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Multi-locus phylogeny reveals the sexual state of *Tiarosporella* in Botryosphaeriaceae

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Abstract – A new taxon of Botryosphaeriaceae associated with *Arrhenatherum* and *Dactylis* was collected in Italy. Single ascospore isolates were obtained and formed asexual morphs in culture. Multi-gene phylogenetic analysis was carried out using ITS, LSU nrDNA and EF1-α sequence data and the resulting phylogenetic tree showed that new species clustered in *Botryosphaeriaceae* together with *Tiarosporella* species with high bootstrap support. The new species is introduced as a sexual morph of *Tiarosporella* and detailed descriptions and illustrations are provided. A discussion of the asexual morphs of *Tiarosporella* is provided.

Asexual morph / Poaceae / New species / Phylogeny / taxonomy

INTRODUCTION

The family Botryosphaeriaceae introduced by Theissen and Sydow (1918) is considered to be one of the largest families in the class Dothideomycetes. The members of this family comprise a wide range of morphologically diverse taxa that are pathogens, endophytes or saprobes, especially on woody hosts in terrestrial habitats (Liu *et al*., 2012; Hyde *et al*., 2013; Phillips *et al*., 2013). The family Botryosphaeriaceae is characterized by uni- to multi-locular, ascostromata,

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sparse hypha-like pseudoparaphyses, 8-spored, bitunicate, asci and hyaline to brown, aseptate to 2-septate ascospores. The asexual morph produces uni to multi-locular pycnidial conidiomata, with hyaline, phialidic conidiogenous cells and hyaline to pigmented, aseptate to septate conidia, sometimes with mucoid appendages or sheaths (Liu *et al*., 2012; Hyde *et al*., 2013).

Tiarosporella was introduced by Höhnel (1919) and is considered as an asexual genus in Botryosphaeriaceae (Jami *et al*., 2012; Phillips *et al*., 2013; Slippers *et al*., 2013) and mainly occurs on grasses, conifers and the members of Asteraceae and Zygophyllaceae (Karadžić 2003; Jami *et al*., 2012). However, the generic type of *Tiarosporella*, *T. paludosa* (Sacc. & Fiori ex P. Syd.) Höhn. is listed as an asexual state of *Darkera* (Helotiales) in *Index Fungorum* (2014) and *Tiarosporella* species have distinct cultural characteristics and conidial morphology as compared to other genera of Botryosphaeriaceae (Jami *et al*., 2012). There are 19 epithets for *Tiarosporella* in *Index Fungorum* (2014) and only *Tiarosporella africana* F. Jami, B. Slippers, M.J Wingf. & M. Gryzenhout, *T. graminis* var. *karoo* B. Sutton & Marasas, *T. madreeya* (Subram. & K. Ramakr.) Nag Raj, *T. tritici* B. Sutton & Marasas and *T. urbis-rosarum* Jami, Gryzenh., Slippers & M.J. Wingf. are resolved in Botryosphaeriaceae (Jami *et al*., 2012, 2014; Phillips *et al*., 2013; Slippers *et al*., 2013). von Arx (1981) introduced *Tiarosporella phaseolina* (Tassi) Aa based on *Macrophoma phaseolina* Tassi and synonymized the genus *Macrophomina* Petr. under *Tiarosporella* Höhn in considering the conidial morphology. However, Crous *et al*., (2006) showed that several isolates of the type of *Macrophomina*, *M. phaseolina* Petr. clustered in Botryosphaeriaceae, albeit separately from *Tiarosporella*. In our phylogenetic analysis, our strains (MFLUCC 13-0276 and MFLUCC 13-0874) clustered in the *Tiarosporella* clade along with other *Tiarosporella* species and they show typical morphological characteristics of Botryosphaeriaceae. Therefore, we introduce a new species of *Tiarosporella* in this paper, which is the sexual state.

MATERIAL AND METHODS

Sample collection, specimen examination and isolation

The specimens were collected from dead leaves of *Dactylis glomerata* (Poaceae) and *Arrhenatherum eliatus* (Poaceae) in Italy. Specimens were observed and examined under a Motic SMZ 168 stereomicroscope. Micro-morphological characters of the taxon were examined under a Nikon ECLIPSE 80i compound microscope and images were captured with a Canon EOS 550D digital camera. Observations and photographs were made from material mounted in water. Measurements were made with the Tarosoft (R) Image Frame Work and images used for figures were processed with Adobe Photoshop CS3 Extended version 10.0 software. Isolates were derived via single spore isolation following the method of Chomnunti *et al*. (2014). Ascospore germination was examined after 24 h and germinating spores were transferred to potato dextrose agar (PDA) media. The obtained pure culture was incubated at 18°C in the dark and the cultural characteristics such as mycelium colour, shape, texture and growth rate were determined. The herbarium specimens of the new species are deposited in the Mae Fah Luang University Herbarium (MFLU) and New Zealand Fungal &

Plant Disease Collection (PDD) while, ex-type culture are deposited at the Mae Fah Luang University Culture Collection (MFLUCC) with duplicates in the International Collection of Microorganisms from Plants (ICMP), Landcare Research, New Zealand.

DNA extraction, PCR amplification and sequencing

Cultures were grown on PDA at 25°C for 7 days. Extraction of genomic DNA from mycelia was carried out following a modified method of Udayanga *et al.* (2012). Polymerase chain reaction (PCR) was carried out using four partial gene portions in this study; LROR and LR5 (Vilgalys and Hester, 1990) for the nuclear ribosomal large subunit (LSU); ITS4 and ITS5 (White *et al*., 1990) for the internal transcribed spacer (ITS); EF1-728F and EF1-986R (Carbone and Kohn1999) for translation elongation factor 1-alpha (EF1-α); NS1 and NS4 (White *et al.*, 1990) the nuclear ribosomal small sub unit (SSU). The amplifications were performed in 25 µL of PCR mixtures containing 9.5 µL ddH2O, 12.5 µL 2 × PCR Master Mix (TIANGEN Co., China), 1 µL of DNA template, 1 µL of each primer (10 µm). The amplification conditions for SSU, LSU and ITS consisted of initial denaturation at 94°C for 4 min; followed by 35 cycles of 45 s at 94°C, 45 s at 56°C and 1 min at 72°C, and a final extension period of 10 min at 72°C. The PCR products were observed on 1% agarose electrophoresis gels stained with Ethidium bromide. Purification and sequencing of PCR products were carried at using the abovementioned PCR primer at Invitrogen Biotechnology Co., China.

Phylogenetic analysis

Sequences generated from LSU, SSU, EF1-α and ITS were identified by BLAST analysis and sequences were analyzed with other sequences obtained from GenBank (Table 1). *Guignardia* (*Phyllosticta*) *citricarpa* (CBS 828.97) was selected as the out group taxon. The LSU, ITS and EF1-α sequence data were aligned and combined using Bioedit (Hall 1999) and MEGA 5.0 (Tamura *et al.*, 2011) and refined visually. The phylogenetic analyses consisted of two methods: The maximum likelihood analysis was performed at the CIPRES webportal using RAxML v.7.2.8 as part of the "RAxML-HPC2 on TG" tool (Stamatakis *et al*., 2008). The general time reversible model (GTR) using proportion of invariable sites was applied with a discrete gamma distribution and four rate classes. The best scoring tree was selected with a final likelihood value of – 10419.281635. All newly generated sequences were deposited in GenBank.

RESULTS AND DISCUSSION

Phylogenetic analysis

Thirty-eight taxa were included in the combined LSU, ITS and EF1-α data set with *Phyllosticta citricarpa* (CBS 828.97) as the outgroup taxon. Maximum Likelihood analysis used 1000 bootstrap replicates and the best scoring RAxML

Table 1. GenBank and culture collection accession numbers of specimens treated in the phylogenetic study. Bold accession numbers are newly generated sequences

Taxon	*Culture Accession No.*	*GenBank Accession No.*		
		LSU	*ITS*	*EF1-α*
Barriopsis fusca	CBS 174.26	DQ377857	EU673330	EU673296
Botryobambusa fusicoccum	MFLUCC 11-0143	JX646809	JX646792	JX646857
Botryobambusa fusicoccum	MFLUCC 11-0657	JX646810	JX646793	JX646858
Botryosphaeria agaves	CBS 133992	JX646808	JX646791	JX646856
Botryosphaeria corticis	CBS 119047	EU673244	DQ299245	EU017539
Botryosphaeria dothidea	CBS 115476	AY928047	AY236949	AY236898
Botryosphaeria fusispora	MFLUCC 10-0098	JX646806	JX646789	JX646854
Cophinforma atrovirens	MFLUCC 11-0425	JX646817	JX646800	JX646865
Cophinforma atrovirens	CBS 117444	DQ377855	KF531822	KF531801
Diplodia cupressi	CBS 168.87	EU673263	DQ458893	DQ458878
Diplodia mutila	CBS 112553	AY928049	AY259093	AY573219
Diplodia mutila	CBS 230.30	EU673265	DQ458886	DQ458869
Diplodia rosulata	CBS 116470	DQ377896	EU430265	EU430267
Dothiorella iberica	CBS 115041	AY928053	AY573202	AY573222
Dothiorella sarmentorum	IMI 63581b	AY928052	AY573212	AY573235
Dothiorella sarmentorum	CBS 115038	DQ377860	AY573206	AY573223
Endomelanconiopsis endophytica	CBS 120397	EU683629	EU683656	EU683637
Endomelanconiopsis microspore	CBS 353.97	EU683628	EU683655	EU683636
Guignardia (=*Phyllosticta*) *citricarpa*	CBS 828.97	KF766334	FJ538318	FJ538376
Lasiodiplodia crassispora	CBS 110492	EU673251	EF622086	EF622066
Lasiodiplodia gonubiensis	CBS 115812	DQ377902	AY639595	DQ103566
Lasiodiplodia lignicola	CBS 134112	JX646814	JX646797	JX646862
Neodeightonia palmicola	MFLUCC 10-0822	HQ199222	HQ199221	–
Neodeightonia phoenicum	CBS 122528	EU673261	EU673340	EU673309
Neodeightonia subglobosa	CBS 448.91	DQ377866	EU673337	EU673306
Neofusicoccum arbuti	CBS 116131	DQ377915	AY819720	KF531792
Neofusicoccum mangiferae	CBS 118531	DQ377920	AY615185	DQ093221
Phaeobotryon mamane	CBS 122980	EU673248	EU673332	EU673298
Phaeobotryon mamane	CPC 12442	DQ377899	EU673333	EU673299
Pseudofusicoccum stromaticum	CBS 117448	DQ377931	AY693974	AY693975
Pseudofusicoccum stromaticum	CBS 117449	DQ377932	DQ436935	DQ436936
Spencermartinsia sp.	ICMP 16827	EU673241	EU673322	EU673289
Spencermartinsia viticola	CBS 117009	DQ377873	AY905554	AY905559
Sphaeropsis porosa	CBS 110496	DQ377894	AY343379	AY343340
Sphaeropsis visci	CBS 186.97	DQ377868	EU673325	EU673293
Tiarosporella africana	CMW38423	KC769990	KC769956	KC769852
Tiarosporella africana	CMW38425	KC769992	KC769958	KC769854
Tiarosporella dactylidis	**MFLUCC 13-0276**	**KM978949**	**KM978944**	**KP031694**
Tiarosporella dactylidis	**MFLUCC 13-0874**	**KM978948**	**KM978945**	**KP031693**
Tiarosporella graminis var. *karoo*	CBS 118718	DQ377939	KF531828	KF531807
Tiarosporella madreeya	CBS 532.76	DQ377940	KC769960	–
Tiarosporella tritici	CBS 118719	DQ377941	KF531830	KF531809
Tiarosporella urbis-rosarum	CBS 130405	JQ239420	JQ239407	JQ239394
Tiarosporella urbis-rosarum	CBS 130406	JQ239421	JQ239408	JQ239395

Abbreviations: CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: FABI, University of Pretoria, South Africa; ICMP International Collection of Micro-organisms from Plants, Landcare Research, New Zealand; CPC Collection of Pedro Crous housed at CBS; IMI International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

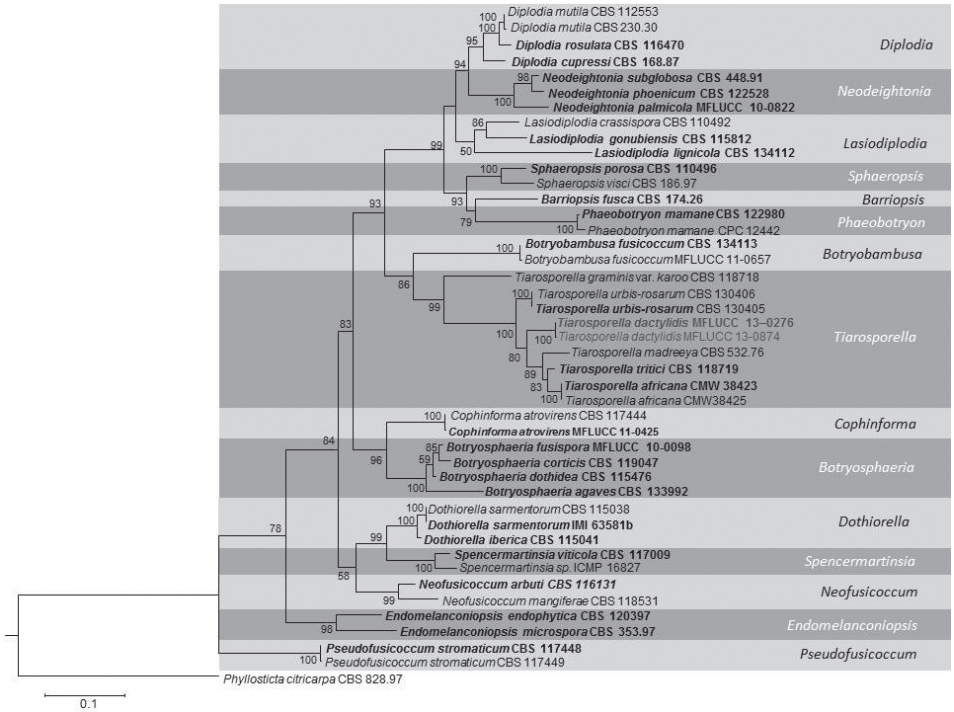

Fig. 1. Best scoring RAxML tree based on a combined dataset of ITS, LSU and EF1-α. RAxML bootstrap support values (equal or greater than 50 %) are given at the nodes. The tree is rooted to *Phyllosticta citricarpa* (CBS 828.97). Ex-type strains are in bold.

tree with a final likelihood value of – 10419.281635 is shown in Figure 1. Maximum Likelihood bootstrap values ≥ 50% are given below or above each node. The phylogenetic tree includes species of *Barriopsis*, *Botryobambusa*, *Botryosphaeria*, *Cophinforma*, *Diplodia*, *Dothiorella*, *Endomelanconiopsis*, *Lasiodiplodia*, *Neodeightonia*, *Neofusicoccum*, *Phaeobotryon*, *Pseudofusicoccum*, *Spencermartinsia*, *Sphaeropsis* and *Tiarosporella* in Botryosphaeriaceae. The *Tiarosporella* species form a distinct clade in Botryosphaeriaceae with high (86%) bootstrap support. The isolates of *T. dactylidis* (MFLUCC 13-0276 and MFLUCC 13-0874) grouped separate from other *Tiarosporella* species with strong BS (80%) support in the combined phylogeny tree and can be considered as a new species (Fig. 1).

Taxonomy

Tiarosporella Höhn., in Weese, Ber. dt. bot. Ges. 37: 159 (1919)

Index Fungorum Number: IF 10233, *Facesoffungi number*: FoF: 00333

Saprobic on grasses and woody substrates in terrestrial habitats. **Sexual morph:** *Ascomata* immersed to erumpent on host tissue, visible as black spots on host tissue, uniloculate, scattered or gregarious, globose to subglobose. *Ostiole* circular, central, papillate. *Peridium* broader at the base, comprising 2 layers: outer layer thin, of small, brown to dark brown cells of *textura angularis*, inner layer thick,

of large, hyaline to lightly pigmented, cells of *textura angularis*. *Hamathecium* comprising hyphae-like, hyaline and sparse pseudoparaphyses. *Asci* 8-spored, bitunicate, fissitunicate, clavate to cylindro-clavate, pedicellate, apically rounded with an ocular chamber. *Ascospores* uni to bi-seriate at the ascus apex, 1-seriate at the base, hyaline, aseptate, ellipsoidal, fusiform, usually wider in the middle, wall rough, surrounded by a mucilaginous sheath. **Asexual morph:** *Pycnidia* solitary, scattered or grouped, globose, dark brown to black, immersed to semi-immersed, pulvinate, unilocular or multilocular, with long necks, ostiolate. Ostiole circular and covered with hyaline to light brown or grey-olivaceous, simple, septate, straight or flexuous, smooth or verruculose pycnidial hairs with obtuse ends. *Pycnidial wall* several cell layers thick, outer layers composed of dark-brown *textura angularis*, becoming thin-walled and hyaline towards the inner region. *Conidiogenous cells* formed from the cells lining the inner walls of the pycnidia, holoblastic, determinate, hyaline, fusiform to cylindrical or ellipsoidal. *Conidia* solitary, ovoid, straight, oval to fusiform, smooth with fine granular content, thin-walled, hyaline, aseptate, apices rounded or with truncate base and obtuse apex, with or without appendages (Nag Raj 1993; Crous *et al*., 2006; Jami *et al*., 2012, 2014; Phillips *et al*., 2013).

Tiarosporella dactylidis K.M. Thambugala, E. Camporesi & K.D. Hyde, **sp. nov.**
Figs 2-3

Facesoffungi number: FoF 00334; Index Fungorum Number: IF550768
Etymology: Referring to the host genus *Dactylis*.

Saprobic on grasses in terrestrial habitats.

Sexual morph: *Ascomata* 170-215 µm high × 160-250 µm diam, pseudothecia, immersed to erumpent on host tissue, visible as black spots on host tissue, uniloculate, scattered or gregarious, globose to subglobose, ostiolate. *Ostiole* circular, central, papillate. *Peridium* up to 26-54 µm wide, broader at the base, comprising of 2 layers: outer layer of thin, small, brown to dark brown cells of *textura angularis*, inner layer of thick, large, hyaline to lightly pigmented, cells of *textura angularis*. *Hamathecium* comprising 2-3 µm wide, hyphae-like, hyaline, sparse pseudoparaphyses. *Asci* 80-160 × 17-21 µm ($\bar{x}$ = 107 × 19.2 µm, n = 15), 8-spored, bitunicate, fissitunicate, clavate to cylindro-clavate, pedicellate, apically rounded with an ocular chamber. *Ascospores* 22.5-28 × 7-8.5 µm ($\bar{x}$ = 25.2 × 8 µm, n = 25), uni to bi-seriate in the upper half, uniseriate at the base, hyaline, aseptate, ellipsoidal to fusiform, usually wider in the middle, thick-walled, smooth-walled, surrounded by a mucilaginous sheath.

Asexual morph: *Conidiomata* up to 500 µm diam, on pine needles, solitary or in groups, scattered, globose to sub globose, dark brown to black, immersed to semi-immersed on PDA, superficial on pine needles, pulvinate, unilocular, ostiolate. *Conidiomatal wall* several cell layers thick, with outer layers composed of dark-brown cells of *textura angularis*, becoming thin-walled and hyaline towards the inner region. *Conidiophores* up to 7 µm long, cylindrical, hyaline, unbranched. *Conidiogenous cells* 6.2-10 × 1.2-2.5 µm ($\bar{x}$ = 7.8 × 1.8 µm, n = 15), formed from the cells lining the inner walls of the conidiomata, phialidic, fusiform to cylindrical, determinate, hyaline. *Conidia* 2-4 × 1-2.2 µm ($\bar{x}$ = 2.7 × 1.4 µm, n = 40), solitary, ovoid, straight, oval to ellipsoidal, producing conidia at their tips, smooth, hyaline, aseptate. *Culture characteristics*: Colonies on PDA, covering 90 mm diam. petri dishes after 4 d in the dark at 25°C; circular, initially white, after 1 week becoming greyish brown to black; reverse grey to dark grayish green, white in the margin; flattened, fluffy, fairly dense, aerial, smooth surface with crenate edge, filamentous and conidia produced on pine needles after 3 weeks at 18°C.

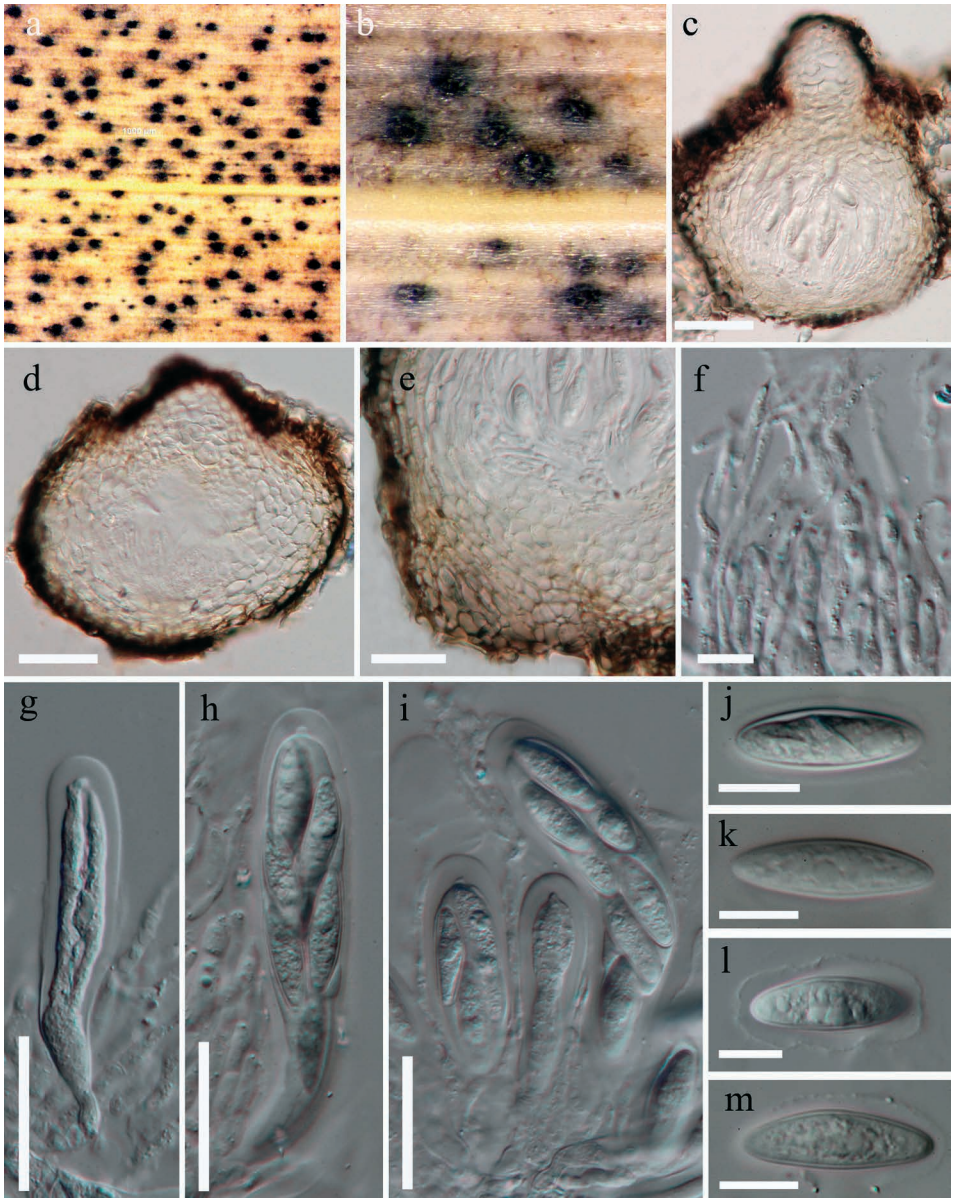

Fig. 2. *Tiarosporella dactylidis* – sexual morph (MFLU 14-0580, **holotype**). **a-b.** Ascomata on host surface. **c-d.** Section through ascomata. **e.** Peridium. **f.** Pseudoparaphyses **g.** Immature ascus. **h-i.** Mature bitunicate asci **j-m.** Ascospores. Note the inconspicuous mucilaginous sheath. Scale bars: c-d = 50 µm, e = 25 µm, f = 10 µm, g-i = 30 µm, j-m = 10 µm.

Material examined: ITALY. Teodorano – Meldola (province of Forlì-Cesena [FC]) – Italy, on dead leaves of *Dactylis glomerata* (Poaceae), 15 December 2012, Erio Camporesi (MFLU 14-0580 = holotype; isotype, PDD), ex-type living cultures = MFLUCC 13-0276 = ICMP 20383 (GenBank Accession

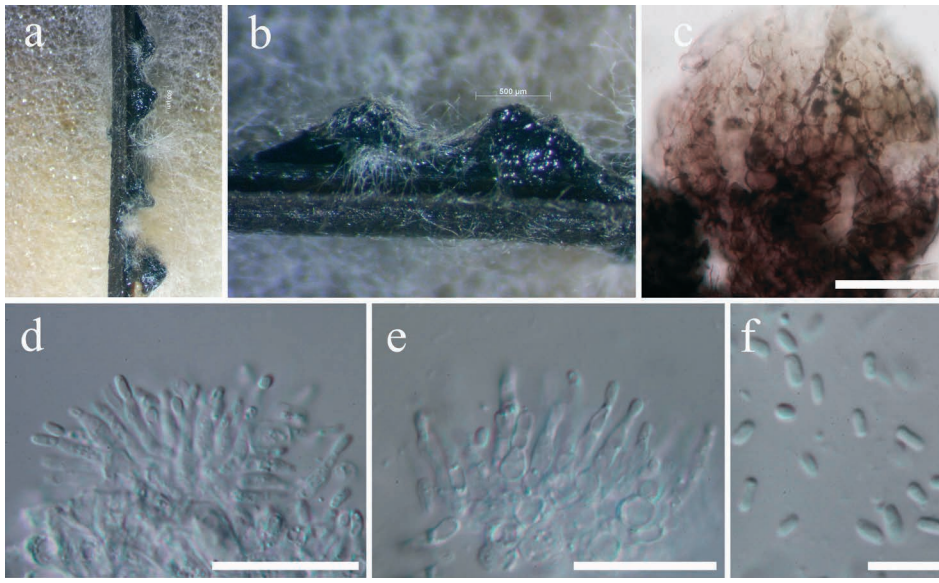

Fig. 3. *Tiarosporella dactylidis* – asexual morph (MFLU 14-0580, **holotype**). **a-b.** Conidiomata on pine needles in culture. **c.** Squash mount of conidiomata. **d-e.** Conidia developing on conidiogenous cells. **f.** Conidia. Scale bars: c = 50 µm, d-e = 20 µm, f = 10 µm, j-m = 10 µm.

No.; SSU = KM 978947); ITALY. San Paolo in Alpe – Santa Sofia (province of Forlì-Cesena [FC]), on *Arrhenatherum eliatus* (Poaceae), 20 April 2013, Erio Camporesi (MFLU 14-0114, PDD, paratypes), living cultures = MFLUCC 13-0874 = ICMP 20382 (GenBank Accession No.; SSU = KM 978948).

Notes: The sexual morph of *Tiarosporella dactylidis* is morphologically similar to *Botryosphaeria* in having globose ascomata with a central ostiole, a two-layered peridium, hyphae-like pseudoparaphyses and hyaline, aseptate, fusoid to ovoid ascospores with a mucilaginous sheath. The asexual morph of *Tiarosporella dactylidis* we observed in the culture looks similar to the spermatial states of *Botryosphaeria* (Phillips *et al*., 2013) and differs from the other asexual morphs of *Tiarosporella* (Crous *et al*., 2006; Jami *et al*., 2012, 2014). Our strains of *T. dactylidis* (MFLUCC 13-0276 and MFLUCC 13-0874) clustered in the *Tiarosporella* clade with 80% bootstrap support and this is the first sexual report for *Tiarosporella*. However, the generic type of *Tiarosporella*, *T. paludosa* has no molecular data and needs recollecting and sequencing to establish a better phylogeny.

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