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ORIGINAL ARTICLE

Colletotrichum gigasporum sp. nov., a new species of *Colletotrichum* producing long straight conidia

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Abstract A new species of *Colletotrichum* was described, based on morphology and phylogeny. The fungus was isolated in Madagascar from healthy leaves of *Centella asiatica*, in Mexico from wild native of *Stylosanthes guianensis* and in Colombia from *Coffea arabica*. The fungus differed from the currently related species in the genus by its longer and wider size of conidia. In potato dextrose agar medium supplemented with sterilized leaf powder of *Ce. asiatica*, the fungus produced fertile perithecia containing asci and unusual long ascospores measuring up to 90 μm. In addition to these morphological characteristics, the maximum parsimony analysis of the ITS region and β-tubulin gene placed the fungus in a distinct clade far from the currently valid *Colletotrichum* species. Based on the morphological and molecular characterization, *Colletotrichum gigasporum* sp. nov. was proposed as a new species in the genus *Colletotrichum* Corda.

Keywords *Colletotrichum gigasporum* · New species · *Centella asiatica* · Fungal endophytes · Taxonomy

Introduction

Colletotrichum Corda is an important fungal genus containing more than 700 species (*Index Fungorum* – www.indexfungorum.org). Several of these species are responsible for the anthracnose diseases on a wide range of plants (Freeman

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and Katan 1997; Martín and García-Figueres 1999; Yang et al. 2009; Nguyen et al. 2010), while a few were repeatedly isolated as endophytes from apparently asymptomatic healthy plants (Bussaban et al. 2001; Lu et al. 2004; Photita et al. 2005; Promputtha et al. 2007). In the past, many *Colletotrichum* species were named solely on morphological characteristics and on host plants (Sutton 1980, 1992; von Arx 1957). The lack of reliable morphological criteria within this genus further resulted in doubtful identifications. It is likely that a number of the several hundred species described in this genus are synonyms (Sutton 1980, 1992; von Arx 1957). Thus, there is a need for taxonomic revision of many *Colletotrichum* species (Hyde et al. 2009).

Recently, Cai et al. (2009) proposed to describe these fungi using a combination of multilocus molecular phylogenetic analysis with morphological and cultural characteristics, physiology and pathogenicity tests. Considerable progress has been made in species delimitation in this genus and many new species and designation of epitypes were reported following this polyphasic approach (Crouch et al. 2009; Damm et al. 2009; Moriwaki and Tsukiboshi 2009; Prihastuti et al. 2009; Weir and Johnston 2010; Wikee et al. 2011; Yang et al. 2011). In parallel, systematic revisions of major *Colletotrichum* species complexes, such as *C. acutatum*, *C. boninense* and *C. gloeosporioides* sensu *lato* have been proposed (Damm et al. 2010, 2012).

Centella asiatica (L.) Urb (Apiaceae) (*Ce. asiatica*) is an ethnomedicinal plant, native to India, Madagascar, Sri Lanka, China, Indonesia, Australia and South Africa. Puja et al. (2006) described a new fungal species, *Echinosphaeria macrospora*, teleomorph of *Vermiculariopsiella endophytica* from the stems of *Ce. asiatica*, while Krishnamurthy et al. (2008) showed that *Chaetomium globosum* was the most frequently isolated fungus from living healthy leaves of *Ce. asiatica* collected from the Malnad region (southern India). Recently, Rakotoniriana et al. (2008) isolated an unusual long straight conidia-producing *Colletotrichum* species (MUCL 44947), morphologically similar to the original description of *C. crassipes* (von Arx 1957). The fungus was isolated as endophyte from *Ce. asiatica* in Madagascar. Single conidia grown on potato dextrose agar (PDA) supplemented with *Ce. asiatica* leaf powder resulted in the production of fertile perithecia. Preliminary genotypic comparisons using BLAST searches for ITS sequence similarities in GenBank yielded many *Colletotrichum* sequences identical or closely related to that of strain MUCL 44947. Interestingly, most of these isolates were sampled as endophytes from a wide range of host plants located in geographically distant tropical areas. However, the species identity of these *Colletotrichum* strains remains unclear. No formal or Latin diagnosis has been reported so far. They likely belong to undescribed species in the genus *Colletotrichum*.

Here we described a new species of *Colletotrichum*, i.e. *Colletotrichum gigasporum* sp. nov., based on morphology and molecular phylogeny. The morphological characteristics of the teleomorph were also described and discussed.

Material and methods

Fungal isolates

Isolate MUCL 44947 was isolated from healthy leaves of *Centella asiatica* (*Ce. asiatica*) collected in the middle-eastern region of Madagascar in 2003 following surface sterilization (Rakotoniriana et al. 2008). Isolates MUCL 42034, 42044 and 42077 were obtained as foliar endophytes from *Stylosanthes guianensis* in Nayarit state in Mexico in 1995 (Gama-López et al. 2007). The isolate MUCL 41702 referred to as *C. macrosporum* was recovered from *Bulbophyllum* sp. (Orchidaceae) collected at Bukit Timah Nature Reserve in Singapore in 1999. All isolates are deposited in the BCCM/MUCL collection, Université catholique de Louvain, Belgium and preserved in vials either under sterile paraffin oil, lyophilized or frozen at -130 °C. Four strains identified as *C. crassipes*, CBS 169.59, CBS 159.75, CBS 112984 and CBS 112988 obtained from the Centraalbureau voor Schimmelcultures (*CBS*), Netherlands, and a *Colletotrichum* sp. strain VEGA 508, an endophytic fungus from *Coffea arabica* (*Co. arabica*) showing identical ITS sequences to isolates from *Ce. asiatica* and *S. guianensis*, were also included. Overall, ten fungal isolates were examined for detailed study of the morphological and cultural characters.

Morphological and cultural studies

Conidial shape and size along with cultural characters including colony morphology (colony color, mycelial characteristics, presence of setae, and spore masses) were recorded from cultures derived from single germinated conidia on potato dextrose agar (PDA, Scharlau Chemie S.A., Barcelona, Spain) and incubated in the dark at 25 °C for 2 weeks. Growth rate was calculated as the 7-day average of mean daily growth (mm per day) (Photita et al. 2005). Experiments were repeated three times, each experiment was performed in four replicates with two measurements per replicate.

Dilutions were made with conidia masses and drops of the suspension were placed on microscope slides and mixed with lactophenol/cotton blue (Shipton and Brown 1962). Length and width of 50 conidia were measured with an Olympus BX 52 under 1,250 × magnification. Appressoria were observed in slide cultures on PDA (Sutton 1980) grown under the same conditions as above.

To induce the production of the teleomorph, single conidia isolates were grown on PDA supplemented with 2 g/l sterilized air-dried leaf powder of *Ce. asiatica* and incubated in the dark at 25 °C for 10 days. Plates were further exposed constantly under near UV light combined with cool white fluorescent light at room temperature till the production of perithecia. A total of 20 perithecia were examined and measured (length × width), with the width of each perithecium measured at its widest point using an ocular micrometer. Length and width of 50 ascospores and asci from various perithecia stained with lactophenol/cotton blue were measured with an Olympus BX 52 under 600–1,250 × magnification.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from lyophilized fungal mycelium derived from single conidia culture using a CTAB-based method (Abang et al. 2002) adapted as follows. Dried mycelium, 50 mg, were put in microtubes and ground to a fine powder with a MagNa Lyser (Roche Diagnostics Gmbh, Germany). Microtubes were filled with 700 µl of CTAB extraction buffer. After incubation at 65 °C for 1 h, 700 µl of chloroform/isoamylalcohol (24/1; vol/vol) were added to the homogenate and mixed by vortexing to form an emulsion. Following centrifugation for 10 min at 13,000 rpm, the aqueous top layer was transferred to a new microcentrifuge tube and incubated with RNase A (100 mg/ml) and proteinase K (10 mg/ml) for 30 min at 37 °C. DNA was precipitated by adding 1.2 volume of isopropanol and incubating at room temperature for 10 min. Supernatant was discarded after centrifugation for 15 min at 13,000 rpm and the pellet was washed twice in 70 % EtOH, air-dried and dissolved in 100 µl of MilliQ water.

PCR amplifications were performed in DNA Thermal Cycler 480 (Perkin Elmer Life and Analytical Sciences, Inc, Milano, Italy) using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990) to amplify the entire ribosomal internal transcribed spacer (ITS) region and primers Bt2a (5′- GGTAACCAAATCGGTGCTGCTTTC -3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTT-3′) (Glass and Donaldson 1995) for the β-tubulin nuclear gene. The PCR reaction mixture of 50 µl included 10 µl of fungal DNA containing 10 ng/µl and 40 µl of master mix consisting of 5 µl buffer, 3 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 1 µl of each primer (10 µM), 0.5 µl Taq DNA polymerase (5 U/µl) and 28.5 µl of sterile distilled water. PCR programs consisted of an initial denaturation step for 3 min at 94 °C followed by 40 cycles of 90 s denaturation at 94 °C, 90 s annealing at 55 °C and 2 min extension at 72 °C. At the end, a final extension step of 72 °C for 10 min was included. PCR products were purified using MSB Spin PCRapace (Invitek, Berlin) according to the manufacturer's protocols. Primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), ITS4 and ITS5 for ITS sequencing and primers Bt2a and Bt2b for β-tubulin sequencing were used to sequence both strands of DNA molecule using a CEQ DTCS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA). Reaction products were run on CEQ 2000XL capillary automated sequencer (Beckman Coulter Inc., USA).

Phylogenetic analysis

For the phylogenetic analysis of ITS region, 38 ITS sequences, of which 26 were from *Colletotrichum* spp. retrieved from EMBL databank, 11 were obtained from this study and one was from *Plectosphaerella cucumerina* NRRL 20430 used as outgroup (see Table 1). Sequences were aligned using the Clustal W2 software (Chenna et al. 2003). All the ITS sequences started at the first base of the ITS1 region and ended at the last base of the ITS2 region.

The final dataset for the phylogenetic analysis of ITS consisted of 32 sequences after selecting representatives of haplotypes from sequence comparison. The aligned ITS sequences matrix was imported into PAUP* 4.0b10 (Swofford 2000) for phylogenetic analysis. Heuristic searches were run with parsimony as the optimality criterion. Alignment gaps were treated as a fifth character, and all characters were unordered and of equal weight. Starting trees were obtained via stepwise addition of 1,000 random-addition sequences with tree-bisection-reconnection branch swapping algorithm and branches were collapsed if maximum branch length is zero. To estimate confidence in the clades of the resulting trees, maximum parsimony bootstrap values were generated from 1,000 replicates. Consistency index (CI), homoplasy index (HI) and retention index (RI) were calculated to obtain the amount of homoplasy in the dataset.

For strains having the same ITS sequences as that of *C. gigasporum*, and of which the β-tubulin sequences were available in public database, a phylogenetic analysis of the β-tubulin gene was performed using the same software with 1,000 parsimony bootstrap replications.

Results

Induction of the teleomorph

Fertile perithecia were readily produced when monoconidial isolates of *Colletotrichum* sp. MUCL 44947 from healthy leaves of *Ce. asiatica* were grown on PDA supplemented with 2 g/l of *Ce. asiatica* leaf powder and exposed constantly under near UV light and cool white fluorescent light. Under

Table 1 Origin of ITS and β-tubulin sequences from GenBank database or sequenced for this study (in bold) used for phylogenetic analyses

Species	Isolate	Country	Host	GenBank no.	
				ITS	β-tubulin
C. acutatum	BBA 70339	The Netherlands	*Vaccinium corymbosum*	AJ301911	
C. boninense	MEP1554	China	*Pachira* sp.	DQ286160	
C. boninense	465	Portugal	*Citrus limon* var. *Lisboa*		FN611025
C. incarnatum	unknown	unknown	unknown	GQ369600	
C. coccodes	STE-U 5301	Zimbabwe	*Lycopersicon esculentum*	AY376528	
C. crassipes	CBS 169.59	The Netherlands	*Oncidium excavatum*	FN557344	
C. crassipes	CBS 159.75	India	Air and stored grains	**FN557345**	
C. crassipes	CBS 112984	Madeira	*Dryandra* sp.	**FN557347**	
C. crassipes	CBS 112988	Switzerland	*Dryas octopetala*	**FN557348**	
C. destructivum	CD-hz 03	China	Cowpea	**EU070913**	
C. dracaenophilum	MEP1537	China	*Dracaena* sp.	DQ286207	
C. fragariae	BBA 70340	unknown	*Fragaria* sp.	AJ301912	
C. fuscum	MAFF 238340	Japan	*Nemesia strumosa*	AB105966	
C. gloeosporioides	AR 4040	Panama	*Theobroma cacao*	DQ286194	
C. gloeosporioides	MCA 2499	Hungary	unknown	DQ286196	
C. higginsianum	IFO 6182	Japan	unknown	AB105957	
C. kahawae	STE-U 5295	Kenya	*Coffea arabica*	AY376540	
C. lagenarium	BBA 71046	Europe	*Cucumis sativus*	AJ301965	
C. lindemuthianum	BBA 65483	unknown	*Phaseolus* sp.	AJ301958	
C. lupini	BBA 70884	Ukraine	*Lupinus albus*	AJ301948	
C. macrosporum	MUCL 41702	Singapore	*Bulbophyllum* sp.	**FM163593**	
C. musae	BBA 62471	Germany	*Musa* sp.	AJ301904	
C. trifolii	BBA 70709	USA	*Trifolium* sp.	AJ301941	
C. gigasporum	MUCL 42034	Mexico	*Stylosanthes guianensis*	**AM982794**	**FN557443**
C. gigasporum	MUCL 42044	Mexico	*Stylosanthes guianensis*	**AM982795**	**FN599823**
C. gigasporum	MUCL 42077	Mexico	*Stylosanthes guianensis*	**AM982796**	**FN599824**
C. gigasporum	MUCL 44947	Madagascar	*Centella asiatica*	**AM982797**	**FN557442**
C. gigasporum	MUCL 52571	Madagascar	*Centella asiatica*	**FN557441**	**FN599825**
C. gigasporum	VEGA 508	Colombia	*Coffea arabica*	**FN557349**	**FN599822**
Colletotrichum sp.	3,386	Panama	*Theobroma cacao*	GU994389	GU994480
Colletotrichum sp.	4,801	Panama	*Theobroma cacao*	GU994386	GU994479
Colletotrichum sp.	E1249	Panama	*Trichilia tuberculata*	GU994388	
Colletotrichum sp.	Z31	China	*Taxus chinensis* var. *mairei*	JN198428	
C. yunnanense	AS3.9616	China	unknown	EF369491	
C. yunnanense	AS3.9617	China	unknown	EF369490	
Glomerella septospora	C040981	Korea	*Capsicum annuum*	GU935907	GU935912
Glomerella septospora	C07005	Korea	*Capsicum annuum*	GU935910	GU935916
Glomerella septospora	C07011	Korea	*Capsicum annuum*	GU935911	GU935915
Glomerella septospora	C07001	Korea	*Capsicum annuum*		GU935913
Glomerella septospora	C07002	Korea	*Capsicum annuum*		GU935914
Plectosphaerella cucumerina	NRRL 20430	Egypt	*Viola odorata*	AF176952	

these conditions, perithecia were consistently produced whereas this strain formed rarely the teleomorph on PDA alone. Perithecia grouped mostly near the inoculum point at the center of the plates (< 2 cm around), and were rarely found in the margin of the colony. The teleomorph was only produced in culture by the isolate MUCL 44947 from *Ce. asiatica* but it was not observed neither for isolates from *S. guianensis* (MUCL 42034, MUCL 42044 and MUCL 42077) nor for isolate from *Co. arabica* (VEGA 508), when grown under the same conditions.

Morphological comparison of *C. gigasporum* with related *Colletotrichum* species

Details of the morphological and cultural characters of the ten fungal isolates observed in the study were provided (Table 2). The mean length of the conidia from *Ce. asiatica* (MUCL 44947) was a bit longer than those from *S. guianensis* (MUCL 42034, MUCL 42044 and MUCL 42077) and from *Co. arabica* (VEGA 508) but the length range overlapped. Conidia from *C. gigasporum* (MUCL 44947, MUCL 42034, 42044, 42077 and VEGA 508) mostly bore resemblance to those identified as *C. crassipes* CBS 169.59. Conidia were hyaline, aseptate, straight, cylindrical and having somewhat similar size except that both apices were obtuse for isolates of *C. gigasporum* whereas conidial base was truncate in isolates identified as *C. crassipes*. Observations showed that isolates CBS 112984 and CBS 112988 had considerably shorter conidia than isolates CBS 15975 and CBS 16959. Overall, *C. macrosporum* MUCL 41702 had the largest conidia measuring (28) 31.5–38 (40)×9–11 μm.

Molecular phylogenetic analysis

The final alignment of the ITS sequences generated a total of 540 characters, 309 of which were constant, 99 were parsimony uninformative and 132 were parsimony informative. One of the 18 parsimonious trees retained is shown (Fig. 1). The tree length was 485, the consistency index (CI) 0.7052, the homoplasy index (HI) 0.2948 and the retention index (RI) 0.8462. Isolates of *C. gigasporum* clustered in a clade with isolates identified as *Glomerella septospora*, *C. incarnatum* and several *Colletotrichum* sp. strains from various host plants, and their grouping was supported by high bootstrap of 99 %. This clade was well separated from other clades containing known, valid *Colletotrichum* species. Isolates identified as *C. crassipes* were resolved into three different clades.

For β-tubulin gene alignment data ten taxa provided 499 characters, of these 411 characters were constant, 77 characters were parsimony uninformative and 11 characters were parsimony informative. The one parsimonious tree resulting from the analysis of the β-tubulin gene is shown (Fig. 2). The tree length was 91, the consistency index (CI) 0.9780, the homoplasy index (HI) 0.0220 and the retention index (RI) 0.8947. The phylogenetic tree of the β-tubulin gene placed isolates of *C. gigasporum* in a distinct clade with a bootstrap support of 86 %.

Taxonomy

Colletotrichum gigasporum E.F. Rakotoniriana & F. Munaut sp. nov. (Fig. 3 a, b, c, d, e, f, g, h and i)

MycoBank no. MB 800175

Etymology. The epithet *gigasporum* refers to the large size of the conidia.

Holotype. MUCL 44947-H. MADAGASCAR. MANGORO (18°52′20″S, 48°06′25″E): isolated from the inner part of healthy leaves of *Centella asiatica*, May 2003, E.F. Rakotoniriana, dried culture. Ex holotype culture: MUCL 44947, deposited at BCCM/MUCL, Louvain-la-Neuve, and preserved under sterile paraffin oil, lyophilized or frozen at -130 °C.

Table 2 Morphological and cultural comparison between *C. gigasporum* and related taxa

Species	Colony diameter [a]	Aerial mycelium	Conidial shape	Conidial length × width (μm)		
Isolates				Mean	Range	SD [b]
C. gigasporum						
MUCL 44947	65.9±1.3	Gray to green	Cylindrical, both apices rounded	26.9×7.7	22–32×7–9	1.9×0.7
MUCL 42044	70.1±1.7	Gray	Cylindrical, both apices rounded	22.1×6.8	18–26×6–8	1.8×0.6
MUCL 42034	67.5±2.7	Gray	Cylindrical, both apices rounded	21.0×6.9	16–24×6–8	1.8×0.7
MUCL 42077	57.1±1.9	Gray	Cylindrical, both apices rounded	20.3×6.9	18–24×6–8	1.4×0.7
VEGA 508	69.2±1.5	Brown	Cylindrical, both apices rounded	22.7×7.8	20–28×7–9	1.7×0.7
Isolates identified as *C. crassipes*						
CBS 159.75	42.8±1.4	Brown	Cylindrical, apex rounded, base truncate	18.2×8.2	14–20×7–9	0.6×0.7
CBS 169.59	77.2±2.7	Brown	Cylindrical, apex rounded, base truncate	22.4×5.8	18–26×5–7	1.7×0.6
CBS 112984	47.6±1.7	Whitish	Cylindrical, apex rounded, base truncate	14.1×4.3	11–16×4–5	1.1×0.5
CBS 112988	49.2±1.3	Whitish	Cylindrical, apex rounded, base truncate	15.2×4.4	12–17×4–5	0.9×0.4
C. macrosporum						
MUCL 41702	73.2±1.2	Dark green	Cylindrical, both apices rounded	34.4×10.5	28–39×9–11	2.3×0.5

[a] Colony diameter (mm) after 7 day-culture, except for *C. macrosporum* for which values were taken after 5 days due to its high growth rate

[b] SD = Standard deviation

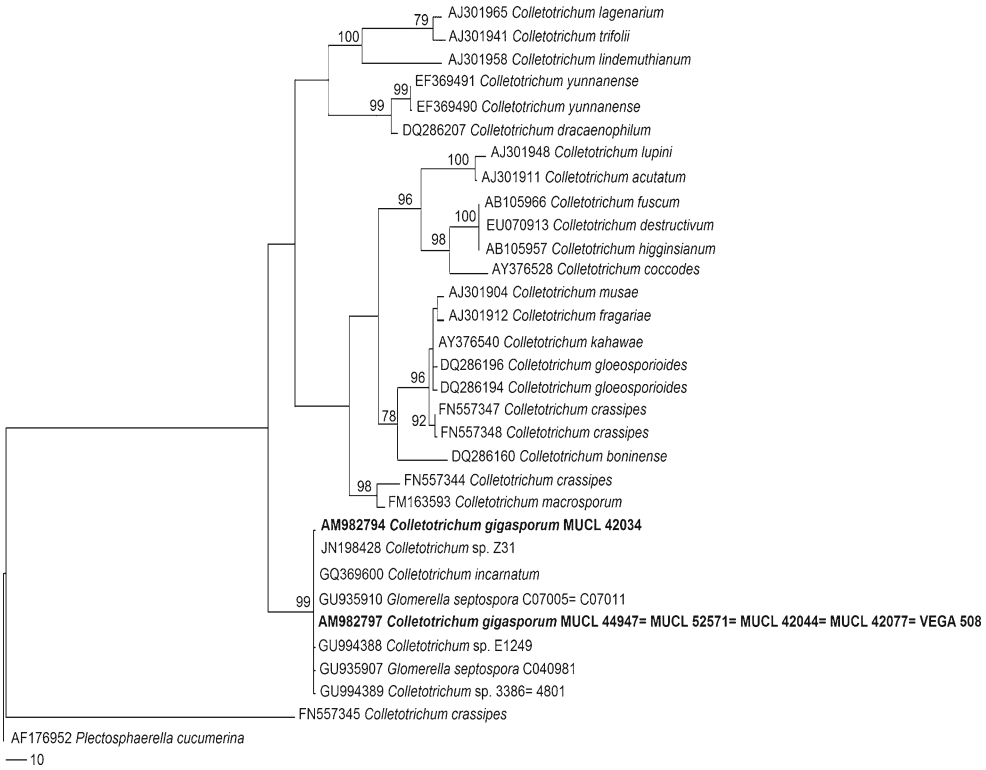

Fig. 1 Phylogenetic tree based on maximum parsimony analysis of the ITS sequences of *Colletotrichum gigasporum* and the closest relatives from GenBank. Percent of 1,000 bootstrap replications greater than 70 % are indicated at the internodes. *Plectosphaerella cucumerina* (accession number AF176952) was used as an outgroup

Paratype. MUCL 52571-H. Dried culture with perithecia produced from single conidia of MUCL 44947 (ex holotype), June 2009, E.F. Rakotoniriana. Ex-paratype culture: MUCL 52571, deposited at BCCM/MUCL, Louvain-la-Neuve, and preserved under sterile paraffin oil, lyophilized or frozen at -130 °C.

Anamorph. Colonies on PDA at 25 °C in complete darkness 6.6 cm diam in 7 days, grayish green margin regular. Aerial mycelium gray to pale green, short, reverse dark green. Sclerotia absent. Setae present, light to dark brown abundant, ruguous, septate, straight becoming narrower, tapered at the apex or rounded 90–140×3.5–4 μm. Conidioma acervular. Appressoria abundant, pale to medium brown with germ pore, clavate or irregular in shape with moderate crenate edges 14–16×10–12 μm, becoming complex. Conidiogenous cells cylindrical to clavate, hyaline or slightly brown, phialidic, cylindrical, 17–28×5–6 μm. In PDA (24 g/l, Duchefa) supplemented with 2 g/l *Centella asiatica* powder, under near UV, sporulation abundant, with conidia formed in a pinkish masses, hyaline, aseptate, straight, cylindrical, guttulate, rounded at both ends, measuring (22)25–29(32)×(6)7–9 μm.

Teleomorph developed on PDA supplemented with 2 g/l sterilized *Centella asiatica* leaf powder. Ascoma an

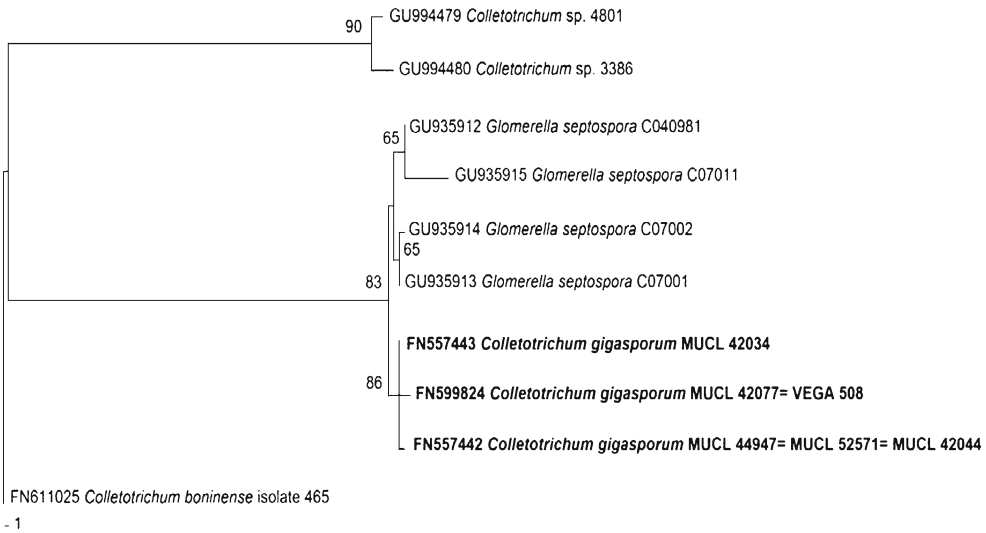

Fig. 2 Phylogenetic tree based on maximum parsimony analysis of the β-tubulin gene of *Colletotrichum gigasporum* and the closest relatives from GenBank. Percent of 1,000 bootstrap replications are indicated at the internodes. *Colletotrichum boninense* (accession number FN611025) was used as an outgroup

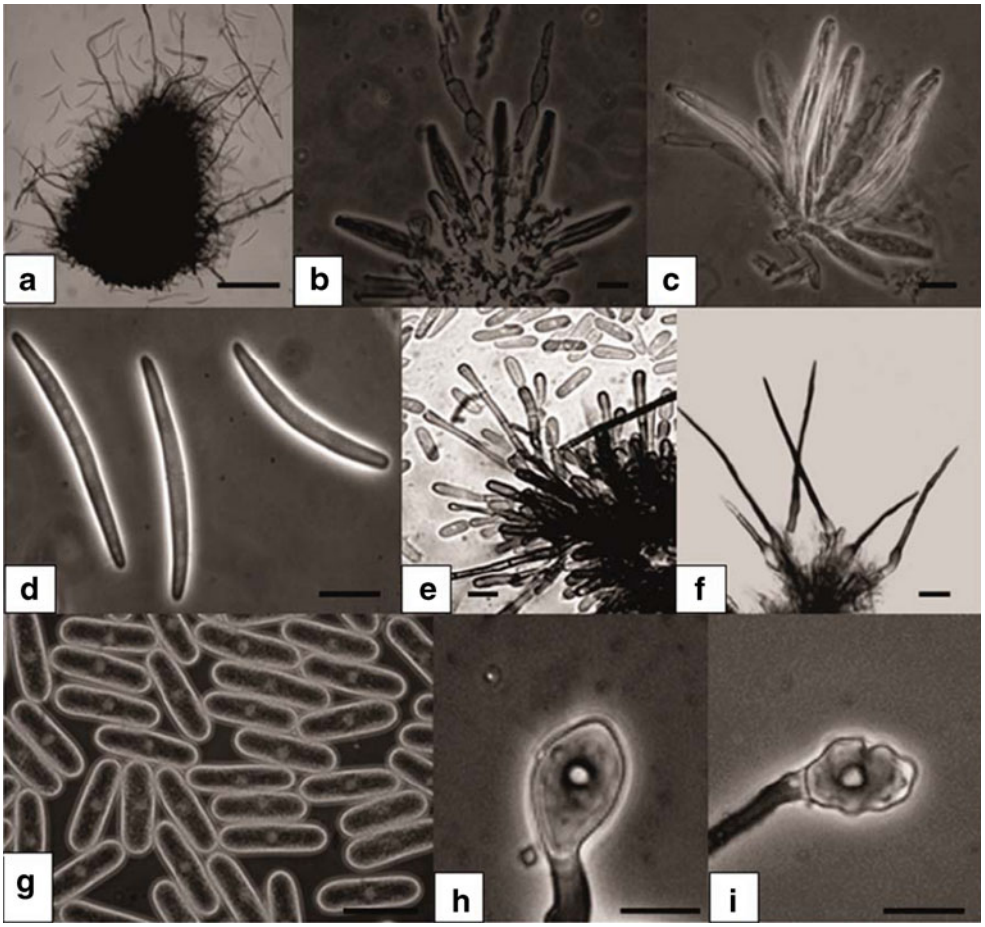

Fig. 3 *Colletotrichum gigasporum* and its teleomorph. **a** Perithecia formed in culture. **b** Immature asci with septate paraphyses. **c** Mature asci. **d** Ascospores. **e** Conidiophores of *C. gigasporum* produced in culture. **f** Setae of *C. gigasporum* formed on PDA. **g** Conidia of *C. gigasporum* formed on PDA at 25 °C in the dark for 2 week. **h**–**i** Appressoria of *C. gigasporum* formed on potato carrot agar slide culture at 25 °C in the dark for 3 d. Bars: 1=200 μm; 2–7=20 μm; 8–9=10 μm

ostiolate perithecium, dark brown, obpyriform to subglobose, 400–640 μm high, 240–320 μm wide, rarely solitary to mostly confluent. Asci unitunicate, eight-spored, broadly cylindrical to clavate, truncate at apex, thin-walled, short-stalked, evanescent, 64–120×8–12 μm. Paraphyses septate. Ascospores hyaline, fusoid to cylindrical, mostly straight to slightly curved, rounded at both ends, aseptate, 1-septate at maturity, septum median, (56)60–84(92)×5–7 μm.

Isolates examined. MADAGASCAR. MANGORO. Isolated from *Centella asiatica*, Mai 2003, E.F. Rakotoniriana, MUCL 44947 (EX HOLOTYPE); MADAGASCAR. MANGORO. Produced in laboratory culture from single conidia of MUCL 44947, June 2009, E.F. Rakotoniriana, MUCL 52571 (EX-PARATYPE); MEXICO. NAYARIT STATE. Isolated from *Stylosanthes guianensis*, Jan 1995, F. Munaut, MUCL 42034; MEXICO. NAYARIT STATE. Isolated from *S. guianensis*, Jan 1995, F. Munaut, MUCL 42044; MEXICO. NAYARIT STATE. Isolated from *Stylosanthes guianensis*, Jan 1995, F. Munaut, MUCL 42077; COLOMBIA. Isolated from *Coffea arabica*, July 2003, F.E. Vega, VEGA 508. A dried culture of each isolates was deposited at the herbarium of MUCL (as, MUCL 42034-H, MUCL 42044-H, MUCL 42077-H and VEGA 508-H), Louvain-la-Neuve, Belgium.

Notes: Isolates MUCL 42034, MUCL 42044, MUCL 42077 and VEGA 508 produce slightly shorter conidia (16)21–24(28)×(6)7–9 μm compared to those of the ex-holotype (MUCL 44947) but they are similar in shape, i.e., hyaline, aseptate, straight, cylindrical, guttulate and rounded at both ends. Teleomorph not produced.

Ascospore-derived colonies displayed the same cultural characters and conidial morphology as those from monoconidial isolates of the parental *Colletotrichum*.

Discussion

Conidial features (both size and shape) are important taxonomic criteria for morphological identification of *Colletotrichum* species. Sutton (1980) informally divided the genus into two groups based on the conidia shape: species with straight and species with falcate conidia. Recently, Crouch et al. (2009) and Damm et al. (2009) investigated the falcate-spored *Colletotrichum* species from graminicolous and herbaceous hosts. Here, we report the description of a new *Colletotrichum* species, *Colletotrichum gigasporum*, based on morphological characters and phylogenetic analysis.

Single conidia cultures of *C. gigasporum* MUCL 44947 produced fertile perithecia with asci bearing 0- to 1-medianly septate, long ascospores measuring (56)60–84 (92)×5–7 μm. Typical common ascospores are usually shorter, less than 20 μm long and aseptate, but they may become 1-septate when old (Hanlin 1990). The supplement of *Ce. asiatica* powder along with constant exposure to near UV and cool white fluorescent lights enhanced the production of the teleomorph by *C. gigasporum* MUCL 44947. Under these conditions, perithecia were consistently produced by this strain whereas it rarely formed the teleomorph on PDA alone. Colonies from single ascospores displayed the same cultural characters and conidial morphology as those from monoconidial isolates of the parental *Colletotrichum*, supposing that *C. gigasporum* may be homothallic. The sexual form of *C. gigasporum* produced ascospores that mostly resembled those of *Glomerella septospora* with respect to the ascospore size [60–95(100)×5.0–7.5 μm]. However, *G. septospora* ascospores were mostly 3-septate and may become up to 6- or 8-septate when mature (Sivanesan and Hsieh 1993). Given these atypical morphological characters the inclusion of *G. septospora* within the genus *Colletotrichum* was still regarded as doubtful (Hyde et al. 2009), even though evidence for its belonging to the genus *Colletotrichum* was earlier demonstrated using maximum parsimony analysis of the nuclear small-subunit ribosomal DNA (18S rDNA) with sequences issued from the type strain (accession number U78779, strain ATCC90830, IMI353024) (Wanderlei-Silva et al. 2003).

Although *C. gigasporum* isolates MUCL 42034, MUCL 42044, MUCL 42077 and VEGA 508 failed to produce teleomorph under the cultural conditions used in this study, their belonging to *C. gigasporum* was proved on the basis of ITS and β-tubulin phylogenetic analysis. Indeed, it was previously demonstrated that MUCL 42034, MUCL 42044 and MUCL 42077 were conspecific, since they displayed very similar RAPD profiles with the random primer OPA-15 (Gama-López et al. 2007). As the type strain of *C. gigasporum*, the isolate MUCL 44947, was morphologically similar to isolates MUCL 42044, MUCL 42034, MUCL 42077 and VEGA 508 and because the ITS and β-tubulin sequences were identical or near-identical (differed in one to two nucleotides position), isolates MUCL 42044, MUCL 42034, MUCL 42077, and VEGA 508 could consequently be referred to as *C. gigasporum*.

Morphologically, *C. gigasporum* differed from currently valid *Colletotrichum* species by its long conidia up to 32 μm (average length 26 μm). The search in the literature for previously identified taxa yielding similar conidial traits in shape and size revealed the existence of *C. macrosporum* (28–32×8–10 μm) (Saccardo 1899) collected from leaves of an orchid in Brazil, *C. echinatum* (22–26×8–9 μm) (Saccardo and Trotter 1913), and *C. vinosum* (20–30×6–8 μm) (Saccardo and Saccardo 1906). But these taxa were considered synonyms to *C. crassipes* by von Arx (1957). Compared to the species original description of *C. crassipes* by von Arx (1957), mainly based on the conidial size (22–31×6–8 μm) and the morphology of the appressoria with deeply lobed margins, some minor morphological differences could be noted with *C. gigasporum*. This latter produced conidia with rounded apices at both ends and the appressoria edges were entire to crenate. Whereas conidia of *C. crassipes* were obtuse at the apex but truncate at the base, and the appressoria edges were deeply lobed. Since no ex type culture was currently available for *C. crassipes* (Hyde et al. 2009) it would be difficult to distinguish closely related taxa to *C. crassipes* based only on morphological characteristics due to the wide range of the conidial size and lack of distinct morphological features. Indeed, further taxonomic and phylogenetic studies are needed to designate an epitype strain in *C. crassipes*, which will serve as the basis for an accurate identification in this species. Anyway, a high level of genetic dissimilarities was observed between *C. gigasporum* and isolates identified as *C. crassipes* obtained from the Centraalbureau voor Schimmelcultures (*CBS*), Netherlands. For instance, the ITS region of *C. gigasporum* MUCL 44947 differed in about 32 bp nucleotides to strain CBS 159.75. Furthermore, morphological comparison (Table 2) combined with the maximum parsimony analysis of the ITS sequences (Fig. 1) provided ample evidence that *C. gigasporum* can be distinguished from *C. macrosporum* and the so-called *C. crassipes* isolates included in this study.

Similar types of conidia as long as those produced by *C. gigasporum*, even longer, were also reportedly produced by an unusual *Colletotrichum* named *C. taiwanense*. However, conidia of *C. taiwanense* may become multiseptate (0–3 septate conidia 22–35(45)×5–8 μm, 4–5 septate conidia 50–58×5–8 μm) with age (Sivanesan and Hsieh 1993) while those of *C. gigasporum* remained aseptate. Interestingly, the teleomorph of *C. taiwanense* (*Glomerella septospora*), appeared to have identical ITS sequence as that of *C. gigasporum* and gathered together in a clade distinct from known *Colletotrichum* species (Fig. 1). Inappropriately, due to the lack of ITS and β-tubulin sequences from the type strain of *G. septospora* (W.H. Hsieh IMI353024, ATCC90830) isolated from stems of *Styrax formosanus* Matsum. in Taiwan, we used available sequences of *G. septospora* isolated from *Capsicum annuum* in Korea to perform the analysis. In any case, the β-tubulin phylogenetic analysis allowed their separation into two different clades with a bootstrap value of 83 %, supporting the hypothesis that they belong to two different species (Fig. 2).

The BLAST searches for ITS sequence similarity also showed that *C. incarnatum* (GQ369600) and *C. gloeosporioides* (AY266402) had the same ITS sequence with *C. gigasporum*. But conidia of *C. gigasporum* were much longer than

those of *C. incarnatum* (14–19×5 μm) (Zimmermann 1901) and those of *C. gloeosporioides* (AY266402) [(12)16.1(20)×(3)4.6(6)] (Photita et al. 2005). The BLAST searches also revealed the occurrence of numerous *Colletotrichum* sp. that had similar ITS sequence as *C. gigasporum*, comprising endophytic isolates from various hosts, such as *Magnolia liliifera* in Thailand (Promputtha et al. 2007); *Taxus chinensis* var. *mairei* in China; *Theobroma cacao*, *Trichilia tuberculata* and *Virola surinamensis* in Panama (Rojas et al. 2010) as well as a *Colletotrichum* sp. isolate LD35b (B2) associated with anthracnose disease of coffee in Vietnam (Nguyen et al. 2010). The identity of these *Colletotrichum* isolates is not very clear at the species level. They likely belong to a taxon distinct from known and accepted *Colletotrichum* species.

Endophytism is an important factor in microbial speciation and biodiversity (Stone et al. 2000). Liu et al. (2007) reported a new *Colletotrichum* endophyte from *Buxus* sp. assigned to *C. yunnanense* with bacilliform or slightly clavate conidia, rounded at both ends, hyaline, measuring (14)16–21×5–6 μm. However, this latter was shorter and narrower than *C. gigasporum*. Hitherto, *Colletotrichum* isolates previously described as endophytes belong mainly to *C. acutatum, C. gloeosporioides*, and *C. boninense* (Bussaban et al. 2001; Lu et al. 2004; Photita et al. 2005; Rojas et al. 2010). However, the conidial size of *C. gigasporum* were clearly longer and wider than that of *C. gloeosporioides* which measured 12–17(–23.5)×4.5–6 μm in water and (10.5–)11–15×4.5–5.5 μm in lactic acid/cotton blue (Cannon et al. 2008) and that of *C. acutatum* 7–14×2.5–3.5 μm (Than et al. 2008). In addition, in *C. gloeosporioides* the conidial shape was generally ovoid to oblong and that of *C. acutatum* was often fusiform, whereas *C. gigasporum* produced cylindrical conidia. All *C. gigasporum* isolates reported in this study were found to be endophytes from various plant sources located from geographically distant areas. To some extent, *C. gigasporum* appeared to have a widespread distribution mostly in tropical regions, being isolated in Madagascar, as foliar endophyte of *Ce. asiatica*, in Mexico, from healthy leaves of *S. guianensis* and in Colombia, as endophyte of *Co. arabica*. Further isolates are required to define its host range and to clarify its true lifestyle if *C. gigasporum* could occur as epiphyte or pathogen in other plants.

Cai et al. (2009) suggested performing multi-gene phylogenetic analysis for a better resolution of the relationships among *Colletotrichum* species and for an accurate and reliable species diagnosis. Consistent with this, using a multi-gene phylogeny of a combined datasets of the partial actin, β-tubulin (tub2), calmodulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase genes and the complete rDNA ITS1-5.8S-ITS2 regions, Prihastuti et al. (2009) was able to distinguish *C. gloeosporioides* from *C. kahawae* supported by high bootstrap support (100 %). In this study, we only carried out sequencing of ITS region and β-tubulin gene since, the taxonomic position of *C. gigasporum* was well resolved among the main groups of straight conidia-producing *Colletotrichum* species in the phylogenetic tree of the ITS sequences. The subsequent phylogenetic analysis based on the β-tubulin gene clearly differentiated *C. gigasporum* from other *Colletotrichum* species sharing identical ITS sequences. Notably, the β-tubulin gene sequences have also been successfully used to solve identification problems of closely related species of other fungal genera. Chung et al. (2006) reported that β-tubulin sequences separated *Ophiostoma breviusculum* from *O. piacea* and *O. canum*, while ITS sequence did not. Thus, based on the integration of the morphological and phylogenetic features, it is concluded that *C. gigasporum* is a distinct species in the genus *Colletotrichum*.

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