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Standard Paper

Multilocus-phylogeny of the lichen-forming genus *Bacidia* s. str. (*Ramalinaceae*, *Lecanorales*) with special emphasis on the Russian Far East

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Abstract

To clarify deep relationships among species lineages within *Bacidia* s. str., and to investigate the robustness of the deeper branches, we combined data from three traditionally used RNA-coding genes (nrITS, nrLSU and mtSSU) with two protein-coding genes (*RPB*1 and *RPB*2). The multigene phylogeny contained 48 newly generated sequences from the Russian Far East and all *Bacidia* s. str. sequences from GenBank (131 sequences). We subjected the alignments for the single and concatenated data sets to Bayesian inference (BI) and two maximum likelihood (ML) analyses (RAxML and IQ-TREE). The topologies of phylogenetic trees recovered from BI and ML analyses were highly concordant. The multilocus phylogeny of *Bacidia* s. str. was congruent with previous results based on nrITS sequences from the Russian Far East but with considerably higher support values for most of the deeper branches. A correlation between the recovered clades and apothecial pigments in the upper part of the hymenium and lateral exciple was observed. Based on morphological and molecular evidence, *Bacidia obtecta* is described as new to science. It was recovered as the sister lineage of *B. elongata*. The two species are alike in having up to four enlarged lumina cells along the exciple edge, but *B. obtecta* differs in the abundant crystals found in the upper hymenium and lateral exciple, and by having spores with fewer septa.

Key words: *Bacidia obtecta*, crustose lichen, diversity, multilocus alignments, nuclear genes, protein-coding genes

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Introduction

Bacidia De Not. is an almost exclusively epiphytic crustose lichen genus, characterized by smooth or warted to granular thalli; acicular multiseptate spores with an average of 30–40, up to 100 µm in length and 2.0–4.0 µm in width; and a pronounced exciple, consisting of radiating hyphae, frequently with enlarged lumina cells at the tips (Ekman 1996; Llop 2007). Until recently, a large variety of lichens were included in the genus *Bacidia* in a broad sense. Zahlbruckner (1921–1940) used the name for crustose lichens with a chlorococcoid photobiont, biatorine apothecia, and ascospores with three or more transverse septa. This circumscription was unnatural and included numerous taxa not closely related to the type species, *Bacidia rosella* (Pers.) De Not. (Gerasimova & Ekman 2017).

The first extensive phylogenetic study of *Bacidia* was based on gene sequences of the nuclear ribosomal internal transcribed spacer (nrITS). It resulted in a re-evaluation of the generic delimitation, suggesting the transfer of several species to genera such as *Biatora* Fr., *Toninia* A. Massal. and *Bacidina* Vězda (Ekman 2001). In more recent phylogenetic studies, several species have been transferred to further genera, such as *Bellicidia* Kistenich *et al.*, *Bibbya* J. H. Willis, *Scutula* Tul. and *Toniniopsis* Frey (Kistenich *et al.* 2018). These phylogenetic studies narrowed the generic concept, although the relationship between species, and support for the species groups, remained unclear. Several additional studies, partially focusing on *Bacidia*, used two or multilocus data sets, combining sequences from nrITS with mitochondrial small subunit rDNA (mtSSU) (Andersen & Ekman 2005; Lendemer *et al.* 2016; Malíček *et al.* 2018), and nuclear ribosomal large subunit rDNA (nrLSU) with mtSSU (Lumbsch *et al.* 2004; Sérusiaux *et al.* 2012), but they covered only a small number of species of *Bacidia* s. str. Additional multilocus phylogenetic studies involving two genes encoding the largest and the second-largest subunit of RNA polymerase II (*RPB*1 and *RPB*2, respectively) also encompassed only six species, namely *B. absistens* (Nyl.) Arnold, *B. arceutina* (Ach.) Arnold, *B. squamulosula* (Nyl.) Zahlbr., *B. schweinitzii* (Fr. ex Tuck.) A. Schneid., *B. rosella* and *B. rubella* (Hoffm.) A. Massal. (James *et al.* 2006; Miadlikowska *et al.* 2006, 2014; Reese Næsborg *et al.* 2007; Ekman *et al.* 2008; Kistenich *et al.* 2018).

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In the first molecular study on *Bacidia* s. str. from the Russian Far East (RFE), taxon sampling was expanded (Gerasimova *et al.* 2018). Four new species were described, and these are currently considered endemic. While well-supported groups within *Bacidia* s. str. were revealed, support in the nodes of basal clades was low and several polytomies remained.

The main goal of this study was to investigate whether a phylogeny of *Bacidia* s. str. inferred from a larger, multilocus data set with a broader taxon sampling would yield higher backbone support than the previous studies based on nrITS sequences. We address the phylogeny of *Bacidia* s. str. based on nrITS data together with newly obtained data from nrLSU, mtSSU and two protein-coding genes, *RPB*1 and *RPB*2, and 131 sequences of *Bacidia* currently available in GenBank.

Material and Methods

Specimens

This study was based on fresh collections of *Bacidia* gathered by the authors in the field from 2013 to 2015 in the southern part of the Russian Far East (Primorskiy and Khabarovskiy Krai), and on Sakhalin and the Kurile Islands (as indicated in Gerasimova *et al.* (2018)). The second author collected the *Bacidia obtecta* specimens on Sakhalin in 2017 (see details under Taxonomy). Voucher specimens are deposited in the herbaria of the Botanische Staatssammlung München (M), Institute of Marine Geology and Geophysics (SAK) and Altai State University (ALTB). The inclusion of *Bacidia squamulosula* was based on a specimen examined in M (M-0012326; K. Kalb, *Lichenes Neotropici* No. 405, *Bacidiopsora squamulosula* (Nyl.) Kalb). Detailed information on the newly obtained sequences, together with their respective voucher information and GenBank Accession numbers, is given in Table 1.

Morphology

Microscopic observations were made using a Zeiss Axioplan light microscope (Oberkochen, Germany) equipped with differential interference contrast. Cross-sections of apothecia were made on a Leica Jung Histoslide 2000 Mikrotom (Heidelberg, Germany), with a thickness of 8–10 µm. Micrographs of cross-sections were taken on a Zeiss Axioplan with an attached AxioCam 512 Color camera and processed with the Zeiss ZEN 2.3 (blue edition) image software. Macrographs of external characters were taken on a Leica Z6 Apo microscope (with a ×1.0 Planapo lens; Leica, Germany) with a Sony Alpha 6400 camera (Sony, Japan) attached and equipped with a StackShot Macro Rail (Cognisys, USA) as detailed in Gerasimova *et al.* (2021). A single image was mounted from 40–100 serial images using Helicon Focus v.7 software (Helicon, USA).

Measurements are given as (min–) average ± SD (–max) (SD = standard deviation, n_1 = number of all observations, n_2 = number of specimens observed). We provide a detailed description of specimens using standard microscopic techniques following Ekman (1996) and use the proper exciple subdivision scheme, differentiating the following structures: the rim, lateral part and medullary part. Pigment characterization follows Meyer & Printzen (2000) and Ekman (1996).

DNA extraction, PCR amplification and DNA sequencing

DNA extraction was carried out using the Stratec Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany) following the manufacturer's instructions. Five to eight apothecia were used from fresh material not older than five years. Thallus fragments were removed to minimize the risk of contamination by, for example, lichenicolous fungi. PCR amplification, purification and sequencing were performed as described in Gerasimova *et al.* (2018). Cycling conditions included initial denaturation at 95 °C for 2 min, 5 cycles of 95 °C for 40 s, 54 °C for 60 s, 72 °C for 90 s, 33 cycles of 95 °C for 40 s, 54 °C for 60 s, 72 °C for 90 s, and a final extension step at 72 °C for 7 min. In those cases when PCR products were insufficient, a second PCR with a reduced number of cycles was conducted: denaturation at 95 °C for 2 min, 5 cycles of 95 °C for 40 s, 54 °C for 60 s and 72 °C for 90 s, 22 cycles of 95 °C for 40 s, 54 °C for 60 s and 72 °C for 90 s, with a final extension step at 72 °C for 7 min. We used five pairs of primers: ITS1F (White *et al.* 1990) and ITS4m (Beck & Mayr 2012), LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990), mtSSU1 and mtSSU3R (Zoller *et al.* 1999), fRPB2-5F and fRPB2-7cR (Liu *et al.* 1999), and newly designed primers for *Bacidia* gRPB1AFba (5′-GAG TGY CCG GGA CAT TTT GG-3′) and fRPB1cRba2 (5′-GSC CRG CAA TRT CGT TAT CCA-3′).

Alignment and phylogenetic analyses

Forty-eight new sequences of *Bacidia* s. str. from three ribosomal RNA-coding (nrITS, nrLSU, mtSSU) and two protein-coding genes (*RPB*1 and *RPB*2) were obtained and combined with 22 nrITS sequences from our previous study and 109 sequences of *Bacidia* s. str. from GenBank (Table 1). *Bacidia schweinitzii* (JG014), *B. areolata* Gerasimova & A. Beck (JG114) and *B. obtecta* sp. nov. (JG139-141) were newly sequenced.

Altogether 85 individuals from 24 OTUs of *Bacidia* s. str. were included. '*Phyllopsora*' *borbonica* Timdal & Krog and *Sporacestra pertexta* (Nyl.) Stapnes & Timdal were selected as the outgroup based on the results of Kistenich *et al.* (2018).

BLAST searches in GenBank were performed to detect and exclude accessory/lichenicolous fungi and potential contaminations. Alignments were carried out using standard settings in MUSCLE v.3.8.31 (Edgar 2004) as implemented in PhyDE-1 v.0.9971 and optimized manually. Positions where a gap had to be inserted in more than 95% of the sequences were excluded. The most variable ITS1 region was aligned using Gblocks v.0.91.1 (https://ngphylogeny.fr/tools/tool/276/form). The alignments are provided in Supplementary Material File S1 (available online).

Sequences of *Bacidia friesiana* (Hepp) Körb., *B. purpurans* R. C. Harris *et al.*, *B. hostheleoides* (Nyl.) Zahlbr. and *B. thiersiana* Lendemer (deposited as *Bacidia lutescens* Malme in GenBank) were revealed as separate lineages. *Bacidia purpurans* is more distantly related to *Bacidia* s. str. than the outgroup (Supplementary Material File S2, Figs S1–S3, available online). According to a BLAST search, *B. friesiana* (MH539765) is closest to *Bacidina* with more than 90% similarity. Given the high morphological variation and the necessity for further sequencing, we refrain from transferring this species to another genus.

We performed analyses for single-locus and concatenated data sets as follows: 1) single-locus data set for each locus separately (trees are included in Supplementary Material File S3, Figs S5–S19, available online); 2) concatenated multilocus data set including 26 taxa (including the outgroup) with sequences from three to all five genes available for each specimen (both newly obtained and GenBank sequences (Fig. 1; Supplementary Material File S3, Figs S1–S2, available online); 3) concatenated all-taxa data set including 87 taxa (including the outgroup) with a minimum

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Table 1. DNA codes and specimen information used in this study for species of *Bacidia* and outgroup taxa, with their respective GenBank Accession numbers. New sequences are in bold.

DNA code (JG)	Name	Country	Specimen voucher/isolate	GenBank Accession number				
				nrITS	nrLSU	mtSSU	*RPB1*	*RPB2*
	Bacidia absistens	Norway	*Ekman* 3223 (BG)	AF282085		MG925845	MG926139	MG926229
	B. albogranulosa	Czech Republic	*Vondrák* 17113 (PRA)	MK158339		MK158334		
	B. albogranulosa	Russia	*Malíček* 9622 (hb. Malíček)	MK158340		MK158335		
	B. albogranulosa	Czech Republic	*Vondrák* 11888 (PRA)	MK158342		MK158332		
	B. albogranulosa	Czech Republic	*Vondrák* 11889 (PRA)	MK158341		MK158333		
	B. albogranulosa	Czech Republic	*Malíček* 8013 (hb. Malíček)			MK158336		
	B. albogranulosa	Ukraine	*Vondrák* 12235 (PRA)			MK158337		
	B. albogranulosa	Czech Republic	*Vondrák* 12865 (PRA)			MK158338		
	B. arceutina	Sweden	*Ekman* 3110 (BG)	AF282083	MG926041	MG925846	MG926140	MG926230
	B. arceutina	Switzerland	*van den Boom* (LG DNA 579)	JQ796851	JQ796842	JQ796829		
	B. arceutina	United Kingdom	E:DNA:EDNA09-01505	FR799125				
	B. arceutina	United Kingdom	E:DNA:EDNA09-01507	FR799126				
	B. arceutina	United Kingdom	E:DNA:EDNA09-01587	FR799127				
JG037	*B. areolata*	Russia	*Gerasimova* M-0182592 (M)	MH048614		**MW506357**	**MW540434**	**MW522875**
JG114	*B. areolata*	Russia	*Davydov* 17428 & *Yakovchenko* (ALTB)	**MW491455**		**MW506358**		
	B. biatorina	Sweden	*Knutsson* 94-148 (hb. Knutsson)	AF282079				
JG083	*B. diffracta*	USA	*Wetmore* 46555-A (M)	MH048620				
	B. diffracta	USA	*Wetmore* 26401 (MIN)	AF282090				
	B. ekmaniana	USA	*Lendemer* 33836 (NY1538)			KX151741		
	B. ekmaniana	USA	*Lendemer* 33920 (NY1543)			KX151743		
	B. ekmaniana	USA	*Lendemer* 30488A (NY1454)			KX151746		
	B. ekmaniana	USA	*Lendemer* 31362 (NY1455)			KX151744		
	B. ekmaniana	USA	*Lendemer* 33783 (NY1537)			KX151745		
	B. ekmaniana	USA	*Lendemer* 34000 (NY1540)			KX151742		
JG007	*B. elongata*	Russia	*Ezhkin* M-0182571 (M)	MH048626				
JG101	*B. elongata*	Russia	*Ezhkin* M-0182625 (M)	MH048627	**MW493329**	**MW506351**	**MW540430**	**MW522870**
JG102	*B. elongata*	Russia	*Ezhkin* M-0182626 (M)	MH048628	**MW493330**	**MW506352**		**MW522871**
JG103	*B. elongata*	Russia	*Ezhkin* M-0182627 (M)	MH048629				
	B. fraxinea	Sweden	*Johansson* 1620 (BG)	AF282088				
	B. hostheleoides	United Kingdom	*Seaward* 108121 (priv. hb.)	AF282081				

(*Continued*)

Table 1. (*Continued*)

DNA code (JG)	Name	Country	Specimen voucher/isolate	GenBank Accession number				
				nrITS	nrLSU	mtSSU	*RPB*1	*RPB*2
JG092	*B. kurilensis*	Russia	*Ezhkin* M-0182620 (M)	MH048610	**MW493325**	**MW506348**	**MW540428**	**MW522868**
JG095	*B. kurilensis*	Russia	*Ezhkin* M-0182621 (M)	MH048611	**MW493326**			
JG096	*B. kurilensis*	Russia	*Ezhkin* M-0182622 (M)	MH048612				
JG091	*B. laurocerasi*	Russia	*Galanina* (424)	MH048609				
	B. laurocerasi subsp. *laurocerasi*	USA	*Wetmore* 74318 (MIN)	AF282078				
JG139	*B. obtecta*	Russia	*Ezhkin* M-0308498 (M)	**MW491457**	**MW493335**	**MW506362**		**MW522877**
JG140	*B. obtecta*	Russia	*Ezhkin* M-0308497 (M)	**MW491458**	**MW493336**	**MW506363**		**MW522878**
JG141	*B. obtecta*	Russia	*Ezhkin* M-0308496 (M)	**MW491459**				
	B. polychroa	Sweden	*Knutsson* 91-215 (hb. Knutsson)	AF282089				
	B. rosella	Sweden	*Ekman* 3117 (BG)	AF282086	AY300829	AY300877	AY756412	AM292755
JG085	*B. rubella*	Russia	*Gerasimova* M-0182581 (M)	MH048630	**MW493331**	**MW506353**	**MW540431**	
	B. rubella	Poland	AFTOL-ID 1793	HQ650644	DQ986793	DQ986808		DQ992422
	B. rubella	Switzerland	*van den Boom* (LG DNA 578)	JQ796852	JQ796843	JQ796830		
	B. rubella	Sweden	*Ekman* 3021 (BG)	AF282087		AY567723		
	B. rubella	Ukraine	*Vondrák* 12200 (PRA)	MK158343		MK158331		
	B. rubella	Switzerland	*van den Boom* (LG DNA 581)			JQ796831		
	B. rubella	Switzerland	LIFU076-16	KX132984				
	B. rubella	Hungary	*Hur* H06122	EU266078				
JG082	*B. sachalinensis*	Russia	*Ezhkin* M-0182619 (M)	MH048621				
JG097	*B. sachalinensis*	Russia	*Ezhkin* M-0182623 (M)	MH048622	**MW493333**	**MW506355**	**MW540433**	**MW522873**
JG098	*B. sachalinensis*	Russia	*Ezhkin* IMGIG 147	MH048623	**MW493334**	**MW506356**		**MW522874**
JG099	*B. sachalinensis*	Russia	*Ezhkin* IMGIG 148	MH048624				
JG100	*B. sachalinensis*	Russia	*Ezhkin* M-0182624 (M)	MH048625				
JG014	*B. schweinitzii*	Russia	*Gerasimova* M-0182579 (M)	**MW491454**	**MW493327**			
JG015	*B. schweinitzii*	Russia	*Gerasimova* M-0182580 (M)	MH048613	**MW493327**	**MW506350**	**MW540429**	**MW522869**
	B. schweinitzii	USA	*Wetmore* 72619 (MIN)	AF282080	MG926045		MG926146	MG926235
	B. schweinitzii	USA	AFTOL-ID 642	DQ782850	DQ782911	DQ972998	DQ782830	DQ782872
	B. schweinitzii	USA	AFTOL-ID 4969		KJ766527	KJ766354		
	B. schweinitzii	USA	*Shaheen* (NY1451)	MG461696				
	B. schweinitzii	USA	*Tripp* 2614 (NY1448)	KX151762		KX151750		

	B. schweinitzii	USA	*Lendemer* 29364 (NY1449)	KX151763		KX151751		
	B. schweinitzii	USA	*Lendemer* 31230A (NY1450)	KX151766				
	B. schweinitzii	USA	*Lendemer* 31238 (NY1451)	KX151764		KX151752		
	B. schweinitzii	USA	*Lendemer* 30548 (NY1452)	KX151761		KX151749		
	B. schweinitzii	USA	*Lendemer* 31855 (NY1453)	KX151765		KX151753		
	B. scopulicola	Sweden	*Ekman* 3106 (BG)	AF282084				
	B. sipmanii	Spain	*Sérusiaux* (LG DNA 361)	JQ796853	JQ796844	JQ796832		
	B. sorediata	USA	*Lendemer* 31692 (NY1389)	KX151768		KX151755		
	B. sorediata	USA	*Lendemer* 31527 (NY1397)	KX151771		KX151758		
	B. sorediata	USA	*Lendemer* 33702 (NY1539)	KX151767		KX151754		
	B. sorediata	USA	*Lendemer* 33787 (NY1544)	KX151772		KX151759		
	B. sorediata	USA	*Lendemer* 33869 (NY1546)	KX151773		KX151760		
	B. sorediata	USA	*Lendemer* 35031 (NY1747)	KX151769		KX151756		
	B. sorediata	USA	*Lendemer* 35386 (NY1748)	KX151770		KX151757		
	B. sorediata	USA	*Lendemer* 38909 (NY2294)	KX151774				
	B. sorediata	USA	*Barton* 658 (NY2496)	KX151775				
	B. squamulosula	Ecuador	*Kalb, Lich. Neotropici* No. 405 (SE-314)	MG925955	MG926051	MG925856	MG926152	
	B. suffusa	USA	*Wetmore* 74771 (MIN)	AF282091				
JG038	*B. suffusa*	Russia	*Gerasimova* M-0182593 (M)	MH048616		**MW506359**	**MW540435**	
JG039	*B. suffusa*	Russia	*Gerasimova* M-0182594 (M)	MH048617		**MW506360**		
JG051	*B. suffusa*	Russia	*Gerasimova* M-0182601 (M)	MH048615		**MW506361**		**MW522876**
JG080	*B. suffusa*	USA	*Tucker* 17000 (M)	MH048618				
JG081	*B. suffusa*	USA	*Wetmore* 40219 (M)	MH048619				
	B. suffusa	USA	*Lumbsch* 19190c (AFTOL-ID 5785)		KJ766528	KJ766355	KJ766836	
	B. thiersiana	USA	*Ekman* L1161 (LD)	AF282082				
	Sporacestra borbonica	Réunion	*Krog & Timdal* RE08,12 (isolate 1040)	MG925988	MG926086	MG925890	MG926184	
	S. pertexta	Cuba	*Pérez-Ortega* s. n. (isolate 511)	MG926000	MG926093	MG925903	MG926194	MG926268

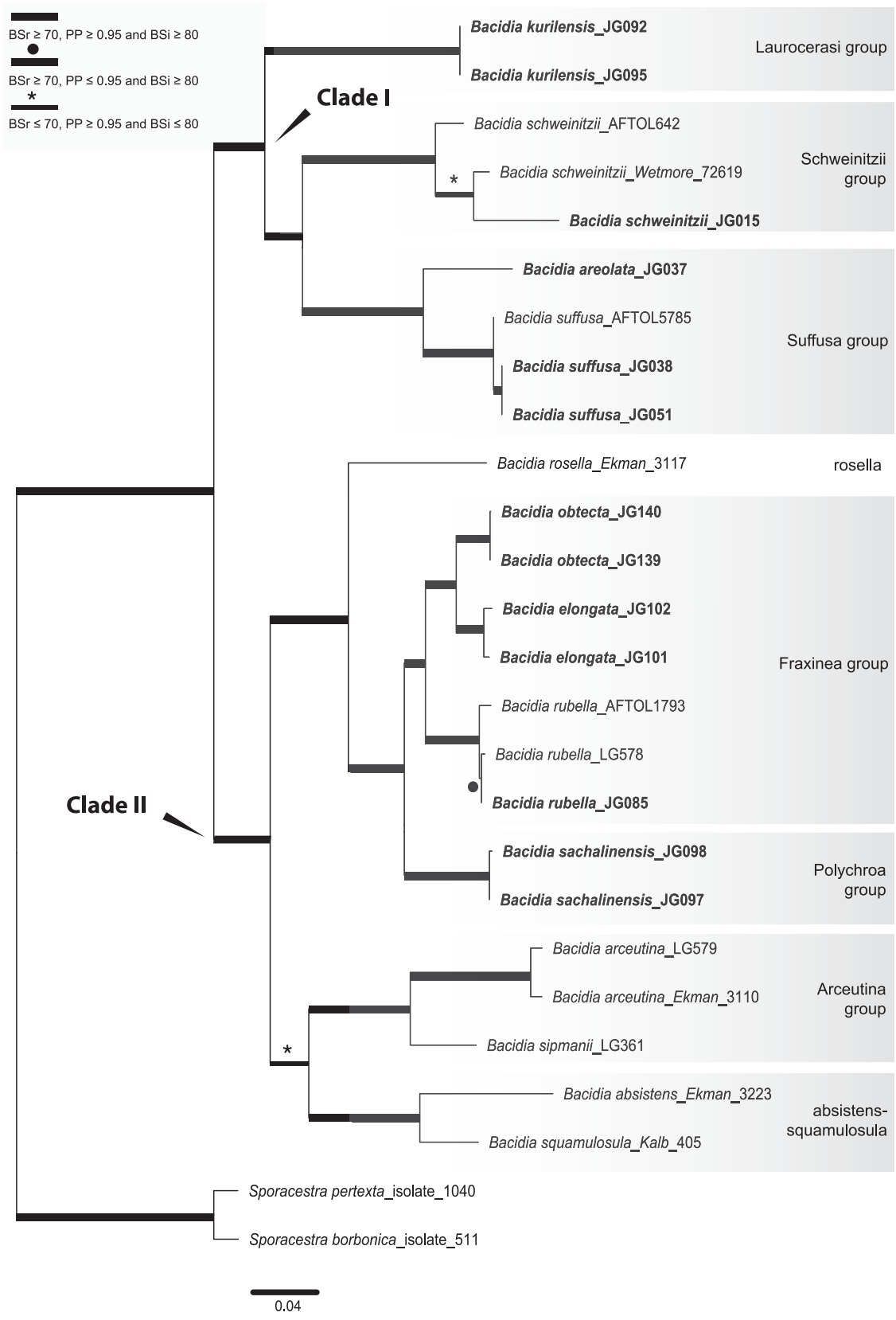

Fig. 1. Maximum likelihood (ML) tree of *Bacidia* s. str. resulting from the RAxML analysis of the concatenated multilocus data set with a minimum of three loci included (out of nrITS, nrLSU, mtSSU, *RPB*1 and *RPB*2). RAxML bootstrap values (BSr), Bayesian posterior probabilities (PP) and IQ-TREE bootstrap values (BSi) are indicated. Highly supported branches with BSr ≥ 70%, PP ≥ 0.95 and BSi ≥ 80% are marked in bold; strongly supported branches with BSr ≥ 70% and BSi ≥ 80% are also marked in bold with a black dot above the branch; branches with PP ≥ 0.95 are marked in narrower bold lines and a star above the branches. *Sporacestra* taxa are the outgroup. *Bacidia lutescens* is referred to as *B. thiersiana* in the text.

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of one sequence available (both newly obtained and GenBank sequences) in order to include all specimens with sequences available (Fig. 2; Supplementary Material File S3, Figs S3–S4, available online).

Based on the support values of the phylogenetic analyses, no supported incongruence between the single-locus tree topologies was found; therefore, the concatenated alignment was analyzed. The number of taxa and alignment details for the single and concatenated data sets are summarized in Table 2. We used the multilocus alignment to produce a robust phylogeny, including the taxa with sequences from a minimum of three available loci. For comparison, we analyzed the all-taxa data set with all *Bacidia* s. str. specimens, even if only the sequence of a single locus was available.

The alignment with nrITS, nrLSU, mtSSU, *RPB*1 and *RPB*2 was subjected to Bayesian inference (BI) and maximum likelihood (RAxML and IQ-TREE) analyses for the single and concatenated data sets separately, as implemented in Gerasimova *et al.* (2021).

Substitution models for the entire (no-partitioned) concatenated and single-locus data sets were selected using jModelTest v.2 (Darriba *et al.* 2012) following the Akaike selection criterion (AIC). The best substitution model was selected in jModelTest with 1-, 2- and 6-model groups with additional options of independent state (nucleotide) frequencies, gamma-distributed rate variations across sites and a proportion of invariable sites (24 possible models).

Bayesian inference was carried out in MrBayes v.3.2.6 (Ronquist *et al.* 2012) using the Markov chain Monte Carlo method (MCMC) and GTR + I + G model. Two parallel runs were performed (two cold chains) with a single tree saved every 10th generation for a total of 1 000 000 generations. According to the trends in likelihood values, the convergence of the Markov chain was reached after 10 000 generations. As a result, the initial 10% was discarded as burn-in and the results summarized as a 50% majority-rule consensus tree.

Maximum likelihood (ML) analysis was performed with RAxML v.8.2.4 on the CIPRES web portal (Miller *et al.* 2010). The GTRGAMMA model with rapid bootstrap analysis searching for the best-scoring ML tree, using a majority-rule consensus tree and 1000 bootstrap iterations, was used for the entire data. The bipartition of the best-scoring tree was drawn onto the most likely tree topology following the recommendation of Stamatakis (2014).

Further tree reconstruction using ML analysis was performed in IQ-TREE v.1.6.12 using standard bootstrap approximation with 1000 bootstraps, specifying the GTR + I + G model as suggested by jModelTest (Nguyen *et al.* 2015).

The phylogenetic trees were visualized using FigTree v.1.4.2 (Rambaut 2009). The ML trees based on the different substitution models from single and concatenated data sets were congruent, therefore only the RAxML trees for the concatenated data sets are shown. Clades that received bootstrap support values (BSr) ≥ 70% in RAxML, posterior probabilities (PP) ≥ 0.95 in BI and bootstrap values (BSi) ≥ 80% in IQ-TREE were considered highly supported. The concatenated and individual gene trees obtained from RAxML, MrBayes and IQ-TREE are provided as Supplementary Material File S3 (Figs S1–S19).

Results

The BI and ML analyses for the single and concatenated data sets recovered highly concordant topologies of the phylogenetic trees. Both multilocus and all-taxa trees resulted in well-supported basal nodes (Figs 1 & 2).

Single-locus trees were congruent with the multilocus and all-taxa trees, but with high support in the terminal groups and low support in the backbone. All clades in the multilocus tree received strong support (i.e. with BSr ≥ 70%, PP ≥ 0.95 and BSi ≥ 80%) both in the terminal and deeper branches with only a small number of exceptions. The clade including *B. arceutina*, *B. absistens* and *B. squamulosula* was supported in BI (BSr/PP/BSi: 66/0.97/62); the subclade with two *B. rubella* from the RFE (JG085) and Switzerland (LG578) was supported in RAxML (BSr/PP/BSi: 87/0.75/71), and the subclade with *B. schweinitzii* from the RFE (JG015) and the USA (*Wetmore* 72619) was supported in BI only (BSr/PP/BSi: 56/0.99/61). The multilocus tree is congruent with the all-taxa phylogeny, forming the same groups with high support values (Fig. 2). The enlarged all-taxa phylogeny comprises all specimens with sequence data available; therefore, we discuss the groups in more detail based on this tree. According to phylogenetic results and morphological characters, six *Bacidia* s. str. groups are recognized: Laurocerasi, Schweinitzii, Suffusa, Fraxinea, Polychroa, and Arceutina. The separate lineages of 1) *B. ekmaniana* R. C. Harris *et al.*, *B. absistens* and *B. squamulosula*, and 2) *B. hostheleoides* and *B. thiersiana*, are discussed individually. Furthermore, a correlation between these phylogenetic groups and the apothecial pigments was observed (see Table 3 and Discussion).

The Laurocerasi group, consisting of the three lineages *B. biatorina* (Körb.) Vain., *B. kurilensis* Gerasimova *et al.* and *B. laurocerasi* (Delise ex Duby) Zahlbr., received strong support (BSr/PP/BSi: 92/1.0/93).

Specimens of *B. schweinitzii* and *B. sorediata* Lendemer & R. C. Harris formed a paraphyletic clade (Schweinitzii group), comprising several separate clades with the highest support (BSr/PP/BSi: 99/1.0/100). Two subclades were present in *B. schweinitzii*, each containing an additional phylogenetic structure. One subclade, with high support (BSr/PP/BSi: 84/0.95/83), includes only GenBank sequences of *B. schweinitzii* from North America (AFTOL642 and NY). The second subclade contains both individuals from the RFE and North America and two subclades of *B. sorediata*, but without significant support (BSr/PP/BSi: 40/0.8/46). Individuals of *B. schweinitzii* from the RFE (JG014 and JG015) were found in two groups in the ITS phylogeny (see Supplementary Material File S3, Figs S5–S7, available online). Given the differences in nrITS sequences (3% = 13 nucleotides), they probably belong to two independent species (see Discussion below). This is also supported by morphological observations which indicate differences in thallus and apothecial characters (details are summarized in Supplementary Material File S4, available online). The Schweinitzii group is sister to the Suffusa group; together, they form a well-supported clade (BSr/PP/BSi: 78/1.0/79).

The clade containing *B. areolata* and *B. suffusa* (Fr.) A. Schneid. (Suffusa group) was highly supported (BSr/PP/BSi: 100/1.0/99). Two separate well-supported lineages in *B. suffusa* represent the North American and RFE populations. For two individuals from the USA, JG080 and JG081, only the nrITS2 sequences were available, most likely due to the age of the specimens (Gerasimova *et al.* 2018). However, compared to the RFE population, they already reveal variations in the relatively conserved nrITS2 region: four transitions at several positions, indicating a 2% difference. For another individual from the USA (AFTOL5785), the nrITS sequence is unavailable, but there are nrLSU, mtSSU and *RPB*1 sequences. In the mtSSU phylogeny, it also forms a separate lineage from the RFE individual, JG051,

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Table 2. Overview of the numbers of taxa and newly produced sequences for each genetic marker and concatenated alignment for *Bacidia* in this study.

	nrITS	nrLSU	mtSSU	*RPB1*	*RPB2*	Multilocus data set	All-taxa data set
Number of taxa (including outgroup species)	75	24	55	17	17	26	87
Newly produced sequences	5	11	14	8	10	13	48
Length with gaps (bp)	434	850	783	639	1084	3786	3785
Parsimony informative sites	174	110	173	284	409	1111	1149

with a 3% (= three nucleotides) difference (Supplementary Material File S3, Figs S11–S13). The Suffusa group formed a strongly supported clade with the Schweinitzii and Laurocerasi groups (BSr/PP/BSi: 90/1.0/87).

The highly supported Fraxinea group (BSr/PP/BSi: 100/1.0/99) comprises sequences representing *B. fraxinea* Lönnr., *B. rubella*, *B. elongata* Gerasimova & A. Beck and a new lineage. Detailed morphological analysis showed that this lineage differs from recently known species in *Bacidia* s. str., forming the highly supported sister group to *B. elongata* (BSr/PP/BSi: 99/1.0/98). Consequently, *Bacidia obtecta* sp. nov. is described here.

The recently described *B. albogranulosa* Malíček *et al.* nested together with *B. polychroa* (Th. Fr.) Körb., *B. diffracta* S. Ekman and *B. sachalinensis* Gerasimova *et al.* (Polychroa group) with the highest support (BSr/PP/BSi: 100/1.0/99). The sister relationship with the *B. polychroa* lineage (*Knutsson* 91-215) received high support in the RAxML phylogeny (BSr/PP/BSi: 71/-/59) but recovered a polytomy in BI.

Bacidia ekmaniana is the sister group of *B. absistens* and *B. squamulosula*, but without significant support (BSr/PP/BSi: 56/0.77/63). Together with *B. arceutina*, *B. scopulicola* (Nyl.) A. L. Sm. and *B. sipmanii* M. Brand *et al.* (Arceutina group) they formed a well-supported clade in RAxML (BSr/PP/BSi: 71/0.77/68). In the multilocus phylogeny, the clade was highly supported in BI only (BSr/PP/BSi: 66/0.97/62). *Bacidia arceutina* from Switzerland (LG579) formed a separate lineage to individuals from the United Kingdom (FR799125–FR799127) and Sweden (*Ekman* 3110), with high support in the ML phylogenies (BSr/PP/BSi: 97/0.67/97). Compared to sequences from the United Kingdom, it contained a 1% (= five nucleotides) difference in nrITS and a 1% (= six nucleotides) difference in nrLSU. Likewise, the sequence from Sweden differed by 1% (= three nucleotides) in the mtSSU compared to one from Switzerland (LG579).

The basal clade with *B. thiersiana* and *B. hostheleoides* received high support but revealed long branches (BSr/PP/BSi: 85/0.96/85).

Three main clades were recovered in *Bacidia* s. str.: clade I comprises the Laurocerasi, Schweinitzii and Suffusa groups; clade II includes the Fraxinea, Polychroa, and Arceutina groups, and clades of *B. ekmaniana*, *B. absistens* and *B. squamulosula*; clade III, sister to the others, includes *B. thiersiana* and *B. hostheleoides*.

Discussion

The multilocus phylogeny resulted in groups congruent with previous results based on nrITS (Ekman 2001; Gerasimova *et al.* 2018), but significantly increased backbone support of the clades in both multilocus and all-taxa phylogenetic trees (Figs 1 & 2).

The phylogenetic trees group the taxa into two large clades: the first clade (I) includes the Laurocerasi, Schweinitzii and Suffusa groups, while the second clade (II) includes the Fraxinea, Polychroa, and Arceutina groups and clades of *B. ekmaniana*, *B. absistens* and *B. squamulosula*. In the phylogeny, they are represented by specimens from the temperate region and can be separated based on apothecial pigment (Table 3). The well-supported third clade (III) includes *B. thiersiana* and *B. hostheleoides*, which are widespread in south-eastern North America and the Neotropics, respectively (Malme 1935; Ekman 1996; Lendemer 2020).

Specimens in clade I have either dark brown, red-brown and green pigments (Laurocerasi-brown and Bagliettoa-green) or a combination of these in the upper part of the hymenium and lateral exciple (Table 3). In contrast, those in clade II have a mixture of yellow, orange and/or brown apothecial pigments (Arceutina-yellow, Polychroa-brown and Rubella-orange). An exception is a clade containing *B. absistens*, the pigmentation of which is highly variable (see groups below). Specimens in clade III are characterized by almost colourless or faintly and diffusely pigmented internal apothecial structures (Ekman 1996).

The taxon sampling in the phylogeny of *Bacidia* s. str. still needs to be completed; therefore, division of the clades based on the apothecial pigment is provisional. Given the available data, the main focus in this discussion is on representatives of *Bacidia* s. str. from the RFE. For convenience, the order of the groups corresponds to the position in the phylogenetic tree from top to bottom.

Laurocerasi group (including B. biatorina, B. kurilensis *and* B. laurocerasi *clades)*

Bacidia laurocerasi is one of the most widespread species of the group, occurring in Europe, Macaronesia, Africa, South and North America, Asia, Australia and New Zealand (Ekman 1996; Llop 2007; Coppins & Aptroot 2009). *Bacidia biatorina* is reported less frequently, and known collection localities include Europe, North America and Asia. In contrast, *B. kurilensis* is known only from the Kuril Islands and is considered endemic (Gerasimova *et al.* 2018). The phylogeny contains *B. biatorina* from Sweden, *B. laurocerasi* from North America and the RFE, and *B. kurilensis* from the RFE.

All three taxa share Laurocerasi-brown in the exciple and upper part of hymenium (Table 3). The mixture of Laurocerasi-brown and Bagliettoa-green found in the upper part of the

Fig. 2. Maximum likelihood (ML) tree of *Bacidia* s. str. resulting from the RAxML analysis of the concatenated all-taxa data set with a minimum of one sequence available (out of nrITS, nrLSU, mtSSU, *RPB1* and *RPB2*). RAxML bootstrap values (BSr), Bayesian posterior probabilities (PP) and IQ-TREE bootstrap values (BSi) are indicated. Highly supported branches with BSr ≥ 70%, PP ≥ 0.95, and BSi ≥ 80% are marked in bold; strongly supported branches with BSr ≥ 70% and BSi ≥ 80% are also marked in bold with a black dot above the branch; branches with PP ≥ 0.95 are marked in narrower bold lines and a star above the branches; branches with PP ≥ 0.95, and/or BSr ≥ 70% and/or BSi ≥ 80% are marked with a white dot. *Sporacestra* taxa are the outgroup. *Bacidia lutescens* is referred to as *B. thiersiana* in the text.

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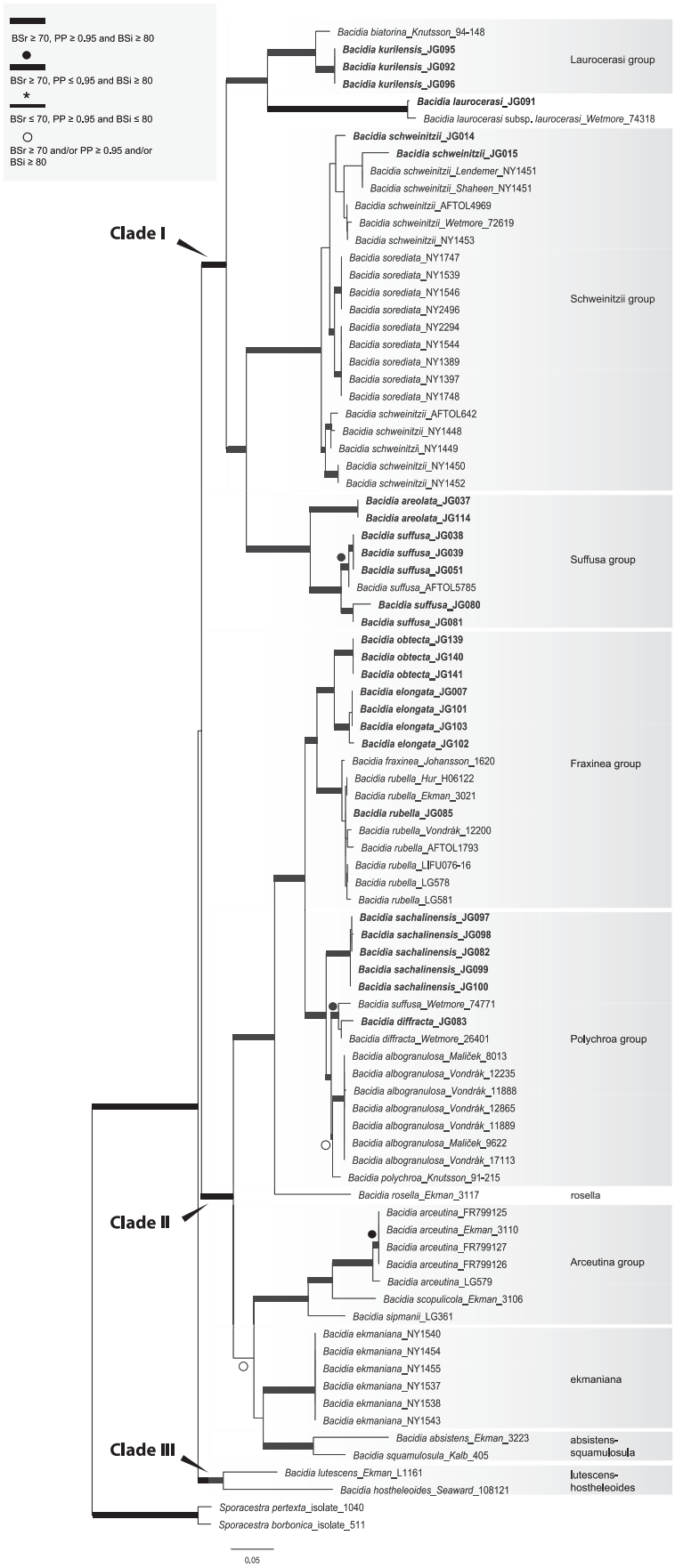

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Table 3. Comparison of the pigmentation in the different phylogenetic groups in *Bacidia* s. str. as shown in Fig. 2. Pigment characterization follows Meyer & Printzen (2000) and Ekman (1996). The three parts of the table represent the pigmentation of the two major clades and separate lineages of *Bacidia* s. str.

Group	Upper hymenium	Exciple edge	Lateral exciple	Hypothecium
Laurocerasi	Laurocerasi-brown + Bagliettoa-green (in *B. kurilensis*)	Laurocerasi-brown	Laurocerasi-brown/ Rubella-orange	Arceutina-yellow
Schweinitzii	Bagliettoa-green	Bagliettoa-green	Schweinitzii-red	Schweinitzii-red
Suffusa	Laurocerasi-brown	Laurocerasi-brown	Rubella-orange	Rubella-orange
Fraxinea	Rubella-orange	Rubella-orange	Rubella-orange	Rubella-orange
Polychroa	Polychroa-brown	Rubella-orange	Rubella-orange	Rubella-orange
Arceutina	Arceutina-yellow	Arceutina-yellow	Arceutina-yellow	Arceutina-yellow
B. ekmaniana	Polychroa-brown	-	Rubella-orange	Rubella-orange
B. absistens	Bagliettoa-green/ Laurocerasi-brown	Bagliettoa-green	Laurocerasi-brown	Arceutina-yellow
B. squamulosula	Rubella-orange	-	Arceutina-yellow	Arceutina-yellow
B. gigantensis	Grey to grey-brown coloration	Arceutina-yellow	Arceutina-yellow	Arceutina-yellow
B. thiersiana	-	-	-	-
B. hostheleoides	No colour or small amounts of Rubella-orange	No colour or small amounts of Rubella-orange	No colour or small amounts of Rubella-orange	No colour or small amounts of Rubella-orange

hymenium in *B. kurilensis* remains unique in this group (Gerasimova *et al.* 2018).

All three lineages formed a strongly supported group that confirms our earlier results based on nrITS locus sequences. Two *B. laurocerasi* individuals formed a rather distant lineage from *B. biatorina* and *B. kurilensis*. However, as they all share the same pigment in the apothecia, we consider them to be one group.

Schweinitzii group (including B. schweinitzii *and* B. sorediata *clades)*

Bacidia schweinitzii occurs in the temperate forests of Canada around the Great Lakes and the Maritimes, in eastern Asia and the eastern parts of the USA, and the temperate region of the southern part of the RFE, in Primorskiy and Khabarovskiy Krai, and Kunashir Island (Ekman 1996; Lendemer *et al.* 2016; Gerasimova *et al.* 2018). *Bacidia sorediata* is so far known only from coastal south-eastern North America, particularly the Mid-Atlantic Coastal Plain (Lendemer *et al.* 2016).

The high variability in morphology of *B. schweinitzii* s. lat. has already been discussed in previous studies (Ekman 1996; Lendemer *et al.* 2016). Two morphotypes, sorediate and esorediate, were recognized based on morphology and were subsequently shown to be concordant with the molecular results, leading to the description of three new species (Lendemer *et al.* 2016). It has been shown that sequences within the *B. schweinitzii* group formed multiple clades within *B. schweinitzii* s. str. (esorediate populations) and *Bacidia sorediata* s. lat. (sorediate morphotype). Sequences derived from sorediate populations were grouped within two strongly supported clades, sister to each other but without support. Sequences derived from esorediate specimens were recovered in a series of clades whose relationships were poorly supported. Thus, data from the nrITS and mtSSU regions appear to be insufficient to resolve the relationships between sorediate and esorediate populations with confidence.

The multilocus phylogeny presented here confirmed that *B. schweinitzii* is paraphyletic and contains lineages of several taxa. Nevertheless, not all branches within *B. schweinitzii* received high support values in both the multilocus and all-taxa phylogenies, maintaining unclear relationships within this group.

Two *B. schweinitzii* representatives, JG014 and JG015, form a grade and are not sister taxa in the ITS phylogeny (see Supplementary Material File S3, Figs S5–S7, available online). Instead JG015, having an abundantly granular thallus forming coral-like structures, was nested within the North American individuals. In addition, its apothecia are black, adpressed to the surface, with a brown to dark brown hypothecium merging into a darker brown exciple below and medullary part which does not form a distinct colourless zone. The spores are acicular and shorter with fewer septa, 37–46–50 × 2.5–3.5 µm, 5–7 septa (Fig. 3B & D; Supplementary Material File S4, available online). According to Lendemer *et al.* (2016), such specimens can be assigned to the 'typical' *B. schweinitzii* morph by the granular thallus and apothecial coloration.

In contrast, JG014 has morphological characters that do not clearly match any species currently recognized in the *B. schweinitzii* group. Morphological observation demonstrated that it differs in the ±smooth to warted thallus; black caliciform apothecia, which rise above the surface; dark brown hypothecium merging into the dark brown-red exciple (K+ purplish), which forms a distinct colourless zone in the lateral part and the base of the medulla; acicular spores, 56–69–80 × 2.5–3.0 µm, 7–15 septa (Fig. 3A & C; Supplementary Material File S4). *Bacidia ekmaniana* also forms a distinct colourless zone in the lateral and medullary parts, in a similar way to JG014, but differs by having orange-brown apothecia and a lighter orange-brown hypothecium and exciple (K+ darker brown). It is thus likely that JG014 belongs to a different species. However,

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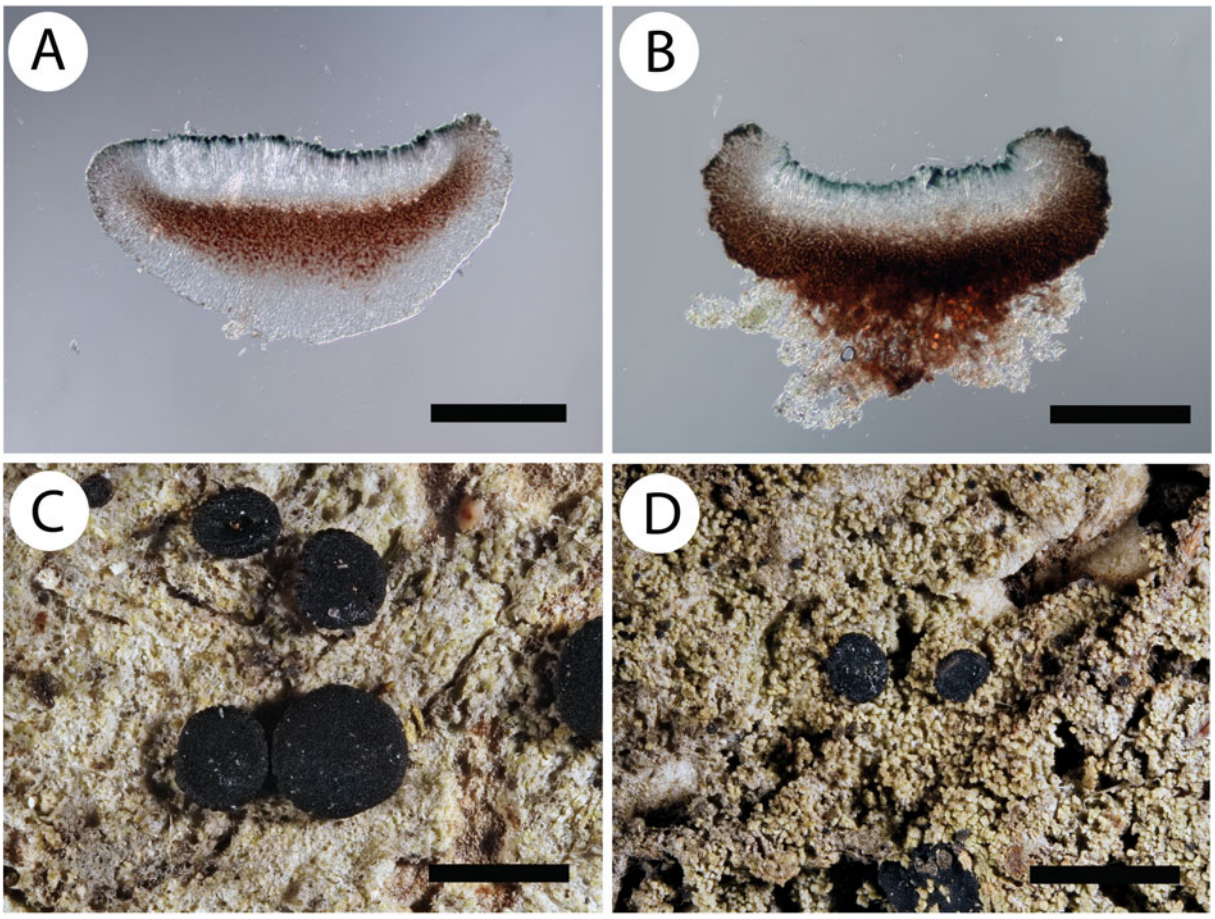

Fig. 3. Cross-sections of apothecia and thallus structure of two individuals of *Bacidia schweinitzii*. A & C, M-0182579 (JG014). B & D, M-0182580 (JG015). A, dark brown hypothecium, merging into the coloration of exciple below. Exciple forms a distinct colourless zone in the lateral and medullary part. B, brown hypothecium merging into the coloration of the exciple. Dark brown exciple not forming a distinct colourless zone. C, thallus smooth to warted, consisting of single or contiguous ±roundish warts. D, thallus granular, consisting of ±globose to subsquamulose granules. Scales: A & B = 200 µm; C & D = 1 mm. In colour online.

further details and careful investigation of all the type material and specimens from GenBank are necessary for making a comprehensive description and species delimitation; therefore, we currently refrain from describing new entities.

Suffusa group (including B. areolata *and* B. suffusa *clades)*

Bacidia suffusa s. lat. occurs in the eastern temperate region of North America, in the North Caucasus and the RFE (Ekman 1996; Gerasimova *et al.* 2018). *Bacidia areolata* is known from the RFE and considered endemic. The phylogeny contains the sequences from North America and the RFE.

The specimens of *B. areolata* and *B. suffusa* formed a strongly supported clade and share Laurocerasi-brown in the exciple edge and upper part of the hymenium. Since our first molecular study, additional sequences of *B. suffusa* from the USA have been submitted to GenBank (AFTOL5785), including nrLSU, mtSSU and *RPB*1 sequences (Table 1). As the two individuals from the USA, JG080 and JG081, are represented by short sequences of nrITS2 only, the relationship to the other *B. suffusa* from North America remains unclear. Nevertheless, *B. suffusa* from the RFE formed a separate lineage from the ones collected in North America in the combined and single-locus trees based on nrLSU, mtSSU and *RPB*1 sequences. We could not study the individual from GenBank and suggest including more specimens from North America and other regions. Thus, we consider individuals of *B. suffusa* from the RFE and North America as different populations but belonging to the same species.

The newly sequenced specimen of *B. areolata* (JG114) represents the second occurrence of this species. Compared to the type specimen (JG037, M-0182592), it also has three layers of enlarged lumina cells along the edge of the exciple. The thallus structure, apothecial pigment and measurements are also in the range found in the type specimen. Nevertheless, JG114 differs from the type specimen by the lack of any pruina on the apothecial margin and the presence of abundant colourless crystals in the lateral exciple, dissolving in KOH with the following colour changes: green → yellow → colourless.

Fraxinea group (including B. obtecta, B. elongata, B. rubella *and* B. fraxinea *clades)*

The most widespread taxon of the group, *B. rubella*, has a Holarctic distribution and is known from Europe, Macaronesia, Africa, Asia and North America (Ekman 1996; Llop 2007; Coppins & Aptroot 2009). *Bacidia fraxinea* is less frequent and occurs in eastern parts of north and central Europe and the northern Mediterranean region (Ekman & Nordin 1993). In contrast, both *B. elongata* and *B. obtecta* are known only from Sakhalin and can be considered endemic (Gerasimova *et al.* 2018).

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Table 4. Main characters separating *Bacidia obtecta* from the closely related *B. elongata* and *B. fraxinea*. Measurements for *B. fraxinea* are based on those from Ekman & Nordin (1993) and measurements of *B. elongata* from Gerasimova *et al.* (2018). Quantitative information of our measurements is given as (min–) average ± SD (–max), while that for *B. fraxinea* was taken from the original manuscript.

	B. obtecta	*B. elongata*	*B. fraxinea*
Thallus	Thick, wrinkled, warted; grey-green to yellowish green	Thin to thick, smooth to areolate, wrinkled and warted; grey to dark grey-green	Thin and almost smooth to thick and verrucose, areolate or irregularly cracked, grey
Apothecial colour	Beige to rusty brown	Orange to dark purple-brown	Orange-brown to dark brown
Margin	Paler than disc, beige to pale brown, distinct; with pruina	Concolorous with or paler than disc, light orange; with pruina	Concolourous with disc; with or without pruina
Apothecial size (mm)	(0.4–)0.85 ± 0.25(–1.5)	(0.25–)0.55 ± 0.15(–0.95)	0.6–1.1
Crystals in exciple	Throughout lateral part of exciple and upper hymenium; K+, N–	Without or with clusters of crystals in exciple; N+, K–	With or without radiating clusters of minute crystals in exciple rim; N+, K–
Hypothecium	Orange-brown	Colourless, pale yellow to orange-brown	Straw-coloured to pale orange
Number of enlarged lumina cells along exciple edge	Up to 4 layers	Up to 4 layers	Without or with single cell layer
Size (µm)	Up to 5 × 13	Up to 7 × 20	Up to 6 × 6
Ascospore length (µm)	(47–)61.2 ± 7.0(–79)	(39–)59 ± 8(–80)	(42–)50–67–85(–109)
Ascospore width (µm)	(2.0–)3.0 ± 0.36(–4.0)	(2.0–)2.5 ± 0.5(–4.0)	(2.5–)2.6–3.0–3.4(–4.3)
Number of septa	(1–)6 ± 2(–11)	(2–)5–7–12(–16)	5–17

The phylogeny contains specimens of *B. rubella* from Europe and the RFE, *B. fraxinea* from Europe, and *B. elongata* and *B. obtecta* from the RFE. The phylogeny recovered the whole clade with high support, but the relationship between *B. rubella* and *B. fraxinea* remains unclear. The only sequence of *B. fraxinea* (*Johansson* 1620) was nested together with other *B. rubella* included in the analysis. Therefore, more specimens of *B. fraxinea* need to be studied.

All four taxa share Rubella-orange in the upper part of the hymenium, hypothecium and exciple. Moreover, they all have a similar coloration of the apothecia (orange to orange-brown), often with white pruina along the margin. Three taxa, *B. obtecta*, *B. elongata* and *B. fraxinea*, are characterized by smooth, warted to wrinkled thalli. This contrasts with the granular thallus of *B. rubella*.

Recent studies showed that *B. elongata* forms a separate entity sister to *B. fraxinea*, differing in its exciple structure that usually has up to four layers of enlarged lumina cells (Gerasimova *et al.* 2018). This feature is also characteristic of the closely related and newly described *B. obtecta*, whereas *B. fraxinea* and *B. rubella* have only a single layer of enlarged lumina cells. Compared with *B. elongata*, *B. obtecta* has abundant colourless crystals in the upper part of the hymenium and lateral exciple, and spores with fewer septa (a detailed description is provided under Taxonomy and in Table 4).

Polychroa group (including B. albogranulosa, B. polychroa, B. diffracta *and* B. sachalinensis *clades)*

Bacidia polychroa represents the most widespread species of the group, occurring in Europe, North and South America, and Asia (Ekman 1996; Llop 2007; Coppins & Aptroot 2009). The known collection localities of *B. albogranulosa* include the Czech Republic, Poland, Russia (Caucasus) and Ukraine (Malíček *et al.* 2018). *Bacidia diffracta* and *B. sachalinensis* are endemics and are so far known from North America and the RFE, respectively (Ekman 1996; Gerasimova *et al.* 2018). The phylogeny contains *B. polychroa* from Sweden, *B. albogranulosa* from Europe, *B. diffracta* from North America, and *B. sachalinensis* from the RFE.

The phylogeny recovered the whole clade with high support and is concordant with the previous results of Gerasimova *et al.* (2018), who provide a detailed discussion. All taxa within the clade share Polychroa-brown in the hypothecium, exciple and hymenium, the K+ purplish reaction in apothecial cross-sections. However, this reaction is unknown for the recently described *B. albogranulosa* since it is known only in its sterile form (Malíček *et al.* 2018).

Arceutina group (including B. arceutina, B. scopulicola *and* B. sipmanii *clades)*

Bacidia arceutina is the most widespread epiphytic species of the group. The known collection localities include Europe, Asia and North America (Coppins & Aptroot 2009). In contrast, *B. scopulicola* and *B. sipmanii* are almost exclusively epilithic, occurring in coastal Europe from Scandinavia to the Azores, and in North America (Coppins & Aptroot 2009). The phylogeny includes the specimens of all three taxa collected in Europe.

All three taxa share Arceutina-yellow in the apothecial structures. So far, none of the three species have been observed from the RFE. *Bacidia arceutina* from Switzerland formed separate lineages from those originating in the United Kingdom (FR799125-FR799127) and Sweden (*Ekman* 3110). However, given the low genetic differentiation in nrITS, nrLSU and mtSSU (1% in each), we regard them as conspecific.

Clades including B. ekmaniana, B. absistens *and* B. squamulosula

Bacidia ekmaniana is known from south-eastern North America, occurring from the Appalachian Mountains to the Coastal Plain

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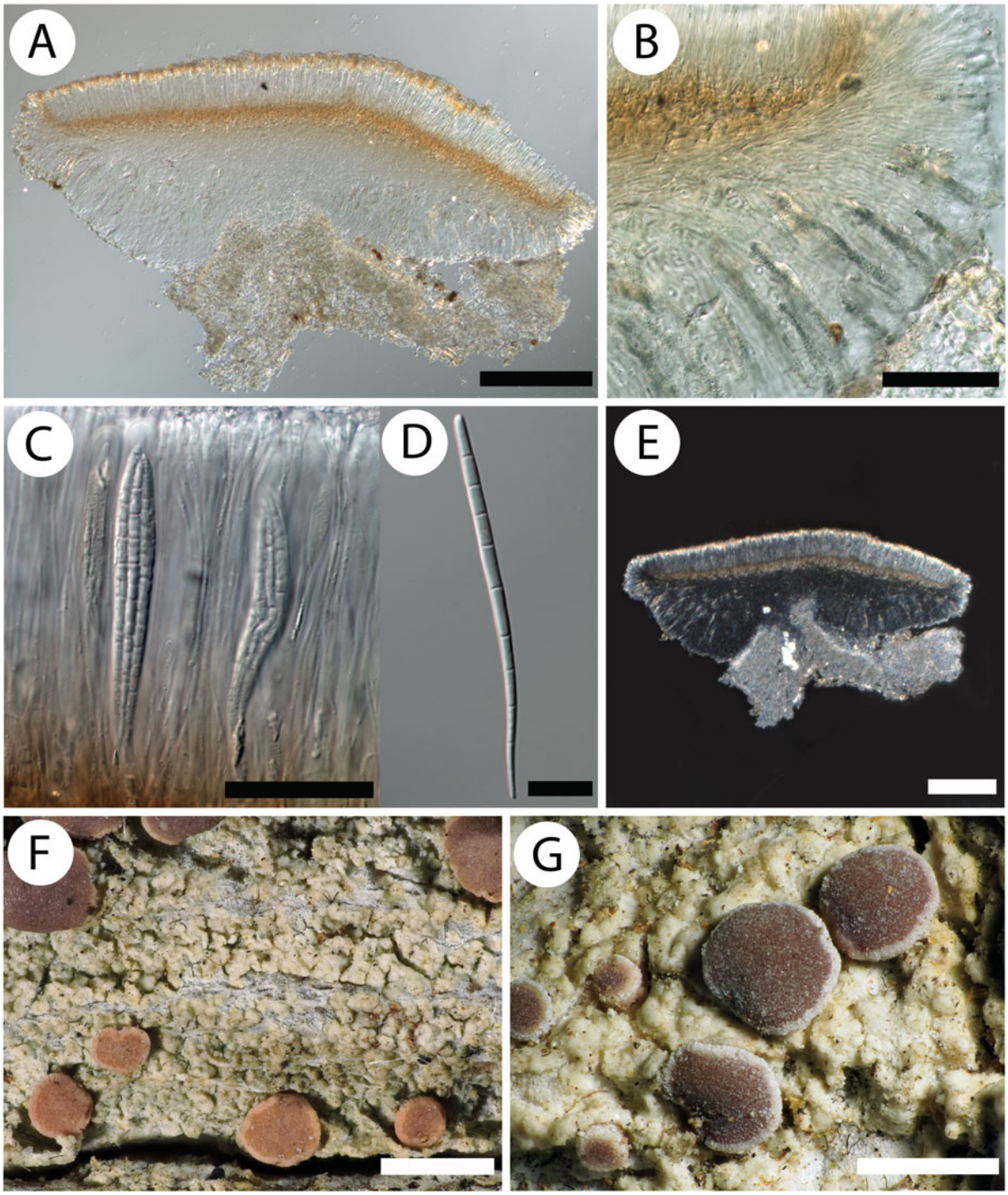

Fig. 4. Cross-section of apothecium and thallus structure of *Bacidia obtecta* (M-0308496, holotype). A, cross-section of apothecium. B, clusters of crystals radially arranged in the lateral part of the exciple. C, asci with ascospores. D, acicular multiseptate ascospore. E, crystals in the cross-section of apothecium visualized using polarized light. F, general overview of apothecia and thallus structure. G, detailed view of apothecia and thallus structure. Thallus wrinkled, irregularly shaped. Rusty brown apothecia with scurfy surface and white pruina along the margin. Scales: A, B & E = 200 μm; C = 50 μm; D = 20 μm; F & G = 1 mm. In colour online.

and southern Interior Highlands (Lendemer *et al.* 2016). The known distribution of *B. absistens* includes Europe, Macaronesia, Africa, Asia and North America (Ekman 1996; Coppins & Aptroot 2009; Gerasimova 2016), whereas *B. squamulosula* is known from South and North America (Dahl 2017; McMullin *et al.* 2020).

Bacidia ekmaniana and *B. squamulosula* share a mixture of Rubella-orange and Arceutina-yellow in the apothecia, a feature they share with other taxa from clade II (Table 3). In contrast, the pigmentation in *B. absistens* is highly variable. It may contain a mixture of Bagliettoa-green, Laurocerasi-brown or grey coloration in the upper hymenium and exciple edge (Gerasimova 2016). Thus, the pigmentation of *B. absistens* is unique in this clade. The significant morphological variation in this species requires further study, involving more specimens.

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The recently published *B. gigantensis* formed a sister clade to *B. absistens* and *B. squamulosula* in the original publication of McMullin *et al.* (2020) and the relationships of these taxa are discussed in detail there. *Bacidia gigantensis* has grey to grey-brown coloration in the upper hymenium and exciple, also observed in *B. absistens* (Gerasimova 2016). The three taxa, *B. absistens*, *B. gigantensis* and *B. squamulosula* contain secondary compounds, 4-O-methylcryptochlorophaeic and homosekikaic acids. This is unusual and is not known for any other *Bacidia* s. str., which contain atranorin as the main secondary compound (Ekman 1996). In addition, all three taxa are characterized as having minute colourless crystals in the exciple.

Clade including B. thiersiana *and* B. hostheleoides

Bacidia thiersiana (deposited as *Bacidia lutescens* in GenBank) is widespread in south-eastern North America, while *B. hostheleoides* is widely distributed in the Neotropics (Malme 1935; Ekman 1996; Lendemer 2020). *Bacidia thiersiana* is characterized by having some oil droplets in the hypothecium and by the production of lobaric acid, both unique characters in *Bacidia* s. str. phylogeny. Both taxa are characterized by almost colourless or faintly and diffusely pigmented internal apothecial structures (Ekman 1996; Lendemer 2020). However, *B. hostheleoides* has small amounts of Rubella-orange in the proper exciple, hypothecium and hymenium when pigmented (Ekman 1996).

These taxa belong to a well-supported clade with long branches, most likely resulting from incomplete species sampling rather than a genuine relationship. Therefore, further sampling in this group is still required to draw reliable conclusions.

Taxonomy

Bacidia obtecta Gerasimova, A. Ezhkin & A. Beck sp. nov.

MycoBank No.: MB 838949

Similar to *Bacidia elongata* but differs by abundant colourless crystals in the upper part of the hymenium and lateral exciple, dissolving in KOH, and by having spores with fewer septa.

Type: Russia, Sakhalin, Tymovskiy District, valley of the Tym River, Zonal'noye village surroundings, 51°04′42.6899″N, 142°44′25.8756″E, *c.* 100 m a.s.l., floodplain forest, on the bark of *Populus maximowiczii*, 4 June 2017, *A. K. Ezhkin* [B4/02.2018] (M M-0308496—holotype; UPS, SAK 1368—isotypes).

(Fig. 4)

Thallus indefinite, continuous or discrete, relatively thick, wrinkled, sinuous, consisting of scattered or continuous wrinkled, irregularly shaped or ±convex warts, grey, grey-green to yellowish green. *Prothallus* white. *Photobiont* chlorococcoid.

Apothecia (0.4–)0.85 ± 0.25(–1.5) mm, ±plane when young, soon becoming markedly convex, then margin is excluded; disc pruinose, with scurfy/incrusted surface; margin of young and medium-aged apothecia often with thick white pruina. *Disc* beige, pale orange, orange-brown to rusty brown. *Margin* distinct, always paler than disc, beige or pale brown. *Epithecium* colourless, interspersed with abundant colourless crystals between the paraphysis apices, less than 1 µm. *Exciple* laterally 86–94.3–98 µm high, with abundant colourless crystals, less than 1 µm. *Rim* pale to intense straw-coloured, along the edge with up to four layers of enlarged lumina cells that are 3–5 µm wide and 5–13 µm long. *Lateral part* pale to intense straw-coloured or yellow. *Medullary part* of the same colour as lateral part, downwards almost colourless. *Hymenium* 73.5–108–135 µm high. *Hypothecium* pale to intense orange-brown (Rubella-orange). *Paraphyses* simple, thin, colourless, sometimes bifurcate, 1.0–1.5 µm wide, ±clavate or only slightly swollen at the apices, 1.5–3.0 µm, without internal pigment. *Ascospores* acicular, straight, (47–)61.2 ± 7.0(–79) µm long ($n_1 = 88$, $n_2 = 3$), (2.0–)3.0 ± 0.36(–4.0) µm wide ($n_1 = 88$, $n_2 = 3$), with (1–)6 ± 2(–11) septa ($n_1 = 88$, $n_2 = 3$).

Pycnidia not observed.

Pigments. Hypothecium K+ intensifying or yellowish, exciple C+ and N+ yellow, subsequently becoming colourless. Rubella-orange in hypothecium and exciple. Crystals dissolve in K, forming the following reaction: green → yellow → colourless; do not dissolve in N or C.

Etymology. Apothecial disc covered with pruina on top, which gives a 'powdery' appearance.

Habitat and distribution. The specimens were collected in the floodplain forest in river valleys on the bark of *Chosenia arbutifolia* and *Populus maximowiczii*. So far, known only from Sakhalin. Probably endemic to the island.

Comments. *Bacidia obtecta* is similar to *B. elongata* and *B. fraxinea* but differs in several distinct features, summarized in Table 4. Compared to *B. elongata* and *B. fraxinea*, it is distinguished by the apothecial colour, which is mainly brown to rusty brown, abundant colourless crystals in the upper part of the hymenium and lateral exciple, which dissolve in KOH but not in HNO_3, and spores with fewer septa. From *B. fraxinea* it can be additionally distinguished by the number of enlarged lumina cells along the exciple edge.

Additional specimens examined. **Russia:** *Sakhalin*: Tymovskiy District, valley of Pilenga River, Adotymovo village surroundings, 51°02′07.6236″N, 142°49′26.5692″E, *c.* 160 m a.s.l, floodplain forest, on bark of *Populus maximowiczii*, 5 vi 2017, *A. K. Ezhkin* [B2/02.2018; GPS 547] (M M-0308497, SAK 1365); *ibid.*, valley of Tym River, Zonal'noye village surroundings, 50°38′51.2951″N, 142°45′48.2868″E, *c.* 160 m a.s.l., floodplain forest, on bark of *Chosenia arbutifolia*, 6 vi 2017, *A. K. Ezhkin* [B1/02.2018; GPS 551] (M-0308498, SAK 1362).

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