© 2013 Plant Management Network.
Accepted for publication 3 January 2012. Published 9 May 2013.

# Identification, Transmission, and Partial Characterization of a Previously Undescribed Flexivirus Causing a Mosaic Disease of Ash (*Fraxinus* spp.) in the USA

**Jose Ernesto Machado-Caballero, Ben E. Lockhart, Shauna L. Mason,** and **Dimitre Mollov,** Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108; and **Jason A. Smith,** School of Forest Resources and Conservation, University of Florida, Gainesville, FL 32611

Corresponding author: Ben E. Lockhart. lockh002@umn.edu

Machado-Caballero, J. E., Lockhart, B. E., Mason, S. L., Mollov, D., and Smith, J. A. 2013. Identification, transmission, and partial characterization of a previously undescribed flexivirus causing a mosaic disease of ash (*Fraxinus* spp.) in the USA. Online. Plant Health Progress doi:10.1094/PHP-2013-0509-01-RS.

## Abstract

A previously undescribed virus with flexuous filamentous particles 720 nm in length was associated with a mosaic disease of ash (*Fraxinus* spp.) occurring in Minnesota, Ohio, New York, and Illinois. The virus was initially identified in white ash in Minnesota and named white ash mosaic virus (WAMV), but also occurred naturally in green, black, and blue ash. The virus was transmitted readily by mechanical inoculation and was determined to be the causal agent of the disease. It infected only ash, and was not transmitted through seed or by *Myzus persicae*. The virus has a ssRNA genome of approximately 9.0 kb and a capsid protein of ~34 kDa based on electrophoretic analysis. One partial and five complete putative ORFs most similar in size, arrangement, and amino acid sequence to the corresponding genomic regions of flexiviruses were identified in a 5227 nt 3′ terminal fragment of the WAMV genome (GenBank accession no. GU906791.1). However, the relatively low levels (27-59%) of amino acid sequence identity suggests that WAMV is not related closely to any known member of the family *Flexiviridae*. White ash mosaic virus was identified in Minnesota in black ash trees showing symptoms of ash decline, a syndrome of wide occurrence and possible multiple etiology. Antibody and PCR-based protocols were developed for reliable detection of WAMV in ash and will be made available upon request.

## Introduction

Ash (*Fraxinus* spp.) is an important component of hardwood forest communities in North America, and is also valued for timber and landscaping. In September 2005 virus-like mosaic symptoms (Fig. 1) were observed in plantings of white ash (*F. americana*) in St. Paul, MN. The foliar symptoms

Fig. 1. Foliar symptoms induced by white ash mosaic virus (WAMV) infection in white ash (*Fraxinus americana*). Healthy leaf at left.

persisted and intensified until leaf fall in November. A similar cycle of disease development was observed between June and November 2006-2011. Numerous filamentous virus-like particles with a modal length of 720 nm (Fig. 2A) were observed by transmission electron microscopy (TEM) in negatively-stained partially-purified leaf tissue extracts (2) from diseased but not from healthy, asymptomatic white ash plants. No other virus-like particles were observed in partially-purified extracts from diseased plants. Three viruses, *Tobacco mosaic virus* (TMV) (3,6,18), *Tobacco ringspot virus* (TRSV) (3,6,14), and *Tomato ringspot virus* (ToRSV) (11,15), have been reported to occur in *Fraxinus* spp. in the USA. Three other viruses, *Arabis mosaic virus* (ARMV) (5,8,24), *Cherry leaf roll virus* (CLRV) (8), and *Tobacco necrosis virus* (TNV) (24,25) have been reported to occur in *Fraxinus* spp. in Europe. No particles resembling those of any of these six viruses were detected by initial TEM examination or by enzyme-linked immunosorbent assay (ELISA) in any of the symptomatic ash trees examined in this study. It was therefore concluded that the disease symptoms observed in white ash were not associated with infection by any of these viruses, and that the filamentous virus-like particles observed by TEM in symptomatic ash were most likely particles of a previously unreported viral pathogen of ash. The objectives of this study were to determine the identity and main properties of this virus and its role in the etiology of the disease. It was also considered important to develop and make available reliable WAMV detection protocols that would hopefully yield additional information on the distribution and incidence of this virus in *Fraxinus* spp. throughout North America.

**Disease incidence and virus identification**. Leaf samples were collected from trees of white (*F. americana*), black (*F. nigra*), blue (*F. quadrangulata*), and green ash (*F. pennsylvanica*) showing mosaic symptoms or symptoms associated with ash decline with or without mosaic. Samples were collected in locations in Minnesota, New York, Ohio, and Illinois. Leaf samples were assayed for presence of WAMV by TEM and immunosorbent electron microscopy (ISEM) using partially-purified extracts, and by ELISA using crude sap extracts as described below.

**Virus source, transmission, host range**. The virus used in this study was obtained from a naturally-infected white ash tree in St. Paul, MN. The only virus-like particles observed by TEM in partially-purified leaf tissue extracts from this plant were flexuous filaments 720 nm in length. Inoculum prepared by grinding young symptomatic leaf tissue from the source plant in 100 mM potassium phosphate, pH 7.4 containing 0.5% (v/v) 2-mercaptoethanol was used to mechanically inoculate Carborundum-dusted leaves of indicator plants. Indicator plants (Table 1) were kept in the greenhouse for up to 10 months post-inoculation. They were observed for symptom development and were assayed for presence of WAMV by TEM, ISEM, and ELISA using partially-purified or crude sap extracts as described below. Seed transmission of WAMV was tested by assay of 100 seedlings of white ash grown from seed collected from the infected source plant. The seedlings were grown in the greenhouse for six months, and were observed for symptom expression and tested by TEM, ELISA, and ISEM for presence of WAMV. Aphid transmission tests were done using *M. persicae* apterae raised on Chinese cabbage. After a 30-min fast, the aphids were allowed a 2-5-min acquisition access period on young symptomatic leaves from the white ash source plant and then transferred in lots of 15 to each of five healthy white ash seedlings and allowed to feed overnight, after which they were killed with insecticide. Aphid-inoculated white ash test seedlings were kept in the greenhouse for up to 10 months post-inoculation. They were observed for symptom development and were assayed for presence of WAMV by TEM, ELISA, and by ISEM using partially-purified extracts as described below.

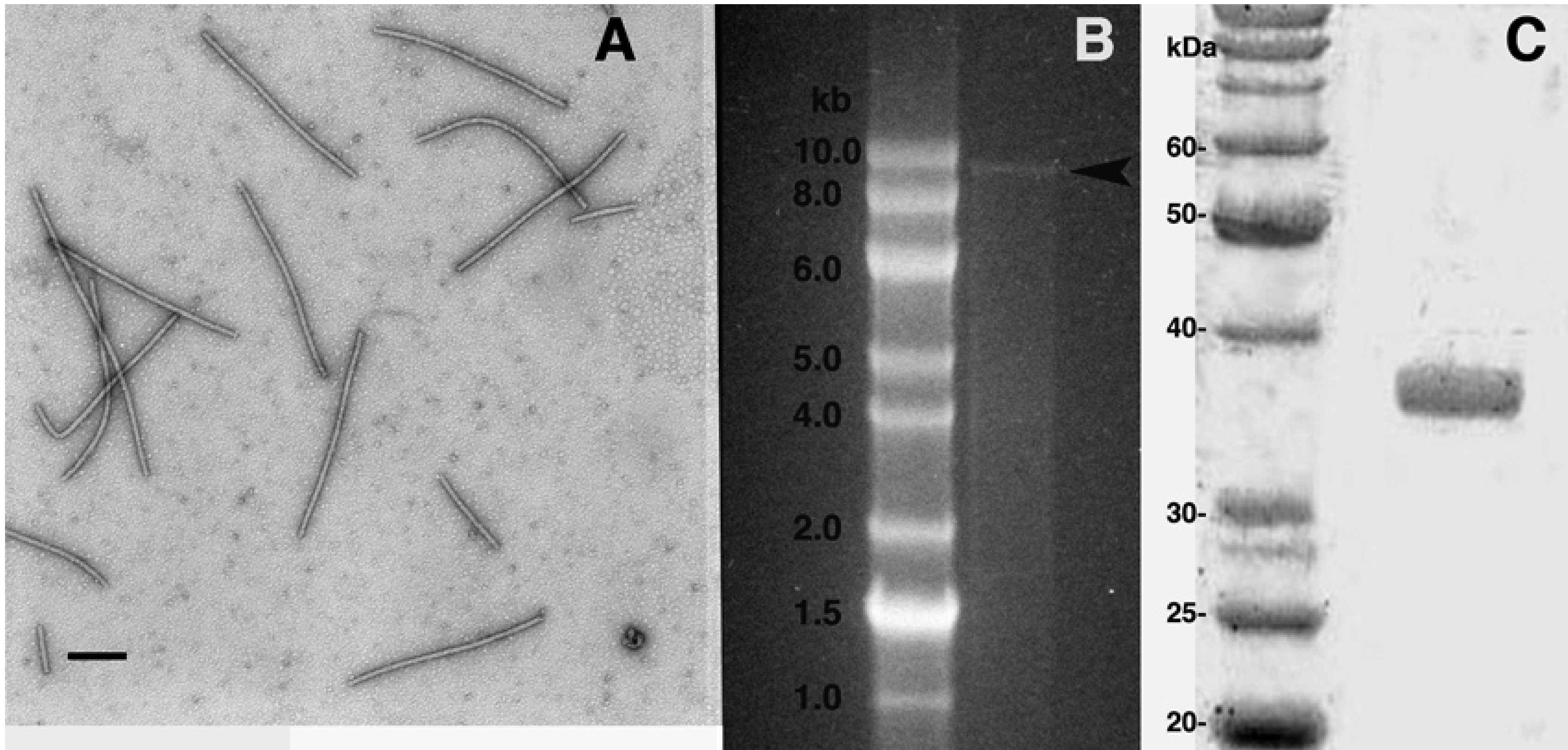


Fig. 2. (**A**) Characterization of white ash mosaic virus (WAMV) virions. Virion morphology in a purified preparation negatively-stained with aqueous uranyl formate. Scale bar equals 200 nm. (**B**) Electrophoretic migration pattern under denaturing conditions of genomic ssRNA extracted from purified WAMV virions. Electrophoresis was conducted in 1.5% agarose in Tris-acetate-EDTA. (**C**) Estimation of WAMV coat protein (CP) subunit molecular mass by SDS-PAGE. Lane 1, BenchMark 10-200 kDa protein ladder (Invitrogen). Lane 2, WAMV coat protein.

Table 1. Experimental host range of white ash mosaic virus (WAMV) based on mechanical inoculation of indicator plants.

| Indicator plant | Family | No. plant infected/ inoculated[x] | Symptoms |
|---|---|---|---|
| *Fraxinus americana* (White ash) | Oleaceae | 10/10 | Systemic mosaic |
| *F. pennsylvanica* (Green ash) | Oleaceae | 10/10 | Systemic mosaic |
| *Olea europaea* (Olive) | Oleaceae | 0/5 | – |
| *Jasminum* (Jasmine) | Oleaceae | 0/5 | – |
| *Syringa* (Lilac) | Oleaceae | 0/5 | – |
| *Nicotiana benthamiana* | Solanaceae | 0/5 | – |
| *N. occidentalis* | Solanaceae | 0/5 | – |
| *N. clevelandii* | Solanaceae | 0/5 | – |
| *Chenopodium quinoa* | Chenopodiaceae | 0/5 | – |
| *Pisum sativum* | Fabaceae | 0/5 | – |
| *Phaseolus vulgaris* | Fabaceae | 0/5 | – |
| *Cucumis sativus* | Cucurbitaceae | 0/5 | – |
| *Spinacia oleracea* | Amaranthaceae | 0/5 | – |
| *Gomphrena globosa* | Amaranthaceae | 0/5 | – |

[x] As determined by immunosorbent electron microscopy (ISEM) and indirect enzyme-linked immunosorbent assay (ELISA).
– = no symptoms, no virus detected.

**Virion purification, characterization, and electron microscopy**. Partially-purified preparations of WAMV were obtained from 2-5-gm leaf tissue samples as described (2) and were used in TEM and ISEM assays as described below. For characterization studies and antiserum production virions of WAMV were extracted from infected leaf tissue and further purified by isopycnic density gradient centrifugation in $Cs_2SO_4$ as described previously (9). Gradient fractions containing virions were identified by TEM examination rather than light scattering (UV absorbance at 240 nm) and fractions containing detectable plant debris were discarded. Virion-containing fractions were pooled and diluted with 5 volumes of distilled water ($dH_2O$). The virions were collected by ultracentrifugation at 184,000 $g_{max}$ for 2 h and the resulting pellets resuspended in $dH_2O$. Purity of purified virion suspensions was assessed by $A_{260}/A_{280}$ ratio and TEM examination. Virus concentration was estimated using an assumed extinction coefficient ($E_{260}^{0\text{-}1\%}$) of 3.0. Partially-purified and purified virion preparations were examined by TEM following negative staining with 2% sodium phosphotungstate, pH 7.0 (PTA) containing 250 µg/ml bacitracin, 2% ammonium molybdate, pH 6.8 (AM), or aqueous uranyl formate (UF). Microscope magnification was calibrated using the lattice spacing of potassium permanganate-stained catalase crystals (28).

Genomic RNA was extracted from purified virion suspensions as described (9). Size estimate of the genomic RNA under denaturing conditions was determined by electrophoresis in native 1.5% Tris-acetate-EDTA (TAE) agarose gels using Superload Denaturing Gel Loading Buffer (Viagen, Austin, TX). Type and strandedness of the WAMV genome were based on sensitivity to RNase and S1 nuclease digestion and resistance to DNase digestion (20). The molecular mass of virion capsid subunits was estimated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (17) using a Benchmark 10-220-kDa protein ladder (Invitrogen, Grand Island, NY) as size markers.

**Antiserum production and serological assays**. An antiserum against WAMV antigen was prepared by immunizing a New Zealand White rabbit with purified virus emulsified 1:1 in Titermax Gold adjuvant (Sigma, St. Louis, MO). Injections were administered subcutaneously at days 0 (1 mg), 14 (0.5 mg), and 21 (0.5 mg). Blood samples were taken at weekly intervals starting at day 56. Specific antibody titer of serum samples was determined by indirect ELISA (16) using crude sap from healthy and WAMV-infected white ash as coating antigen. Subsequent virus detection assays were done by indirect ELISA and by ISEM (21).

**Cloning and genomic sequence analysis**. The sequence of a 1.3 kb amplicon comprising the 3' terminus of the WAMV genome was obtained initially using total RNA extracted from WAMV-infected white ash leaf tissue using a Qiagen RNeasy Plant Mini Kit. Synthesis of cDNA was done using the primer M4T and Superscript III reverse transcriptase (Invitrogen), followed by PCR amplification using the primer pair M4 and pCar1 as described by Chen et al. (7). Following PCR clean-up using the Pure-Link PCR purification kit (Invitrogen) the amplicon was cloned using a TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions. Additional WAMV genomic sequences were subsequently obtained using RNA extracted from purified virions using random hexamers and Superscript III reverse transcriptase as described by Tzanetakis et al. (27). Following restriction enzyme digestion and A-tailing with Taq DNA polymerase (27) the cDNA fragments were cloned using a TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions.

Sequences were assembled and aligned using Sequencher, version 4.2 (Gene Codes Corporation, Ann Arbor, MI). Gaps were filled using primers designed based on the sequences of the initial genomic fragments. Final nucleotide sequences were aligned and translated using Sequencher. Similarity searches of non-redundant sequences on the GenBank database were performed using BLAST (www.ncbi.nih.gov/BLAST). The sequence was aligned with the corresponding sequences of other species in the family *Flexiviridae*. Nucleic acid alignments were constructed using CLUSTAL X1.81 with default parameters (19). Alignments were refined using MacClade 4 version 4.6 (22). The coding capacity of the WAMV partial sequence was screened looking for all reading frames using the ORF Finder program (www.ncbi.nih.gov/gorf/gorf.html).

## Identification, Transmission, and Partial Characterization

**Disease and virus incidence.** White ash mosaic virus was identified in symptomatic white, black and blue ash in St. Paul and Chanhassen, MN, in white ash in the Bronx, NY, in black ash in Columbiana County, OH, and in green ash in Milton Township, IL. The virus was also identified in Minnesota in several black ash trees showing decline symptoms but mild or no mosaic symptoms. Presence of WAMV was verified in all cases by ISEM using partially-purified leaf tissue extracts.

**Virus and disease transmission and experimental host range**. White ash mosaic virus was transmitted readily by sap inoculation from infected white ash to healthy white and green ash seedlings. Initial symptoms appeared in new growth at 20 days post-inoculation under greenhouse conditions (24-28°C). Disease symptoms similar to those observed in the source plant were observed in mechanically-inoculated ash seedlings 2-3 months post-inoculation. As indicated in Table 1, WAMV was transmitted by mechanical inoculation only to *Fraxinus* spp. None of the other indicator plants, including members of three genera in the family Oleaceae, developed symptoms, and all tested negative for WAMV infection by ISEM. White ash mosaic virus was not detected by ISEM or ELISA in any of 100 white ash seedlings grown from seed collected from the infected source plant. No symptoms were observed in, nor virus detected in, any of 5 healthy ash seedlings on which *M. persicae* was allowed to feed following a short (205-min) acquisition access period on the infected white ash source plant.

**Virion purification and characterization**. Suspensions of virions of WAMV purified by isopycnic density gradient in $Cs_2SO_4$ contained no detectable host plant contaminants in negatively-stained preparations examined by TEM

(Fig. 2A). The only particles observed in these preparations were slightly flexuous filaments with a modal length of 720 ± 14 nm based on measurement of 100 randomly selected particles. Purified virions of WAMV contained a single structural (capsid) protein having an estimated molecular mass of 34kDa as determined by SDS-PAGE (Fig. 2B). Genomic nucleic acid extracted from purified WAMV virions consisted of a single migrating species of ssRNA approximately 9 kb in size as determined by agarose gel electrophoresis under denaturing conditions (Fig. 2C). Type and strandedness of the WAMV genome were based on sensitivity to RNase and S1 nuclease digestion and resistance to DNase digestion as described above (20).

**Serological tests**. The rabbit antiserum prepared against purified WAMV antigen readily detected the virus in infected ash by DAS-ELISA, indirect ELISA, and ISEM assays (*data not shown*).

**Genomic sequence analysis**. A partial sequence of the WAMV genome was obtained. This sequence (GenBank accession no. GU906791.1) is 5242 nt in length (5227 nt excluding the 3' terminal poly-A tract). Based on the estimated genome size (9 kb) determined empirically by agarose gel electrophoresis (Fig. 2B) this fragment represents approximately 60% of the WAMV genomic sequence. This sequence contains one partial 5' terminal ORF and five complete ORFs. The nucleotide positions of these ORFs, sizes of the predicted proteins they encode and the highest level of their amino acid sequence similarity to known viral genomic sequences are summarized in Table 2. The number and arrangement of these six ORFs are similar to these of members of the family *Flexiviridae* (1,10,23). The data presented in this table are consistent with the conclusion that WAMV is related most closely to members of the family *Flexiviridae*, and has the genome structure typical of this family, consisting of a large 5' terminal ORF encoding the replicase, followed by the ORFs encoding the triple gene clock (TGB) proteins, and terminating with an ORF encoding the CP. It is of interest to note that the size of the CP predicted by sequence analysis (34 kDa) closely approximates the value (~35 kDa) determined empirically by SDS-PAGE of purified virions (Fig. 2C).

Table 2. Highest degree of amino acid sequence identity between white ash mosaic virus (WAMV) and known members of the family *Flexiviridae* in the putative protein coding regions identified in the partial genomic sequence of WAMV (5,227 nt, GU906791.1)

| ORF | Size (amino acids) kDa | Nucleotide position | Highest identity | Identities |
|---|---|---|---|---|
| 1 | 692/79 | 1-2078 | *Garlic virus B1* Replicase ABQ96641 | 399/676 (59%) |
| 2 | 244/26 | 2128-2829 | Garlic virus D 26 kDa protein BAA74945 | 105/233 (45%) |
| 3 | 105/11 | 2826-3140 | *Garlic virus C* 11kDa protein NP_569134 | 44/85 (52%) |
| 4 | 75/8 | 3053-3275 | *Lily virus X* TGB protein 3 YP_263306 | 18/54 (33%) |
| 5 | 315/35 | 3257-4201 | *Garlic virus B* 40 kDa protein BAA61815 | 98/365 (27%) |
| 6 | 326/34 | 4194-5120 | Blackberry virus E coat protein YP_004659204 | 108/197 (55%) |

## WAMV and Reliable Detection Protocols

From the data presented above it was concluded that ash mosaic is a previously unreported disease of *Fraxinus* spp. and is caused by a previously undescribed virus that was named white ash mosaic virus (WAMV). Ash mosaic and WAMV were identified in relatively limited sampling in four states in the USA. This suggests that this disease may occur elsewhere in North America, and it would be of interest to conduct wider surveys to determine the range of this disease. This would also be of practical interest in addressing the possible role of WAMV in the syndrome known as ash decline, which is of widespread occurrence but undetermined etiology. Ferris et al. (12) concluded that infection by phytoplasmas (MLO) but not by viruses (TMV, TRSV, ToRSV) was associated with suppressed growth in ash, and that "neither synergistic nor additive effects of multiple pathogen infection on growth or symptom development was detected." Ash decline symptoms in *F. velutina* in Arizona were also attributed to phytoplasma (MLO) infection (4). Ash trees in Minnesota showing symptoms of decline were found to be infected by WAMV, and tested negative for phytoplasma infection using either direct (26) or nested (13) PCR that gave expected positive results with elm yellows and aster yellows positive controls (*data not shown*). These observations suggest that the syndrome referred to as ash decline may be due to multiple causes, and that WAMV infection may possibly be involved in some instances. A long-term field trial installed in 2007 at the University of Minnesota, St. Paul, was designed to examine the effect of WAMV infection on white and black ash growth over a 10-15-year period, and data from this field trial will therefore not be available for some time. In the interim, there is a need for more systematic surveys to evaluate the distribution, incidence and possible impact of WAMV infection in ash in N. America. Reliable methods for detection of WAMV by serological (ELISA, ISEM) genome-based (RT-PCR, immunocapture RT-PCR) are currently available and would greatly facilitate these types of investigations. These detection methods could also be used in the shade tree industry to index both rootstock and scion sources in order to prevent dissemination of the virus by graft transmission in budded trees. Further research is also needed to answer the question concerning the natural modes of transmission of WAMV, which is of great practical interest. The virus was identified in New York in (ungrafted) white ash, indicating that virus transmission can occur in nature via either seed or vector(s). The results of seed transmission tests reported above were based on a sample size of 100 seeds, and indicated that seed transmission of WAMV, should it occur, would be <1%. Seed transmission tests using larger sample sizes (250-500) would provide useful and statistically valid data on the probability of seed-transmission of WAMV in ash. A research priority of equal practical importance is the search for a possible vector of WAMV. The negative results obtained with the green peach aphid, *Myzus persicae*, are not highly significant since this aphid species does not normally colonize *Fraxinus* spp. The consistent albeit relatively low level of amino acid sequence identity between WAMV and the mite-transmitted garlic-infecting allexiviruses suggests that it could be worthwhile to investigate the possibility of an arachnid (i.e., eriophyid mite) vector of the virus.

On the basis of virion properties, genome organization and amino acid sequence similarities, WAMV most closely resembles members of the family *Flexiviridae* (1,10,23). The relative low levels of highest amino acid sequence similarity (27-59%, Table 2) suggest that based on current criteria for species demarcation in currently recognized genera in the family Flexiviridae (10), WAMV may represent a new and distinct member of this family. However, the taxonomic status of WAMV cannot be clearly ascertained until additional data are obtained. An indispensible element would be the completion of the genomic sequence. Other desirable elements would include additional biological data (e.g., type of vector) and construction of an infectious genomic clone to validate the fidelity of the proposed genomic sequence and structure.

## Literature Cited


1. Adams, M. J., Antoniw, J. F., Bar-Joseph, M., Brunt, A. A., Candresse, T., Foster, G. D., Martelli, G. P., Milne, R. G., and Fauquet, C. M. 2004. The new plant virus family *Flexiviridae* and assessment of molecular criteria for species demarcation. Arch Virol. 149:1045-1060.
2. Ahlawat, Y. S., Pant, R. P., Lockhart, B. E. L., Srivastava, M., Chakraborty, N. K., and Varma, A. 1996. Association of a badnavirus with citrus mosaic disease in India. Plant Dis. 80:590-590.
3. Amico, L. A., O'Shea, M. T., Castello, J. D., and Hibben, C. R. 1985. Transmission of tobacco mosaic and tobacco ringspot viruses from Moraine ash in New York. Plant Dis. 69:542.
4. Bricker, J. S., and Stutz, J. C. 2004. Phytoplasmas associated with ash decline. J. Arboric. 30:193-199.
5. Casalicchio, G. 1965. La punteggiatura cloronecrotica de Fragsino maggiore. Monti Boschi 16:39-46.
6. Castello, J. D., Amico, L. A., and O'Shea, M. T. 1984. Detection of *Tobacco mosaic and Tobacco ringspot* viruses in white ash trees by enzyme-linked immunosorbent assay. Plant Dis. 68:787-790.
7. Chen, J., Chen, J., and Adams, M. J. 2002. Characterization of some carla- and potyviruses from bulb crops in China. Arch. Virol. 147:419-428.
8. Cooper, J. I., and Sweet, J. B. 1976. The detection of viruses with nematode vectors in six woody hosts. Forestry 49:73-78.
9. Currier, S., and Lockhart, B. E. L. 1996. Characterization of a potexvirus infecting Hosta spp. Plant Dis. 80:1040-1043.
10. Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U., and Ball, L. A., eds. 2005. Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, UK.
11. Ferris, M. A., and Castello, J. D. 1988. Detection of *Tomato ringspot virus* in white ash and adjacent vegetation in central New York. Can J. For. Res. 18:813-817.
12. Ferris, M. A., Castello, J. D., and Sinclair, W. A. 1989. Effects of virus and mycoplasmalike organism infection on green and white ash. Phytopathology 79:579-583.
13. Gundersen, D. E., and Lee, I.-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathology Mediterr. 35:144-151.
14. Hibben, C. R., and Bozarth, R. F. 1972. Identification of an ash strain of *Tobacco ringspot virus*. Phytopathology 62:1023-1029.
15. Hibben, C. R., Reese, J., and Castello, J. D. 1988. Identification of *Tomato ringspot virus* in ash in New York. Plant Dis. 72:175.
16. Koenig, R., and Paul, H. L. 1982. Variants of ELISA in plant virus diagnosis. J. Virol. Methods 5:113-1250.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685.
18. Lana, A., and Agrios, G. 1974. Properties of a Strain of *Tobacco mosaic virus* isolated from white ash trees. Phytopathology 64:1490-1495.
19. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. 2007. Clustal W and Clustal X, Vers. 2.0. Bioinformatics 23:2947-2948.
20. Lockhart, B. E. L. 1990. Evidence for a double-stranded circular DNA genome in a second group of plant viruses. Phytopathology 80:127-131
21. Lockhart, B. E. L., Autrey, L. J-C., and Comstock, J. C. 1992. Partial purification and serology of Sugarcane mild mosaic virus, a mealy-bug transmitted closterolike virus. Phytopathology 82:691-695.
22. Maddison, W. P., and Maddison D. R. 2000. MacClade: Analysis of Phylogeny and Character Evolution, Vol. 4. Sinauer, Sunderland, MA.
23. Martelli. G. P., Adams, M. J., Kreuze, J. F., and Dolja, V. V. 2007. Family Flexiviridae: A Case Study in Virion and Genome Plasticity. Annu. Rev. Phytopathol. 45:73-100.
24. Nienhaus, F., and Castello, J. D. 1989. Viruses in forest trees. Annu. Rev. Phytophathol. 27:165-186.
25. Nienhaus, F., and Hamacher, J. 1990. Virosen in Eschen. Allg. Forstzeitg. 16:385-386.

26. Smart, C. D., Schneider, B., Blomquist, C. L., Guerra, L. J., Harrison, N. A., Ahrens, U., Lorenz, K.-H., Seemüller, E., and Kirkpatrick, B. C. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Appl. Environ. Microbiol. 62:2988-2993.
27. Tzanetakis, I. E., Keller, K. E., and Martin, R. R. 2004. The use of reverse transcriptase for efficient first- and second-strand cDNA synthesis from single- and double-stranded RNA templates. J. Virol. Methods 124:73-77.
28. Wrigley, N. G. 1968. The lattice spacing of crystalline catalase as an internal standard of length in electron microscopy. J. Ultrastruct. Res. 24:454.