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A new circumscription of the genus *Varicellaria* (Pertusariales, Ascomycota)

Imke Schmitt[1,2], Jürgen Otte[1], Sittiporn Parnmen[3], Anna D. Sadowska-Deś[1,2], Robert Lücking[3], H. Thorsten Lumbsch[3]

1 *Biodiversity and Climate Research Centre (BiK-F), Forschungsinstitut Senckenberg, Senckenberganlage 25, D-60325 Frankfurt, Germany* **2** *Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Goethe Universität, Max-von-Laue-Str. 13, D-60438 Frankfurt, Germany* **3** *Department of Botany, Field Museum of Natural History, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA*

Corresponding author: *Imke Schmitt* (imke.schmitt@senckenberg.de)

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Abstract

The lichen-forming genus *Pertusaria* under its current circumscription is polyphyletic and its phylogenetic affiliations are uncertain. Here we study the species of the genera *Pertusaria* and *Varicellaria* which contain lecanoric acid as major constituent, have disciform apothecia, strongly amyloid asci, non-amyloid hymenial gel, 1-2-spored asci, and 1- or 2-celled ascospores with thick, 1-layered walls. We infer phylogenetic relationships using maximum likelihood and Bayesian analyses based on four molecular loci (mtSSU, nuLSU rDNA, and the protein-coding, nuclear *RPB1* and *MCM7* genes). Our results show that the lecanoric acid-containing species form a well-supported, monophyletic group, which is only distantly related to *Pertusaria* s.str. The phylogenetic position of this clade is unclear, but placement in *Pertusaria* s.str. is rejected using alternative hypothesis testing. The circumscription of the genus *Varicellaria* is enlarged to also include species with non-septate ascospores. Seven species are accepted in the genus: *Varicellaria culbersonii* (Vězda) Schmitt & Lumbsch, **comb. nov.**, *V. hemisphaerica* (Flörke) Schmitt & Lumbsch, **comb. nov.**, *V. kasandjeffii* (Szatala) Schmitt & Lumbsch, **comb. nov.**, *V. lactea* (L.) Schmitt & Lumbsch, **comb. nov.**, *V. philippina* (Vain.) Schmitt & Lumbsch, **comb. nov.**, *V. rhodocarpa* (Körb.) Th. Fr., and *V. velata* (Turner) Schmitt & Lumbsch, **comb. nov.** A key to the species of *Varicellaria* is provided.

Key words

Agyriales, Ascomycota, lichen-forming fungi, molecular phylogeny, Ostropomycetidae, *Pertusaria,* Pertusariales, taxonomy

Introduction

Generic classifications in lichen-forming fungi have changed dramatically since the introduction of molecular data. Numerous genera have been shown to be polyphyletic or nested within larger genera (e.g., Amo de Paz et al. 2010a, b; Blanco et al. 2004a, b, 2005, 2006; Crespo et al. 2007, 2010; Crewe et al. 2006; Divakar et al. 2006; Ertz and Tehler 2011; Gueidan et al. 2009; Högnabba 2006; Muggia et al. 2010; Printzen 2010; Rivas Plata and Lumbsch 2011; Rivas Plata et al. 2012; Tehler and Wedin 2008; Wedin et al. 2005; Westberg et al. 2010). A further example of incongruence of current classification and phylogenetic relationships as inferred from DNA sequences is the heterogeneous genus *Pertusaria.* It is the largest genus within Pertusariales*,* with possibly over 1000 species (Archer and Elix 2011, Messuti and Archer 2009). However, it has been shown to be polyphyletic with species belonging even to different families within the order (Lumbsch and Schmitt 2001, 2002; Schmitt and Lumbsch 2004; Schmitt et al. 2006, 2010).

Schmitt and Lumbsch (2004) identified a combination of phenotypical characters to distinguish between three of the clades of *Pertusaria.* These characters include secondary metabolites, ascoma-morphology, amyloidity of ascus walls and hymenial gel, number of ascospores per ascus, and ascospore wall thickness and layers. Later, Schmitt et al. (2010) identified a fourth clade with gyalectoid ascomata and found it to be related to Coccotremataceae. The latter clade was distinguished as the genus *Gyalectaria* and was placed in Coccotremataceae. However, the two remaining major clades that are not closely related to *Pertusaria* s.str., the *Variolaria* and *Varicellaria* groups identified in Schmitt and Lumbsch (2004), have not yet been reclassified. In continuation of our studies on pertusarialean fungi, we are here addressing the issue of monophyly and classification of the so-called *Varicellaria* clade of *Pertusaria.* This is a group of pertusarialean lichenized fungi characterized by disciform apothecia, non-amyloid hymenial gel, strongly amyloid asci, 1-2-spored asci, and 1- or 2-celled ascospores with more or less thick, 1-layered walls (Schmitt and Lumbsch 2004). Chemically, the clade is characterized by the presence of lecanoric acid as major metabolite. Recent collections of *Pertusaria culbersonii,* a neotropical species with lecanoric acid, prompted us to address the phylogeny of this group and to classify those *Pertusaria* species belonging to the *Varicellaria* group. We have compiled a data set of 29 pertusarialean fungi including all but two species (*P. kasandjeffii* and *P. philippina* – no fresh material available) that were thought to belong to the *Varicellaria* group based on phenotypical evidence.

Materials and methods

Taxon sampling and molecular methods

We assembled a four-locus data set consisting of mtSSU rDNA, nuLSU rDNA, and the protein-coding genes *RPB1* and *MCM7.* The alignment contained 31 species. Specimens and sequences used for molecular analyses are listed in Table 1. Two sequences

Table 1. Species and sequences used in this study. New sequences are indicated in bold.

Name	Phylogenetic lineage	Family	nuLSU	mtSSU	*1RPB*	*7MCM*
*Varicellaria culbersonii**	Varicellaria	?	**JX101871**	**JX101873**	**JX101875**	**JX101874**
Varicellaria hemisphaerica	Varicellaria	?	AF381556	AF381563	DQ902341	GU980998
Varicellaria lactea	Varicellaria	?	AF381557	AF381564	DQ870971	GU981000
Varicellaria rhodocarpa	Varicellaria	?	AF381559	AF381569	N/A	N/A
Varicellaria velata	Varicellaria	?	AY300855	GU980981	DQ870982	GU981005
"Pertusaria" amara	Variolaria	?	AF274101	AY300900	DQ870965	GQ272423
"Pertusaria" corallina	Variolaria	?	AY300850	AY300901	DQ870967	GU980997
"Pertusaria" scaberula	Variolaria	?	AF274099	AF431959	DQ870980	GU981003
"Pertusaria" subventosa	Variolaria	?	AY300854	AY300905	DQ870981	GU981004
Circinaria contorta		Megasporaceae	DQ986782	DQ986876	DQ986852	GU980989
Circinaria hispida		Megasporaceae	DQ780305	HM060722	DQ870933	DQ780273
Lobothallia radiosa		Megasporaceae	DQ780306	DQ780274	DQ870954	GQ272397
Ochrolechia parella		Ochrolechiaceae	AF274097	GU980977	DQ870959	GQ272421
Ochrolechia subpallescens		Ochrolechiaceae	GU980985	GU980978	GU981008	GU980994
Ochrolechia upsaliensis		Ochrolechiaceae	GU980986	GU980979	GU981009	GU980995
Coccotrema cucurbitula		Coccotremataceae	AF274092	AF329161	DQ870939	GU980990
Coccotrema maritimum		Coccotremataceae	AF329164	AF329163	N/A	GU980991
Coccotrema pocillarium		Coccotremataceae	AF274093	AF329166	DQ870940	GU980992
Gyalectaria diluta		Coccotremataceae	GU980982	GU980974	N/A	N/A
Gyalectaria gyalectoides		Coccotremataceae	GU980983	GU980975	GU981006	GU980993
Gyalectaria jamesii		Coccotremataceae	GU980984	GU980976	GU981007	N/A
Thamnolia vermicularis		Icmadophilaceae	AY961599	AY853345	DQ915599	N/A
Icmadophila ericetorum		Icmadophilaceae	DQ883694	DQ986897	DQ883723	N/A
Dibaeis baeomyces		Icmadophilaceae	AF279385	AY300883	DQ842011	N/A
Agyrium rufum		Agyriaceae	EF581826	EF581823	EF581822	GU980988
*Miltidea ceroplasta***		Miltideaceae	HQ391558	HQ391557	**JQ900620**	N/A
*Pertusaria hermaka****	Pertusaria s. str.	Pertusariaceae	DQ780334	DQ780299	**JX101872**	GU980999
Pertusaria paramerae	Pertusaria s. str.	Pertusariaceae	DQ780328	GU980980	GU981012	GU981001
Pertusaria pustulata	Pertusaria s. str.	Pertusariaceae	DQ780332	DQ780297	GU981013	GU981002
Parmeliopsis hyperopta	outgroup	Parmeliaceae	AY607823	AY611167	EF092142	GQ272426
Everniopsis trulla	outgroup	Parmeliaceae	EF108290	EF108289	EF105429	GQ272396

*source: Costa Rica, *R. Lücking 15424* (F)

**source: Australia, *H.T. Lumbsch 20004b, S. Parnmen & T. Widhelm* (F)

***source: Australia, *A. Mangold,* 22 March 2005 (MIN) of Parmeliaceae (Lecanoromycetes) were used as outgroup, since Lecanoromycetes was shown to be a sister-group of Ostropomycetidae to which Pertusariales belongs (Grube et al. 2004; Miadlikowska et al. 2006; Schmitt et al. 2009). Molecular methods were the same as in a previous study (Schmitt et al. 2010).

Sequence alignments and phylogenetic analysis

We assembled partial sequences using Geneious Pro 5.4.3 (Drummond et al. 2011) and edited conflicts manually. We aligned the sequences using Clustal W (Thompson et al. 1994) (nuLSU, *RPB1*, *MCM7*) or PRANK (Loytynoja and Goldman 2005, 2010) (mtSSU). MtSSU sequences are highly variable and contain substantial length polymorphisms that disrupt the alignment. Thus, we eliminated unreliably aligned sites from the mtSSU alignment using the program Aliscore 2.0 (Misof and Misof 2009). Aliscore settings were: window size of six positions, and gaps treated as ambiguous characters (-N option invoked). After cutting 1084 unreliably aligned positions, 698 positions (39%) of the original mtSSU alignment were left.

We analyzed the alignments using maximum likelihood (ML) and Bayesian inference. To test for potential conflict between data sets, we performed ML analyses on the individual alignments and examined the trees for conflicts supported by 75% bootstrap support. ModelTest (Posada and Crandall 1998) selected the following models as best fits for our data: GTR+G+I for nuLSU, *RPB1*, *MCM7*, and GTR+G for mtSSU. The individual alignments were analyzed in Geneious using MrBayes 3.1 (Huelsenbeck and Ronquist 2001) with the following settings: 1,100,000 generations starting with a random tree and employing 12 simultaneous chains. Two runs were executed, and every 1000th tree was saved into a file. The first 100 trees were discarded as burn in. We checked the traces in Geneious to ensure that stationarity was achieved after the first 100,000 generations. MrBayes settings for the concatenated alignment were the same as above but with 8,000,000 generations and the data split into 8 partitions (mtSSU, nuLSU, and each codon position of *RPB1* and *MCM7*). We used the model GTR+I+G and the burn in was set to 1000. Of the remaining trees, a majority rule consensus tree with average branch lengths was calculated. Posterior probabilities were obtained for each clade. Only clades with posterior probabilities equal or above 0.95 in the Bayesian analysis or bootstrap support equal or above 75 % under ML were considered as strongly supported.

The ML analysis of the concatenated alignment was performed with the program RAxML (Stamatakis 2006) using the default rapid hill-climbing algorithm. The model of nucleotide substitution chosen was GTRMIX. The data set was partitioned into eight parts (mtSSU, nLSU and each codon position of *RPB1* and *MCM7*). Rapid bootstrap estimates were carried out for 2000 pseudoreplicates. Phylogenetic trees were visualized using the program TreeView (Page 1996).

As in previous studies (e.g. Schmitt and Lumbsch 2004) the lecanoric acid-containing species of *Pertusaria* clustered outside *Pertusaria* s.str., and instead with the genus *Varicellaria*, hence contradicting current classification. Thus, we tested whether our data are sufficient to reject monophyly of *Pertusaria* s.str. + lecanoric acid containing *Pertusaria* spp. For hypothesis testing, we used two different methods: i) Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 2001) and ii) expected likelihood weight (ELW) test (Strimmer and Rambaut 2002). The SH and ELW test were performed using Tree-PUZZLE 5.2 (Schmidt et al. 2002) with the combined data set, comparing the best tree agreeing with the null hypotheses, and the unconstrained ML tree. These trees were inferred in Tree-PUZZLE using the GTR+I+G nucleotide substitution model.

Results

We obtained six new sequences indicated in Table 1. The combined alignment of the nuLSU, mtSSU rDNA, *RPB1,* and *MCM7* included 2790 unambiguously aligned nucleotide position characters, 1226 of which were variable. The single locus ML topologies did not show any conflicts and hence a concatenated analysis was performed. The maximum likelihood tree did not contradict the Bayesian tree topologies and thus only the majority-rule consensus tree of the Bayesian tree sampling is shown here (Fig. 1). In the phylogenetic tree, species of the *Varicellaria*-group form a strongly supported monophyletic group, including *P. culbersonii.* The *Varicellaria*-group is sister to the *Variolaria*-group, but this relationship lacks support. The genus *Ochrolechia* is a well-supported sister-group to Megasporaceae (*Circinaria* and *Lobothallia*), and this clade is sister to the *Varicellaria*- and *Variolaria*-groups, but again this relationship lacks support. *Agyrium* and *Miltidea* form a supported sister-group, which is strongly supported sister to the well-supported, monophyletic *Pertusaria* s.str. The well-supported, monophyletic genera *Coccotrema* and *Gyalectaria* have a well-supported sister-group relationship. The sister-group relationship of Coccotremataceae and the clade including *Agyrium, Miltidea,* and *Pertusaria* s.str. lacks support. A placement of the *Varicellaria* clade in *Pertusaria* s.str. is rejected significantly ($p \leq 0.001$ in both tests) using alternative hypothesis testing.

Discussion

The current study confirms previous results on the polyphyly of *Pertusaria* (Lumbsch and Schmitt 2001, 2002; Lumbsch et al. 2006; Schmitt and Lumbsch 2004; Schmitt et al. 2006, 2010). It also confirms that species with lecanoric acid as major constituent and disciform apothecia are closely related to *Varicellaria rhodocarpa* and therefore should be included in the genus *Varicellaria*. Our taxon sampling included all but two species putatively belonging to the *Varicellaria*-group and hence we feel confident to draw formal nomenclatural consequences.

We will address the issue of the phylogeny and classification of the species-rich *Variolaria*-group in the future using an extended and geographically balanced taxon sampling. Our study shows that additional, molecular markers will be necessary to elu-

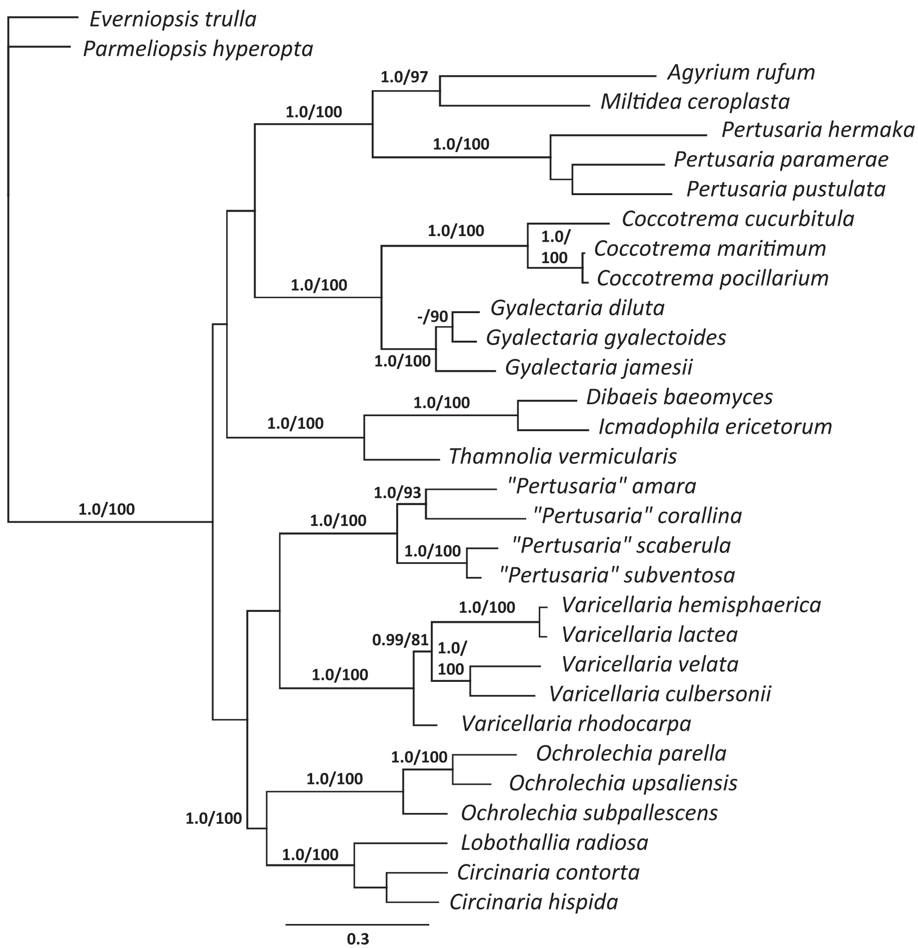

Figure 1. Phylogeny of pertusarialean fungi based on mtSSU, nuLSU, *RPB1* and *MCM7* sequences. This is a 50% majority rule consensus tree based on 14,000 trees from a Bayesian analysis. Values above the branches are posterior probabilities/ML bootstrap support (ML based on 2000 replicates).

cidate the phylogenetic relationships of major clades within Pertusariales (incl. Agyriales) (Hodkinson and Lendemer 2011), since the backbone of the phylogeny of the order almost entirely lacks support.

Taxonomic consequences and key to the species

Varicellaria Nyl. Mém. Soc. Imp. Sci. Nat. Cherbourg 5: 119. 1858.

Type species. *Varicellaria microsticta* Nyl. Mém. Soc. Imp. Sci. Nat. Cherbourg 5: 119. 1858. [=*V. rhodocarpa* (Körb.) Th.Fr.]

=*Clausaria* Nyl. Annls Sci. Nat., Bot., sér. 4 15: 45. 1861.

Type species. *Clausaria fallens* Nyl., Ann. Sci. Nat., Bot., sér. 4 15: 45. 1861. [=*Varicellaria velata* (Turner) Schmitt & Lumbsch]

The genus in its enlarged circumscription includes species with disciform ascomata, non-amyloid hymenial gel, strongly amyloid, 1-2-spored asci, and 1- or 2-celled ascospores with thick, 1-layered walls. All species contain lecanoric acid, and may also contain lichexanthone or variolaric acid. Currently, we accept seven species in this genus. The accepted names and authorities are listed below.

***Varicellaria culbersonii* (Vězda) Schmitt & Lumbsch, comb. nov.**
Mycobank: MB 800038

Basionym. *Pertusaria culbersonii* Vězda. Lich. sel. exs. 60: 4 (no. 1487). 1977. Type. Costa Rica, San José, Cerro de la Muerte, 3330m alt., 1976, on soil, *W.L. Culberson 13195J* (holotype PRA-V).

***Varicellaria hemisphaerica* (Flörke) Schmitt & Lumbsch, comb. nov.**
Mycobank: MB 800039

Basionym. *Variolaria hemisphaerica* Flörke. Deutsche Lich. 2: 6. 1815. Type. Germany, Berlin [Flörke, Deutsche Lichenen exs. 29] (isotype BM).

Synonym. *Pertusaria hemisphaerica* (Flörke) Erichsen. Hedwigia 72: 85. 1932.

***Varicellaria kasandjeffii* (Szatala) Schmitt & Lumbsch, comb. nov.**
Mycobank: MB 800040

Basionym. *Pertusaria kasandjeffii* Szatala. Magy. Bot. Lapok 29: 83. 1930. Type. Bulgaria, Cepelarska planina, in monte Turluka, par Pamsakli, 1500m alt., 6.1929, *Szatala* (isotype HBG-1233).

This species is only known from a few localities in Bulgaria and Romania (Hanko 1983). Since no fresh material was available, we could not generate molecular data. However, the species agrees morphologically and chemically with the *Varicellaria*-group (Fig. 2) and in fact its distinction from *P. lactea* is not entirely clear. Both taxa contain lecanoric and variolaric acid, but *P. kasandjeffii* differs in being esorediate and having a thick, bulbate thallus. Additional collections are required to test whether *P. kasandjeffii* is indeed different from *P. lactea.*

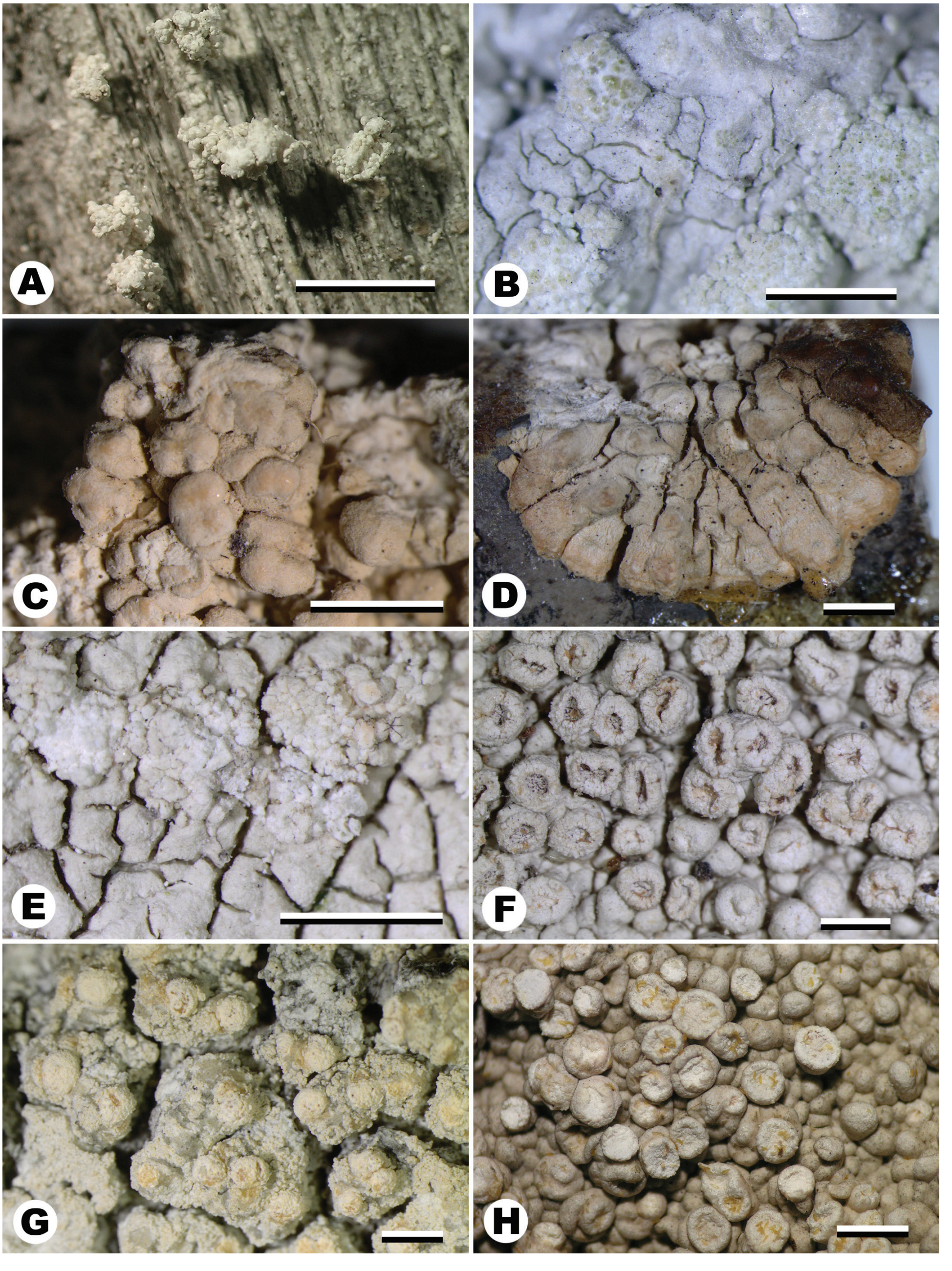

Figure 2. The species of *Varicellaria*. **A** *V. culbersonii*. Costa Rica, Buck 44182 (F) **B** *V. hemisphaerica*. Germany, 15.4.2004, Schmitt (FR) **C, D** *V. kasandjeffii*. Isotype. Bulgaria, Cepalarska planina: in monte Turluka, par Pasmakali, 1500 m, 9.6.1929, *Szatala* (HBG-1233) **E** *V. lactea*. Spain, Schmitt 5.6.2003 (FR) **F** *V. philippina*. Holotype. Philippines, Mindanao Dist. Lanao, Camp Keithley by lake Lanao, Sept. 1907, M.S. Clemens, (TUR-V-0006709) **G** *V. rhodocarpa*. Sweden, Printzen 6908 (FR) **H** *V. velata*. Colombia, Moncada & Davila 1537 (F). Scale bar: 1mm. Images were taken with an Olympus SC30 camera under an Olympus SZX7 stereomicroscope.

***Varicellaria lactea* (L.) Schmitt & Lumbsch, comb. nov.**
Mycobank: MB 800041

Basionym. *Lichen lacteus* L., Mant. Pl. 1: 132. 1767. Type. Sweden, Västergötland, Mularp, 6.08.1922, *Vrang* [=Malme, Lich. Suec. Exs. 848] (neotype UPS, designated by Jørgensen et al. (1994)).

Synonyms. *Lepra lactea* (L.) F.H.Wigg. Prim. fl. Holsat.: 97. 1780. *Variolaria lactea* (L.) Pers. Ann. Bot. 1: 24. 1794. *Psora lactea* (L.) P.Gaertn., G.Mey. & Scherb. Ökonom.-techn. Fl. Wetterau 3: 214. 1801. *Zeora lactea* (L.) Arnold. Flora, Jena 53: 214. 1870. *Pertusaria lactea* (L.) Arnold. Verh. zool.-bot. Ges. Wien 22: 283. 1872. *Ochrolechia lactea* (L.) Matzer & Hafellner. Bibl. Lichenol. 37: 101. 1990.

***Varicellaria philippina* (Vain.) Schmitt & Lumbsch, comb. nov.**
Mycobank: MB 800589

Basionym. *Pertusaria philippina* Vain. Philipp. J. Sci., C, Bot. 8: 131. 1913. Type. Philippines, Mindanao, Lanao, Castra Keithley at Lake Lanao, 1907, *Clemens 1302* (holotype TUR-V 6391!).

This species is only known from the Philippines (Wainio 1913) and Papua New Guinea (Elix et al. 1997). We could not generate molecular data since no fresh material was available. Morphologically and chemically the species agrees with *P. velata* (Fig. 2), but differs in having 2-spored asci.

***Varicellaria rhodocarpa* (Körb.) Th.Fr. Lich. Scand. (Uppsala) 1: 322. 1871.**
Basionym. *Pertusaria rhodocarpa* Körb. Syst. lich. germ.: 384. 1855.

Synonyms. *Varicellaria microsticta* Nyl. Mém. Soc. Imp. Sci. Nat. Cherbourg 5: 119. 1858. *Varicellaria kemensis* Räsänen. Ann. Soc. zool.-bot. Fenn. Vanamo 3: 295. 1926.

***Varicellaria velata* (Turner) Schmitt & Lumbsch, comb. nov.**
Mycobank: MB 800042

Basionym. *Parmelia velata* Turner. Trans. Linn. Soc. London 9: 143. 1808. Type. Great Britain, England, Sussex, 1805, *Borrer* (holotype BM-4109).

Synonyms. *Lichen velatus* (Turner) Sm. & Sowerby. Engl. Bot. 29: tab. 2062. 1809. *Variolaria velata* (Turner) Ach. Lich. univ.: 696. 1810. *Pertusaria velata* (Turner) Nyl. Lich. Scand. (Uppsala): 179. 1861.

Pertusaria conglobata (Ach.) Th.Fr. Lichenogr. Scand. 1: 321. 1871. *Variolaria conglobata* Ach. Syn. Lich.: 132. 1814.

Pertusaria haematommoides Zahlbr., Feddes Rep. 33: 50. 1933. Type. Taiwan, Rengechi, *Asahina 263* (W – holotype!).

Pertusaria obvelata Nyl. Bih. K. svenska Vetensk. Akad. Handl. 3: 1–156. 1888.

Key to the species of Varicellaria

1a	Ascospores 2-celled, thallus esorediate or rarely sorediate, containing lecanoric acid, growing on soil, detritus or mosses in arctic-alpine habitats of the northern Hemisphere .... ***V. rhodocarpa***
1b	Ascospores 1-celled, thallus esorediate or sorediate, chemistry and habitat various **2**
2a	Thallus esorediate........ **3**
2b	Thallus sorediate **6**
3a	Thallus thin, coarsely wrinkled to rimose-cracked, containing lecanoric acid, ± lichexanthone, and ± variolaric acid **4**
3b	Thallus thick, bullate, apothecia rare or unknown, when present 1-1.5 mm in diam., lacking lichexanthones, Neotropical or restricted to eastern Europe.... 5
4a	Asci 1-spored, cosmopolitan .... ***V. velata***
4b	Asci 2-spored, so far only known from Philippines and Papua New Guinea... .... ***V. philippina***
5a	Growing on siliceous rocks, known only from the Balkan region of Europe . ... ***V. kasandjeffii***
5b	Growing on soil, detritus or mosses, known from high altitudes in Central America .... ***V. culbersonii***
6a	Thallus containing lecanoric acid, on bark, rarely on rocks .... ***V. hemisphaerica***
6b	Thallus containing lecanoric acid and variolaric acid, on rocks, rarely on bark .... ***V. lactea***

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