


Biatriospora (Ascomycota: Pleosporales) is an ecologically diverse genus including facultative marine fungi and endophytes with biotechnological potential

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Abstract *Biatriospora* (Ascomycota: Pleosporales, Biatriosporaceae) is a genus with unexplored diversity and poorly known ecology. This work expands the *Biatriospora* taxonomic and ecological concept by describing four new species found as endophytes of woody plants in temperate forests of the Czech Republic and in tropical regions, including Amazonia. Ribosomal DNA sequences, together with protein-coding genes (*RPB2*, *EF1 α*), growth rates and morphology, were used for species delimitation and description. Ecological data gathered by this and previous studies and the inclusion of sequences deposited in public databases show that *Biatriospora* contains species that are endophytes of angiosperms in temperate and tropical

regions as well as species that live in marine or estuarine environments. These findings show that this genus is more diverse and has more host associations than has been described previously. The possible adaptations enabling the broad ecological range of these fungi are discussed. Due to the importance that *Biatriospora* species have in bioprospecting natural products, we suggest that the species introduced here warrant further investigation.

Keywords Antibiotics · Biatriosporaceae · Cryptic species · Halotolerance · Multigene phylogeny

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Introduction

Endophytic fungi live asymptotically in plant tissues (Hyde and Soyong 2008). They can influence the physiology and biochemistry of plants (Arnold and Engelbrecht 2007; Rodriguez et al. 2009; Khan et al. 2015), and many species have been shown to be useful in agriculture or drug discovery (Dreyfuss and Chapela 1994; Schulz et al. 2002; Higginbotham et al. 2013). Obligate endophytes such as *Epichloë* are not known to have other ecological roles, but in most other taxa, the endophytic stage represents only a part of their lifecycle. Non-obligate endophytes have the ability to switch from endophytic and asymptomatic to saprotrophic or parasitic on the same or different plant host (Hyde and Soyong 2008). Endophytic fungi of terrestrial plants have been also found in marine or estuarine habitats, where they can be found on submerged plant material such as mangrove plants or drifting wood (Sakayaroj et al. 2012). These fungi are classified as facultative or secondary marine fungi, meaning that they have originated in terrestrial habitat but have the ability to grow (and possibly to reproduce) in marine environments. This ecology

contrasts with obligate marine fungi, which grow and reproduce exclusively in marine or estuarine habitats (Kohlmeyer 1981; Jones et al. 2009).

Many fungal species with a marine-associated ecology have been shown to be a rich source of structurally unique and biologically active secondary metabolites (Bugni and Ireland 2004; Santos et al. 2004; Rateb and Ebel 2011; Navarri et al. 2016). The same is true for endophytes (Schulz et al. 2002; Higginbotham et al. 2013; Chagas et al. 2015; Spakowicz and Strobel 2015), and thus, fungal groups that present lineages associated with both ecologies (e.g., endophytes of marine plants) are considered favorites for bioprospecting projects (Smith et al. 2008; Debbab et al. 2012). *Biatriospora* represents one of these lineages, as it contains species that have been isolated as endophytes of terrestrial and marine-associated plants. Until this study, only two species of *Biatriospora* were known: *B. marina*, a species consistently found inhabiting tropical mangrove wood in Southeast Asia (Hyde and Borse 1986; Chinnaraj 1993; Jones et al. 2006) and *B. mackinnonii* (syn. *Pyrenochaeta mackinnonii*, *Nigrogana mackinnonii*) a species reported to produce human mycetoma (Borelli 1976; de Gruyter et al. 2013; Ahmed et al. 2014). Additionally, unidentified *Biatriospora* spp. have been reported from elm trees (Stodůlková et al. 2015) and marine sponges (Pasarini et al. 2013).

Biatriospora is known to produce an extraordinary diverse set of metabolites, including potent antibiotics (Shaw et al. 2015; Stodůlková et al. 2015). Despite the demonstrated importance in drug discovery and in human health, relatively little is known about the diversity, ecology, biogeography and bioprospecting potential of Biatriosporaceae. The aim of this study was to expand the taxonomic and ecological diversity of Biatriosporaceae by describing new species and novel host associations, mining public sequence databases recording host and habitat collection data, revising the genus taxonomy and systematics using a multigene phylogenetic approach, and summarizing the knowledge regarding the natural history of this taxa and their secondary metabolite production capabilities.

Materials and methods

Source of isolates and culturing

Nine isolates were used in this study. *Biatriospora mackinnonii* type strain CBS 674.75 was obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection. The other isolates were obtained as part of other studies targeting the endophytic diversity of diverse tree species distributed in the Czech Republic (Pažoutová et al. 2010, 2012; Koukol et al. 2015), Ecuador (Rundell et al.

2015) and Peru (Gazis and Chaverri 2015; Table 1). The material (cryopreserved cultures) from Ecuador is deposited in the Yale University Herbarium (YU) in the Peabody Museum of Natural History. Dried type specimens of other species are deposited in the National Museum in Prague (PRM) and respective ex-type cultures in the Culture Collection of Fungi in Prague (CCF). Isolates were cultured in three types of media: malt extract agar [MEA; malt extract (Oxoid) 20 g, glucose 20 g, peptone (Difco) 1 g, agar 15 g, 1000 ml distilled water, pH 5.0–6.0], potato dextrose agar (PDA; HiMedia, pH 5.7) and oatmeal agar (OA, Difco, pH 6.0). To promote sporulation, we increased salinity of MEA medium (see below), added autoclaved lupine stems to the culturing media, prolonged the incubation period (up to 1 year) and exposed the cultures to desiccation, UV light periods (254, 365 nm, 7d) and temperature shocks (5 and 10 °C for 14 days). Growth rates were measured after 7 and 14 days of incubation at 24 °C on MEA, PDA and OA. Color codes were determined according to Kelly and Judd (1976).

Tolerance to salinity

The effect of NaCl on the growth of five selected *Biatriospora* strains was determined by measuring colony diameter on MEA (Online Resource 1). Within the selected strains were representatives of *B. antibiotica*, *B. carollii*, *B. peruviansis* and *B. mackinnonii*. Nutrient media was amended with four NaCl concentrations: 0.02 M (1.2 g NaCl/l), 0.2 M (12 g NaCl/l), 0.5 M (29 g NaCl/l), 2.5 M (146 g NaCl/l) and 5 M (303 g NaCl/l), based on the scale described by Kushner (1978). According to this scale, non-halophiles have the growth optimum below the concentration of 0.2 M. Seawater has an average NaCl concentration of 0.5 M. The triplicate plates were inoculated with mycelial plugs, incubated at 24 °C and measured after 7 and 14 days (Online Resource 1). The ability to grow at the simulated sea water conditions was tested in Petri dishes with sea water agar as recommended in Kjer et al. (2010). Growth medium (pH 6.0) was prepared using malt extract (Oxoid) 20 g, glucose 20 g, peptone (Difco) 1 g, agar 15 g, 1000 ml of artificial sea water (CaCl₂·2H₂O [1.36 g L⁻¹], MgCl₂·6H₂O [9.68 g L⁻¹], KCl [0.61 g L⁻¹], NaCl [30.0 g L⁻¹], Na₂HPO₄ [0.014 mg L⁻¹], Na₂SO₄ [3.47 g L⁻¹], NaHCO₃ [0.17 g L⁻¹], KBr [0.10 g L⁻¹], SrCl₂·6H₂O [0.04 g L⁻¹] and H₃BO₃ [0.03 g L⁻¹]). An agar plug (5 mm Ø) of a test fungus, grown previously on respective agar medium, was used for inoculation in all growing tests.

DNA analyses and phylogenetic reconstruction

Genomic DNA was isolated by using the ArchivePure DNA Yeast & Gram-+Kit (5 PRIME, Hamburg). The

Table 1 List of strains studied in this study

Species	Strain	Isolation source	Location	Collector and date of isolation	Sequences accession number				
					ITS	LSU	SSU	<i>EF1α</i>	<i>RPB2</i>
<i>B. antibiotica</i>	CCF 4378 ^T	<i>Ulmus laevis</i> , phloem endophyte	Czech R., Velký Osek	A. Kubátová, K. Prášil and M. Kolařík, May 2008	JX570932.2	KF925327	KF925328	JX570934	LN626661
<i>B. antibiotica</i>	CCF 4998	Wood of living <i>Acer pseudoplatanus</i>	Czech R., Prague, Kinského zahrada	I. Kelharová, Oct 2014	LT221894	LT221895			
<i>B. carollii</i>	CCF 4884	<i>Hevea brasiliensis</i> , sapwood endophyte	Peru, Loreto, Napo	R. Gazis, Jun 2009	LN626657	LN626682	LN626674	LN626668	LN626662
<i>B. mackinnonii</i>	CBS 674.75 ^T	Black grain mycetoma, man	Venezuela	D. Borelli, Aug 1975	KF015654	KF015612	GQ387552	KF407986	KF015703
<i>B. mackinnonii</i>	CBS 110022	Mycetoma, human	Mexico	R. Arenas, Feb 2002	KF015653	KF015609	GQ387553	KF407985	KF015704
<i>B. mackinnonii</i>	YU.101027 (E9303e)	<i>Malvaviscus concinnus</i> , endophyte	Ecuador	S. Kuan, Mar 2011	JN545759	LN626681	LN626678	LN626673	LN626666
<i>B. mackinnonii</i>	YU.100463 (E5202 h)	<i>Guazuma ulmifolia</i> , stem endophyte	Ecuador, Guayaquil, Cerro Blanco	R. Dalal, Mar 2009	JX264157.2	KI605422	JX264155	JX264154	JX264156
<i>B. marina</i>	CY 1228	Mangrove wood	Singapore, Singapore mangrove forest	E.B.G. Jones	–	GQ925848	GQ925835	GU479848	GU479823
<i>B. peruviana</i>	CCF 4885 ^T	<i>Virola</i> sp., sapwood endophyte	Peru, Loreto, Napo	D. Skaltsas, Jun 2010	LN626658	LN626683	LN626677	LN626671	LN626665
<i>B. yasuniana</i>	YU.101026 ^T (E8604b)	<i>Conceveiba guianensis</i> , endophyte	Ecuador, Orellana, La Selva Lodge	C.E. Portero, Mar 2010	HQ108005	LN626684	LN626676	LN626670	LN626664
<i>Biatriospora</i> sp.	YU.101028 (E11301d)	<i>Erythrina velutina</i> , branch endophyte	Ecuador, Guayas, Guayaquil, Cerro Blanc	S.G. Lee, Mar 2010	LN626659	LN626685	LN626675	LN626669	LN626663
<i>Biatriospora</i> sp.	YU.101025 (E8502c)	<i>Tabernaemontana columbiensis</i> , endophyte	Ecuador, Guayaquil	D. Thakral, Mar 2010	HQ117845	LN626680	LN626679	LN626672	LN626668
<i>Trematosphaeria pertusa</i>	CBS 122371	<i>Platanus</i> sp.	France	M. Delponi, Nov 2006	KF015669	FJ201992	FJ201993	KF015702	GU371801
<i>Medicopsis romeroi</i>	CBS 122784	<i>Hordeum vulgare</i>	Not provided	M.M.J. Dorenbosch	KF366447	EU754208	EU754109	KF015679	KF015707

The strains analyzed for the taxonomic assessment using morphology, physiology and multigene sequencing are printed in bold. The sequences codes beginning with “LN” and “LT” were obtained during this study

nuclear ribosomal genes ITS (internal transcribed spacer), LSU (large subunit) and SSU (small subunit), and the protein-coding genes *EF1 α* (elongation factor alpha) and *RPB2* (RNA polymerase II gene) were amplified and sequenced using the primer sets listed in Table 2. The reaction mixtures and amplification protocols are described in Kolařík and Jankowiak (2013). PCR product purification and sequencing was performed at Macrogen Inc. (Seoul, South Korea). The EMBL accession numbers are listed in Table 1. Three datasets were assembled and used in the phylogenetic analyses. The first dataset was comprised of LSU sequences generated from our isolates and curated sequences representing the main lineages within the Pleosporales. This dataset was used to place our isolates in the broader context of Pleosporales and was generated based on an alignment published by de Gruyter et al. (2013) and deposited in the TreeBASE (www.treebase.org) under the code M14603 and complemented with other representative Pleosporales sequences gathered from NCBI nucleotide database (Fig. 1).

The second dataset was comprised of ITS sequences generated from our isolates and sequences obtained through the mining of NCBI nucleotide database. This dataset was used to explore the geographical and ecological range of the targeted taxa. Sequences were obtained by blasting our sequences to the NCBI database or by querying “*Biatriospora*.” The “distance tree of results” option on the BLAST results page was used to determine limits of Biatriosporaceae. Sequences that were at least 93 % similar (>90 % coverage) to our query sequences were attributable to Biatriosporaceae and were downloaded

Fig. 1 Bayesian phylogenetic tree based on LSU rDNA sequences showing the relatedness of *Biatriospora* within main lineages of Pleosporales. The dataset was generated based on an alignment published by de Gruyter et al. (2013) and complemented with close relatives gathered from NCBI nucleotide database (taxa with GenBank accession numbers). The tree is rooted with *Sporormiella minima*. Assignment to the families is according to de Gruyter et al. (2013) and Liu et al. (2014). Bayesian PP followed by Maximum likelihood BS supports are indicated. Branches with PP \geq 0.99 and BS \geq 95 are shown in *bold*

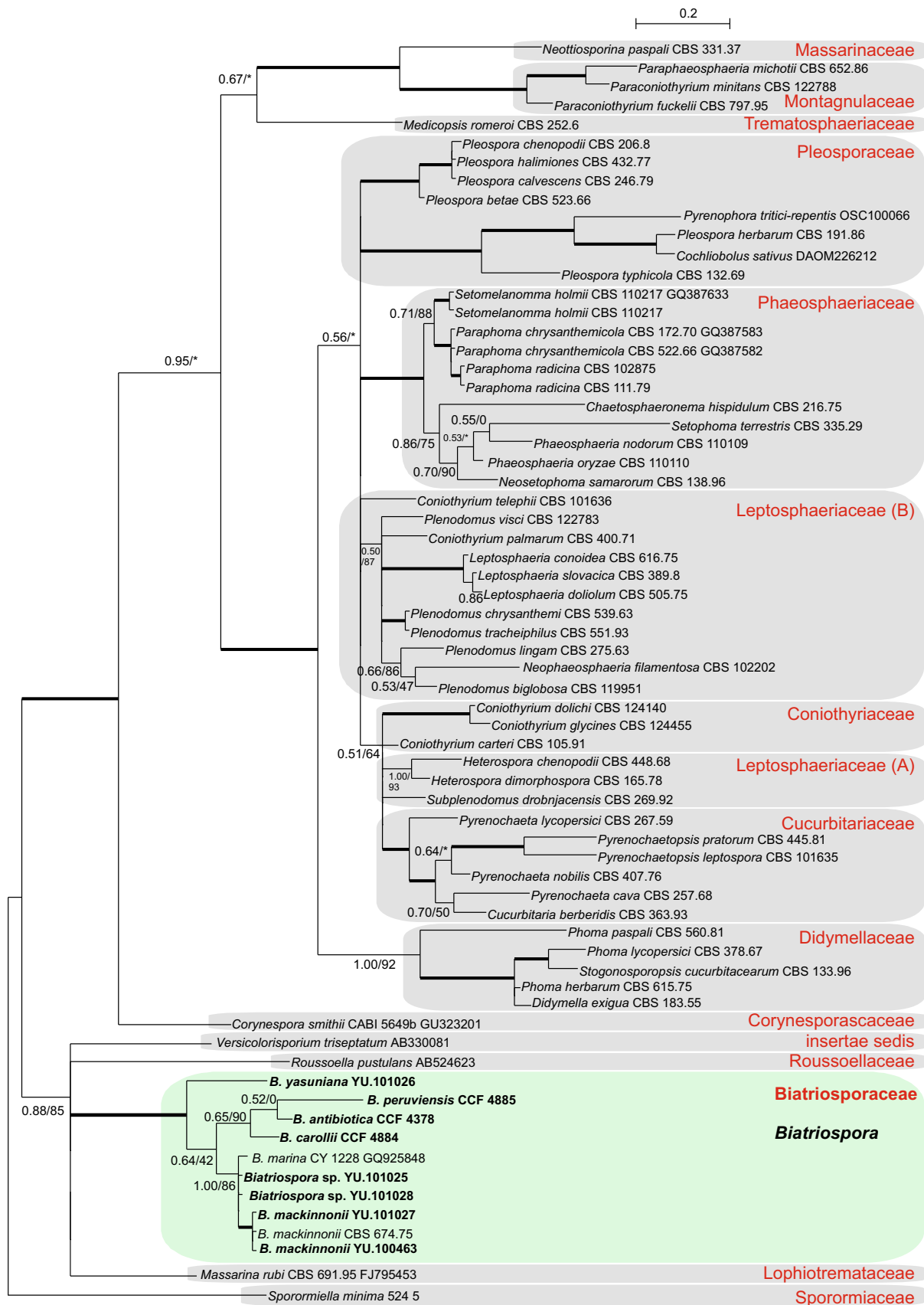
and added to the ITS dataset. Maximum likelihood phylogenetic analysis (ML) was performed to identify sequences localized outside of the Biatriosporaceae family. Such distant sequences, except for the two most related outgroup species, *Medicopsis romeroi* and *Trematosphaeria pertusa*, were removed from the final ITS dataset. From the two previously described species, *B. marina* and *B. mackinnonii*, only the sequence of the type strain from the latter was available (KF015654) (Fig. 2, Online Resource 2).

Finally, the third dataset was comprised of a multigene alignment (ITS, LSU, *RPB2*, *EF1 α*) which was used to infer phylogenetic species boundaries. Topologies of single gene ML phylogenetic trees were assessed visually for congruence of species-rank clades. Mutually exclusive, strongly supported clades (Bootstrap support, BS, \geq 60 %) were considered indicative of significant topological incongruence, and because there were no such conflicts, the alignments were concatenated.

All sequence alignments were done in MAFFT 6 using the G-INS-i strategy (Katoh et al. 2009). ML phylogenetic

Table 2 Primers used for PCR and sequencing

Locus	Primer	Primer sequence 5' to 3'	Reference
Nuclear ITS rDNA	ITS1F	CTTGGTCAATTTAGAGGAAGTAA	Gardes and Bruns (1993)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
Nuclear LSU rDNA	NL1	GCATATCAATAAGCGGAGGAAAAG	O'Donnell (1993)
	NL4	GGTCCGTGTTTCAAGACGG	O'Donnell (1993)
Nuclear SSU rDNA	NS1	GTAGTCATATGCTTGCTC	White et al. (1990)
	NS4	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
	NS5	AACTTAAAGGAATTGACGGAA	White et al. (1990)
	NS24	AAACCTTGTTACGACTTTTA	White et al. (1990)
Translation elongation factor-1 α	EF1-983F	GCYCCYGGHCA YCGTGAYTTYAT	Rehner and Buckley (2005)
	EF1-2218R	ATGACACCRACRGCACRGTYTG	Rehner and Buckley (2005)
	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)
RNA polymerase II second largest subunit	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)
	fRPB2-7cR	CCCATRGTGTYTTRCCCAT	Liu et al. (1999)
β -Tubulin	T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)
	T10	ACGATAGGTTACCTCCAGAC	O'Donnell and Cigelnik (1997)
	Bt2b	ACCCTCAGTGTAGTGACCCCTTGGC	Glass and Donaldson (1995)



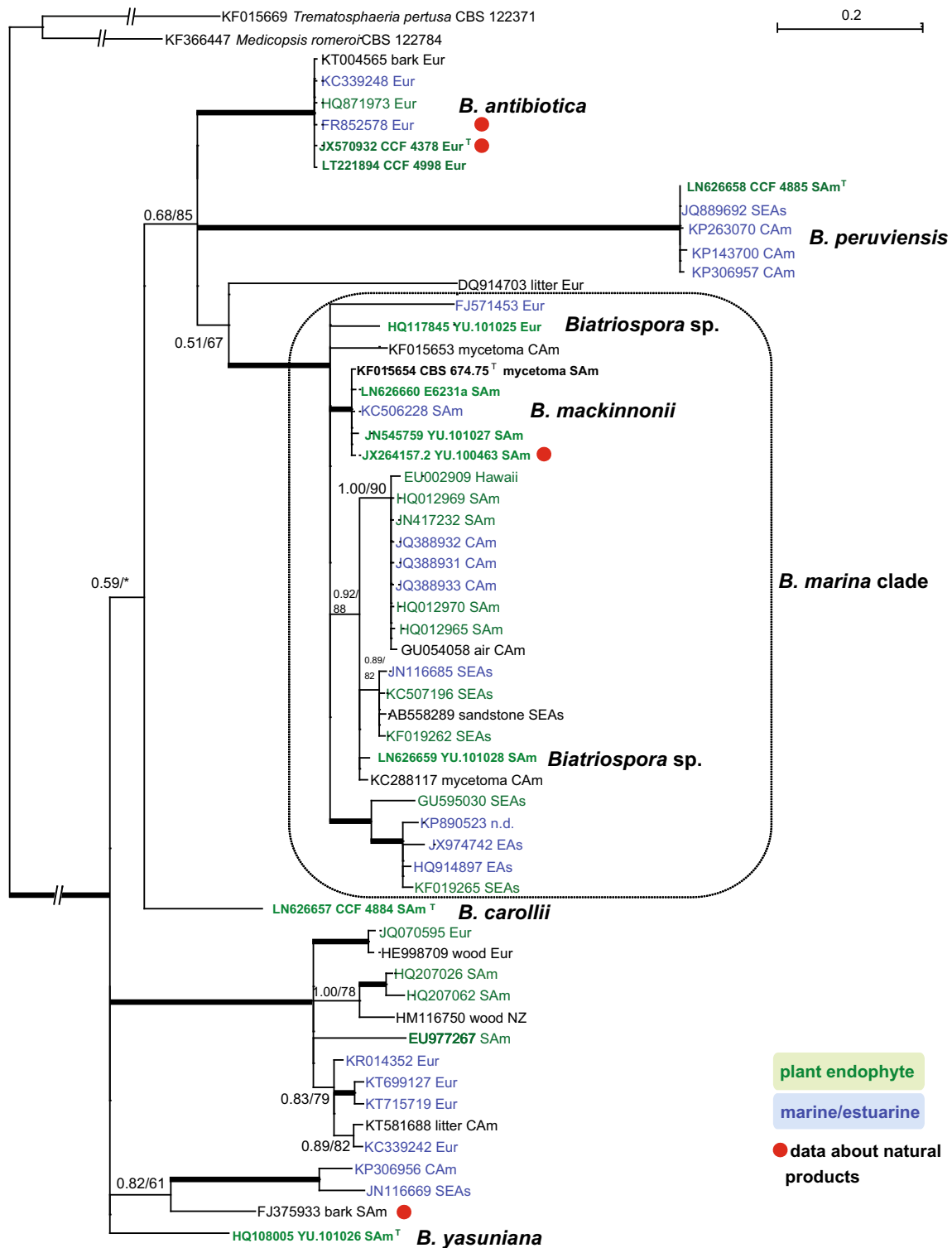


Fig. 2 Bayesian phylogenetic tree based on ITS rDNA sequences showing the relatedness of studied *Biatriospora* isolates and the most similar sequences from NCBI database. The branch leading to the outgroup (*Medicopsis romeroi* and *Trematosphaeria pertusa*) has been five times shortened. The isolates printed in bold were analyzed as part of this study. For the full details see Online Resource 1.

Bayesian PP followed by Maximum likelihood BS supports are indicated. Branches with PP ≥ 0.99 and BS ≥ 95 are shown in **bold**. Region of sequence origin is indicated. *CAM* Central America and Caribbean, *EAs* Eastern Asia, *Eur* Europe, *NAm* Northern America and Caribbean, *SEAs* Southeast Asia, *NZ* New Zealand

analyses were done in PhyML 3.1. (Guindon et al. 2010) using 500 bootstrap replicates. Bayesian phylogenetic (MB) analyses were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). A metropolis-coupled Markov chain Monte Carlo search algorithm with 2,000,000 generations was used. Trees were sampled every 1000 generations. Chain convergence was determined with Tracer 1.4 (<http://tree.bio.ed.ac.uk/software/tracer>), and the first 20 % trees were discarded as burn-in. Evolutionary models (K2 + G model for ITS and LSU, TN93 + G model for multigene dataset) were determined for all datasets using MEGA 6.06 (Tamura et al. 2013). In MB analysis of multigene dataset, the eight data partitions were recognized which include ITS, LSU and splitting of each codon position to separate partitions in *RPB2* and *EF1 α* .

The LSU alignment had 34 sequences and 3818 characters, from which 477 were variable and 272 were parsimony informative. The ITS alignment had 58 sequences and 548 characters, from which 69 were variable and 39 were parsimony informative. The multigene dataset had 13 sequences and 2777 characters from which 92 were variable and 82 were parsimony informative.

Results

Phylogeny and species delimitation

Phylogenetic analysis of the LSU rDNA dataset containing representative members of the families within Pleosporales showed that our strains can be placed in a phylogenetically well-defined clade. This lineage is sister to *Masarina rubi*, *Rousoella pustulans* and *Versicolorisporium triseptatum* (Fig. 1). Phylogenetically, the sequences can be placed in the genus *Biatriospora*, within the monotypic family Biatriosporaceae. The ITS region did not have enough informative characters to resolve relationships among species within *Biatriospora*. A total of nine distinct lineages were recovered, which included clades containing sequences from culture collections, as well as GenBank sequences (Fig. 2, Online Resource 2). However, four lineages were represented by only one sequence. Two of them consisted of the single strains CCF 4884 and YU.101026 studied during this study. Among the well-supported clades comprised by several sequences, the largest contained the type strain of *B. mackinnonii*. ITS sequence from *B. marina* was not available, but other data suggested its close relativity with *B. mackinnonii* (see below) and the whole clade could be termed as “*Biatriospora marina*” clade. In this clade, the type strain of *B. mackinnonii*, isolates YU.101027, E6231a and YU.100463 formed well-supported lineage, whereas strains YU.101025, YU.101028 and sequences gathered from GenBank formed eight independent

lineages. The *Biatriospora marina* clade is resolved as sister to the clades represented by the strains CCF 4378 and CCF 4885.

A multigene dataset of representative strains was further analyzed to delimit phylogenetic species boundaries and to resolve the relationships among the main lineages (Fig. 3). Phylogenetic reconstruction revealed four well-supported and genetically distant lineages outside the *B. marina* clade, which we describe as new species: *Biatriospora antibiotica* (CCF 4378^T), *B. carollii* (CCF 4884^T), *B. yasuniana* (YU.101026^T) and *B. peruviansis* (CCF 4885^T). *Biatriospora antibiotica* and *B. carollii* were resolved as sister clades with Bayesian posterior probability (PP) 0.92, but the relationships among the other two novel *Biatriospora* taxa were not clear due to low statistical support (PP < 80; Fig. 3). The rest of isolates, together with *B. mackinnonii* and *B. marina*, formed a well-defined clade with apparently low genetic variability, as suggested by the relatively short branch length (*B. marina* clade, Fig. 3). In this clade, *B. mackinnonii* type strain CBS 674.75 and isolates YU.101027 and YU.100463 formed a monophyletic group (PP: 1.00). Two additional strains within the *B. marina* clade, YU.101028 and YU.101025, represented separate lineages with unique haplotypes in all genes studied (except of SSU rDNA) with similarity of 98.7 and 99.5 % in ITS rDNA with the *B. mackinnonii* type strain. The other lineage is represented by the strain CBS 110022, which was not available for this study. This strain, together with two strains from the *B. mackinnonii* lineage (CBS

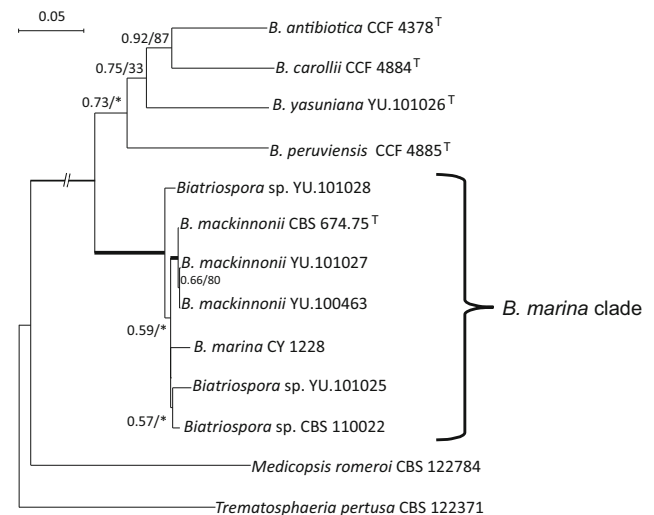


Fig. 3 Bayesian phylogenetic tree based on concatenated ITS, LSU, *RPB2*, *EF1 α* sequences showing the relationships among the lineages within the genus *Biatriospora*. The branch leading to the outgroup (*Medicopsis romeroi* and *Trematosphaeria pertusa*) has been six times shortened. Bayesian PP followed by Maximum likelihood BS supports are indicated. Branches with PP \geq 0.99 and BS \geq 95 are shown in **bold**

674.75, YU.100463), was characterized by the presence of a 334 bp insert in the SSU rDNA sequence (Fig. 3). A homologous insert having sequence similarity of 86 % or lower was found in *Flavomyces fulophazii* KP184081, *Neosetophoma samarorum* GQ387519 and several other genera from the related families within Pleosporales.

All the strains in our study remained sterile, forming smooth, hyaline, 4.5–5.0 µm wide mycelium that was sparsely branched and sometimes fragmented. The rapid growth on three types of media visually distinguished *B. peruviansis* (strain CCF 4885^T) and *B. yasuniana* (strain YU.101026^T) from the other strains; the other strains showed relatively similar growth rates (Online Resource 3). *Biatriospora antibiotica* and *B. carollii* produced a soluble red or brown pigment on agar media, whereas the other strains did not. Inside the *B. marina* clade, all strains studied had slow growth on MEA (Online Resource 3). Colonies on MEA were plane, floccose to lanose, dark gray to olive gray, without soluble pigment and with the reverse moderate olive to olive black in the colony center. Colonies on PDA were floccose, slightly wrinkled radially with a heaped cottony center, olive brown, without soluble pigment and with the reverse side a dark grayish olive color. Colonies on OA were plane to slightly wrinkled, floccose, olive gray to dark olive gray within a colony center, with the reverse olive gray.

Ecology and biogeography based on database mining

The mining of ITS sequences from the NCBI nucleotide database resulted in a total of 66 sequences that were identified as *Biatriospora* through phylogenetic analyses (Fig. 2, Online Resource 2). The majority of sequences were from Americas (25 sequences, Ecuador, French Guiana, Mexico, Panama, Peru, USA, Venezuela) or tropical parts of Asia (10 sequences, China, Cambodia, Hawaii, Thailand). Ten sequences, from which five were attributable to *B. antibiotica*, were from Europe (Czech R., Germany, Italy, Germany, Mediterranean sea, Poland) and one from New Zealand. Most of the sequences originated from terrestrial habitats (33), four from estuarine (e.g., mangrove soil and plants) and 21 from marine habitats (sea sediment, marine sponges, corals, seagrass). Based on their habitats of origin, 24 sequences were isolated as plant endophytes, 10 sequences from marine sponges or algae, nine from dead organic matter (wood, plant litter), five from roots and soil, two from a human mycetoma and three from other sources (water, air, stone). From the 37 sequences associated with plants, 28 originated from terrestrial plants and nine from estuarine plants or algae. Taxonomically, all of the host plants were angiosperms.

Tolerance to salinity

All five selected *Biatriospora* strains were able to grow in salinity concentrations of 0.5 M NaCl and in the sea water agar, although their growth optima were in lower salt concentrations (Online Resource 2). Notable was the stimulation of soluble pigment production by *B. antibiotica* and *B. carollii* on sea water agar.

Discussion

Identification and species delimitation

The family Biatriosporaceae was recovered as a distinct clade (1/100) within the larger clade (0.88/0.85) containing the families Lophiotremataceae, Roussoellaceae and *Vericolorisporium triseptatum* (Fig. 1), as previously shown by Hyde et al. (2013) and Liu et al. (2014). In this study, we introduce four new species: *B. antibiotica*, *B. carollii*, *B. peruviansis* and *B. yasuniana*. The species can be unambiguously delimited based on their phylogenetic distinctiveness and partially on their differential growth rates and pigment production. In addition, we identified several well-supported lineages within the *B. marina* clade that are morphologically undistinguishable.

Cultures of *Biatriospora* are sterile and lack complex morphological structures, which complicates their taxonomic assignment and the delimitation of species boundaries. The production of fertile pycnidia was observed in the ex-type strain of *B. mackinnonii*, CBS 674.75 (de Gruyter et al. 2013). Nevertheless, studies performed later on the same strain by us and Ahmed et al. (2014) did not observe such structures. The absence of reproductive structures in the anamorph was also reported in *B. mackinnonii* E5202 h (=YU.100463 from this study) (Shaw et al. 2015), *B. antibiotica* (Shushni et al. 2009, 2011) and *Biatriospora* sp. IBWF77-89A (Opatz et al. 2008). The whole genus seems to reproduce mainly vegetatively and seems to produce conidia only under specific conditions. A sexual state was observed only in the filed collections of *B. marina* (Hyde and Borse 1986) and *Biatriospora* sp. IBWF77-89A (Opatz et al. 2008). The faster growth rates of *B. peruviansis* and *B. yasuniana* on the three tested nutrient media set them apart from the rest of *Biatriospora* species. The rest of the strains have relatively similar growth rates, and thus, this character has limited taxonomic utility (Online Resource 3). Another potential diagnostic character is the production of a red pigment, which has been previously characterized as mix of pleorubines (Stodůlková et al. 2015). This character is present only in *B. antibiotica* and *B. carollii*, reaffirming their evolutionary relatedness as suggested by multi-loci analyses (Fig. 3).

The *B. mackinnonii* clade was distinct from the *B. marina* CY 1228 (Fig. 3), which was used by Suetrong et al. (2009) to represent this species. The same strain was used by Ahmed et al. (2014), together with CBS 110022 and *B. mackinnonii* type strain CBS 674.75, concluding that both species cannot be unambiguously separated, even by using five genes. Our analysis conducted on the larger set of sequences suggests both species to be genetically distinct. Additional strains, including the *B. marina* type specimen, and potentially additional markers, are necessary for resolving the taxonomy in this lineage.

An SSU rDNA insert was found in strains within the *B. marina* clade. The presence of an insert in the SSU rDNA, mostly identified as group I introns, is widespread in fungi, and their sporadic distribution among closely related fungi suggests that the inserts can be gained and lost relatively rapidly (Nikoh and Fukatsu 2001; Haugen et al. 2005). Based on the inconsistency of its presence among *Biatriospora* species, we believe that this insert has no taxonomic value.

This study expands the concept of *Biatriospora* by reporting a diversity that was previously unknown. By analyzing our fungal collections and mining ITS databases, we have uncovered at least 20 lineages that potentially represent taxa at the species level (Fig. 2). Given our findings, we believe there is yet more diversity to be discovered in *Biatriospora*.

Ecology and biogeography of *Biatriospora*

Biogeographically, species from *Biatriospora* are primarily inhabitants of tropical regions worldwide, with an apparent higher diversity in the Americas, where some species [*B. mackinnonii*, *Biatriospora* sp. CBS 110022 (Ahmed et al. 2014) and *Biatriospora* sp. L3396 (Santos et al. 2004)] have been isolated as human pathogens. Our results show that *Biatriospora* contains species that can live as endophytes of plants in terrestrial ecosystems (in both tropical and temperate forests) and also as species associated with plant material found in marine habitats. A large proportion of the sequences obtained through the ITS mining originated from fully marine environments (e.g., sea sediments and sponges) and were isolated using sea water agar (Paz et al. 2010; Xing and Guo 2011; Panno et al. 2013; Passarini et al. 2013; Supaphon et al. 2014; Bolaños et al. 2015) (Online Resources 1). The isolation methods suggest that these fungi were actively growing in the marine habitat where they were found and indeed the isolates grew on media containing 0.5 M NaCl and sea water and can be considered halotolerant (Zak and Wildman 2004). This pattern has also been reported for other terrestrial fungi (e.g., *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Trichoderma*) that are frequently isolated from

marine sponges, corals and deep sea sediments habitats using media based on sea water agar (Paz et al. 2010; Panno et al. 2013; Passarini et al. 2013; Zhang et al. 2014; Bolaños et al. 2015; Navarri et al. 2016; Xu et al. 2016). Most of the terrestrial ascomycetes tolerate NaCl concentrations that are similar to sea water conditions (Tresner and Hayes 1971), but the possibility that some species can actively live in both terrestrial and marine environments has been under debate (Kohlmeyer and Volkmann-Kohlmeyer 2003). However, the studies on the model species *Aspergillus flavus* (Ramírez-Camejo et al. 2012) and *A. sydowi* (Rypien et al. 2008) suggested that true marine populations (e.g., parasites of corals) are undistinguishable from terrestrial populations. As a result, the facultative marine fungi such as *Aspergillus*, *Fusarium* or *Penicillium* are considered terrestrial, but adapted to marine environments (Damare et al. 2012). These results are consistent with *Biatriospora* as primarily terrestrial genus that is also compatible with marine habitats. The halotolerance of these fungi could be linked to their pathogenic potential, as has been previously suggested by De Hoog et al. (2005).

Biotechnological potential

Marine-derived and endophytic fungi have been shown to be a rich source of structurally unique and biologically active secondary metabolites (Bugni and Ireland 2004; Chagas et al. 2015). Factors suggested to have contributed to the high number of diverse and unique secondary metabolite gene clusters present in marine and endophyte fungi include the need to interact and evade their host's immune system (e.g., plant, sea sponge, corals) (Ravindran et al. 2012a), to deter herbivores and other microbes (Kusari et al. 2012) or to cope with abiotic stresses posed by their environment (Debbab et al. 2012). Consequently, these habitats are usually occupied by diverse assemblages of fungi but are dominated by a limited group of genera (e.g., *Acremonium*, *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*). These taxa have been called the “creative fungi” (Dreyfuss and Chapela 1994) from which the majority of novel compounds have been described (Debbab et al. 2012). These “creative fungi” typically have numerous and diverse secondary metabolite gene clusters in their genomes. For example, *F. avenaceum* has 24 polyketide synthase (PKS) clusters, which is the largest number among all known *Fusarium* species (Hansen et al. 2015), and the model organism, *A. nidulans*, has 29 PKS clusters (Ahuja et al. 2012). *Biatriospora mackinnonii*, with 32 PKS clusters, exceeds the number of PKS clusters of any known *Fusarium* or *Aspergillus* isolate (Shaw et al. 2015). Furthermore, many of these gene clusters encode previously unknown and unique secondary metabolites. *Biatriospora antibiotica*, is known to produce 20 different

secondary metabolites, of which 12 were first described in this fungus (Opatz et al. 2008; Shushni 2009; Shushni et al. 2009, 2011) (Online Resource 2). The structural diversity of the produced secondary metabolites is vast and includes a macrolide (balticolid) (Shushni et al. 2011), naphthalenes (balticols), a benzopyrane (altechromone A) (Shushni et al. 2009), an azaanthraquinone (6-deoxybostrycoidine) (Shushni 2009; Stodůlková et al. 2015) and various naphthoquinones, including a rich set of pyranonaphthoquinone antibiotics (Stodůlková et al. 2015). The biological activities of *B. antibiotica* metabolites include antiviral (balticolid, balticols) (Shushni et al. 2009, 2011), antibacterial (6-deoxybostrycoidine, herbarin) (Schuffler et al. 2009; Shushni 2009), antifungal (ascomycone A, B, herbarin) (Opatz et al. 2008; Schuffler et al. 2009), anti-inflammatory (Balticol D) and cytotoxic against animal cells (6-deoxybostrycoidine, pleorubrin B, herbarin) (Gu 2009; Heimberger et al. 2015; Stodůlková et al. 2015). Potentially positive effectors of the plant–endophyte symbiosis include altechromone A, which promotes plant root growth (Kimura et al. 1992). Other identified products such as balticofuran and herbarin have antioxidant properties (Shushni 2009; Heimberger et al. 2015), reducing oxidative stress that can arise as a result of salinity stress affecting the fungi itself or its plant host (Ravindran et al. 2012a, b). Other secondary metabolites with potential industrial applications include the odd chain polyene produced by *B. mackinnonii*, which has a suggested application as a biofuel (Shaw et al. 2015) and the heptaketides-ascomycone A-C produced by *Biatriospora* sp. IBWF77-89A, which has antifungal activities (Fig. 2, Online Resource 2). The latter demonstrates the diversity of secondary metabolites produced by *Biatriospora*, and it prompts the hypothesis that this broad activity of chemical production is involved in enabling this fungus to live as plant endophyte as well as facultative marine fungus. In summary, this is the first study to map the biogeography, ecology and diversity of *Biatriospora*. These mostly tropical fungi have a broad ecology, spanning from plant endophytes to facultative marine fungi. Our study describes four new species of *Biatriospora* and provides a framework for further taxonomic evaluations of this important genus with significant medical and biotechnological potential.

Taxonomic treatment

Biatriospora antibiotica M.Kolařík & Kubátová, **sp. nov.** —TYPE: Czech republic, Velký Osek, Libický luh forest, 50°5'52.5" N, 15°10'5.9" W, 190 m a. s. l., from phloem of living *Ulmus laevis* (Ulmaceae), May 2008, A. Kubátová, K. Prášil and M. Kolařík AK165/08 (holotype: PRM

933240, dried ex-type culture CCF 4378T; isotype: PRM 933241). [Mycobank # MB 815910] (Fig. 4).

Diagnosis: Dark gray colonies on MEA, 14 mm in diameter (24 °C, 14 days), producing soluble reddish pigment on MEA and dark brown pigment on PDA, mycelium sterile and gray colored.

Description: Colonies on MEA after 14 days at 24 °C reaching a diameter of 14 mm, dark gray (ISCC-NBS No. 266), plane to slightly furrowed with heaped central part, effuse with slightly ruffled margin, soluble moderate reddish brown (No. 43) pigment, reverse dark grayish reddish brown (No. 47) to dark reddish brown (No. 44). Colonies on PDA reaching a diameter of 20.5 mm more light colored and with dark brown (No. 59) soluble pigment. Colonies on OA at 24 °C, 14 days reaching a diameter of 24.5 mm, plane, floccose, grayish olive (No. 110) to dark gray (No. 266) in colony center, no soluble pigment, reverse grayish olive. Mycelium sterile, gray colored, smooth, 2.3–3.9 µm wide, sparsely branched, often fragmenting. Teleomorph unknown.

The above description is based on the ex-type culture and corresponds with the other strain, CCF 4998 which has an identical ITS–LSU sequence.

Etymology: From the Latin adjective “*antibiotica*” (fem.), antibiotic, intended to mean producing antibiotic.

Habitat and distribution: *Ulmus*, *Fraxinus* and *Vitis* phloem and wood, *Phragmites* roots, isolated from various marine habitats in the Europe (Czech Republic, Italy, and Germany) and the USA (California).

Additional material examined: Czech Republic, Prague, Kinského zahrada, 50°04'46.6"N, 014°23'54.2"W, 220 m a. s. l., from wood of living *Acer pseudoplatanus*, 8 Oct 2014, I. Kelnarová, culture CCF 4998.

Notes: The distinctive pigment production on PDA is shared with *B. carollii*, which also has similarly slow growth rates. These two species differed by pigment production on MEA and can be reliably separated by molecular data (sequence similarity: ITS: 95.7 %, LSU: 99.5 %). The holotype was isolated at the frequency of 0.002 % from all isolates obtained from *Ulmus laevis* wood and phloem during 2005–2008. The morphologically identical strain CCF 4998 was isolated from single wood/phloem drill core out of 112 cores taken from *Acer pseudoplatanus* stems. Its ITS rDNA sequence is identical with GenBank entries KT004565, KC339248, HQ871973 and FR852578, which were isolated from necrotic lesion on a *Fraxinus excelsior* stem in Poland (Kowalski et al. 2016), from a *Posidonia oceanica* root mat in Italy (Panno et al. 2013), roots of an littoral plant *Phragmites australis* plant in Italy (Angelini et al. 2012) and from a drifting wood from the Baltic, Germany (Shushni et al. 2009). The unpublished GenBank entry KR909174 (1 bp

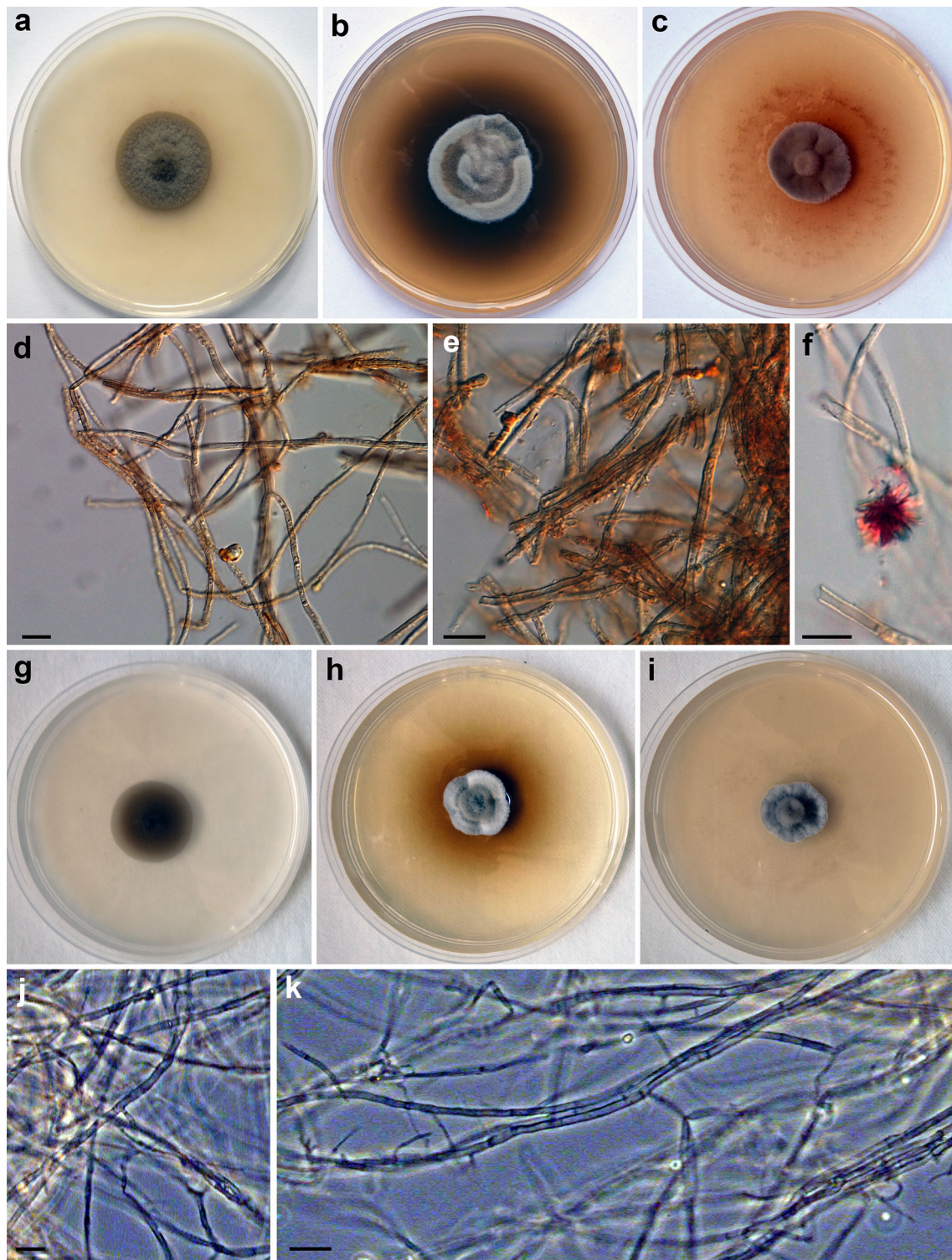


Fig. 4 Morphology of *Biatriospora antibiotica* CCF 4378^T. Colony grown on OA (a), PDA (b) and MEA (c) after 14 days in 25 °C; micromorphology on MEA after 14 days (d, e); crystals produced on

MEA medium (f). Morphology of *B. carollii* CCF 4884^T. Colony grown on OA (g), PDA (h) and MEA (i) after 14 days in 25 °C; micromorphology on MEA after 14 days (j, k). Bar 10 μm

difference in ITS rDNA), isolated from the wood of *Vitis vinifera* in California, shows that the fungus occurs also outside of Europe. Twenty different natural products have

been identified from this species (Shushni et al. 2009, 2011, 2013; Stodůlková et al. 2015) (Online Resources 2).

Biatriospora carollii M.Kolařík & R.Gazis, **sp. nov.** — TYPE: Peru, Loreto, Napo, Amazon Conservatory of Tropical Studies (ACTS) biological station, 3°14'52.3"S, 72°54'53.8"W, ~100 m a. s. l., endophytic on living sapwood of wild *Hevea brasiliensis* (Euphorbiaceae), Jun 2009, R. Gazis IQ89 (holotype: PRM 933239, dried ex-type culture CCF 4884T). [Mycobank # MB 815911] (Fig. 4).

Diagnosis: Dark gray colonies on MEA, 16.25 mm in diameter (24 °C, 14 days), producing dark brown soluble pigment on PDA, but not on MEA, mycelium sterile and gray colored.

Description: Colonies on MEA after 14 days at 24 °C, reaching a diameter of 16.25 mm, plane, floccose with heaped central part, medium gray (ISCC-NBS No. 265) to dark gray (No. 266) with paler margin, no soluble pigment, reverse dark grayish brown (No. 62). Colonies on PDA reaching a diameter of 16 mm, plane, effuse, light gray (No. 264) and with strong brown (No. 55) soluble pigment, reverse brownish black (No. 65). Colonies on OA at 24 °C, 14 days reaching a diameter of 21 mm, plane, sparse mycelium, deep yellowish brown (No. 75), no soluble pigment and reverse deep yellowish brown. Mycelium sterile, hyaline to light gray, smooth, 1.6–3.3 µm wide, often fragmenting. Teleomorph unknown.

Etymology: Named in honor of George C. Carroll for his significant contribution to endophyte research.

Habitat and distribution: Endophyte of *Hevea brasiliensis* in Peru.

Notes: See *B. antibiotica* for distinguishing characters. The description of this species is only based on the holotype, which was isolated in a frequency of 0.5 % from a total of ~200 isolates, collected from 15 individual trees during a survey of phloem and leaf endophytes of *Hevea brasiliensis* trees in Peru in 2009.

Biatriospora peruviansis M.Kolařík & R.Gazis, **sp. nov.** —TYPE: Peru, Loreto, Napo, Amazon Conservatory of Tropical Studies (ACTS) biological station, 3°14'56.78"S, 72°54'32.84"W, ~100 m a. s. l., endophytic on living sapwood of wild *Virola* sp., Jun 2010, D. Skaltsas PNB-30-5-6A (holotype: PRM 933263, dried ex-type culture CCF 4885T). [Mycobank # MB 815913] (Fig. 5).

Diagnosis: Dark gray colonies on MEA, 34.75 mm in diameter (24 °C, 14 days), without soluble pigment, mycelium sterile and gray colored.

Description: Colonies on MEA after 14 days at 24 °C reaching a diameter of 34.75 mm, plane, floccose with slightly heaped colony center, dark gray (ISCC-NBS No. 266) with blackish green (No. 152) colony center, no soluble pigment, reverse brownish black (No. 65). Colonies on PDA reaching a diameter of 39.4 mm, plane, floccose to

lanose, dark greenish gray (No. 156) with paler margin, no soluble pigment, reverse greenish black (No. 157). Colonies on OA at 24 °C, 14 days reaching a diameter of 34.75 mm, plane, floccose, dark grayish yellowish brown (No. 81), no soluble pigment, dark grayish yellowish brown. Mycelium sterile, smooth to granulose, 2.1–3.2 µm wide, brownish gray colored, producing mycelial tufts, sparsely branched. Teleomorph unknown.

Etymology: The epithet “*peruviansis*” refers to the Peru (Peruvia in Latin) where the holotype was collected.

Habitat and distribution: Endophyte of *Virola* sp. (Peru) and *Melia toosendan* (China), mangrove soil (China) and four species of marine sponges (Pacific and Caribbean Panama).

Notes: It resembles *B. yasuniana* by the fast growth rate. The sequence similarity with *B. yasuniana* was of 94.9 % in ITS and 96.7 % in LSU rDNA. The description of this species is only based on the holotype, which was isolated in a frequency of 0.25 % from a total of ~400 isolates, collected from 50 individual trees during a survey of phloem and leaf endophytes of woody plants in Peru in 2010.

The ITS rDNA of the strain CCF 4885^T is identical to GenBank entry HF12700 (unpublished) isolated from the mangrove soil in China. It also clustered with several sequences (e.g., KP322773, KP143700, JN903539) obtained from marine sponges in the Caribbean and Pacific side of Panama (Bolaños et al. 2015) and Brazil (Passarini et al. 2013) and sequence KF881748 isolated as endophyte of *Melia toosendan* in China (unpublished) (Fig. 2, Online Resource 2). The isolates from the marine sponges were isolated using the agar medium with artificial sea water and were considered to be actively growing in the seawater environment (Bolaños et al. 2015, Passarini et al. 2013).

Biatriospora yasuniana M.Kolařík & D.Spakowicz, **sp. nov.**—TYPE: Ecuador, Orellana, Yasuni National Park, La Selva Lodge, 0°49'00"S, 76°37'00"W, 241 m a. s. l., endophyte on *Conceveiba guianensis* (Euphorbiaceae), 10 Mar 2010, Carolina E. Portero E8604b (holotype: YU.101026T). [Mycobank # MB 815914] (Fig. 5).

Diagnosis: Dark gray colonies on MEA, 35.5 mm in diameter (24 °C, 14 days), without soluble pigment, mycelium sterile and gray colored.

Description: Colonies on MEA after 14 days at 24 °C reaching a diameter of 35.5 mm, plane, floccose to funiculose with heaped and lanose colony center, dark gray (ISCC-NBS No. 266) to olive gray (No. 113), no soluble pigment, reverse dark grayish brown (No. 62). Colonies on PDA at 24 °C, 14 days reaching a diameter of 38 mm, slight radially wrinkled, light brownish gray (No. 63) to olive gray (No. 113) in some parts, no soluble pigment, reverse olive black (No. 114). Colonies on OA at 24 °C,

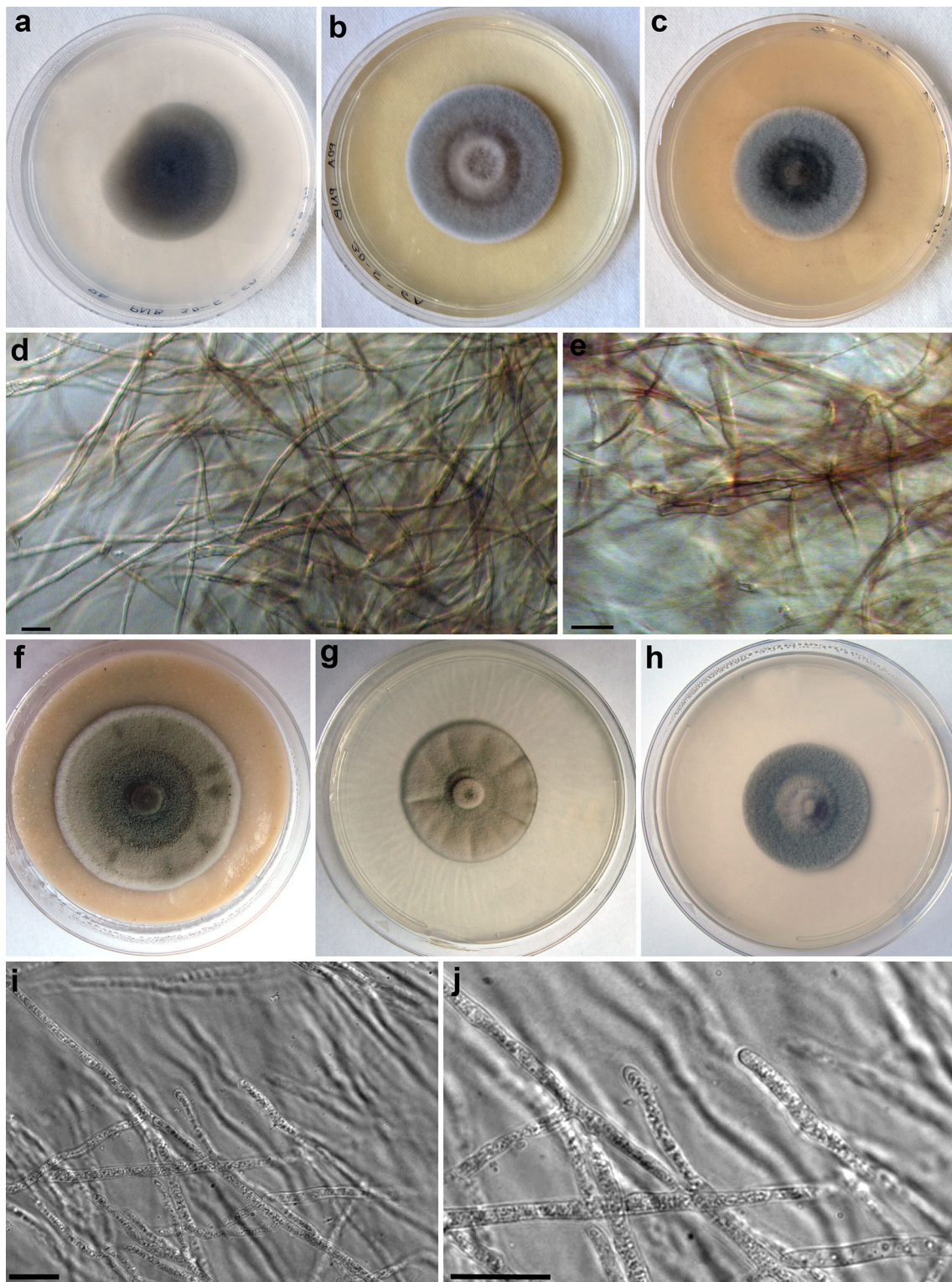


Fig. 5 Morphology of *Biatriospora peruviana* CCF 4885^T. Colony grown on OA (a), PDA (b) and MEA (c) after 14 days in 25 °C; micromorphology on MEA after 14 days (d, e). Morphology of *B.*

14 days reaching a diameter of 52.5 mm, plane, floccose, grayish gray (No. 155) with paler 2 mm margin and dark gray (No. 266) colony center, no soluble pigment, reverse

yasuniana YU.101026^T. Colony grown on OA (f), PDA (g) and MEA (h) after 14 days in 25 °C; micromorphology on MEA after 14 days (i, j). Bar 10 μm

grayish green (No. 150). Mycelium sterile, hyaline, smooth, 2.0–2.5 μm wide, fully filled with granules, often fragmenting. Teleomorph unknown.

Etymology: Referring to area of origin, Yasuni National Park.

Notes: For distinguishing characters see *B. peruviansis* description. The description of this species is only based on the holotype, which was isolated at the frequency of 0.07 % from all isolates during the survey of phloem and wood endophytes of 640 woody plants in Ecuador from 2008 to 2014.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic supplementary material

Online Resource 1. Growth response to increasing NaCl concentration and growth on sea water agar in five isolates of *Biatrispora*.

Online Resource 2. List of ITS rDNA sequences used in phylogenetic comparisons (Fig. 2). Source of origin, geographical location and data about secondary metabolite production is provided.

Online Resource 3. Growth of *Biatrispora* spp. on three different media.

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