ORIGINAL ARTICLE

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

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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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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,
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 Camarosporum 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 Camarosporum 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 Camarosporum sensu stricto, we introduce it as a new species of Camarosporum 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 55) 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. 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, dH2O 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. cbc.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 (Swoford, 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 dolichum (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 Camarosporum 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.
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. (x = 449 ± 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 (x = 199 ± 13 μm, n = 40), 8-spored, bitunicate, fissitunicate, cylindrical, short-pedicellate, apex rounded with a minute ocular chamber. Ascospores 19–28 × 9–15 μm (x = 26 ± 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 obduens* (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 stains 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  *Camerosprium 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: $b = 200 \mu m$, $c = 50 \mu m$, $d–i = 20 \mu m$, $j–n = 10 \mu m$. 
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                       |                          |      |      |      |
| Camarosporium aloes          | CPC 21572                  | KF77142                  |      |      |      |
| Camarosporium clematidis     | MFLUCC 13-0336             | KJ589414                 | KJ562213 | KJ562188 | KF77198 |
| 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 | KJ589413 |
| Camarosporium spartii        | MFLUCC 13-0548             | –                        |      |      | –      |
| Cochliobolus heterostrophus  | ATCC 64121                 | –                        |      |      | –      |
| Coniothyrium palmarum        | CBS 400.71                 | EU754054                 |      |      | JX681084 |
| Decorospora gaudefoyi        | CBS 332.63                 | AF394542                 |      |      | –      |
| Leptosphaeria doliolam       | CBS 541.66                 | –                        |      |      | JF740206 |
| Pleospora herbarum           | CBS 191.86                 | GU238232                 | –    |      | GU238160 |
| Pleospora typica             | CBS 132.69                 | JF740105                 | –    |      | JF740325 |
| Pyrenochaetopsis phaeocomes  | AFTOL-ID283                | –                        |      |      | –      |
| Pyrenophora tritici-repentis | DAOM 226213                | –                        |      |      | JF740721 |
| Cucurbitaria berberidis      | CBS 363.93                 | GQ387545                 |      |      | GQ387606 |
| Cucurbitaria berberidis      | MFLUCC 11-0387             | KC506800                 | –    |      | KC506796 |
| Cucurbitaria berberidis      | MFLUCC 11-0386             | KC506799                 | –    |      | KC506796 |
| Pyrenochaeta nobilis         | CBS 407.76                 | EU754107                 | –    |      | EU754206 |
| Pyrenochaetopsis decipiens   | CBS 343.85                 | GQ387563                 | –    |      | GQ387624 |
| Pyrenochaeta quericina       | 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   | Black, semi-immersed,         | Thick, comprising 8–10 layers,   | Comprising numerous,       | 8-spored, bitunicate,          | Partially overlapped, mostly   |
| (In this study)             | scattered beneath the host    | outer layer heavily pigmented,   | filamentous, branched      | fissitunicate, cylindrical,    | ellipsoidal, mutiform, with    |
|                             | periderm or on decorticated    | thick-walled, comprising         | septate, pseudoparaphyses  | short-pedicellate, apex        | 5–7 transverse septa, 4–6       |
|                             | wood, fully or partly         | blackish to dark brown cells of  | overlapping uniseriately or| rounded with a minute ocular   | longitudinal septa, constricted |
|                             | erumpent, globose, rough or   | textura angularis, inner         | biseriately                | chamber                       | at the central septum, initially|
|                             | hairy, with an ostiole        | layer composed of hyaline,       |                            |                               | hyaline, becoming brown at    |
|                             |                               | thin-walled cells of textura     |                            |                               | maturity, with slightly        |
|                             |                               | angularis                       |                            |                               | paler ends, conical and        |
|                             |                               |                                 |                            |                               | narrowly rounded at the ends,  |
|                             |                               |                                 |                            |                               | not surrounded by a mucilaginous|
|                             |                               |                                 |                            |                               | sheath                         |
| Cucurbitaria ahmadi         | Erumpent, globose to           | Uniform on sides, made up of     | Well developed, light-brown | Long stipitate, 4–8 spores,    | Golden-brown, 3–7              |
|                             | subgbose or obovate, papilla   | dark-brown polygonal cells       | densely interwoven hyphae  | spore overlapped uniseriately  | transverse septa, one          |
|                             | bearing a comparatively wide   |                                  | Poorly developed, a        | or biseriately                 | longitudinal septum            |
|                             | ostiole                       |                                  | subiculum of dark-brown    |                               |                                |
| Cucurbitaria ononidis       | Globose to subgbose, forming   | Slightly rough surface           | Well developed, brown      | Long stipitate, 4–8 spores,    | Brown, 5–9 transverse septa,   |
|                             | a slight depression bearing   | sometimes provided with hair-like|                            | spore overlapped uniseriately  | 1–3 longitudinal septa         |
|                             | ostiole, papilla lacking       | structures                      |                            | or biseriately                 |                                |
| Cucurbitaria elaeagni       | Erumpent, globose to           | Slightly rough surface,          | Well developed, brown      | Long stipitate, 4–8 spores,    | Golden to dull brown, 5–7      |
|                             | subgbose                      | made up of elongated             |                            | spore overlapped uniseriately  | transverse septa, up to 2       |
|                             |                               | polygonal cells, hyaline        |                            | or biseriately                 | longitudinal septa             |
4. Discussion

Pleosporales is the largest order of Dothideomycetes (Kirk et al., 2008) and several studies have been carried out using multigene 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, 2014; Chomnunti et al., 2009; Boonmee et al., 2011, 2012, 2014; Chomnunti et al., 2008). A recent molecular phylogenetic analysis by Kirk et al. (2009) recognised the suborders Camarosporium and Cucurbitaria providing the groundwork towards a natural classification of the class. Several studies have been carried out using multigene phylogeny, with high bootstrap support 71% (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. 2014a, b), 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. In their molecular data analyses, Wijayawardene et al. (2014a,b) showed that Camarosporium sensu stricto clusters as a distinct phylogenetic lineage in Pleosporaceae. In our molecular data analyses (Fig. 1) we also show Camarosporium sensu stricto is not related to Cucurbitariaceae, Pleosporaceae or, and Leptosphaeriaceae.

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| Characters | Cucurbitaria cytisi (Mirza, 1968) | Cucurbitaria laburni (Pers.) De Not. 1862 | Cucurbitaria obducanus (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 | Pseudothecia 300–700 μm × 350–610 μm diam., black or blackish brown, erumpent in clusters seated on a scantly 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 | Prominently rough 75–160 μm |
| Asci | Dark- to light-golden brown, 18–26 × 7.5–10 μm, muriform, 3 to 7 transverse septa, constricted at the central septum, longitudinal sepa 1 or continuous or dis-continuous | Prominently rough up to 130 μm | Prominently rough up to 130 μm | Prominently rough up to 130 μm | Prominently rough 30–70 μm |
| Spore | C. pendulinus, C. scoparius, C. sessilifolius | C. alpinss, C. laburnum, C. radiatus | C. scaparius | C. capitatus, C. scaparius, Cyttis sp. | Cytisus sp. |
| Host species (Cytisus sp.) | C. cytisi, C. laburni, C. obducens | C. scaparius | C. capitatus | C. capitatus | Cytisus sp. |
| Country | Portugal, Spain, France, Germany, England, Italy, Switzerland | Germany, England, Spain, Sweden | Germany, Portugal, Spain, Sweden | 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 |
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