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Whole genome characterization of *Potato virus Y* isolates collected in the western USA and their comparison to isolates from Europe and Canada

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Summary. Potato virus Y (PVY) is a serious potato pathogen that affects potato seed and commercial production crops. In recent decades, novel PVY strains have been described that cause necrotic symptoms on tobacco foliage and/or potato tubers. The major PVY strains that affect potato include PVY^{O} and PVY^{N}, which have distinct serotypes that can be differentiated by immunoassay. Other economically important strain variants are derived from recombination events, including variants that cause tuber necrotic symptoms (PVY^{NTN}) and PVY^{O} serotypes that cause tobacco veinal necrosis (PVY^{N}-W, $PVY^{N:O}$). Although the PVY^{NTN} and PVY^{N}-W variants were first reported in Europe, apparently similar strains have been appearing in North America. Confirmation of the existence of these recombinant strains in North America is important, as is whether they spread from a common source or were derived by independent recombination. Whole genome sequencing can be used to positively identify strain variants and begin to address the issue of origins. Symptomology, serology, RT-PCR, and partial sequencing of the coat protein region were used to identify isolates of the PVY^{NTN}, PVY^{N}, $PVY^{NA\text{-}N}$, and $PVY^{N:O}$ for whole-genome sequencing. Sequencing confirmed the presence of PVY^{NTN} and PVY^{N} isolates that were >99% identical to European sequences deposited in GenBank in the 1990's. Sequences of the $PVY^{NA\text{-}N}$ and $PVY^{N:O}$ types were 99.0% and 99.5% identical to known sequences, respectively. There was no indication that recombinant strains PVY^{NTN} or $PVY^{N:O}$ had different parental origins than recombinant strains previously sequenced. This is the first confirmation by whole-genome sequencing that "European"-type strain variants of PVY^{N} and PVY^{NTN} are present in

North America, and the first reported full-length sequence of a tuber necrotic isolate of $PVY^{N:O}$.

Introduction

Potato virus Y (PVY) is one of the most common viral pathogens found in potato, and is the type member of the genus *Potyvirus* (family *Potyviridae*). PVY is distributed globally and causes loss in the form of reduced yield or quality [22]. Distinct strains can be differentiated through the use of bioassay, serology, or nucleic acid-based techniques. The predominant strain throughout the world has been the common strain (PVY^{O}), with reports of the necrotic strain (PVY^{N}) more common in Europe, Africa, New Zealand, and South America; PVY^{C} has been reported in Europe, Australia/New Zealand, the Americas, and South Africa [7, 13]. Isolates of PVY^{O} induce systemic mottle or mosaic symptoms in tobacco and moderate to distinct systemic mottle or mosaic in most potato cultivars [7, 13]. Visual symptoms caused by these strains are the primary means of detection to ensure clean potato seed [17]. Suspected infection by PVY^{O} is often confirmed by the use of immunoassay.

The tobacco veinal necrosis strain of the virus (PVY^{N}) causes systemic veinal necrosis and stem lesions in tobacco, yet its potato foliar symptoms range from not visible to relatively mild mosaic in potato cultivars [13]. This strain had not been observed in the potato growing regions of North America until recently [11, 31]. An additional strain of PVY with PVY^{N}-type serological characteristics but distinct symptomology and pathology is termed PVY^{NTN} [27, 28]. The PVY^{NTN} isolates induce external and internal rings, arcs, or discolorations on tubers of some potato cultivars [27, 28], termed potato tuber necrotic ringspot disease (PTNRD). These PVY^{NTN} variants are apparently derived from recombination between PVY^{O} and PVY^{N} [19, 20, 40]. An additional tuber necrotic type, termed NA-PVY^{NTN}, was reported to have originated from mutation and not recombination [36]. The authors of that paper defined strain types according to continent of detection, and differentiated European strains of PVY^{N} or PVY^{NTN} from North American necrotic strains [35, 36], hereafter referred to as $PVY^{NA\text{-}N/NTN}$.

Another set of PVY strain types has been isolated from potato in Europe and also represents recombination between PVY^{O} and PVY^{N}. These new strains are designated $PVY^{N}W$ (or PVY^{N}-Wi) [4, 9, 10]. The $PVY^{N}W$ isolates produce systemic necrosis in tobacco and relatively mild foliar symptoms in potato similar to the PVY^{N} pathotype, yet these isolates are of PVY^{O} serotype. Similar isolates with nearly identical genetic structure were termed $PVY^{N:O}$ and have now been isolated from potato in Canada and the north-central US [36, 39]. $PVY^{N:O}$ isolates have recently been reported in the western USA; some $PVY^{N:O}$ isolates also cause an atypical PTNRD [12, 39].

The PVY^{NTN} strain variant of the Hun (M95491, 50) type contains three recombinant junctions, whereas the $PVY^{N}W$ or $PVY^{N:O}$ strains contain only one recombinant junction, allowing differentiation of these strains by reverse transcription-polymerase chain reaction (RT-PCR) assays [19, 20, 36]. Other

apparent recombinant isolates of PVY have been detected in Europe [6, 40]. Indeed, genetic recombination may be quite common in the potyviruses in general and in PVY in particular [40].

An incursion of PVY^N was reported in seed potatoes in eastern Canada in the early 1990's, and eradication was attempted [31]. However, several recent reports of necrotic PVY isolates in potato in North American indicate a widespread introduction of these isolates into the potato seed producing areas [11, 12, 32, 33, 36, 39]. These new isolates present many challenges to North American seed potato growers, since certification of seed potatoes in the USA is based on limiting the incidence of PVY in advanced seed generations, not on eradication of the pathogen. Since the symptomology of the necrotic strain variants often varies from that of PVY^O, and detection in the seed crop is often based on visual symptoms during field inspections, control of these isolates presents additional challenges.

The methodology of PVY detection for certification of potato seed has continued to evolve [17]. The production of PVY-specific polyclonal antibodies and the adoption of ELISA facilitated PVY detection and tracking, since immunoassays are sensitive, fast, and more amenable to high-throughput utilization than bioassays. Monoclonal antibodies (MAbs) are available in strain-neutral and strain-specific versions [25], including PVY^N-specific [15, 42] and PVY^O-specific [15, 16] MAbs. However, the appearance of PVY^N-W/$PVY^{N:O}$ necrotic strain variants with PVY^O serotypes eliminated positive confirmation by ELISA of PVY^O, causing significant problems for certification agencies responsible to recognize and regulate viruses in seed potatoes.

Characterization of newly emerged recombinant strain variants has included analysis of nucleic acid sequence data [20, 24, 38, 48, 50], enabling development of new detection assays, often based on RT-PCR techniques. These RT-PCR assays have become common for detection and characterization of virus isolates by researchers and diagnostic laboratories. Several RT-PCR assays have been developed to differentiate between PVY strain variants [11, 12, 35, 36, 43]. Some RT-PCR assays target the coat protein (CP) region of the genome [e.g. 2, 3, 5, 6, 8, 14, 46, 53, 54]. Such assays can corroborate immunological data, and more sequence data are available for this region of the genome. However, neither the CP epitope nor its coding sequence are suitable for unambiguous determination of strain phenotype because PVY^N and PVY^{NTN} strain variants cannot be readily differentiated from this information [5, 9, 35, 53], and PVY^N-W/$PVY^{N:O}$ isolates have emerged that cause tobacco necrosis and PTNRD phenotypes but have PVY^O serotypes [12, 20, 38, 39]. Another region useful for PVY strain delineation is the P1 cistron, which has the most genetic variability [41, 45, 49] and is immediately adjacent to the HCP cistron, reported to contain the key determinant for the necrotic response of tobacco [52].

In order to better understand the attributes associated with these new strain variants, and to gain insight as to the origin of each variant, it would be useful to acquire complete nucleotide sequences for PVY strain variants recently found in North America. This information will allow comparisons with complete sequences

of other European and North American PVY isolates and would help develop robust assays to unambiguously identify each major biological strain. Utilization of infectious clones for identifying specific pathogenicity determinants would also be aided by complete sequences that could direct reverse genetics and targeted mutagenesis approaches.

The objectives of this study were to characterize and confirm by whole-genome sequencing the presence of European and North American variants of necrotic PVY strains found in seed potatoes in the western USA to determine whether they represent novel types or share common origins with strain variants reported elsewhere. We report here the sequencing and characterization of four such isolates. These isolates were first characterized via serology, PCR assays, and sequencing of PCR amplicons of the CP region to represent the following classes: PVY^{N} (similar to N605, X97895), PVY^{NTN} (similar to Hun NTN, M95491), $PVY^{NA\text{-}N}$ (similar to N-Jg, AY166867), and $PVY^{N:O}$ recombinant isolate (similar to Mb112, AY745491).

Materials and methods

Virus isolates

The PVY isolates used in this study were derived from infected potatoes grown in the Pacific Northwest, some of which have been described in earlier reports [11, 12]. Isolate Mont (= RR1) was isolated from potato cv. Ranger Russet in early 2001 and was classified as a PVY^{N} strain based on infectivity bioassay, immunoassay results, and molecular characteristics. Isolate RRA-1 was also isolated from Ranger Russet in 2001, and has characteristics most closely associated with $PVY^{NA\text{-}N/NTN}$ isolates. Isolate 423-3 was from leaf tissue of a potato cultivar (Alturas) with symptomatic (necrotic) tubers recovered in a 2002 field trial and was classified as a 'European' NTN isolate ($PVY^{Eu\text{-}NTN}$). Isolate Alt (= AL1) was from symptomatic (necrotic) tubers of cv. Alturas and characterized in early 2002 as a $PVY^{N:O}$ recombinant. General characteristics of each isolate are summarized in Table 1.

Table 1. Characterization of isolates reported in this study by biological, serological, and molecular (PCR) assays

Virus isolate	Serological group	Tobacco bioassay	Chenopodium bioassay	Tuber symptoms[1]	Molecular grouping		
					P1	CP	3′-UTR
O (control)	O	Mosaic	L.L.[2]	no	O	O	O
Mont	N	V.N.[3]	N.S.[4]	no	N	N	N
RRA-1	N	V.N.	N.S.	no	NA-N	N	N
423-3	N	V.N.	N.S.	yes	Eu-NTN	N	O
Alt	O	V.N.	N.S.	yes[5]	Eu-NTN	O	O

[1]Virus isolated from plants with characteristic tuber symptoms
[2]L.L. = chlorotic local lesions appearing 12 days post inoculation
[3]V.N. = veinal necrosis
[4]N.S. = No symptoms
[5]Symptoms observed only on original source tubers of cv. Alturas

Characterization of isolates

Biological characterization: Viruses were isolated from living tobacco or potato leaf tissue after initial serological characterization as previously described [12]. Infected symptomatic tubers were stored until they sprouted to collect sufficient leaf tissue for inoculations. Initial inoculations were made by grinding leaf tissue in a 1:10 dilution (w/v) of 0.05 M sodium phosphate buffer containing 0.02 M sodium sulfite onto newly expanded leaves of tobacco *cvs*. Samsun NN and Burley 21, and *Chenopodium quinoa*. Isolates from tissues without detectable traces of other viruses were chosen for further study. Inoculations for biological characterization were made from symptomatic tobacco leaves onto tobacco *cvs*. Samsun NN and Burley 21; potato *cvs*. Russet Burbank, Desiree, Gem Russet, and Ranger Russet; and *C. quinoa*. Each virus isolate was assayed on two test plants of every cultivar and species. Data were recorded at 8, 10, and 35 days after inoculation for foliar symptoms. Tubers were harvested after approximately 90 d and observed for tuber symptoms immediately and after an additional four weeks storage in a dark vernalization chamber at 6–8 °C.

Serological and molecular characterization

The serological identity of each virus isolate was confirmed by ELISA using a panel of monoclonal antibodies (4C3, 1F5, and 295-5) that can identify and differentiate PVY strains as previously described [12]. Molecular characteristics of these isolates were determined by a combination of RT-PCR-restriction enzyme analysis of the P1 cistron and nucleic acid sequence comparisons of the CP cistron [12]. The P1 molecular assay is also capable of detecting mixed infections of PVY strains.

Virus purification

Although full-length RT-PCR products can be obtained from crude viral RNA isolated from PVY- infected plant tissues, we most consistently amplified strong, distinct RT-PCR products from RNA derived from purified virions (unpublished results). All PVY isolates sequenced in this study were purified using previously described methods [1]. Approximately 200 g of infected, symptomatic tobacco leaf tissue from *N. tabacum* cv. 'Samsun NN' or 'Burley 21' were triturated in 2 volumes (per fresh tissue weight) of 0.5 M sodium borate buffer, pH 8.0, amended with 0.15% sodium thioglycollate and 50 ml of chloroform, and centrifuged for 10 min at 10,000 g at 4 °C. The supernatant was filtered through a large Kimwipe and Triton X-100 was added to 0.5% (vol/vol) while the extract was stirring, a separation funnel being used to remove residual chloroform. Polyethylene glycol (M.W. 8000) was added to 4% and NaCl was added to a 0.2 M final concentration while stirring. The extract was allowed to stir at 4 °C for 2.5 h and then was centrifuged for 15 min at 10,000 g. The supernatant was discarded and the pellets re-suspended in approximately one-quarter of the original volume in 0.5 M sodium borate buffer, pH 8.0, overnight at 4 °C.

Triton X-100 was added to 0.5% (vol/vol) to the re-suspended virus extract the next day, while stirring. The extract was centrifuged for 10 min at 10,000 g and the supernatant retained. The supernatant was centrifuged at 80,000 g for 1.5 h, the supernatant discarded, and the pellets re-suspended in 10 ml of 0.05 M sodium borate buffer, pH 8.0. Sucrose density gradient centrifugation was performed by layering the virus solution on 10–40% sucrose gradients, prepared in 0.05 M sodium borate buffer, pH 8.0, and centrifuged at 100,000 g in a swinging bucket rotor for 2.5 h at 6 °C. After gradient centrifugation, virion-containing bands were visualized by shining a dissecting microscope light through the centrifuge tube. The virion-containing band was removed using a glass Pasteur pipette, and diluted by addition of at least two volumes of 0.05 M sodium borate buffer, pH 8.0, and pelleted by high speed centrifugation as described above. The final pellet was resuspended in 1 ml of 0.05 M sodium

borate buffer and the UV absorbance of the product analyzed using a Beckman DU-50 spectrophotometer.

Generation of RT-PCR products

Reverse transcription of viral RNA to DNA and amplification of full-length cDNA products was accomplished using the Takara RNA LA PCR Kit, Ver. 1.1 (Takara Mirus Bio, Madison, WI) as per manufacturer's instructions. First-strand synthesis utilized an oligo-dT primer, since RT using PVY-specific reverse primers gave substantially more background smearing (unpublished data). The thermocycler profile for generating full-length PCR products consisted of denaturing at 94 °C for 2 min, 14 cycles of 92 °C for 10 s, 68 °C for 10 min, and 16 cycles of 92 °C for 10 s and 68 °C for 10 min + 30 s/cycle, followed by a final extension for 15 min at 70 °C. If the initial cDNA template was insufficient to achieve complete sequence coverage, shorter products were generated by RT-PCR using appropriate primers and primer-specific cycling conditions to fill sequence gaps.

Sequencing and sequence analysis

Sequencing primers were designed to cover approximately 500 bp intervals for each strand, based on the full-length sequence for PVY isolate N605 (X97895). Primers were designed using the program, Primer Designer 2.0 (Scientific & Educational Software), or FastPCR [25]. Additional primers were designed as needed, based on existing sequence for the respective isolate, to obtain complete sequence of both strands. All sequencing was done using PCR products as template. Sequencing reactions were performed using an MJ PTC100 or PTC200 (MJ Products, Watertown, MA) with BigDye 3.0 or 3.1 according to the manufacturer's instructions for "1/8" reactions (Applied Biosystems, Foster City, CA). Sequencing products were purified as per manufacturer's instructions using ethanol precipitation prior to resuspension in formamide and injection in an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). Assessment of sequence quality and assembly of contig alignments were performed using the programs Phred/Phrap/Consed/Polyphrap [21], with a minimum Phred score of 25 for each strand. Aligned sequences were visually inspected, especially with regard to Polyphrap-flagged polymorphic base calls that could indicate mixed infections.

Initial alignment and subsequent production of bootstrapped phylogenetic trees were performed by the program ClustalX [51]. Bootstrap analysis was conducted using a neighbor-joining analysis with 1000 replications. Output was edited to collapse nodes with less than 50% bootstrap support to polytomies. Phylogenetic trees were drawn using the program TreeView. Recombination analysis was conducted with the RDP suite of recombination programs [29, 30]. The initial analysis utilized the embedded programs RDP, BootScan, MaxChi, and SiScan; identified recombinant events were re-tested with those programs, Chimaera, and GeneConv for confirmation. Recombination plots were generated from output data from the program SiScan [18] with set values: window = 200, step = 40, and the nearest outlier as the fourth sequence.

Results

Isolate characterization

Viruses isolated from either tobacco or potato tissue produced mosaic or veinal necrosis symptoms on tobacco between 8 and 12 days after inoculation. None of the potato isolates produced local lesions after inoculation on *C. quinoa*, indicating that none of the samples tested were infected with PVX, PVM, PVS,

or other common viruses. Evidence of mixed infection by multiple PVY strains was not detected by strain-specific MAbs or the P1 molecular assay. The results of preliminary characterization of the selected isolates are summarized in Table 1. The only isolate with a PVY^{O} serotype (besides the reference PVY^{O}) was Alt; isolates Mont, RRA-1, and 423-3 displayed PVY^{N} serotypes. All of these isolates except PVY^{O} produced veinal necrotic reactions on tobacco. Only one potato plant was observed to have "typical" PTNRD symptoms when mechanically inoculated under greenhouse conditions. Two tubers of cv. Ranger Russet inoculated with isolate 423-3 ($PVY^{Eu\text{-}NTN}$) displayed external necrotic rings typical of PTNRD after tubers were collected. Although foliar symptoms were observed on plants of Ranger Russet and other cultivars, no tuber symptoms were observed on the other cultivars with this isolate. No tuber symptoms were observed on any inoculated cultivar with the other isolates (Table 1). The only "typical" potato tuber necrotic ringspot disease (PTNRD) symptoms were observed with isolate 423-3. Isolate Alt has been associated with atypical tuber symptoms on cv. Alturas and Caribe. These cultivars were not tested with isolated viruses in these studies because certified seed wasn't available for the bioassay.

Virus purification

All isolates in this study were successfully purified to homogeneity through sucrose density gradient centrifugation using the procedure described above. Virus yield was generally 1 mg of virus from every 23–40 g infected leaf tissue with an $A^{260/280}$ ratio of between 1.20 and 1.37.

Sequence analysis

Sequence traces of the four isolates were assembled into single "contigs" to give complete full-length sequence for each isolate. Analysis of aligned sequences by the program Polyphrap did not detect polymorphic peaks that would be indicative of mixed strains. The length of the four isolates (excluding poly-A tail) and their corresponding GenBank accession numbers were: 423-3: 9702 bp (AY884982), Mont: 9700 bp (AY884983), RRA-1 9700 bp (AY884984), Alt: 9702 bp (AY884985). All four completely sequenced isolates showed high similarity to GenBank sequences, some of which were from European sources submitted in the previous decade. Two of the sequences were most similar to isolates characterized and first sequenced in Europe. Isolate Mont (RR1) was 99.2% identical to Swiss PVY^{N} isolate N605 (X97895, 24) and 98.5% identical to SCRI-N (AJ585197); the next nearest sequence homology was with the $PVY^{NA\text{-}NTN/N}$ type isolates SASA-61, TU660, and N-Jg (96.2%, 96.1%, 96.0%, respectively). Amino acid identities for these isolates were 99.5%, 99.1%, 98.1%, 98.0%, and 98.1%, respectively. Isolate 423-3 was 99.3% identical to the Hungarian PVY^{NTN} isolate (M95491, 50) and 98.8% identical to NIB-NTN (AJ585342). The amino acid identities were 99.3% and 99.5%, respectively. No other PVY sequence in the GenBank database showed >95% nucleotide identity to this isolate.

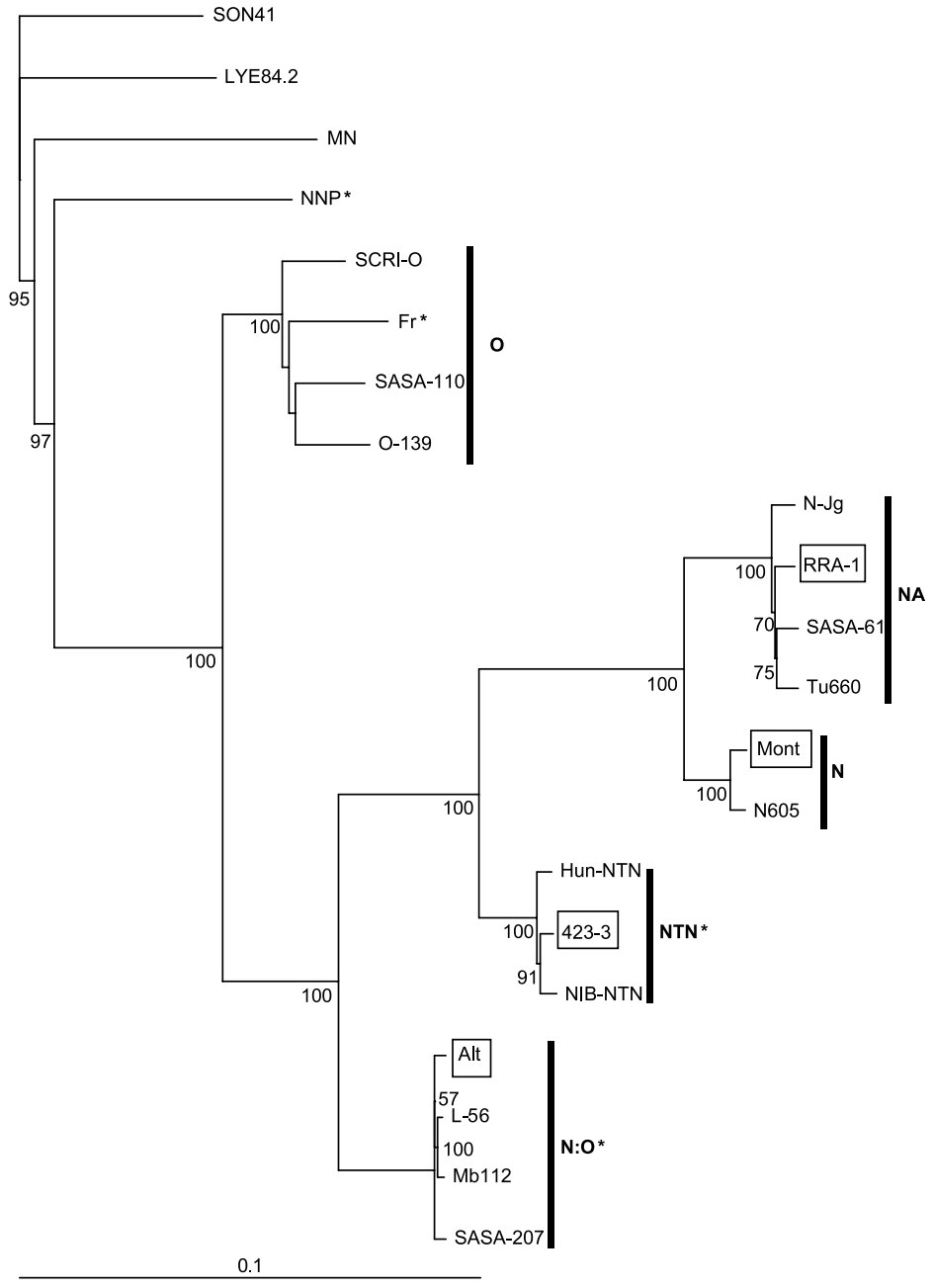

Fig. 1. Rooted dendogram showing phylogenetic relationships (nt) among full-length PVY isolates. Bootstrap values (%) are listed; nodes with less than 50% bootstrap support have been collapsed. Group classification is given to the right of vertical bars. Sequences with known recombination junctions that could affect apparent genetic distance are indicated by "*". Sequence identifiers for isolates are given in parentheses: N-Jg (AY166867), SASA-61 (AJ585198), Tu660 (AY166866), N605 (X97895), SCRI-N (AJ585197), Hun-NTN (M95491), NIB-NTN (AJ585342), SASA-207 (AJ584851), L-56 (AY745492), Mb112 (AY745491), SASA-110 (AJ585195), SCRI-O (AJ585196), O-139 (U09509), Fr (X12456), SON41 (AJ439544), LYE84.2 (AJ439545), NNP (AF237963), MN (AF463399)

The sequence of isolate RRA-1 was 99.1% identical to SASA-61 (AJ585198, Barker et al. unpubl.), 99.0% identical to the Canadian sample TU660 (AY166866, $PVY^{NA\text{-}NTN}$; 36), 98.9% identical to isolate N-Jg (AY166867, $PVY^{NA\text{-}N}$; ibid.). The next most related sequence (96.3%) in the database was Swiss isolate N605 (X97895, PVY^{N}, 24). The amino acid identities of RRA-1 to these isolates were 99.6%, 99.4%, 99.4%, and 98.2%, respectively. The Alt sequence was 99.5% identical to $PVY^{N:O}$ isolates L56 and Mb112 (AY745492, AY745491, 38), and 99.4% identical to isolate SASA-207 (AJ584851). The amino acid identities for these comparisons were 99.7%, 99.6%, and 99.7%, respectively. No other

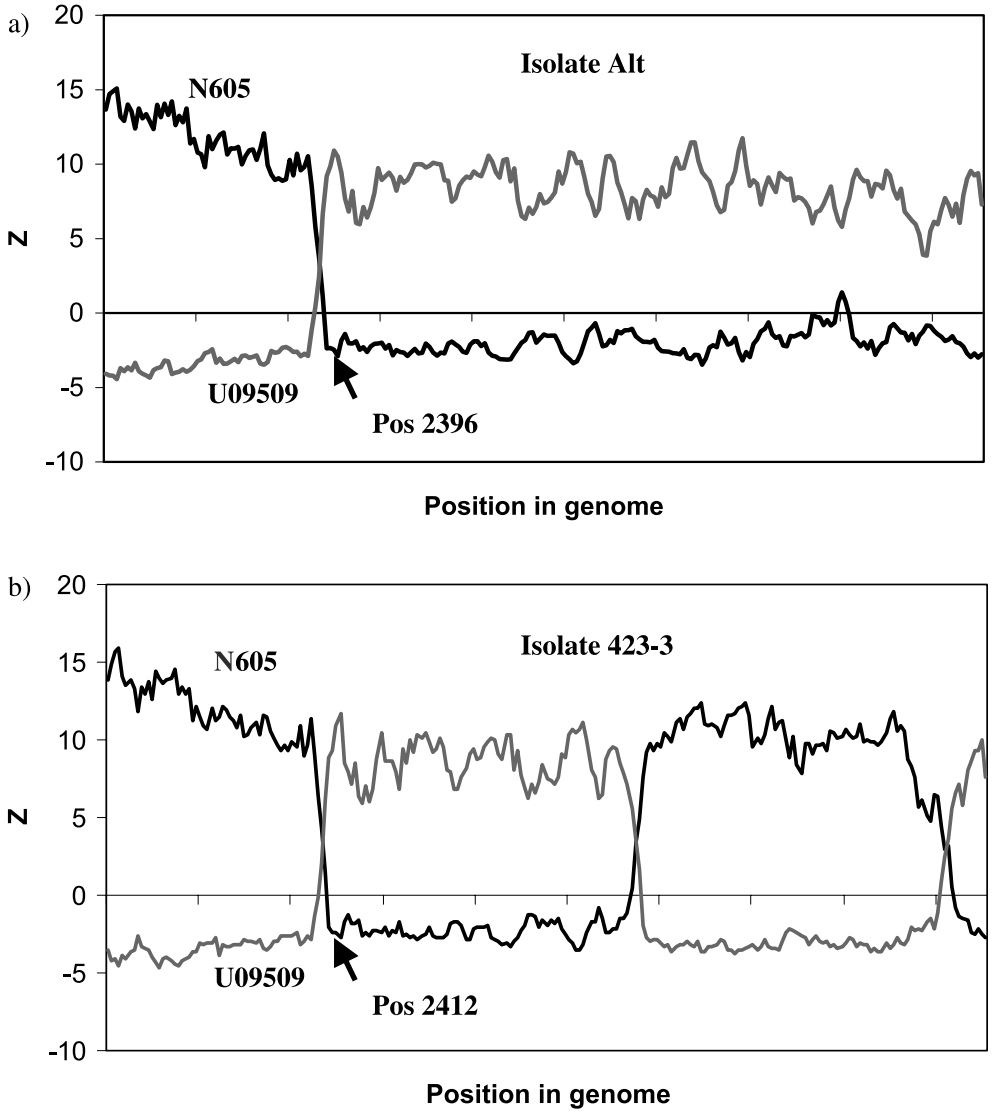

Fig. 2. Recombination analysis for isolates Alt and 423-3. Comparison between PVY^{N} (dark) and PVY^{O} (light) for (**a**) Alt and (**b**) 423-3 using the program SiScan with window = 200, step = 40. Assumed parent for each analysis is indicated

isolates were within 95% nucleotide identity of this sequence. Genetic distances are indicated by the dendogram in Fig. 1, which is based on the full-length nucleic acid sequences. Sequences affected by known recombination events are indicated as such in Fig. 1.

An analysis of recombination indicated a single break point for isolate Alt (Fig. 2a) at position 2412, with assumed parents N605 and PVY^{O} (U09509).

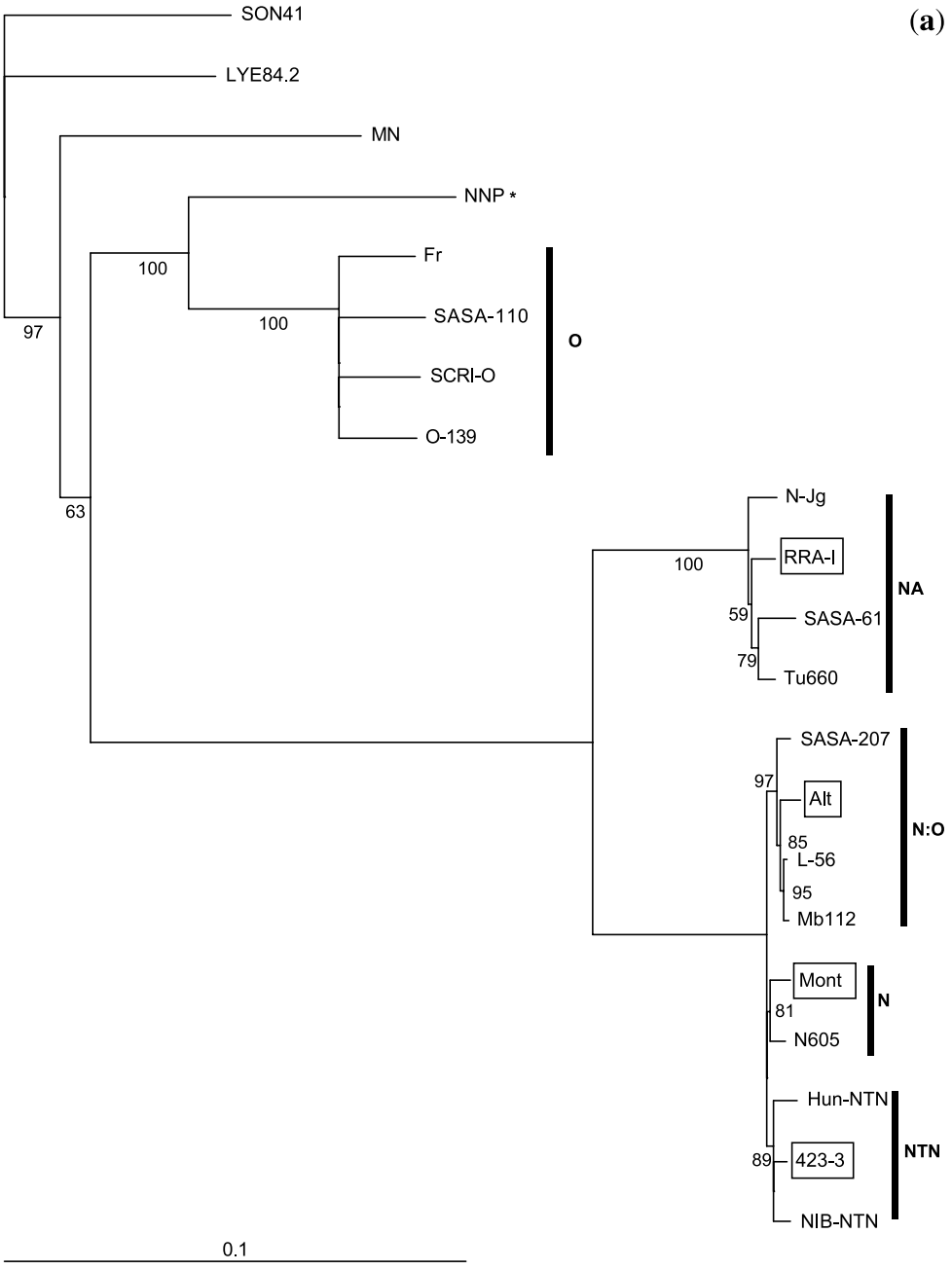

Fig. 3 (*continued*)

The break point was identified by programs RDP ($p = 1.9 \times 10^{-202}$), Bootscan ($p = 1.8 \times 10^{-194}$), MaxChi ($p = 3.8 \times 10^{-200}$), Chimaera ($p = 1.0 \times 10^{-80}$), and SiScan ($p = 1.2 \times 10^{-23}$). Recombination analysis identified two additional break points in isolate 423-3, at identical points as for the Hungarian PVY^{NTN} isolate, at positions 5897 and 9170 (Fig. 2b), with assumed parents N605 and ALT. This

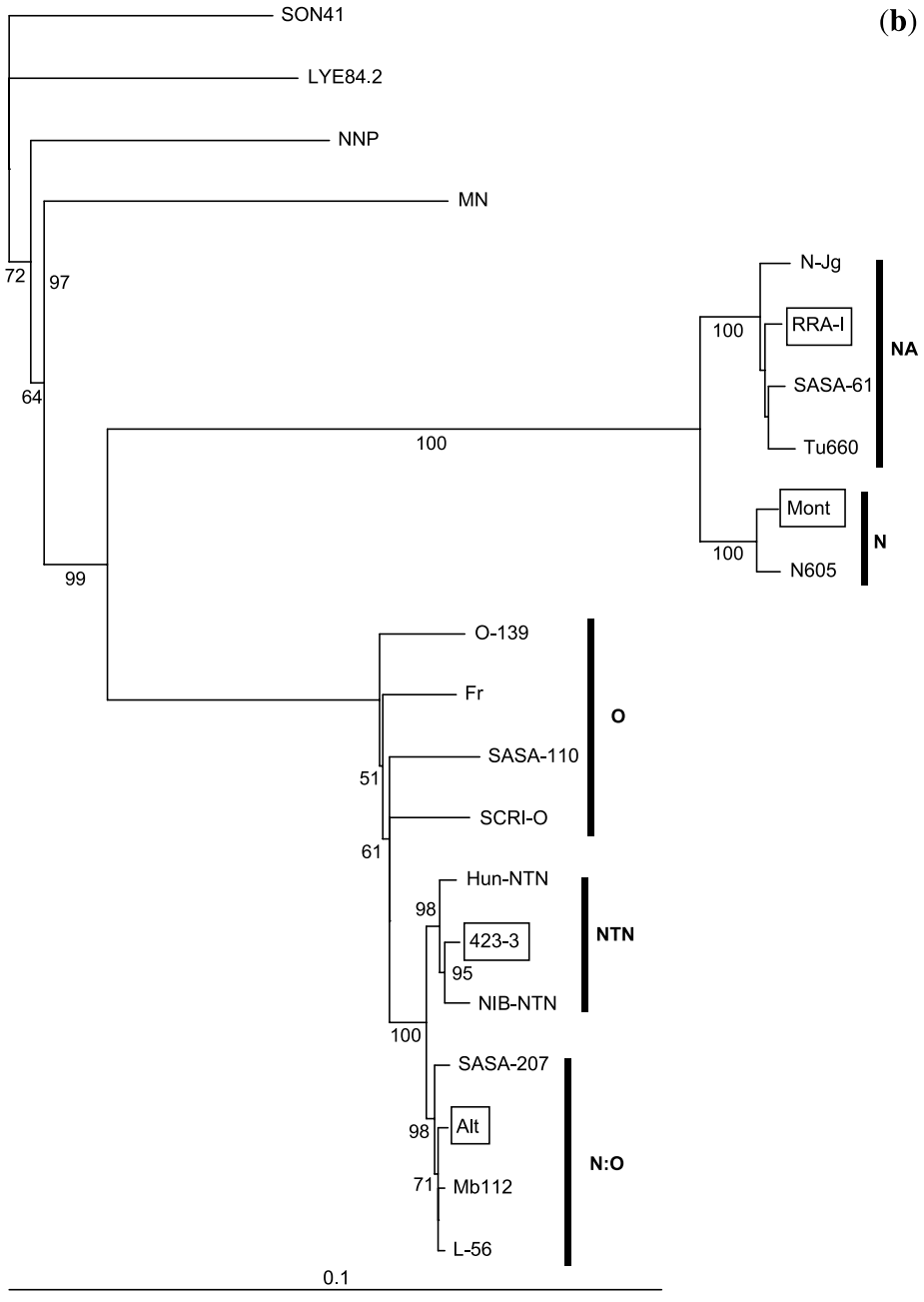

Fig. 3 (*continued*)

recombinant segment was also predicted by each of the same set of detection programs (p range: 1.0×10^{-22} to 2.2×10^{-141}).

The apparent genetic distances in Fig. 1 are affected by known recombination events. The four genomic segments (1 to 2412, 2413 to 5842, 5843 to 9170, and 9171 to 9700) defined by the recombination events of Hun-NTN (and 423-3) were aligned and phylogenetic distances of each segment are presented in Fig. 3a to d. Isolates Alt, Mont, and 423-3 were closely aligned with PVY^N sequences for the initial 2412 bp, although each was clustered tightly within its

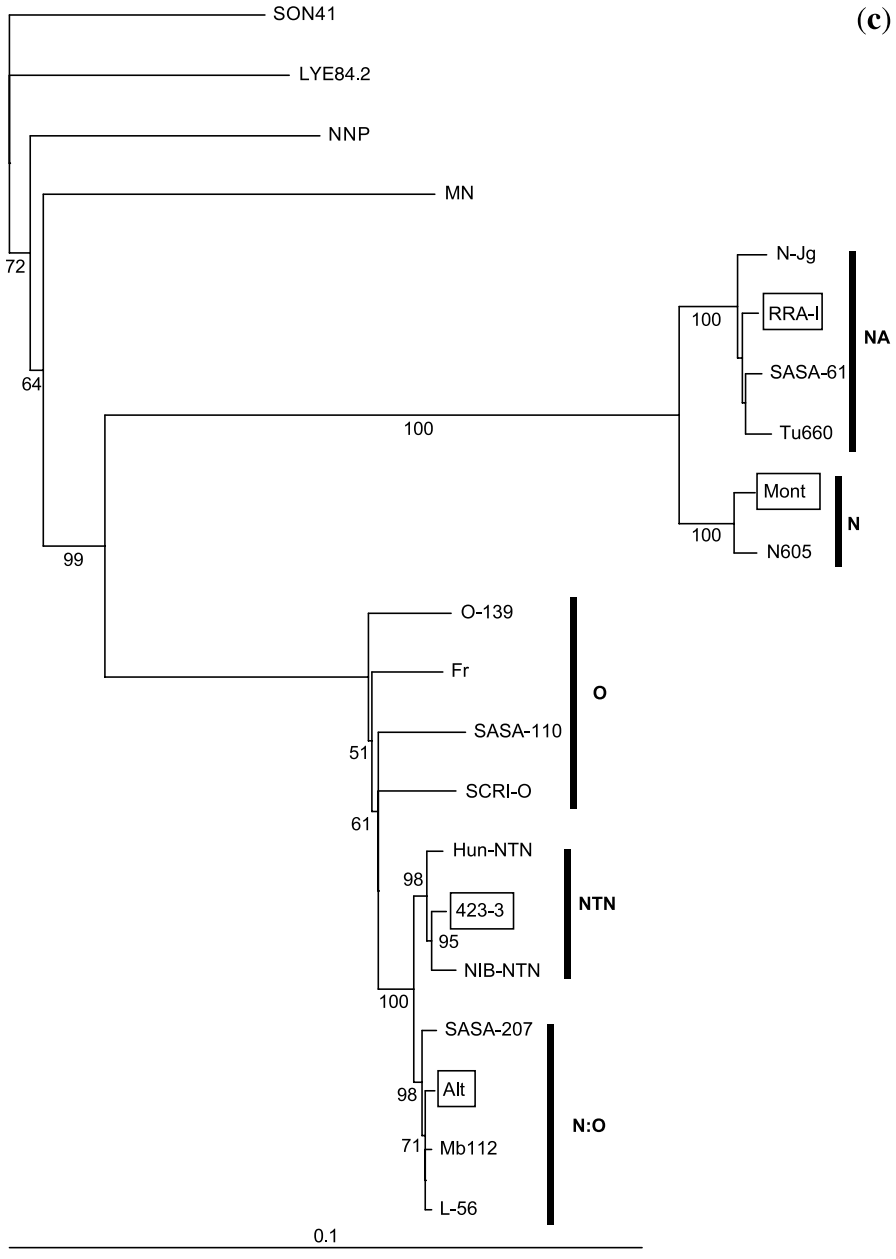

Fig. 3 (*continued*)

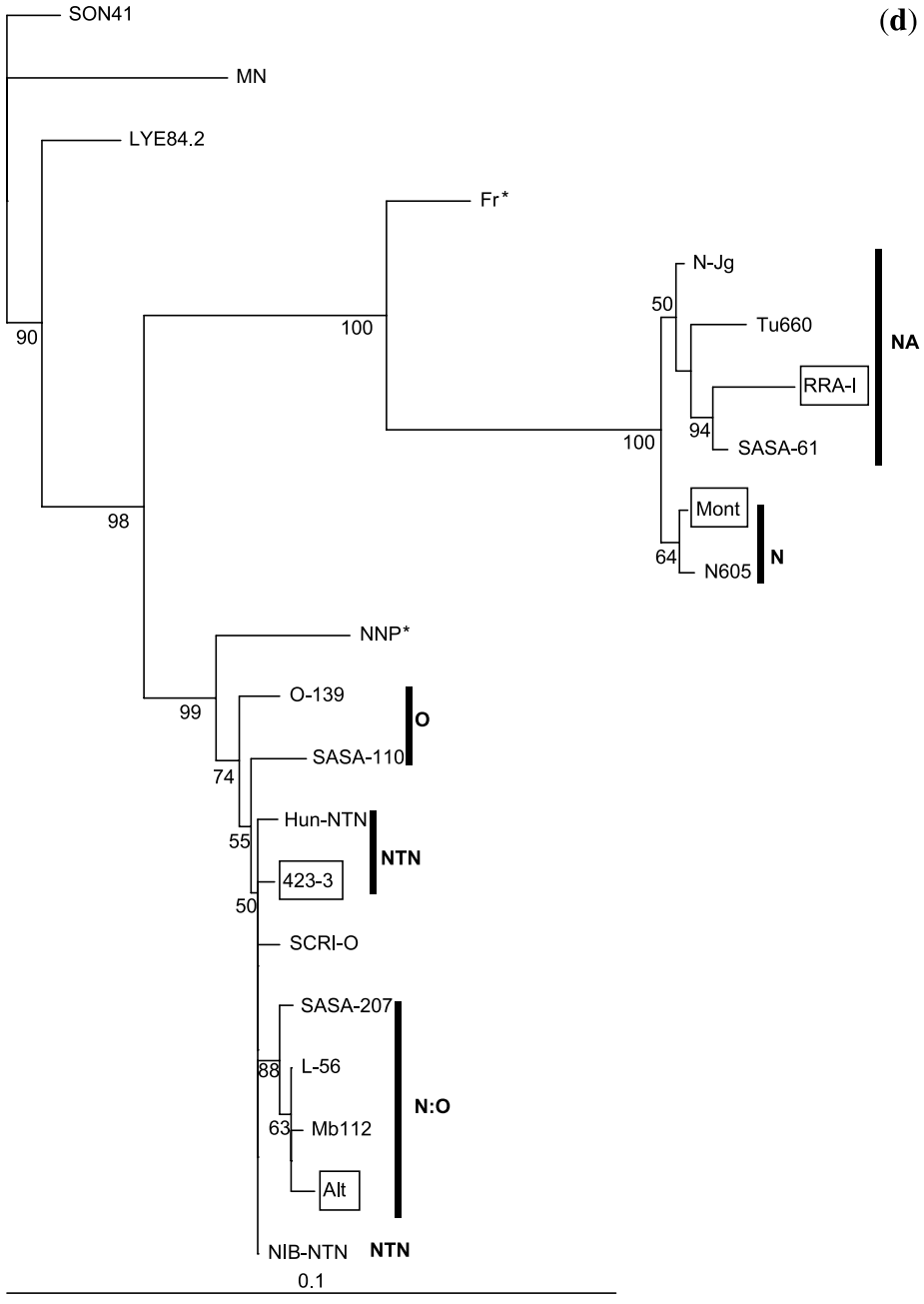

Fig. 3. Rooted dendograms showing phylogenetic relationships (nt) among PVY isolates as defined by recombinant break-points for genomic regions (**a**) 1 to 2411, (**b**) 2412 to 5840, (**c**) 5841 to 9164, and (**d**) 9165 to 9702 (in bp, numbering based on M95491). Bootstrap thresholds are as indicated for Fig. 1. Sequences with known recombination junctions that could affect apparent genetic distance are indicated by "*"

respective $PVY^{N:O}$, PVY^{N}, or PVY^{NTN} subgroup (Fig. 3a). Isolate RRA-1 and the $PVY^{NA\text{-}N}$ and $PVY^{NA\text{-}NTN}$ isolates were most distant from the other PVY^{N} isolates in this segment, which consists of the 5′UTR, P1, and HCP cistrons. In

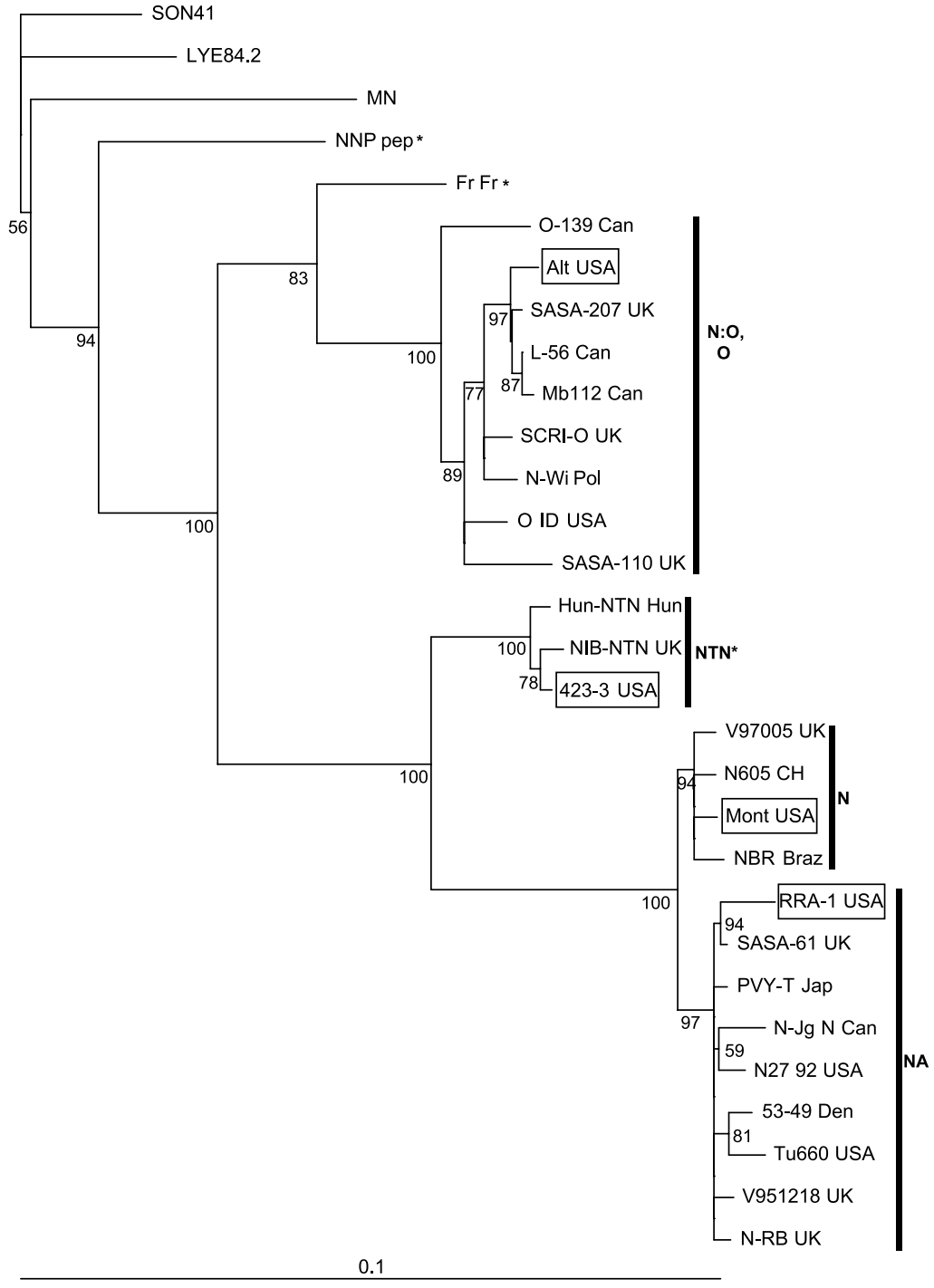

Fig. 4. Rooted dendograms showing phylogenetic relationships (nt) among PVY isolates in the CP-3′UTR region, including additional GenBank accessions similar to RRA-1 and Mont, or that represent the diversity among PVY strain types. Country of origin is indicated. Sequences with known recombination events that could affect apparent genetic distance within this genomic region are indicated by "*". Bootstrap thresholds are as indicated for Fig. 1. Sequence identifiers for isolates not presented in Fig. 1 are listed in parentheses: N-Wi (Z70238), PVI3 (AJ390307), V951218 (AJ390287), PVY-T (D12570), 53-49 (AJ390299), N-RB (AJ390285), N27-92 (U09508), V97005 (AJ390303), NBR (AF255660), PMB21 (AJ390306), O_ID (M81435)

the second segment, which consists of the P3, 6K1, CI, 6K2, and beginning of the VPG cistrons, the recombinant Alt and 423-3 isolates were generally clustered with the group of PVY^{O} isolates, but most closely with their respective strain variant classes $PVY^{N:O}$ or PVY^{NTN}, respectively (Fig. 3b). Mont and RRA-1 were clustered with their respective PVY^{N} and $PVY^{NA\text{-}N/NTN}$ subsets, which were more similar than in the first 2412 bp. In the third segment, representing the VPG/Nia, Nib, and much of the CP cistrons, Alt and the $PVY^{N:O}$ isolates remained with the PVY^{O} group, but 423-3 and the PVY^{NTN} isolates were quite close to the PVY^{N} isolates (Fig. 3c). For the final genomic segment, representing the 3′ end of the CP cistron and the 3′UTR, the $PVY^{N:O}$ and PVY^{NTN} variants were clustered with the PVY^{O} isolates (Fig. 3d). The PVY^{N} and $PVY^{NA\text{-}N/NTN}$ strains were most similar in this region (genetic distance of ∼0.015).

The GenBank database includes many more PVY sequences for the CP-3′UTR region than for any other region. A BLASTn search using the CP-3′UTR portion of isolate RRA-1 identified several sequences from Europe and Japan with very high genetic similarity to RRA-1. These isolates included 53-29 (not shown) and 53-49 from Denmark (5); V9511218, V951156-1 (not shown), and N-RB from the UK (5); and PVY-T from Japan (Hidaka et al. unpublished, Fig. 4). The clade of $PVY^{NA\text{-}N/NTN}$ isolates including RRA-1, recognized $PVY^{NA\text{-}N/NTN}$ isolates Tu660 and N-Jg, and SASA-61 with the above isolates formed a clade that was separated from PVY^{N} isolates with good bootstrap support (Fig. 4). In addition to Mont, a South American isolate, NBR [23] was very closely related to PVY^{N} isolate N605 (Fig. 4).

Discussion

The biological and molecular characterization confirmed that isolate Mont should be classified as PVY^{N} (= $PVY^{Eu\text{-}N}$), RRA-1 is a $PVY^{NA\text{-}N/NTN}$ strain, and that 423-3 is typical of a PVY^{NTN} ($PVY^{Eu\text{-}NTN}$) strain. The Alt isolate was recovered from tubers showing "atypical" symptoms very similar to those reported by Piche et al. [39] and is apparently very similar to those $PVY^{N:O}$ isolates with regard to recombination junction and biological and serological reactions. Strains represented by isolates 423-3, Mont, and Alt are descended from isolates introduced from Europe or another common source without extensive genetic change.

Appearance of these isolates in the region within the same time period suggests that they may have been introduced within a close time frame. It is unlikely that the PTNRD symptoms produced by isolates 423-3 and Alt would have gone unnoticed over many field generations. Although it is nearly impossible to know route of transit, a reasonable hypothesis might be that the necrotic isolates reported in this paper are descended from the original (or subsequent) outbreak of PVY^{N} reported in eastern Canada [31].

Modern reports of PVY^{N} and its economic impact first came from Europe [13], although they were preceded by a decade by similar strains collected from Andean potatoes and analyzed in Brazil [44]. Similarly, the first reports of PVY^{NTN}

and PVY^{N}W (PVY$^{N:O}$) isolates came from Europe [10, 50]. The first sequences reported for "North American" strains of necrotic PVY indicated a significant difference between the PVY$^{NA\text{-}N}$/PVY$^{NA\text{-}NTN}$ strains and the European PVY^{N} [36]. The probable presence of the "European" versions of necrotic PVY in North America has been indicated by PCR assays, as has the presence in North America of PVY recombinants with one and three recombinant junctions [37, 39]. However, this report is the first confirmation via whole-genome sequencing that strains nearly identical to the "European" variants of PVY^{N} and PVY^{NTN} exist in North America.

The high similarity between Mont and N605 [24], and 423-3 and Hun PVY^{NTN} [50] indicate the probability of common origins rather than convergent evolution and/or independent recombination. While the current data do not exclude the latter for 423-3, the high degree of sequence homology relative to relationships among sequenced PVY^{O} strains makes an independent recombination event seem less plausible (Figs. 1, 3, 4). Although detection of PVY$^{N:O}$ isolates has been previously reported [19, 20, 36, 47], and partial sequences for European PVY^{N}W isolates have been reported [20], the first complete sequences of such PVY$^{N:O}$ isolates with a single recombinant junction were posted in 2004 ([37], Barker and McGeachy, unpublished). Although these PVY$^{N:O}$ isolates and Alt share this common recombination junction with PVY^{N}W and high sequence homology with available 3′-sequences of PVY^{N}W, defining the exact relationship with European PVY^{N}W isolates must await the publication of the complete sequences of authenticated PVY^{N}W isolates. Genomic sequence for the central portion of PVY^{N}W isolates has not been reported. RFLP analysis indicated that the central portion (CI cistron) of PVY^{N}W types was very similar to PVY^{NTN} and resembled PVY^{O} [20]. Downstream (3′) of this portion where PVY$^{Eu\text{-}NTN}$ sequences are characterized by PVY^{N}-type sequence, PVY^{N}W sequences types were more similar to PVY^{O} than to PVY^{NTN}. The same description applies to Alt in this report. However, Singh et al. [47] reported that PVY$^{N:O}$ recombinant isolates in Manitoba, Minnesota, and North Dakota differed from PVY^{N}W strains in symptomology. Furthermore, Alt caused atypical PTNRD symptoms in at least two potato cultivars (Table 1, Ref. [12]), similar to those reported in other PVY$^{N:O}$ isolates on cv. Yukon Gold [39]. Such tuber symptoms have not been reported for PVY^{N}W or previously sequenced PVY$^{N:O}$ isolates. Sequence differences between Alt and previously sequenced PVY$^{N:O}$ isolates are very subtle (0.5%) and do not immediately suggest candidate residues associated with the atypical PTNRD symptoms.

Figures 1, 3, and 4 clearly indicate that isolates with molecular genotypes of the "North American" PVY$^{NA\text{-}N/NTN}$ strain variant class are also present in Europe and Asia. The full-length sequence of SASA-61 (UK) is more closely related to RRA-1 and the PVY$^{NA\text{-}N/NTN}$ strain variants than it is to "European" PVY^{N}. The CP/3′UTR sequences of V951218, PVY-T, 53-49, N-RB, N27-92, and V97005 fall in a PVY$^{NA\text{-}N/NTN}$ clade that is well separated from the "European" PVY^{N} strain (Fig. 4). Presumably the rest of their genomes are of the same molecular genotype, although this remains to be validated. This clade includes variants described as both PVY^{N} and PVY^{NTN} with regard to pathotype on potato

tubers [5, 35]. Therefore, although the "Eu" and "NA" prefixes may be useful for identifying molecular genotype, they are not particularly illuminating with regard to geographic distribution or pathotype on potato. The globalization of the seed potato trade may have provided a vehicle for the widespread distribution of PVY strain variants that are pathogenic to potato.

Recombination analysis suggested that Alt or a similar $PVY^{N}W/PVY^{N:O}$ isolate may be a more likely parent of Hun PVY^{NTN}/423-3 than are available PVY^{O} sequences. Therefore, it is possible that the similar recombination junction that Alt shares with Hun PVY^{NTN}/423-3 and $PVY^{N}W$ near position 2400 [20] represents the original $PVY^{N}W/PVY^{N:O}$ recombination junction, with a subsequent recombination event defining additional junctions at 500 (20), 5840 (20), and 9160 (40). Alternatively, the RJ near position 2400 bp may represent a recombination "hotspot" that recreated similar strains through independent recombination events. Evidence for the presence of such a recombination hotspot has been presented for the CP cistron [5, 40]. Regardless of the relationship between the $PVY^{N:O}$ and PVY^{NTN} types, the limited diversity (99.5% identity) among the sequenced $PVY^{N:O}$ genomes strongly suggests common origin.

Previously reported recombination events are suggested in the phylogenetic dendograms that represent the genomic segments defined by the 3 recombination junctions of the PVY^{NTN} strain variants. The NNP isolate is intermediate to the non-potato and PVY^{O} strains in Fig. 3a and d, and has been previously reported to have recombination events with a PVY^{O} type in the P1 and 3′UTR regions [16]. Similarly, isolate Fr is intermediate to the PVY^{O} and PVY^{N} strain types in Fig. 3c and d, and has been reported to contain small portions of PVY^{N} sequence in the Nia and 3′UTR regions [34].

If the 423-3 and Mont isolates were derived from the type isolates represented by European sequences in GenBank, data on the accumulation of mutations from these isolates can be estimated. Assuming that the 423-3 and Mont isolates diverged from the PVY^{NTN} and PVY^{N} strains around the time of their collection and sequence deposition in GenBank, accumulation of non-deleterious mutations appears to be around 60 per 10 years. The Alt sequence generally corroborates that estimate, diverging from sequences of recently deposited $PVY^{N:O}$ isolates by fewer mutations in a shorter time frame. However, since the actual time of physical separation is unknown, such estimates are speculative at best. About 75 to 80% of the mutations were silent, indicating the proteins coded for by these nucleic acids are conserved and that mutations are under purifying or negative selection as opposed to diversifying or positive selection [34].

In conclusion, whole genome sequencing of four PVY isolates collected in N. America confirmed that strains previously considered to be "European" (PVY^{N}, PVY^{NTN}) are present in North America. Conversely, isolates with molecular genotypes more consistent with the "North American" $PVY^{NA\text{-}N/NTN}$ strain group have been reported from Europe and Asia. The high genetic similarity in both cases indicates common origins. Both types of recombinant isolates reported in this study have the capability to induce PTNRD, although the symptoms caused by isolate Alt on some potato cultivars could be termed, "atypical". Assays

developed for "European" necrotic strains of PVY should be equally useful in North America, and *vice versa*.

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