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BRIEF REPORT

Molecular characterization of yam virus X, a new potexvirus infecting yams (*Dioscorea* spp) and evidence for the existence of at least three distinct potexviruses infecting yams

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Abstract The genome of yam virus X (YVX), a new member of the genus *Potexvirus* from yam (*Dioscorea trifida*), was completely sequenced. Structural and phylogenetic analysis showed that the closest relative of YVX is nerine virus X. A prevalence study found YVX only in plants maintained in Guadeloupe and showed that it also infects members of the complex *D. cayenensis rotundata*. This study provides evidence for the existence of two additional potexviruses, one of which infects *D. nummularia* in Vanuatu and the other, *D. bulbifera* and *D. rotundata* in Haiti and *D. trifida* and *D. rotundata* in Guadeloupe. This work also shows that existing potexvirus-specific degenerate primers targeting the ORF1-encoded polymerase domain are well suited for the identification of the three potexviruses reported here.

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The nucleotide sequences reported in this work have been deposited in the GenBank database under accession numbers KJ711908, KJ729592 to KJ729598, and KJ815096 to KJ815104.

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Yams (*Dioscorea* spp) are one of the most important food commodities in the tropics and subtropics [4]. Edible yams, which represent about 10 of the ca. 600 known species of yams, produce starchy tubers with high nutritional value and play a key role in food security, accounting for a substantial part of food consumption in Asia, Africa, the Pacific and the Americas, including the Caribbean. As cash crops, yams also play an important role in the local economy of numerous developing countries. Several yam species are also used in traditional medicine as a source of antioxidants [6] and in the pharmaceutical industry for the extraction of steroids including diosgenin, a steroidal sapogenin with estrogenic and antitumor properties [16].

Yams are vegetatively propagated, thus promoting the accumulation of phytoviruses. To date, several viruses of the genera *Aureusvirus*, *Badnavirus*, *Carlavirus, Comovirus*, *Cucumovirus*, *Fabavirus*, *Macluravirus*, *Potexvirus* and *Potyvirus*, have been reported and characterized in *Dioscorea* spp [8–10, 13–15, 18–21, 26]. However, the diversity of viruses infecting yams remains largely unexplored. The impact of viral infections on tuber yields and quality can be important and sometimes threatens yam cultivation itself. For example, cultivation of *Dioscorea trifida* is undergoing a strong decline in the Caribbean due to its high susceptibility to potyviruses. Efforts are being made worldwide to promote the sanitation of infected yam germplasm in order to produce and distribute clean tuber/plantlets in an effort to control yam viruses and to restore production and productivity. However, these efforts are hampered by our limited knowledge of the diversity of viruses infecting yams, thus preventing the development of appropriate and cost-effective diagnostic tools. These efforts are also impacted by the recent discovery of endogenous badnavirus sequences in the genome of yams of the *Dioscorea cayenensis-rotundata* complex [22, 24].

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D. trifida accession #551 from Guadeloupe's Biological Resource Centre for Tropical Plants (CRB-PT) yam collection, showing mild leaf chlorosis symptoms, was positively indexed for the presence of potexviruses by RT-PCR performed on total nucleic acids (TNAs) extracted from leaf tissues [5], using the potexvirus-specific degenerate primer pair Potex-2RC/Potex-5 [25]. These primers target the conserved polymerase active domain of the replicase encoded by potexviruses open reading frame 1 (ORF1). The amplification product was cloned into the pGEM-T Easy Vector (Promega, France) and sequenced. Analysis of the sequence showed that it was homologous to the corresponding region of other potexviruses but that it was nevertheless distant from any sequence in the GenBank database, suggesting that it corresponded to an as yet uncharacterized potexvirus. In order to amplify and sequence the 3′ half of its genome, the primer Potex-Yam-GSP4 (5′ GGACGAGACCCTCTATCGAGCAACCATT 3′) was designed based on the sequence of the amplification product (Fig. 1A) and used with the LDprim oligonucleotide in long-distance reverse-transcription PCR as described by Youssef *et al.* [27]. This generated a 3,150-bp amplification product. For the 5′ genome part, primer Potex-Yam-rev (5′ GAGATTGGCTTCTTCAGAGC 3′) was designed based on the originally obtained internal sequence, while an upstream primer Potex-Yam-fw2 (5′ TACGCYACMRTGGTDCTHCC 3′) was designed based on potexviral sequences observed in *D. trifida* expressed sequence tags (EST) data generated by the Agropolis Resource Center for Crop Conservation, Adaptation and Diversity (ARCAD) project (unpublished). These primers were used in a long RT-PCR amplification experiment, generating a 2,487-bp product overlapping the 3′ product by 67 nucleotides. Finally, based on the 5′ region sequence thus obtained, primer potex-5′-rev (5′ GCGTGTGCGCCA GGTATGTACTGAAACC 3′) was designed and used in a 5′ RACE experiment, generating a 704-bp product covering the 5′ end of the viral genome and overlapping the long PCR amplification product by 116 nucleotides. Amplification products were cloned into pGEM-T Easy Vector and sequenced by Beckman Coulter Genomics (Takesley, UK). Sequence assembly and analysis, including phylogenetic studies, were performed using MEGA 6.0 [23].

The complete genome of the potexvirus isolate infecting *D. trifida* accession #551 was reconstituted by assembling the three overlapping sequences, which displayed 100 % homology in the overlap regions, suggesting that accession #551 was infected by a single viral isolate. The size of the viral genome is 6,158 nucleotides, excluding the 3′ polyA tail. Its organization (Fig. 1A) is typical of members of the genus *Potexvirus*, with five open reading frames (ORFs). A large ORF (ORF1, nt 79-4236) encodes an RNA-dependent RNA polymerase (RdRp or replicase) including the characteristic conserved RdRp core motif ([11], S/TGx3Tx3NS/Tx22GDD, nt 3667-3802). The first initiation codon at position 79 is in a favorable context for initiation (GGTATGGC), with a G at position −3, a purine at position +4 and a GC after the start codon [12, 17]. Three smaller and overlapping ORFs, ORF 2 (nt 4270-4933), ORF 3 (nt 4926-5250) and ORF 4 (nt 5179-5380) encode putative triple gene block proteins TGBp1, TGBp2 and TGBp3, whose sequences, arrangement and function in viral movement are typical of and conserved among potexviruses [2]. ORF2 and ORF3 overlap by 7 nt, whereas ORF3 and ORF4 overlap over a longer sequence (71 nt). ORF5 (nt 5384-6029), encodes the putative coat protein (CP), which contains the conserved amphipathic core CP motif (AAFDxFx2Vx4A, nt 5798-5837) shared among members of the family *Alphaflexiviridae* [1, 3]. The 5′ and 3′ untranslated regions are 78 nt and 128 nt long, respectively, and the latter comprises a putative polyadenylation signal (AATAAA, nt 6053-6059).

BLAST analysis against the GenBank database and phylogenetic analysis performed using RdRp and CP amino acid sequences (Fig. 1B and C) confirmed that this virus is a member of the genus *Potexvirus* but has no close counterpart in the database. Indeed, the highest percentage of sequence identity for any of the five viral proteins never reached more than 51.9 % with any of the potexviruses analyzed, clearly indicating that the studied isolate represents a previously unsequenced potexvirus (Table 1), for which the name yam virus X (YVX) is proposed. These and additional phylogenetic analyses performed on the amino acid sequences of the other putative proteins encoded by the genome of YVX showed that the *Potexvirus* genus member most closely related to YVX is nerine virus X (NVX; see Table 1).

Mechanical transmission of the viral isolate to herbaceous hosts, including *Nicotiana benthamiana*, *N. clevelandii*, *Chenopodium quinoa* and *C. amaranticolor*, was attempted, but no local or systemic infection was observed, and no YVX could be detected in the indicators when tested by RT-PCR performed on TNAs using the potexvirus-specific degenerate primer pair Potex-2RC/Potex-5. A study of the prevalence of the virus was performed by RT-PCR as described above on 170 accessions from Guadeloupe's CRB-PT yam collection. These accessions were originally collected in 14 countries (Barbados, Brazil, Dominica, Dominican Republic, French Guyana, Guadeloupe, Haiti, Ivory Coast, Jamaica, Martinique, New Caledonia, Nigeria, Philippines, and Puerto Rico) but have been conserved in Guadeloupe under field conditions for many years. An additional 213 accessions from the yam quarantine facility of the Centre International de Recherche en Agronomie pour le Développement (CIRAD) in Montpellier (France) were similarly analyzed. These accessions were collected in 20 countries (Benin, Brazil, Burkina

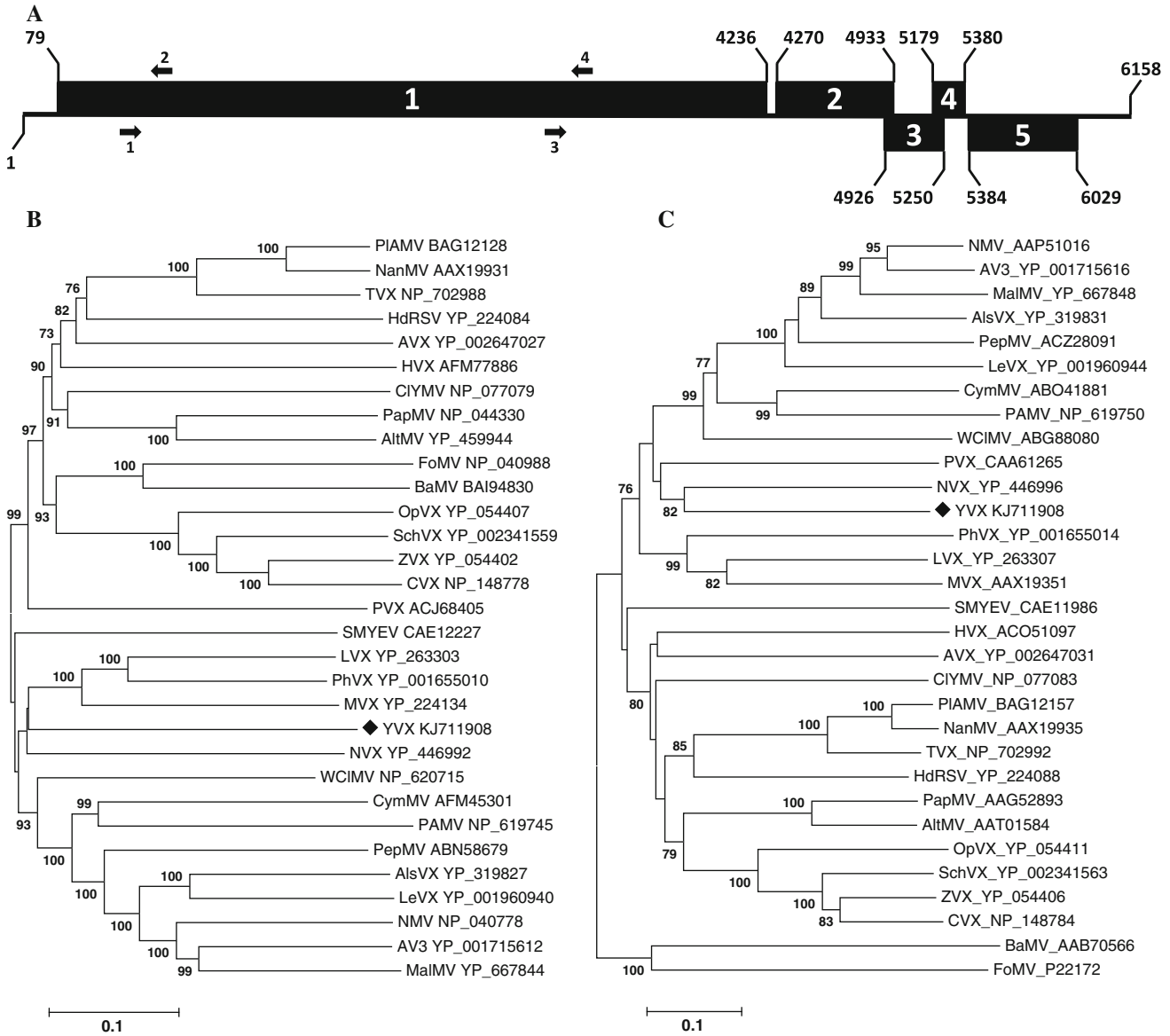

Fig. 1 Schematic representation of the genome organization of YVX (A) and neighbor-joining phylogenetic tree of potexvirus RdRp (B) and coat protein (C) amino acid sequences constructed using strict identity distances. Bootstrap analysis (1,000 replicates) was used to evaluate the significance of nodes. **A.** Position of the primers used to amplify and sequence the YVX genome. 1, Potex-yam-fw2; 2, potex 5′ rev; 3, GSP4; 4, Potex-yam-rev. **B, C.** Bootstrap values above 70 % are indicated at nodes. The scale bar shows the number of substitutions per site. The sequence derived from the full-length genome sequence of yam virus X (YVX) is indicated by a black diamond. Accession numbers are shown for the reference potexviruses used. Acronyms are as follows: AlsVX, alstroemeria virus X; AltMV, alternanthera mosaic virus; AV3, asparagus virus 3; AVX, allium virus X; BaMV, bamboo mosaic virus; ClYMV, clover yellow mosaic virus; CVX, cactus virus X; CymMV, cymbidium mosaic virus; FoMV, foxtail mosaic virus;.HdRSV, hydrangea ringspot virus; HVX, hosta virus X; LeVX, lettuce virus X; LVX, lily virus X; MalMV, malva mosaic virus; MVX, mint virus X; NanMV, nandina mosaic virus; NMV, narcissus mosaic virus; NVX, nerine virus X; OpVX, opuntia virus X; PaMV, potato acuba mosaic virus; PapMV, papaya mosaic virus; PepMV, pepino mosaic virus; PhVX, phaius virus X; PlAMV, plantago asiatica mosaic virus; PVX, potato virus X; SchVX, schlumbergera virus X; SMYEV, strawberry mild yellow edge virus; TVX, tulip virus X; WClMV, white clover mosaic virus; ZVX, zygocactus virus X

Faso, Cameroon, Cuba, Fiji, France, French Guyana, Ghana, Guadeloupe, Guinea, Haiti, India, Ivory Coast, Madagascar, Martinique, Papua New Guinea, Togo, Vanuatu, Vietnam) and are conserved under greenhouse conditions. A total of 17 samples were indexed positive: 12 samples from Guadeloupe's CRB-PT collection and five samples from CIRAD's yam quarantine facility. Amplification products were cloned and sequenced, and the corresponding amino acid sequences were compared. A yam expressed sequence tag (EST) sequence from the ARCAD

Table 1 Percentages of amino acid sequence identity between the proteins of yam virus X and those of other members of the genus *Potexvirus*

	POL	TGB1	TGB2	TGB3	CP
NVX	50.1	**33.2**	41.7	25.4	**47.9**
PVX	45.8	26.4	42.9	31.3	42.7
NanMV	44.3	27.1	41.9	22.2	39.8
WClMV	**51.7**	26.2	35.0	25.0	39.6
PlAMV	45.1	27.6	43.8	20.6	38.3
CymMV	47.7	22.9	31.1	34.8	37.6
AlsVX	45.8	21.7	27.5	19.7	37.3
TVX	45.3	30.4	41.3	21.3	37.3
SMYEV	47.8	25.1	46.2	26.7	37.1
LVX	50.5	30.0	39.8	27.0	36.9
PhVX	51.4	28.2	42.9	26.9	36.7
PepMV	48.0	23.6	28.7	**36.4**	36.7
ClYMV	46.2	28.2	**51.9**	22.2	36.7
AltMV	46.7	26.9	45.7	35.7	35.7
NMV	46.2	25.1	33.3	23.1	35.3
AV3	45.3	25.6	35.2	24.6	35.3
HdRSV	44.1	30.5	42.3	17.0	35.1
LeVX	45.3	20.8	32.7	32.3	34.9
MVX	51.3	31.6	37.3	23.1	34.8
PapMV	45.4	25.8	37.5	23.3	34.0
PAMV	44.0	21.3	28.8	26.2	33.7
SchVX	43.4	27.8	41.3	23.6	33.5
AVX	44.0	28.3	41.7	6.6	33.0
MalMV	45.1	26.5	38.0	18.5	32.9
ZVX	42.6	25.5	40.6	22.8	32.5
HVX	44.3	28.5	39.8	21.7	31.7
OpVX	42.7	26.9	44.2	17.2	31.3
CVX	42.2	28.3	43.3	18.6	31.2
BaMV	43.1	24.1	40.2	28.3	23.1
FoMV	44.1	26.0	36.2	19.1	18.8

Pol, RNA-dependent RNA polymerase; TGB1-2-3, triple gene block proteins 1, 2 and 3; CP, capsid protein. For each protein, the potexvirus showing the highest percentage of homology with YVX is indicated by bold. Virus acronyms and GenBank accession numbers are provided in the legend to Figure 1

project corresponding to the same viral genomic region was also included in the analysis. The reconstructed phylogenetic tree (Fig. 2) showed that variants closely related to the sequenced YVX isolate infect *D. trifida* and *D. rotundata* accessions of the CRB-PT collection. The yam EST sequence, obtained from accession #554 (*D. trifida*) of the same collection, also displays significant homology to the fully sequenced isolate and clusters with it with 100 % bootstrap support, suggesting that it corresponds to a viral variant belonging to the same viral species. There is therefore currently no indication that YVX could be present in yam species other than *D. trifida* and *D. rotundata*.

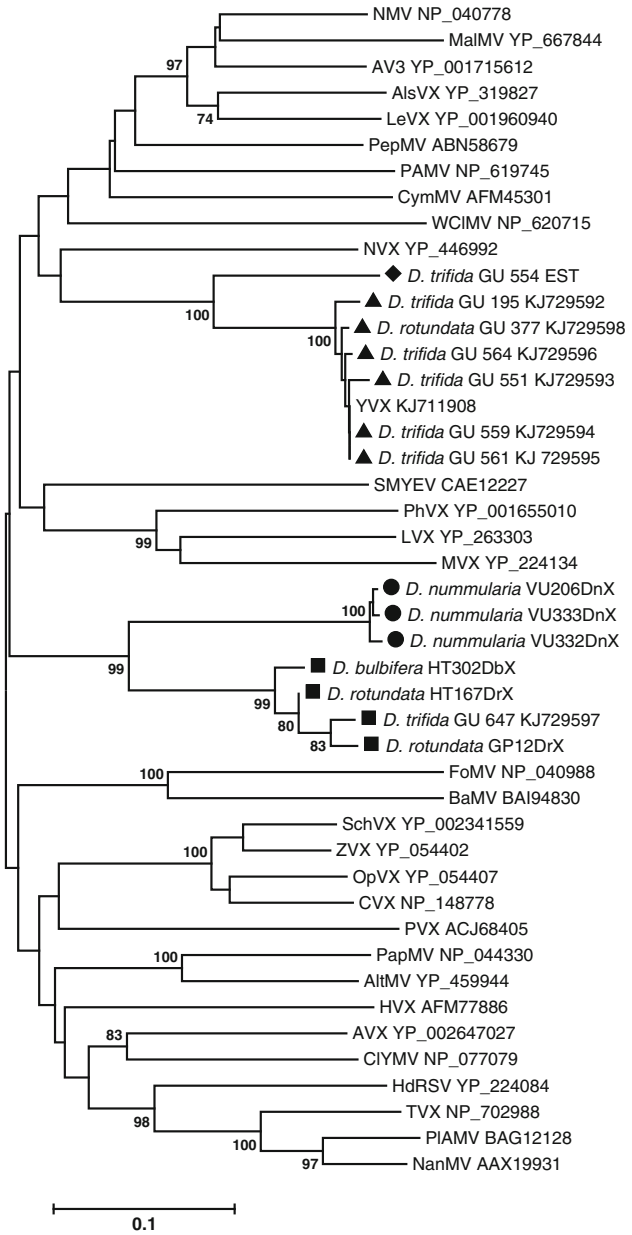

Fig. 2 Neighbor-joining phylogenetic tree of partial RdRp sequences of potexviruses corresponding to YVX genomic RNA positions 3015-3561, including two additional groups of potexvirus isolates infecting yams. The tree was constructed using strict amino acid distances and 1,000 bootstrap replicates. Bootstrap values above 70 % are shown at nodes. Sequences corresponding to the three potexviruses identified are indicated by black triangles (yam virus X, YVX), black squares, and black dots, respectively. A yam (*D. trifida* from Guadeloupe) EST sequence is indicated by a black diamond. The scale bar shows the number of substitutions per base. Virus acronyms are as in the legend to Figure 1, and relevant accession numbers are shown. The geographical origin of the samples from which sequences were amplified is specified: GU, Guadeloupe; HT, Haïti; VN, Vanuatu

Surprisingly, the phylogenetic tree shown in Figure 2 also provides evidence for the existence of two additional and clearly distinct groups of potexvirus sequences

amplified from the analyzed samples. The first group includes three sequences amplified from *D. nummularia* samples originating from Vanuatu (VU206DnX, VU332DnX, VU333DnX) and displaying 98.9-99.4 % amino acid sequence homology to each other in the encoded protein. A second group comprises four sequences amplified from *D. bulbifera* and *D. rotundata* samples from Haiti (HT302DbX and HT167DrX) and from *D. trifida* and *D. rotundata* samples from Guadeloupe (*D. trifida* accession #647 and GP12DrX) displaying 94.2-97.7 % amino acid sequence homology to each other. The average amino acid divergence level between the sequences from these three groups of potexviral sequences from yam ranged between 26.5 and 36.6 %. As illustrated by the phylogenetic tree in Figure 2, these values are well above those observed between some members of approved species within the genus *Potexvirus*, such as the 9.9 %-14.4 % values between members of the cactus virus X (CVX), opuntia virus X (OpVX), schlumbergera virus *X* (SchVX) and zygocactus virus X (ZVX) cluster or the 17.1-20.4 % values between asparagus virus 3 (AV3), malva mosaic virus (MalMV) and narcissus mosaic virus (NMV). These results therefore strongly suggest that members of two additional *Potexvirus* species were also present in the analyzed yams.

So far, only one potexvirus, dioscorea latent virus (DLV), has been reported in yams [13]. Although no sequence data are available for DLV, it has been characterized in detail [20] and shown to have serological cross-relationships with a few definite potexviruses, and its taxonomic status therefore seems to be firmly established. DLV was identified in symptomless *D. floribunda* in Puerto Rico, where it was shown to spread naturally [7, 13, 21], but not in any of the 37 cvs of *D. alata, D. bulbifera, D. esculenta* or *D. rotundata* collected by Phillips *et al.* [20] in eight countries (Barbados, Indonesia, Malaysia, Nigeria, Papua New Guinea, Philippines, Puerto Rico and Solomon Islands). DLV was detected serologically in *D. alata* samples from several South Pacific islands but could not be detected by RT-PCR in any of these samples using degenerate potexvirus-specific primers [14]. This suggests that DLV might be a member of the genus *Potexvirus* that is distinct from those reported in this work, although this needs to be investigated further when molecular data become available for DLV. Moreover, DLV has been reported to be readily transmissible by mechanical inoculation to a range of herbaceous hosts and to induce symptomless infection only in inoculated leaves of most experimental hosts, but symptomless systemic infection in *Nicotiana benthamiana* and *N. megalosiphon* [7, 20, 21]. In contrast, the inoculation experiments performed in the present work showed that the YVX isolate from yam #551 is unable to infect, locally or systemically, the various experimental hosts tested, including *N. benthamiana*. Given the difference in host range, and the inability of Lebas [14] to amplify DLV using degenerate potexvirus-specific primers, it is likely that the YVX isolate analyzed here is different from DLV. It remains to be evaluated whether the mild symptoms observed in the original yam #551 accession, which tested negative for badnaviruses, closteroviruses, macluraviruses and sadwaviruses but tested positive for potyviruses, were caused by YVX or by a co-infecting agent, considering that all other YVX-infected samples were symptomless.

No specific immunological or molecular reagent is currently available for the reliable diagnosis of the three yam potexviruses described in this work. The results reported here, however, demonstrate that RT-PCR performed on TNAs extracted from leaf tissues using the potexvirus-specific degenerate primer pair Potex-2RC/Potex-5 [25] is well suited for the detection of the three potexviruses reported here and could be used to improve the sanitary status of yam germplasm repositories and prevent further spread of these agents. Additional work is needed to further characterize the diversity of potexviruses infecting yams in order to develop generic molecular diagnosis tools for the detection of these viruses, including DLV.

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