Characterization by Deep Sequencing of Divergent *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) Isolates and Development of a Broad-Spectrum PBNSPaV Detection Assay

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ABSTRACT

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Plum bark necrosis stem pitting-associated virus (PBNSPaV), the causal agent of plum bark necrosis stem pitting disease, belongs to the genus *Ampelovirus* in the family *Closteroviridae*. The complete genome sequence of PBNSPaV isolates from four *Prunus* sources was determined by pyrosequencing. All isolates showed the same genomic organization as the PBNSPaV reference isolate. The least divergent isolate, found in a peach tree from China, showed an overall 91.8% of nucleotide identity

with the type isolate. Two closely related isolates, defining a second cluster of diversity, were found in two Japanese plum lines from France and showed only 82.8% identity with the type isolate. On the other hand, they were highly homologous with two recently described PBNSPaV divergent isolates from China. The fourth and most divergent isolate, from a Chinese peach, showed only 71.2% identity to other PBNSPaV isolates and was not detected by currently available PBNSPaV reverse-transcription polymerase chain reaction detection assays. Complete sequencing of the divergent isolates allowed the development of a more broad-spectrum detection test targeting a conserved region in the P61 gene. Taken together, these results indicate a much broader diversity of PBNSPaV than previously thought and provide for a more robust detection of this still poorly characterized pathogen.

Plum bark necrosis stem pitting-associated virus (PBNSPaV) belongs to the genus *Ampelovirus* in the family *Closteroviridae*. The associated disease, plum bark necrosis stem pitting disease (PBNSPD), was first reported in 1986 on a 'Black Beaut' Japanese plum (*Prunus salicina* Lindl.) in the United States (25), and the putative causal agent was identified as PBNSPaV in 2001 and 2002 (1,18). The complete PBNSPaV genome was sequenced in 2007 (2) and showed an organization comparable with that of other members of the genus *Ampelovirus*. The virus was further reported in Europe (1,11,16,17), North Africa (5,9), the Middle East (21), and Asia (7,24). The PBNSPaV natural host range includes cultivated (almond, apricot, cherry, peach, and plum) and ornamental *Prunus* spp. (1,3,11,15,24,25). The typical symptoms of PBNSPD seem to vary greatly depending on the susceptibility of the hosts but generally consist of bark gummosis, necrosis, and stem pitting (4). Symptoms on leaves are not systematically observed. Moreover, latent infections have been reported in some hosts or in specific cultivars of otherwise susceptible hosts (4,11). The virus is not mechanically transmitted and has no known vector, although natural virus spread has been reported (8). Its transfer from plant to plant is thought to occur through vegetative propagation techniques. As with other *Ampelovirus* spp., PBNSPaV is not known to be seed transmitted.

Our knowledge of the molecular diversity of PBNSPaV is still limited, with few, closely related sequences available in databases and only one complete genome sequence (1,2,11,18,20). In all these studies, the results revealed a high level of homology between the studied isolates, suggesting a recent origin or transfer to *Prunus* of the virus populations studied or the involvement of a factor or factors constraining the molecular evolution of the virus. More recently, an investigation of the incidence of PBNSPaV on stone fruit trees in China provided a first indication that the diversity of PBNSPaV could be wider than suggested by previous sequencing efforts (7). The partial heat shock protein 70 homolog (HSP70h) gene sequences from 18 isolates were obtained and shown to share between 94 and 100% amino acid identity with the corresponding region of already known PBNSPaV isolates.

Preliminary analysis of *Prunus* samples from various surveys, using a range of polyvalent, virus-genus-specific polymerase chain reaction (PCR) assays (14), has indicated the presence of divergent PBNSPaV isolates (17), suggesting that the diversity of PBNSPaV could be broader than expected. In the present study, the molecular diversity of PBNSPaV was investigated by determining by pyrosequencing the complete genome sequence of four divergent isolates. The results obtained confirm a much broader diversity of PBNSPaV than previously known and allowed the development of a new, more broad-spectrum reverse-transcription (RT)-PCR assay, which extends the range of detectable PBNSPaV isolates.

MATERIALS AND METHODS

Plant material and virus isolates. Peach (*P. persica*) 'Ta Tao 25' (Q-375-02) originally from China was provided by Dr. A. Hadidi (United States Department of Agriculture, Beltsville, MD). The peach 'Nanjing' source as well as the *P. mume* sources

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S3 and S4 were collected during surveys in China in 2007 and 2009. Two Japanese plum (*P. salicina*) lines, 'Pair' and 'PR258', the latter one affected by the peach red marbling disease (12), and two domestic plum (*P. domestica*) lines, '387-07-4' and '369-04-7', were from a germplasm collection in France (CTIFL Lanxade). Various *Prunus* sources (*P. mume*, *P. domestica*, *P. salicina*, *P. persica*, *P. armeniaca*, *P. sibirica*, *P. cerasus*, and *P. avium*) were obtained during several surveys in Azerbaidjan, China, Iran, and Czech Republic in 2005, 2007, 2008, 2009, and 2013. All *Prunus* spp. used in the present study are listed in Table 1. Except for *P. mume* S3 and S4, all the isolates were propagated by grafting on GF305 peach seedlings under level-three containment greenhouse conditions.

Total nucleic acids extraction and PBNSPaV detection by RT-PCR. Total nucleic acid (TNA) extraction was performed according to the procedure described by Foissac et al. (10). PBNSPaV was detected by two-step RT-PCR assays. TNA samples (5 µl) were first submitted to a reverse transcription initiated by pdN₆ primers and using the Superscript II Reverse Transcriptase in a 20-µl volume reaction, following the manufacturer's recommendations (Invitrogen/Fisher Scientific, Illkirch, France). In a second step, the cDNAs (5 µl) were amplified in independent reactions using four different primer pairs (Table 2): two primer pairs targeting the HSP70h gene (PBNSPa-3f/PBNSPa-2r [18] and PBNSPaVdet-F/PBNSPaVdet-R [2]), a primer pair targeting the capsid protein (CP) gene (PBCP-F and PBCP-R primers) (2), and the degenerate primer pair designed in the present study and targeting the P61 gene (PBN-F1 and PBN-R1). The first three assays involved amplifications performed in a 50-µl volume containing 10 mM Tris-HCl (pH8.8), 1.5 mM MgCl₂, 50 mM KCl, 250 µM dNTPs, primers at 1 µM each, and 1 U of DyNAzyme II DNA polymerase (Finnzymes/Fisher Scientific) and 45 cycles of 95°C for 30 s, the prescribed annealing temperature (Table 2) for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 10 min. For the assay using the PBN-F1/PBN-R1 primer pair, all conditions were similar, except that the MgCl₂ concentration was increased to 4 mM, the annealing temperature set at 42°C, and the number of cycles reduced to 40. Amplification products were analyzed by nondenaturing agarose gel electrophoresis and ethidium bromide staining. When needed, PCR products were either directly sequenced or, in the case of mixed infections, identified by mixed sequencing signals, cloned into the pGEM-T Easy vector (Promega, Charbonnières-Les Bains, France) prior to sequencing (GATC Biotech, Mulhouse, France).

Determination of complete genome sequences by pyrosequencing. Double-stranded RNAs (dsRNAs) extracted from leaves of GF305 grafted with the various PBNSPaV sources (Ta Tao 25, PR258, Pair, and Nanjing) were analyzed by 454 pyrosequencing, using the strategy described by Candresse et al. (6). Following assembly, PBNSPaV contigs were identified by identity to the PBNSPaV reference genome (PL186 isolate,

GenBank accession number EF546442). Based on the mapping of contigs on this reference genome, primer pairs were designed to amplify cDNA fragments spanning the remaining small gaps in the assembled genomes. The sequences of the primers as well as the annealing conditions used are detailed in Table 2. The PCR products thus obtained were then submitted to Sanger sequencing (GATC Biotech) and used to assemble and validate the complete genome sequences. The 5' genomic regions for Pair-2 and PR258-2 isolates were determined using the 5' random amplification of cDNA ends (RACE) strategy and a specific reverse primer designed from the pyrosequencing data (Table 2). Amplifications were performed following the kit supplier's recommendations (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). The amplified fragments were then sequenced (GATC Biotech).

Sequence analysis, comparisons, and phylogenetic analyses. All analyses and manipulations of 454 pyrosequencing data were performed as described by Candresse et al. (6) using the CLC Genomics Workbench 4.8 (<http://www.clcbio.com>). Multiple alignments of nucleotide or amino acid sequences were performed using the ClustalW program (23) implemented in Mega 5.1 (22). Phylogenetic trees were reconstructed using the neighbor-joining technique with strict nucleotide or amino acid distances and randomized bootstrapping for the evaluation of branching validity. Mean diversities and genetic distances (p-distances calculated on nucleotide or amino acid identity) were calculated using Mega 5.1.

RESULTS

Pyrosequencing of dsRNAs purified from PBNSPaV sources. dsRNAs were extracted from fresh leaves of GF305 peach plants that had been graft-inoculated with the Ta Tao 25, Pair, PR258, or Nanjing *Prunus* sources and submitted to 454 pyrosequencing. The sequences were treated as described in a previous work (6). In the PR258 source, 38 contigs were annotated as belonging to two distinct PBNSPaV isolates and represented 52.5% of the reads. The corresponding isolates were called PBNSPaV PR258-1 and PBNSPaV PR258-2, respectively (Table 1). BLAST analysis of the contigs obtained from the Pair sample allowed the identification of three viruses: a *Prunus necrotic ringspot virus* isolate, a new virus belonging to the family *Betaflexiviridae* (under characterization), and two variants of PBNSPaV, representing 1.3, 10.8, and 60.9% of the reads, respectively. As above, the PBNSPaV isolates were called PBNSPaV Pair-1 and PBNSPaV Pair-2 (Table 1). Multiple infections were also found in the Ta Tao 25 and Nanjing sources. Four viruses were identified in Ta Tao 25 source: *Apple chlorotic leaf spot virus* (0.6% of the reads), *Cherry green ring mottle virus* (3.4%), *Asian prunus virus 1* (62.8%), and one isolate of PBNSPaV (20.1%). Finally, BLAST analysis of the contigs reconstructed for the Nanjing source showed sequence similarities to *Asian prunus virus 3* (6.7% of the reads), *Plum pox virus* (52%), and one isolate of PBNSPaV (34.3%).

TABLE 1. List of *Plum bark necrosis stem pitting-associated virus* sources used in the present study, together with their original host, country of origin, and the relevant sequence accession numbers

Source	Host	Country	Accession numbers
PR258 ^a	<i>Prunus salicina</i>	France	PR258-1; KC590330, KC590341, PR258-2; KC590346 (complete sequence)
Pair ^b	<i>P. salicina</i>	France	Pair-1; KC590331, KC590339, Pair-2; KC590345 (complete sequence)
S3 ^c	<i>P. mume</i>	China	S3-1; KC590335, KC590342, S3-2; KC590343
S4	<i>P. mume</i>	China	KC590333, KC590340
Iran1	<i>P. persica</i>	Iran	KC590334, KC590336
387-07-4	<i>P. domestica</i>	France	KC590332, KC590338
369-04-7	<i>P. domestica</i>	France	KC590337
Ta Tao 25	<i>P. persica</i>	China	KC590344 (complete sequence)
Nanjing	<i>P. persica</i>	China	KC590347 (complete sequence)

^a Tree infected by both isolates PR258-1 and PR258-2.

^b Tree infected by both isolates Pair-1 and Pair-2.

^c Tree infected by both isolates S3-1 and S3-2.

For each source, the contigs identified as belonging to a PBNSPaV isolate were then further manually assembled into scaffolds. For the Nanjing and Ta Tao 25 sources, 6 and 15 scaffolds were finally reconstructed spanning the full PBNSPaV genome, with 5 and 14 internal gaps, respectively. The two assembled sequences started at the first 5' nucleotide and extended to the last 3' nucleotide, as compared with the PBNSPaV reference genome (2). In the case of Pair and PR258 sources, which contained a mixed infection of two PBNSPaV isolates, only the genomes of the most divergent isolates were reconstructed. For the PBNSPaV Pair-2 isolate, three scaffolds were assembled from positions 43 to 13,759 of the reference genome, with only two internal gaps. The PR258-2 isolate genome was reconstructed using nine scaffolds spanning the reference genome from positions 351 to 13,285, with eight internal gaps.

The four divergent PBNSPaV sequences were completed and polished by direct sequencing of RT-PCR fragments generated from TNA extracts of the respective sources, using primer pairs spanning the remaining gaps in the assembled genomes or targeting regions for which sequence confirmation was needed (Table 2). The 5' part of Pair-2 and PR258-2 genomes was obtained using a RACE strategy while the 3' part was obtained by RT-PCR between an internal primer and a specific reverse primer designed from 3' end sequences available for the reference (EF546442), Ta Tao 25, and Nanjing isolates (Table 2). Essentially, no differences were observed between the initially assembled scaffolds and the resequenced regions.

Genomic organization of the PBNSPaV isolates, phylogenetic relationships, and sequence comparisons. The genome sizes for the four isolates sequenced here are very close to that of

TABLE 2. Primers used in the present study, together with their annealing temperature in polymerase chain reaction assays (T_m) and the target region on the *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) genome using the numbering of the reference isolate (PL186, GenBank accession number EF546442)

Primer name	Primer sequence (5'–3') ^a	T _m (°C)	Target ^b
Detection			
PBNSPa-3f ^c	CTTCGGCACTACTTTCAGCAGT	48	HSP70h (9,078–9,618)
PBNSPa-2r ^c	AGTCGCACCACCACTTCT	...	...
PBNSPaVdet-F ^d	TACCGAAGAGGGTTTGGATG	56	HSP70h (9,219–9,618)
PBNSPaVdet-R ^d	AGTCGCACCACCACTTCT	...	...
PBCP-F ^e	TCACTCTGGTTCACGTGGCA	56	CP (12,153–12,578)
PBCP-R ^e	CCACCTGTCGGACAGCTTCTT	...	...
PBN-F1	AAYACWAATACYGTYAGGTATGT	42	P61 (11,801–12,119)
PBN-R1	CARYTCHGCMARYAAMACCTCTC	...	...
Sequencing			
PR258-F1	AGGCCTATCAGGAGTACCGCTA	53	ORF1a (720–1,419)
PR258-R1	CGACGTCCTTATCATAACAT	...	...
PR258-F2	CAAACAGCAGTGGAGAGGG	53	ORF1a (2,660–3,645)
PR258-R2	TCGTGAAGGACGCAGCTGATC	...	...
PR258-F3	AATCTGACAGAGGGCTGATC	53	P61 (10,684–10,935)
PR258-R3	AATTCCGTGCGATACCGTAA	...	...
PR258-F4	GGCTTTGTACTGAGGGTGAG	53	P61 (11,162–11,592)
PR258-R4	CCGGTAACCTAGAAATAGTC	...	...
PR258-F6	GTTCAGTGAGGTGGAGTATG	52	P61 (11,843–12,137)
PR258-R6	TGTCCTAAACCTGTTGAGCA	...	...
PR258-F7	GATTCCCCATTTGTTGAAGA	50	ORF1a (5,223–5,677)
PR258-R7	ACAACATTACAGCCTGAAC	...	...
Pair-F1	CTTACAAATTCGTGGCCGGG	55	ORF1a-ORF1b (7,039–7,704)
Pair-R1	CACCAAGGATGGTTCGTAATGAG	...	...
Pair-F2	GATCGAAGTCTACGACATCCA	50	ORF1a (5,566–5,824)
Pair-R2	GTAGACTCTTCATCATCATCA	...	...
Race-PBvar ^f	GTGGTACCGCGAGTCTTATCCACGGCA	72	5'NCR-ORF1a (1–559)
PBfin-F1 ^g	TTGGTCGTGGATTCTCGTGTG	55	CP-3'NCR (13,147–14,219)
PBfin-R1 ^g	TCCTTGATTTTTCCCAAGGGTAG	...	...
Nanj-F1	CTTCACTTCGACATGAAGGGAA	55	ORF1a (2334–2674)
Nanj-R1	CTCCACTGTTGCTTAAACTCC	...	...
Nanj-F2	GTGTCAGATTTCGACTCTTGG	55	ORF1a (4,536–4,899)
Nanj-R2	AACTGACAGCTGCACGTGC	...	...
Nanj-F3	CGGTTGTGAGCGAGGGCACT	55	ORF1a (5,344–5,956)
Nanj-R3	CAGGAGAGTCCAGAACAGCG	...	...
Nanj-F4	CGCGAATCCTTGACTGTAGC	55	ORF1b-HSP70 (8,390–9,197)
Nanj-R4	TCCTTTCGCAAACCAAGATG	...	...
TT25-F1	TTTCTCTTACGGAATACGCA	55	5'NCR-ORF1a (4–537)
TT25-R1	ACGGCATTCTACCACTAGA	...	...
TT25-F2	CTCTTCCTCCCCTTCTTCCA	55	ORF1a (871–1,769)
TT25-R2	TGAGAGTGCAAGCAACATCCCT	...	...
TT25-F3	CCAGAGCAGCTCTCATTCAGA	50	ORF1a (3,936–4,143)
TT25-R3	CTCAGAAGCCGCCATAGACGT	...	...
TT25-F4	GAAGTTACTGGCTGTACAGAA	54	ORF1a-ORF1b (6,364–7,680)
TT25-R4	AACGATTGGTCTCAACACTTC	...	...

^a Y = C or T; W = A or T; R = A or G; M = A or C; H = A, C or T.

^b Target gene and the positions on the PBNSPaV genome are indicated. HSP70h = heat shock protein 70 homolog, CP = capsid protein, ORF = open reading frame, and NCR = noncoding region.

^c From Marini et al. 2002 (18).

^d From Al Rwahnih et al. 2007 (2).

^e From Matic et al. 2010 (20).

^f This reverse primer was used in conjunction with the universal primer provided by the 5' random amplification of cDNA ends kit (Takara Bio Europe/Clontech). It was used for both PBNSPaV isolates Pair-2 and PR258-2.

^g Amplification with these primers was performed for both PBNSPaV isolates Pair-2 and PR258-2.

the PL186 reference isolate (14,214 nucleotides [nt]). There are 14,211 nt for Ta Tao 25, 14,219 nt for the Pair-2 and PR258-2 isolates, and 14,234 nt for Nanjing. The gene order and the overall genomic organization are conserved between the four sequences and those of PL186 (Fig. 1). Seven open reading frames (ORFs) are encoded from 5' to 3', encoding the replication related proteins (ORF1a and ORF1b), the P6 protein of unknown function (ORF2), the HSP70 homolog protein (ORF3), the P61 protein of unknown function (ORF4), the CP (ORF5), and the minor CP (CPm) (ORF6). Two noncoding regions (NCRs) were identified: a 5'NCR of 301 nt (Ta Tao 25) or 302 nt (Pair-2, PR258-2, and Nanjing, with an extra G at the 5' end) and a 3'NCR of 234 nt for all isolates but Nanjing, which genome is characterized by a 13-nt insertion immediately after the CPm ORF stop codon. An intergenic region (59 to 60 nt long, depending on the isolates) is located between the P61 and CP ORFs, accumulating a few indel mutations between isolates. Among the seven ORFs, five are conserved in size: P6 (171 nt, 57 amino acids [aa]), HSP70h (1,587 nt, 529 aa), P61 (1,638 nt, 546 aa), CP (975 nt, 325 aa), and CPm (654 nt, 218 aa). The two remaining ORFs (ORF1a and ORF1b) were found to contain one extra amino acid in the Pair-2, PR258-2, and Nanjing isolates compared with 2,344 and 524 aa in PL186 and Ta Tao 25, respectively.

The neighbor-joining phylogenetic tree shown in Figure 2 was reconstructed using strict amino acid sequence identity distances for the HSP70h proteins of representative members of the family *Closteroviridae* and shows clearly that the four new isolates clustered together with the PL186 PBNSPaV reference isolate with a 100% bootstrap support within the genus *Ampelovirus* cluster. The same clustering was observed whatever the ORF analyzed, confirming the clear relationships between the new isolates and the PBNSPaV reference isolate (data not shown).

Compared with the PL186 isolate, the genome of each isolate showed an overall nucleotide identity of between 91.8% (Ta Tao 25) and 71.2% (Nanjing) (Table 3). The higher similarity observed between Ta Tao 25 and PL186 confirmed the close relationship observed in the HSP70h phylogenetic tree (Fig. 2). Depending on the ORF considered, the amino acid identity between the PL186 and Ta Tao 25 protein was 91.5 to 98.1%, with the highest divergence in ORF1a (Table 3). In contrast, the same comparison performed between the Nanjing isolate and PL186 showed amino acid identities of between 70.6% (ORF1a) and 93% (P6, Table 3) but overall, and with the exception of ORF1a, all computed protein divergence values fall within the 25% sequence-based species demarcation criterion accepted for the genus *Ampelovirus* (19). The situation for the closely related Pair-2 and PR258-2 isolates (overall nucleotide identity of 99.2%) (Table 3) seems to be intermediate, because the amino acid identities with PL186 were between 84.6% (ORF1a) and 100% (P6, Table 3).

Evaluation of available PBNSPaV detection assays and development of a polyvalent PBNSPaV-specific RT-PCR assay. Considering the large PBNSPaV molecular diversity uncovered above, the ability of available molecular detection assays to detect PBNSPaV divergent isolates was evaluated. The four sequenced PBNSPaV isolates were tested with three published PBNSPaV-specific RT-PCR assays, which target either the HSP70h gene or the CP gene (2,18,20). None of these assays was able to detect all

four isolates (Table 4). In particular, and as expected from in silico analyses showing mutations in the various primer binding sites, the Nanjing isolate was not detected with any assay. Surprisingly, the Ta Tao 25 isolate, although closest to the reference isolate, also was not detected by two of the assays evaluated (Table 4).

Following their cloning, the three PCR fragments obtained with PBNSPaV-3f/2r primer pair were sequenced. In the case of the Ta Tao 25 isolate, a single sequence was obtained with 100% nucleotide identity with the genomic sequence determined by pyrosequencing. As expected, for the Pair and PR258 sources, two types of sequences were found, one close to the reference isolate sequence and the other matching the divergent genomic sequence determined by pyrosequencing, thus confirming the mixed infection status of these two PBNSPaV sources.

The comparison of the full-length genomic sequences determined here and of the reference isolate allowed the identification of conserved motifs in the P61 protein gene, from which a pair of degenerate primers was designed (PBN-F1/PBN-R1) (Table 2). The broad specificity of this primer pair was evaluated using the Ta Tao 25, Pair, PR258, and Nanjing sources as controls. A fragment with the expected size was amplified from all four samples (Fig. 3, lanes 6 to 9), whereas no amplification was obtained using a virus-free GF305 extract (Fig. 3, lane 10). The specificity of the amplification was verified by sequencing of the obtained products. A single sequence was obtained from the Nanjing and

Fig. 2. Unrooted phylogenetic tree constructed with the heat shock protein 70 homolog (HSP70h) protein sequences of representative members of family *Closteroviridae*. Trees were constructed by the neighbor-joining method and the statistical significance of branches was evaluated by bootstrap analysis (1,000 replicates). Only bootstrap values >70% are indicated. The scale bar represents 10% strict amino acid divergence. The PR258-2, Pair-2, Ta Tao 25, and Nanjing isolates are indicated by a star (*). Genera to which the particular viruses belong are indicated on the right. Abbreviations followed by the relevant accession numbers are PBNSPaV = Plum bark necrosis stem pitting-associated virus, LChV-2 = Little cherry virus 2, GLRaV-1 = Grapevine leafroll-associated virus 1, GLRaV-3 = Grapevine leafroll-associated virus 3, CTV = Citrus tristeza virus, GLRaV-2 = Grapevine leafroll-associated virus 2, BYV = Beet yellows virus, GLRaV-7 = Grapevine leafroll-associated virus 7, LChV-1 = Little cherry virus 1, CYSDV = Cucurbit yellow stunting disorder virus, TICV = Tomato infectious chlorosis virus, LIYC = Lettuce infectious yellows virus.

Fig. 1. Schematic representation of the genome of *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) isolate PL186. ORF = open reading frame, ORF1b = RNA-dependent RNA polymerase, P6 = protein of unknown function, HSP70h = heat shock protein 70 homolog, P61 = protein of unknown function, CP = capsid protein, CPm = minor capsid protein.

Ta Tao 25 samples, identical to that determined by pyrosequencing; whereas, for the Pair and PR258 samples, two distinct sequences were determined corresponding to the two PBNSPaV isolates identified by pyrosequencing, demonstrating that the two isolates in co-infection in those samples were detected by the new assay.

Validation of the new PBNSPaV detection assay and analysis of PBNSPaV diversity. In order to validate the new detection assay and to estimate the prevalence of PBNSPaV in a range of *Prunus* spp. collected during various surveys, 130 trees (symptomatic or not) were tested with primers PBNF1/PBN-R1, including *P. mume*, *P. domestica*, *P. salicina*, *P. persica*, *P. armeniaca*, *P. sibirica*, *P. cerasus*, and *P. avium* samples. Five samples (lanes 1 to 5) among the 130 tested were found to be infected by PBNSPaV, giving an overall prevalence of 3.9% (Fig. 3). The infected trees were two plum (*P. domestica*) trees from France, a peach (*P. persicae*) tree from Iran, and two *P. mume* trees from China. These positive samples were also submitted to the three published detection assays and some of them were either not detected or gave only very weak signals (Table 4), confirming the limited detection spectrum of these assays.

The nucleotide sequences of the PBNSPa-3f/2r (HSP70h) and PBN-F1/R1 (P61) fragments were determined and used for phylogenetic analyses, together with corresponding sequences available in GenBank (Fig. 4). As illustrated by the unrooted neighbor-joining tree constructed using the partial HSP70h nucleotide sequences (Fig. 4A), the diversity of PBNSPaV is structured into three distinct clusters supported by 100% bootstrap values. Besides the major group of isolates (group 1) which contains the original PBNSPaV type isolate (EF546442), two additional groups can be defined. Group 2 comprises four isolates: the PR258-2 and Pair-2 isolates and two isolates from China recently reported by Cui et al. (7). Finally, group 3 is composed of only one isolate, Nanjing, which seems to be the most divergent PBNSPaV isolate known to date.

A phylogenetic analysis was also performed using the partial P61 nucleotide sequences generated in the present study (Fig. 4B). Except for the complete sequence of the PBNSPaV type isolate, no corresponding sequence is available in GenBank. Based on these sequences, the PBNSPaV diversity could be divided again into the same three clusters supported by high bootstrap

values (97 to 100%) but with a higher intragroup diversity for group 2 typified by the PR258-2 and Pair-2 isolates, due to the identification of a divergent isolate in the *P. mume* sample from China (isolate S3-2) for which no equivalent could be identified using the PBNSPa-3f/2r (HSP70h) assay.

DISCUSSION

The four complete PBNSPaV genome sequences determined in the present study provide evidence for a significantly wider diversity than previously known (3). The Ta Tao 25 isolate is the least divergent one when compared with the reference PL186 (8.2% of overall nucleotide divergence). Two isolates found in two *P. salicina* sources from France (Pair-2 and PR258-2 isolates) are very closely related to each other (98.2% overall nucleotide identity) but significantly more distant from the reference isolate (83.2 and 83.4% overall nucleotide identity, respectively). Based on the limited sequence information available of other isolates available, these two isolates seem to be close to some of the Chinese isolates for which only a small fragment of the HSP70h gene has been sequenced (7). Therefore, the Pair-2 and PR258-2 sequences are likely to represent the first complete genomic sequences available for this group of divergent PBNSPaV isolates.

Fig. 3. Detection of *Plum bark necrosis stem pitting-associated virus* isolates by polyvalent reverse-transcription polymerase chain reaction assay based on the degenerate primers PBN-F1/PBN-R1. Samples analyzed were lane 1, Iran1; lane 2, 369-04-7; lane 3, 387-07-4; lane 4, S3; lane 5, S4; lane 6, Nanjing; lane 7, Ta Tao 25; lane 8, Pair; lane 9, PR258; lane 10, negative control; lane L, 100-bp ladder (Invitrogen). A precise description of the *Prunus* sources used is presented in Table 1.

TABLE 3. Percentages of divergence observed between the various genomic regions of the four *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) isolates identified in the Ta Tao 25, PR258, Pair, and Nanjing sources, and the reference PBNSPaV isolate (PL186, GenBank accession number EF546442)^a

Isolate pair ^b	5'NC		ORF1a		ORF1b		P6		HSP70h		P61		CP		CPm		3'NC	Overall
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	nt
Pair-PR258	0.7	0.9	0.9	0.3	0	1.2	1.8	0.3	0.2	0.9	0.5	1.2	1.5	0.8	0.5	0.9	0.8	0.8
PL186-TT25	8.2	9.6	8.5	7.6	3.1	2.9	1.9	6.3	3.4	6.7	4.6	7.5	4	5	5.5	0.9	8.2	8.2
PL186-Nanj	28.8	32.7	29.4	25.5	16.2	26.3	7	24.8	12.9	28.3	16.3	23.8	8.9	21.7	22.9	5.1	28.8	28.8
PL186-PR258 ^c	21.4	19.1	15.4	14.9	5.9	11.1	0	14.8	5.7	15.8	8.8	14.2	6.2	9	10.6	1.3	16.6	16.6
TT25-Nanj	28.6	32.5	28.7	26.3	17	24.6	5.3	24.6	12.7	27.7	15.9	23.7	7.7	20.6	22	5.1	28.6	28.6
TT25-PR258 ^c	16.9	19.4	14.9	15	7.3	10.5	1.8	14.8	6.2	15.4	8.6	14.6	6.8	9	8.7	1.3	16.9	16.9
Nanj-PR258 ^c	28.8	32.9	29.1	26.2	17	25.1	7	23.9	12.7	28	17.9	23.8	10.8	21.7	22.9	5.6	28.8	28.8

^a Abbreviations: nt = nucleotides, aa = amino acids, 5'NC = 5' noncoding region, ORF = open reading frame, HSP70h = heat shock protein 70 homolog, CP = capsid protein, CPm = minor CP, and 3'NC = 3' noncoding region.

^b Pair = Pair-2 sequence; PR258 = PR258-2 sequence; TT25 = Ta Tao 25 sequence; Nanj = Nanjing sequence; PL186 = reference isolate, EF546442.

^c Same or very similar values were obtained for the comparison with Pair-2 sequence.

TABLE 4. Detection of *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) in various *Prunus* sources using primer pairs PBNSPa-3f/2r (18), PBNSPaV-detF/R (2), PBCP-F/R (20), and PBN-F1/R1 (this work)^a

Primer pair	Iran1	369-04-7	387-07-4	S3	S4	Nanjing	Ta Tao 25	Pair	PR258
PBNSPa-3f/2r	+/-	-	+	+	+	-	+	+	+
PBNSPaV-detF/R	+	-	+/-	+	+	-	-	+	+
PBCP-F/R	+/-	+/-	+/-	+	+	-	-	+	+
PBN-F1/R1	+/-	+/-	+	+	+	+	+	+	+

^a Symbol: +/- indicates faint band.

The Nanjing isolate was recovered from a peach tree from China (Nanjing province) and showed the highest divergence (28.8%) from the reference isolate but also from the PR258-2, Pair-2, and Ta Tao 25 isolates (similar level of overall nucleotide divergence) (Table 3). In the genus *Ampelovirus*, an amino acid divergence of more than 25% in the polymerase, CP, or HSP70h is considered as one of the species demarcation criteria (19). Except for the ORF1a of the Nanjing isolate, which showed an amino acid divergence of 29.4% compared with the reference isolate, the levels of divergence observed in all ORFs of the sequences reported here fall within the species demarcation criteria (Table 3). Therefore, the PR258-2 and Pair-2 isolates as well as the Ta Tao 25 isolate should clearly be considered as belonging to the PBNSPaV species. Considering that the level of divergence between the Nanjing isolate and PBNSPaV was found in all ORFs (with the exception of ORF1a) to be above the 25% species demarcation criterion and considering that there is no further evidence that the Nanjing isolate may belong to a distinct species, we propose that it, too, should be considered as a member of the PBNSPaV species.

Due to the level of divergence of the Nanjing sequence compared with the reference isolate sequence, it is not so surprising that it was not detected by the three RT-PCR assays evaluated in this work. It seems that the S3-2 isolate identified in a *P. mume* sample from China was not detected, either, by the three assays,

because only one sequence corresponding to the S3-1 isolate was retrieved from the HSP70h amplification, even though two sequences corresponding to S3-1 and S3-2 isolates were retrieved from the P61 amplification (Fig. 4). However, we cannot totally exclude the possibility that the S3-2 isolate was positively amplified by the HSP70h RT-PCR assay but that no corresponding clone was sequenced. The 369-04-7 isolate was detected only by the test of Matic et al. (20) and the new broad-spectrum test. Although the intensity of the band obtained was low in both cases, the new RT-PCR assay seems to be more sensitive, because only 40 cycles were applied instead of 45 in Matic's test. In the literature, four other PBNSPaV detection tests have been published (1,7,11). Their performances were not evaluated in the present work; however, in silico, considering the numbers of mismatches between the primer sequences and the Nanjing sequence (from one to nine mismatches depending on the primer considered), it seems very improbable that the Nanjing isolate could be detectable by one of those assays. In contrast, the new broad-spectrum primers reported here allowed the detection of representative members of all PBNSPaV clusters known to date and, therefore, seem to represent a significant improvement over the existing PBNSPaV detection techniques in terms of broad specificity and sensitivity.

In this work, only five *Prunus* samples were found to be infected by PBNSPaV, in addition to the four isolates fully se-

Fig. 4. Unrooted phylogenetic trees for a range of *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) sources calculated from **A**, a partial fragment (499 nucleotides [nt]) of the heat shock protein 70 homolog (HSP70h) gene generated using the PBNSPa-3f and PBNSPa-2r primers or **B**, a partial fragment (265 nt) of the P61 gene generated using PBN-F1 and PBN-R1 primers. Isolates for which the corresponding sequences are available from GenBank were included in the analysis. Accession numbers and the hosts are indicated. Black triangle (▲): the Nanjing isolate was not detected by the reverse-transcription polymerase chain reaction targeting the HSP70h gene; therefore, the sequence was retrieved from its genomic sequence. Trees were constructed using the neighbor-joining method and the statistical significance of branches was evaluated by bootstrap analysis (1,000 replicates). Only bootstrap values >70% are indicated. The scale bar represents 2% nucleotide divergence. Identified phylogenetic groups are indicated on the right side of each figure.

quenced, representing 3.9% of incidence, which is in agreement with the idea that this virus has a relatively low incidence (3). Although Koch's postulates have not been fulfilled, PBNSPaV infection is tentatively associated with necrosis and pitting symptoms in susceptible trees. However, their severity or even existence seems to depend largely on the host plant cultivar (3,11). From our work, it is still difficult to associate PBNSPaV infection with specific symptoms. Indeed, all the samples found to be infected by PBNSPaV displayed various symptoms but were simultaneously infected by others viruses, such as *Plum pox virus*, *Prunus necrotic ringspot virus*, or *Apple chlorotic leaf spot virus*, or viroid (*Peach latent mosaic viroid*, data not shown), so that our results show no correlation between the symptoms displayed and PBNSPaV infection.

The PR258 sample is affected by the peach red marbling disease (PRMaD), one of the disorders reported on stone fruit trees for which the etiology remains unclear (12,13). This sample was found to be infected by two isolates of PBNSPaV and by a new member of *Betaflexiviridae* (still under characterization). Ten further *Prunus* PRMaD sources were assayed for the presence of PBNSPaV but the results obtained failed to confirm an association between PRMaD and PBNSPaV infection; therefore, further efforts are needed to understand the etiology of the PRMaD and, more broadly, to understand the contribution of PBNSPaV to syndromes affecting stone fruit trees.

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