Microbiol. Cult. Coll. **29**(1): 13-23, 2013

Molecular re-identification of strains in NIAS Genebank belonging to phylogenetic groups A2 and A4 of the *Colletotrichum acutatum* species complex

Toyozo Sato[1)]* and Jouji Moriwaki[2)]

[1)] Genetic Resources Center, National Institute of Agrobiological Sciences (NIAS)
2-1-2, Kannondai, Tsukuba, Ibaraki 305-8602, Japan
[2)] Horticultural Research Institute Toyama Prefectural Agricultural, Forestry & Fisheries Research Center
288, Goromaru, Tonami, Toyama 939-1327, Japan

INTRODUCTION

An anthracnose pathogen, *Colletotrichum acutatum* J.H. Simmonds is known as a plurivorous and cosmopolitan fungus. Since it was first described to have fusiform conidia and reddish colonies in Australia (Simmonds, 1965), its distribution, host plants, macroscopic and microscopic morphology have been reported to vary in the extreme (Aa, *et al*., 1990; Sato, 1997; Sato *et al*., 1998; Vinnere *et al.*, 2002). A 'larger spored form', which Simmonds (1965) described in the first report, appeared to be one of the morphologically distinct groups of *C. acutatum*. Because some strains of the fungus that are pathogenic to anemone were morphologically similar to *Colletotrichum gloeosporioides* (Penzig) Penzig & Saccardo, they were misidentified as the latter in Japan (Sato *et al*., 1996). Recently, several researchers recognized genetic groups A1 to A8 within the species based on sequences of the ribosomal DNA internal transcribed spacer region (rDNA-ITS) (Johnston & Jones, 1997; Lardner *et al.*, 1999; Vinnere *et al.*, 2002; Sreenivasaprasad & Talhinhas, 2005). Group A9 was added based on randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and β-tubulin-2 (TUB2) gene partial sequences (Whitelaw-Weckert *et al*., 2007). Some of the researchers proposed dividing the groups into distinct species (Guerber *et al.*, 2003; Sreenivasaprasad & Talhinhas, 2005; Vinnere *et al*., 2002), indicating that *C. acutatum* sensu lato is a typical species complex.

Colletotrichum fioriniae (Marcelino & Gouli) R.G. Shivas & Y.P. Tan (=group A3) and *Colletotrichum simmondsii* R.G. Shivas & Y.P. Tan (=group A2, A9) were separated from the *C. acutatum* species complex and also defined *C. acutatum sensu stricto* (=group A5) based on their morphological characteristics in culture and molecular phylogenetic analyses of the ITS region and the β-tubulin-2 gene sequences (Shivas & Tan, 2009). *Colletotrichum carthami* (Fukui) S. Uematsu, Kageyama, Moriwaki & Toy. Sato was revived as a member of the species complex. This species had distinct β-tubulin-2 gene sequences and specific pathogenicity for asteraceous plants (Uematsu *et al*., 2012). Although each of the three species except for *C. carthami* was reported to have characteristic colony color (Shivas & Tan, 2009; Uematsu *et al.*, 2012), differences in microscopic morphology of the four species were still unclear.

Quite recently, the species complex was split into 29 species based on phylogenetic studies with the 5.8S nuclear ribosomal gene including the two internal transcribed spacers (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase 1 (CHS-1), histone 3 (HIS3), actin (ACT) and TUB2 gene sequences of 331 strains (Damm *et al*., 2012). Twelve of the 29 species were separated from group A2 and *Colletotrichum godetiae* Neergaard was assigned to the monophyletic group A4. All of the member species including 21 new ones that have been studied in culture were characterized morphologically (Damm *et al.*, 2012). On the other hand, 170 strains deposited as *C. acutatum* in the NIAS Genebank, Japan were re-identified as *C. fioriniae*, *C. simmondsii*, *C. carthami*, *C. acutatum* group A2-P, group A4 and other species of the *C. acutatum* species complex based on phylogenetic analysis with TUB2 (Sato *et al.*, 2013). Morphological differences such as colony color, conidial shape and size of each

*Corresponding author
E-mail: s1043@affrc.go.jp

species and group were clarified in the latest paper. However, group A2 appeared to be polyphyletic, because the strains in the group were divided into a few clades of *C. simmondsii*, *C. carthami* and group A2-P. Therefore, group A2 as well as the monophyletic group A4 remained to be re-identified according to the latest taxonomic system of the *C. acutatum* species complex. We carried out re-identification of strains belonging to the groups preserved in the NIAS Genebank based on the 6 genes mentioned above. This study is necessary to determine the host range of each member species of the complex in Japan and will lead to an appropriate evaluation of pathogenicity to host plants, as shown in case of '*C. simmondsii*' and *C. fioriniae*, which cause quite different symptoms in celery (Fujinaga *et al.*, 2011).

MATERIALS AND METHODS

Molecular re-identification

Seventy three, five and seven strains of *C. acutatum* group A2, group A4 and other *Colletotrichum* spp., respectively preserved in the NIAS Genebank (Table 1) were re-identified based on phylogenetic analyses with the ITS, and GAPDH, CHS-1, HIS3, ACT and TUB2 gene partial sequences. Genomic DNA extracted according to the procedure of Moriwaki *et al.* (2002) was used as a template for the following polymerase chain reaction (PCR) analysis. The 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 the ITS were 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 sec − 55℃ for 1 min − 72℃ for 1 min, and a final step at 72℃ for 5 min. Those for the five genes were 94℃ for 5 min, followed by 40 cycles of 94℃ for 30 sec − 52℃ for 30 sec − 72℃ for 30 sec, and a final step at 72℃ 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 web pages, "Detailed information of microorganism genetic resources of Microorganism Search System", NIAS Genebank (http://www.gene.affrc.go.jp/databases-micro_search_en.php).

For phylogenetic analysis, sequence data of the ITS region, GAPDH, CHS-1, HIS3, ACT and TUB2 gene sequences for the 85 strains examined in this paper as well as 31 strains comprising an additional 29 species and *Colletotrichum* sp. in the *C. acutatum* species complex (Damm *et al.*, 2012) downloaded from DDBJ/EMBL/GenBank databases were also included as references (Table 2). Those of two strains of *Colletotrichum orchidophilum* Damm, P.F. Cannon & Crous deposited in the databases were also used as an outgroup. Multiple sequence alignments were carried out using the FFT-NS-i strategy of MAFFT version six (Katoh *et al.*, 2002). The alignments of all sequences were further optimized manually. Gaps were treated as missing data. A phylogenetic tree was constructed from sequences of the six genes combined, by maximum likelihood (ML) methods using shotgun searches with Treefinder 2011-March version (Jobb *et al.*, 2004). The search was repeated until the maximum likelihood. The optimum substitution models for each partition were selected by Kakusan4 (Tanabe, 2011), based on the Akaike information criterion (Akaike, 1974). The models for different genes (proportional model among regions and among codons; ITS: K80+Gamma; GPDH: GTR+Gamma; CHS-1 first codon: HKY85+Homogeneous; CHS-1 second codon: K80+Gamma; CHS-1 third codon: HKY85+Gamma; ACT: SYM+Gamma; HIS3: GTR+Gamma; TUB2: GTR+Gamma) were used in the tree searches. The reliability of the inferred tree was estimated by bootstrap analysis (Felsenstein, 1985).

Morphological observation

Two kinds of media, potato dextrose agar (PDA, Difco laboratories, Detroit, MI, USA) and synthetic nutrient-poor agar medium (SNA; Nirenberg, 1976) were used to produce conidia. Autoclaved filter paper pieces were placed onto the surface of the latter medium to enhance sporulation (Damm *et al.*, 2012). Mycelial discs (6 mm diameter) of strains of *C.*

Table 1 Re-identification of Strains in NIAS Genebank (MAFF[a]) belonging to phylogenetic groups A2 and A4 of *Colletotrichum acutatum s. lato*

Re-identified species	Isolation sources (Host plant)[b]	MAFF accession[c]	Reference[d]
C. acutatum A2-S[e]	*Fragaria*×*ananassa*	238555, 744062, 744063	Sato *et al*., 2013
C. carthami s. str.[f]	*Calendula officinalis*	239358–239361	**Uematsu *et al*., 2012**
C. chrysanthemi[g]	*Calendula officinalis*	239356, 239357	**Uematsu *et al*., 2012**
	Carthamus tinctorius	239370–239374	**Uematsu *et al*., 2012**
	Chrysanthemum coronarium var. *spatiosum*	239362, 239364–239369	**Uematsu *et al*., 2012**
C. godetiae[h]	***Anemone coronaria****	306506	Sato *et al*., 2013
	*Cydonia oblonga**	241296	Sato *et al*., 2013
	Malus pumila* var. *domestica*	241297	Sato *et al*., 2013
	Prunus domestica*	241295	**Hagita, 2006**
	Sanguisorba officinalis*	240289	**Sugiyama *et al*., 2008**
C. nymphaeae[i]	***Anemone coronaria***	306487, 306488, 306507–306509	**Sato *et al*., 1996**, 2013
	Apium graveolens*	242590	**Fujinaga, *et al*., 2011**
	Eriobotrya japonica*	306406, 306407	**Sato *et al*., 1997**
	Fragaria*×*ananassa	239773, 306647, 306682, 731068, 731069	Sato & Moriwaki, 2003
	Gentiana scabra var. *buergeri**	712289	Sato *et al*., 2013
	*Kadsura japonica**	241261	Sato *et al*., 2013
	Malus pumila* var. *domestica	306546–306548	**Sato *et al*., 1998**, 2013
	Matthiola incana*	712311	**Sugawara *et al*., 2009**
	*Petroselinum crispum**	242413–242419	Sato *et al*., 2013
	Prunus domestica*	241294, 306503, 306505	**Hagita, 2006**
			Sato *et al*., 1996, 2013
	Prunus persica*	306430, 306522–306524	Sato *et al*., 1998, 2013
			Kanno & Moriwaki, 1999
	*Vigna radiata**	242581	Sato *et al*., 2013
C. scovillei[j]	***Capsicum annuum* (var. '*grossum*'*)**	242420–242423, 242425–242428, 242592, 242692, 242693, 243021, 243022, 243038	**Kanto *et al*., 2010** **Tsukamoto *et al*., 2010**
C. sloanei[k]	Woody plant	239736	Sato *et al*., 2013
Colletotrichum sp.(B)	*Annona squamosa*	306172	Sato, 1997, Sato *et al*., 2013
	Bischofia javanica	237894	Sato & Moriwaki, 2003
Colletotrichum sp.(C)	*Prunus*×*yedoensis*	240237	Sato *et al*., 2013
***Colletotrichum* sp.(J)**	***Stewartia pseudo-camellia***	237922, 306725, 306726, 410809	**Kaneko *et al*., 1998** Sato *et al*., 1998, 2013

[a] Acronym of microbe strains in the Genebank, National Institute of Agrobiological Sciences, Japan.
[b] Bold names were reported as host plants of diseases caused by *Colletotrichum acutatum* in Japan, *new host.
[c] Profile and DNA sequences of each strain appears in the website, http://www.gene.affrc.go.jp/databases-micro_search_en.php
[d] Bold references are first reports on diseases caused by *Colletotrichum acutatum* in Japan, in which the strains listed were described.
[e] *Colletotrichum acutatum* group A2-S, newly designated in this paper
[f] *Colletotrichum carthami sensu stricto* Toy. Sato & Moriwaki
[g] *Colletotrichum chrysanthemi* (Hori) Sawada
[h] *Colletotrichum godetiae* Neergaard (the group A4)
[i] *Colletotrichum nymphaeae* (Passerini) Aa
[j] *Colletotrichum scovillei* Damm, P.F. Cannon & Crous (the group A2-P)
[k] *Colletotrichum sloanei* Damm, P.F. Cannon & Crous

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

Species[b]	Strain[c]	DNA sequence accession number in DDBJ/EMBL/GenBank					
		ITS[d]	GAPDH[e]	CHS-1[f]	HIS3[g]	ACT[h]	TUB2[i]
C. acerbum	**CBS 128530**	JQ948459	JQ948790	JQ949120	JQ949450	JQ949780	JQ950110
C. acutatum	**CBS 112996**	JQ005776	JQ948677	JQ005797	JQ005818	JQ005839	JQ005860
C. australe	**CBS 116478**	JQ948455	JQ948786	JQ949116	JQ949446	JQ949776	JQ950106
C. brisbanense	**CBS 292.67**	JQ948291	JQ948621	JQ948952	JQ949282	JQ949612	JQ949942
C. chrysanthemi	IMI 364540	JQ948273	JQ948603	JQ948934	JQ949264	JQ949594	JQ949924
	CBS 126518	JQ948271	JQ948601	JQ948932	JQ949262	JQ949592	JQ949922
C. cosmi	**CBS 853.73**	JQ948274	JQ948604	JQ948935	JQ949265	JQ949595	JQ949925
C. costaricense	**CBS 330.75**	JQ948180	JQ948510	JQ948841	JQ949171	JQ949501	JQ949831
C. cuscutae	**IMI 304802**	JQ948195	JQ948525	JQ948856	JQ949186	JQ949516	JQ949846
C. fioriniae	**CBS 128517**	JQ948292	JQ948622	JQ948953	JQ949283	JQ949613	JQ949943
C. godetiae	**CBS 133.44**	JQ948402	JQ948733	JQ949063	JQ949393	JQ949723	JQ950053
C. guajavae	**IMI 350839**	JQ948270	JQ948600	JQ948931	JQ949261	JQ949591	JQ949921
C. indonesiense	**CBS 127551**	JQ948288	JQ948618	JQ948949	JQ949279	JQ949609	JQ949939
C. johnstonii	**CBS 128532**	JQ948444	JQ948775	JQ949105	JQ949435	JQ949765	JQ950095
C. kinghornii	**CBS 198.35**	JQ948454	JQ948785	JQ949115	JQ949445	JQ949775	JQ950105
C. laticiphilum	**CBS 112989**	JQ948289	JQ948619	JQ948950	JQ949280	JQ949610	JQ949940
C. limetticola	**CBS 114.14**	JQ948193	JQ948523	JQ948854	JQ949184	JQ949514	JQ949844
C. lupini	**CBS 109225**	JQ948155	JQ948485	JQ948816	JQ949146	JQ949476	JQ949806
C. melonis	**CBS 159.84**	JQ948194	JQ948524	JQ948855	JQ949185	JQ949515	JQ949845
C. nymphaeae	**CBS 515.78**	JQ948197	JQ948527	JQ948858	JQ949188	JQ949518	JQ949848
C. orchidophilum	**CBS 632.80**	JQ948151	JQ948481	JQ948812	JQ949142	JQ949472	JQ949802
	IMI 309357	JQ948153	JQ948483	JQ948814	JQ949144	JQ949474	JQ949804
C. paxtonii	**IMI 165753**	JQ948285	JQ948615	JQ948946	JQ949276	JQ949606	JQ949936
C. phormii	**CBS 118194**	JQ948446	JQ948777	JQ949107	JQ949437	JQ949767	JQ950097
C. pyricola	**CBS 12853**	JQ948445	JQ948776	JQ949106	JQ949436	JQ949766	JQ950096
C. rhombiforme	**CBS 129953**	JQ948457	JQ948788	JQ949118	JQ949448	JQ949778	JQ950108
C. salicis	**CBS 607.94**	JQ948460	JQ948791	JQ949121	JQ949451	JQ949781	JQ950111
C. scovillei	**CBS 126529**	JQ948267	JQ948597	JQ948928	JQ949258	JQ949588	JQ949918
C. simmondsii	**CBS 122122**	JQ948276	JQ948606	JQ948937	JQ949267	JQ949597	JQ949927
C. sloanei	**IMI 364297**	JQ948287	JQ948617	JQ948948	JQ949278	JQ949608	JQ949938
C. tamarilloi	**CBS 129814**	JQ948184	JQ948514	JQ948845	JQ949175	JQ949505	JQ949835
C. walleri	**CBS 125472**	JQ948275	JQ948605	JQ948936	JQ949266	JQ949596	JQ949926
Colletotrichum sp.	CBS 129823	JQ948192	JQ948522	JQ948853	JQ949183	JQ949513	JQ949843

[a] Cited from Damm *et al.* (2012)
[b] *C.*=*Colletotrichum*
[c] Bold strains are type or ex-type
[d] Ribosomal DNA internal transcribed spacer
[e] Glyceraldehyde-3-phosphate dehydrogenase
[f] Chitin synthase 1
[g] Histone3
[h] Actin
[i] Beta-tubulin-2

carthami, *Colletotrichum chrysanthemi* (Hori) Sawada and *Colletotrichum* sp. from strawberry listed in Table 3 were cultured on plates (55 mm in diam.) of both media at 25℃ under black light for 7 days. The length and width of each fifty conidia of the strains were measured with a phase contrast microscope (Nikon Eclipse 80i with an image analyzer, Nikon Digital Sight; Nikon, Tokyo, Japan). The conidia of the representative strains were photographed with a digital camera attached to the microscope.

RESULTS AND DISCUSSION

Molecular Re-identification

An ML tree with log likelihood of −6,542.8 was obtained from the phylogenetic analysis with the

sequences of 85 strains uploaded to the NIAS Genebank website and those downloaded from the DDBJ/EMBL/GenBank databases (Fig. 1). Fourteen, 37 and 1 strains in group A2 constituted clades with ex-type strains of *Colletotrichum scovillei* Damm, P. F. Cannon & Crous, *Colletotrichum nymphaeae* (Passerini) Aa and *Colletotrichum sloanei* Damm, P. F. Cannon & Crous, respectively. Approximately 50% of 73 strains belonging to *C. acutatum* group A2 were re-identified as *C. nymphaeae*, which was described as an anthracnose pathogen of strawberry fruits and other horticultural plants (Damm *et al.*, 2012). Almost all of the strains were once identified as *C. simmondsii sensu lato* based on a phylogenetic study using only TUB2 partial sequences (Sato *et al.*, 2013). In that study none of the strains were located in the clade with the ex-type strain of *C. simmondsii sensu stricto*, which is consistent with the results in this study. The pathogen of the stunt anthracnose of celery, MAFF 242590, which was identified as *C. simmondsii* (Fujinaga *et al.*, 2011), was clearly re-identified as *C. nymphaeae* based on the present multi-gene analysis. Nine plants including celery are new hosts for *C. nymphaeae* (Table 1). Group A2-P, which was designated as sweet pepper pathogens by Sato *et al.* (2013) corresponded to *C. scovillei*, which is known to be an anthracnose pathogen of *Capsicum annuum* (chili fruit) in Thailand (Damm *et al.*, 2012). Sweet pepper is a new host for *C. scovillei*, although it is closely related to chili pepper. MAFF 239736, isolated from a woody plant leaf on Iriomote Is., a subtropical island in Okinawa, Japan, was re-identified as *C. sloanei*, which was described based on material from a leaf of *Theobroma cacao* in Borneo, Malaysia (Damm *et al.*, 2012).

Twenty one strains once identified as *C. carthami* based on TUB2 (Uematsu *et al.*, 2012; Sato *et al.*, 2013) were divided into 3 clades. Fourteen of them were clustered with reference strains of *C. chrysanthemi* accepted by Damm *et al.* (2012). The bootstrap value 92 of the branches indicates that they are genetically distinct from two other sister lineages. Four isolated from pot marigold and three from strawberry were made their own clades, respectively. The former clades are recognized as *C. carthami sensu stricto*, because they were combined with the type specimen of *C. carthami* based on TUB2 sequences (Uematsu *et al.* 2012; Sato *et al.*, 2013). The latter is tentatively classified as '*C. acutatum* (group) A2-S' newly designated in this paper, because it does not corresponds to any species listed by Damm *et al.* (2012).

Bedlan (2012) reported strains causing safflower anthracnose in Austria and transferred *Gloeosporium carthami* to *Colletotrichum*. But he did not give correct basionym citation to the new combination (Art 33.4). Moreover, the TUB2 sequence of the representative strain (Bedlan, personal communication) is identical with those of our *C. chrysanthemi* strains, MAFF 239364–239374, and have 99.7% homology with that of the neotype specimen of the species designated below. The Austrian strain seems to belong not to *C. carthami s. str.* but to *C. chrysanthemi*, because TUB2 sequences was regarded as a typical barcode gene for the *C. acutatum* species complex (Cannon *et al.*, 2012).

Five strains once identified as group A4 by Sato *et al.* (2013) belonged to a single clade of *C. godetiae*, indicating that the group is monophyletic. The species is a plurivorous anthracnose pathogen in Europe, Africa, North and South America (Damm *et al.*, 2012). This is the first report from the Far East. Five plants are all new hosts for *C. godetiae* (Table 1).

Although seven other strains did not belong to any clades containing reference strains, four of them from Japanese stewartia and two from other woody plants constituted their own clades (Fig. 1). The two lineages, *Colletotrichum* sp. (J) and (B), should be given names except 29 of the *C. acutatum* species complex recognized by Damm *et al.* (2012), after detailed morphological and physiological studies. Strain MAFF 240237 from a cherry tree, *Colletotrichum* sp. (C) isolated alone on a branch needs to be examined further in order to clarify its novelty.

Morphological observation

There are some differences in conidial morphology on both PDA and SNA between six representative strains of *C. chrysanthemi*, and 4 of *C. carthami s. str.* Average conidial length of the former was obviously longer than those of the latter (Table 3, Fig. 2). IMI 364540, the strain of *C. chrysanthemi* examined by Damm *et al.* (2012) produced similar size of conidia on SNA to those of the Japanese strains combined with it in the phylogram, although CBS 126518 has exceptionally shorter and broader conidia (Table 3). *Colletotrichum chrysanthemi*, therefore, was demonstrated to be a distinct species

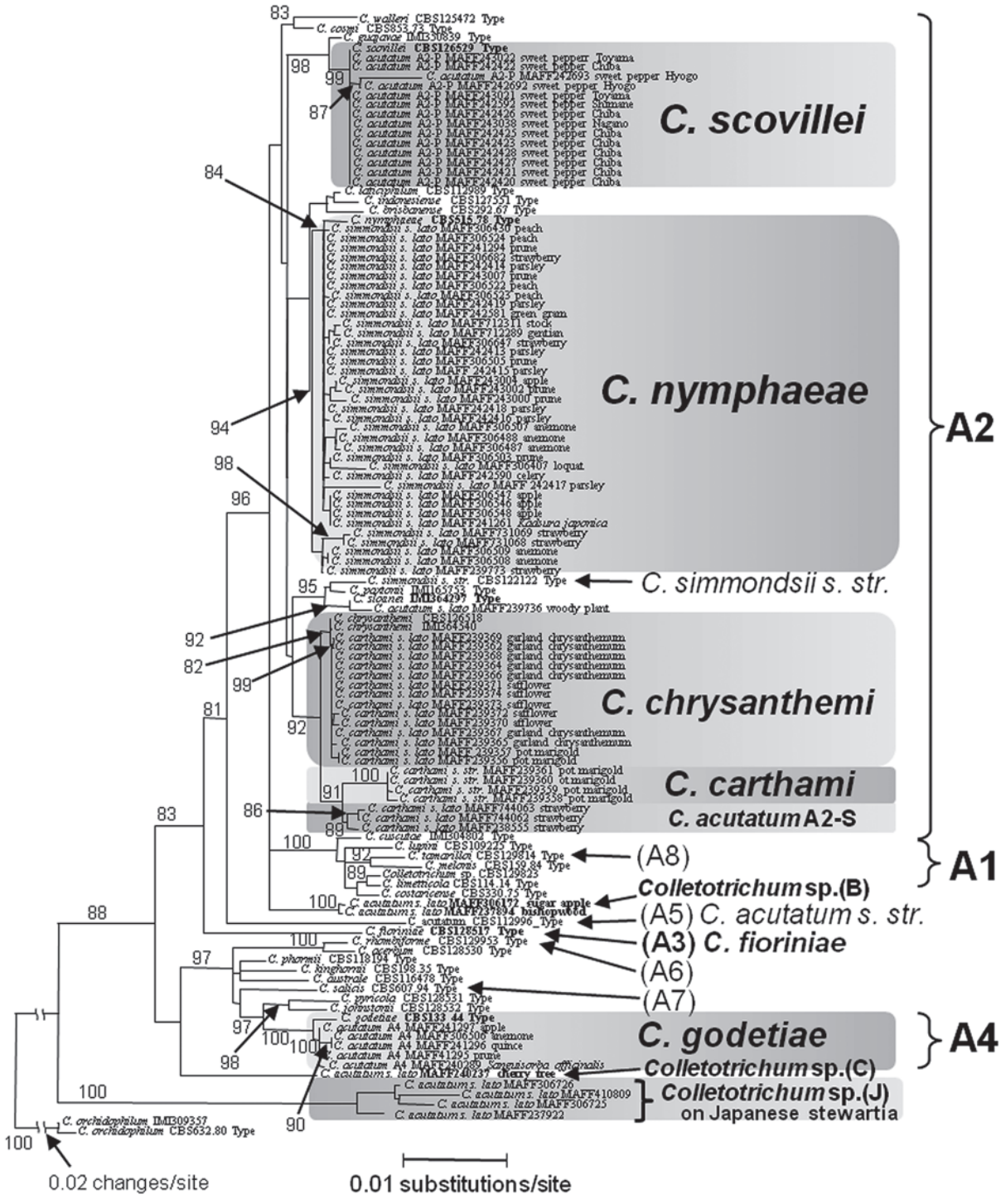

Fig. 1 Maximum likelihood tree of 2011 bp of rDNA-ITS region, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase 1 (CHS-1), histone 3 (HIS3), actin (ACT), and beta-tubulin-2 (TUB2) partial gene sequences of 85 strains belonging to the *Colletotrichum acutatum* species complex preserved in NIAS Genebank and 33 strains comprising 31 species downloaded from the DDBJ/EMBL/GenBank databases. Numbers on the branches represent the percentage of congruent clusters in 100 bootstrap trials when the values were greater than 80%.

also in morphology, although Uematsu *et al.* (2012) treated it a synonym of *C. carthami*. Because neither specimens nor figures have been found for the original description of the species, we designated its neotype here and revised conidial sizes as follows;

Colletotrichum chrysanthemi (Hori) Sawada, Rep Govt Res Inst Dep Agric, Formosa 85: 81 (1943) ≡ *Gloeosporium chrysanthemi* Hori in Takimoto S, Nihon-engei-zasshi 36 (9):27 (1924).

Neotypus: *Chrysanthemum coronarium* L. var. *spatiosum* L.H. Baily, Suwon, Kyonggi, Korea, June, 1919, Seito Takimoto (SAPA100010 as *Gloeosporium chrysanthemi*, Hokkaido University Museum). Conidia on PDA were 7.3–15.8 (–17.3) × 2.6–5.3 (–6) μm, average 10.8 × 4 μm in size, L/B ratio 2.73, and those on SNA 7.5–15.6 (–20) × 2.6–6.2 (–6.6) μm, average 11.9 × 4.1 μm in size, L/B ratio 2.93 (Fig. 2a–c).

We also revised the conidial size of *C. carthami* as follows; Conidia on PDA were 4.5–9.8 (–10.9) × 2.4–4.6 μm, average 7 × 3.2 μm in size, L/B ratio 2.18, and those on SNA 5.8–11.4 × 2.6–4.8 μm, average 8.4 × 3.5 μm in size, L/B ratio 2.47 (Fig. 2d).

Conidial sizes of *C. acutatum* A2-S strains varied widely depend on the strains. Conidia on PDA were 5.3–14.3 (–16.8) × 2.6–5.5 μm, average 8.6 × 4.1 μm in size, L/B ratio 2.17, and those on SNA 5.8–13.0 (–15.8) × 2.8–5.6 (–6.5) μm, average 8.7 × 4.2 μm in size, L/B ratio 2.14. They produced some snowman-shaped conidia among ovoid to broadly ellipsoid ones in common, which were unique to the *C. acutatum* species complex (Fig. 2e). More strains belonging to the lineage are expected to collect in other locations, because the host plant, strawberry, is one of popular crops in temperate region. They should be also examined for host range to clarify the identity before a new name will be given.

The results of this study and the report by Sato *et al.* (2013) revealed that NIAS Genebank kept six

Table 3 Conidial sizes of strains of *Colletotrichum carthami*, *C. chrysanthemi* and *C. acutatum* A2-S (*Colletotrichum* sp. from strawberry)

Species, strain[b]	Conidium on PDA (μm)			Conidium on SNA[a] (μm)		
	Range	Average	L/B ratio	Range	Average	L/B ratio
C. carthami	**4.5–9.8 (–10.9) × 2.4–4.6**	**7 × 3.2**	**2.18**	**5.8–11.4 × 2.6–4.8**	**8.4 × 3.5**	**2.47**
MAFF 239358[c]	5.3–9.1 × 2.4–4.4	7.2 × 3.3	2.22	6.1–10.7 × 2.8–4	8.3 × 3.4	2.45
MAFF 239359[c]	5.3–9.4 (–10.9) × 2.5–4.6	7.4 × 3.4	2.21	6.6–10.9 × 2.7–4.8	8.2 × 3.6	2.3
MAFF 239360[c]	5.3–8.9 × 2.4–3.92.4–3.9	6.7 × 3.1	2.16	5.8–9.9 × 2.6–4	8.5 × 3.3	2.61
MAFF 239361[c]	4.5–9.8 × 2.4–4.2	6.6 × 3.1	2.14	6–11.4 × 2.7–4.3	8.6 × 3.5	2.5
C. chrysanthemi	**7.3–15.8 (–17.3) × 2.6–5.3 (–6)**	**10.8 × 4**	**2.73**	**7.5–15.6 (–20) × 2.6–6.2 (–6.6)**	**11.9 × 4.1**	**2.93**
MAFF 239356[c]	7.8–15 × 3–5 (–6)	10.9 × 4	2.76	7.5–15.6 × 2.6–4.6	10.5 × 3.6	2.9
MAFF 239357[c]	7.3–13.8 × 2.6–5.3	9.6 × 3.8	2.56	7.9–14.9 (–18) × 3–4.5	11.3 × 3.6	3.11
MAFF 239367[d]	7.4–13.5 × 2.4–4.3 (–5.4)	9.5 × 3.5	2.72	10–14.1 (–17.9) × 3.1–5.2	12.7 × 4.1	3.14
MAFF 239369[d]	10.1–14.7 (–17.3) × 3.1–5.1	12.1 × 4	3.08	7.7–14.2 (–17.2) × 3.4–4.9	12.7 × 4.2	3.02
MAFF 239370[e]	8–15.8 (–16.9) × 3.2–5.1 (–5.7)	10.8 × 4.3	2.55	8.1–13.5 (–16.4) × 3.6–6.2 (–6.6)	11.4 × 4.7	2.46
MAFF 239374[e]	9.1–13.6 × 3.5–5.2	11.7 × 4.4	2.68	10–15.1 (–20) × 3–6.1	13 × 4.4	2.97
(IMI 364540[f]	not examined			(4.5–)6.5–13(–26) × (3–)3.5–5(–11)	9.8 × 4.3	2.3)
(CBS 126518[g]	not examined			(6–)7–9.5(–12) × (3–)4–5.5(–6)	8.3 × 4.8	1.7)
***C. acutatum* A2-S**	**5.3–14.3 (–16.8) × 2.6–5.5**	**8.6 × 4.1**	**2.17**	**5.8–13.0 (–15.8) × 2.8–5.6 (–6.5)**	**8.7 × 4.2**	**2.14**
MAFF 238555	5.3–8.4 (–9.5) × 2.6–5.4	7 × 4.2	1.69	5.8–10.8 × 3.6–5.6 (–6.5)	7.7 × 4.5	1.74
MAFF 744062	(6.1–) 7.3–14.3 (–16.8) × 2.6–4.8	10.1 × 3.6	2.83	7–13.0 (–15.8) × 2.8–4.8 (–5.1)	9.8 × 3.7	2.68
MAFF 744063	5.8–12.2 × (3–) 3.6–5.5	8.8 × 4.4	1.99	6.4–10.9 (–12.8) × 2.9–5.3 (–6.2)	8.7 × 4.4	2.01

[a] Pieces of autoclaved filter paper were placed onto the plate surface.
[b] Cultured at 25℃ under black light for 7 days.
[c] Isolated from *Calendula officinalis* (pot marigold).
[d] Isolated from *Chrysanthemum coronarium* var. *spatiosum* (garland chrysanthemum).
[e] Isolated from *Carthamus tinctorius* (safflower).
[f] Isolated from *Chrysanthemum coronarium* in China (Damm *et al.*, 2012)
[g] Isolated from *Carthamus* sp. in Netherlands (Damm *et al.*, 2012)

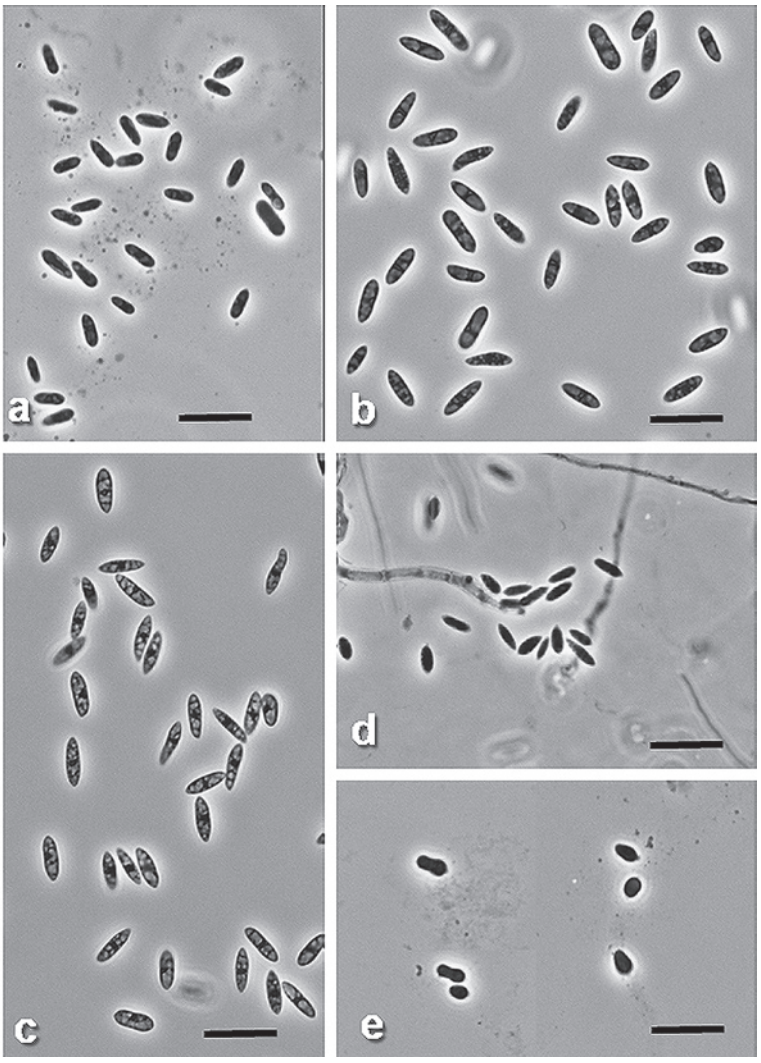

Fig. 2 Conidia of strains of *Colletotrichum chrysanthemi* (a: MAFF 239356 isolated from pot marigold, b: MAFF 239374 from safflower, c: MAFF 239369 from garland chrysanthemum), *C. carthami sensu stricto* (d: MAFF 239360 from pot marigold) and *C. acutatum* group A2-S (e: MAFF 238555 from strawberry) produced on PDA 25℃, 7 days after transplant (bar: 20 μm, phase contrast optics).

species of 29 accepted by Damm *et al.* (2012), i.e., *C. chrysanthemi*, *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. sloanei*, *C. scovillei*, as well as *C. carthami* and four nameless taxa in the *C. acutatum* species complex. Damm *et al.* (2012) found five of 21 new species among only 16 strains from Asian regions outside of Japan, which is 4.8% of all the strains examined. *Colletotrichum scovillei* and *C. sloanei* of the Asian species are also present in Japan. It is expected that more new taxa will be found in Asian strains of the species complex, as we suggested a few new ones in Japanese strains above.

Sato *et al.* (2013) clarified the differences in conidial size and shape among the three species, groups A2-P and A4 of the *C. acutatum* species complex by detailed investigation and proposed a morphological key. *Colletotrichum carthami sensu lato* was found to contain *Colletotrichum chrysanthemi* and *C. acutatum* A2-S in addition to *C. carthami s. str.* We update it as follows;

1. Reverse colony color reddish on PDA or WSH
…… *C. fioriniae*

1. Reverse colony color not reddish on PDA or WSH ····· 2
  2. Some conidia more than 18 μm in length ·····*C. godetiae*
  2. Conidial length less than 18 μm ····· 3
    3. Average of conidial length less than 11 μm on PDA ····· 4
    3. Average of conidial length more than 11 μm on PDA ····· 5
      4. Most conidial L/B ratio less than 3 on PDA ····· 6
      4. Most conidial L/B ratio more than 3 on PDA ····· *C. nymphaeae*
        5. Average of conidial width less than 3.5 μm ·····*C. scovillei*
        5. Average of conidial width more than 3.5 μm ····· *C. fioriniae*
          6. Some conidia snowman-shaped to ovoid ·····*C. acutatum* A2-S
          6. Conidia fusiform, subcylindrical, clavate ····· 7
            7. Average of conidial length less than 8 μm on PDA ·····*C. carthami s. str.*
            7. Average of conidial length more than 9 μm on PDA ····· *C. chrysanthemi*

According to Damm *et al.* (2012), *C. sloanei* sometimes forms polyphialides on SNA plates with filter paper, whereas 6 other species including *C. carthami s. str.* have not produced such conidiogenous cells to date. *Colletotrichum simmondsii s. str.* is absent from the key because of the current lack of any strain of this species in NIAS Genebank. The species was described as forming colonies with a grey cottony on PDA and in reverse pale grey to pale orange sometimes with dark flecking (Shivas & Tan, 2009) like certain strains of *C. nymphaeae* (=*C. simmondsii*; Sato *et al.*, 2013), and small setae on autoclaved *Anthriscus* stem placed on SNA (Damm *et al.*, 2012). Some host plants such as strawberry, kiwifruit and tomato in Japan possibly bear *C. simmondsii s. str.*, which appears to be easily misidentified as *C. nymphaeae*. Therefore, molecular identification with β-tubulin-2 gene sequences is recommended when *C. nymphaeae*-like strains will be isolated.

Update of pathogen names

Many pathogenic strains of anthracnose first found in Japan are present in the material examined here. They have been identified "*C. acutatum*" except for those of parsley (Uematsu *et al.*, 1991), celery (Fujinaga *et al.*, 2011) and three asteraceous plants (Uematsu *et al.*, 2012) in Japan. Sato *et al.* (2013) previously updated the strain names as *C. carthami*, *C. fioriniae*, *C. simmondsii*, groups A2-P or A4. We update again the strains of *C. carthami sensu lato*, *C. simmondsii sensu lato*, groups A2-P and A4. Updated pathogen names and their host plants are as follows (Table 1):

C. carthami and *C. chrysanthemi* ····· pot marigold, safflower
C. chrysanthemi ····· garland chrysanthemum
C. godetiae····· burnet, quince
C. nymphaeae ··· celery, loquat, parsley, peach, stock, strawberry
C. nymphaeae and *C. godetiae* ····· anemone, apple, prune
C. scovillei····· sweet pepper

Tentative host ranges of the member species of groups A2 and A4 were clarified in this study (Table 1), although they will expand gradually whenever strains and isolates from other host plants are identified as member species. The restricted host ranges make it easier to identify the source of infection and to control the diseases caused by the member species than previously.

ACKNOWLEDGEMENTS

We are grateful to Mr. Seiji Uematsu and several Japanese researchers for depositing "*C. acutatum*" strains into the NIAS Genebank, Japan; Professor Dr. Hideki Takahashi, Hokkaido University Museum, for loaning the herbarium specimen of *G. chrysanthemi*; Dr. Ulrike Damm, Centraalbureau voor Schimmelcultures, for providing valuable suggestions; and Ms. Hiromi Nakajima and Ms. Kyoko Yabunaka, NIAS Genebank, for assistance with the experiments.

REFERENCES

Aa, H.A. van der, Noordeloos, M.E. & Gruyter, J. de 1990. Species concepts in some larger genera of the Coelomycetes. Stud. Mycol. **32**: 3-19.

Akaike, H. 1974. A new look at the statistical model identification. IEEE T. Automat. Contr. **19**: 716-723.

Bedlan, G. 2012. *Colletotrichum carthami* comb. nov. on safflower (*Carthamus tinctorius*). J. Kulturpflanzen **64**: 309-313.

Cannon, P.F., Damm, U., Johnston, P.R. & Weir B.S. 2012. *Colletotrichum* — current status and future directions. Stud. Mycol. **73**: 181-213.

Carbone, I. & Kohn, L.M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia **91**: 553-556.

Crous, P.W., Groenewald, J.Z., Risede, J.M. & Hywel-Jones, N.L. 2004. *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. Stud. Mycol. **50**: 415-430.

Damm, U., Cannon, P.F., Woudenberg, J.H.C. & Crous, P.W. 2012. The *Colletotrichum acutatum* species complex. Stud. Mycol. **73**: 37-113.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783-791.

Fujinaga, M., Yamagishi, N., Ogiso, H., Takeuchi, J., Moriwaki, J. & Sato, T. 2011. First report of celery stunt anthracnose caused by *Colletotrichum simmondsii* in Japan. J. Gen. Plant Pathol. **77**: 243-247.

Glass, N.L. & Donaldson, G. 1995. Development of primer sets designed for use with CR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. **61**: 1323-1330.

Guerber, J.C., Liu, B., Correll, J.C. & Johnston, P.R. 2003. Characterization of diversity in *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. Mycologia **95**: 872-895.

Hagita, T. 2006. Occurrence of prune (*Prunus domestica*) anthracnose caused by *Colletotrichum acutatum* in Hokkaido. Ann. Rept. Plant Prot. North Japan **57**: 78-81 (in Japanese).

Jobb, G., von Haeseler, A. & Strimmer, K. 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. **4**: 18.

Johnston, P.R. & Jones, D. 1997. Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. Mycologia **89**: 420-430.

Kaneko, S., Sato, T., Tomioka, K., Watanabe, N. & Isaka, T. 1998. Anthracnose of *Stewartia pseudocamellia*. J. Tree Health **2**: 17-20 (in Japanese).

Kanno, H. & Moriwaki, J. 1999. Bitter rot of pear (*Pyrus communis*) and anthracnose of peach (*Prunus persica*), apricot (*Prunus armenica*) and Mume (*Prunus mume*) caused by *Colletotrichum acutatum*. Ann. Phytopathol. Soc. Jpn. **65**(3): 407-408 (abstract in Japanese).

Kanto, T., Usami, T, Sato, T, Moriwaki, J., Aino, K., Maekawa, K., Iwamoto, Y. & Matsuura, K. 2010. Anthracnose of sweet pepper caused by *Colletotrichum acutatum sensu lato* in Japan. Jpn. J. Phytopathol. **76**: 157 (abstract in Japanese).

Katoh, K., Misawa, K., Kuma, K., & Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. **30**: 3059-3066.

Lardner, R., Johnston, P.R., Plummer, K.M. & Pearson, M.N. 1999. Morphological and molecular analysis of *Colletotrichum acutatum* sensu lato. Mycol. Res. **103**: 275-285.

Moriwaki, J., Tsukiboshi, T. & Sato, T. 2002. Grouping of *Colletotrichum* species in Japan based on rDNA sequences. J. Gen. Plant Pathol. **68**: 307-320.

Nirenberg, H.I. 1976. Untersuchungen über die morphologische und biologische Differenzierung in der Fusarium-Sektion Liseola. Mitt. Biol. Bundesanst. Land- Forstw. Berlin-Dahlem **169**: 1-117.

O'Donnell, K. & Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. **7**: 103-116.

Sato, T. 1997. Characters and identification of a plurivorous anthracnose fungus, *Colletotrichum acutatum*. Proc. Assoc. Plant Prot. Shikoku **32**: 1-19 (in Japanese with English summary).

Sato, T., Ueda, S., Iijima, A. & Tezuka, N. 1996. Re-identification of pathogens of anemone and prune anthracnose. Ann. Phytopathol. Soc. Jpn. **62**: 170-174.

Sato, T., Uematsu, S., Mizoguchi, H., Kiku, T. & Miura, T. 1997. Anthracnose of prairie gentian and loquat caused by *Colletotrichum acutatum*. Ann. Phytopathol. Soc. Jpn. **63**: 16-20.

Sato, T., Uematsu, S., Iijima, A. & Koganezawa, H. 1998. Occurrence of apple bitter rot by grayish colony form of *Colletotrichum acutatum* in Japan and pathogenicity to apple fruits of *C. acutatum* and *Glomerella cingulata* isolated from other plants. Trans. Mycol. Soc. Japan **39**: 35-44 (in Japanese with English summary).

Sato, T. & Moriwaki, J. 2003. Plurivorous plant anthracnose fungus, *Colletotrichum acutatum*. MAFF Microorg. Gene. Resour. Manual **13**: 1-12 (in Japanese).

Sato, T., Moriwaki, J. & Misawa, T. 2013. Molecular Re-identification of Strains of the *Colletotrichum acutatum* species complex deposited in the NIAS Genebank and morphological characteristics of its member Species. JARQ **47**: 295-305.

Shivas, R.G. & Tan, Y.P. 2009. A taxonomic re-assessment of *Colletotrichum acutatum*, introducing *C. fioriniae* comb. et stat. nov. and *C. simmondsii* sp. nov. Fungal Diversity **39**: 111-122.

Simmonds, J.H. 1965. A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. Queens. J. Agric. Anim. Sci. **22**: 437-459.

Sreenivasaprasad, S. & Talhinhas, P. 2005. Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. Mol. Plant Pathol. **6**: 361-378.

Sugawara, K., Matsudate, A., Ito, Y. & Namai, T. 2009. Anthracnose of smoke tree and petal anthracnose of stock caused by *Colletotrichum acutatum*. Jpn. J. Phytopathol. **75**: 119-122 (in Japanese with English summary).

Sugiyama, S., Sato, T., Moriwaki, J., Kageyama, K. & Chu, E. 2008. First report on anthracnose of *Sanguisorba officinalis* caused by *Colletotrichum acutatum*. Ann. Rept. Plant Prot. North Japan **59**: 86-89 (in Japanese).

Tanabe, A.S. 2011. Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. Mol. Ecol. Resour. **11**: 914-921.

Tsukamoto, T., Moriwaki, J. & Sato, T. 2010. Sweet pepper anthracnose caused by *Colletotrichum acutatum* Simmonds ex Simmonds and identification of *Sarcandra glaber* anthracnose pathogen as *C. gloeosporioides* (Penzig) Penzig & Saccardo. Jpn. J. Phytopathol. **76**: 200 (abstract in Japanese).

Uematsu, S., Tsuzaki, Y., Osawa, T., Horie, H., Mihira, T., Sogo, K., Ozaki, J., Sekiyama, K. & Homma, H. 1991. Incidence of parsley anthracnose. Ann. Phytopathol. Soc. Jpn. **57**(3): 398 (abstract in Japanese).

Uematsu, S., Kageyama, K., Moriwaki, J. & Sato, T. 2012. *Colletotrichum carthami* comb. nov., an anthracnose pathogen of safflower, garland chrysanthemum and pot marigold, revived by molecular phylogeny with authentic herbarium specimens. J. Gen. Plant Pathol. **78**: 316-330.

Vinnere, O., Fatehi, J., Wright S.A.I. & Gerhardson, B. 2002. The causal agent of anthracnose of Rhododendron in Sweden and Latvia. Mycol. Res. **106**: 60-69.

White, T.J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, *In* Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (eds.), PCR Protocols: A Guide to Methods and Applications, p. 315-322, Academic Press, San Diego.

Whitelaw-Weckert, M.A., Curtin, S.J., Huang, R., Steel, C.C., Blanchard, C.L. & Roffey, P.E. 2007. Phylogenetic relationships and pathogenicity of *Colletotrichum acutatum* isolates from grape in subtropical Australia. Plant Pathol. **56**: 448-463.