

## Novel taxa in the *Fusarium fujikuroi* species complex from *Pinus* spp.

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**Abstract:** The pitch canker pathogen *Fusarium circinatum* has caused devastation to *Pinus* spp. in natural forests and non-natives in commercially managed plantations. This has drawn attention to the potential importance of *Fusarium* species as pathogens of forest trees. In this study, we explored the diversity of *Fusarium* species associated with diseased *Pinus patula*, *P. tecunumanii*, *P. kesiya* and *P. maximinoi* in Colombian plantations and nurseries. Plants displaying symptoms associated with a *F. circinatum*-like infection (i.e., stem cankers and branch die-back on trees in plantations and root or collar rot of seedlings) were sampled. A total of 57 isolates were collected and characterised based on DNA sequence data for the translation elongation factor 1- $\alpha$  and  $\beta$ -tubulin gene regions. Phylogenetic analyses of these data allowed for the identification of more than 10 *Fusarium* species. These included *F. circinatum*, *F. oxysporum*, species within the *Fusarium solani* species complex and seven novel species in the *Fusarium fujikuroi* species complex (formerly the *Gibberella fujikuroi* species complex), five of which are described here as new. Selected isolates of the new species were tested for their pathogenicity on *Pinus patula* and compared with that of *F. circinatum*. Of these, *F. marasasianum*, *F. parvisorum* and *F. sororula* displayed levels of pathogenicity to *P. patula* that were comparable with that of *F. circinatum*. These apparently emerging pathogens thus pose a significant risk to forestry in Colombia and other parts of the world.

**Key words:** *Fusarium*, Morphology, Pathogenicity, Phylogenetics, *Pinus kesiya*, *P. maximinoi*, *P. patula*, *P. tecunumanii*.

**Taxonomic novelties: New species:** *F. fracticaudum* Herron, Marinc. & M.J. Wingf., *F. marasasianum* Herron, Marinc. & M.J. Wingf., *Fusarium parvisorum* Herron, Marinc. & M.J. Wingf., *F. pininemorale* Herron, Marinc. & M.J. Wingf., *F. sororula* Herron, Marinc. & M.J. Wingf..

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### INTRODUCTION

Over the last two decades, the incidence of plant diseases in forest ecosystems has increased dramatically (Orwig 2002, Fisher *et al.* 2012). This is primarily due to anthropogenic activities (e.g., Anagnostakis 2001, Wingfield *et al.* 2001, 2008b, Garnas *et al.* 2012) and the disruption of forest ecosystems (Liebhold *et al.* 1995, Jactel *et al.* 2009). The disease levels are particularly increasing where native ecosystems have been disrupted by the planting of extensive areas to forest monocultures, especially exotic species (Chou 1991, Bradshaw *et al.* 2000, Wingfield *et al.* 2001, Scholthof 2006, Jactel *et al.* 2009). For example, in the Southern Hemisphere, large areas are planted with monocultures of exotic *Pinus* or *Eucalyptus* spp. (Wingfield 2003), which are typically located within or near natural woodlands and forests (Richardson *et al.* 1994, Ayala *et al.* 2005, Sano *et al.* 2010, da Silva *et al.* 2011). In such areas where native and commercial forestry ecosystems co-occur, the risks associated with new plant diseases are significantly increased, particularly where trees in the two ecosystems are related (Perkins & Matlack 2002, Tommerup *et al.* 2003, Wingfield *et al.* 2010, Blitzer *et al.* 2012).

The forests in Colombia, together with those in Brazil, Peru, Bolivia and Venezuela make up approx. 84 % of South America's total forested area (FAO 2012). Of the ca. 60.5 M ha of forests in Colombia, only 405 000 ha represent commercially managed plantations (FAO 2005, FAO 2010). *Pinus* spp. represent approximately 35 % of the commercially planted species in this country (IDEAM 2009). Although commercial forestry in Colombia

is relatively young, a number of diseases and insect pests that damage *Pinus* spp. have been reported. Rodas (1998) recorded 30 different native species of defoliating insects occurring on exotic plantation species in the Andean region of Colombia. More recently, *Fusarium circinatum*, the causal agent of pitch canker, was also reported from diseased seedlings and established *Pinus* spp. in Colombia (Steenkamp *et al.* 2012). As time passes, the number of emerging pests and pathogens will likely increase, particularly as native organisms adapt to infest/infect non-native trees and where new organisms are accidentally introduced into the country.

Many *Fusarium* spp. have a global distribution and are economically important as producers of toxic secondary metabolites and infective agents of plants, animals and humans (Leslie & Summerell 2006). Notable examples include *Fusarium poae*, *F. verticillioides* and members of the *F. solani* species complex (FSSC), *F. oxysporum* species complex (FOSC) and the *F. graminearum* species complex (FGSC) (Matuo & Snyder 1973, Marasas 2001, Nucci & Anaissie 2002, Pietro *et al.* 2003, Zhang *et al.* 2006, Streit *et al.* 2012). Although most cultivated plants are host to one or more pathogens in this genus (Leslie & Summerell 2006), the only *Fusarium* sp. known to severely affect *Pinus* spp. is *F. circinatum* (Wingfield *et al.* 2008a). In general, however, limited information is available regarding the diversity of *Fusarium* spp. associated with commercially propagated *Pinus* spp. or the possible diseases they cause in this setting.

Steenkamp *et al.* (2012) explored the presence of the pitch canker fungus on *Pinus* spp. in Colombia but also found a number of other *Fusarium* spp. that were frequently and/or

consistently encountered (unpubl. data). All of these other fungi were also isolated from either *Pinus* seedlings or established plantation trees, showing symptoms typical of infection with *F. circinatum*. On seedlings, the symptoms included wilt, root and collar rot, and on established trees they included stem cankers and branch and tip die-back (Steenkamp *et al.* 2012). Knowledge regarding the identity and pathogenicity of these isolates is important in order to realistically quantify the risks they pose to *Pinus*-based plantation forestry in Colombia and other parts of the world. They could also represent threats to *Pinus* spp. where these trees grow naturally, as has been the case with *F. circinatum* in native forests in the United States (Gordon *et al.* 2001, Gordon 2006, Wingfield *et al.* 2008a).

The aim of this study was, firstly, to identify the *Fusarium* spp. associated with diseased *P. patula* seedlings and with *P. patula*, *P. tecunumanii*, *P. kesiya* and *P. maximinoi* trees in plantations showing symptoms of pitch canker in Colombia. This was accomplished using conventional morphology and culture-based approaches together with the DNA sequence information for portions of the genes encoding translation elongation factor 1- $\alpha$  (*tef1*) and  $\beta$ -tubulin (*tub2*). Descriptions were provided for the new *Fusarium* spp. recognised. A second aim was to evaluate the pathogenicity of the identified fungi to *Pinus* and to determine whether they could have been responsible for the symptoms observed.

## MATERIALS AND METHODS

### Isolates

The *Fusarium* isolates used in this study were collected from a number of different locations and *Pinus* spp. in Colombia (Table 1). These included *Pinus kesiya*, *P. maximinoi*, *P. patula* and *P. tecunumanii* trees exhibiting canker-like infections in plantations in or near Calima, Aguacalara, La Cumbre (Valle de Cauca), Angela Maria (Risaralda), El Darién (Valle del Cauca), El Guasimo (Antioquia), Campania, Riosucio (Caldas), and Volconda (Valle de Cauca). Isolates were also obtained from symptomatic (*i.e.*, wilting, root rot, root collar and stem discoloration) *P. patula* seedlings collected in nurseries [Bandeja (Valle de Cauca), Canaletta (Valle de Cauca) and Peñas Negra (Valle de Cauca)].

Diseased plant tissue was surface-disinfected for 1 min in a solution containing 1.5 % (v/v) sodium hypochlorite, rinsed with sterile distilled water, immersed in 70 % (v/v) ethanol for 1 min and air-dried. Small pieces of tissue, cut from the leading edges of lesions, were plated directly onto half-strength potato dextrose agar medium ( $1/2$  PDA) and *Fusarium* selective medium (FSM, Nash & Snyder 1962). Following incubation at 27.5 °C, isolates resembling *Fusarium* were transferred to fresh PDA and grown for 7 d at 23 °C, after which pure cultures were prepared. This was done by washing the conidia from the mycelium using a 2.5 % (v/v) Tween 60 (Sigma–Aldrich, St Louis, Missouri, USA) solution and spreading 1 mL of the spore suspension across the surface of water agar medium (WA; 20 g/L PDA; Biolab Diagnostics). Following incubation at 16 °C for 2 d, single germinating conidia were transferred to fresh PDA and incubated for 7 d at 23 °C. All of the cultures collected for this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria,

South Africa and representative isolates representing novel species were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS). Dried cultures of novel species were deposited in the fungarium of the Agricultural Research Council (ARC), Pretoria, South Africa (PREM).

### DNA extraction, PCR amplification and sequencing

Fungal DNA was extracted from 7-d-old cultures using a modified CTAB (hexadecyltrimethylammonium bromide) method (Steenkamp *et al.* 1999) and mycelium was scraped directly from the surface of the growth media. Specific regions of *tef1* and *tub2* were amplified with a Bio-Rad iCycler (Bio-Rad, California, USA) using, respectively, primers EF-1 and EF-2 (O'Donnell *et al.* 1998, Geiser *et al.* 2004) and primers T1 and T2 (O'Donnell & Cigelnik 1997). Each amplification reaction contained 2–4 ng/ $\mu$ L DNA, 0.25  $\mu$ M of each primer, 200  $\mu$ M dNTPs (Fermentas, Nunningen, Germany), 2.5 mM MgCl<sub>2</sub>, 0.04 U/ $\mu$ L of Supertherm Taq polymerase and reaction buffer with KCl (10 $\times$ ) (Southern Cross Biotechnology, Cape Town, South Africa). The PCR started with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 54 °C (*tub2*) or 56 °C (*tef1*) for 45 s and 72 °C for 1 min. A final extension step at 72 °C for 10 min was used to conclude the PCR.

Amplified PCR products were purified using polyethylene glycol (Steenkamp *et al.* 2006) or G50 Sephadex columns (Sigma, Steinheim, Germany). The purified samples were then sequenced in both directions using the original PCR primers, an ABI PRISM BigDye<sup>®</sup> Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and an ABI PRISM<sup>®</sup> 3100 Genetic Analyzer (Applied Biosystems). Electropherogrammes were examined and manually corrected where necessary using Chromas Lite v. 2.1.1 (Technelysium, Australia) and BioEdit v. 7.2.5 (Hall 1999). The *tef1* nucleotide sequences were compared to those in the *Fusarium*-ID identification database (Geiser *et al.* 2004; <http://isolate.fusariumdb.org>) using the basic local alignment search tool (BLAST) search algorithm (Altschul *et al.* 1990).

Multiple sequence alignments were generated with MAFFT v. 7.0 (<http://mafft.cbrc.jp/alignment/software/>) with the L-INS-i option selected (Katoh *et al.* 2002, 2005, Katoh & Toh 2008, Katoh & Standley 2013) and corrected manually where necessary. The datasets constructed for *tef1* and *tub2* contained all the sequences generated in this study and the recognised species and phylogenetic lineages in the *F. fujikuroi* complex (FFSC, previously known as the *Gibberella fujikuroi* species complex) (Geiser *et al.* 2013), as well as the outgroup species *F. oxysporum* (Table 2). To infer phylogenies, the *tef1* and *tub2* datasets were analysed separately, as well as combined as previously described (O'Donnell *et al.* 1998, O'Donnell *et al.* 2000, Geiser *et al.* 2005). MrBayes v. 3.2.1 (Heulemans *et al.* 2001) and PhyML v. 3.0 (Guindon *et al.* 2010) were used to generate phylogenies based on Bayesian inference (BI) and Maximum Likelihood (ML), respectively. The best-fit parameters, as indicated by jModelTest v. 2.1.3 (Guindon & Gascuel 2003, Posada 2008, Darriba *et al.* 2012), for ML analyses of the *tef1* and *tub2* datasets included gamma correction (G) to account for among site rate variation and the TIM2ef and TIM2 (Posada 2003) models, respectively. BI analysis of these datasets utilised the General Time Reversible (GTR) model

**Table 1.** Host and geographic origin of the *Fusarium* isolates used in this study.

<i>Fusarium</i> species <sup>1</sup>	Accession number <sup>2</sup>	<i>Pinus</i> species <sup>3</sup>	Area in Colombia	Provenance	GPS co-ordinates
<i>Fusarium</i> sp.	CMW 25516; FCC 5428	<i>P. patula</i> (T)	Angela María (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
<i>Fusarium</i> sp.	Colombia 18	<i>P. patula</i> (T)	Angela María (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
<i>F. circinatum</i>	CMW 25239; FCC 5379	<i>P. tecunumanii</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25240; FCC 5380	<i>P. tecunumanii</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25251; FCC 5391	<i>P. maximinoi</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25255; FCC 5395	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25256; FCC 5396	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25257; FCC 5397	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25258; FCC 5398	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25259; FCC 5399	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25260; FCC 5400	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25262; FCC 5402	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25263; FCC 5403	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25264; FCC 5404	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25265; FCC 5405	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25266; FCC 5406	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25271; FCC 5411	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25272; FCC 5412	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25273; FCC 5413	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25274; FCC 5414	<i>P. patula</i> (S)	Vivero, Peñas Negras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25518; FCC 5430	<i>P. kesiya</i> (T)	Aguaclara, La Cumbre	Valle del Cauca	76°1'33" W 3°44'33" N
	CMW 25519; FCC 5431	<i>P. patula</i> (T)	Angela María (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25520; FCC 5432	<i>P. patula</i> (T)	El Guasimo (Santa Rosa de Osos)	Antioquia	75°26'30" W 6°52'04" N
<i>F. falciforme</i> *	CMW 25507; FCC 5419	<i>P. maximinoi</i> (S)	Vivero Canaleta	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25514; FCC 5426	<i>P. tecunumanii</i> (T)	La Suiza, Restrepo	Valle del Cauca	76°29'33" W 3°50'55" N
<i>F. fracticaudum</i> sp. nov.	CMW 25237; FCC 5377; CBS 137233	<i>P. tecunumanii</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25238; FCC 5378	<i>P. tecunumanii</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25241; FCC 5381	<i>P. maximinoi</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25242; FCC 5382	<i>P. maximinoi</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	<b>CMW 25245; FCC 5385; CBS 137234</b>	<i>P. maximinoi</i> (T)	Angela María (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25249; FCC 5389	<i>P. maximinoi</i> (T)	Angela María (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25250; FCC 5390	<i>P. maximinoi</i> (T)	Angela María (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25511; FCC 5423	<i>P. tecunumanii</i> (T)	Volconda (Calima El Darién)	Valle del Cauca	76°25'06" W 4°01'47" N
<i>F. keratoplasticum</i> *	CMW 25505; FCC 5417	<i>P. tecunumanii</i> (S)	Vivero Bandeja	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25515; FCC 5427	<i>P. tecunumanii</i> (T)	La Suiza, Restrepo	Valle del Cauca	76°29'33" W 3°50'55" N
<i>F. marasasianum</i> sp. nov.	CMW 25246; FCC 5386	<i>P. tecunumanii</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
	CMW 25248; FCC 5388	<i>Pinus</i> sp. (T)	Colombia	n/a	n/a
	CMW 25252; FCC 5392	<i>Pinus</i> sp. (T)	Colombia	n/a	n/a
	CMW 25253; FCC 5393; CBS 137237	<i>Pinus</i> sp. (T)	Colombia	n/a	n/a
	<b>CMW 25261; FCC 5401; CBS 137238</b>	<i>P. patula</i> (S)	Vivero, Peñas Negra	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25512; FCC 5424	<i>P. tecunumanii</i> (T)	Volconda (Calima, El Darién)	Valle del Cauca	76°25'06" W 4°01'47" N
<i>F. parvisorum</i> sp. nov.	<b>CMW 25267; FCC 5407; CBS 137236</b>	<i>P. patula</i> (S)	Vivero, Peñas Negra	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25268; FCC 5408; CBS 137235	<i>P. patula</i> (S)	Vivero, Peñas Negra	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25269; FCC 5409	<i>P. patula</i> (S)	Vivero, Peñas Negra	Valle del Cauca	76°29'49" W 3°51'45" N

(continued on next page)

Table 1. (Continued).

<i>Fusarium</i> species <sup>1</sup>	Accession number <sup>2</sup>	<i>Pinus</i> species <sup>3</sup>	Area in Colombia	Provenance	GPS co-ordinates
<i>F. pininemorale</i> sp. nov.	<b>CMW 25243; FCC 5383; CBS 137240</b>	<i>P. tecunumanii</i> (T)	Angela Maria (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25244; FCC 5384; CBS 137239	<i>P. tecunumanii</i> (T)	Angela Maria (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25247; FCC 5387	<i>P. tecunumanii</i> (T)	Calima, El Darién	Valle del Cauca	76°26'03" W 3°56'57" N
FSSC 5*	CMW 25509; FCC 5421	<i>P. maximinoi</i> (S)	Vivero Canaleta	Valle del Cauca	76°29'49" W 3°51'45" N
FSSC 20*	CMW 25506; FCC 5418	<i>P. maximinoi</i> (S)	Vivero Canaleta	Valle del Cauca	76°29'49" W 3°51'45" N
<i>F. sororula</i> sp. nov.	CMW 25254; FCC 5394; CBS 137241	<i>Pinus</i> sp. (T)	Colombia	n/a	n/a
	CMW 25513; FCC 5425	<i>P. tecunumanii</i> (T)	Angela Maria (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25517; FCC 5429	<i>P. patula</i> (T)	Campania, Riosucio	Caldas	75°49'18" W 5°21'45" N
	Colombia 8	<i>P. patula</i> (T)	Volconda (Calima, El Darién)	Valle del Cauca	76°25'06" W 4°01'47" N
	Colombia 19	<i>P. patula</i> (T)	Angela Maria (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
<i>F. oxysporum</i>	<b>CMW 40578; CBS 137242</b>	<i>P. patula</i> (T)	Angela Maria (Santa Rosa)	Risaralda	75°36'21" W 4°49'18" N
	CMW 25503; FCC 5415	<i>P. tecunumanii</i> (S)	Vivero Eras	Valle del Cauca	76°29'49" W 3°51'45" N
	CMW 25504; FCC 5416	<i>P. tecunumanii</i> (S)	Vivero Bandeja	Valle del Cauca	76°29'49" W 3°51'45" N

<sup>1</sup> Five novel *Fusarium* species were described in this study. The FSSC species and lineages (indicated with \*) were recognised according to O'Donnell *et al.* (2008) and Short *et al.* (2013). n/a = not available.

<sup>2</sup> CMW: Culture collection at the FABI, University of Pretoria, South Africa. FCC, original numbers of the *Fusarium* culture collection at FABI, University of Pretoria, South Africa. CBS, Culture collection at the CBS-KNAW Fungal diversity Centre, Utrecht, Netherlands. Isolate numbers in **boldface** represent ex-type cultures.

<sup>3</sup> Letters in brackets indicate whether the isolates came from seedlings (S) or mature trees (T).

(Tavare 1986) with G. Bayesian inference and ML analysis of the combined dataset also utilised GTR+G. The ML branch support was estimated using bootstrap analyses based on 1 000 pseudoreplicates and model parameters as described above. The BI analyses were based on 6 M generations using one cold and three heated chains, and Bayesian posterior probabilities were calculated after discarding a burn-in corresponding to approximately 75 000 generations post-stationarity. The BI-based analysis of the combined dataset utilised separate model parameters for each gene (Heulsenbeck *et al.* 2001). Phylogenetic trees were viewed and edited using MEGA v. 5 (Tamura *et al.* 2011). All novel sequences were deposited in GenBank (see Table 2 for accession numbers), while the alignments and phylogenetic trees were deposited in Tree BASE.

## Morphology

The morphological characters of 10 isolates, two for each of the five purportedly novel species as determined by the phylogenetic analyses (see below), were studied. These isolates were as follows: *F. fracticaudum* (CMW 25237; CMW 25245), *F. marasasianum* (CMW 25253; CMW 25261), *F. parvisorum* (CMW 25267; CMW 25268), *F. pininemorale* (CMW 25243; CMW 25244) and *F. sororula* (CMW 25254; CMW 40578).

The morphological characteristics examined included those of the microconidia, macroconidia, and conidiophores. Measurements of microconidia and macroconidia were made using 7-d- and 14-d-old cultures grown on carnation leaf agar (CLA; 20 g/L agar Biolab Diagnostics, 5–6 carnation leaf pieces). Microscope slides were prepared for each isolate by mounting structures in 85 % (v/v) lactic acid (Sigma–Aldrich, St Louis, Missouri, USA) and 25–50 measurements were recorded for each characteristic. Microconidia and macroconidia sizes were recorded as

minimum–maximum (average). Characteristics of the specimens were described based on the species descriptions of Leslie & Summerell (2006).

For all isolates, the colony reverse colour was observed on full-strength PDA after incubation at room temperature, in the dark and under near-UV light. Colony colours (surface and reverse) were described using the colour charts of Rayner (1970). Colony growth rates were assessed on full-strength PDA in 90 mm Petri plates at 10–35 °C at 5 °C intervals. Three plates were used for each culture and two measurements of colony diameter perpendicular to each were made during 8 d of incubation in the dark, after which averages were computed. Descriptions and nomenclature were deposited in MycoBank (Crous *et al.* 2004).

## Pathogenicity

Two isolates of each of the five novel species were inoculated onto 6-mo-old *P. patula* seedlings in a glass house (Table 3). Isolate FCC 3579, which is a virulent strain of *F. circinatum* used in routine screening trials (Porter *et al.* 2009), was used for comparative purposes as a positive control. The inocula for the pathogenicity trial were prepared by growing the isolates on full-strength PDA for 10 d at 25 °C, after which spores were washed from the cultures using a sterile 15 % glycerol solution. These spore suspensions were filtered through cheese cloth and adjusted to a concentration of  $5 \times 10^4$  spores/mL using a haemocytometer. Each isolate was inoculated on 16 seedlings by first cutting the growth tips from the tops of the seedlings, approximately 1 cm from the top, and then placing a 1  $\mu$ L drop of the spore suspension onto the cut end using a pipette (Porter *et al.* 2009). The seedlings used for the negative controls were treated in the same manner, except that a 15 % glycerol solution

**Table 2.** The species names and their GenBank accession numbers for all the *Fusarium* isolates included in the phylogenetic analyses.

Species <sup>1</sup>	Host/substrate	Origin	Culture collection <sup>2</sup>	GenBank accession <sup>3</sup>	
				<i>tub2</i>	<i>tef1</i>
<i>Fusarium acutatum</i>	–	India	NRRL 13308	U34431 <sup>a</sup>	AF160276 <sup>b</sup>
<i>F. ananatum</i>	<i>Ananas comosus</i>	England	NRRL 22945	U34420 <sup>a</sup>	AF160297 <sup>a</sup>
<i>F. anthophilum</i>	<i>Hippeastrum</i> sp.	Germany	NRRL 13602	U61541 <sup>a</sup>	AF160292 <sup>a</sup>
<b><i>F. bactridioides</i></b>	<i>Cronartium conigenum</i>	USA	NRRL 20476	U34434 <sup>a</sup>	AF160290 <sup>a</sup>
<b><i>F. begoniae</i></b>	<i>Begonia elatior</i>	Germany	NRRL 25300	U61543 <sup>a</sup>	AF160293 <sup>a</sup>
<b><i>F. brevicatenulatum</i></b>	<i>Striga asiatica</i>	Madagascar	NRRL 25446	U61545 <sup>a</sup>	AF160265 <sup>a</sup>
<b><i>F. bulbicola</i></b>	<i>Nerine bowdenii</i>	Netherlands	NRRL 13618	U61546 <sup>a</sup>	AF160294 <sup>a</sup>
<b><i>F. circinatum</i></b>	<i>Pinus radiata</i>	USA	NRRL 25331	U61547 <sup>a</sup>	AF160295 <sup>a</sup>
<b><i>F. concentricum</i></b>	<i>Musa sapientum</i>	Costa Rica	NRRL 25181	U61548 <sup>a</sup>	AF160282 <sup>a</sup>
<i>F. denticulatum</i>	<i>Ipomoea batatas</i>	USA	NRRL 25302	U61550 <sup>a</sup>	AF160269 <sup>a</sup>
<b><i>F. dlaminii</i></b>	Soil	South Africa	n/a	n/a <sup>k</sup>	n/a <sup>k</sup>
<b><i>F. fracticaudum</i></b> *	<i>Pinus maximinoi</i>	Colombia	CBS 137233	KJ541051	KJ541059
	<i>Pinus maximinoi</i>	Colombia	CBS 137234	KJ541048	KJ541058
<b><i>F. fractiflexum</i></b>	<i>Cymbidium</i> sp.	Japan	NRRL 28852	AF160315 <sup>c</sup>	AF160288 <sup>c</sup>
<i>F. fujikuroi</i>	<i>Oryza sativa</i>	Taiwan	NRRL 13566	U34415 <sup>a</sup>	AF160279 <sup>a</sup>
<i>F. globosum</i>	<i>Zea mays</i>	Central America	NRRL 26131	U61557 <sup>a</sup>	AF160285 <sup>a,l</sup>
<i>F. guttiforme</i>	<i>Ananas comosus</i>	South America	NRRL 22945	U34446 <sup>a</sup>	AF160297 <sup>a, l</sup>
<i>F. inflexum</i>	<i>Vicia faba</i>	Germany	NRRL 20433	U34435 <sup>a</sup>	AF008479 <sup>a</sup>
<i>F. konzum</i>	<i>Andropogon gerardii</i>	North America	MRC 8854	EU220234 <sup>j</sup>	EU220235 <sup>j</sup>
<b><i>F. lactis</i></b>	<i>Ficus carica</i>	USA	NRRL 25200	U61551 <sup>a</sup>	AF160272 <sup>a</sup>
<b><i>F. lyarnte</i></b>	Soil	Australia	F19374	EF107122 <sup>f</sup>	EF107118 <sup>f</sup>
<b><i>F. mangiferae</i></b>	<i>Mangifera indica</i>	India	NRRL 25226	U61561 <sup>a</sup>	AF160281 <sup>a</sup>
<b><i>F. marasasianum</i></b> *	<i>Pinus patula</i>	Colombia	CBS 137238	KJ541054	KJ541063
	<i>Pinus patula</i>	Colombia	CBS 137237	KJ541052	KJ541062
<b><i>F. mexicanum</i></b>	<i>Mangifera indica</i>	Mexico	NRRL 53147	GU737494 <sup>e</sup>	GU737282 <sup>e</sup>
<b><i>F. musae</i></b>	<i>Musa</i> sp.	Honduras	MUCL 52574	FN545368 <sup>h</sup>	FN552086 <sup>h</sup>
<b><i>F. napiforme</i></b>	<i>Pennisetum typhoides</i>	South Africa	NRRL 13604	U34428 <sup>a</sup>	AF160266 <sup>a</sup>
<b><i>F. nygamai</i></b>	<i>Sorghum bicolor</i>	Australia	NRRL 13448	U34426 <sup>a</sup>	AF160273 <sup>a</sup>
<i>F. oxysporum</i>	<i>Pseudotsuga menziesii</i>	USA	NRRL 22902	U34424 <sup>a</sup>	AF160312 <sup>a</sup>
<b><i>F. phylophilum</i></b>	<i>Dracaena deremensis</i>	Italy	NRRL 13617	U34432 <sup>a</sup>	AF160274 <sup>a</sup>
<b><i>F. parvisorum</i></b> *	<i>Pinus patula</i>	Colombia	CBS 137236	KJ541055	KJ541060
	<i>Pinus patula</i>	Colombia	CBS 137235	KJ541056	KJ541061
<b><i>F. pininemorale</i></b> *	<i>Pinus tecunumanii</i>	Colombia	CBS 137240	KJ541049	KJ541064
	<i>Pinus tecunumanii</i>	Colombia	CBS 137239	KJ541050	KJ541065
<i>F. proliferatum</i>	<i>Cattleya</i> sp.	Germany	NRRL 22944	U34416 <sup>a</sup>	AF160280 <sup>a</sup>
<i>F. pseudoanthophilum</i>	<i>Zea mays</i>	Zimbabwe	NRRL 25206	U61553 <sup>a</sup>	AF160264 <sup>a</sup>
<b><i>F. pseudocircinatum</i></b>	<i>Solanum</i> sp.	Zimbabwe	NRRL 22946	U34427 <sup>a</sup>	AF160271 <sup>a</sup>
<b><i>F. pseudonygamai</i></b>	<i>Pennisetum typhoides</i>	Ghana	NRRL 13592	U34421 <sup>a</sup>	AF160263 <sup>a</sup>
<b><i>F. ramigenum</i></b>	<i>Ficus carica</i>	Nigeria	NRRL 25208	U61554 <sup>a</sup>	AF160267 <sup>a</sup>
<i>F. sacchari</i>	<i>Saccharum officinarum</i>	USA	NRRL 13999	U34414 <sup>a</sup>	AF160278 <sup>a</sup>
<b><i>F. sororula</i></b> *	<i>Pinus patula</i>	Colombia	CBS 137242	KJ541057	KJ541067
	<i>Pinus patula</i>	Colombia	CBS 137241	KJ541053	KJ541066
<b><i>F. sterilihyphosum</i></b>	<i>Mangifera indica</i>	India	MRC 2802	AF160316 <sup>a</sup>	AF160300 <sup>a</sup>
<b><i>F. subglutinans</i></b>	<i>Zea mays</i>	USA	NRRL 22016	U34417 <sup>a</sup>	AF160289 <sup>a</sup>
<i>F. succisae</i>	<i>Succisa pratensis</i>	Germany	NRRL 13613	U34419 <sup>a</sup>	AF160291 <sup>a</sup>
<b><i>F. temperatum</i></b>	<i>Zea mays</i>	Belgium	MUCL 52450	HM067695 <sup>g</sup>	HM067687 <sup>g</sup>
<i>F. thapsinum</i>	<i>Sorghum bicolor</i>	South Africa	NRRL 22045	U34418 <sup>a</sup>	AF160270 <sup>a</sup>

(continued on next page)

**Table 2.** (Continued).

Species <sup>1</sup>	Host/substrate	Origin	Culture collection <sup>2</sup>	GenBank accession <sup>3</sup>	
				<i>tub2</i>	<i>tef1</i>
<b><i>F. tuiense</i></b>	<i>Mangifera indica</i>	Brazil	CML 262	DQ445781 <sup>i</sup>	DQ452859 <sup>j</sup>
<i>F. udum</i>	–	Germany	NRRL 22949	U34433 <sup>a</sup>	AF160275 <sup>a</sup>
<i>F. verticillioides</i>	<i>Zea mays</i>	Germany	NRRL 22172	U34413 <sup>a</sup>	AF160262 <sup>a</sup>
<b><i>F. werrikimbe</i></b>	<i>Sorghum leiocladum</i>	Australia	F19350	EF107133 <sup>f</sup>	EF107131 <sup>f</sup>
<b><i>F. xylarioides</i></b>	<i>Coffea</i> sp.	Ivory Coast	NRRL 25486	AY707118 <sup>d</sup>	AY707136 <sup>d</sup>
<i>Fusarium</i> sp.	<i>Striga hermonthica</i>	Madagascar	NRRL 26061	AF160319 <sup>a</sup>	AF160303 <sup>a</sup>
	–	Niger	NRRL 26152	AF160349 <sup>a</sup>	AF160306 <sup>a</sup>
	<i>Sorghum bicolor</i> seed	Tanzania	NRRL 26064	AF160346 <sup>a</sup>	AF160302 <sup>a</sup>
	<i>Zea mays</i>	Central America	NRRL 25221	U61560 <sup>a</sup>	AF160268 <sup>a</sup>
	<i>Striga hermonthica</i>	Africa	NRRL 26793	AF160324 <sup>a</sup>	AF160309 <sup>a</sup>
	<i>Oryza sativa</i>	Southeast Asia	NRRL 25615	AF160320 <sup>a</sup>	AF160304 <sup>a</sup>
	Soil	Australia	NRRL 25807	U61542 <sup>a</sup>	AF160305 <sup>a</sup>
	–	–	NRRL 25195	U61558 <sup>a</sup>	AF160298 <sup>a</sup>
	<i>Ipomoea batatas</i>	Central America	NRRL 25346	U61564 <sup>a</sup>	AF160296 <sup>a</sup>
	Ornamental reed	South Africa	NRRL 26756	AF160322 <sup>a</sup>	AF160307 <sup>a</sup>
	Ornamental reed	South Africa	NRRL 26757	AF160323 <sup>a</sup>	AF160308 <sup>a</sup>
	Palm	–	NRRL 25204	U61559 <sup>a</sup>	AF160299 <sup>a</sup>
	<i>Bidens pilosa</i>	South America	NRRL 29124	AF160326 <sup>a</sup>	AF160311 <sup>a</sup>
	<i>Zea mays</i>	Central America	NRRL 25622	DQ448031 <sup>a</sup>	AF160301 <sup>a</sup>
	<i>Triticum</i> sp.	South Asia	NRRL 25309	U61563 <sup>a</sup>	AF160284 <sup>a</sup>
	<i>Oryza sativa</i>	Southeast Asia	NRRL 25303	U61562 <sup>a</sup>	AF160283 <sup>a</sup>
	Soil	Papua New Guinea	NRRL 26427	AF160313 <sup>a</sup>	AF160286 <sup>a</sup>

n/a = not available.

<sup>1</sup> Species for which type strains were included in the study are in **boldface**. The new species described in this study are indicated with \*.<sup>2</sup> The abbreviations for the culture collections: CBS (Centraalbureau voor Schimmelcultures) Culture collection at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; CML (Coleção Micológica de Lavras) Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; F (University of Sydney) Sydney, New South Wales, Australia; MRC (Medical Research Center) Tygerberg, Cape Town, South Africa; MUCL (Mycothèque de l'Université Catholique de Louvain), Louvain-la-Neuve, Belgium and NRRL (National Center for Agricultural Utilization Research) Peoria, Illinois, USA.<sup>3</sup> References for studies where DNA sequences were generated: <sup>a</sup>O'Donnell *et al.* 1998; <sup>b</sup>O'Donnell *et al.* 2000; <sup>c</sup>Aoki *et al.* 2001; <sup>d</sup>Geiser *et al.* 2005; <sup>e</sup>Otero-Colina *et al.* 2010; <sup>f</sup>Walsh *et al.* 2010; <sup>g</sup>Scaufflaire *et al.* 2011; <sup>h</sup>Van Hove *et al.* 2011; <sup>i</sup>Lima *et al.* 2012; <sup>j</sup>Kvas *et al.* 2009; <sup>k</sup>Marasas *et al.* 1985; <sup>l</sup>Nirenberg & O'Donnell 1998.**Table 3.** The results of pathogenicity tests with *Fusarium* spp. on *Pinus patula* seedlings.

<i>Fusarium</i> species <sup>1</sup>	Isolate	Mean lesion length (mm) <sup>2</sup>			Standard error (combined)
		Replicate 1	Replicate 2	Combined	
<i>F. circinatum</i>	FCC 3579	49.00 (14) c	52.53 (15) b	50.83 (29) b	1.76
<i>F. fracticaudum</i>	CMW 25237	1.00 (15) f	1.42 (14) f	1.21 (29) f	1.76
	<b>CMW 25245</b>	1.85 (14) f	1.85 (13) f	1.85 (27) f	1.82
<i>F. marasasianum</i>	CMW 25253	1.86 (14) f	1.71 (14) f	1.75 (28) f	1.79
	<b>CMW 25261</b>	48.33 (15) c	41.13 (15) c	44.73 (30) c	1.73
<i>F. parvisorum</i>	<b>CMW 25267</b>	43.36 (14) d	32.07 (15) d	37.53 (29) d	1.76
	CMW 25269	50.33 (15) b	52.79 (14) b	51.52 (29) b	1.76
<i>F. pininemorale</i>	<b>CMW 25243</b>	1.5 (14) f	1.33 (15) f	1.41 (29) f	1.76
	CMW 25244	1.8 (15) f	1.33 (15) f	1.57 (30) f	1.73
<i>F. sororula</i>	CMW 25254	12.38 (13) e	12.80 (15) e	12.61 (28) e	1.79
	<b>CMW 40578</b>	59.86 (15) a	54.73 (15) a	57.30 (30) a	1.73
n/a	Control	1.46 (16) f	1.18 (16) f	1.31 (32) f	1.68

<sup>1</sup> Strain numbers in **boldface** indicate the ex-type strains.<sup>2</sup> Values in parentheses represent total number of measurements for each treatment from which the means were calculated. A one-way ANOVA (Analysis of Variance) indicated significance between all inoculum treatments. The observed F-value was 187.48 and the significance probability associated with the F-statistic was <0.0001. Individual means were compared and grouped according to the Duncan Multiple Range Test with a confidence level of 95 %. Means that were not significantly different are indicated with the same letter.

replaced the spore suspension. The seedlings were arranged using a randomised block design and maintained in a greenhouse. After 6 wk, disease severity was evaluated by measuring the lesion lengths from the inoculation site to the leading edge of the lesions down the stems. The entire trial was repeated once.

Analysis of Variation (ANOVA) was used to determine significant differences within and between treatments for the first pathogenicity test and the Duncan Multiple Range Test was used to compare treatment differences (Onofri 2006, Zaiantz 2013, [www.real-statistics.com](http://www.real-statistics.com)). After conclusion of the pathogenicity trial, Koch's postulates were confirmed with re-isolations from the diseased seedling tissues and using *tef1* sequence data for a representative set of isolates to confirm that the inoculated fungi were indeed responsible for the observed lesions.

## RESULTS

### Isolates

A total of 57 isolates resembling those of the genus *Fusarium* were recovered from the diseased plant material. All isolates were collected in Colombia either from *P. patula* seedlings in nurseries or from trees in established plantations of *P. kesiya*, *P. maximinoi* and *P. tecunumanii* (Table 1). All of the trees and seedlings sampled showed similar symptoms to trees and seedlings typically infected with *F. circinatum* (Wingfield *et al.* 2008a).

### Sequence analysis

Comparison of the *tef1* sequences against those in the NCBI database and the *Fusarium* Identification Database (Fusarium-ID) (<http://isolate.fusariumdb.org/>) revealed that 49 of the 57 *Fusarium* isolates examined in this study represented members of the FFSC. Of these, 21 isolates displayed 97–99 % *tef1* sequence similarity to *F. circinatum*. The sequences for six isolates were 98–99 % similar to that of *F. begoniae* and those for 22 isolates were 97–99 % similar to that of *F. sterilihyphosum*. Among the remaining eight isolates, two shared 98–100 % *tef1* sequence similarity with members of the FOSC while six shared 98–100 % *tef1* sequence similarity to members of the FSSC.

### Phylogenetic analysis

The aligned *tef1* and *tub2* datasets consisted of 675 and 552 nucleotides, respectively. Maximum Likelihood and BI analyses of these datasets generated trees (Figs 1–3) with topologies resembling those previously recovered from these gene regions (O'Donnell *et al.* 1998, O'Donnell *et al.* 2000, Geiser *et al.* 2005) in which the FFSC is separated into three large clades (*i.e.*, the so-called “American”, “Asian” and “African” clades). All of the 49 FFSC isolates examined in this study formed part of the “American” clade.

Analyses of the combined sequence dataset separated the isolates from Colombia into eight distinct groups. Of these, only one corresponded to a known species (*i.e.*, *F. circinatum*). The remaining seven lineages appeared to represent novel species based on the fact that the isolates did not cluster with any known FFSC species. Because of the limited resolving power of most

single-gene phylogenetic analyses of the FFSC, not all eight groups were recovered from the respective individual *tef1* and *tub2* phylogenies (Figs 2 and 3), although they were not incongruent with those supported by the combined dataset (Fig. 1). Application of a modified version of Nixon & Wheeler's (1990) phylogenetic species concept indicated that the seven lineages identified for the Colombian isolates could be recognised as distinct species. This species concept essentially defines species as diagnosable groups on phylogenetic trees, for example, and is commonly employed for taxonomic studies on the FFSC (O'Donnell *et al.* 1998, O'Donnell *et al.* 2000, Geiser *et al.* 2005). In this study, descriptions are provided for five lineages that included multiple representatives (see below). Lineages 1 and 2 were represented by inordinately few isolates to justify describing them at the present time.

In general, the results of the BLAST analyses were not mirrored in the phylogenies, because isolates that had sequences similar to those of *F. sterilihyphosum* did not group closely with this species and were rather scattered into five phylogenetic lineages throughout the American clade (Fig. 1). Also, isolates that had sequence similarity to *F. begoniae* formed part of a group that did not include this species. Isolates that had a 99–100 % sequence similarity with *F. circinatum* were the only isolates that grouped with the type strain of any species. This general lack of consistency between the results of BLAST and phylogenetic analyses highlights the limitations associated with using sequence similarity alone for diagnosing novel species (*e.g.*, Kang *et al.* 2010, Hibbett *et al.* 2011, Boykin *et al.* 2012).

### Taxonomy

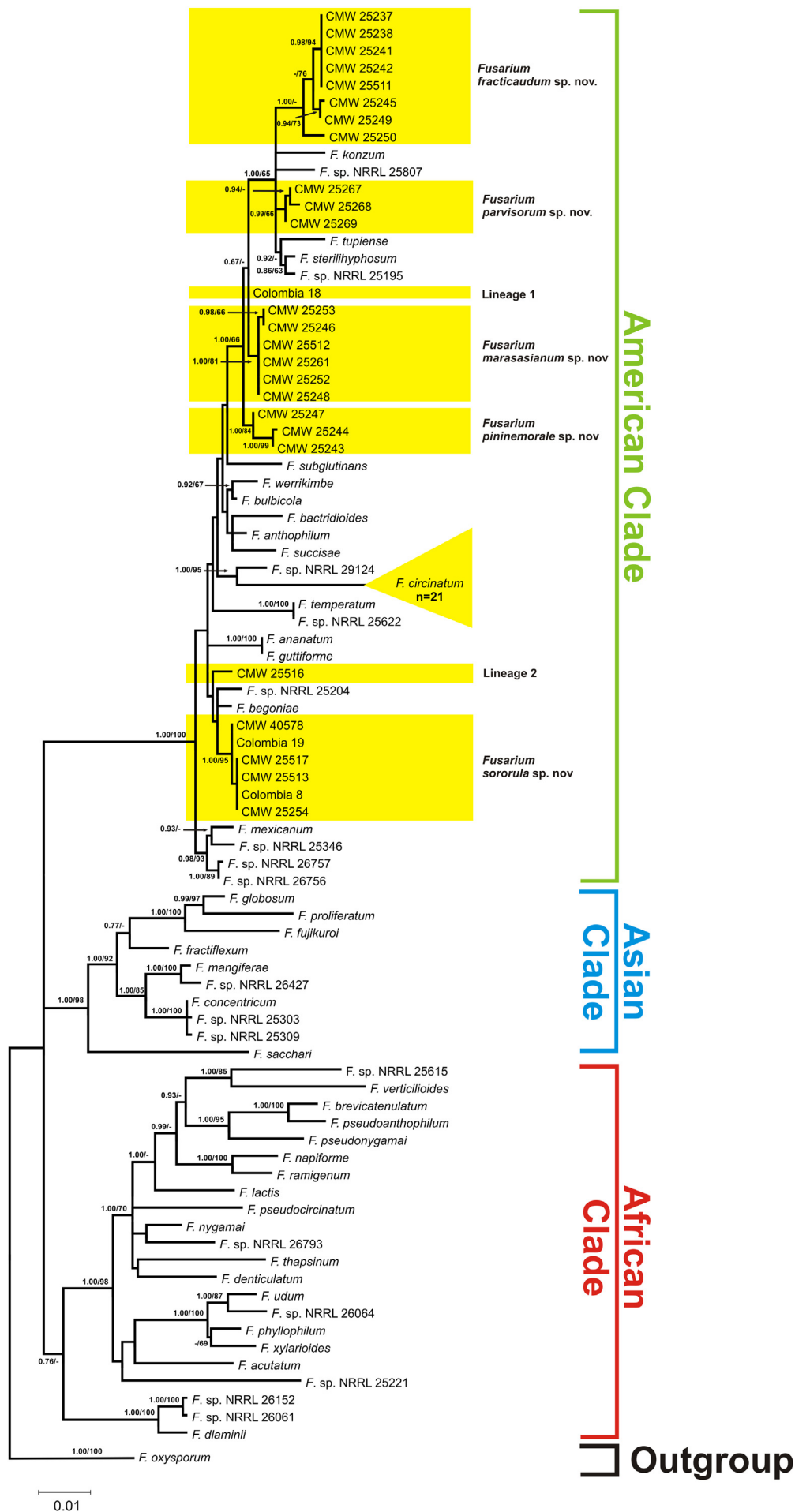
Morphological characters used to distinguish the five novel species included colony colour and conidial size, shape, septation and arrangement (Figs 4–8). Although the isolates shared an optimum growth temperature (*i.e.*, 25 °C), there were differences in their average growth/d, which ranged from 6 to 15.4 mm/d (Table 4). The isolates also differed morphologically from *F. sterilihyphosum*, *F. begoniae* and *F. circinatum*. Based on the results of both the phylogenetic and morphological analyses, five distinct novel species in the FFSC are described below.

***Fusarium fracticaudum*** Herron, Marinc. & M.J. Wingf., **sp. nov.**  
Mycobank MB809885. Figs 4A–C, 5A–C, 6A–C, 7A–D, 8A–B.

**Etymology:** From *fracti* (Latin for broken or bent) and *caudum* (Latin for tail) to describe the “broken tail” of the skewed macroconidial foot cell.

**Macroconidia** abundant, elongate, straight, 38–63.5 × 2.5–4.5 µm (av. 47.6 × 3.3 µm), with 3–5 septa, apical cells tapering, curved, 9–15 µm long (av. 12.2 µm), basal cells distinctly notched to foot-shaped, 9–14.5 µm long (av. 11.8 µm). **Microconidia** abundant, fusiform to obovoid, occasionally curved, 8–13 × 1.5–3 µm (av. 9.9 × 2.3 µm), with 0–1 septum. **Conidiogenous cells** monophialidic or polyphialidic, 11–23.5 µm long, microconidia arranged in false heads.

**Culture characteristics:** Colonies showing optimal growth at 25 °C with an average growth rate of 6.9 mm/d (CMW 25237) and 9.1 mm/d (CMW 25245). Colony reverse in the dark more or less uniformly fulvous or in near-UV uniformly buff.



**Fig. 1.** Maximum likelihood (ML) phylogeny of the *Fusarium fujikuroi* species complex (FFSC), including *Fusarium* isolates collected from Colombia, inferred from the combined *tef1* and *tub2* sequence data. The tree is rooted to *F. oxysporum*. A similar topology was generated using Bayesian inference (BI). The FFSC taxa are grouped into the so-called “American,” “African” and “Asian” clades (O’Donnell *et al.* 1998). The blocks indicate the five novel species and two phylogenetic lineages identified in this study. Bootstrap support values (>60 %) for ML and Bayesian posterior probabilities (>0.6) are indicated at the internodes in the order BI/ML. Branches with bootstrap support values less than 60 % or posterior probability values less than 0.6 are indicated with a “-”. NRRL, ARS Culture collection Peoria, IL, USA.





**Fig. 2.** Maximum likelihood phylogeny of the *Fusarium fujikuroi* species complex (FFSC), including the isolates collected from Colombia, inferred from the *tef1* sequence data. The tree is rooted to *F. oxysporum* and a similar topology was obtained using Bayesian inference. Branch support, as well as clade and isolate information are indicated as detailed in the legend of Fig. 1.

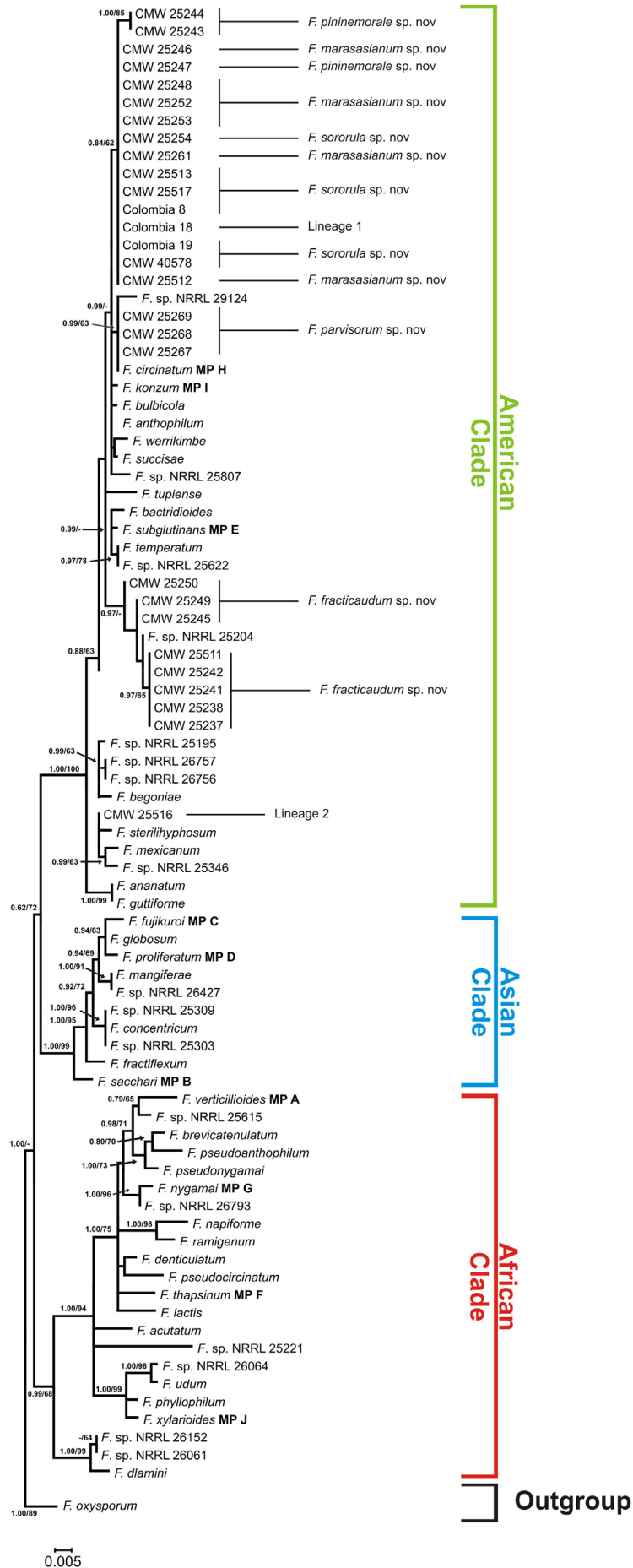
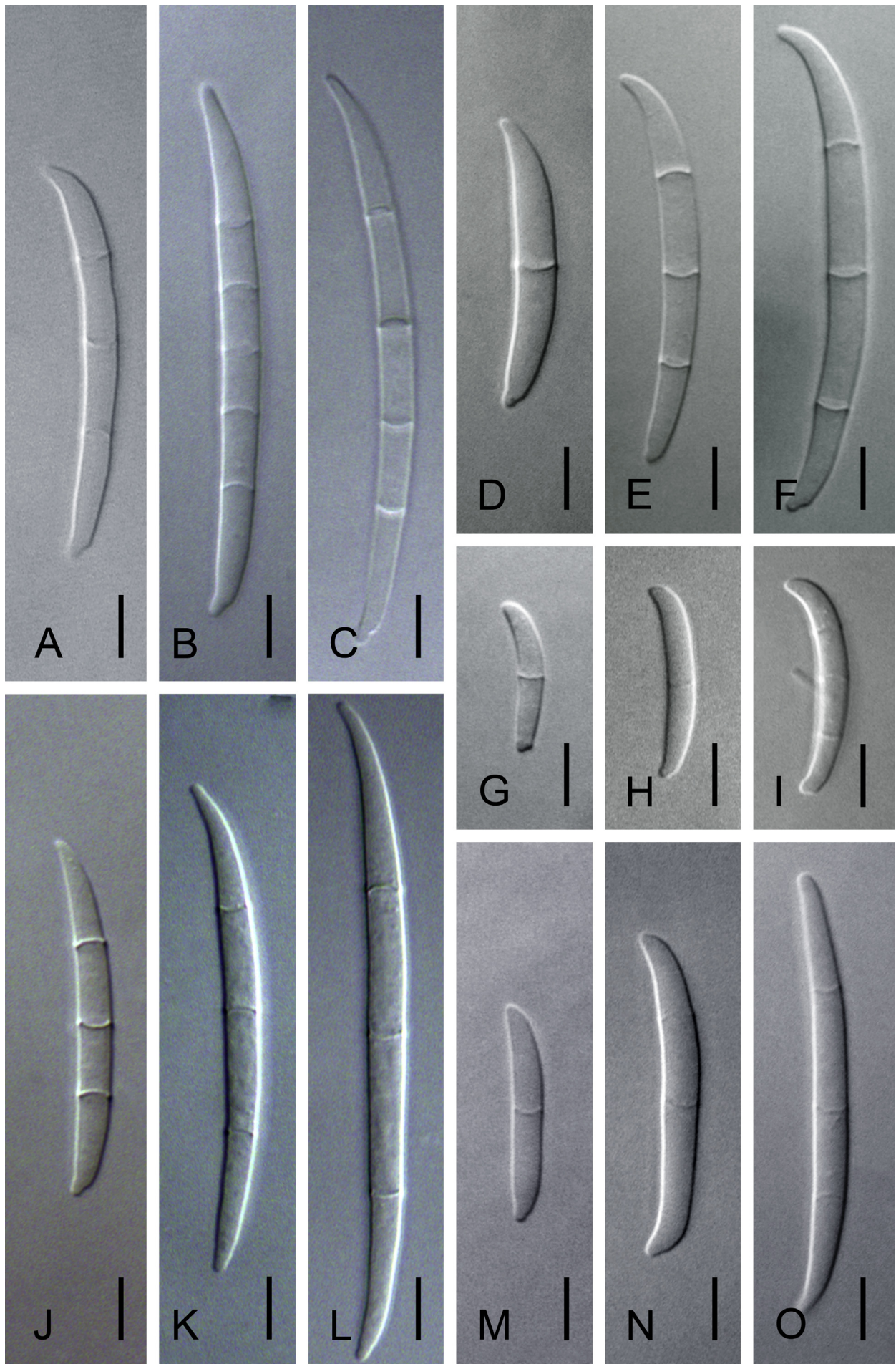
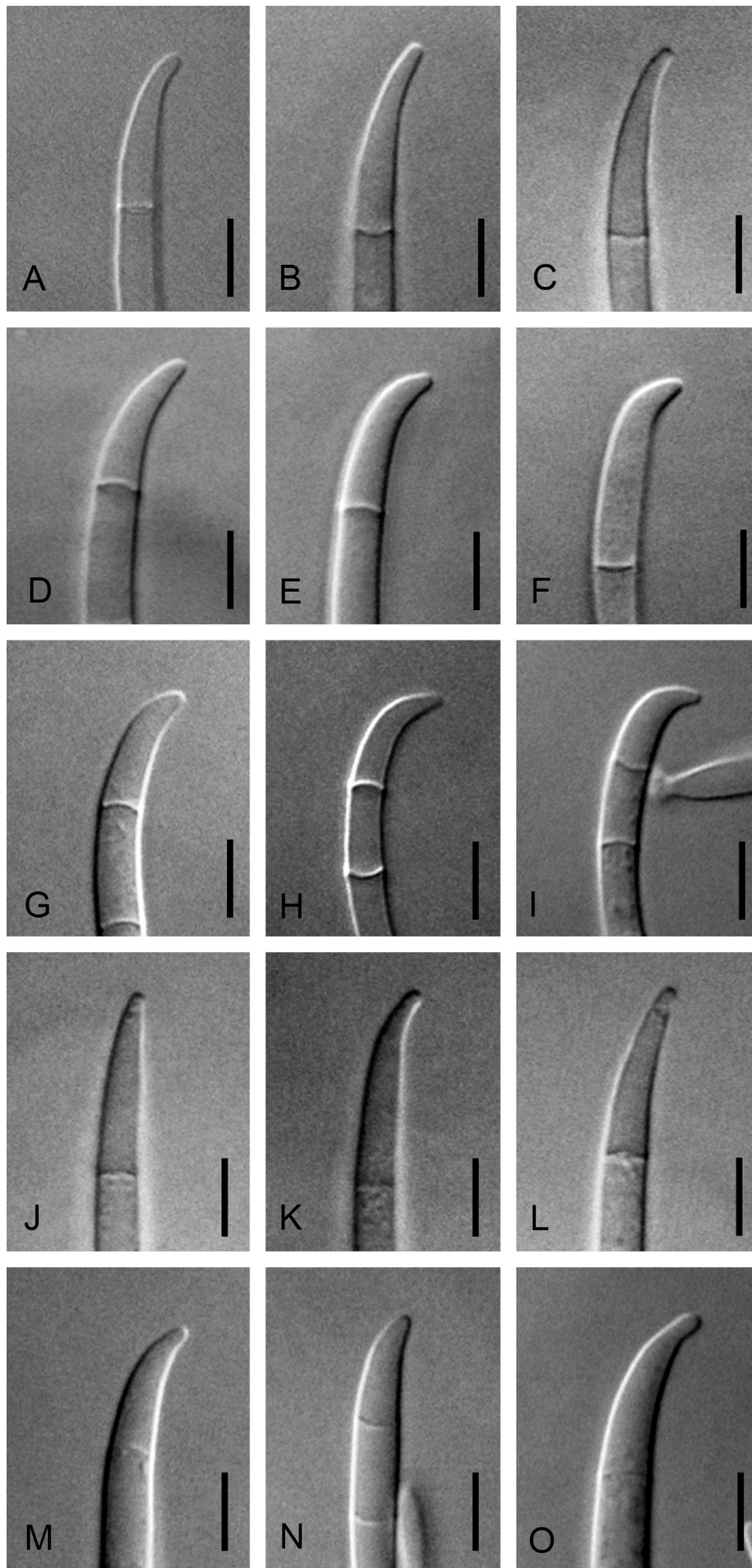


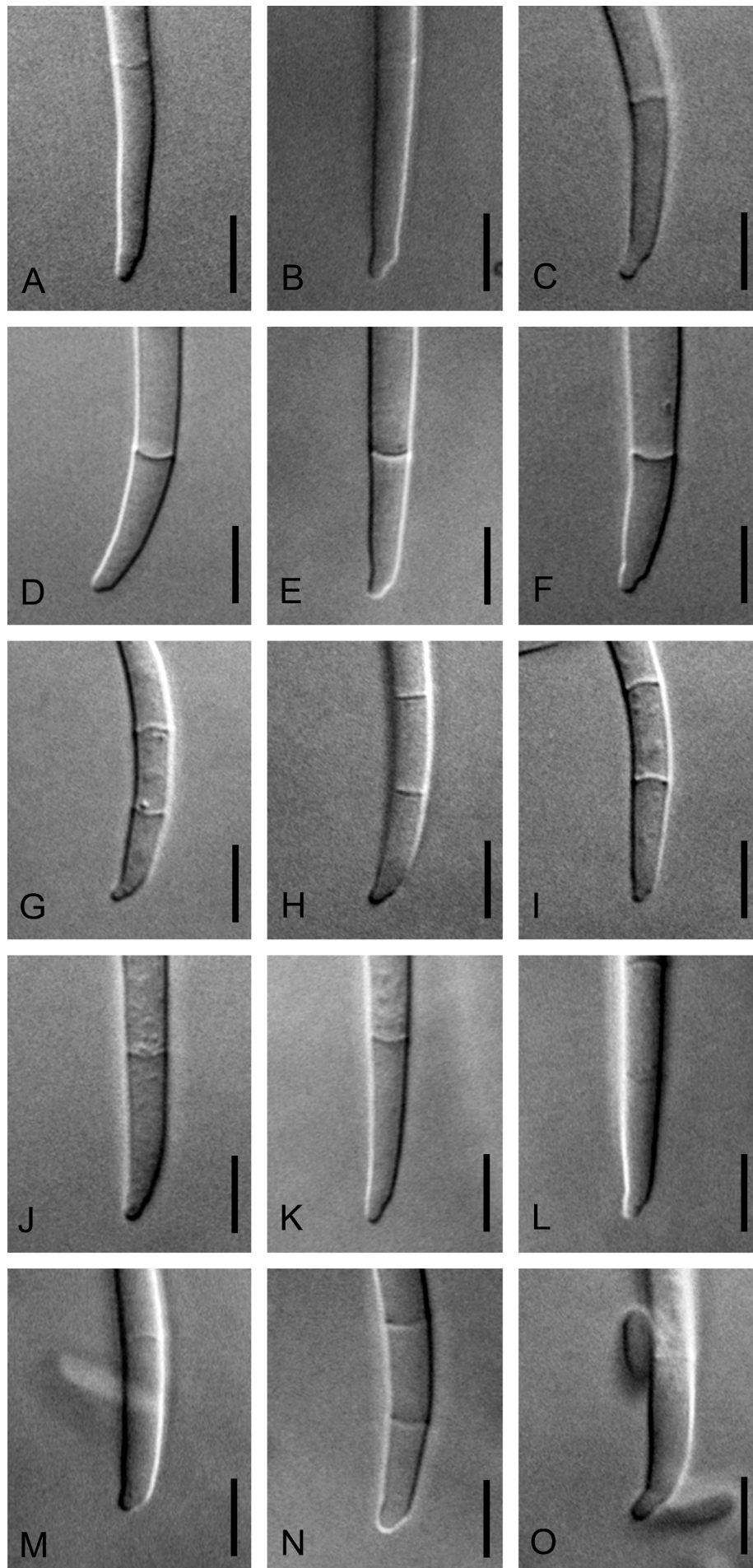
Fig. 3. Maximum likelihood phylogeny of the *Fusarium fujikuroi* species complex (FFSC), including the isolates collected from Colombia, inferred from the *tub2* sequence data. The tree is rooted to *F. oxysporum* and a similar topology was obtained using Bayesian inference. Branch support, as well as clade and isolate information are indicated as detailed in the legend of Fig. 1.



**Fig. 4.** Variation observed in size and shape of macroconidia produced by *Fusarium fracticaudum* sp. nov. (A–C), *Fusarium marasasianum* sp. nov. (D–F), *Fusarium parvisorum* sp. nov. (G–I), *Fusarium pininemorale* sp. nov. (J–L) and *Fusarium sororula* sp. nov. (M–O). Scale bar = 5  $\mu$ m.



**Fig. 5.** Variation observed in apical cells produced by *Fusarium fracticaudum* sp. nov. (A–C), *Fusarium marasasianum* sp. nov. (D–F), *Fusarium parvisorum* sp. nov. (G–I), *Fusarium pininemorale* sp. nov. (J–L) and *Fusarium sororula* sp. nov. (M–O). Scale bar = 5  $\mu$ m.



**Fig. 6.** Variation observed in basal foot cells produced by *Fusarium fracticaudum* sp. nov. (A–C), *Fusarium marasasianum* sp. nov. (D–F), *Fusarium parvisorum* sp. nov. (G–I), *Fusarium pininemorale* sp. nov. (J–L) and *Fusarium sororula* sp. nov. (M–O). Scale bar = 5  $\mu$ m.

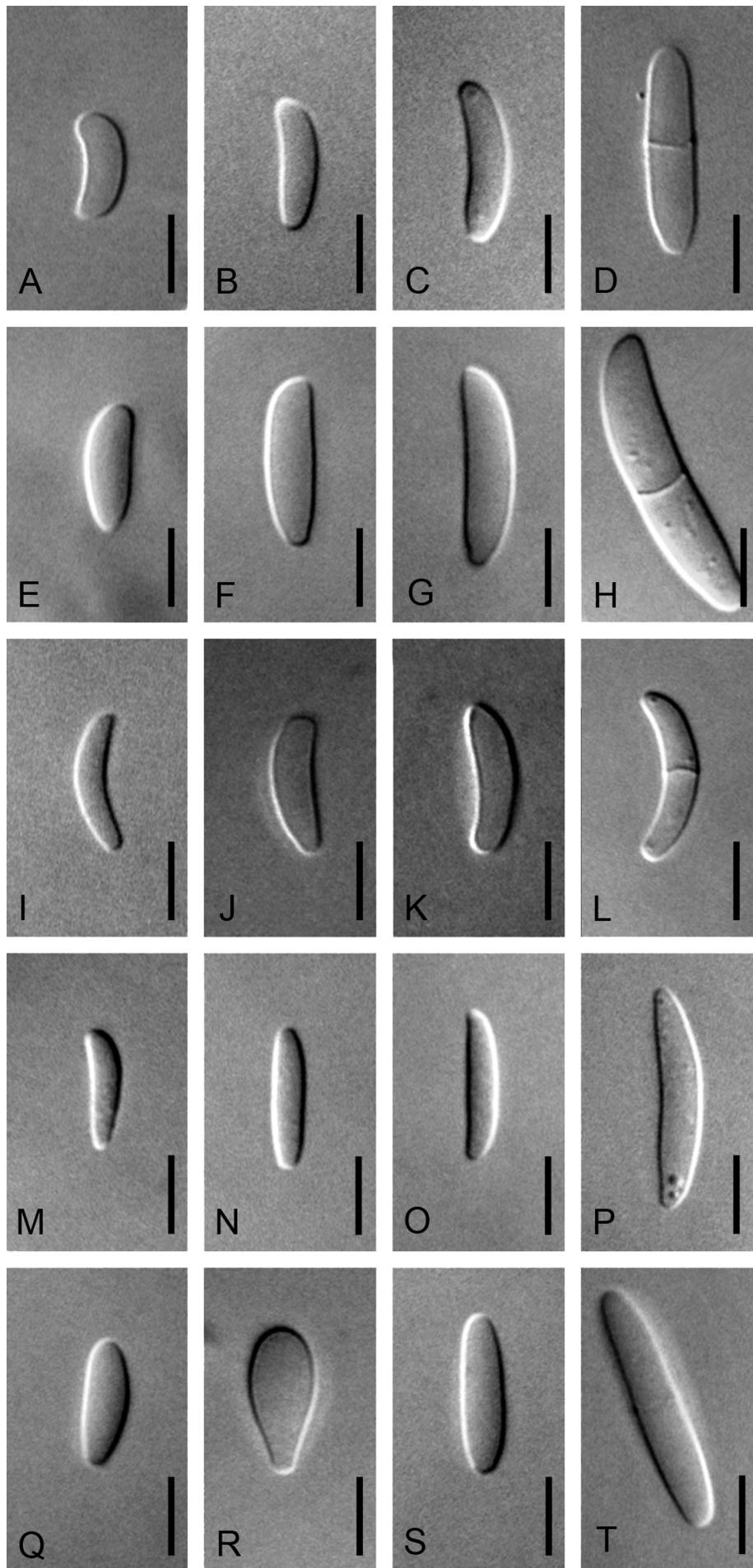
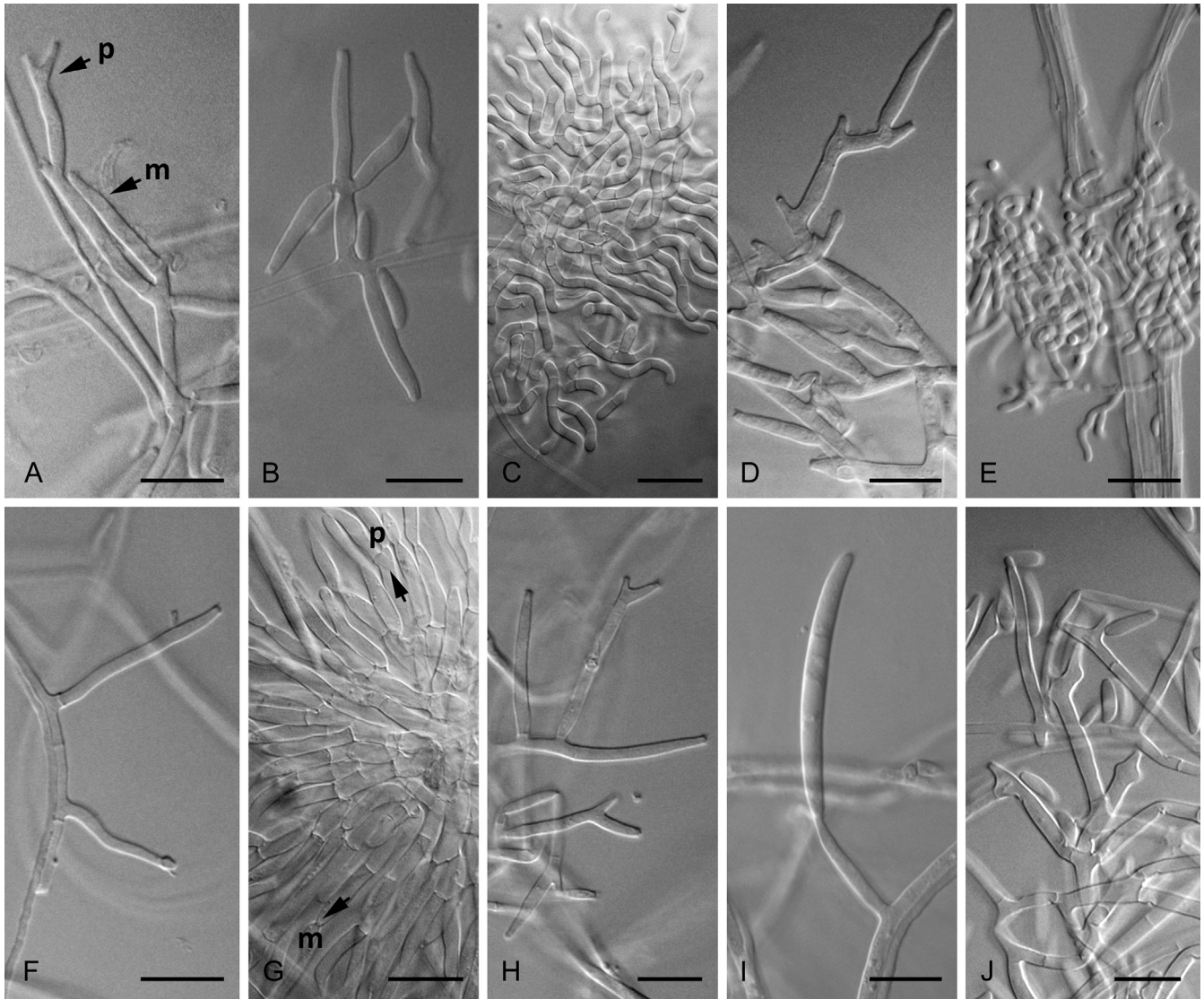


Fig. 7. Variation observed in the size and shape of microconidia produced by *Fusarium fracticaudum* sp. nov. (A–D), *Fusarium parvisorum* sp. nov. (E–H), *Fusarium marasasianum* sp. nov. (I–L), *Fusarium pininemorale* sp. nov. (M–P) and *Fusarium sororula* sp. nov. (Q–T). Scale bar = 5  $\mu$ m.



**Fig. 8.** Monophialidic (m) and polyphialidic (p) conidiogenous cells, as well as circinate hyphae of the species described in this study. Microconidia produced by *Fusarium fracticaudum* sp. nov. on mono- and polyphialides (A, B), circinate hyphae and microconidia produced by *Fusarium parvisorum* sp. nov. (C, D) and *Fusarium marasasianum* sp. nov. (E, F), macroconidia borne on mono- and polyphialides (G) and microconidia borne on monophialides (H) produced by *Fusarium pininemorale* sp. nov., and the conidiogenous cells of *Fusarium sororula* sp. nov. bearing macroconidia (I) and microconidia (J). Scale bar: A, B, D, F–J = 10  $\mu$ m, C, E = 25  $\mu$ m.

**Table 4.** The results of the growth studies conducted on *F. fracticaudum*, *F. marasasianum*, *F. parvisorum*, *F. pininemorale* and *F. sororula*.

Species <sup>1</sup>	Isolate number	Growth (mm) at various incubation temperatures after 8 d <sup>2</sup>					Growth/d 25 °C <sup>3</sup>	
		10 °C	15 °C	20 °C	25 °C	30 °C		35 °C
<i>Fusarium fracticaudum</i>	CMW 25237	20.83	32	51.83	46.33	20.83	0	6.89
	<b>CMW 25245</b>	11.96	36.5	56.65	67	13.33	0	9.09
<i>F. marasasianum</i>	CMW 25253	15.8	45.83	68.34	80	52.33	0	11.43
	<b>CMW 25261</b>	13.17	46.83	80	80	48.67	0	15.43
<i>F. parvisorum</i>	<b>CMW 25267</b>	15.83	41.67	71.83	80	52.17	0	11.43
	CMW 25268	15.97	43.83	75.5	80	44	0	13.33
<i>F. pininemorale</i>	<b>CMW 25243</b>	17	33.75	45.6	51.83	41.33	0	6
	CMW 25244	22.17	40.33	57.13	76.75	44	0	10.17
<i>F. sororula</i>	CMW 25254	14.66	31.33	48.8	62	40.66	0	7.48
	<b>CMW 40578</b>	11.33	44	66.83	80	47.16	0	11.43

<sup>1</sup> Strain numbers in **boldface** indicate the ex-type strains.

<sup>2</sup> Agar plates are 80.0 mm diam.

<sup>3</sup> Average growth per day was recorded at 25 °C, the optimum temperature for growth.

**Habitat:** Stem canker on mature *Pinus maximinoi* trees.

**Distribution:** Angela Maria (Santa Rosa) and Calima (Darien Valle) Colombia, South America.

**Materials examined:** Colombia, Angela Maria (Santa Rosa), Risaralda (75°36'21" W and 4°49'18" N), *P. maximinoi*, 2007, M.J. Wingfield & C.A. Rodas (**holotype** PREM 60895, ex-type culture CMW 25245 = CBS 137233); Calima (Darien Valle), Colombia (76°26'03" W and 3°56'57" N), *P. maximinoi*, 2007, M.J. Wingfield & C.A. Rodas, (**paratype** PREM 60894, living culture, CMW 25237 = CBS 137234).

***Fusarium marasasianum*** Herron, Marinc. & M.J. Wingf. **sp. nov.** MycoBank MB809887. Figs 4D–F, 5D–F, 6D–F, 7E–H, 8E–F.

**Etymology:** Named for the late Professor W.F.O. Marasas who dedicated the greater part of his professional life to the study of *Fusarium* spp. and mentored many students, including the authors of this study.

**Macroconidia** abundant, elongate, straight, 23.5–44.5 × 2.5–4 µm (av. 34.8 × 3.1 µm), with 0–3 septa, apical cells tapering, curved or hooked, 7–14 µm long (av. 10.4 µm), basal cells not well-developed, barely to distinctly notched or foot-shaped, 6.5–12 µm long (av. 9.2 µm). **Microconidia** scarce, fusiform to obovoid, 7.5–18 × 2–3.5 µm (av. 11.4 × 2.7 µm), with 0–1 septum. **Conidiogenous cells** monophialidic or polyphialidic, 9–27 × 2–3.5 µm long, microconidia arranged in false heads. Other characteristics include the presence of circinate hyphae.

**Culture characteristics:** Colonies showing optimal growth at 25 °C with an average growth rate of 11.4 mm/d (CMW 25253) and 15.4 mm/d (CMW 25261). Colony reverse in the dark unpigmented with spots of purple or in near UV light entirely dark purple but with less intensity.

**Habitat:** Diseased roots of *Pinus patula* seedlings.

**Distribution:** Vivero, Peñas Negra, Valle del Cauca, Colombia, South America.

**Materials examined:** Colombia, Vivero, Peñas Negra, Valle del Cauca, (76°29'49" W and 3°51'45" N), *Pinus patula*, 2007, M.J. Wingfield & C.A. Rodas (**holotype** PREM 60899, ex-type culture, CMW 25261 = CBS 137238); *Pinus patula* 2007, M.J. Wingfield & C.A. Rodas, (**paratype** PREM 60898, ex-paratype culture, CMW 25253 = CBS 137237).

***Fusarium parvisorum*** Herron, Marinc. & M.J. Wingf., **sp. nov.** MycoBank MB809886. Figs 4G–I, 5G–I, 6G–I, 7I–L, 8C–D.

**Etymology:** From *parvi* (Latin for small) and *sorum* (Latin for spore), describing the small macroconidia produced by this species.

**Macroconidia** not abundant, squat, straight, 12.5–29.5 × 1.5–3 µm (av. 19.1 × 2.3 µm), with 1–3 septa, apical cells hooked, 4.5–10.5 µm long (av. 7.1 µm), basal cells not well developed, barely to distinctly notched, 4.5–12 µm long (av. 7.6 µm). **Microconidia** not abundant, fusiform to obovoid, 7–13 × 1.5–3 µm (av. 9.7 × 2.5 µm), with 0–1 septum. **Conidiogenous cells** monophialidic or polyphialidic, 5.5–27 × 1.5–3 µm long, microconidia arranged in false heads. Other characteristics include the presence of circinate hyphae.

**Culture characteristics:** Colonies showing optimal growth at 25 °C with an average growth of 11.4 mm/d (CMW 25267) and 13.3 mm/d (CMW 25268). Colony reverse in the dark and near-UV light unpigmented.

**Habitat:** Diseased roots of *Pinus patula* seedlings.

**Distribution:** Vivero, Peñas Negra, Valle del Cauca, Colombia, South America.

**Materials examined:** Colombia, Vivero, Peñas Negra, Valle del Cauca, (76°29'49" W and 3°51'45" N), *Pinus patula*, 2007, M.J. Wingfield & C.A. Rodas (**holotype** PREM 60897, ex-type culture CMW 25267 = CBS 137236); *P. patula*, 2007, M.J. Wingfield & C.A. Rodas, (**paratype** PREM 60896, ex-paratype culture, CMW 25268 = CBS 137235).

***Fusarium pininemorale*** Herron, Marinc. & M.J. Wingf., **sp. nov.** MycoBank MB809888. Figs 4J–L, 5J–L, 6J–L, 7M–P, 8G–H.

**Etymology:** From *pin* (from pine), the host of this species and *nemorale* (from *nemoralis* which is Latin for a “collection” or “group”), thus describing the fact that this species was isolated from a group of pines or pine plantation.

**Macroconidia** abundant, elongate, straight, 35–52 × 2–3.5 µm (av. 42.2 × 2.9 µm), with 3–4 septa, apical cells tapering, curved, 8.5–14 µm long (av. 12.0 µm), basal cells foot-shaped, elongated foot shape, barely to distinctly notched, 9–14 µm long (av. 11.0 µm). **Microconidia** scarce, fusiform to obovoid, 5–16.5 × 1.5–3 µm (av. 10.1 × 2.2 µm), 0–1 septa. **Conidiogenous cells** monophialidic or polyphialidic, 6.5–32 × 2–3.5 µm long, microconidia arranged in false heads.

**Culture characteristics:** Colonies showing optimal growth at 25 °C with an average growth rate of 6 mm/d (CMW 25243) and 10.2 mm/d (CMW 25244). Colony reverse in the dark and near-UV light unpigmented.

**Habitat:** Stem canker on *Pinus tecunumanii*.

**Distribution:** Angela Maria (Santa Rosa), Risaralda, Colombia, South America.

**Materials examined:** Colombia, Angela Maria (Santa Rosa), Risaralda (75°36'21" W and 4°49'18" N), *Pinus tecunumanii*, 2007, M.J. Wingfield & C.A. Rodas, (**holotype** PREM 60901, ex-type culture, CMW 25243 = CBS 137240); *Pinus tecunumanii*, 2007, M.J. Wingfield & C.A. Rodas, (**paratype** PREM 60900, ex-paratype culture, CMW 25244 = CBS 137239).

***Fusarium sororula*** Herron, Marinc. & M.J. Wingf., **sp. nov.** MycoBank MB809889. Figs 4M–O, 5M–O, 6M–O, 7Q–T, 8I–J.

**Etymology:** From *soror-* (Latin for sister) and *sororula* (diminutive: little sister). This name depicts the fact that this species produces small macroconidia similar to its sister species, *F. parvisorum*, also described in this study.

**Macroconidia** scarce, elongate, straight, 20–42.5 × 2–4 µm (av. 28.7 × 2.9 µm), with 1–3 septa, apical cells hooked, 7.5–12.5 µm long (av. 9.3 µm), basal cells foot-shaped, elongated foot shape, barely to distinctly notched, 7–12.5 µm long (av. 9.1 µm), some producing secondary conidia. **Microconidia** abundant, fusiform to obovoid or pyriform, 5.5–15.5 × 1.5–3 µm



(avg.  $8.1 \times 2.2 \mu\text{m}$ ), with 0–1 septum. *Conidiogenous cells* monophialidic or polyphialidic, 10.9–34  $\mu\text{m}$  long, microconidia arranged in false heads.

**Culture characteristics:** Colonies showing optimal growth at 25 °C at an average growth rate of 7.5 mm per d (CMW 25254) and 11.4 mm per d (CMW 40578). Colony reverse in the dark with patches, sectors or entire area of purple or dark purple or in near-UV light with patches of partly covered with purple or dark purple.

**Habitat:** Stem canker on *Pinus patula*.

**Distribution:** Angela Maria (Santa Rosa), Risaralda, Colombia, South America.

**Materials examined:** Colombia, Angela Maria (Santa Rosa), Risaralda (75°36'21" W and 4°49'18" N), *Pinus patula*, 2007, M.J. Wingfield & C.A. Rodas (holotype PREM 60903, ex-type culture CMW 40578 = CBS 137242); *Pinus patula*, 2007, M.J. Wingfield & C.A. Rodas, (paratype PREM 60902, ex-paratype culture, CMW 25254 = CBS 137241).

## Pathogenicity

In the pathogenicity trial, five of the 11 *Fusarium* isolates used to inoculate 6-m-old *P. patula* trees produced lesions that were significantly larger ( $P < 0.0001$ ) than those recorded for the negative controls (Table 3). These included the two examined isolates of *F. parvisorum* (CMW 25269 and CMW 25267) and *F. sororula* (CMW 25254 and CMW 40578), as well as one isolate of *F. marasasianum* (CMW 25261). In most of these cases, the lesions produced were within the same size range as those observed for *F. circinatum* (Table 3). All of these fungi were re-isolated from the inoculated plants, fulfilling Koch's postulates, while no *Fusarium* spp. were isolated from control plants.

## DISCUSSION

In this study, more than 10 distinct *Fusarium* spp. were recovered from *Pinus* tissue showing symptoms of infection similar to those found for *F. circinatum*. These included the pitch canker fungus itself, the five newly described species *F. fracticaudum*, *F. marasasianum*, *F. parvisorum*, *F. pininemorale*, *F. sororula*, and two undescribed species in the FFSC and isolates belonging to the FOSC (Baayen *et al.* 2000) and FSSC (O'Donnell 2000, Zhang *et al.* 2006). Of these, only *F. circinatum* was known as a primary pathogen having an established association with *Pinus*, prior to the present study (Nirenberg & O'Donnell 1998, Gordon 2006, Wingfield *et al.* 2008a). Given the small number of sites examined in this study and the recovery of five new species, an expanded survey of the fusaria associated with *Pinus* in Colombia and surrounding countries would likely yield even more novel taxa, of which some might represent significant threats to forestry worldwide.

The distribution of the *Fusarium* species examined in this study varied in terms of host and tissue type from which they were recovered. Like *F. circinatum*, we isolated *F. marasasianum* and FSSC spp. from nursery seedlings and from cankers on established plantation trees. Isolates of *F. fracticaudum*, *F. pininemorale* and *F. sororula* were isolated from plantation

trees only and isolates of *F. oxysporum* and *F. parvisorum* only from nursery seedlings. Also, *F. circinatum*, *F. fracticaudum*, *F. marasasianum*, *F. sororula* and FSSC spp. were isolated from more than one *Pinus* spp., while *F. pininemorale* and FOSC spp. were restricted to *P. tecunumanii*. Apart from the two putative novel *Fusarium* spp. represented by single isolates, all species examined here were also recovered from more than one location in Colombia.

The recovery of isolates residing in the FOSC and FSSC complexes was not unexpected as they are known to harbour plant pathogens (Baayen *et al.* 2000, O'Donnell *et al.* 2000, Zhang *et al.* 2006). For example, isolates from both complexes have been associated with diseased *Pinus strobus* seed and seedlings (Rifle & Strong 1960, Enebak 1988, Ocamb & Juzwik 1995) and *Pinus radiata* seedlings in bare root nurseries (Dick & Dobbie 2002). However, the symptoms induced by these fusaria typically do not resemble those of the pitch canker fungus (Ocamb & Juzwik 1995, Dick & Dobbie 2002, Wingfield *et al.* 2008a). Their recovery from the *Pinus* tissues in this study is likely to be a consequence of the fact that members of these two species complexes are often saprobes with ubiquitous distributions (Burgess 1981).

Apart from FOSC and FSSC isolates, all of the *Fusarium* spp. included in this study form part of the "American" clade (*sensu* O'Donnell *et al.* 1998) of the FFSC. The emergence of this clade, together with the so-called "Asian" and "African" clades was initially suggested by O'Donnell *et al.* (1998) to be due to fragmentation of Gondwana during the upper Cretaceous through to the Paleocene. However, the same authors later reported that the complex emerged more recently (*ca.* 8.8 M yr ago) and that the apparent biogeographic clustering is probably due to long distance dispersal from South America to Africa and then to Asia in the late Miocene (O'Donnell *et al.* 2013). Nevertheless, the members of the respective clades are generally associated with hosts that have their origin in the specific geographic areas. For example, the "American" clade species *F. circinatum* and *F. subglutinans* are thought to have evolved with their hosts (*i.e.*, *Pinus* and *Zea* spp., respectively) in Mexico and Central America (Gaut & Doebley 1997, Iltis 2000, Wikler & Gordon 2000). These fungi were then introduced with their hosts to other regions as part of the global development and expansion of agriculture and forestry (Desjardins *et al.* 2000, Wingfield *et al.* 2008a). Following this view, it is possible that the new *Fusarium* spp. identified in this study also originated from Mexico and Central America, because these regions represent centres of origin for many *Pinus* spp. (Millar 1993).

An alternative hypothesis is that the new species recognised in this study are native on other host plant species in Colombia. This would then suggest that the *Fusarium* spp. have undergone host shifts to *Pinus* spp. from other hosts. This is plausible as the phenomenon of host jumping (Slippers *et al.* 2005) occurs frequently in environments where native ecosystems and exotic monoculture-based forestry or agriculture exist in close association (Burgess & Wingfield 2004, Stenlid *et al.* 2011). Furthermore, these host jumps occur more readily when the host species are related. This has been shown, for example, for *Chrysosporthe austroafricana* (Gryzenhout *et al.* 2004), which is native to southern Africa and associated with native *Myrtales* (Heath *et al.* 2006), but can cause comparable (and often more severe) symptoms on exotic *Eucalyptus* spp. planted in intensively managed plantations (Nakabonge *et al.* 2006). Another example is myrtle rust caused by *Puccinia psidii*, which is native

on many *Myrtaceae* in Latin America but has jumped to *Eucalyptus* spp. planted as exotics to establish forest plantations (Coutinho *et al.* 1998, Glen *et al.* 2007). Future studies should thus seek to understand the host range and centres of origin of the newly identified species, which would in turn reveal the potential risks these fusaria pose to conifers and other gymnosperms native to the Colombia.

Results of this study showed that the new species, *F. marasianum*, *F. parvisorum* and *F. sororula* all have the ability to cause disease in *P. patula* seedlings. These fungi induced lesions in seedlings that were as large as or larger than those caused by a virulent isolate of *F. circinatum*. However, we observed some level of variation in pathogenicity and virulence between the isolates of the same species. Although such variation in the ability of isolates to cause disease is well documented for plant pathogenic *Fusarium* spp. (Burgess 1981, Gordon & Okamoto 1992, Appel & Gordon 1995, Miedaner *et al.* 2001, Carter *et al.* 2002), an important aspect of our results is that *Pinus* spp. in Colombia are infected with additional *Fusarium* pathogens as aggressive as *F. circinatum*. This has significant implications for commercial forestry in Colombia and elsewhere where *Pinus* spp. are planted as non-natives or where they occur naturally. In general, the susceptibility of planting stock to these new pathogens will need to be evaluated, by following approaches similar to those used for *F. circinatum* (e.g., Roux *et al.* 2007, Mitchell *et al.* 2011). Suitable control strategies will also have to be developed, although this will require detailed knowledge regarding the distribution, host range and ecology of the newly recognised pathogens.

Studies such as this one where new *Fusarium* pathogens of *Pinus* spp. have been discovered are important, not only to diagnose new diseases, but to improve global quarantine measures and thus contain their potential spread to new areas. For example, strategies can now be developed to identify and track the possible movement of the apparently aggressive pathogens *F. marasianum*, *F. parvisorum* and *F. sororula* in Colombia and possibly elsewhere in the world. Active monitoring of these areas is of particular importance for forest industries where rotation periods are especially long. This implies that they are exposed to pests and pathogens for extended periods of time and where problems emerge, the consequences can be dire. But even where early detection is achieved, the appearance of new tree diseases is difficult to treat or prevent. In the case of *F. circinatum*, 70 yr after its discovery, it has spread to more than ten countries on five different continents (reviewed by Steenkamp *et al.* 2012) and losses remain very serious in some areas.

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