Characterization of a New Tymovirus Causing Stunting and Chlorotic Mosaic in Naranjilla (*Solanum quitoense*)

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Abstract

Naranjilla (“little orange”), also known as lulo (*Solanum quitoense* Lam.), is a perennial shrub species cultivated in the Andes for fresh fruit and juice production. In 2015, a naranjilla plant exhibiting stunting, mosaic, and chlorotic spots was sampled in the Pastaza province of Ecuador and maintained under greenhouse conditions. An infectious agent was mechanically transmitted to indicator plants and was subjected to biological and molecular characterization. Spherical particles approximately 30 nm in diameter, composed of a single 20-kDa capsid protein, were observed under an electron microscope in infected naranjilla plants. High-throughput sequencing conducted on inoculated *Nicotiana benthamiana* plants produced a single sequence contig sharing the closest relationship with several tymoviruses. The entire 6,245-nucleotide genome of a new

tymovirus was amplified using reverse-transcription polymerase chain reaction and resequenced with the Sanger methodology. The genome had three open reading frames typical of tymoviruses, and displayed a whole-genome nucleotide identity level with the closest tymovirus, *Eggplant mosaic virus*, at 71% (90% coverage). This tymovirus from naranjilla was able to systemically infect eggplant, tamarillo, *N. benthamiana*, and naranjilla. In naranjilla, it produced mosaic, chlorotic spots, and stunting, similar to the symptoms observed in the original plant. The virus was unable to infect potato and tobacco and unable to systemically infect pepper plants, replicating only in inoculated leaves. We concluded that this virus represented a new tymovirus infecting naranjilla, and proposed the tentative name Naranjilla chlorotic mosaic virus (NarCMV).

Naranjilla, also called lulo (*Solanum quitoense* Lam.), is a perennial shrub species cultivated in subtropical areas of Ecuador and Colombia. The naranjilla fruit pulp is rich in vitamins A, C, and K, and provides an important dietary supplement, which is nutritionally similar to orange juice (Acosta et al. 2009; Gancel et al. 2008). In Ecuador, naranjilla is grown on approximately 10,000 ha and constitutes the main income source for thousands of families in the provinces of Tungurahua, Napo, Pastaza, and Morona Santiago in the Amazon region. Production has increased significantly in the past two decades and naranjilla is now widely available as fresh fruit, juice, and a flavoring for other products such as ice cream. Pests and diseases affect naranjilla production and limit potential benefits and income from the crop by local farmers. Vascular wilt caused by the soilborne pathogen *Fusarium oxysporum* and the root knot nematode (*Meloidogyne* spp.) are the major problems in “common naranjilla” (*S. quitoense*) production (Monteros et al. 2004; Ochoa et al. 2001). The severity of these pathogens led to important changes in the cropping systems such as grafting scions onto resistant rootstock and development of interspecific hybrids (Integrated Pest Management Innovation Lab 2013). One of these hybrids, known as Puyo (*S. sessiliflorum* × *S. quitoense*), has become the “cultivar” of choice in most production areas of the Amazon region (Dennis and Herner

1985; Heiser and Anderson 1999). However, the small size of its fruit has challenged breeders to develop hybrids with both good agronomic and disease resistance traits.

Thus far, there have been no reports of viral infections in naranjilla. Among the possible reasons for this could be the lack of prominent symptoms, latent infections, or simply an underestimation of the potential damage caused by these pathogens, especially when present in mixed infections. Furthermore, the vegetative propagation nature of common interspecific hybrids poses a direct threat to the sustainability of this crop due to the lack of naranjilla certification programs. In addition, the continuous search for hybrids and varieties endowed with optimal production traits may trigger the appearance of new or reemerging viruses, which are usually symptomless in wild species. Symptomatic (mosaic, mottling, leaf deformation, and stunting) tamarillo (*S. betaceum*), a fruit crop commonly grown side-by-side with naranjilla and other solanaceous crops in the Amazon region, was recently reported as infected with *Potato virus Y*, *Potato virus V*, and *Potato leafroll virus*, and others traditionally considered potato viruses (Espinoza et al. 2017; Insuasti et al. 2016).

This prompted us to investigate the virus status of naranjilla hybrid Puyo in the main production areas of Ecuador, using several approaches. This study reports a novel tymovirus, provisionally named Naranjilla chlorotic mosaic virus (NarCMV). The objectives of this study were to characterize the host range of this new virus, determine its genome organization and phylogenetic relationship with known viruses, and develop methods for its detection.

Materials and Methods

Virus origin and maintenance. In 2015, several naranjilla cultivars, including the Puyo hybrid, were collected from Pastaza province and greenhouse propagated at the Ecuadorean Agricultural Research Institute (Instituto Nacional Autónomo de Investigaciones Agropecuarias) in Santa Catalina, Quito, Ecuador. Plants were monitored for virus-like symptoms. One sample from the Puyo hybrid exhibited mosaic and chlorotic spots. Symptomatic leaves were ground in 50 mM sodium phosphate buffer, pH 7.0, plus 20 mM sodium sulfite, and mechanically inoculated onto *Nicotiana benthamiana* plants,

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which developed virus-like symptoms. The isolate was maintained in *N. benthamiana* through periodic reinoculations into young healthy plants at the four- to six-leaf stage.

Physicochemical characterization and antisera production.

The virus was purified using a modification of the protocol described by Dunn and Hitchborn (1965). Briefly, 50 g of systemically infected *N. benthamiana* leaves were homogenized in 100 ml of cold bentonite preparation mixed with 0.1 M MgSO_4 (pH 7.4). Leaf homogenate was squeezed through cheesecloth and subjected to two cycles of differential centrifugation consisting of low speed centrifugation for 15 min at $21,000 \times g_{\text{max}}$ in a JLA16.250 rotor (Beckman Avanti J-25) to separate the bentonite and 70 min at $64,000 \times g$ in a Ti45 rotor (Beckman) to pellet the virus. The final pellet was resuspended in buffer (0.05 M MgSO_4 + 0.01 M Na_2HPO_4 + 0.01 M KH_2PO_4 , pH 7.4), and stored at 4°C with 0.1% sodium azide.

Dilutions of the purified virus were separated in 4 to 20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels (Bio-Rad) and stained using Coomassie Brilliant Blue G-250 (Bio-Rad). Two protein marker sets were used: an SDS polyacrylamide gel electrophoresis (PAGE) broad-range standard (Bio-Rad catalog number 161-0318) and a Precision Plus Protein Dual Color standard (Bio-Rad catalog number 161-0374). The major protein band was excised from the gel, subjected to in-gel trypsin digestion, and analyzed using the liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomics instrument at the Food Research Center of the University of Idaho, according to the previously described protocol (Hanson et al. 2016). Mascot v2.5 software (Matrix Science) was used for MS/MS ion matching and protein identification.

The bentonite-purified virus preparation was also used for antisera production by Cocalico Biologicals in rabbit and in mouse, after a series of three intramuscular and subcutaneous injections with Freund's adjuvant. The resulting antisera were tested against infected and healthy *N. benthamiana* plants using a triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) according to the methodology of Clark and Adams (1977), with some modifications as described by Nikolaeva et al. (2012). Specificity of polyclonal antibodies was initially tested against symptomatic (inoculated) or healthy (noninoculated) *N. benthamiana* leaf tissue in TAS-ELISA. The two antisera, UID-41 (rabbit) and UID-M28 (mouse), were used to develop a TAS-ELISA-based detection protocol. Plant extracts were obtained by grinding leaf tissue in an ELISA extraction buffer (Nikolaeva et al. 2012). These extracts, as well as purified virus preparations, were analyzed using 4 to 20% gradient polyacrylamide gel electrophoresis (PAGE) (Bio-Rad) followed by Coomassie staining as described above, or by Western blots. For Western blot, proteins separated by PAGE were transferred to nitrocellulose membranes and probed with the putative NarCMV antibody, as described previously (Karasev et al. 2010).

Biological characterization. Infected *N. benthamiana* plants were used as a source to inoculate six plant species: *S. quitoense* Lam. (naranjilla), *S. melongena* 'Fairy Tale' (eggplant), *S. betaceum* (tamarillo), and three additional solanaceous species: *N. tabacum* 'Burley' (tobacco), *Capsicum annuum* 'Early California Wonder' (pepper), and *S. tuberosum* 'King Edward' (potato). All plants were grown in an insect-proof growth room at 23 to 26°C before and after inoculations, under artificial light provided by fluorescent and incandescent lamps with a day-and-night cycle of 18 and 6 h, respectively. Plants were mechanically inoculated at the four- to six-leaf stage and observed for symptoms every 4 to 5 days after inoculation for 6 to 8 weeks. Three plants per species or cultivar were tested in a single experiment which always included healthy control plants. RT-PCR was used to confirm the results from visual observations using two primers used for sequencing, tymo1294F and tymo2278R, which amplify a 1-kb fragment within the overlapping 69- to 200-kDa polyprotein (69K to 200K) genes. Total RNA was extracted from 200-mg samples of inoculated (7 to 10 days postinoculation) and upper noninoculated (3 to 4 weeks postinoculation [wpi]) leaves using the Dellaporta extraction protocol (Dellaporta et al. 1983).

Transmission electron microscopy. Symptomatic leaves (3 to 5 g) were harvested from mechanically inoculated naranjilla, tamarillo, eggplant, and *N. benthamiana* 18 wpi and used for partial virus

purification and transmission electron microscope (TEM) observations. Leaves were ground in liquid nitrogen and suspended in four volumes (wt/vol) of 0.5 M sodium phosphate buffer, pH 7.4, containing 1 M urea, 5% (wt/vol) PVP-40, and 0.5% (vol/vol) 2-mercaptoethanol. Plant extracts were filtered and the liquid was centrifuged at $19,800 \times g_{\text{max}}$ for 10 min. Triton X-100 (5%, vol/vol) was added to the supernatant and was left at 4°C overnight to allow additional precipitation. Samples were centrifuged again at $19,800 \times g_{\text{max}}$ for 10 min. The supernatant was layered over a 30% sucrose cushion and centrifuged at $109,000 \times g_{\text{max}}$ for 2 h at 10°C. The resulting pellets were resuspended in 0.3 ml of 0.1 M sodium phosphate buffer, pH 7.4, and vortexed with an equal volume of chloroform. The resulting emulsion was centrifuged at $18,400 \times g_{\text{max}}$ for 20 min and the upper aqueous phase containing virions was applied to formvar-coated carbon grids. Grids were stained with 2% sodium phosphotungstate, pH 7.0, and visualized on a Hitachi 7700 transmission electron microscope at the Electron and Confocal Microscopy Unit, United States Department of Agriculture-Agricultural Research Service, Beltsville Agricultural Research Center.

High-throughput sequencing, Sanger sequencing, and sequence analysis. Symptomatic *N. benthamiana* leaves were used as a source for the high-throughput sequencing (HTS) analysis. Total RNA was extracted by a Qiagen RNA plant mini kit and sent for cDNA library construction and sequencing to SeqMatic. HTS was performed on a NextSeq 500 Illumina platform as single 75-bp reads. The raw HTS reads were trimmed and assembled into contigs by CLC Workbench 9.0.

Sanger sequencing was performed on a series of overlapping RT-PCR fragments amplified on total RNA extracted from the infected *N. benthamiana* plants via the Dellaporta method, essentially as described previously (Green et al. 2017). Primers used to amplify these DNA fragments are listed in Supplementary Table S1. PCR fragments were treated with ExosapIt (Affymetrix) and submitted for sequencing to Genewiz, Inc. The 5' terminus was amplified using the 5' RACE Kit (Roche) according to the manufacturer's instructions using primers tymo1008R and tymo180R. To amplify the 3' terminus of the genome, total RNA was polyadenylated using *Escherichia coli* Poly(A) Polymerase (New England Biolabs) according to manufacturer's instructions, the first cDNA strand was synthesized using an anchored oligo(dT) primer, and then PCR was conducted using the tymo5975F and tymo5566F forward primers in combination with the anchored oligo(dT) reverse primer. The sequence contigs obtained were assembled using the SeqMan Pro program of the Lasergene 9 program suite (DNASTAR).

For the multiple sequence alignment, MUSCLE (Edgar 2004) implemented in the program MEGA, version 7 (Kumar et al. 2016), was used with the default parameters. Complete genomes (one representative isolate per species), available at the National Center for Biotechnology Information GenBank, from all genera within the family *Tymoviridae* were used for analysis (Table 1). Phylogeny inference was conducted based on the aligned whole genomes using the maximum-likelihood method implemented in MEGA 7, after using its model selection function to change the appropriate model parameters (Kumar et al. 2016). All nodes with <70% support were collapsed. The tRNA secondary structure was predicted using RNAfold (Lorenz et al. 2011).

Results

Biological characterization. The new virus from naranjilla systemically infected eggplant, *N. benthamiana*, tamarillo, and naranjilla (Fig. 1; Table 2). The infection in Fairy Tale eggplant was essentially symptomless, producing only transient mild mosaic 3 to 5 wpi. Nevertheless, virus titer remained high in infected eggplant, easily detected by RT-PCR and also by TEM up to 18 wpi. In *N. benthamiana*, the virus induced severe mosaic, vein clearing, and chlorotic spots, which became visible at 2 wpi (Fig. 1A) and later developed into blistering, curling, and other leaf deformations. In common naranjilla, the virus produced mosaic and stunting, visible by 4 wpi; chlorotic spots formed on upper uninoculated leaves, which eventually necrotized and fell off, leaving a perforated leaf surface (Fig. 1B). The symptoms in inoculated naranjilla maintained in the

growth room were similar to those observed in the plant from which the virus was originally isolated. In tamarillo, the systemic spread of the virus was very rapid, inducing visible mosaic 7 to 10 days post-inoculation. Later, at 3 to 5 wpi, the virus produced bright chlorotic mosaic and yellowing in upper uninoculated leaves of the plant, with small necrotic lesions becoming visible at 8 wpi (Fig. 2C). No stunting associated with the virus symptoms was observed in infected tamarillo plants for the 18 weeks of observation (Table 2). The virus did not systemically infect potato, tobacco, or pepper, although virus was easily detected in inoculated leaves of pepper plants. Interestingly, these inoculated pepper plants had retarded growth despite the apparent absence of the virus in upper, uninoculated leaves. The results of the host range study are summarized in Table 2.

TEM, virus purification, LC-MS/MS analysis, and immunodetection. Spherical particles, approximately 30 nm in diameter, were observed under TEM in infected naranjilla (Fig. 2A), tamarillo, eggplant, and *N. benthamiana* plants (not shown). The particles, revealed by negative staining, contained a mixture of intact and empty isometric particles, typical of tymovirus morphology (Koenig and Lesemann 1979). The purification protocol utilizing bentonite applied to infected *N. benthamiana* plants resulted in a virus preparation producing a single major protein band of approximately 20 kDa in SDS-PAGE (Fig. 2B). When this protein band was subjected to the LC-MS/MS analysis, a 9-amino-acid (aa) peptide (LSSPLLQAS) was identified by Mascot among the products of digestion with the highest score of 31. This peptide matched the C-terminal peptide in the capsid protein (CP) of *Chiltepin yellow mosaic virus* (ChiYMV), a tymovirus isolated from pepper (Pagán et al. 2010). The combination of morphological characteristics (Fig. 2A), the approximately 20-kDa size of the CP (Fig. 2B), and the presence of the 9-aa peptide in the CP matching the C-terminus of a known tymovirus, ChiYMV, indicated that the virus isolated from naranjilla in Ecuador may belong to the genus *Tymovirus*.

The purified virus preparation was used as an antigen for the production of two antisera, in rabbit and in mouse. The antisera displayed high titers against the virus in the infected *N. benthamiana* plants, allowing the use of the rabbit antiserum for coating at the 1:20,000 dilution and the mouse antiserum for detection at the 1:30,000 dilution. This TAS-ELISA format gave a ratio (infected/healthy) of absorbance at 405 nm of up to 29 at 3 h after the substrate was added, increasing to approximately 35 at 5 h in the TAS-ELISA format. It allowed detection of the virus in infected leaf tissue extract of *N. benthamiana*, defined as infected plant signal exceeding the healthy plant signal threefold, following dilution of up to 1:1,000,000 (Fig. 2C). When used as a detecting antibody in Western blot analysis, the rabbit antiserum bound a single 20-kDa protein band in plant extract from infected *N. benthamiana* (Fig. 2D).

Sequencing and genome analysis. HTS analysis conducted on inoculated *N. benthamiana* plants produced 20,189,606 raw data reads that assembled into 4,221 contigs longer than 500 nucleotides (nt). BLASTn and BLASTx analyses and assembly (Altschul et al. 1990) of these contigs revealed a single contig of 6,214 nt with closest identities to several tymoviruses, particularly *Eggplant mosaic virus* (EMV). To confirm and complete the sequence of this novel tymovirus, a set of primers was designed for Sanger sequencing based on this initial HTS sequence. The entire 6,245-nt genome of this novel tymovirus was amplified using RT-PCR, as previously described (Green et al. 2017), and resequenced with the Sanger methodology. The sequence has been deposited into GenBank under accession number MG323924.

The sequenced genome was conceptually translated and found to encode three open reading frames (ORF) typical of tymoviruses (Fig. 3A). The largest ORF starts at position 113 and terminates at position 5,560, encoding a 200K protein containing replication-related domains with presumed methyltransferase, protease, helicase, and RNA-dependent RNA-polymerase (RdRp) activities. A

Table 1. Complete genomes of the virus species from the family *Tymoviridae* used for phylogenetic analysis^a

Number	Viruses (standard abbreviation)	GenBank accession	Reference
1	<i>Eggplant mosaic virus</i> (EMV)	KJ690172	...
2	Naranjilla chlorotic mosaic virus (NarCMV)	MG323924	This article
3	<i>Andean potato mild mosaic virus</i> (APMMV)	JX508290	Kreuze et al. 2013
4	<i>Chiltepin yellow mosaic virus</i> (ChiYMV)	FN563124	Pagán et al. 2010
5	<i>Tomato blistering mosaic virus</i> (ToBMV)	KC840043	...
6	<i>Andean potato latent virus</i> (APLV)	JX508291	Kreuze et al. 2013
7	<i>Tomato yellow blotch virus</i> (ToYBV)	EU779803	...
8	<i>Physalis mottle tymovirus</i> (PhyMV)	Y16104	...
9	<i>Chayote mosaic tymovirus</i> (ChaMV)	AF195000	...
10	<i>Nemesia ring necrosis virus</i> (NeRV)	AY751778	Koenig et al. 2005a,b
11	<i>Anagryis vein yellowing virus</i> (AVYV)	AY751780	Koenig et al. 2005b
12	<i>Ononis yellow mosaic virus</i> (OYMV)	J04375	Ding et al. 1989
13	<i>Plantago mottle virus</i> (PIMV)	AY751779	Koenig et al. 2005b
14	<i>Scrophularia mottle virus</i> (ScMV)	AY751777	Koenig et al. 2005b
15	<i>Dulcamara mottle virus</i> (DuMV)	AY789137	Tzanetakis et al. 2009
16	<i>Turnip yellow mosaic virus</i> (TYMV)	X07441	Morch et al. 1988
17	<i>Kennedya yellow mosaic virus</i> (KYMV)	D00637	Ding et al. 1990a
18	<i>Okra mosaic virus</i> (OkMV)	EF554577	Stephan et al. 2008
19	<i>Erysimum latent virus</i> (ErLV)	AF098523	Srifah et al. 1992
20	<i>Poinsettia mosaic virus</i> (PnMV)	AB550790	Okano et al. 2010
21	<i>Bombyx mori macula-like virus</i> (BoMV)	AB186123	Katsuma et al. 2005
22	<i>Grapevine fleck virus</i> (GFkV)	AJ309022	Sabanadzovic et al. 2001
23	<i>Grapevine Syrah virus 1</i> (GSyV-1)	KP221256	Glasa et al. 2015
24	<i>Olive latent virus 3</i> (OLV-3)	FJ444852	Alabdullah et al. 2010
25	<i>Peach virus D</i> (PeVD)	KY084481	...
26	<i>Maize rayado fino virus</i> (MRFV)	KM523134	Edwards et al. 2015
27	<i>Blackberry virus S</i> (BIVS)	FJ915122	Sabanadzovic and Abou Ghanem-Sabanadzovic 2009
28	<i>Oat blue dwarf virus</i> (OBDV)	U87832	Edwards et al. 1997
29	<i>Nectarine virus M</i> (NeVM)	KT273411	Villamor et al. 2016
30	<i>Citrus sudden death associated virus</i> (CSDaV)	AY884005	Maccheroni et al. 2005

^a A reference is given if the authors who deposited the respective sequence listed a publication in the GenBank description; ... indicates sequences deposited without a reference. The new tymovirus, Naranjilla chlorotic mosaic virus (NarCMV) characterized in this article, is highlighted in bold.

smaller ORF almost completely overlaps with the 200K protein ORF, starting at position 106 and ending at position 1,998, encoding a 69K protein. This 69K protein is required for virus spread and also functions as a gene-silencing suppressor (Bozarth et al. 1992; Chen et al. 2004). The 3'-proximal ORF starts at position 5,563 and ends at position 6,129, encoding the 188-aa CP with an estimated molecular weight of 20 kDa (Fig. 3A). The 9-aa C-terminal peptide LSSPLLQAS of this CP matched the partial sequence for the major protein in the purified virus preparation, as determined by LC-MS/MS (described above). A conserved 16-nt tymoviral sequence known as the "tymobox" was found between positions 5,531 and 5,546, upstream of the CP start codon (Fig. 3A)—this sequence is believed to be involved in the synthesis of the subgenomic RNA expressing the CP (Ding et al. 1990b). The 3'-terminal RNA sequence of 199 nt was predicted to form an extensive secondary structure as revealed by the RNAfold software (Lorenz et al. 2011), resembling tRNA structures characteristic of other tymovirus genomes (Dreher 2009); the CAC valine anticodon was also identified in an exposed loop (Fig. 3B).

When the whole genome of the new tymovirus was compared with the tymovirus sequences available in the GenBank database using the BLASTn program package, the closest match, EMV (Osorio-Keese et al. 1989), had a nucleotide sequence identity level of 71% (90% coverage). This is below the 80% threshold for the species demarcation in the family *Tymoviridae* (Dreher et al. 2012). Pairwise comparisons of the amino acid sequence for the 200K protein with the corresponding proteins of other tymoviruses produced the highest

identity level of 73% with the EMV protein (93% coverage), while the RdRp domain of the same 200K protein produced the highest identity level of 77% with the EMV RdRp (100% coverage). The amino acid sequence of the CP was found only 83% identical (87%

Table 2. Summary of the host range studies of the Naranjilla chlorotic mosaic virus (NarCMV)

Host tested	Symptoms, systemic ^b	Virus detection, RT-PCR ^a	
		Inoculated leaves	Uninoculated leaves
Naranjilla	ChlMos, LDef, LPerf, St	+	+
Tamarillo	ChlMos, LDef, syst. NL	+	+
<i>Nicotiana benthamiana</i>	Mos, blist	+	+
Eggplant, Fairy Tale	Mild mos	+	+
Tobacco, Burley	NS	—	—
Pepper, Early California Wonder	St, NS	+	—
Potato, King Edward	NS	—	—

^a Virus presence was detected using reverse-transcription polymerase chain reaction (RT-PCR) with primers tymo1294F and tymo2278R; + = presence of the 985-nucleotide band and — indicates no band in the gel.

^b Abbreviations of symptoms: ChlMos = chlorotic mosaic, LDef = leaf deformations, LPerf = leaf perforations, St = stunting, syst. NL = systemic necrotic lesions, Mos = mosaic, blist = blistering, and NS = no symptoms.

Fig. 1. A, Symptoms of mosaic, blistering, and leaf deformations induced by the new tymovirus in *Nicotiana benthamiana*, 2 weeks after mechanical inoculation. B, Symptoms of stunting, chlorotic mosaic, leaf deformations, and leaf perforations induced by the new tymovirus in naranjilla (*Solanum quitoense*), 10 weeks after mechanical inoculation. Healthy plant (H) is on the left and an infected plant (I) is on the right. C, Symptoms of chlorotic mosaic, leaf deformations, and systemic necrotic lesions induced by the new tymovirus in tamarillo (*S. betaceum*), 9 weeks after mechanical inoculation. Healthy plant (H) is on the left and an infected plant (I) is on the right.

coverage) to the closest match, ChiYMV, also below the 90% threshold for the *Tymovirus* sp. demarcation (Dreher et al. 2012). Phylogenetic analysis of the whole genome of this new virus species placed it within the lineage of the genus *Tymovirus*, with EMV, another tymovirus infecting solanaceous hosts, as the closest relative (Fig. 4).

Discussion

During the last two decades, the production of naranjilla has increased significantly in Ecuador. The commercialization of the fruit has gone from small farmers' markets to supermarket chains and juice and ice cream industries. In efforts to develop better cultivars, and considering the low intraspecific variation observed in the "common"

naranjilla (*S. quitoense*), growers and researchers have developed interspecific hybrids that are now widely cultivated. The potential damage caused by virus diseases, however, has not been considered in these new hybrids. The present study reports the complete characterization of a new virus isolated from a symptomatic naranjilla plant found in cultivar Puyo grown in the Pastaza province of Ecuador. The virus was found to be mechanically transmissible, in a very efficient way, to several solanaceous hosts, inducing a range of symptoms from very mild to severe depending on the host. Sequencing and biological data indicated that the virus is a member of the genus *Tymovirus* in the family *Tymoviridae*. Nucleotide and amino acid sequence comparisons to the closest relative (EMV) showed percent identities below

Fig. 2. **A**, Electron micrograph of the partially purified Naranjilla chlorotic mosaic virus (NarCMV) particles from naranjilla (*Solanum quitoense*), 18 weeks postinoculation. Negative staining, magnification $\times 120,000$. **B**, Gradient 4 to 20% sodium dodecyl sulfate (SDS) polyacrylamide gel analysis of the purified NarCMV preparation, 10 μ g and 1 μ g loaded per lane, respectively. M1 and M2 are lanes with protein markers; individual protein sizes are listed next to the bands. **C**, Titration curves for NarCMV in *Nicotiana benthamiana* leaves. Detection by triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA); virus antigen was captured by the rabbit UID41 polyclonal antibody (PAb) at 1:20,000 dilution. The mouse UID-M28 PAb was used as detecting antibody at 1:30,000 dilution. The plant extract was titrated along the X-axis: leaves of three individual infected (I) and healthy (H) plants were subjected to extraction in a sodium carbonate buffer at 1:10 (wt/vol) ratio; these extracts were diluted prior to loading onto the ELISA plate as indicated along the x-axis. OD₄₀₅ = optical density at 405 nm. **D**, Western blot analysis of the *N. benthamiana* healthy (H) and infected (I) leaf tissue. Plant extract (3 μ l) diluted 1:100 (corresponds to the '100' point in C) was loaded onto the gradient 4 to 20% SDS-polyacrylamide gel, and, after separation, transferred to the nitrocellulose membrane and probed with the rabbit UID41 PAb at 1:20,000 dilution. M designates the lane with protein markers; individual protein sizes are listed next to the bands.

Fig. 3. A, Schematic diagram of the Naranjilla chlorotic mosaic virus (NarCMV) genome organization. Positions of the three open reading frames coding for the 69-kDa (69K) protein, 200-kDa (200K) protein, and capsid protein (CP) are shown as lightly shaded rectangles, with nucleotide numbers for the start and stop codons indicated. Solid rectangles indicate positions of the known protein domains identified in the 200K protein: methyltransferase (MT), protease (PRO), helicase (HEL), and RNA-dependent RNA polymerase (RdRp). Below the diagram, the sequence fragment between the 200K protein and CP genes is shown with the 16-nucleotide (nt) conserved 'tymobox' sequence underlined. **B**, Secondary structure prediction for the 3'-terminal 150-nt segment of the NarCMV genome, generated using the RNA-fold program (Lorenz et al. 2011). Positions of the 3' terminus and the anticodon loop with the CAC valine anticodon are indicated with the arrow and the bracket, respectively.

Fig. 4. Maximum-likelihood phylogenetic tree generated for whole-genome nucleotide sequences of the viruses from the family Tymoviridae. Bootstrap values exceeding 70% are shown at the respective nodes; nodes with values below 70% were collapsed. Brackets to the right designate established genera in the family. EMV = Eggplant mosaic virus, NarCMV = Naranjilla chlorotic mosaic virus, APMNV = Andean potato mild mosaic virus, ChiYMV = Chiltepín yellow mosaic virus, ToBMV = Tomato blistering mosaic virus, APLV = Andean potato latent virus, ToYBV = Tomato yellow blotch virus, PhyMV = Physalis mottle tymovirus, ChaMV = Chayote mosaic tymovirus, NeRNV = Nemesia ring necrosis virus, AVYV = Anagallis vein yellowing virus, OYMV = Ononis yellow mosaic virus, PIMV = Plantago mottle virus, ScMV = Scrophularia mottle virus, DuMV = Dulcamara mottle virus, TYMV = Turnip yellow mosaic virus, KYMV = Kennedy yellow mosaic virus, OkMV = Okra mosaic virus, ErLV = Erysimum latent virus, PnMV = Poinsettia mosaic virus, BoMV = Bombyx mori macula-like virus, GfKV = Grapevine fleck virus, GSyV-1 = Grapevine Syrah virus 1, OLV-3 = Olive latent virus 3, PeVD = Peach virus D, MRFV = Maize rayado fino virus, BIVS = Blackberry virus S, OBDV = Oat blue dwarf virus, NeVM = Nectarine virus M, and CSDaV = Citrus sudden death associated virus.

the values for species demarcation in the family. This is the first report and complete characterization of a virus from naranjilla. The name Naranjilla chlorotic mosaic virus (NarCMV) is proposed, based on the symptoms induced in the original host.

The identification of NarCMV facilitates implementation of screening schemes for both breeding and virus-free certification programs, which are especially beneficial for vegetatively propagated crops. Determining the natural mode of NarCMV transmission requires further investigation. Several tymoviruses are known to be transmitted by flea beetles (Jones and Fribourg 1977; Markham and Smith 1949; Walters 1969; Weidemann and Bode 1973). In some known cases, however, mechanical transmission is more efficient than transmission by beetles, as demonstrated for *Andean potato latent virus*, a related tymovirus, infecting another solanaceous host (Jones and Fribourg 1977). The detection tools developed here, both ELISA and RT-PCR based, can now be applied to surveys of the viruses infecting naranjilla, and to studies of epidemiology of NarCMV.

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