A NEW POTYVIRUS SPECIES IN *COTYLEDON ORBICULATA* IN MIXED INFECTION WITH A NUCLEORHABDOVIRUS

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SUMMARY

Several species and varieties of the family Crassulaceae have been introduced and cultivated for ornamental as well as medicinal purposes in Brazil, more prominently *Cotyledon orbiculata* (pig's ear). This work reports the identification and characterization of viruses associated with foliar mosaic and distortion of *C. orbiculata* from the succulent plant collection of the garden of the University of São Paulo, Brazil. *In situ* analyses revealed enveloped bacilliform particles in the perinuclear space of infected *C. orbiculata* cells, and cytoplasmic cylindrical inclusions, typical of the family *Potyviridae.* Viruses were mechanically transmitted and separated by differential indicator hosts and aphid transmission (*Myzus persicae*). *Chenopodium amaranticolor* and *Datura stramonium* served as differential hosts for the potyvirus and the bacilliform virus, respectively. Positive serological reactions were obtained when leaf extracts from naturally infected *C. orbiculata* and experimental hosts were exposed to an antiserum to *Sonchus yellow net virus* (genus *Nucleorhabdovirus*). Identification of the potyvirus by RT-PCR, cloning into pGEM-T vector and sequencing generated a 1,752 nt fragment corresponding to part of the nuclear inclusion protein b (NIb), the complete coat protein (CP) and the untranslated region (UTR). Amino acid identities below 80% and phylogenetic analyses indicated that the potyvirus of *C. orbiculata* may be a new species belonging to the *Potato virus Y* subgroup, for which the name Cotyledon virus Y (CotVY) is suggested.

Key words: Ornamentals, medicinal plant, plant virus, phylogeny, nucleorhabdovirus, potyvirus

INTRODUCTION

The flowering plant family Crassulaceae comprises 35 genera, that include *ca.* 1,500 species of succulent herbs and small shrubs (Mort *et al*., 2005). Distribution of Crassulaceae is almost cosmopolitan, and most often its members occur in arid and mountainous habitats of temperate and sub-tropical regions. The family presents a particularly high diversity in South Africa, Mexico, Macronesia, the Mediterranean area, and Himalaya (Gontcharova and Gontcharov, 2009). As currently defined, the genus *Cotyledon* includes 10 species that occur primarily in South Africa (Mort *et al*., 2005). Several Crassulaceae genera, like *Cotyledon*, *Echeveria*, *Kalanchoë*, *Sedum* and *Sempervivum*, have been introduced in Brazil as ornamentals (Souza and Lorenzi, 2005).

Cotyledon orbiculata L. (pig's ear), a succulent shrub native to South Africa, has thick leaves green to grey in colour, often showing a red line around the margin and comprises five varieties differing by leaf morphology and biogeography. This species is cultivated as an ornamental plant, for medicinal purposes to treat epilepsy, boils, corns and warts, earache and toothache, and is used to expel worms (Harris and Reynolds, 2004; Rowley, 2007). Like other succulent plants, *C. orbiculata* is propagated by cuttings, which increases the risk of introduction and dissemination of new viruses.

In fact, viruses like *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV), both members of the genus *Tospovirus*, and a strain of *Tobacco streak virus* (TSV, genus *Ilarvirus*) have been reported in crassulaceous species, especially *Kalanchoë* spp. The occurrence of TSWV in *Sedum* sp. has also been reported (Hausbeck *et al*., 1992). In addition, *Kalanchoe mosaic virus* (KMV, *Potyvirus*), *Kalanchoe latent virus* (KLV, *Carlavirus*), *Kalanchoe top-spotting virus* (KTSV, *Badnavirus*), *Sonchus yellow net virus* (SYNV, *Nucleorhabdovirus*), and other viruses of minor importance like the *Alfalfa mosaic virus* (AMV, *Alfamovirus*), Kalanchoe isometric virus (KIV) and *Potato yellow dwarf virus* (PYDV, *Nucleorhabdovirus*) have been detected in *K. blossfeldiana* (Burnett and Long, 1962; Hearon, 1982; Lockhart and Ferji, 1988; Husted *et al*., 1994; Bech and Husted, 1995; Brunt *et al*., 1997; Bouwen *et al*., 2002). Isometric virus-like particles have also been detected in crude sap from *K. pinnata*, *K. daigremontiana* and *K. tubiflora* (Izaguirre-Mayoral *et al*., 1990). Recent works have described the occurrence of *Tobacco rattle virus* (TRV, *Tobravirus*) in *Sedum* sp. (Lockhart and Mason, 2010), and *Aeonium ringspot virus* (AeRSV, *Nepovirus*) in *Aeonium* spp. (Sorrentino *et al*., 2013). The occurrence of two or more plant viruses in mixed infections is common

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Table 1. Symptoms induced by *Cotyledon orbiculata* viruses in artificially inoculated hosts

Indicator plants	Flexuous virus	Bacilliform virus	Mixed infection
Amaranthaceae			
Chenopodium amaranticolor	**L**: NS	-	**L**: NS
C. murale	**L**: CS	-	**L**: CS
C. quinoa	**L**: NS	-	**L**: NS
Gomphrena globosa	-	**L**: NR	**L**: NR
Asteraceae			
Sonchus oleraceus	-	-	-
Zinnia elegans	**S**: M, LD, CB	**S**: VC	**S**: M, LD, CB
Crassulaceae			
Kalanchoë blossfeldiana	-	**S**: La	**S**: La
Gentianaceae			
Eustoma grandiflorum	**S**: Y, N, PD	**L**: PP **S**: M, LD, PP	**L**: PP **S**: Y, N, PD
Solanaceae			
Datura metel	-	-	-
D. stramonium	-	**L**: PP	**L**: PP
Nicotiana benthamiana	**S**: M, LD, B	**L**: CS **S**: M, LD, B	**L**: CS **S**: M, LD, B, St
N. clevelandii	**S**: M, LD, B	**S**: M, LD, B	**S**: M, LD, B
N. debneyi	-	**L**: PP	**L**: PP
N. glutinosa	-	**L**:PP **S**: MN, LD, Y	**L**:PP **S**: MN, LD, Y
N. megalosiphon	**S**: M, LD, B	**S**: VB	**S**: M, LD, B
N. tabacum 'Samsun'	-	-	-
N. tabacum 'White Burley'	-	**L**: La	**L**: La
Petunia x hybrida	-	-	-

L = local symptoms; S = systemic symptoms; - = not infected; B = blistering; CB = colour breaking; CS = chlorotic spots; La = latent infection; LD = leaf deformation; M = mosaic; N = necrosis; NR = necrotic rings; NS = necrotic spots; PD = plant death; PP = pinpoint lesions; St = stunt; VB = vein banding; VC = vein clearing; Y = yellowing

(Dietrich and Maiss, 2003). Lockhart and Ferji (1988) reported that the KLV (carlavirus) is commonly found in mixed infection with KTSV (badnavirus) in *Kalanchoe* sp. However, in Brazil only an uncharacterized potyvirus has been recorded in *Kalanchoë* sp. (Braz *et al*., 1996).

The aim of this work was to identify and characterize viruses associated with foliar mosaic and distortion of *C. orbiculata* from the succulent plant collection of São Paulo University garden, São Paulo state, Brazil.

MATERIALS AND METHODS

Virus sources, mechanical and aphid transmission tests. Leaf tissues from naturally infected *C. orbiculata* plants were ground in 0.01 M phosphate buffer pH 7.0 supplemented with 0.04 M sodium sulfide and mechanically inoculated onto species of Amaranthaceae, Asteraceae, Crassulaceae, Gentianaceae and Solanaceae. Aphid transmission tests were conducted with *Myzus persicae* maintained on healthy *Nicotiana tabacum*. At least 50 aphids were transferred to Petri dishes for a 1 h starvation period. After 10 min of virus acquisition feeding on infected *C. orbiculata*, batches of 5 aphids were transferred to 10 virus-free plants of *N. benthamiana* (one batch per plant). Aphids were killed with insecticide after 24 h. Aphid transmission test were repeated twice.

Electron microscopy. Foliar extracts from infected *C. orbiculata* and experimental hosts were negatively stained with 2% uranyl acetate. Tissues of naturally infected *C. orbiculata* and *Datura stramonium* were processed and used for cytological analyses, according to the methods described by Martelli and Russo (1984).

Serological tests. An antiserum to SYNV was used for DAS-ELISA. Leaf extracts of naturally infected *C. orbiculata* and experimental hosts were prepared by grinding 1 g of tissues in 10 ml of sample buffer (PBST plus 2% PVP). SYNV-infected and healthy plants of *K. blossfeldiana* were used as positive and negative controls, respectively.

Molecular methods and sequence analyses. Total RNA was extracted from naturally infected *C. orbiculata* and experimentally infected *N. benthamiana* using Trizol LS reagent according to the manufacturer's instructions (Gibco BRL, USA). Reverse transcription was performed using an oligo dT as a complementary primer. PCR amplification was performed using an oligo dT and Poty 2 primer: GGB AAY AAY AGY GGD CAR CC (Gibbs and Mackenzie, 1997). Amplified DNA fragments were cloned into the pGEM-T vector (Promega, USA) and sequenced by the ABI Prism, using the Big Dye Terminator System (PE Applied Biosystems, USA). The nucleotide (nt) and the predicted amino acid (aa) sequences were compared

with those deposited in GenBank using BLAST (Altschul *et al*., 1990), aligned with the corresponding sequences of 32 *Potyvirus* species and isolates. The percentage of identity at the nt level between sequences was determined by PAUP v. 4.0b10 for Macintosh. Maximum parsimony (MP) analysis was made with nt and aa sequences using PAUP v. 4.0b10 with heuristic search and equal weighting (Swofford, 2002). The stepwise addition algorithm was also used, and bootstrap values were determined using the branch-and-bound method (Thompson, 1987). Neighbor-joining (NJ) and maximum likelihood (ML) analyses with nt sequences was done with PAUP v. 4.0b10, after estimating the nucleotide substitution and gamma distribution with the software Modeltest v. 3.06 (Posada and Crandal, 1998).

RESULTS AND DISCUSSION

Although *C. orbiculata* had been introduced in Brazil as an ornamental species, it has also been used for medicinal purposes (Castellucci *et al*., 2000; Amabeoku *et al*., 2007). Despite the introduction and vegetative propagation of Crassulacaeae species, in Brazil only one uncharacterized potyvirus has been reported in *Kalanchoë* sp. (Braz *et al*., 1996).

Electron microscopic examination of leaf extracts from naturally infected *C. orbiculata* and experimentally infected *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana benthamiana*, *N. clevelandii* and *N. megalosiphon* revealed the presence of flexuous particles *ca.* 780 nm in length. Bacilliform particles were also observed in leaf extracts from *Datura stramonium* and *N. benthamiana* (not shown). The simultaneous presence of bacilliform particles and of bundles of filamentous particles in foliar tissues of *C. orbiculata* (Fig. 1a) clearly indicates co-infection by both viruses in the same cell. The same condition was rarely observed by Fránová *et al.* (2006) in *Daphine mezereum* infected with *Daphne mosaic virus* (DapMV) as well as rhabdovirus-like particles.

Although the occurrence of two or more plant viruses in mixed infections is common and well documented for many viruses it is unclear whether they generally multiply within the same cells or if co-existence is restricted to a few cells because of spatial separation (Dietrich and Maiss, 2003).

Using light microscopy, McWhorter and Price (1949) reported that the different viruses could occur together within the same cells. Studies have demonstrated that populations of identical viruses replicated predominantly in discrete areas and remained separately distributed in both inoculated and upper uninoculated leaves in co-inoculated plants. In contrast, distinct virus populations in mixed infection were observed in the same area of *N. benthamiana* leaves, strongly suggesting that both viruses were replicated in the same tissues of infected leaves (Dietrich and Maiss, 2003; Takahashi *et al*., 2007).

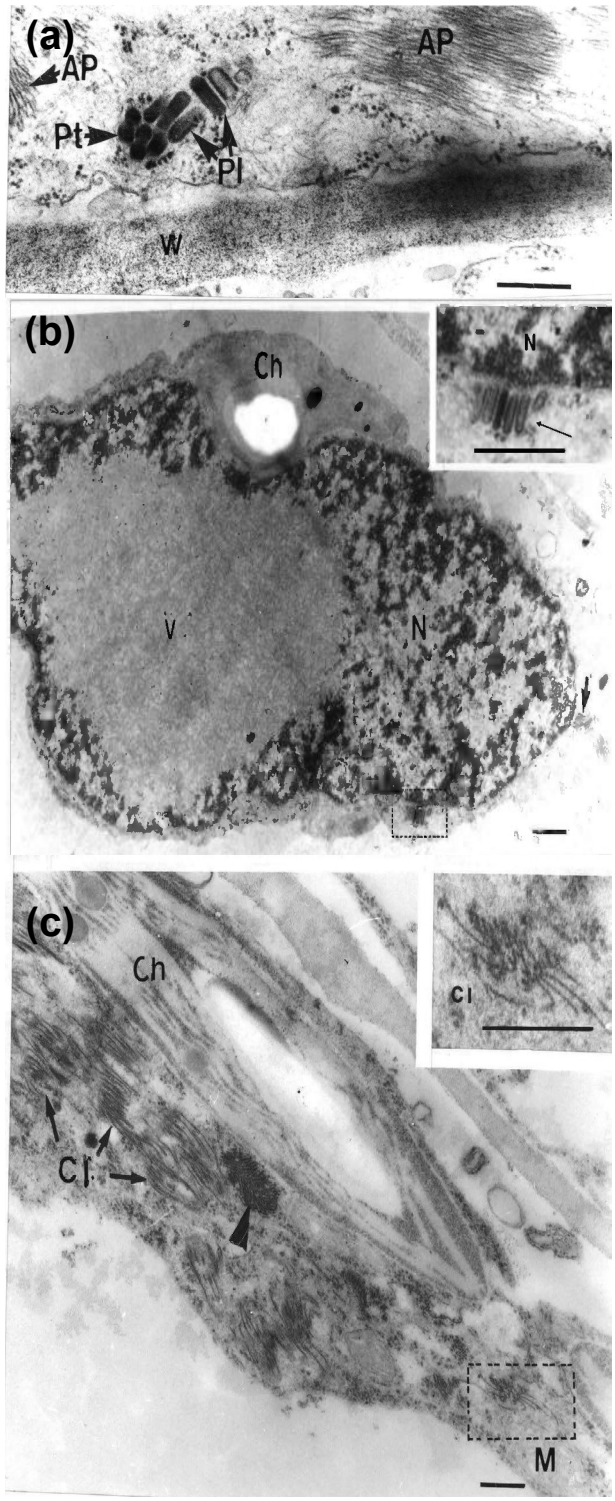

Fig. 1. Ultrathin sections of leaf tissue of naturally infected *Cotyledon orbiculata*. **(a)** Transversally (Pt) and longitudinally (Pl) sectioned bacilliform particles and elongated virus particles arranged in bundles (AP). W = cell wall. Bar = 300 nm. **(b)** Nucleus (N) containing a translucent viroplasm-like inclusion (V) and virus particles in the perinuclear space (arrow). Detail of bacilliform particles in the perinuclear space (arrow). Bars = 700 nm. **(c)** Cytoplasmic cylindrical inclusions (CI) sectioned in different planes and aggregates of viral particles transversally sectioned (arrow). Detail of cytoplasmic cylindrical inclusion. Ch = Chloroplast, M = Mitochondria, W = Cell wall. Bars = 300 nm.

The occurrence of translucent inclusion in the nucleus representing viroplasms (Jackson *et al*., 2005) and accumulation of bacilliform particles in the perinuclear space (PE) in cells of naturally infected *C. orbiculata* (Fig. 1b) have been associated with the *Nucleorhabdovirus* genus of plant rhabdoviruses (Jackson *et al*., 2005; King *et al*., 2012). These observations were also reported for *Kalanchoë blossfeldiana* infected with SYNV (Bouwen *et al*., 2002). Furthermore, as it occurs with members of the genus *Nucleorhabdovirus* (Dietzgen *et al*., 2012) in the cells of experimentally infected *D. stramonium* the three stages of virus maturation were recognized, i.e. presence of nucleocapsids within the nuclei, budding processes from the inner nuclear membrane, and virion accumulation in PE (data not shown). These findings agree with *Nucleorhabdovirus* replication in cell nuclei (Martins *et al*., 1998; Ammar *et al*., 2009). The presence of cytoplasmic cylindrical inclusions (CI) sectioned at different planes and pinwheels (Fig. 1c) provides strong evidence of infection caused by a virus belonging to family *Potyviridae*, as this is one of the main characteristics used in the demarcation of virus in the family (King *et al*., 2012).

The viruses isolated from *C. orbiculata* were separated by differential host and transmission by *M. persicae* in a non-persistent manner to *N. benthamiana* from naturally infected plants of *C. orbiculata*. Symptoms appeared in 70% of the plants two weeks after exposure of the plants to the aphids. The flexuous virus induced local lesions in *Chenopodium* and mosaic, leaf deformation and blistering in *Nicotiana benthamiana*, *N. clevelandii* and *N. megalosiphon* but did not infect *Datura* spp., *Gomphrena globosa*, *K. blossfeldiana* and *Petunia x hybrida* (Table 1). The occurrence of a few viruses has been reported for Crassulaceae species. Of these, the *Kalanchoe mosaic virus* (KMV) is a definitive potyvirus species, which has been reported in *Kalanchoe* spp. (Husted *et al*., 1994). However, unlike *C. orbiculata* flexuous virus, KMV did not induce symptoms on *N. benthamiana* and *N. clevelandii*. The bacilliform virus was not transmitted to *Chenopodium* species, but it caused local lesions in *G. globosa* and *D. stramonium*, systemic symptoms in *N. benthamiana*, latent systemic infection in *K. blossfeldiana* and latent local infection in *N. tabacum* White Burley. It should be underlined that the nucleorhabdovirus host range was determined after its isolation from *D. stramonium*, and the infection was confirmed by DAS-ELISA with SYNV antiserum, which gave consistent positive reaction with absorbance at 405 nm ranging between 0.800 and 1.200. Potyvirus infection was confirmed by electron microscopy and differential host.

Co-infection caused by both potyvirus and nucleorhabdovirus on *N. benthamiana* was more drastic as compared to that caused by viruses separately, since they also induced stunting of plants. The same condition has been described for mixed infection caused by SYNV and the *Bidens mottle virus* (BiMoV) in *Bidens pilosa* (Christie *et al*., 1974).

Synergism normally occurs in mixed infections, when the two viruses involved are unrelated, and the resulting increase in symptom severity have important practical implications for some diseases, especially those caused by the direct outcome of a synergistic interaction (Zhang *et al*., 2001; Martin *et al*., 2004). According to Pagán *et al*. (2010), most reported examples of synergism in different hosts involve a potyvirus and another unrelated species, and synergism may be strain-specific. One aspect that deserves attention concerns the fact that *C. orbiculata* is an introduced species. Also, there were no healthy plants in the collection site that could be inoculated in order to determine whether the original symptoms are caused by one virus or by both.

The identification and characterization of the *C. orbiculata* potyvirus were carried out by RT-PCR using universal primers (Gibbs and Mackenzie, 1997), and sequencing of the fragments obtained. The product amplifed by RT-PCR from the flexuous virus isolated from *C. orbiculata*, consisted of a fragment 1,752 nt in size comprising 639 nt from the 3' end of NIb (nuclear inclusion protein b), 921 nt from the coat protein (CP) coding region, 192 nt from the 3' UTR, and the poly (A) tail (accession No. JN572103) of the genome of what appeared to be a putative potyvirus. The dipeptide QG located at aa position 213-214 was selected as the putative cleavage site between the NIb and CP. Consensus sequence motifs GDD and QPSTVVDN were found in the NIb protein, 165 and 200 aa upstream of the putative NIb/CP cleavage site. Consensus motifs DAG required for aphid transmission of potyviruses (King *et al*., 2012), MVWCIENGTSP, AFDF and QMK APAL at the amino acids 5, 117, 200 and 220, respectively, from the Q/G cleavage site were found on the putative CP protein.

BLAST analysis confirmed that the sequence of the *C. orbiculata* virus was comparable to that of potyvirus species. The highest identities in nt and aa were observed in the Bidens mosaic virus (BiMV), *Alstroemeria mosaic virus* (AlMV), *Pepper severe mosaic virus* (PepSMV), *Potato virus Y* (PVY^{NTN}) and Sunflower chlorotic mottle virus (SuCMoV), when compared with homologous sequence of the *C. orbiculata* potyvirus (70.1 to 78.3%). CP sequences were also compared to those available for members of the PVY subgroup, and a Brazilian isolate of BiMV described by Inoue-Nagata et al. (2006) shared the highest nt and aa identities (72.3% and 78.3%, respectively). CP aa sequence identity under ca. 80% may be an indicator of different species in the same genus (King *et al*., 2012). Adams *et al*. (2005), studying molecular criteria for genus and species discrimination within the *Potyviridae*, suggested that 76–77% nt identity is the optimal species demarcation criterion for the CP, when complete sequences of the polyprotein are not available.

The topology of the tree constructed under MP using nt and aa sequences of CP and NIb was similar (not shown) and indicated that *C. orbiculata* potyvirus belongs to the PVY subgroup, supported by a high bootstrap

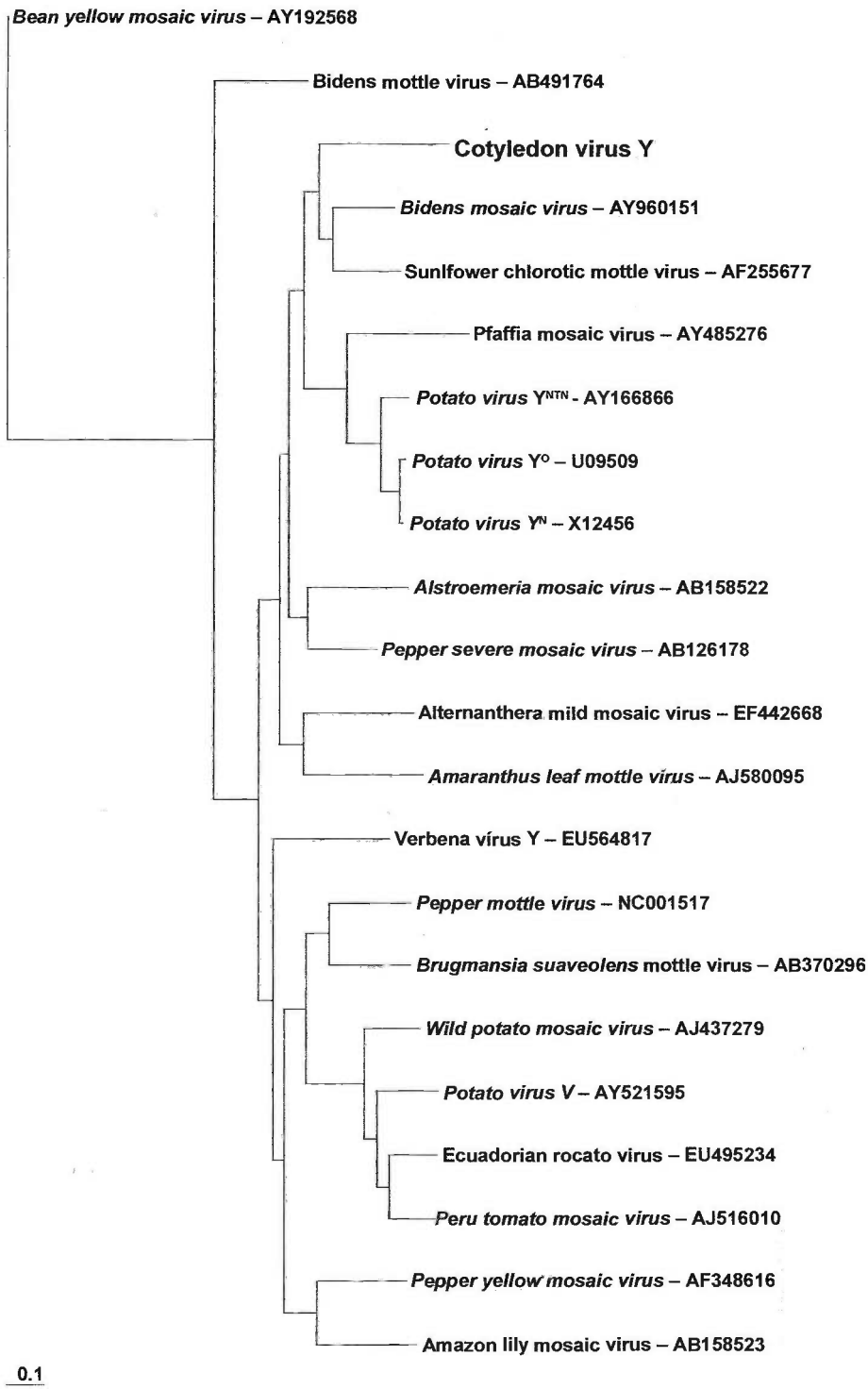

Fig. 2. Maximum likelihood tree constructed with coat protein sequences of Cotyledon virus Y (JN572103) and comparable *Potato virus Y* (PVY) subgroup sequences retrieved from GenBank using the PAUP program with the F81 substitution model and gamma-distributed rate (G=1.63). *Bean yellow mosaic virus* (BYMV) was used as outgroup.

value, and therefore was initially named Cotyledon virus Y (CotVY). *Ryegrass mosaic virus* (RGMV) was used as outgroup, as rymoviruses are consistently a close sister group to potyviruses (Gibbs and Ohshima, 2010).

As revealed by the phylogenetic tree constructed under ML condition using CP and NIb sequences of PVY subgroup species, tree topologies were similar. The putative Cotyledon virus Y shared the same common ancestor with BiMV and SuCMoV (Fig. 2). On the other hand, when NIb sequences were compared, CotVY formed a sister group with AlMV; additionally, CotVY and AlMV share the same common ancestor with PepSMV. Although

C. orbiculata is a species native to South Africa, the infection caused by this potyvirus may have occurred after its introduction in Brazil. According to Quenouille *et al.* (2013), the species PVY belongs to a large clade of nineteen potyvirus species, sometimes referred to as the 'PVY group' or 'PVY clade, which are mostly present in America and, more particularly, in South America. Furthermore, they were isolated from plants native to that continent, and there is a significant association between the potyvirus species of the PVY clade that are found outside the American continents and their occurrence in crops propagated by vegetative propagation (Gibbs and Ohshima, 2010; Quenouille *et al*., 2013). Thus, it is clear that the PVY group diverged in the Americas and, more specifically, South America, and that a worldwide dispersal of some of its members has involved mostly human activities such as trade of potato tubers and bulbs or rhizomes of ornamental plants (Gibbs and Ohshima, 2010; Quenouille *et al*., 2013).

Members of this aphid-transmitted virus group generally infect Solanaceae, but other natural hosts have been discovered in Alstroemeriaceae, Amaranthaceae, Amaryllidaceae, Asteraceae (Giolitti *et al*., 2010) and Verbenaceae (Kraus *et al*., 2010). The lineage that eventually gave rise to CotVY subsequently became better adapted to infect members of Crassulaceae, as described for SuCMoV (Inoue-Nagata *et al*., 2006).

The percent identity between CotVY and other potyviruses, in line with phylogenetic analyses, indicates that CotVY is a putative new potyvirus species belonging to the PVY subgroup, one of the most important groups of plant viruses in agriculture and floriculture.

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