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Anthracnose Fungi with Curved Conidia, *Colletotrichum* spp. belonging to Ribosomal Groups 9-13, and Their Host Ranges in Japan

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Abstract

Ninety fungal strains with falcate conidia belonging to *Colletotrichum* spp. classified into the ribosomal groups 9-13 (the RG 9-13 spp.) and preserved at the NIAS Genebank, Japan were re-identified based on molecular phylogenetic analysis of the internal transcribed spacer (ITS) region of the rRNA gene, sequences of the glyceraldehyde 3-phosphate dehydrogenase, chitin synthase 1, histone3, and actin genes, and partial sequences of β-tubulin-2 (TUB2) genes, or by BLASTN searches with TUB2 gene sequences. Seventy strains were reclassified into nine recently revised species, *C. chlorophyti*, *C. circinans*, *C. dematium* sensu stricto, *C. lineola*, *C. liriopes*, *C. spaethianum*, *C. tofieldiae*, *C. trichellum* and *C. truncatum*, whereas 20 strains were grouped into four unidentified species. RG 9, 10 and 12 corresponded to the *C. spaethianum*, *C. dematium* and *C. truncatum* species complex, respectively, while RG 11 and 13 agreed with *C. chlorophyti* and *C. trichellum*, respectively. Phylograms derived from a six-locus analysis and from TUB2 single-locus analysis were very similar to one another with the exception of the association between *C. dematium* s. str. and *C. lineola*. Thus, TUB2 partial gene sequences are proposed as an effective genetic marker to differentiate species of RG 9-13 in Japan except for *C. dematium* s. str. and *C. lineola*. Thirty-two plant species were identified as new hosts for seven of the species found in this study except for *C. circinans* and *C. trichellum*; and two unidentified species. *Colletotrichum chlorophyti*, *C. lineola*, *C. liriopes*, *C. spaethianum*, and *C. truncatum* were regarded as polyphagous, whereas *C. trichellum* and *Colletotrichum* sp. (Ra), designated tentatively in this study, appeared to have specific pathogenicity to single hosts, *Hedera rhombea* and *Raphanus sativus* var. *hortensis*, respectively. Conidial curvature properties, "outer curvature," "inner curvature" and "height/width ratio" successfully represented conidial shape parameters. Conidial curvature properties of the species in RG 9, 10 and 12 were found to correlate with the species complexes.

Discipline: Agricultural Environment
Additional key words: β-tubulin-2, conidial curvature properties, molecular phylogenetic analyses, re-identification, species complex

Introduction

The genus *Vermicularia* Tode (1790) was established for anthracnose fungi with falcate conidia and setae in acervuli. More than 280 species have been described with nearly every host plant identified (Anonymous 2014). More species with falcate conidia were also described as species in *Colletotrichum* Corda (1831). Since Arx (1957) synonymized many *Vermicularia* as well as *Colletotrichum* species with *Colletotrichum graminicola* (Ces.) G.W. Wilson and *Colletotrichum dematium* (Pers.) Grove in addition to its three forma based on morphology, *C. graminicola* and *C. dematium* have been regarded as representatives of pathogens on the Poales grasses and other plants, respectively (Arx 1981, 1987, Sutton 1980, 1992). More than 230 strains of *Colletotrichum* in Japan, including those with falcate conidia and setae, were classified into 20 ribosomal groups (RG) based on molecular phylogenetic analyses of the

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internal transcribed spacer (ITS) region of the rRNA gene (Moriwaki *et al.* 2002). This analysis showed that six and three species were closely related to *C. graminicola* and *C. dematium*, respectively. The latter three species, *Colletotrichum circinans* (Berk.) Voglino, *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore, and *Colletotrichum trichellum* (Fr.) Duke, were placed on blanches of RG 10, 11 and 13 in the phylogenetic tree, respectively, while strains of *C. dematium* were classified into both RG 9 and 12. These species belonging to ribosomal groups 9-13 have been tentatively designated as "the RG 9-13 spp." in this study. After Moriwaki *et al.* (2002), strains pathogenic to the Poales grasses and that are related to *C. graminicola* were re-classified into 14 species based on a molecular phylogenetic analysis with the ITS, the DNA lyase gene (Apn2), the mating type Mat1-2 (Mat1/Apn2) gene and the manganese superoxide dismutase gene (Sod2) (Crouch *et al.* 2009). Conversely, strains pathogenic to other plants and related to *C. dematium* were examined based on a molecular phylogenetic analysis with six genes/regions as described below and re-classified into 20 species, including two previously undescribed species (Damm *et al.* 2009).

Approximately 40 and 90 strains putatively belonging to the *C. graminicola* species complex and the RG 9-13 spp., respectively, are preserved at the NIAS Genebank, National Institute of Agrobiological Sciences, Japan. Accessions in RG 9-13 spp. are more important economically in Japan because they contain numerous horticultural and food crop pathogens (The Phytopathological Society of Japan & National Institute of Agrobiological Science 2012). Thus, in order to appropriately control anthracnose caused by these species, it is important that we re-identify these fungi based on the latest methods of molecular phylogenetic analysis and clarify host ranges and morphological differences among species. We, therefore, re-identified a number of Japanese strains belonging to RG 9-13 according to the method described by Damm *et al.* (2009) to characterize the distribution of the species revised by Damm *et al.* (2009) and their host plants. The geometry of conidial morphology, especially the curvature of conidia, was also examined to quantify the morphological characteristics of the re-identified species.

Materials and Methods

1. Molecular phylogenetic analyses and re-identification of strains

Eighty-three strains belonging to RG 9-13 preserved at the NIAS Genebank (Table 1) were re-identified based on phylogenetic analyses using the ITS region of the rRNA gene, sequences of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), chitin synthase 1 (CHS-1), histone3 (HIS3) and actin (ACT) genes, and partial sequences of the β-tubulin-2 (TUB2) genes. Genomic DNA extracted according to the procedure described by Moriwaki *et al.* (2002) was used as a template for the following polymerase chain reaction (PCR) analyses. ITS, GAPDH, CHS-1, HIS3, ACT and TUB2 genes were amplified and sequenced using the primer pairs ITS5 & ITS4 (White *et al.* 1990), GDF1 & GDR1 (Guerber *et al.* 2003), CHS-354R & CHS-79F (Carbone & Kohn 1999), CYLH3F & CYLH3R (Crous *et al.* 2004), ACT-512F & ACT-783R (Carbone & Kohn 1999) and T1 (O'Donnell & Cigelnik 1997) & Bt2b (Glass & Donaldson 1995), respectively. Each gene region was amplified with *Taq* polymerase (TaKaRa, Otsu, Japan) in a GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA). Cycling conditions for amplification of ITS were 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec- 55°C for 1 min - 72°C for 1 min, and a final step at 72°C for 5 min. Conditions for the remaining five genes were 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec- 52°C for 30 sec- 72°C for 30 sec, and a final step at 72°C for 7 min. PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Chatsworth, CA, USA) and were sequenced directly with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing reactions were conducted according to the manufacturer's instructions. Extension products were analyzed on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All sequences were uploaded to the database, "Microorganism Search System", NIAS Genebank (http://www.gene.affrc.go.jp/databases-micro_search_en.php).

For phylogenetic analyses, sequence data of the ITS region and the GAPDH, CHS-1, HIS3, ACT and TUB2 gene sequences for the 83 strains examined in this paper, as well as 22 strains comprising an additional 18 species revised by Damm *et al.* (2009) downloaded from DDBJ/EMBL/GenBank databases, were included as references (Table 2). Sequences for strains of *Glomerella lindemuthiana* Shear (*Colletotrichum* lindemuthianum (Sacc. & Magnus) Briosi & Cavara) deposited in the database were also used as an outgroup. Multiple sequence alignments were carried out using the FFT-NS-i strategy of MAFFT version 6 (Katoh *et al.* 2002). The alignments of all sequences were further optimized manually, and gaps were deleted. A phylogenetic tree was constructed from sequences of the six genes combined by maximum likelihood (ML) methods using shotgun searches with RAxML version 8 (Stamakakis 2014). The search was repeated until the maximum likelihood was identified. Base composition homogeneity tests were conducted using Kakusan4 (Tanabe 2011). The model: GTR + Gamma was used in the tree searches. The reliability of the inferred tree was estimated by bootstrap analysis (Felsenstein 1985) repeated 100 times. Another ML tree was constructed with TUB2 gene partial sequences

only to estimate the efficacy of using a single gene for the molecular identification of species belonging to RG 9-13.

Partial sequences of the TUB2 gene from the seven strains listed in Table 1 were used in "Standard Nucleotide BLAST" searches on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&BLAST _PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=) to identify species of RG 9-13 based on identity with TUB2 sequences deposited by Damm *et al.* (2009).

2. Host plants of the re-identified species

Sources for the strains registered by depositors were determined by search with the "Microorganism Search System" on the NIAS Genebank website (http://www.gene.affrc.go.jp/databases-micro_search_en.php). Plants reported to be susceptible to the fungal strains were determined by search of the references listed on the "Detailed information of microorganism genetic resources" website [http://www.gene.affrc.go.jp/databases-micro_search_detail_en.php?maff=239500]. Plants from which deposited fungal strains were isolated as well as susceptible plants were recognized as host plants of the re-identified species to which the strains belong.

3. Morphological observations

Potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) plates were used to produce conidia.

Table 1. Re-identification of *Colletotrichum* strains at the NIAS Genebank (MAFF[a]) belonging to RG 9-13 spp.

Re-identified species	Isolation sources (Host plant)[b]	MAFF accession[c]	Reference
C. chlorophyti	***Ipomoea batatas***	**238700**	Moriwaki *et al.* 2002
	Prunus* × *yedoensis	**240236**	
	Vigna radiata	305748	Sato *et al.* 2014
C. circinans	*Allium cepa*	237304	Sato *et al.* 2012
C. dematium s. str.	***Armeria maritima***	712331	
C. lineola	***Dianthus* sp.**	237705	
	Helleborus niger	712313, 712314	
	Isotoma axillaris	712332	
	Sanguisorba officinalis	243332	
	Saponaria officinalis	240431	Sato *et al.* 2012
	Taraxacum officinale	238064	
	Vigna angularis	306708	
C. liriopes	***Erigeron philadelphicus***	238029	
	Gnaphalium affine	238063	
	Helleborus niger	**239544**	
	Rohdea japonica	238703, 240189, 242679	Sato *et al.* 2012
	Rumex acetosa	238060	
C. spaethianum	***Allium fistulosum***	237488, 242675	Moriwaki *et al.* 2002
	Crinum latifolium	238702	Sato *et al.* 2012
	Dianthus chinensis	238023	
	Hosta montana	238062, 238067	
	Iris* × *germanica	238024, 238025, 238026	Sato *et al.* 2012
	Kniphofia northiae	238061	
	Polygonatum falcatum	239500, 242741	Tomioka *et al.* 2008
C. tofieldiae	***Ornithogalum umbellatum***	712333, 712334	Sato *et al.* 2012
C. trichellum	*Hedera rhombea*	237918, 237991, 237992, **237993**, 238020, **238022**, 238027, 410046	Moriwaki *et al.* 2002 Sato *et al.* 2012

[a] Acronym for microbe strains at the NIAS Genebank, National Institute of Agrobiological Sciences.
[b] Bold names are host plants of diseases caused by the RG 9-13 spp. in Japan.
[c] Profile and DNA sequences of each strain appears on the website, http://www.gene.affrc.go.jp/databases-micro_search_en.php
Strains shown in bold font were re-identified based on BLASTN with β-tubulin-2 gene sequences and strains shown in normal font were based on a phylogenetic analysis with six genes/region.

Table 1. (continued) Re-identification of *Colletotrichum* strains at the NIAS Genebank (MAFF[a]) belonging to RG 9-13 spp.

Re-identified species	Isolation sources (Host plant)[b]	MAFF accession[c]	Reference
C. truncatum	***Brassica rapa* var. *chinensis***	238716, 305969	Moriwaki *et al.* 2002
	Brassica sp.	240540	
	Capsicum annuum var. *annuum*	242674, 243068	
	Carica papaya	241269	
	Cucumis sativus	**242676**	
	Dendranthema grandiflorum	238500	
	Dieffenbachia sp.	238717	Moriwaki *et al.* 2002
	Euphorbia pulcherrima	239896, 239897	Sato *et al.* 2008
	Fagopyrum esculentum	306552	Moriwaki *et al.* 2002
	Glycine max	239536, 305754	Moriwaki *et al.* 2002
	Hippeastrum* × *hybridum	238718	Moriwaki *et al.* 2002
	Houttuynia cordata	238066	
	Hylocereus undatus	240532	
	Passiflora edulis	237989, 305982	Moriwaki *et al.* 2002
	Plumeria rubra	240492, 240494	
	Salsola komarovii	726762, 726763	Kubota *et al.* 2011
	Sansevieria sp.	238104	
	Solanum melongena	240523, 240525	
	***Syngonium* sp.**	240453	Sato *et al.* 2012
	Vigna subterranea	306411	Moriwaki *et al.* 2002
Colletotrichum sp. (F)[d]	*Fagus crenata*	410758, 410759	Sasaki 1977
Colletotrichum sp. (PS)[d]	***Prunus* × *yedoensis***	240235, **240236**	
	Sanguisorba officinalis	243333, 243334, 243337	Sugawara *et al.* 2012
Colletotrichum sp. (Ra)[e]	***Raphanus sativus***	238704-238715	Sato *et al.* 2005
Colletotrichum sp. (S)	*Shibataea kumasaca*	239098	

[a] Acronym for microbe strains at the NIAS Genebank, National Institute of Agrobiological Sciences.
[b] Bold names are host plants of diseases caused by the RG 9-13 spp. in Japan.
[c] Profile and DNA sequences of each strain appears on the the website, http://www.gene.affrc.go.jp/databases-micro_search_en.php Strains shown in bold font were re-identified based on BLASTN with β-tubulin-2 gene sequences and strains shown in normal font were based on a phylogenetic analysis with six genes/region.
[d] Belonging to the *C. dematium* species complex.
[e] Belonging to the *C. spaethianum* species complex.

Mycelial discs (6 mm diameter) of 15 representative strains of each *Colletotrichum* species listed in Table 3 were cultured on the agar plates (55 mm in diam.) at 25°C under black light for 7 to 14 days. The length (l), width (w), height (h), horizontal distance from the apical tip to the peak of the convex (a) and the horizontal distance from the basal tip to the peak of the convex (b) for 20 conidia of each strain (Fig. 1) were measured using an image analyzer (Nikon Digital Sight; Nikon, Tokyo, Japan) attached to a microscope with differential interference contrast (DIC) illumination (Nikon Eclipse 80i; Nikon, Tokyo, Japan). "Outer curvature," "inner curvature," "curvature deviation" and the "height/width ratio" of the conidia were calculated with the formulae, h/l × 100, (h–w)/l × 100, b/a and h/w, respectively, to quantify the curvature properties. The conidia of representative strains were photographed with a digital camera attached to the microscope with DIC illumination or phase contrast optics.

Results

1. Molecular phylogenetic analyses and re-identification of strains

A ML tree based on five gene sequences and a DNA region with a log likelihood of -12956. 452967 was obtained from the phylogenetic analysis of sequences from 83 strains uploaded to the NIAS Genebank website and downloaded from the DDBJ/EMBL/GenBank databases

Table 2. DNA sequence data[a] used in this study

Species	Strain[d]	DNA sequence accession number at the DDBJ/EMBL/GenBank					
		ITS[e]	GAPDH[f]	CHS-1[g]	HIS3[h]	ACT[i]	TUB2[j]
C. anthrisci	**CBS 125334**	GU227845	GU228237	GU228335	GU228041	GU227943	GU228139
C. chlorophyti	**IMI 103806**	GU227894	GU228286	GU228384	GU228090	GU227992	GU228188
C. circinans	**CBS 221.81**	GU227855	GU228247	GU228345	GU228051	GU227953	GU228149
C. curcumae	**IMI 288937**	GU227893	GU228285	GU228383	GU228089	GU227991	GU228187
C. dematium s. str.	**CBS 125.25**	GU227819	GU228211	GU228309	GU228015	GU227917	GU228113
C. dematium s. str.	CBS 115524	GU227826	GU228218	GU228316	GU228022	GU227924	GU228120
C. fructi	CBS 346.37	GU227844	GU228236	GU228334	GU228040	GU227942	GU228138
C. lilii	CBS 109214	GU227810	GU228202	GU228300	GU228006	GU227908	GU228104
C. lineola	**CBS 125337**	GU227829	GU228221	GU228319	GU228025	GU227927	GU228123
C. lineola	CBS 124959	GU227842	GU228234	GU228332	GU228038	GU227940	GU228136
C. liriopes	**CBS 119444**	GU227804	GU228196	GU228294	GU228000	GU227902	GU228098
C. phaseolorum 1[b]	CBS 157.36	GU227896	GU228288	GU228386	GU228092	GU227994	GU228190
C. phaseolorum 2[b]	CBS 158.36	GU227897	GU228289	GU228387	GU228093	GU227995	GU228191
C. rusci	**CBS 119206**	GU227818	GU228210	GU228308	GU228014	GU227916	GU228112
C. spaethianum	CBS 167.49	GU227807	GU228199	GU228297	GU228003	GU227905	GU228101
C. spinaciae	CBS 128.57	GU227847	GU228239	GU228337	GU228043	GU227945	GU228141
C. tofieldiae	CBS 495.85	GU227801	GU228193	GU228291	GU227997	GU227899	GU228095
C. tofieldiae	IMI 288810	GU227803	GU228195	GU228293	GU227999	GU227901	GU228097
C. trichellum	CBS 217.64	GU227812	GU228204	GU228302	GU228008	GU227910	GU228106
C. truncatum	**CBS 151.35**	GU227862	GU228254	GU228352	GU228058	GU227960	GU228156
C. truncatum	CBS 345.70	GU227867	GU228259	GU228357	GU228063	GU227965	GU228161
C. verruculosum	**IMI 45525**	GU227806	GU228198	GU228296	GU228002	GU227904	GU228100
G. lindemuthiana	CBS 151.28	GU227800	GU228192	GU228290	GU227996	GU227898	GU228094

[a] Cited from Damm *et al.* (2009)
[b] Tentatively designated by Damm *et al.* (2009)
[c] *C.* = *Colletotrichum*
[d] Bold strains are type or ex-type
[e] Ribosomal DNA internal transcribed spacer
[f] Glyceraldehyde-3-phosphate dehydrogenase
[g] Chitin synthase 1
[h] Histone3
[i] Actin
[j] β-Tubulin-2

(Fig. 2). Sixty-four strains constituted clades with ex-type or reference strains of nine species revised by Damm *et al.* (2009): *C. chlorophyti* S. Chandra & Tandon, *C. circinans* (Berk.) Voglino, *C. dematium* (Pers.) Grove sensu stricto, *C. lineola* Corda, *C. liriopes* Damm, P.F. Cannon & Crous, *C. spaethianum* (Allesch.) Damm, P.F. Cannon & Crous, *C. tofieldiae* (Pat.) Damm, P.F. Cannon & Crous, *C. trichellum* (Fr.) Duke and *C. truncatum* (Schwein.) Andrus & W.D. Moore, whereas 18 strains formed three additional clades without the ex-type or reference strains and the remaining single strain was placed on an isolated branch. The unidentified clades and the branch were tentatively designated as *Colletotrichum* sp. (F), (PS), (Ra) and (S) in this study (Table 1, Figs. 2, 3, 4).

Another ML tree based on phylogenetic analyses using TUB2 gene sequences with a log likelihood of -3342.819522 was obtained with the same strains used in the six gene/region sequence phylogenetic analysis (Fig. 3). The topology of both trees derived from TUB2 single-locus and the six-locus analyses were nearly the same except for the inseparability of *C. dematium* s. str. and *C. lineola*.

Six strains not examined in the phylogenetic analyses were re-identified as *C. chlorophyti*, *C. liriopes*, *C. trichellum* and *C. truncatum* based on a BLASTN search (Table 1).

Table 3. Conidial Morphology of *Colletotrichum* strains re-identified as revised species in RG 9-13 based on molecular phylogenetic analyses

Colletotrichum spp.	MAFF[b] accession	Outer curvature[c]	Inner curvature[d]	Curvature deviation[e]	Height/ width ratio[f]	Length/ width ratio[g]	Conidial size (μm)		
							Average length[h]	Average width[i]	Range of length × width
C. chlorophyti	305748	30.9	12.2	1.37	1.67	5.44	23.4	4.3	(17-) 20.6-28.5 × 3.6-5.1
C. circinans	237304	18.4	3.1	1.06	1.23	6.79	22.4	2.9	20.2-25.1 × (1.9-) 2.6-3.4
C. dematium s. str.	712331	21.7	4.9	1.60	1.30	6.00	21.9	3.7	20.2-24.9 × 3.3-4.1
C. lineola	306708	19.9	2.9	2.12	1.18	5.99	18.4	3.1	16.6-20.0 × 2.5-3.8
C. liriopes	242679	31.6	5.1	1.49	1.21	3.87	17.8	4.7	14.7-20.8 × (3.4-) 4.2-5.6
C. spaethianum	238026	25.4	3.6	1.18	1.18	4.65	13.8	3.0	12.7-16.3 × (2.2-) 2.7-3.7
C. tofieldiae	712333	30.4	3.8	1.16	1.17	3.87	13.8	3.6	11.5-15.8 (19.8) × 3.1-4.3 (-4.8)
C. trichellum	238027	19.0	2.0	1.12	1.14	6.04	23.8	4.0	21.7-25.6 (-28) × 3.8-4.9 (-5.4)
C. truncatum	305969	29.9	10.8	1.35	1.58	5.32	19.4	3.7	(16.7-) 17.9-22.4 × 2.9-4.6
	240453	26.3	14.2	1.21	2.19	8.41	20.4	2.5	(15.9-) 18-23.2 × (1.8-) 2.1-2.8
	238500	27.2	14.3	1.72	2.15	7.92	22.4	2.9	21.2-25.1 × (1.9-) 2.6-3.6
Colletotrichum sp. (Ra)[a]	238705	34.8	14.5	1.17	1.73	4.99	19.5	3.9	16.6-21.6 × 3.3-4.5
	238713	32.4	10.9	0.81	1.51	4.71	19.9	4.3	17.6-22.5 × 3.6-5
	238706	29.6	10.4	1.22	1.56	5.31	21.5	4.1	18.9-24.6 × 3.4-5
	238710	26.1	11.1	1.34	1.75	6.75	22.9	3.4	20.5-26.7 × 2.8-4.3

[a] the anthracnose pathogen of *Raphanus sativus* var. *hortensis* (Sato *et al.* 2005)
[b] acronym for microbe strains at the NIAS Genebank, National Institute of Agrobiological Sciences
[c] height/length × 100 ([c]–[g] see Fig. 1)
[d] (height–width)/length×100
[e] horizontal distance from the basal tip to the peak of the convex/horizontal distance from the apical tip to the peak of the convex
[f] height/width
[g] length/width
[c]–[f] colors indicate larger values than colorless ones

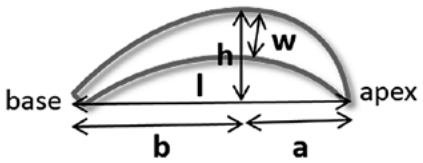

Fig. 1. Factors for calculating conidial curvature; l: length, w: width, h: height, a: horizontal distance from the apical tip to the peak of the convex, b: horizontal distance from the basal tip to the peak of the convex.

Strain MAFF 240235 had the same TUB2 sequence as that of MAFF 240236, *Colletotrichum* sp. (PS).

Greater than 30% of the 90 strains examined were re-identified as *C. truncatum*. Approximately 13, 9 and 7% were *C. spaethianum*, and *C. lineola* and *C. liriopes*, respectively. One or two strains corresponded to *C. circinans*, *C. dematium* s. str. or *C. tofieldiae*, whereas 12 strains pathogenic to *Raphanus sativus* var. *hortensis* formerly identified as *C. dematium* sensu lato (Sato *et al.* 2005) were reclassified as an unidentified species of the *C. spaethianum* complex.

2. Host plants of the re-identified species

Isolation sources of the re-identified strains and/or plants susceptible to the strains are listed in Table 1. References describing pathogenicity of the strains and new hosts found in this study are indicated in bold letters (Table 1). Thirty-two plant species were identified as new hosts for the seven re-identified species and two unidentified species of the RG 9-13. Of the species containing more than one strain, *C. chlorophyti*, *C. lineola*, *C. liriopes*, *C. spaethianum*, and *C. truncatum* had several host plants, whereas strains of *C. tofieldiae*, *C. trichellum* and *Colletotrichum* sp. (F, Ra) were isolated from single hosts.

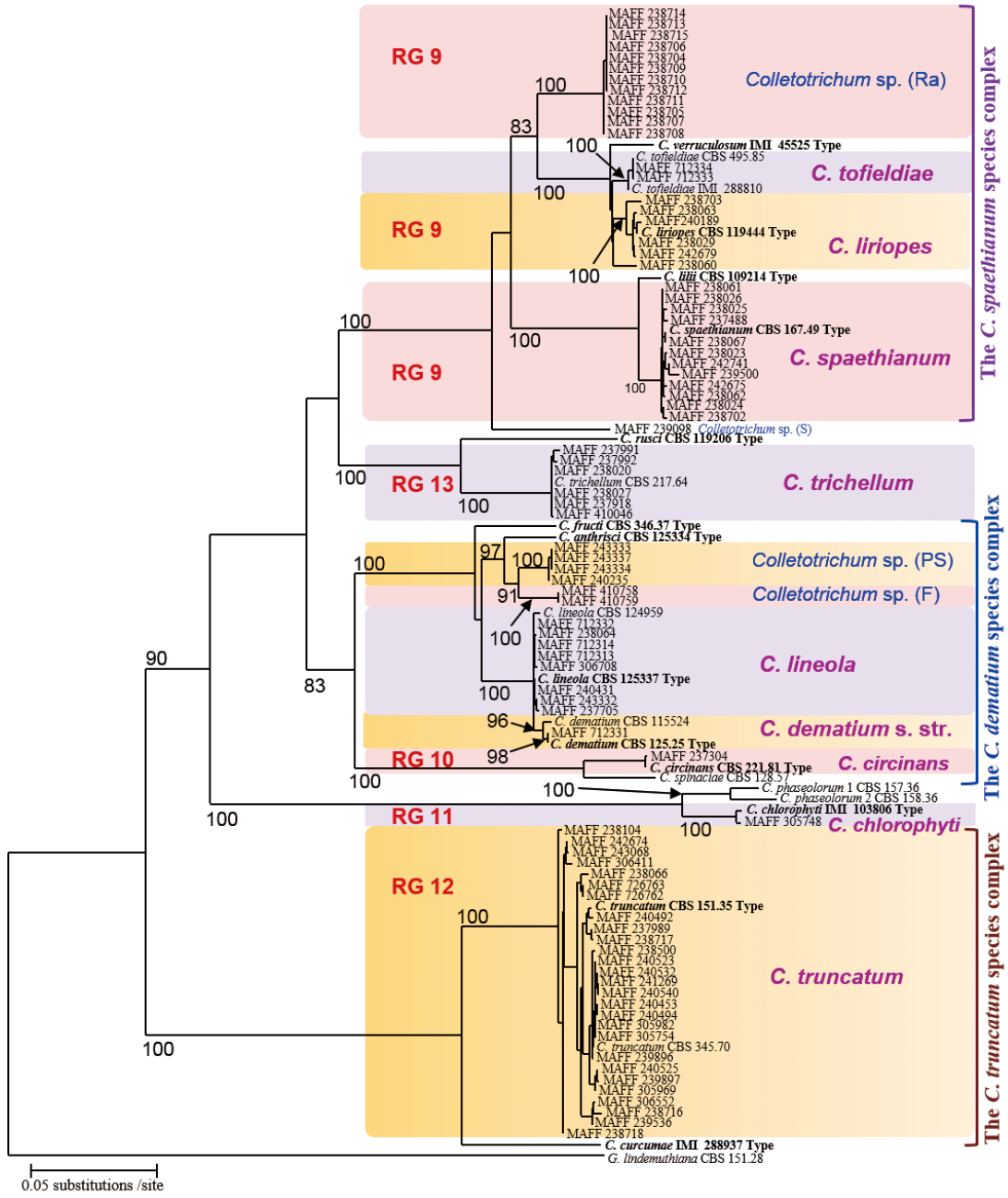

Fig. 2. Maximum likelihood tree of the rDNA-ITS region, GAPDH, CHS-1, HIS3, ACT, and TUB2 partial gene sequences of 83 *Colletotrichum* strains belonging to RG 9-13 spp. preserved at the NIAS Genebank (MAFF) and 23 strains comprising 19 species downloaded from the DDBJ/EMBL/GenBank databases. Numbers on the branches represent the percentage of congruent clusters in bootstrap trials repeated 100 times when the values were greater than 80%. RG means "ribosomal group" proposed by Moriwaki *et al.* (2002) and the species complexes defined by Cannon *et al.* (2012)

3. Conidial Morphology

The conidial morphology of 15 representative strains re-identified as the nine species and *Colletotrichum* sp. (Ra) from Japanese radish are shown in Table 3 and Fig. 4. The conidial curvature properties, "outer curvature," "inner curvature," "curvature deviation" or "height/width ratio" in addition to sizes and length/width ratio were significantly different among the strains examined (Table 3), although values for some strains were similar to each other in terms of individual properties. The conidial curvature properties were rather consistent although some variation was found among strains of *C. truncatum* or *Colletotrichum* sp. (Ra).

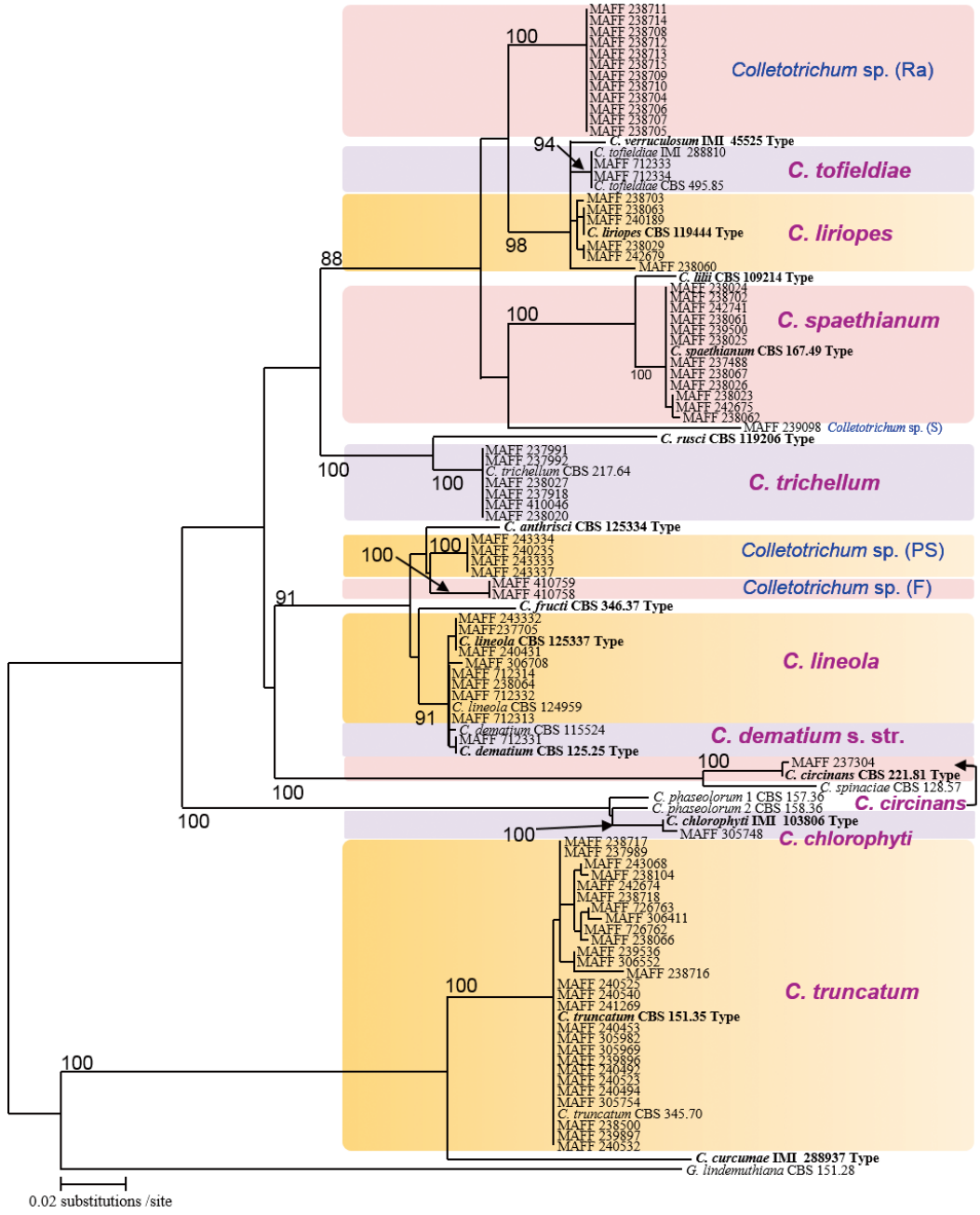

Fig. 3. Maximum likelihood tree of β-tubulin-2 partial gene sequences of *Colletotrichum* strains belonging to RG 9-13 spp. preserved at the NIAS Genebank (MAFF) and 23 strains comprising 19 species downloaded from the DDBJ/EMBL/GenBank databases. Numbers on the branches represent the percentage of congruent clusters bootstrap trials repeated 100 times when the values were greater than 80%.

Discussion

The nine species originally defined by Damm *et al.* (2009) and the four unidentified species belonging to RG 9-13 were identified in this study. The largest group, *C. truncatum*, contains 28 strains of various origins, though only five strains had been named *C. truncatum* prior to this study. One of the factors for strain inflation is that *Colletotrichum capsici* (Syd. & P. Syd.) E.J. Butler & Bisby, a common and global species with falcate conidia, was previously synonymized with *C. truncatum* (Damm *et al.* 2009). Sixteen of the 28 strains of *C. truncatum* were identified originally as *C. capsici* (http://www.gene.affrc.go.jp/databases-micro_search_en.php). *Colletotrichum spaethianum*, a species new to Japan, had twelve strains,

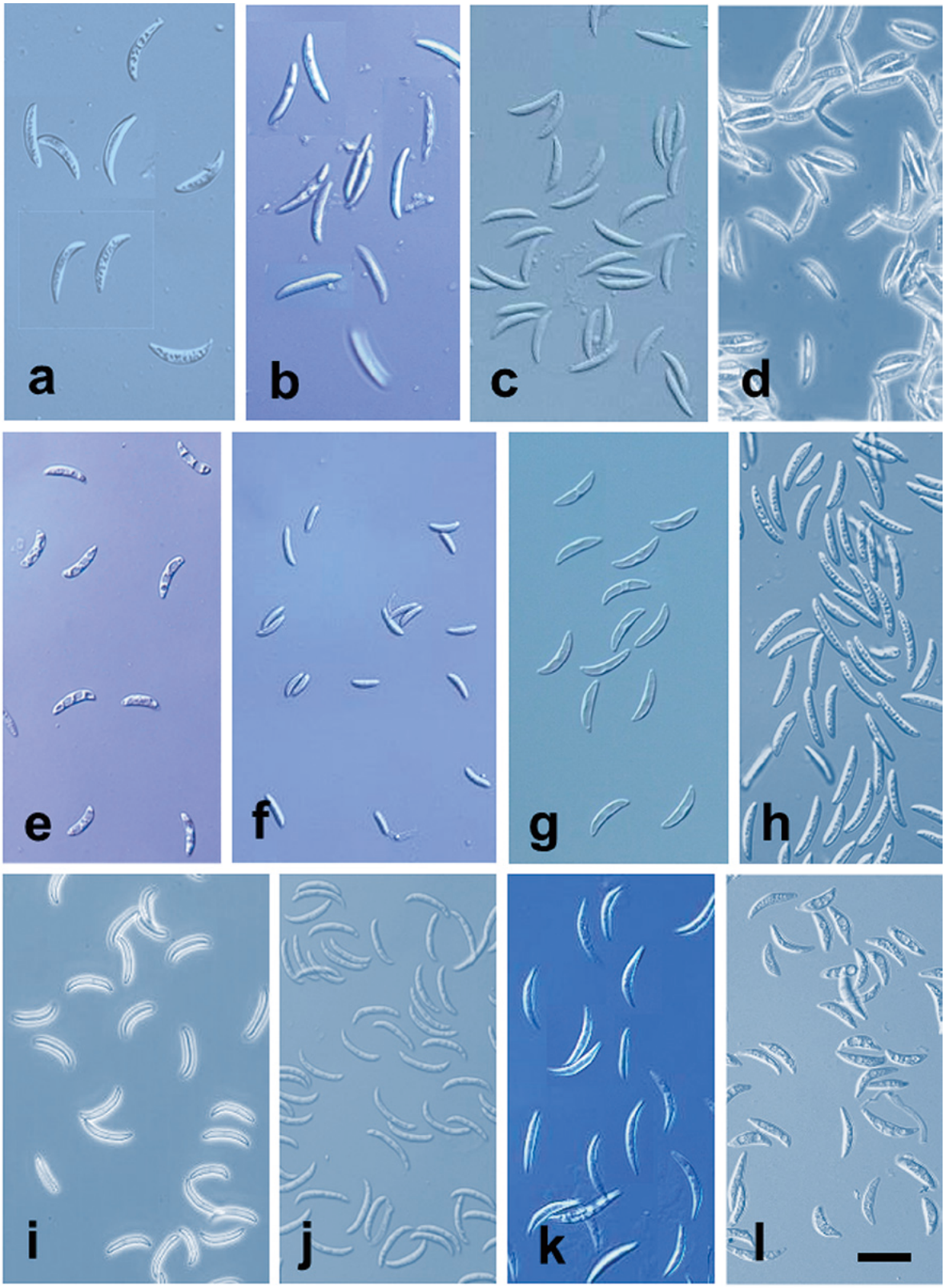

Fig. 4. Conidia of strains re-identified as revised *Colletotrichum* species in RG 9-13 based on the molecular phylogenetic analyses. a: MAFF 305748 (*C. chlorophyti*), b: MAFF 237304 (*C. circinans*), c: MAFF 712331 (*C. dematium* sensu stricto), d: MAFF 306708 (*C. lineola*), e: MAFF 242679 (*C. liriopes*), f: MAFF 238026 (*C. spaethianum*), g: MAFF 712333 (*C. tofieldiae*), h: MAFF 238027 (*C. trichellum*), i: MAFF 240453 (*C. truncatum*), j: MAFF 238500 (*C. truncatum*), k: MAFF 238710 and l: MAFF 238706 (*Colletotrichum* sp. from *Raphanus sativus* var. *hortensis*), (d, i: photographed with phase contrast optics, others: with differential interference contrast (DIC) illumination, bar: 20μm)

of which nine were re-identified from *C. dematium* s. lato, whereas only one strain from *Armeria maritima* labeled as *Colletotrichum* sp. when deposited was identified as *C. dematium* s. str. (see above website). Conversely, four strains formerly named *C. truncatum* were re-identified as *C. lineola* in our study. Strict attention should be paid to the changes in definition of the scientific names accompanied with the splitting of RG 9-13 spp.

Colletotrichum phaseolorum S. Takim. was first described as the anthracnose pathogen of adzuki bean (*Vigna angularis*) in Japan. The species was once regarded erroneously as synonymous with *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., a typical species with cylindrical conidia (Arx 1957). Furthermore, Arx misspelled the name as "*C. phascorum*" Takimoto and cited an incorrect reference as "Ann. Phytopath. Soc. Japan 5, 21 (1934)". Two reference strains of *C. phaseolorum*, identified tentatively as "*C. phaseolorum* 1" and "2," were placed on branches adjacent to the clade of *C. chlorophyti*, as in the previous phylogenetic tree (Damm *et al.* 2009). Contrary to our expectations, MAFF 306708, a strain isolated from *V. angularis* belongs to the *C. lineola* clade (Fig. 2, 3). The authentic strains of CBS 157.36 (*C. phaseolorum* 1) and CBS 158.36 (*C. phaseolorum* 2) were isolated from *Phaseolus radiatus* var. *aureus* (adzuki bean: *V. angularis* at present) and *Vigna sinensis* (black-eyed pea: *Vigna unguiculata* at present), respectively, according to the CBS web catalogue. The fungus attacks *V. unguiculata* as well as *V. angularis*, as shown by reciprocal inoculations, and is also slightly pathogenic to *Phaseolus vulgaris* (common bean) (Takimoto 1934). The morphology and pathogenicity of MAFF 306708 should be carefully re-examined in comparison with *C. phaseolorum*.

In this study, RG 9-13 (Moriwaki *et al.* 2002) were connected with the latest phylogenetic taxonomy proposed by Cannon *et al.* (2012). They defined the *C. dematium*, *C. spaethianum* and *C. truncatum* species complexes for each clades consisting of closely related species reported by Damm *et al.* (2009). RG 9, 10 and 12 corresponded to the *C. spaethianum*, *C. dematium* and *C. truncatum* species complex, respectively, while RG 11 and 13 agreed with *C. chlorophyti* and *C. trichellum*, respectively, based on common strains used both in this study and by Moriwaki *et al.* (2002) (Fig. 2). Consequently, *Colletotrichum* sp. (Ra) belonging to RG 9 classified into the *C. spaethianum* species complex. *Colletotrichum* sp. (PS) and *Colletotrichum* sp. (F) were recognized as members of the *C. dematium* species complex because of their close relationships with the complex in the phylogram, while *Colletotrichum* sp. (S) seemed distinct from any of the three species complexes (Fig. 2).

The partial gene sequences for β-tubulin-2 (TUB2) were effective genetic markers for differentiation of the re-identified species of RG 9-13 except for *C. dematium* s. str. and *C. lineola* because phylogenetic analysis with TUB2 only clearly classified 7 species as in the 6-locus analysis. *Colletotrichum dematium* s. str. and *C. lineola*, can be distinguished not only by conidial morphology (Table 3, Fig. 4) but also by their actin, HIS3 or GAPDH sequences, as has been pointed out (Damm *et al.* 2009).

Thirty-two of 51 plants were new hosts for the seven re-identified species and two unidentified spp. in RG 9-13 found in this study. *Colletotrichum chlorophyti*, *C. lineola*, *C. liriopes*, *C. spaethianum*, and *C. truncatum* were regarded as polyphagous because of their wide host ranges. Caryophyllaceous and iridaceous plants in addition to liliaceous plants were found to be hosts of *C. spaethianum* in this study, though Damm *et al.* (2009) noted only a few liliaceous plants infected by the species. More than 30% of the 90 strains examined were re-identified as *C. truncatum*, a species known as an anthracnose pathogen predominately of leguminous and solanaceous plants (Damm *et al.* 2009). In contrast, *C. trichellum* and *Colletotrichum* sp. (Ra) appeared to be pathogenic to single hosts, *Hedera rhombea* and *Raphanus sativus* var. *hortensis*, respectively, because many strains of the former with diverse origins were isolated from a single plant species. The latter were obtained from two areas and found to demonstrate strong pathogenicity to Japanese radish (Sato *et al.* 2005). Two strains of *Colletotrichum* sp. (F) isolated from *Fagus crenata* seemed to be host specific to the tree, although this fungus was reported to be virulent to *F. crenata* seedlings only when the environment was not suitable for growth (Sasaki 1977). One strain each of *Colletotrichum circinans* and *C. dematium* s. str. was found in this study. Furthermore, *C. tofieldiae* that had two strains of the same origin do not have a narrow host range since the strains were reported or isolated from other plants (Damm *et al.* 2009). *Colletotrichum* sp. (PS) consisting of strains from *Prunus* × *yedoensis* and *Sanguisorba officinalis* is probably compatible with rosaceous plants since the latter plant became diseased after inoculation with the strains (Sugawara *et al.* 2012). *Colletotrichum* sp. (S), placed on an isolated branch, was isolated from *Shibataea kumasaca*, an endemic small bamboo of Japan (Ohwi & Kitagawa 1983). The unidentified fungal species might be specialized in association with the host species.

The conidial curvature properties, especially "outer curvature," "inner curvature" and "height/width ratio" successfully represented the nature of the conidial shape. These curvature properties were stable within species as shown in the cases of *C. truncatum* and *Colletotrichum* sp. (Ra) as well as often different in combination of the parameters among the species (Table 3, Fig. 4). The re-identified species of RG 9-13 in Japan were classified into three groups with characteristics of the conidial curvature properties as follows:

1. All large outer curvature (> 25), inner curvature (> 10)

and height/width ratio (> 1.5): *C. chlorophyti*, *C. truncatum*, *Colletotrichum* sp. (Ra)

2. Large outer curvature (> 25), small inner curvature (< 6) and small height/width ratio (< 1.4): *C. liriopes*, *C. spaethianum*, *C. tofieldiae*
3. All small outer curvature (< 23) and inner curvature (< 6) and height/width ratio (< 1.4): *C. circinans*, *C. dematium*, *C. lineola*, *C. trichellum*

Groups 1, 2 and 3 defined above are obviously correlated with the *C. truncatum*, *C. spaethianum* and *C. dematium* species complex, respectively, because the re-identified species are common with each other except for *C. chlorophyti* and *C. trichellum* belonging to none of the species complexes (Fig. 2).

The conidial curvature properties should be examined in more strains than the 15 representative and in the revised species not found in this study (Damm *et al.* 2009). The quantified phenotypes newly characterized by this study probably more clearly describe the morphological characteristics of the revised species than any other parameter examined to date. It is also worth confirming phylogenetic implications of the curvature properties.

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