pepper, as was shown for *Melon necrotic spot virus*-N isolate in resistant melon (Miras et al. 2014).

The only possible way to positively distinguish between PeVYV, PYLCV, and PeYV is with the unique sequence of each species in the variable part of ORF5. Therefore, the characterization of isolates as PeVYV by using sequence data only from the RdRp-CP region, even though they should be considered as "PeVYV-like" isolates, has led to an overestimation of its spread. For this reason, even though isolate PY from Turkey is almost identical to Pi21, the inability to amplify the ORF5 3′NCR part (data not shown) implies the existence of variability in this region, thus resulting in its

characterization as PeYV-like. Although the members of this group cause similar symptoms and are transmitted by the same aphid species, only the study of their biological characteristics such as host range can finally prove whether they are, in fact, separate taxonomic units or should all be characterized as PeVYV strains.

In conclusion, it has been well established that, like the yellow dwarf disease of cereals and the yellowing diseases in beet and cucurbits, PYD is also caused by a group of closely related but distinct polerovirus species Even though PYD can also be attributed to the presence of BWYV, major outbreaks of this virus in pepper crops have not been reported in Greece or elsewhere and the symptoms induced by BWYV has not been reported to include leafroll and vein yellowing (Buzkan et al. 2013; Timmerman et al. 1985) (personal observations). In Greece, the appearance of the disease in three subsequent years as well as the identification of the new polerovirus in pepper crops in Chania, the furthermost prefecture of Crete from Lasithi, indicates the emergence of this virus that is becoming endemic on the island as an important threat to pepper crop cultivation. Moreover, the small number of symptomatic samples from Heraklion, Kastoria, and Rhodes that were found negative for poleroviruses indicate that other viruses, pathogens, or abiotic factors might exist that can induce symptoms similar to poleroviruses. However, the NGS did not reveal any other known virus to be present in the PX3 sample, except for some caulimo-like contigs that most probably represent endogenous viruses.

Many questions still remain unanswered about the PYD group, including the exact evolutionary process that gave rise to all these closely related viral species. Apart from the significance of the disease induced by these viruses on the cultivation of pepper, we believe that, in the future, this virus group will become an excellent case study of poleroviruses speciation and modular evolution.

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