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

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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

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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 gylecticoid ascomata and found it to be related to Coccomyces. The latter clade was distinguished as the genus *Gyalectaria* and was placed in Coccomyces. 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-amylloid 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 licanoric acid as major metabolite. Recent collections of *Pertusaria culbersonii*, a neotropical species with licanoric 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.

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<tr>
<th>Name</th>
<th>Phylogenetic lineage</th>
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<td>GQ272396</td>
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</table>

*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 unreliable 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
A new circumscription of the genus *Varicellaria* (Pertusariales, Ascomycota) 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 Coccotremae 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≤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 pertusarial 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. Agyri-
ales) (Hodkinson and Lendemer 2011), since the backbone of the phylogeny of the order almost entirely lacks support.

Taxonomic consequences and key to the species


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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


**Varicellaria hemisphaerica** (Flörke) Schmitt & Lumbsch, comb. nov.

Mycobank: MB 800039


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

**Varicellaria kasandjeffii** (Szatala) Schmitt & Lumbsch, comb. nov.

Mycobank: MB 800040


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


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


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.


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


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

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 ......................................................... \textit{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 ...................................................................... \textit{V. velata}

4b Asci 2-spored, so far only known from Philippines and Papua New Guinea... .................................................................................................. \textit{V. philippina}

5a Growing on siliceous rocks, known only from the Balkan region of Europe . ............................................................................................. \textit{V. kasandjeffii}

5b Growing on soil, detritus or mosses, known from high altitudes in Central America .............................................................................................. \textit{V. culbersonii}

6a Thallus containing lecanoric acid, on bark, rarely on rocks .... \textit{V. hemisphaerica}

6b Thallus containing lecanoric acid and variolaric acid, on rocks, rarely on bark ............................................................................................... \textit{V. lactea}

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We thank Armin Mangold (Berlin) and Todd Widhelm (Omaha) for collecting material used in this study, and Miklós Bálint (Frankfurt) for assisting with running phylogenetic analyses on the “FUCHS” computing cluster of the Center for Scientific Computing, Frankfurt am Main. Matthias Schultz (Hamburg), Seppo Huhtinen (Turku), and Anton Igersheim (Vienna) kindly sent type material on loan, and Klaus Kalb (Neumarkt) provided literature. This study was funded by ‘LOEWE, Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz’ of Hesse’s Ministry of Higher Education, Research, and the Arts. A.S-D. was supported by a stipend from Deutsche Bundesstiftung Umwelt (DBU).

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