Saudi Journal of Biological Sciences (2016) 23, 1–8

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Camarosporium arezzoensis on *Cytisus* sp., an addition to sexual state of *Camarosporium sensu stricto*

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Received 10 November 2014; revised 25 January 2015; accepted 25 January 2015
Available online 11 February 2015

KEYWORDS

Morphology;
Multi-gene analysis;
Sexual state

Abstract During a study of saprobic fungi from Bagno di Cetica Province, Italy, we collected a pleosporoid ascomycete on stems of *Cytisus* sp. In morphology, our collection is similar to *Cucurbitaria* species, but molecular analysis of SSU, LSU and ITS genes reveals it can be referred to *Camarosporium*. In this study we compare all other *Cucurbitaria* species from *Cytisus* sp. and based on both morphology and molecular data, we introduce our collection as a new species in *Camarosporium viz. C. arezzoensis*.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

1. Introduction

The genus *Camarosporium* was introduced by Schulzer (1870) with *Camarosporium quaternatum* (Hazsl.) Schulz. as the type species. Index Fungorum (2015) lists 508 records as *Camarosporium* which was formerly recognised as asexual morphs in *Botryosphaeriales*, *Cucurbitariaceae*,

http://dx.doi.org/10.1016/j.sjbs.2015.01.018
1319-562X

Phaeosphaeriaceae and related genera (Kirk et al., 2008; Wijayawardene et al., 2012; Doilom et al., 2013; Hyde et al., 2013). However, Wijayawardene et al. (2014a,b) showed that *Camarosporium sensu stricto* belongs to *Pleosporineae*, *Pleosporales* and has cucurbitaria-like sexual morphs.

During our on-going studies, we found a new taxon with bitunicate asci and muriform ascospores which is morphologically similar with members in *Cucurbitariaceae*, *Pleosporales* (Doilom et al., 2013; Hyde et al., 2013). The blast results of small subunit rDNA (SSU), large subunit rDNA (LSU) and internal transcribed spacer (ITS) showed this taxon is related to *Camarosporium* sp. Thus we have carried out molecular analyses *viz*. maximum-parsimony (MP) and confirmed its placement in *Pleosporineae*, *Pleosporales*. As our new collection groups with *Camarosporium sensu stricto*, we introduce it as a new species of *Camarosporium viz*. *C. arezzoensis*.

2. Materials and methods

2.1. Sample collection and morphological study

Fresh fungal specimens were obtained from recent collections made in Italy. Morphological structures were examined under a Carl Zeiss microscopy GmbH (AxioCam ERC 5S) stereo microscope. To observe the fungal structures, ascomata were picked up and put into rehydrated water or lactoglycerol. For hand cross sections 5% KOH was added prior to examination. Microscopic fungal structures were mounted in water for observation under a Nikon ECLIPSE80i compound microscope and photographs were taken with a Cannon 550D digital camera fitted to the microscope. All micro morphologies were measured using Tarosoft® Image Framework program v.0.9.0.7.

2.2. Isolation

Single spore isolation was carried out following the method described in Chomnunti et al. (2014) on potato-dextrose agar (PDA). Germinated spores were transferred to fresh PDA media and incubated at 16 °C. Culture characteristics were observed after four weeks and these cultures were also used for molecular study. The specimens are deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand. Living cultures are deposited at the Mae Fah Luang University Culture Collection (MFLUCC) Chiang Rai, Thailand, Centraalbureau Voor Schimmelcultures, Netherlands (CBS) and International Collection of Microorganisms from Plants, New Zealand (ICMP).

2.3. DNA extraction, PCR amplification and sequencing

Mycelia grown on PDA media at 16 °C for four weeks were used for DNA extraction. Total DNA extraction was established by using a Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, PR China). The concentration of DNA was determined using an ultraviolet spectrophotometer. PCR reactions were carried out according to Telle and Thines (2008) with the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) to amplify the complete internal transcribed spacer (ITS) region. Twenty micro litres (20 µl) of the reaction mixture contained 2 Mix 10 µl, ITS1-F 0.35 µl, ITS4 0.35 µl, 50 ng/µl DNA 0.6 µl, ddH_2O 8.7 µl for each sample. The PCR programme was set according to Douanla et al. (2005) with the following modifications: an initial denaturation at 94 °C for 3 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and a final elongation step of 7 min at 72 °C. To check the PCR products, 1% agarose gel electrophoresis (AGE) for 30 min at 220V was used. All PCR products were sent to Shanghai Majorbio Bio-Pharm Technology Co., Ltd. for purification and sequencing.

2.4. Molecular phylogenetic analysis

BLAST searches of LSU, SSU and ITS sequence data were carried out to reveal the closest taxa to our strain in GenBank (http://www.ncbi.nlm.nih.gov/). Combined analyses of LSU, SSU and ITS dataset of the closest relatives in *Coniothyriaceae*, *Cucurbitariaceae* and *Pleosporaceae* were used to carry out phylogenetic analyses. Bioedit v.7.2.5 (Hall, 2004), ClustalW v.1.6 (Thompson et al., 1997) and MAFFT v.6 (Katoh et al., 2002; Katoh and Toh., 2008) online sequence alignment editor under the default settings (mafft.cbrc.jp/alignment/server/) were used for aligning the sequences separately for each gene region. The individual datasets were finally combined into one dataset and used PAUP v. 4.0b10 (Swofford, 2002) to perform maximum-parsimony (MP) analysis by bootstrap analysis with 10,000 replicates. All multiple, equally parsimonious trees were saved and descriptive tree statistics for parsimony consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated. The robustness of the best parsimonious tree was estimated by a bootstrap (BT) value with 10,000 replicates, each with 10 replicates of random stepwise addition of taxa (Liu et al., 2011; Phookamsak et al., 2013), and the trees were figured in Treeview v.1.6.6.

3. Results

3.1. Phylogenetic analysis

The combined gene data set of SSU, ITS and LSU rDNA consists of 23 taxa including our strain of IT 791 (MFLUCC 14-0238) and the outgroup taxon *Leptosphaeria doliolum* (CBS 541.66). The dataset consists of 2092 characters including coded alignment gaps; 1835 are constant, and 114 are parsimony informative in the MP analysis. A best scoring tree is shown in Fig. 1. Bootstrap support (BS) values of MP (equal to or above 50% based on 10,000 replicates) are shown above branches (TL = 447, CI = 0.694, RI = 0.700, RC = 0.486, HI = 0.306). Our strain of MFLUCC 14-0238 belongs to the genus *Camarosporium sensu stricto* and were separated from representative species of the genus with a relatively higher bootstrap values as circumscribed by Wijayawardene et al. (2014b).

3.2. Taxonomy

Camarosporium arezzoensis Tibpromma, Wijayawardene, Camporesi & K.D. Hyde, sp. nov.

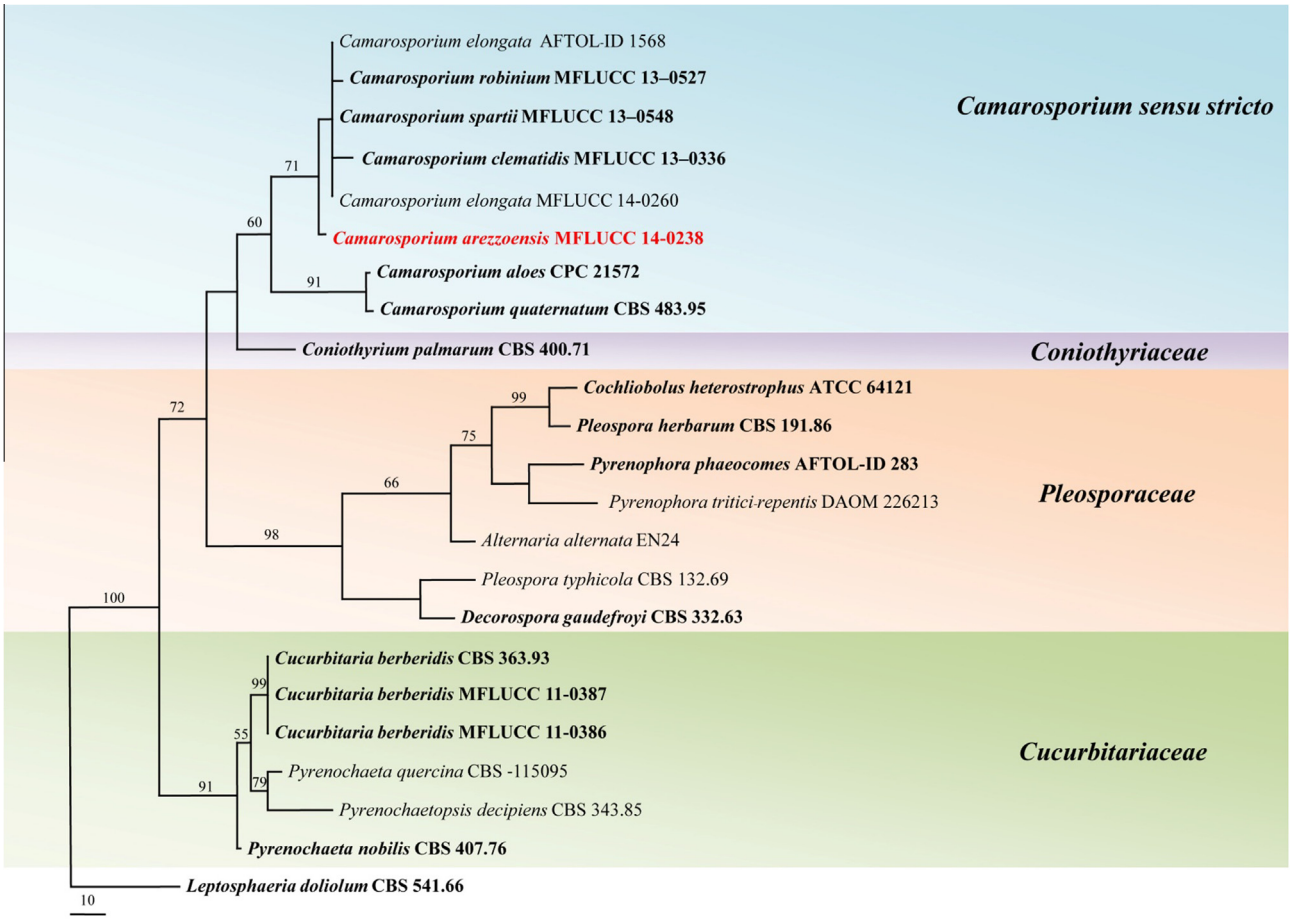

Figure 1 One of the most parsimonious trees generated with SSU, ITS and LSU rDNA combined data analysis. The tree is rooted with *Leptosphaeria doliolum* (CBS 541.66). Type and ex-type strains are in bold. Newly introduced species in red.

Index Fungorum Number: IF550877; Facesoffungi number: 00382

Etymology: Refers to the name of the province in Italy where the fungus was collected

Saprobic on decaying plant stems of *Cytisus* sp. *Sexual morph: Ascomata* 400–500 µm high, 450–550 µm diam. ($\bar{x} = 449 \times 482$ µm, $n = 10$), black, semi-immersed, scattered beneath the host periderm or on decorticated wood, fully or partly erumpent, globose, rough or hairy, with an ostiole. *Ostiole* central, short, slightly sunken, minute and inconspicuous at the surface, smooth, ostiolar canal filled with hyaline cells. *Peridium* 30–45 µm wide at the base, 35–70 µm wide in sides, thick, comprising 8–10 layers, outer layer heavily pigmented, thick-walled, comprising blackish to dark brown cells of *textura angularis*, inner layer composed of hyaline, thin-walled cells of *textura angularis*. *Hamathecium* comprising numerous, 5.5 µm ($n = 40$) wide, filamentous, branched septate, pseudoparaphyses. *Asci* 180–240 × 10–15 µm ($\bar{x} = 199 \times 13$ µm, $n = 40$), 8-spored, bitunicate, fissitunicate, cylindrical, short-pedicellate, apex rounded with a minute ocular chamber. *Ascospores* 19–28 × 9–15 µm ($\bar{x} = 26 \times 12$ µm, $n = 50$), partially overlapping, mostly ellipsoidal, muriform, with 5–7 transverse septa, with 4–6 longitudinal septa, constricted at the central septum, initially hyaline, becoming brown at maturity, with slightly paler ends, conical and narrowly rounded at the ends, not surrounded by a mucilaginous sheath.

Culture characteristics: on PDA reaching 2 cm diam. after 4 weeks at 16 °C, later with dense mycelium, circular, rough margin white at first, iron-grey after 6 weeks, reverse cinnamon, flat on the surface, without aerial mycelium. Hyphae septate, branched, hyaline, thin (see Fig. 2).

Material examined: ITALY, Arezzo Province, Bagno di Cetica, on stems of *Cytisus* sp., 1 October 2012, *Erio Camporesi* IT791 (MFLU14-0636, holotype), extype living cultures, MFLUCC 14-0238, CBS, ICMP (see Table 1).

Notes: Mirza (1968) and Ellis and Ellis (1985) have listed *Cucurbitaria cytisi* Mirza, *Cucurbitaria laburni* (Pers.) De Not., *Cucurbitaria obducens* (Schumach.) Petr. and *Camarosporium spartii* (Nees ex Fr.) Ces. & De Not. on *Cytisus* sp. We compared our collection with those species (Table 2). Molecular data analysis confirms our stain groups with *C. quaternatum* Schulzer (Schulzer, 1870), the type species of *Camarosporium* and other *Camarosporium* spp. *C. arezzoensis* however, differs in having 180–240 × 10–15 µm asci and 19–28 × 9–15 µm brown ascospores. Our new species should be considered as *Camarosporium sensu stricto* and it is not congeneric with *Cucurbitaria sensu stricto* (*Cucurbitariaceae*) (Fig. 1).

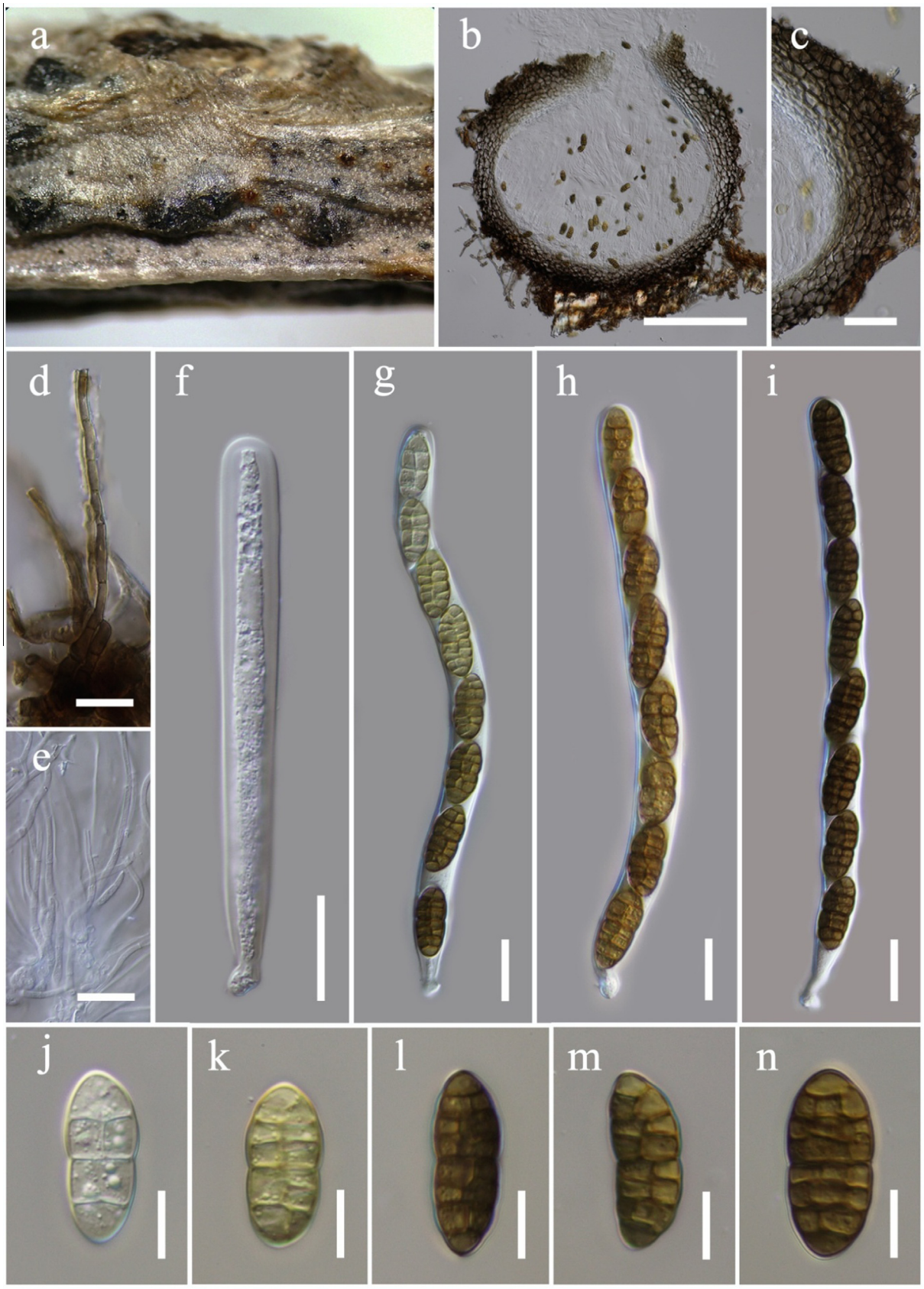

Figure 2 *Camerosporium arezzoensis* (holotype). (a) Ascomata on host substrate. (b) Section of ascoma. (c) Section of peridium. (d) Light brown hyphae around ascomata. (e) Pseudoparaphyses. (f–i) Asci. (j–n) Ascospores. Scale bars: *b* = 200 μm, *c* = 50 μm, *d–i* = 20 μm, *j–n* = 10 μm.

Table 1 Strains used in this study (Type and ex-type strains are in bold, the new taxon is indicated with an asterisk).

Taxon	Culture collection number	GenBank Accession number		
		SSU	ITS	LSU
Alternaria alternata	EN24	–	FJ809940	–
Camarosporium aloes	**CPC 21572**	–	**KF777142**	**KF777198**
Camarosporium clematidis	**MFLUCC 13-0336**	**KJ589414**	**KJ562213**	**KJ562188**
Camarosporium elongata	AFTOL-ID 1568	DQ678009	–	DQ678061
Camarosporium elongata	MFLUCC 14-0260	–	–	KJ724249
*Camarosporium arezzoensis**	**MFLUCC 14-0238**	**KP120928**	**KP120926**	**KP120927**
Camarosporium quaternatum	**CBS 483.95**	**GU296141**	–	**GU301806**
Camarosporium robinium	**MFLUCC 13-0527**	**KJ589415**	**KJ562214**	**KJ589412**
Camarosporium spartii	MFLUCC 13-0548	KJ589416	–	KJ589413
Cochliobolus heterostrophus	**ATCC 64121**	–	**JX094779**	**JX094789**
Coniothyrium palmarum	**CBS 400.71**	**EU754054**	**AY720708**	**JX681084**
Decorospora gaudefroyi	**CBS 332.63**	**AF394542**	**AF394541**	–
Leptosphaeria doliolum	**CBS 541.66**	–	**JF740206**	**JF740284**
Pleospora herbarum	**CBS 191.86**	**GU238232**	–	**GU238160**
Pleospora typhicola	CBS 132.69	JF740105	–	JF740325
Pyrenophora phaeocomes	**AFTOL-ID283**	–	**DQ491507**	**DQ499596**
Pyrenophora tritici-repentis	DAOM 226213	–	**JN943670**	JN940071
Cucurbitaria berberidis	**CBS 363.93**	**GQ387545**	JF740191	**GQ387606**
Cucurbitaria berberidis	**MFLUCC 11-0387**	**KC506800**	–	**KC506796**
Cucurbitaria berberidis	**MFLUCC 11-0386**	**KC506799**	–	**KC506795**
Pyrenochaeta nobilis	**CBS 407.76**	**EU754107**	–	**EU754206**
Pyrenochaetopsis decipiens	CBS 343.85	GQ387563	–	GQ387624
Pyrenochaeta quercina	CBS 115095	GQ387558	–	GQ387619

Table 2 Comparison of our strain with the morphologically similar species in Mirza (1968).

Name	Ascomata	Peridium	Hypostoma	Asci	Ascospore
Camarosporium arezzoensis (In this study)	Black, semi-immersed, scattered beneath the host periderm or on decorticated wood, fully or partly erumpent, globose, rough or hairy, with an ostiole	Thick, comprising 8–10 layers, outer layer heavily pigmented, thick-walled, comprising blackish to dark brown cells of *textura angularis*, inner layer composed of hyaline, thin-walled cells of *textura angularis*	Comprising numerous, filamentous, branched septate, pseudoparaphyses	8-spored, bitunicate, fissitunicate, cylindrical, short-pedicellate, apex rounded with a minute ocular chamber	Partially overlapped, mostly ellipsoidal, muriform, with 5–7 transverse septa, with 4–6 longitudinal septa, constricted at the central septum, initially hyaline, becoming brown at maturity, with slightly paler ends, conical and narrowly rounded at the ends, not surrounded by a mucilaginous sheath
Cucurbitaria ahmadi	Erumpent, globose to subglobose or obovate, papilla bearing a comparatively wide ostiole	Uniform on sides, made up of dark-brown polygonal cells	Well developed, light-brown densely interwoven hyphae	Long stipitate, 4–8 spores, spore overlapped uniseriately or biseriately	Golden-brown, 3–7 transverse septa, one longitudinal septum
Cucurbitaria ononidis	Globose to subglobose, forming a slight depression bearing ostiole, papilla lacking	Slightly rough surface sometimes provided with hair-like structures	Poorly developed, a subiculum of dark-brown	Short stipitate, 4–8 spores, spore overlapped uniseriately	Brown, 5–9 transverse septa, 1–3 longitudinal septa
Cucurbitaria elaeagni	Erumpent, globose to subglobose	Slightly rough surface, made up of elongated polygonal cells, hyaline	Well developed, brown	Long stipitate, 4–8 spores, spore overlapped uniseriately or biseriately	Golden to dull brown, 5–7 transverse septa, up to 2 longitudinal septa

Table 3 Comparison of *Cucurbitaria* species on *Cytisus* sp.

Characters	*Cucurbitaria cytisi* (Mirza, 1968)	*Cucurbitaria laburni* (Pers.) De Not. 1862	*Cucurbitaria obducens* (Schumach.) Petr. 1927	*Cucurbitaria spartii* (Nees ex Fr.) Ces. & De Not. 1863	*Camarosporium arezzoensis* MFLUCC 14-0238
Fruiting bodies (Ascomata)	Pseudothecia 300–700 µm, gregarious in groups of 2–8, erumpent, papilla	Pseudothecia 500–700 µm, black, papillate, usually in large groups seated on a black hyphalsubiculum	Pseudothecia 300–500 µm, black, papillate, usually in large groups seated on a black hyphalsubiculum	Pseudothecia300–700 × 350–610 µm diam., black or blackish brown, erumpent in clusters seated on a scanty brown subiculum	Pseudothecia 450 × 480 µm, black, semi-immersed, scattered beneath the host
Peridium	Prominently rough 55–100 µm	Prominently rough 60–100 µm	Prominently rough up to 130 µm	Prominently rough 75–160 µm	Prominently rough 30–70 µm
Asci	140–200 × 13–15 µm	156–260 × 11–16 µm	100–160 × 17–22 µm	140–200 × 13–15.5 µm	180–240 × 10–15 µm
Spore	Dark- to light-golden brown, 18–26 × 7,5–10 µm, muriform, 3 to 7 transverse septa, constricted at the central septum, longitudinal septa 1 or continuos or dis-continuos	Golden brown, 25–35 × 9–15 µm, muriform, 5 to 7 transverse septa, constricted at the central septum, 1 to 2 longitudinal septa	Olive brown, 21–30 × 8.5–13 µm, muriform, 3 to 7 transverse septa, usually 5–7 transverse septa, constricted at the central septum, 1 to 2 longitudinal septa	Golden brown, 25–30 × 11–12 µm, muriform, 5 to 7 transverse septa, constricted at the central septum, with 1 longitudinal septa	Brown 19–28 × 9–15 µm, muriform, mostly ellipsoidal, 5–7 transversely septate, with 4–6 vertical septa, constricted at the central septum, with 1–2 longitudinal septa
Host species (*Cytisus* sp.)	*C. pendulinus*, *C. scoparius*, *C. sessilifolius*	*C. alpinss*, *C. laburnum*, *C. radiatus*	*C. scoparius*	*C. capitatus*, *C. scoparius*, *Cytisus* sp.	*Cytisus* sp.
Country	Portugal, Spain, France, Italy, Sweden	Germany, England, Italy, Switzerland	Spain	Germany, Portugal, Spain, Sweden	Italy
References	Mirza (1968),Ellis and Ellis (1985)	Mirza (1968), Ellis and Ellis (1985)	Mirza (1968), Ellis and Ellis (1985)	Mirza (1968), Ellis and Ellis (1985)	This study

4. Discussion

Pleosporales is the largest order of *Dothideomycetes* (Kirk et al., 2008) and several studies have been carried out using multi-gene phylogeny, providing the groundwork towards a natural classification of the class (Nelsen et al., 2009, 2011; Schoch et al., 2009; Boonmee et al., 2011, 2012, 2014; Chomnunti et al., 2011, 2014; Liu et al., 2011, 2012; Zhang et al., 2011, 2012; Hyde et al., 2013; Wijayawardene et al., 2014c). Schoch et al. (2009) recognised the suborders *Pleosporinae* and *Massarinae* in *Pleosporales* and Zhang et al. (2012) confirmed it in their molecular data analyses. In their molecular data analyses, Wijayawardene et al. (2014a,b,c) showed that *Camarosporium sensu stricto* clusters as a distinct phylogenetic lineage in *Pleosporinae*. In our molecular data analyses (Fig. 1) we also show *Camarosporium sensu stricto* is not related to *Cucurbitariaceae*, *Pleosporaceae* or/and *Leptosphaeriaceae*.

Our combined LSU, SSU and ITS analyses show that our stain clusters with *C. quaternatum*, the type species of *Camarosporium*, with high bootstrap support 71% (Fig. 1). Recently introduced species of *Camarosporium* have been treated as host-specific (Wijayawardene et al. 2014b), but it is essential to re-collect and carry out generic revision. There are about 500 species epithets of *Camarosporium* and *Cucurbitaria* in Index Fungorum (2015) but most of the species lack good illustrations and descriptions, thus it is difficult to compare all the species with our collection. However, Mirza (1968) has accepted only 28 species based on morphological characteristics. We have compared our collection with accepted species in Mirza (1968) which have closer morphologies with our collection i.e. *Cucurbitaria ahmadi* Mirza, *Cucurbitaria ononidis* Massenot and *Cucurbitaria elaeagni* Mirza. (Table. 2). Furthermore, we compared the morphology of *C. cytisi*, *C. laburni*, *C. obducens* and *C. spartii* on *Cytisus* sp. (Mirza, 1968; Ellis and Ellis, 1985) with our strain (Table 3). Our collection has narrowly fusiform didymosporous ascospores, with mostly ellipsoidal, 5–7 transversely septate, with 4–6 vertical septa, constricted at the central septum, with 1–2 longitudinal septa, with acute ends constricted at the septum.

In this study we used morphology and phylogenetic analyses for the identification of our collection. Thus it is important to carry out molecular analyses to confirm the taxonomic and phylogenetic placement. According to the morphological and phylogenetic analysis results, we introduce our taxon (MFLUCC 14-0238) as a new species of *Camarosporium sensu stricto*. Other *Cucurbitaria* spp. should be recollected and subjected to morphological and molecular analyses as *Camarosporium sensu stricto* has cucurbitaria-like sexual states (Wijayawardene et al. 2014a,b).

Acknowledgements

Mae Fah Luang University Grant "Taxonomy and Phylogeny of selected families of *Dothideomycetes* (Grant number: 56101020032)" is thanked for supporting this study and

Plant Germplasm and Genomics Center in Germplasm Bank of Wild Species, Kunming Institute of Botany is thanked for the help with the molecular work. Erio Camporesi thanks Gigi Stagioni for his invaluable field assistance. Saowaluck Tibpromma would like to thank the Mushroom Research Foundation (MRF), Chiang Rai Province, Thailand for its continuous support. Dhanushka Udayanga, Samantha C. Karunarathna, Belle Damodara Shenoy, Jian-Kui Liu, and Hiran A. Ariyawansa are thanked for their valuable suggestions and help.

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