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Additions to *Pseudocamarosporium*; two new species from Italy

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Abstract

Two coelomycetous taxa with muriform conidia were collected from Italy, and subjected to morpho-molecular taxonomic analyses. A mega blast search showed that the new taxa had a close relationship with *Pseudocamarosporium*. Maximum-likelihood and Bayesian analyses of combined LSU, SSU and ITS sequence data also showed that these strains reside in *Didymosphaeriaceae* and cluster with *Pseudocamarosporium sensu stricto*. Following detailed morphological and molecular analyses, these are introduced as new species in *Pseudocamarosporium*. The new taxa are illustrated and compared with other known species in the genus.

Key words – *Camarosporium* – Coelomycetous fungi – Muriform – Phylogeny

Introduction

Phylogenetic studies based on analyses of DNA sequence data showed that camarosporium-like taxa are polyphyletic in *Pleosporales* and therefore Wijayawardene et al. (2014c) introduced two new genera, *viz*. *Paracamarosporium* and *Pseudocamarosporium*, to accommodate camarosporium-like taxa in *Didymosphaeriaceae*. Both *Paracamarosporium* and *Pseudocamarosporium* have pycnidial conidiomata, enteroblastic and phialidic conidiogenesis with percurrent proliferation and muriform conidia (Wijayawardene et al. 2014c). However, *Paracamarosporium* is distinct in having hyaline, smooth-walled, guttulate, bacilliform to subcylindrical microconidia (Crous et al. 2013 as *Camarosporium psoraleae* Crous & M.J. Wingf.). Crous et al. (2015) showed that *Paraconiothyrium africanum* Damm et al. grouped in *Pseudocamarosporium sensu stricto*, thus, they treated it as a species of *Pseudocamarosporium*.

We collected two coelomycetous taxa with muriform conidia from Italy, on *Quercus pubescens* and *Pinus nigra*. These taxa are morphologically, similar to *Camarosporium sensu stricto*, but a megablast search using LSU rDNA sequence data, showed that they belong in

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Didymosphaeriaceae, *Massarineae*. Maximum-likelihood and Bayesian analyses of combined LSU, SSU and ITS gene regions show these strains grouping with *Pseudocamarosporium sensu stricto*. Hence, two new species are introduced based on morphological characters and phylogenetic analyses.

Materials & methods

Collection, isolation and morphological studies

Decayed plant material collected in Italy, were placed in paper bags and/or Zip-lock bags, and brought to the laboratory. The samples were observed first under a stereoscope to locate fungal taxa. Squash mounts were made to reveal the morphology of conidiophores, type of conidiogenous cells and conidiogenesis and morphology of conidia (Sutton 1980). Thin hand-sections of conidioma were made by razor blade to examine the shape of conidiomata and arrangement of conidiophores and conidiogenous cells. Morphological characters were examined under a compound microscope (Nikon Eclipse E600 DIC microscope and a Nikon DS-U2 camera or a Nikon Eclipse 80i compound microscope fitted with a Canon 450D digital camera).

Single conidial isolation used the method in Chomnunti et al. (2014) and germinating conidia were transferred aseptically to potato dextrose agar (PDA). Germinating conidia were transferred to PDA plates and incubated at 18 °C for further growth. Colony colour and other characters were assessed after 1 to 2 weeks. The holotypes are deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand, Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP) and Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P.R. China (HKAS). Ex-type cultures are also deposited in Culture Collection at Mae Fah Luang University (MFLUCC) and Department of Plant Pathology, Agriculture College, Guizhou University, China (GUCC). Facesoffungi (Jayasiri et al. 2015) and Index Fungorum (2016) numbers were provided for new taxa.

DNA extraction, *PCR amplification and sequencing*

Colonies generated from germinated single conidia were further grown on PDA for 14 days at 18 °C. Fresh fungal mycelia were scraped from PDA using sterilized scalpels. A BIOMIGA Fungus Genomic DNA Extraction Kit (GD2416) was used to extract DNA from the scraped mycelia. The amplification of rDNA regions of the internal transcribed spacers (ITS), small subunit rDNA (SSU) and large subunit (LSU) genes was carried out by using primers ITS5 and ITS4, NS1 and NS4 and LROR and LR5 (Vilgalys & Hester 1990, White et al. 1990). Optimum conditions for amplification of ITS and LSU regions are as described in Alves et al. (2004, 2005) and for the SSU region as demonstrated in Phillips et al. (2008). Amplified PCR fragments were checked on 1% agarose electrophoresis gels stained with ethidium bromide. Purified PCR products (by minicolumns, purification resin and buffer according to the manufacturer's protocols Amersham product code: 27-9602-01) were sent to SinoGenoMax Co., Beijing, China for DNA sequencing. The nucleotide sequence data obtained are submitted to GenBank (Table 1).

Phylogenetic analyses

A megablast search was carried out to confirm the placement of the new strains in *Pleosporales* and therefore phylogenetically related sequences were downloaded from GenBank (Table 1). Since, both strains showed a closer relationship with *Pseudocamarosporium*, molecular data analyses in Hyde et al. (2013), Ariyawansa et al. (2014) and Wijayawardene et al. (2014c, 2016) were used to select c strains in *Didymosphaeriaceae*. Sequences for each gene region (LSU, SSU and ITS) were aligned using MAFFTv6 (Katoh et al. 2002, Katoh & Toh 2008), and online sequence alignment was edited under the default settings (mafft.cbrc.jp/alignment/server/). All absent genes were coded as missing data.

Both datasets were performed using maximum likelihood (ML) and Bayesian Posterior Probabilities (BYPP). Maximum-likelihood (ML) analyses was performed in RAxML (Stamatakis

2006) implemented in raxmlGUI v.0.9b2 (Silvestro & Michalak 2010). The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004). Independent Bayesian phylogenetic analyses were performed in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001) using a uniform [GTR+I+G] model, lset nst = 6 rates = invgamma; prsetstatefreqpr = dirichlet (1,1,1,1). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 10,000,000 generations and trees were sampled every 100th generation (resulting in 10,000 total trees). Phylogenetic trees were visualized with FigTree (Rambaut 2012). BS values of ML (equal or above 70%) and BYPP with those equal or greater than 0.95 of each node are shown on the upper branches.

Table 1 Strains used in this study. Type strains are in bold and newly generated sequences are in bold and marked with an asterisk

Taxon	Culture collection number	GenBank Accession number LSU	SSU	ITS
Alloconiothyrium aptrooti	**CBS 981.95**	**JX496235**		
Alloconiothyrium aptrootii	CBS 980.95	JX496234		JX496121
Austropleospora archidendri	CBS 168.77	JX496162		JX496049
Austropleospora osteospermi	LM-2009a			FJ481946
Deniquelata barringtoniae	**MFLUCC 11-0422**	**JX254655**	**JX254656**	**JX254654**
Deniquelata barringtoniae	MFLUCC 11-0257	KM214000	KM214003	KM213997
Didymosphaeria rubi-ulmifolii	**MFLUCC 14-0023**	**KJ436586**	**KJ436588**	
Didymosphaeria rubi-ulmifolii	MFLUCC 14-0024		KJ436587	
Kalmusia ebuli	**CBS 123120**	**JN644073**	**JN851818**	**KF796674**
Kalmusia italica	MFLUCC 13-0066	KP325441	KP325442	KP325440
Massarina eburnea	CBS 13969	GU301840	GU296170	
Neokalmusia brevispora	CBS 120248	JX681110		
Neokalmusia scabrispora	MAFF 239517	AB524593	AB524452	LC014575
Paracamarosporium fagi	CPC 24892	KR611905		KR611887
Paracamarosporium fagi	CPC 24890	KR611904		KR611886
Paracamarosporium fungicola	CBS 113269	JX496133		JX496020
Paracamarosporium psoraleae	CPC 21632	KF777199	KF777143	
Paraconiothyrium brasiliense	CBS 254.88	JX496171		JX496058
Paraconiothyrium cyclothyrioides	CBS 432.75	JX496201		JX496088
Paraconiothyrium estuarinum	**CBS 109850**	**JX496129**	**AY642522**	**JX496016**
Paraconiothyrium nelloi	MFLUCC 13-0487	KP711365	KP711370	KP711360
Paraphaeosphaeria angularis	CBS 167.70	JX496160		JX496047
Paraphaeosphaeria michotii	MFLUCC 13-0349	KJ939282	KJ939285	KJ939279
Pseudocamarosporium corni	MFLUCC 13-0541	KJ813279	KJ819946	KJ747048
Pseudocamarosporium lonicerae	MFLUCC 13-0532	KJ813278	KJ819947	KJ747047
Pseudocamarosporium pinicola*	**MFLUCC 14-0457**	KT211629	KT211626	
Pseudocamarosporium propinquum	**MFLUCC 13-0544**	**KJ813280**	**KJ819949**	**KJ747049**
Pseudocamarosporium quercinum*	**MFLUCC 14-0456**	**KT211628**	**KT211625**	
Pseudocamarosporium tilicola	**MFLUCC 13-0550**	**KJ813281**	**KJ819950**	**KJ747050**
Verrucoconiothyrium nitidae	CBS 119209			EU552112

Results

Phylogenetic analyses

A combined dataset of LSU, SSU and ITS gene regions is used for taxa phylogenetically close to *Pseudocamarosporium* (Wijayawardene et al. 2016). Wijayawardene et al. (2014c) introduced *Pseudocamarosporium* with five species *viz*. *P. corni* Wijayaw. et al., *P. lonicerae* Wijayaw. et al., *P. piceae* Wijayaw. et al., *P. propinquum* (Sacc.) Wijayaw. et al. and *P. tiliicola* Wijayaw. et al. (Wijayawardene et al. 2014c). Liu et al. (2015) introduced *P. cotinae* Norphanphoun et al. and Crous et al. (2015) introduced *P. africanum* (Damm et al.) Crous and *P. brabeji* (Marincowitz et al.) Crous. Hence, the genus comprises eight species.

The combined LSU, SSU and ITS data set consists of 29 strains with *Massarina eburnea* (CBS 13969) as outgroup taxon. *Pseudocamarosporium sensu stricto* grouped as a distinct clade in *Didymosphaeriaceae* with high bootstrap value in ML analysis and high PP value in Bayesian analyses (98% and 1.00 respectively) (Fig. 1).

The taxon from *Quercus* sp. closely grouped with *Pseudocamarosporium tilicola* with high bootstrap and PP values (85% and 1.00 respectively). The taxon from *Pinus* sp. grouped with *P. barbeji* and *P. corni* with low bootstrap and PP values. *Pseudocamarosporium africanum* and *P. brabeji* do not have SSU sequence data, thus, we could not include them in the analyses. The short branch lengths could resulted from lack of SSU sequence data. Moreover, Crous et al. (2015) used only LSU sequence data which also resulted in poor species distinction in Bayesian analysis.

Paraconiothyrium fungicola resides in *Paracamarosporium sensu stricto* (Wijayawardene et al. 2014c, Crous et al. 2015) and thus, we transfer it to the genus *Paracamarosporium* as a new combination.

Taxonomy

Paracamarosporium fungicola (Verkley & Wicklow) Wijayaw. & K.D. Hyde, ***comb. nov.***
Index fungorum number: IF551932

Basionym: *Paraconiothyrium fungicola* Verkley & Wicklow, in Verkley, da Silva, Wicklow & Crous, Stud. Mycol. 50(2): 331 (2004)

Pseudocamarosporium quercinum Wijayaw., Camporesi & K.D. Hyde, ***sp. nov.***
Index fungorum number: IF551300
Facesoffungi Number: FoF 00877

Etymology – Named after the generic name of host

Saprobic on dead branches and stems of *Quercus pubescens*. **Sexual morph:** Undetermined. **Asexual morph:** *Conidiomata* 420–450 µm diam., 250–280 µm high, pycnidial, immersed, erumpent, solitary, globose, unilocular, black, with a long neck. *Pycnidial wall* 50–70 µm wide, multi–layered, with 3−5 outer layers of brown–walled cells of *textura angularis*, with inner layer thin, hyaline. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 2–7 × 3–5 µm, blastic, phialidic, discrete, determinate, hyaline, smooth. *Conidia* 15–18 × 6–7.5 µm (mean = 16.41 × 6.79 µm, n = 20), oblong, mostly straight, occasionally slightly curved, muriform, with 1–4 transverse and 1–2 longitudinal septa, continuous, initially hyaline, later becoming brown to dark brown at maturity, narrowly rounded at both ends, smooth-walled.

Culture characteristics – Colonies on PDA slow growing, reaching 1.5 cm diam. after 1 week at 18 °C, dense mycelium, circular, rough margin white at first, greenish white after 1 week, flat or effuse on the surface. Hyphae septate branched, hyaline, thin.

Material examined – Italy, Province of Forlì-Cesena [FC], Pian di Spino - Civitella di Romagna, on dead branches of *Quercus pubescens* (*Fagaceae*), 2 December 2013, Erio Camporesi, IT 1552 (MFLU 15–0733, **holotype**), (HKAS 88741, **isotype**); living cultures MFLUCC 14–0456 = GUCC 54.

Notes – There have been several camarosporium-like taxa reported on *Quercus* spp. *viz*. *Camarosporium betulinum* Died. (14–20 × 5–7.5 µm), *C. juglandis* Ellis & Barthol. (12–25 × 8–12 µm), *C. kursanovii* Mekht. (7–16.3 × 7–9 µm), *C. quercus* Sacc. & Roum. (25–28 × 8–10 µm), *C. variabile* (Berk. & M.A. Curtis) Sacc. (30–60 µm long) (Ellis & Ellis 1985, Farr & Rossman 2016). Our collection is morphologically distinct from all above taxa and in molecular analyses resides in *Didymosphaeriaceae* with other *Pseudocamarosporium* species (Fig. 1)

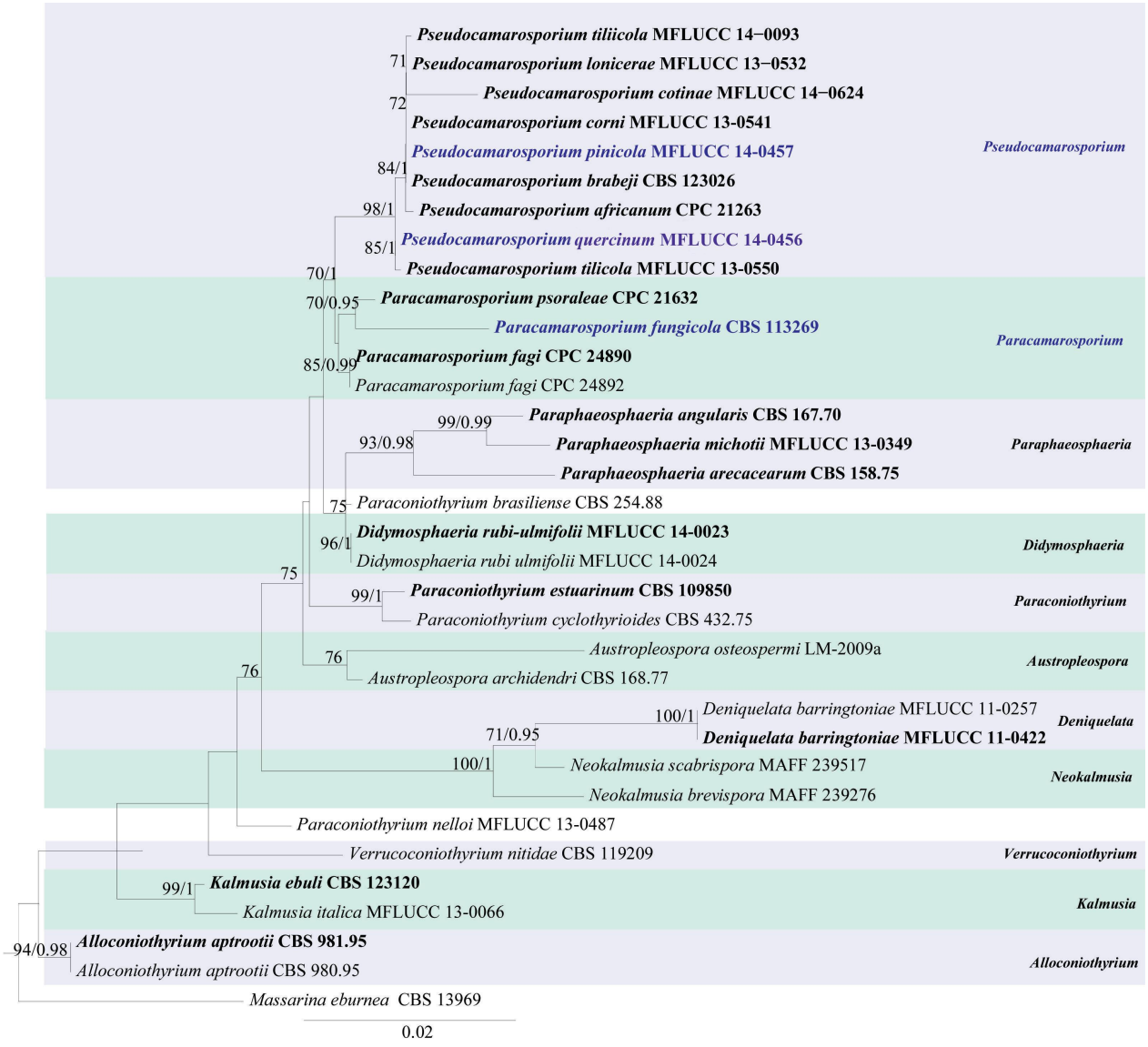

Fig. 1 – The best scoring RAxML tree resulting from the combined analyses of LSU, SSU and ITS sequence data. Bootstrap support values (>70%) for maximum-likelihood (ML) and Bayesian posterior probabilities (BYPP) (above 0.95) are given above the nodes. Ex-type strains are in bold and newly introduced species and combinations are in blue.

Pseudocamarosporium pinicola Wijayaw., Camporesi & K.D. Hyde, ***sp. nov***.
Index fungorum number: IF551302
Facesoffungi Number: FoF 00879

Etymology – Named after the generic name of host

Endophytic or saprobic on dead branch of *Pinus nigra.* **Sexual morph:** Undetermined. **Asexual morph:** *Conidiomata* 1000−1300 µm diam., 800−1100 µm high, pycnidial, immersed, globose to subglobose, unilocular, solitary, black, with a papillate, centrally located ostiole. *Pycnidial wall* multi-layered, with a thick outer layer, composed of dark brown cells of *textura*

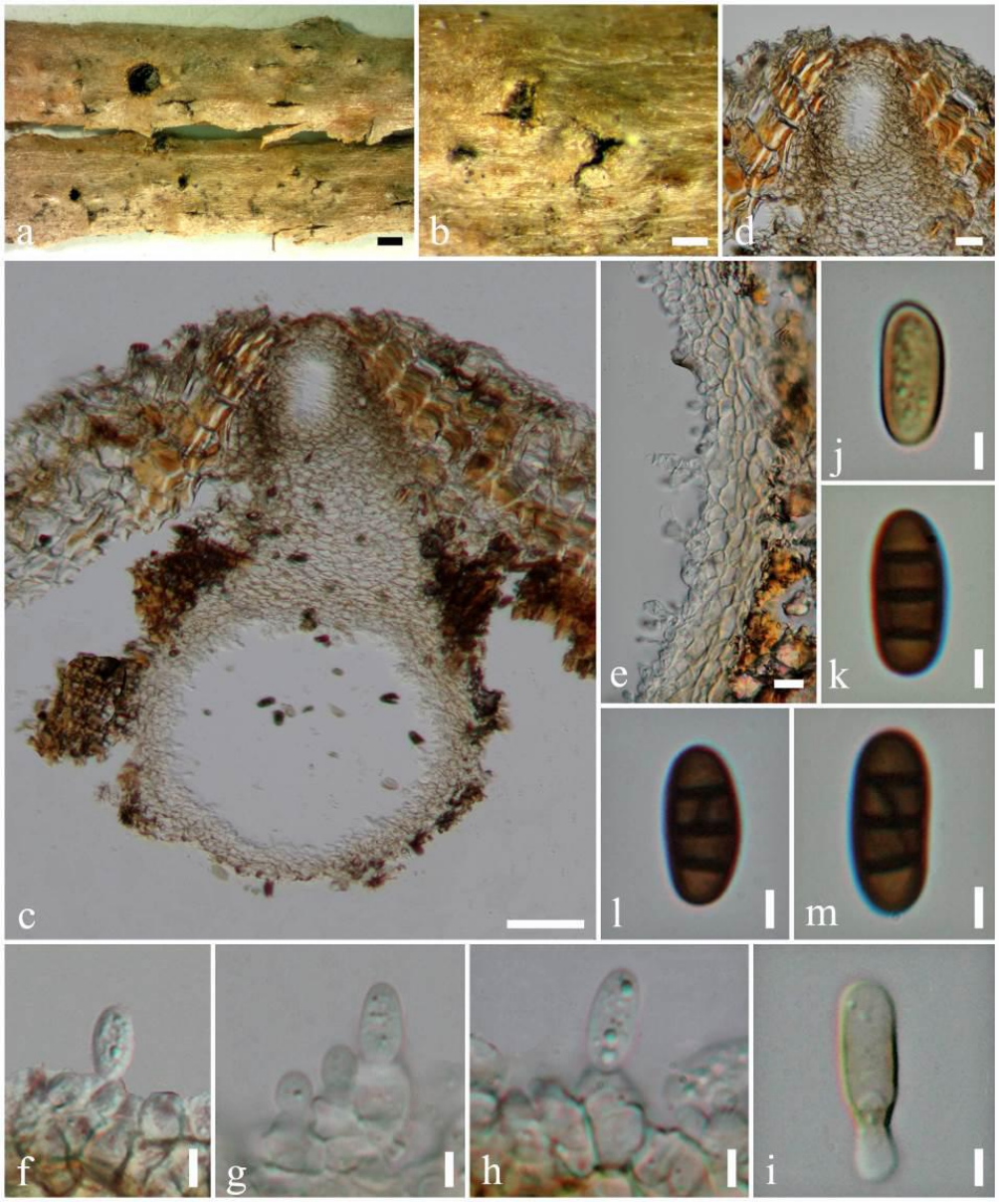

Fig. 2 – *Pseudocamarosporium quercinum* (**holotype**) **a, b.** Immersed conidiomata on dead branches of *Quercus pubescens*. **c.** Vertical section of pycnidium. **d.** Vertical section of the neck of pycnidium. **e.** Conidioma wall. **f-i.** Developing conidia attach to conidiogenous cells. **j-m.** Conidia. Scale bars: a = 500 µm, b = 200 µm, c = 50 µm, d = 20 µm**,** e = 10 µm, f-m = 5 µm.

angularis, with a thick, hyaline inner layer with cells of *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 19−21 × 16−20 µm, cylindrical to ampulliform, holoblastic, phialidic, discrete, determinate, hyaline, smooth, formed from the inner layer of the pycnidial wall. *Conidia* 35−46 × 19−24 µm (mean = 40.38 × 22.02 µm, n = 20), ellipsoid to oblong, muriform, with 3 transverse and 2 longitudinal septa, continuous, straight, occasionally slightly curved, golden to dark brown, smooth-walled.

Culture characteristics – Colonies on PDA slow growing, reaching 2 cm diam. after 1 week at 18 °C, thin mycelium, circular, rough margin white at first, white from the top, pale brown from reverse after 1 week, flat or effused on the surface. Hyphae septate branched, hyaline, thin.

Material examined – Italy, Province of Forlì-Cesena [FC], Corniolo - Santa Sofia on dead branch of *Pinus nigra* (Pinaceae), 7 December 2013, Erio Camporesi, IT 1564 (MFLU 15–0735, **holotype**) (HKAS 88743); living cultures MFLUCC 14–0457, GUCC 60.

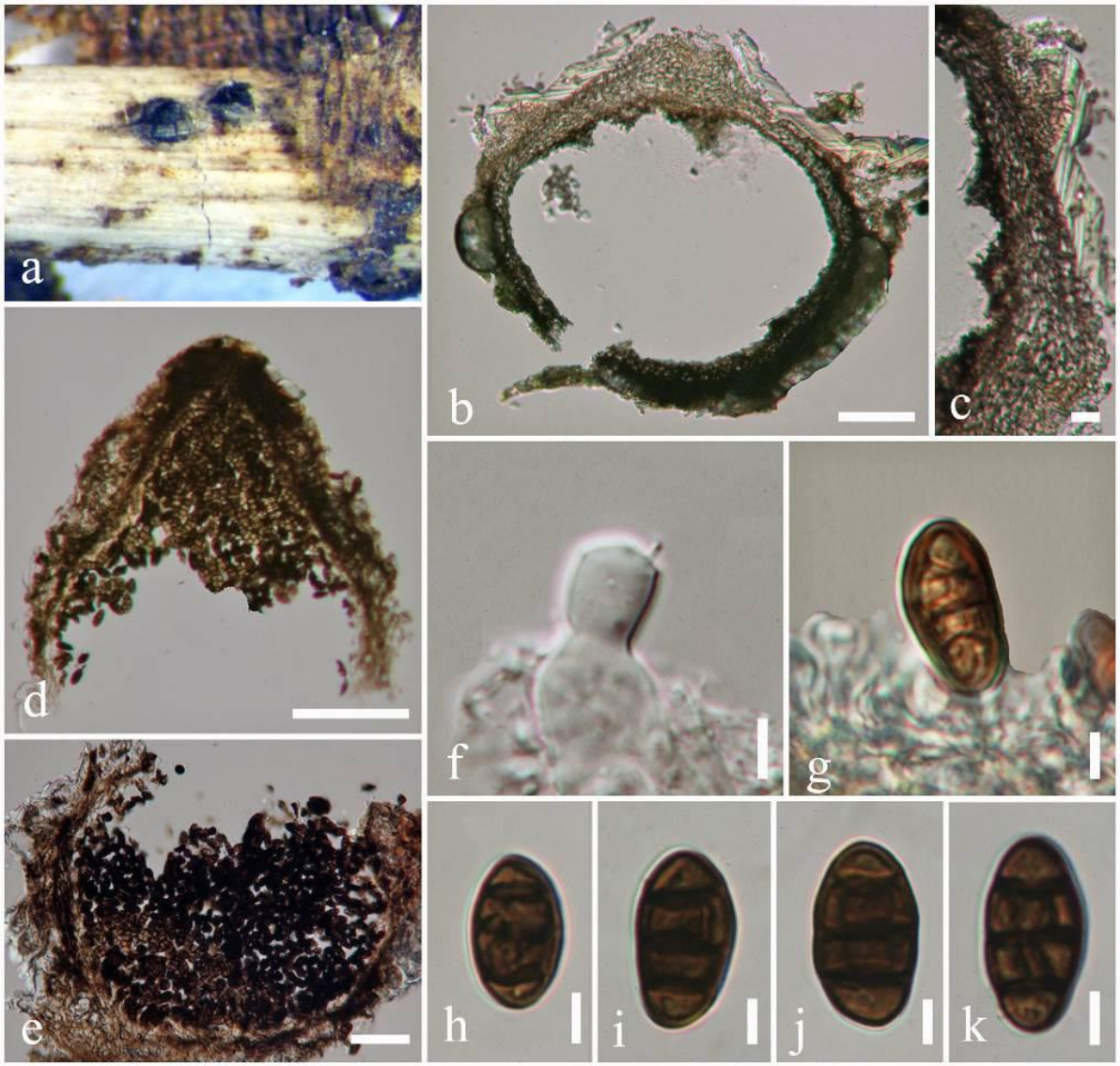

Fig. 3 – *Pseudocamarosporium pinicola* (holotype) **a.** Conidiomata on dead stem of *Pinus nigra*. **b, d, e.** Vertical sections of pycnidia. **c.** Conidioma wall. **f-g.** Conidiogenous cells and conidiogenesis. **h-k.** Conidia. Scale bars: b = 200 µm, c = 40 µm, d-e = 100 µm, f-k = 10 µm.

Notes – *Pseudocamarosporium brabeji* Marinc. et al. (14−18.5 × 7−9 µm *fide* Marincowitz et al. 2008). *Camarosporium pini* (Westend.) Sacc. (18−20 × 9−10 µm *fide* Barna et al. 2010) and *Camarosporium propinquum* (Sacc.) Sacc. have been reported previously from *Pinus* spp. (both species on *Pinus halepensis fide* Farr & Rossman 2016). Both these species are morphologically distinct from our collection. In molecular data analyses, our new strain resides in *Pseudocamarosporium sensu stricto*, but is phylogenetically distinct from *P. propinquum* (Fig. 1). Therefore, our collection is introduced as a new species.

Discussion

Morphological plasticity in coelomycetous genera confuses the classification and has been discussed by Wijayawardene et al. (2012a). Thus genera such as *Camarosporium*, *Coniothyrium*, and *Phoma* have been treated as polyphyletic in Dothideomycetes (Wijayawardene et al. 2012b). The heterogenic nature of camarosporium-like taxa was discussed by Sutton (1980) who pointed out the difference in conidiogenesis in *C. quaternatum* (Hazsl.) Schulz, the type species of *Camarosporium* and *C. propinquum* (Sacc.). Wijayawardene et al. (2014c) confirmed Sutton's observation through DNA sequence analyses, which showed that *C. propinquum* grouped in

Didymosphaeriaceae, *Massarineae*, while *Camarosporium sensu stricto* groups in *Pleosporinae* (Wijayawardene et al. 2014a). *Pseudocamarosporium* was therefore introduced to accommodate *C. propinquum* with four other species. Molecular analyses indicated that camarosporium-like taxa formed distinct phylogenetic lineages in *Pleosporales* hence *Neocamarosporium*, *Paracamarosporium* and *Suttonomyces* were introduced (Crous et al. 2013, Wijayawardene et al. 2014c, 2015). In this paper, we introduce two new species of *Pseudocamarosporium* which are distinct in both morphology and phylogeny (Fig. 1).

Crous et al. (2015) showed that *Paraconiothyrium africanum* clustered with *Pseudocamarosporium* species, thus, they transferred to *Pseudocamarosporium*. Hence, the *Pseudocamarosporium* comprises both camarosporium-like and paraconiothyrium-like species.

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