Arch Virol (2015) 160:2093–2098
DOI 10.1007/s00705-015-2462-7

BRIEF REPORT

# *Fusarium circinatum* isolates from northern Spain are commonly infected by three distinct mitoviruses

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Received: 4 March 2015 / Accepted: 20 May 2015 / Published online: 31 May 2015


**Abstract** Pitch canker is a serious disease of pines caused by the ascomycete fungus *Gibberella circinata* (anamorph = *Fusarium circinatum*). Three distinct mitovirus strains have been described in this fungus: Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. Here, we investigated the frequency and population variation of these viruses and closely related sequence variants in northern Spain using RT-PCR and sequencing. Each virus strain and similar sequence variants shared >95 % sequence identity and were collectively designated as virus types. All virus types were relatively common in Spain, with estimated prevalence of 18.5 %, 8.9 % and 16.3 % for FcMV1, FcMV2-1 and FcMV2-2, respectively.

**Keywords** *Gibberella circinata* · Pitch canker · ssRNA · *Narnaviridae* · Virus population

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s) KP726378–KP726394.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00705-015-2462-7) contains supplementary material, which is available to authorized users.

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The ascomycete fungus *Gibberella circinata* Nirenberg & O'Donnell (anamorph = *Fusarium circinatum*) causes a serious disease known as pitch canker on *Pinus* spp. The pathogen initiates resinous bleeding cankers on the tree trunks or large branches, and infected trees can eventually die due to girdling or stem breakage. The fungus was first reported in North Carolina [6] and Mexico, and has since spread into Haiti, South Africa, Japan, Chile, Korea, Southern Europe, Uruguay [reviewed in reference 12], Colombia [17] and Brazil [15]. *F. circinatum* has moved between plantations areas via contaminated seed and seedlings, and the fungus spreads further through airborne spores, insect vectors and water splash. Mechanical damage that provides infection courts increases the risks of infection [1]. The fungus is considered to be native in Mexico [24], the Caribbean and the southeastern USA.

Fungi of the genus *Fusarium* are hosts to diverse viruses, including members of the families *Chrysoviridae*, *Hypoviridae*, *Partitiviridae* and *Totiviridae* [3], as well as the yet unassigned Fusarium graminearum virus 1, which reduces the growth rate and virulence of its host [4]. We recently characterized three distinct strains of mitoviruses co-infecting *F. circinatum* isolate FcCa070: Fusarium circinatum mitovirus 1 (FcMV1) and two strains of Fusarium circinatum mitovirus 2 (FcMV2-1 and FcMV2-2) [13]. Fungal viruses in the genus *Mitovirus* belong to the family *Narnaviridae*, which are the simplest known viruses. They have a positive-sense ssRNA genome of ~2.5-4.4 kb, are located in mitochondria, and utilize the mitochondrial translation table [7, 22]. Mitoviruses have been found in many phytopathogenic fungi, including both ascomycetes and basidiomycetes. Some mitoviruses appear to mediate reduced virulence (hypovirulence) in important plant pathogens, including *Botrytis cinerea*, *Chalara elegans*, *Ophiostoma novo-ulmi*, *Rhizoctonia solani* and *Sclerotinia*

*homeocarpa* [reviewed in reference 7]. In this study, we examined the occurrence of mitoviruses in *F. circinatum* isolates collected from northern Spain, as well as 29 isolates of *F. circinatum* from South Africa, and five isolates of other species of *Fusarium* from Cantabria (northern Spain). Each new virus sequence determined in this study closely resembled that of one of the previously determined strains (FcMV1, FcMV2-1 or FcMV2-2), and we classified mitovirus sequence variants sharing >95 % similarity with one of these strains as mitovirus 'types'.

Fungal cultures were established from infected plant or insect tissues as described previously [11], and collection data for the *Fusarium* isolates used for virus screening are listed in Tables S1 and S2. Two different approaches were used for virus screening: CF11 cellulose affinity chromatography was used to detect viral dsRNA elements, and specific mitovirus types were detected by RT-PCR with selective primers. CF11 cellulose affinity chromatography was conducted using lyophilized cells as described previously [13, 19]. Complementary DNA for the RT-PCR was generated by random priming using previously described techniques [9, 20]. The following PCR primers were designed for virus screening and sequence determination: FMC3F1 (5′-GAY AGA ACT TTT ACT CAA GAT CC-3′), FMC3Rev1 (5′-ATT CAT CTY TTG GCA AAT TCA TA-3′), FMC1F1 (5′-CGT GGA TTA AAA CCC ACA AA-3′) and FMC1Rev1 (5′-TGG TAA TCT ACC ATA GCA ATT AYT C-3′). Primer pair FMC1F1/FMC1Rev1 is specific for FcMV1, whereas primer pair FMC3F1/FMC3Rev1 preferably detects virus types FcMV1, FcMV2-1 or FcMV2-2, depending on the DNA polymerase used (fusion-type versus conventional) and annealing temperature. The amplification products cover two different genome locations: the product obtained with primer pair FMC1F1/FMC1Rev1 covers nt 511-935 in the FcMV1 genome, whereas the product obtained with FMC3F1/FMC3Rev1 covers nt 1000-1478. PCRs were conducted in volumes of 50 µl, including 1-2 µl of the cDNA product, 25 pmol of each primer, 10 nmol of dNTPs and 1-1.25 units of a DNA polymerase as recommended by the manufacturer. The amplification conditions used for primer pair FMC1F1/FMC1Rev1 were as follows: 10 min at 95 °C, followed by 37 cycles of 30 s at 95 °C, 45 s at 53 °C, 2 min at 72 °C; and a final extension of 7 min at 72 °C. The same PCR program was used for primer pair FMC3F1/FMC3Rev1, but the annealing temperature was adjusted according to the polymerase enzyme used. The amplification of FcMV2-1 from isolates with FcMV2 co-infections was carried out using a conventional DNA polymerase – Dynazyme II (Thermo Scientific), DreamTaq (Thermo Scientific) or Biotools Native DNA polymerase (Biotools B & M Labs) – and an annealing temperature of 53 °C, whereas FcMV2-2 was amplified using a fusion domain containing thermostable DNA polymerase – Phusion (Thermo Scientific), PhireII (Thermo Scientific), or Q5 (New England Biolabs) – and an annealing temperature of 59 °C.

The amplicons were sequenced at Macrogen Inc. (www.macrogen.com). Regardless of the polymerase used, the sequencing results were constant for single viral sequence variants. Sequence variation was examined and phylogenetic analysis was performed using Geneious Pro 5.5.8 (Biomatters Ltd.) and MEGA 5 [18]. The frequency of sequence variants (='haplotypes') at each collection site was compared to an estimated random distribution using AMOVA, implemented in Arlequin 3.5 [5], in order to examine the level of sequence differentiation among the collection sites (i.e., 'population differentiation'). Putative recombination events were identified with RDP4 (v.4.16) [10] using the recombination detection methods RDP, GeneConv, Chimaera, MaxChi, SiScan and 3Seq (highest probability was set as 0.1).

Using CF11 chromatography, we detected dsRNA elements in 14 of the 135 *F. circinatum* isolates analyzed (Table S1). No dsRNA elements were detected among the 29 South African isolates of *F. circinatum* or the five isolates of other species of *Fusarium* (Table S2). RT-PCR using the mitovirus primers yielded amplification products from 43 *F. circinatum* isolates (31.9 %). Therefore, most of the mitovirus infections were not detectable as dsRNA. This is a common phenomenon, likely resulting from the fact that mitoviruses have ssRNA genomes, and the replicative dsRNA intermediates may have a low titer [e.g., 14, 16, 22]. Notably, co-infections by two or three mitovirus types were common, and resulted in a total of 59 virus incidences among the Spanish host isolates. Co-infections with FcMV1 and FcMV2-2 were found in nine isolates, and five isolates hosted both FcMV2-1 and FcMV2-2. One isolate hosted all three virus types.

Genetic variation within the mitovirus population was assessed by sequence analysis. The amplicon length was 425 nt for FMC1F1/FMC1Rev1 and 479 nt for FMC3F1/FMC3Rev1. The partial sequences were deposited in the GenBank database with accession numbers KP726378–KP726394 (Table 1). Based on BlastN and phylogenetic analysis including 312–351 nt sites (Fig. 1), each sequence was highly similar to one of the three mitovirus strains from *F. circinatum* FcCa070 characterized previously. This suggests that FcMV1, FcMV2-1 and FcMV2-2 are separate genetic entities (i.e., virus 'types'), each of which consists of highly similar sequence variants. Sequence identity among variants representing single virus types was >95 % (Table 1). At the protein level, the sequences were highly conserved. All three variants of FcMV2-2 were 100 % identical at the protein level, whereas three different variants of FcMV2-1 shared identical protein sequences, and

**Table 1** Viral sequence characteristics

| Virus strain | Variant (haplotype)[a] | Host isolate | GenBank accession no. | Primer pair | Identity (% nt) with FcMV-strains from isolate 070[b] | Sequence length | Host isolates harboring the same sequence variant (sequence length in bp) |
|---|---|---|---|---|---|---|---|
| FcMV1 | a | 070 | KF803546 | FMC1F1/Rev1 | 100 % | 2419 | **047**(337), **072**(351), **VA104**(325) |
| | a | 070 | KF803546 | FMC3F1/Rev1 | 100 % | 2419 | **042**(146)[c], **045**(386), **048**(433) |
| | b | 60_2010 | KP726378 | FMC1F1/Rev1 | 98.5 % | 332 | - |
| | c | Saint1.7 | KP726379 | FMC1F1/Rev1 | 98.4 % | 322 | - |
| | d | 069 | KP726380 | FMC1F1/Rev1 | 98.3 % | 354 | **067**(322) |
| | d | 069 | - | FMC3F1/Rev1 | 100 % | 392[d] | - |
| | e | Saint1.189 | KP726381 | FMC1F1/Rev1 | 98.0 % | 322[d] | - |
| | f | 22_2010 | KP726382 | FMC1F1/Rev1 | 97.9 % | 327 | - |
| | g | VA221 | KP726383 | FMC1F1/Rev1 | 97.8 % | 348[d] | - |
| | h | 100 | KP726384 | FMC1F1/Rev1 | 97.6 % | 327 | **099**(322) |
| | i | 001 | KP726385 | FMC1F1/Rev1 | 97.2 % | 326 | - |
| | j | VA215 | KP726386 | FMC1F1/Rev1 | 95.4 % | 327 | - |
| | j | VA215 | KP726387 | FMC3F1/Rev1 | 98.3 % | 372[d] | **043**(56)[cd], **050**(285)[e] |
| | k | 052 | KP726388 | FMC3F1/Rev1 | 99.8 % | 405 | **051**(415)[e] |
| | l | 041 | KP726389 | FMC3F1/Rev1 | 99.2 % | 392 | **068**(390), **073**(376) |
| FcMV2-1 | a | 070 | KF803547 | FMC3F1/Rev1 | 100 % | 2193 | **053**(132)[c] |
| | b | 009 | KP726390 | FMC3F1/Rev1 | 99.7 % | 369 | **005**(390)[cd], **007**(356), **010**(354)[cd], **020**(364) |
| | c | 018 | KP726391 | FMC3F1/Rev1 | 99.5 % | 388 | **019**(395)[c], **026**(371)[c], **032**(399) |
| | d | 046 | KP726392 | FMC3F1/Rev1 | 99.5 % | 389 | - |
| FcMV2-2 | a | 070 | KF803548 | FMC3F1/Rev1 | 100 % | 1973 | **010**(359), **020**(363), **026**(363), **033**(340), **035**(360), **042**(117)[c], **054**(359)[c], **073**(321), **099**(358), **100**(372), **VA221**(394), **VA253**(333), **Saint1.7**(60)[c] |
| | b | 004 | KP726393 | FMC3F1/Rev1 | 99.5 % | 398 | **001**(424), **002**(364)[d], **003**(405), **005**(394), **007**(402)[e], **22_2010**(224) |
| | c | 60_2010 | KP726394 | FMC3F1/Rev1 | 99.3 % | 355[d] | - |

[a] FcMV1 sequence variants obtained using the two different primer pairs are considered distinct unless they were obtained from the same host isolate (variants FcMV1-a, FcMV1-j)

[b] Variants of FcMV1 are compared to FcMV1-a; variants of FcMV2-1, to FcMV2-1-a; and variants of FcMV2-2, to FcMV2-2-a

[c] Sequence is in one direction only. All other sequence variants have sequence coverage of at least two times. Sequences determined in one direction only (N = 10) were assigned to haplotypes according to the most reliable sequence region (56-395 nt, depending on sequence quality)

[d] The sequence included one ambiguous nucleotide site (double base calls), and the haplotype assignment was made according to the dominant nucleotide character

[e] The sequence included 2-3 ambiguous nucleotide sites (double base calls), and the haplotype assignment was made according to the dominant nucleotide character

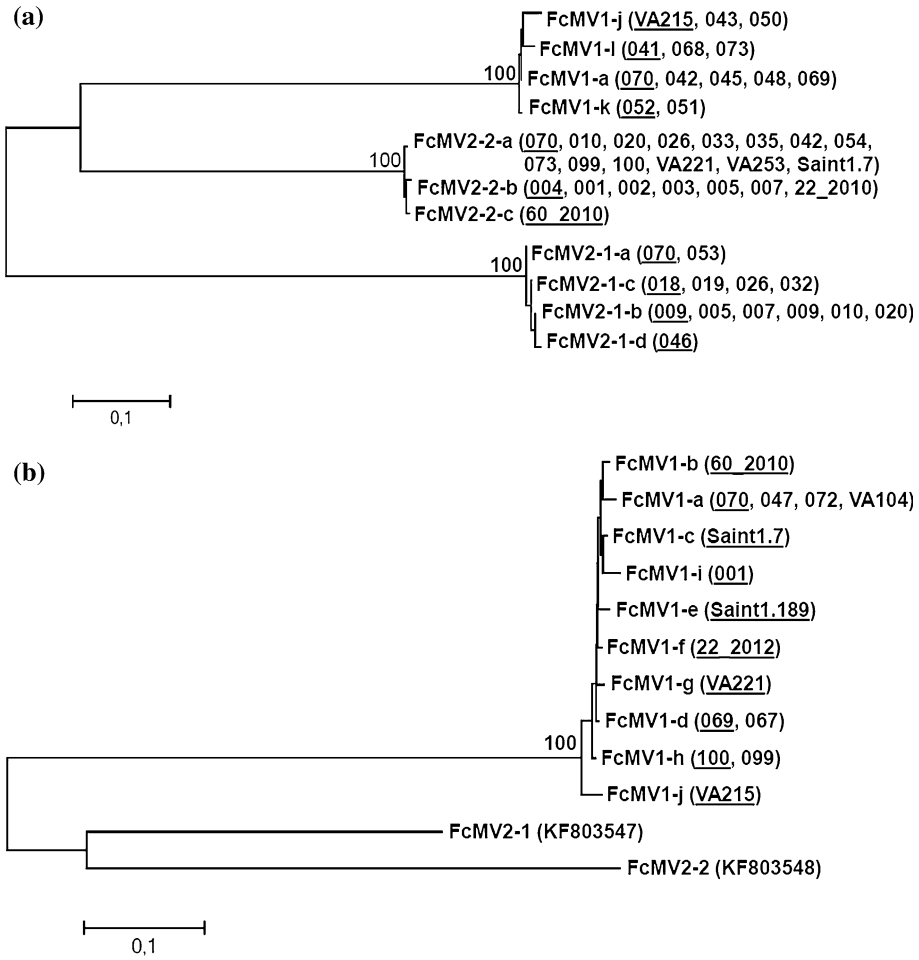


**Fig. 1** Neighbor-joining dendrograms for sequence variants obtained using primer pair FMC3F1/FMC3Rev1 (**a**) or FMC1F1/FMC1Rev1 (**b**). *F. circinatum* isolates harboring the same virus haplotype (identical sequences) are shown in parentheses (see Table 1), and the representative sequence selected for dendrogram construction is underlined. Bootstrap probabilities of >90 % (1000 replicates) are shown next to branches. Distances are shown as the number of substitutions per site. Positions containing gaps or missing data were eliminated, and the number of positions used was 351 for FMC3F1/FMC3Rev1 (**a**) and 312 for FMC1F1/FMC1Rev1 (**b**)

variant FcMV2-1-c differed from them by one amino acid residue. In the case of FcMV1, different variants shared 96.9-99.3 % identity in the sequence region flanked by FMC3F1/FMC3Rev1 (1-3 aa mismatches) and 95.4-100 % in the region flanked by FMC1F1/FMC1Rev1.

The majority of the *F. circinatum* isolates investigated were from Cantabria (Fig. 2). Viruses occurred in 26 (39.4 %) of the 66 isolates from Cabezón de la Sal in Cantabria, and co-infections were found in seven isolates, resulting in a total of 33 viral infections (Table S1). In Rionansa, five (83.3 %) of the six isolates were co-infected, and the number of viral infections was 10. In Comillas, six (85.7 %) of the seven host isolates were virus-infected, and there was one double and one triple co-infection. The two isolates from Castro-Urdiales were both infected by FcMV1. However, isolates from some locations were devoid of viral infections: 17 isolates from Villafufre, 11 isolates from Mazcuerras and one isolate from Cabuérniga in Cantabria were all virus-free. Moreover, in Santiurde de Toranzo, we observed unknown dsRNA elements of ca. 2.5 kb in four *F. circinatum* isolates (Table S1). Whether they represent additional mitoviruses or members of other virus species is still unknown, but they remained undetected using our set of primers. Similarly, two unknown dsRNA virus infections were observed in Cabezón de la Sal. Outside the Cantabria region, we found two FcMV1 infections in five isolates from Asturias and one FcMV2-2 infection in seven isolates from Galicia. No viruses were detected in the four *F. circinatum* isolates from País Vasco, and none were found in the South African isolates or members of other *Fusarium* species (Table S2). It should be noted that many FcMV1 sequence variants were only detected with one of the primer pairs used, which suggests that the mitovirus frequencies reported here are conservative estimates.

Many sequence variants (i.e., haplotypes; see Table 1) were found at more than one collection site: FcMV1-a from Cabezón de la Sal, Comillas and Asturias; FMC1-j from Cabezón de la Sal and Asturias; FcMV1-l from Cabezón de la Sal and Comillas; FcMV2-2-a from Cabezón de la Sal, Comillas, Rionansa, and Galicia; and FcMV2-2-b from Rionansa, Cabezón de la Sal and Castro-Urdiales. Even though the sequences are partial, this suggests that highly similar viral sequence variants occur throughout northern

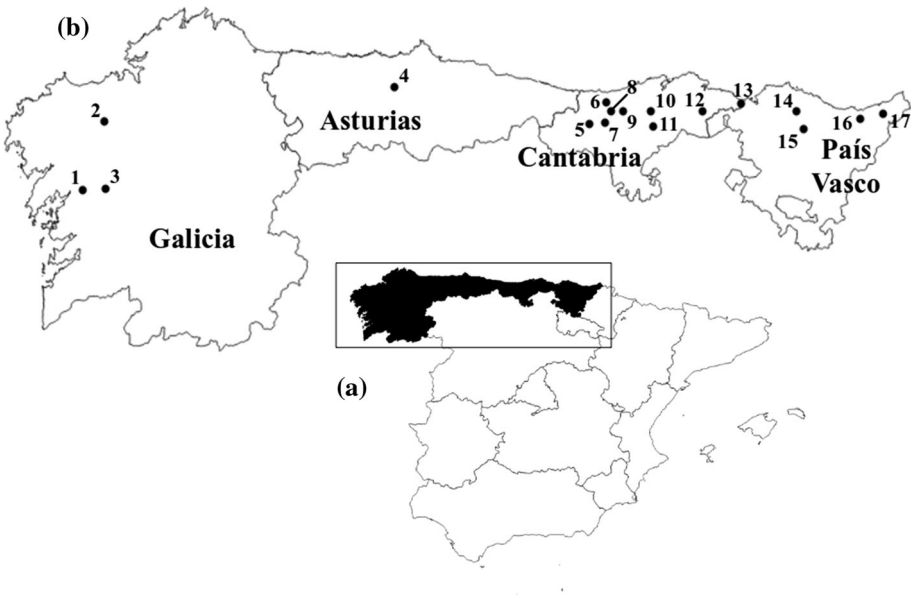


**Fig. 2** Collection sites of *F. circinatum* isolates in Spain (**a**) and in the autonomous communities of Galicia, Asturias, Cantabria and País Vasco (**b**). The numbers refer to municipalities: 1 = Valga, 2 = Ordes, 3 = A Estrada, 4 = Grado, 5 = Rionansa, 6 = Comillas, 7 = Cabuérniga, 8 = Cabezón de la Sal, 9 = Mazcuerras, 10 = Villafufre, 11 = Santiurde de Toranzo, 12 = Ramales de la Victoria, 13 = Castro Urdiales, 14 = Muxika, 15 = Iurreta, 16 = Aia and 17 = Hernani

Spain. Moreover, host mating type compatibility did not seem to restrict the occurrence of mitoviruses. While the vast majority of *F. circinatum* isolates analyzed encompassed mating type idiomorph *MAT1-2* [23], which is the only mating type found in Cantabria [2], there were a total of eight host isolates with the *MAT1-1* mating type in Asturias or Galicia (Table S1). FcMV1 or FcMV2-2 was detected in two of the *MAT1-1* isolates, namely *F. circinatum* VA104 and VA253, respectively. Notably, the latter isolate originated from Galicia and hosted a sequence variant that was identical over 333 nt to FcMV2-2-a, hosted by isolate 070 (=FcCa070) from Comillas, Cantabria. These collection sites are located more than 250 kilometers apart.

Single collection sites typically harbored more than one virus sequence variant, and all three virus types occurred in Cabezón de la Sal and Comillas. Moreover, based on AMOVA analysis, there seemed to be a low-level geographical differentiation between the locations in terms of viral polymorphism. Considering all haplotypes obtained using primer pair FMC3F1/FMC3Rev1, 85.6 % of the variation was attributable to within-population variation, and only 14.4 % was attributable to between-population variation ($P = 0.03$). Individual analysis of haplotypes representing each virus type showed similar results: the level of within-population variation was 79.8-91.3 % in FcMV1 (sequence regions flanked by FMC3F1/FMC3Rev1 and FMC1F1/FMC1Rev1, respectively), 64.0 % in FcMV2-1, and 85.2 % in FcMV2-2, but these results were only tentative ($0.27 \geq P \geq 0.10$).

The number of sequence variants was higher for the sequence region flanked by primer pair FMC1F1/FMC1Rev1 than for that flanked by primer pair FMC3F1/FMC3Rev1. This is due to differences in the level of sequence polymorphism in the sequence regions analyzed: the FcMV1, FcMV2-1 and FcMV2-2 genomes share ~52.2-58.2 % identity at the sequence region flanked by primer pair FMC1F1/FMC1Rev1, whereas sequence identity in the sequence region flanked by primer pair FMC3F1/FMC3Rev1 is higher, and the three virus types share 62.4-67.1 % identity. The sequence region covered by FMC3F1/FMC3Rev1 spans the conserved amino acid sequence 'motif IV' in the RdRp-like proteins encoded by mitochondrial viruses, as determined by Hong et al. [8], whereas the sequence region covered by FMC1F1/FMC1Rev1 is more variable, with only the last 5-14 amino acids overlapping with conserved motif I.

Two putative recombination events were detected using the RDP program among the FcMV1 sequences obtained using primer pair FMC1F1/FMC1Rev1. One putative breakpoint (recombination event) was located at nucleotide site 645 in the FcMV1 genome and was identified by MaxChi, Geneconv and SiScan to have occurred among sequence variants FcMV1-a, FcMV1-g and FcMV1-j ($P = 8.2^{-03}$). Another putative breakpoint detected by 3Seq was located at site 625 in the FcMV1 genome and seemed to have occurred among sequence variants FcMV1-j, FcMV1-b and FcMV1-f ($P = 2.4^{-02}$). No recombination events were detected in the conserved sequence region amplified using primer pair FMC3F1/FMC3Rev1.

North America (USA) is considered to be the area of origin of the Spanish population of *F. circinatum*, and there seem to have been at least two introductions of the pathogen into Spain, followed by clonal spread [2]. As a result, only two to three *F. circinatum* clones occur in Spain. The South African population is considerably more

diverse than the Spanish population and probably originates from Mexico [24]. The *F. circinatum* mycovirus diversity in the United States remains to be investigated and might shed more light on the dispersal pattern of this pathogen, i.e., finding identical virus strains at these geographically distant locations might confirm speculations about the origin of the fungal host. In this study, we observed vast differences in the incidence of viral infection of *F. circinatum* between nearby collection sites. For example, viruses were absent in eleven isolates in Mazcuerras, whereas viruses were common in the nearby ($\sim$8 km distance) Cabezón de la Sal. In contrast, single virus types in Spain appeared to have a wide geographical distribution. Because the presence or absence of viruses in sexual and asexual spores may have a profound effect on the dispersal patterns of mycoviruses [21], future studies are needed to investigate whether viruses are present in the conidia or ascospores of *F. circinatum*.

*F. circinatum* mitoviruses seem to be common and polymorphic in northern Spain. The occurrence of several viral types and variants at single collection sites is consistent with the occurrence of single clonal genotypes of *F. circinatum* throughout a wide geographical region in northern Spain.

**Acknowledgements** This study was financially supported by the Academy of Finland (decision numbers 251193 and 258520), the Spanish Ministry of Economy and Competitiveness (Project: AGL2012-39912), the Government of Cantabria, EU COST Actions FP1002 and FA1103 and the University of Valladolid. Instituto Agroforestal Mediterráneo and the Forestry and Agricultural Biotechnology Institute of the University of Pretoria provided strains for mitovirus screening. We are grateful to M.-L. Santanen, S. Sarsila, E. Simón Vicente, M. de Vallejo and J. Blanco for technical assistance, and Prof. M. Wingfield for commenting on the manuscript.

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