A novel plant virus with unique properties infecting Japanese holly fern

Rodrigo A. Valverde¹ and Sead Sabanadzovic²

¹Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA
²Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA

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A novel RNA virus with a bipartite genome has been found associated with an emerging disease affecting Japanese holly fern (Cyrtomium falcatum). Diseased Japanese holly fern plants showed a variety of foliar symptoms and reduction in size. The virus was transmitted by grafting, as well as through spores from an infected plant. Partially purified preparations of the virus from infected ferns contained quasi-spherical particles that ranged from 30 to 40 nm in diameter. Double-stranded RNA (dsRNA) analyses from diseased plants yielded two major molecules of approximately 6.2 and 3.0 kbp in size, together with three other dsRNAs ascertained to be the replicative forms of subgenomic RNAs. The organization of RNA1 of this novel virus resembles that of raspberry bushy dwarf virus (genus Idaeovirus), whereas the genomic RNA2 showed a distinct organization and evolutionary origin. Results of this study indicate that the virus detected in diseased ferns is an undescribed phytovirus, for which the name Japanese holly fern mottle virus (JHFMoV) is proposed. Furthermore, we postulate that JHFMoV has enough distinguishing features to represent the type species of a novel genus of plant viruses. Taking into account the original host of the virus, we propose the name Pteridovirus for this taxon.

INTRODUCTION

Japanese holly fern (JHF) (Cyrtomium falcatum C. Presl) is used as a landscape plant in the southern USA. JHF plants showing yellow mottle, mosaic, ring spots, oak leaf pattern and necrosis were observed in home gardens, public landscapes and local nurseries in Louisiana (LA) and Mississippi (MS) (Fig. 1). Some diseased plants also exhibited reduced growth and premature senescence of the leaves. The nature of the symptoms, together with preliminary double-stranded RNA (dsRNA) analyses and observations of an apparent increase in the incidence of the disease over the last few years, suggested that a virus was the causal agent (Valverde & Sabanadzovic, 2008).

Several virus-like diseases and/or viruses have been reported in ferns. The earliest report described a putative virus disease on Bird’s nest fern (Asplenium nidus) and was based mainly on symptom observations (Rangone-Gallucci, 1956). Canova & Casalicchio (1961) transmitted a putative virus from diseased common polypody fern (Polypodium vulgare) and Hart’s tongue fern (Scolopendrium vulgare, synonym Asplenium scolopendrium) onto a range of herbaceous hosts, but did not characterize the pathogen further. Several years later, a putative tobravirus was reported from a diseased Hart’s tongue fern (Hull, 1968) and a potyvirus was detected infecting male fern (Dryopteris filix-mas Schott) and common polypody fern (Nienhaus et al., 1974). However, the characterization of these viruses/diseases has never been completed and their final identities are still uncertain. More recently, cucumber mosaic virus has been reported from Northern maidenhair fern (Adiantum pedatum) (Nameth & Steininger, 1997).

The goal of this investigation was to identify and characterize the putative virus causing an emerging disease affecting JHF in the southern USA. In this paper, we provide data supporting the viral aetiology of the disease and characterize the pathogen as a novel RNA virus, for which we propose the name Japanese holly fern mottle virus (JHFMoV).

METHODS

Plant materials and virus sources. A JHF plant (JHF-JM) from a private landscape in Baton Rouge, LA, USA, was selected among several plants showing yellow mottle symptoms and kept in a greenhouse. The virus isolated from this plant was designated JHFMoV-JM. Two other JHF specimens (JHF-DI and JHF-HR) were
obtained from central and northern MS, respectively. The viruses isolated from these plants were designated JHFMoV-DI (central MS) and JHFMoV-HR (northern MS). These plants were used as sources of infected tissue for dsRNA extraction, graft transmission, virus purification, molecular cloning and sequencing experiments.

Other diseased JHF plants obtained from public and private landscapes and retail stores in LA, MS and neighbouring states were used to study virus distribution and incidence. Healthy JHF plants used as controls in all experiments were obtained from Santa Rosa Tropicals. These plants were symptomless and, prior to their use in any experiment, were continuously tested for virus infection by dsRNA analysis and/or RT-PCR following procedures described below.

**Extraction and analysis of dsRNA.** dsRNA was purified from foliar samples of diseased and healthy JHF plants from various locations by using a modification of the Morris & Dodds (1979) CF-11 cellulose column chromatography procedure (Valverde et al., 1990). In some experiments, dsRNA was extracted from sori and spores of JHF-JM-infected plants. Purified dsRNA was stained with ethidium bromide/acidine orange/silver nitrate and resolved by 1.2% agarose or 6% polyacrylamide gel electrophoreses. Selected dsRNAs were gel-purified by using a MiniElute kit (Qiagen) and used for cloning and other experiments. To confirm the nature of the purified nucleic acids, aliquots in nucleic-acid-free water or 0.2 M sodium chloride, 0.1 M Tris, 0.01 M MgCl₂ (pH 7.3) were treated with RNase A (Sigma; 10 μg ml⁻¹) and DNase I (Sigma; 2 μg ml⁻¹) and analysed in 1.2% agarose gels or in 6% polyacrylamide gels. Replicative forms of turnip vein-clearing virus (TVCV; genus *Tobamovirus*) and peanut stunt virus (PSV; genus *Cucumovirus*) were used as references.

**Transmission experiments.** Virus-containing fern sap and/or partially purified virus preparations obtained from accession JHF-JM were rubbed onto cellite-dusted leaves of healthy, 6-month-old JHF plants and seedlings of *Nicotiana benthamiana*, *Nicotiana clevelandii*, *Nicotiana tabacum* cv. Turkish, *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Goephrena globosa* and *Datura stramonium* in order to investigate the mechanical transmissibility of JHFMoV. Inoculated plants were kept in a greenhouse at 25–30 °C and observed for symptom expression for 8 weeks.

For graft inoculations, 15 healthy, 1-year-old JHF plants were used as rootstock. Scions, which consisted of partially unfolded leaves from infected JHF-JM, were grafted on the petioles of partially unfolded leaves of the rootstock (see Supplementary Fig. S1, available in JGV Online). Grafted plants were covered with plastic bags and kept inside screen cages in the greenhouse at temperatures that ranged from 25 to 30 °C. Ten days later, bags were removed and plants were observed for virus-like symptoms for a period of 8 weeks. Eight weeks after inoculations, plants were tested for the presence of dsRNA by PAGE and for virus sequences by RT-PCR using tissue from fronds other than the ones used for grafting purposes.

In order to study the possible vertical transmission of the virus, spores were collected from healthy JHF and infected (JHF-JM) plants and placed on the surface of steam-sterilized topsoil/sand mix (3:1, v/v) in 30 × 40 cm metal trays. After watering, the trays were covered with transparent plastic and kept inside caged greenhouses. One to two months later, gametophytes were produced, followed by sporophytes. When sporophytes were approximately 2–4 cm high, they were transplanted individually in black plastic, 64-cavity seedling flats containing Jiffy Mix (Jiffy Products). Fifty plants from each group (infected and healthy) were selected randomly and transplanted into 10 × 12 cm clay pots. Plants were kept in a large screen cage within the greenhouse and symptom development was monitored for 4 months. Plants that developed symptoms and 10 symptomless plants were tested for the virus by RT-PCR and/or dsRNA analysis.

**Virus purification.** Several attempts were made to purify virions from infected JHF-JM plants using buffers of different chemical nature (borate, sodium phosphate, potassium phosphate and citrate buffers), molarities and pH values, alone or in combination. The extraction step was followed by different attempts to clarify and concentrate the viral preparation using organic solvents, PEG 6000 precipitation, followed by a cycle of density-gradient centrifugation in 10–40% sucrose linear gradients and concentration by high-speed centrifugation at 90 000 g for 2 h. The purification method that yielded the best outcome is described in the Results.

The adopted purification procedures were evaluated by transmission electron microscope (TEM) observations of partially purified preparations after each step of the protocol. Virus preparations were negatively stained with 2% uranyl acetate prior to TEM observations.

**Cloning strategy and sequence analyses.** Cloning of the viral genome was performed as described for rose cryptic virus 1 (Sabanadzovic & Abou Ghanem-Sabanadzovic, 2008). dsRNAs extracted from JHFMoV-DI, -JM and -HR-infected plants were reverse-transcribed with random primers and amplified by degenerate oligonucleotide primers (DOP) by using a commercial kit (Roche). Generated amplicons were cloned into pGEM-T Easy plasmid (Promega) and selected plasmids were custom-sequenced (MWG Biotech). Initial sequences were used to design specific primers to generate the rest of the virus genome. Viral ends were generated by ligation of 5'-phosphorylated/3'-amino-blocked oligonucleotides to target dsRNAs prior to cDNA synthesis (Lambden et al., 1992). Sequences were assembled with Lasergene software (DNASTAR). Comparisons with sequences available in GenBank were performed by using the BLAST (Altschul et al., 1997) and CDD (Marchler-Bauer et al., 2007) online resources of NCBI. Protein sequences were aligned with MUSCLE software (Edgar, 2004), using default parameters with subsequent adjustment by Gblocks software (Castresana, 2000).
Bayesian inference of phylogeny using a variant of Monte Carlo Markov Chain (MCMC) was implemented by the MrBayes program (Huelsenbeck & Ronquist, 2001) and a 50 % majority rule consensus tree was constructed. The tree was visualized with the TreeDyn program (Chevenet et al., 2006). The RNAfold web resource (Gruber et al., 2008) was used to predict the secondary structure of the 3’ ends of JHFMoV genomic RNAs 1 and 2.

**Northern blots.** After electrophoresis in 1 % TAE agarose gels (TAE: 40 mM Tris/acetate, 1 mM EDTA, pH 8.0), dsRNAs of isolate JHFMo-DI were denatured, neutralized and double-rinsed with twice-distilled RNAse/DNase-free water prior to overnight transfer to a Hybond-N+ nylon membrane as described previously (Sabanadzovic et al., 2009). Membranes were hybridized with digoxigenin (DIG)-labelled JHFMoV-specific probes designated pJHFV-1 and pJHFV-2, complementary to 3’-proximal portions of genomic segments 1 and 2, respectively. A standard procedure, outlined by the manufacturer (Roche), was applied to visualize the presence of molecules homologous to the applied probes. DIG-labelled DNA Molecular Weight Marker III (Roche) was used as a molecular mass reference. The same probe was used in hybridization tests on total RNAs extracted from JHFMoV-infected tissue and healthy controls.

**RT-PCR detection.** Total RNAs were extracted from leaf tissue of symptomatic and healthy ferns following the Plant RNeasy Extraction kit protocol (Promega). Samples (5 μl) of total RNAs were heat-denatured at 70 °C for 5 min and reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Promega) in a total reaction volume of 30 μl. PCR was performed with a virus-specific primer pair, HFV-F (5’-GGAGCATGATATGACTATGGT-3’), HFV-R (5’-GGAAAGCCGAACCATGGG-3’), and JHFMo-DI (5’-GGAAAGCCGAACCATGGG-3’), based upon sequences of isolate JHFMo-DI and designed to amplify a 485 nt portion of the virus RNA1. The protocol, once initially set up on the three main isolates (JHFMo-DI, -JM and -HR), was applied for routine virus detection.

Virus detection by RT-PCR using spores was also attempted. For these tests, spores (appro 25 mg) from sori of infected and healthy fern specimens were collected on a sheet of circular filter paper and transferred into a 1.5 ml microcentrifuge tube, and RNA was extracted by using an RNeasy kit (Promega).

The following PCR conditions were applied for JHFMoV detection: (i) initial denaturation at 94 °C for 2 min, (ii) denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, extension for 45 s at 72 °C (40 cycles) and (iii) final extension for 10 min at 72 °C. PCR products were analysed by electrophoresis in 1.5 % TAE agarose gels, stained with ethidium bromide and visualized under UV light.

During the initial stages of this investigation, fern samples were tested for the presence of dsRNAs in order to ascertain virus presence. Once the RT-PCR method became available, it was used to investigate the distribution of JHFMoV by examining 45 symptomatic JHF specimens collected from different locations in LA, MS and neighbouring states.

### RESULTS

#### Symptoms and associated dsRNAs

Diseased JHF plants from various locations exhibited a variety of symptoms consisting of yellow mottle, mosaic, ring spots, oak leaf pattern, and necrosis (Fig. 1a–c). In some cases, overall stunting and premature death of lower leaves were observed in affected specimens.

Agarose and polyacrylamide gel analyses of dsRNA isolated from symptomatic JHF plants yielded a similar banding profile, consisting of two major dsRNAs of approximately 6.2 and 3.0 kbp accompanied by several minor molecules (Fig. 2a). dsRNA profiles of JHFMoV-JM, DI and HR were indistinguishable (Fig. 2b), indicating the presence of the same virus in all three specimens. Similar dsRNA profiles were obtained from all diseased JHF plants of different geographical origins, supporting their association with the symptoms as well as the relatively wide distribution of the virus. In addition to leaf tissue, dsRNAs were detected readily from sori collected from infected JHF-JM plants (results not shown).

#### Transmission studies

Attempts to transmit the virus by mechanical inoculations to several plant species, including young JHF plants, failed. Mechanically inoculated plants did not develop symptoms and tested negative for virus dsRNAs.

Graft transmission of JHFMoV-JM to healthy JHF plants was successful. Typical symptoms developed 4–6 weeks after inoculation in 11 of 15 plants inoculated, and virus dsRNAs were detected in the inoculated plants by PAGE analysis and RT-PCR.

Six of 50 plants grown from spores of an infected mother plant developed yellow mottle and mosaic symptoms resembling those observed on the mother plant 2 months after sporophyte germination. Virus-specific RT-PCR and dsRNA analyses confirmed the presence of the virus. None of the 50 plants from spores of a healthy plant developed symptoms (Fig. 3).
symptoms, nor did 10 randomly chosen specimens from this group of plants react in RT-PCR with JHFMoV (RNA1)-specific primers.

**Purification**

The purification method employing potassium phosphate buffer, followed by PEG precipitation, concentration and sucrose density-gradient centrifugation yielded the best results, showing the presence of quasi-spherical virions. One broad sedimenting band was observed in sucrose gradients after centrifugation. Several fractions corresponding to this band were collected and all contained quasi-spherical virus particles that ranged from 30 to 40 nm in diameter and were mixed with impurities (results not shown).

**Nucleotide sequences and genome organization**

The complete genomic RNA1 and RNA2 of the isolate JHFMoV-DI were 6228 and 3007 nt long, respectively, and both terminated with an unusual string of cytosine residues at the 3′ end (Figs 3, 4a). The length of the 3′ end-proximal oligocytosine stretch varied among sequenced clones from six to 11 residues. The clones containing the longest oligo-C tail were assumed to represent the complete 3′-proximal sequences and were counted as such in reporting the sizes of the two genomic RNA molecules.

Both genomic segments started with an identical hexanucleotide sequence (5′-GAUAAA...3′) and terminated with a highly conserved 62 nt region at the very 3′ end. The 3′ non-coding regions (NCRs) of RNAs 1 and 2 shared 92% identical nucleotides potentially capable of forming conserved secondary structures with apparently unpaired nucleotides at the extreme 3′ ends (Fig. 4a, b).

The organization of genomic RNA1 resembled that of raspberry bushy dwarf virus (RBDV). It contained two putative open reading frames (ORFs 1a and b) preceded and followed by 75 and 70 nt long 5′- and 3′-end NCRs, respectively. The first ORF spanned 1909 codons between nt 76 and 5805 and encoded a putative replication-associated polyprotein with an estimated Mr of 214 000. Analyses showed the presence of conserved motifs of viral methyltransferase (pfam01660), viral RNA helicase superfam-ily 1 (pfam01443) and RdRp superfamily 2 (pfam00978). Whilst the viral helicase and RdRp domains were comparable in size and organization with the corresponding regions of RBDV and members of the family Bromoviridae, the JHFMoV methyltransferase domain was considerably larger than orthologues of RBDV and bromo-viruses, which were of similar size. Conserved motifs of viral methyltransferase appeared to be organized as two ‘subdomains’ (grouped at the N and C termini of the putative protein) and separated by a relatively long chain of amino acids that appeared not to be conserved [see Supplementary Fig. S2(a), available in JGV Online]. The difference in size of this genomic portion was confirmed by RT-PCR, applying two sets of degenerate primers designed on conserved regions of JHFMoV and RBDV methyltransferase domains [see Supplementary Fig. S2(b)]. The viral helicase domain of JHFMoV shared 38% identical amino acids with those of both RBDV and cowpea chlorotic mottle virus, and somewhat less (30–35%) with several viruses belonging to the genera Bromovirus, Cucumovirus and Ilarvirus of the family Bromoviridae. The RNA-dependent RNA polymerase domain shared comparable levels of amino acid identity (30–35%) with RBDV and members of the family Bromoviridae. Despite the apparent similarity in organization, the overall identity of amino acids between the whole polyproteins encoded by ORF1 of JHFMoV and RBDV was rather limited (approx. 27%).

An additional putative ORF (ORF1b) spanning 321 nt, starting from position 5835 and potentially encoding a protein of 106 aa in length and an estimated molecular mass of approximately 12 kDa (p12), was present at the 3′ end of the viral RNA-1. A BLASTP search did not reveal significant homology of this putative product with any
protein currently deposited in GenBank. A similar ORF is also present in the RBDV genome, but is reported as probably not expressed, due to the apparent absence of a corresponding subgenomic RNA (Ziegler et al., 1992).

Direct comparison with the putative protein encoded by RBDV ORF1b revealed low levels of common amino acids (15 %), with no conserved motifs.

Genomic segment 2 (RNA2) contains three ORFs separated by 70–100 nt intergenic regions (Fig. 3). Following a short NCR, ORF 2a starts at nt 46 and extends for 298 codons, encoding a putative product with an estimated molecular mass of 32 kDa (p32) containing conserved motifs of the 3A movement protein superfamily (pfam00803). Curiously, this protein had higher levels of identities (32–35 %) to orthologues from groundnut rosette virus, pea enation mosaic virus 2 and other members of the genus Umbravirus, as well as with various viruses in the family Bromoviridae (15–20 %), than to 2a protein encoded by RBDV, with which it shared 10 % identity. ORF2b starts after a 120 nt intergenic region and encodes a 37 kDa putative polypeptide (p37). The most 3′-end-proximal ORF, 2c, encodes a putative 266 aa protein (p29). Both proteins showed no significant similarity to any of the proteins in GenBank. Whilst the function of p37 is unknown, the putative protein encoded by ORF 2c could be a viral coat protein (CP), as in genomes of RBDV and viruses in the family Bromoviridae. However, pairwise comparisons of p29 with CPs of RBDV and bromoviruses did not reveal significant similarities or conserved motifs between the two proteins, confirming that the RNAs 2 of JHFMoV and RBDV have a different evolutionary origin.

For comparative purposes, complete sequences of the RNA2 of JHFMoV were generated for two other isolates (JHFMoV-JM and JHFMoV-HR). Interestingly, the two isolates from MS appeared almost identical in size (3007 nt) and nucleotide content (96 %). Isolate JHFMoV-JM from LA shared only 83–84 % common nucleotides with JHFMoV-DI and JHFMoV-HR, and had a slightly longer RNA2 molecule (3018 nt). The putative product of ORF2a was 1 aa shorter than p32 of JHFMoV-DI and HR and shared 85 % identical residues with the two isolates from MS, which in turn shared 93 % identity with each other. The product of ORF2b was the most diversified among the three isolates: amino acid identity between JHFMoV-DI and JHFMoV-JM was 83 ‰, whilst the two isolates from MS shared 95 % identical residues. The putative protein encoded by ORF2c was the most conserved. JHFMoV-DI and -HR shared 99 % identical amino acid sequences and only differed by 11 ‰ from deduced amino acid sequences of the isolate JHFMoV-JM.

**Northern blotting**

Northern blot analyses performed on purified dsRNA unveiled the origin and nature of the multiple banding patterns observed in polyacrylamide and/or agarose gel electrophoreses. The specific probe to RNA1 hybridized with two molecules: the replicative form of full-size RNA1 and with another dsRNA molecule with an estimated size of approximately 410 bp. This molecule was also visible in dsRNA gels (see Fig. 2, asterisks) and its size matches the length of replicative (dsRNA) form of a putative subgenomic molecule (sgRNA) that may serve as a template for the expression of ORF1b (Fig. 4c). Furthermore, an RNA2-specific DIG-labelled probe recognized three dsRNAs, corresponding to the full-size replicative form of RNA2 and replicative forms of two subgenomic molecules with estimated sizes of approximately 2.0 and 1.0 kbp, which are in agreement with the sizes of putative sgRNAs deduced from generated sequence data. Hybridization signals corresponding to full genomic size and both putative subgenomic RNAs were obtained when total RNA extracts were exposed to the RNA2-specific probe (results not shown), confirming data from dsRNA blots.

**Phylogenetic analyses**

Phylogenetic analyses, based upon Bayesian inference, showed independent origins of RNAs 1 and 2 of
JHFMoV. Whilst all domains of the RNA1-encoded putative replication-associated polyprotein grouped with RBDV-encoded proteins (Fig. 5a), ORF2a-encoded protein 2a is related more closely to movement proteins of umbraviruses and several other plant virus taxa than to idaeoviruses (Fig. 5b). Similar tree topologies were observed with maximum-likelihood (ML) and neighbour-joining (NJ) methods (results not shown).

**JHFMoV detection**

The primer set designed from a conserved portion of viral RNA1 amplified a single DNA fragment of the expected size (485 nt) from several dsRNA-positive ferns, including JHF-JM and JHF-HR, in the initial phases of the experiment set-up, with no amplicons in negative controls (dsRNA-free fern plants). Further tests detected this virus in a number of diseased Japanese holly ferns from different locations in MS and LA, as well as from some neighbouring states (Tennessee, Texas, Arkansas and Alabama). A correlation between virus infection and disease incidence was observed, supporting the role of JHFMoV in the disease aetiology. In addition, the virus was detected readily in reverse-transcribed extracts from spores collected from symptomatic plants, but not in those from healthy controls (see Supplementary Fig. S3, available in JGV Online).

**DISCUSSION**

An emerging disease of viral origin was recently observed on JHF plants in LA and MS. Disease symptoms varied slightly in different geographical locations and consisted of yellow/chlorotic mosaic, mottling, line patterns and necrosis. In some cases, the virus affected the size of the plant and caused premature senescence of the leaves.

In this paper, we provide evidence of the association of a previously undescribed virus, for which we propose the name Japanese holly fern mottle virus (JHFMoV), with the disease of JHF. As the virus appears not to be mechanically transmissible, we could not fulfill Koch’s postulates to prove experimentally its causal role in the disease, but our results indicate a strong correlation between JHFMoV infection and symptoms. All symptomatic JHF plants obtained from six different states and tested during the investigation contained this virus, supporting the hypothesis of its causative role.

Although the virus was not mechanically transmissible to JHF or to several herbaceous plant species tested, it was transmitted to healthy JHF plants by grafting and by spores from infected plants. Our results represent the first report of spore transmission of a plant virus. In addition to spore transmission, we propose the probable involvement of a yet-to-be-identified aerial vector in the spread of the virus in the southern USA. Our hypothesis is based upon several cases of JHF in plants that were planted almost a decade ago and remained symptomless (and dsRNA-free) for several years until a sudden and recent disease outbreak (i.e. plants growing at the Louisiana State University campus in Baton Rouge, LA, and in several private yards in LA and MS). In LA, thrips and several species of mite were often found infesting JHF plants and therefore should be tested as potential vectors of this virus.

Several purification protocols failed to reveal the presence of virions in infected tissue. However, a method for virus purification similar to that used for RBDV (Jones & Mayo, 1998) yielded a broad sedimenting band in sucrose-density gradients. The band contained partially disrupted quasi-spherical virions mixed with plant-cell residues. The virion swelling/disruption could be an artefact of the purification method, as reported previously for RBDV (Jones & Mayo, 1998).

**Fig. 5.** Bayesian inference-based phylograms based on conserved amino acid sequences of RNA-dependent RNA polymerases (a) and putative movement proteins encoded by ORF2a (b) of JHFMoV and members of several taxa. Branch support values (%) are indicated. Abbreviations: AMV, alfalfa mosaic virus; BMV, brome mosaic virus; CCMV, cowpea chlorotic mottle virus; CMoMV, carrot mottle mimic virus; CMV, cucumber mosaic virus; GLRaV-9, grapevine leafroll-associated virus 9; GRV, groundnut rosette virus; OGSV, oat golden stripe virus; PBNSPaV, plum bark necrosis and stem pitting-associated virus; PEMV-2, pea enation mosaic virus 2; PMMV, pepper mild mottle virus; PSV, peanut stunt virus; RBDV, raspberry bushy dwarf virus; SBCMV, soil-borne cereal mosaic virus 2; SBWMV, soil-borne wheat mosaic virus; TAV, tomato aspermy virus; TBT, tomato bushy top virus; TMV, tobacco mosaic virus; TRV, tobacco rattle virus. Citrus idaeovirus refers to a single partial sequence of a putative idaeovirus available in GenBank.

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JHFMoV has a bipartite genome with a unique arrangement of the five putative ORFs and a peculiar string of 6–11 cytosine residues at the 3’ end of both viral nucleic acids. Such a long homopolymeric cytidine stretch has not, to our knowledge, previously been reported in plant viruses. In addition, RNAs 1 and 2 shared highly conserved sequences at the 3’ end that are capable of forming peculiar secondary structures. 

Northern blot analyses showed the abundant presence of subgenomic RNAs in infected tissues. Non-radioactive probes originating from the 3’-proximal regions of RNA1 recognized full genomic size molecules, as well as an additional molecule of approximately 0.4 kb, whilst an RNA2-specific probe generated a signal with three RNAs corresponding to sizes of the entire genomic RNA2 and two putative subgenomic molecules that are likely to be templates for the expression of ORFs 2b and 2c.

At first sight, the organization of RNA1 of JHFMoV resembles that of RBDV. However, they share very limited levels of conserved amino acids. Unlike RBDV, the presence of a putative 3’-proximal subgenomic RNA corresponding to ORF1b strongly suggests that this cistron is indeed expressed during infection. Experiments on expression of p12 in bacteria and production of anti-p12 antibodies are ongoing in order to test this hypothesis. The second genomic segment of JHFMoV contains three putative ORFs, whereas the RNA2 of RBDV is bicistronic (Natsuaki et al., 1991). The product of ORF2a appears to share common origins with 3A movement proteins of viruses belonging to the genus Umbravirus, as well as with viruses belonging to the family Bromoviridae. Putative proteins encoded by ORFs 2b and 2c currently do not have statistically significant matches with proteins deposited in GenBank.

In this study, the RNA2 sequences of the LA isolate (JHFMoV-JM) were distinct from that of the two MS isolates (JHFMoV-DI and -HR), indicating that considerable genetic variability among isolates from different geographical areas may exist. Despite relatively high differences in RNA2, partial sequences of RNA1 obtained for JHFMoV-JM show a high degree of identity (90% and more), with corresponding regions of JHFMoV-DI confirming that they are indeed the same virus. We are currently cloning and sequencing additional JHFMoV isolates, collected from different locations during this investigation, in order to gain better knowledge of the population structure of this virus in the southern USA. It is unknown whether this virus is limited only to fern species or whether it also infects higher plants. If limited only to ferns, this virus may represent an ‘ancient’ lineage of plant viruses, adapted and co-evolved with its pteridophyte host(s). We have tested additional ornamental and/or wild plant species collected from nearby infected ferns, but we could detect the virus only in infected JHF specimens.

The biological and molecular properties of JHFMoV and its phylogenetic relationships with members of the genera Idaevirus (Jones, 2005) and Umbravirus (Talianisky et al., 2005) and with members of the family Bromoviridae (Roossinck et al., 2005) provide evidence that JHFMoV is a unique and previously undescribed, bipartite RNA virus species.

Based upon the results of this work, we propose that JHFMoV represents the type species of a novel genus of plant viruses. Considering that JHFMoV is the first truly characterized virus from ferns (Planta: Pteridophyta), we propose the name Pterovirus (pteris Gr. n. fern) for this novel phytovirus genus.

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