The Lichenologist **46**(3): 441–453 (2014)
doi:10.1017/S0024282913000935

A molecular phylogeny of the lichen genus *Biatora* including some morphologically similar species

Christian PRINTZEN

Abstract: The lichen genus *Biatora* comprises inconspicuous crustose lichens that are typically found on organic substrata such as tree bark, bryophytes and detritus. During the last 20 years many new species have been added to the genus making its delimitation more and more difficult. The infrageneric relationships of the 42 species have never been investigated thoroughly. Using DNA sequences from three gene loci (ITS, RPB2, mrSSU) and 59 OTUs, an attempt was made to reconstruct the phylogenetic relationships of *Biatora* and its infrageneric groups. *Cliostomum* appears to be the closest relative of *Biatora*. The position of *Mycobilimbia* in the *Lecania*-clade is confirmed. Phylogenetic relationships within *Biatora* are poorly supported, but six different species groups that are also phenotypically distinguished are more or less well supported: the *vernalis*-, *meiocarpa*-, *hertelii*-, *ocelliformis*-, *beckhausii*- and *rufidula*- groups. The analysis also confirms the presence of several undescribed taxa. *Biatora subduplex* as currently circumscribed appears to be heterogeneous, as does *B. helvola*. Based on the phylogeny, the distributional range of *B. alaskana* is extended to Japan. The new combinations *Biatora ementiens* (Nyl.) Printzen and *Biatora hemipolia* (Nyl.) S. Ekman & Printzen are made and both names are typified.

Key words: *Bacidia*, ITS, *Lecidea* s. lat., mrSSU, *Mycobilimbia*, *Ramalinaceae*, RPB2

Accepted for publication 6 *December* 2013

Introduction

Species of the crustose lichen genus *Biatora* have a green-algal photobiont, biatorine apothecia and unpigmented ascopores with no or few transverse septa. This places some of them in Zahlbruckner's genus *Lecidea* (Zahlbruckner 1926), others in *Bacidia* sect. *Weitenwebera*. If *Biatora* were just a modern segregate of these genera, its circumscription would probably not cause much trouble. However, over the years a growing number of species have been newly described or combined into *Biatora* and others excluded, which for now makes a delimitation of the genus rather difficult.

Biatora was described by Elias Fries in 1817 (Fries & Sandberg 1817) as a heterogeneous assemblage of taxa, some of which are today ascribed to, for example, *Caloplaca, Dimerella* or *Pannaria*. *Biatora* was recognized by most authors until about the middle of the 19th century, when, after a dispute about its independence, Nylander (1855) subsumed it under *Lecidea*. With few exceptions (e.g. Räsänen 1926; Choisy 1949), most subsequent authors followed this move. While more and more species were added to *Lecidea*, *Biatora* became one of the many groups hidden within this huge genus. By the 1860s, *Lecidea* had already become unmanageably large, so that several authors attempted to segregate it into subgenera and smaller taxonomic units [e.g. the "stirpes" of Fries (1874)]. These subgeneric systems were mainly based on ascospore characters, pigmentation of apothecia, paraphyses and hypothecium, and in some cases came close to a modern circumscription of *Biatora*, for example the "section *Lecidea vernalis*" of Vainio (1934). Nevertheless, it took about 50 years until Coppins (1983) and Hafellner (1984) almost simultaneously resurrected *Biatora vernalis* (L.) Fr.,

C. Printzen: Senckenberg Forschungsinstitut und Naturmuseum, Abteilung Botanik und Molekulare Evolutionsforschung, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany. Email: cprintzen@senckenberg.de

the type species of the genus, from the mass grave *Lecidea*.

In his revision of the European species, Printzen (1995) defined the genus narrowly and excluded *Biatora carneoalbida* (Müll. Arg.) Coppins, *B. epixanthoides* (Nyl.) Diederich, *B. sphaeroides* (Dicks.) Körb., *B. tetramera* (de Not.) Coppins (today treated as *Mycobilimbia*, Printzen *et al.* 2009), *Lecidea albohyalina* (Nyl.) Th. Fr. and *L. meiocarpa* Nyl. because of a deviating apothecial ontogeny. In *Biatora*, paraphyses and excipular hyphae typically originate from the same, strongly gelatinized initial hyphae, and the first asci grow scattered among these hyphae. In *Mycobilimbia* and *Lecidea albohyalina* (*L. meiocarpa* was not studied), the initial gelatinized hyphae develop into the excipulum, while paraphyses and asci develop *de novo* in the centre of young apothecia. In this circumscription, *Biatora* comprised 18 species with narrowly ellipsoid unicellular ascospores and pale ochre or brownish apothecia. However, a few taxa with transversely septate ascospores [*B. aegrefaciens* Printzen, *B. rufidula* (Graewe) S. Ekman & Printzen] and with distinctly pigmented apothecia [*B. mendax* Anzi, *B. ocelliformis* (Nyl.) Arnold] were already included. Meanwhile the number of species has increased to 42, partly due to the description of new species (e.g. Printzen & Tønsberg 1999, 2003, 2004; Spribille *et al.* 2009) and partly to the recombination of already described species into *Biatora* (e.g. Printzen 2004). *Biatora* now comprises species with ellipsoid ('lecideoid') and elongate ('bacidioid'), simple to multiseptate ascospores, different excipular anatomies and a large variety of secondary metabolites and insoluble pigments that result in differently coloured apothecia. As a result, it has become difficult to delimit the genus from similar or closely related taxa such as *Bacidia*, *Cliostomum*, *Lecania* or *Mycobilimbia*.

Three published molecular phylogenies that included *Biatora* have already found support for the monophyly of the genus, but included only few species (Reese Næsborg *et al.* 2007) or were based on only one or two genetic markers (Printzen & Lumbsch 2000; Spribille *et al.* 2009). The aims of this study are 1) to clarify the circumscription of *Biatora* by phylogenetic analyses based on three different gene loci and an extended sample of taxa (most of the currently accepted *Biatora* species), 2) to find out whether infrageneric evolutionary units correspond to morphologically defined groups of species, and 3) to ascertain whether some morphologically similar, and partly unnamed, taxa belong to *Biatora* and what their closest relatives are.

Material and Methods

Taxon sampling

An attempt was made to include as many accepted species of *Biatora* as possible and superficially similar taxa that had formerly been excluded from the genus, such as *Mycobilimbia* and "*Lecidea*" *albohyalina*, were added to the dataset. In order to verify their taxonomic position, supposed close relatives of accepted *Biatora* species such as *Bacidia beckhausii* Körb., *B. hemipolia* (Nyl.) Malme and "*Lecidea*" *ementiens* Nyl. were included. The results of Reese Næsborg *et al.* (2007) showed that *Lecania*, *Bilimbia* and *Cliostomum* were close relatives of *Biatora*. In order to root the tree and to detect a possible polyphyly of *Biatora*, some species from these groups were therefore added to the dataset as outgroup taxa. Finally, collections were added that phenotypically belonged in *Biatora* but could not be assigned to any described species (*"B. radicicola"*, *Biatora* species from Norway) or that resembled known species but differed in minor details (*B.* "*orientalis*", *B.* "*alaskana*" and *B.* "cf. *helvola*" from Japan). The complete dataset comprised 59 OTUs.

DNA extraction and PCR amplification

DNA was extracted from 2–3 apothecia per thallus using the QIAquick™ Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Three gene loci were amplified with the following primers: ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) for the internal transcribed spacer region of the ribosomal DNA (ITS), mrSSU1 (Zoller *et al.* 1999) and MSU7 (Zhou & Stanosz 2001) for part of the small subunit of the mitochondrial ribosomal DNA (mrSSU), and RPB2-5f and RPB2-7Cr (Liu *et al.* 1999) for part of the second largest subunit of RNA polymerase II (RPB2).

The 50 µl PCR reactions contained 10 µl of aqueous DNA extract, 5 µl Herculase™ reaction buffer (Stratagene), dNTPs (2·5 µM), 2·5 U polymerase (Herculase™, Stratagene), and 0·8 µM (ITS, mrSSU) or 1·4 µM (RPB2) of each forward- and reverse-primer. Some 25 µl reactions were carried out using PCR-PuReTaq Ready-to-Go Beads™ (GE Healthcare) containing 5 µl of DNA extract and 0·4 µM (ITS, mrSSU) or 1·4 µM (RPB2) of each forward- and reverse-primer. Cycling conditions for ITS and mrSSU included initial denaturation at

94°C for 5 min, 5 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, 33 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. For RPB2, initial denaturation at 92°C for 2 min was followed by 8 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 2 min, and 33 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 2 min, and a final extension step at 72°C for 10 min. PCR products were run on agarose gels, bands cut out and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified DNA was labelled with the BigDye™ Terminator Cycle Sequencing Kit (either version II or v3.1, Applied Biosystems) and cycle sequenced at 94°C for 30 s, and 29 cycles of 95°C for 15 s, 45°C for 15 s, and 60°C for 4 min using the PCR primers. Sequences were determined on ABI PRISM® 3700 or 3730 DNA Analyzers (Applied Biosystems), and either edited using SeqMan™ II, version v.5.07 (DNASTAR Inc.) and assembled in BioEdit version 7.0.9.0 (Hall 1999), or assembled and edited using Geneious Pro, version 6.0.4 (Biomatters Inc.).

Phylogenetic analyses

BLAST searches in GenBank were performed to ascertain that all sequences used in the phylogenetic analyses originated from the lichens and not from contaminating organisms such as parasymbiotic fungi. Single gene datasets containing the sequences listed in Table 1 were aligned in Geneious Pro, version 6.0.4, using the Muscle algorithm with default settings. Regions of uncertain alignment were removed using GBlocks, version 0.91b (Castresana 2000), applying default settings but allowing gap positions in half of the sequences. Final alignments comprised 56 sequences, 367 bp (ITS), 47 seq., 804 bp (mrSSU) and 38 seq., 1062 bp (RPB2). These datasets were concatenated to yield an alignment of 59 sequences and 2233 bp length. The optimal partitioning scheme of the concatenated dataset and substitution models for each data partition were inferred with the help of PartitionFinder, version 1.0.1 (Lanfear *et al.* 2012) using the Bayesian Information Criterion and the greedy algorithm (default settings) and suggesting seven data blocks (ITS1, 5.8S rDNA, ITS2, mrSSU, and three independent codon positions for RPB2). Results are shown in Table 2.

Phylogenetic trees for the single gene datasets were inferred with the Markov chain Monte Carlo (MCMC) approach implemented in MrBayes, version 3.2 (Ronquist *et al.* 2012), applying the substitution models and partitioning schemes inferred with PartitionFinder. Default settings of MrBayes were used with the following exceptions: a proportional model on partition-specific rates, gamma-distributed site-specific rates modelled as six rate categories with an exponential prior of mean 1 and an unconstrained, exponential branch length prior. The mean of the branch length prior was inferred by calculating ML trees for all single gene datasets and the concatenated dataset using raxmlGUI version 0.9 beta 2 (Stamatakis 2006; Silvestro & Michalak 2010), and applying either an unpartitioned GTRGAMMAI model (mrSSU) or a partitioned GTRGAMMAI model (ITS, RPB2) and 10 runs. The mean branch lengths of the ML trees were then used as means of the exponential distributions for branch length priors. Parameters of substitution models were unlinked between data partitions. MrBayes was set to sample every 500th tree out of 100 million generations using three independent runs, each with four chains that were incrementally heated by 0·15. To infer convergence of the Markov chains, the average standard deviation of bipartition frequencies among runs was calculated every 1 million generations, discarding the first 50% of the trees sampled as burn-in and including only those bipartitions with a frequency of at least 10%. The analyses were stopped when the standard deviation dropped below 0·01.

The resulting single gene MCMC trees were compared to identify conflicting phylogenetic signal between datasets (see Supplementary Figures S1–S3, available online). Four supported conflicts were detected, which concerned the positions of *B. chrysantha* (Zahlbr.) Printzen, *B. pontica* Printzen & Tønsberg, *B. printzenii* Tønsberg and *Lecania brialmontii* (Vain.) Zahlbr. In mrSSU, *B. chrysantha* was the sister taxon of *B.* cf. *helvola* from Japan, in ITS it was the closest relative of *B. vernalis*. *Biatora pontica* formed a well-supported clade with *B. printzenii* and *B. hertelii* Printzen & Etayo in RPB2, but grouped with *B. bacidioides* Printzen & Tønsberg and "*Lecidea*" *ementiens* in mrSSU, where *B. printzenii* appeared as sister of *Biatora* sp. from Norway. In the ITS analysis, *L. brialmontii* was the closest relative of the two *Lecidea albohyalina* collections, which in mrSSU grouped with *Mycobilimbia*. The four problematic taxa were removed from the dataset and all analyses repeated with the reduced datasets, after which no conflict was found between single-gene phylogenies. The three datasets were then combined into a single alignment for further analysis using the above-mentioned partitioning scheme, substitution models and settings for the Markov chains. The inferred branch length prior for the MCMC analysis of this dataset followed an exponential distribution with mean 1/20. The analysis was stopped after 9 million generations when the standard deviation had dropped below 0·01. A ML bootstrap tree with 1000 replicates was calculated for the concatenated dataset using the 'rapid bootstrap' option in raxmlGUI and unlinked GTRGAMMAI models for the five partitions inferred by PartitionFinder. Newly generated DNA sequences were submitted to Genbank (Table 1), the concatenated dataset used in the final analyses, and the ML tree and the consensus tree of the MCMC analysis were submitted to Treebase (http://purl.org/phylo/treebase/phylows/study/TB2:S15023).

Results and Discussion

For this study, 104 new DNA sequences were generated. Figure 1 shows the midpoint-rooted maximum likelihood tree inferred by raxmlGUI with ML bootstrap values and posterior probabilities. The length of this tree was 0·0487 and that of the Bayesian

TABLE 1. *Taxa, geographical origin of samples and GenBank numbers of sequences used in this study. GenBank numbers in bold indicate newly generated sequences*

Species	Origin	GenBank Accession No.		
		ITS	mrSSU	RPB2
Cliostomum corrugatum	Sweden, Skåne, Trolle-Ljungby par., *S. Ekman* 3115 (BG)	n/a	AY567722	**KF662436**
C. griffithii	GenBank	AF282076	GU138667	n/a
Bilimbia sabuletorum	GenBank	AM292670	AY567721	AM292761
Lecania croatica	Turkey, Trabzon Prov., *C. Printzen* 5946 (BG)	**KF650949**	**KF662397**	**KF662437**
L. cyrtella	Sweden, *S. Ekman* 3017 (BG)	AF282067	AY567720	AM292767
"*Lecidea*" *albohyalina*	Sweden, Hälsingland, *F. Jonsson* 6:29 (hb. Mellansel)	**KF650950**	**KF662398**	**KF662438**
"*Lecidea*" cf. *albohyalina*	Czech Rep., S-Bohemia, Šumava Mts., *Z. Palice* 839 (FR)	**KF650951**	**KF662399**	**KF662439**
"*Lecidea*" *sphaerella*	Czech Rep., S-Bohemia, Šumava Mts., *Z. Palice* 4621 (FR)	**KF650952**	**KF662400**	**KF662440**
Mycobilimbia epixanthoides	Finland, Uusima Prov., *C. Printzen & M. Kuusinen* s.n. (FR)	**KF650953**	**KF662401**	**KF662441**
M. pilularis	Norway, Lindås, *T. Tønsberg* 39665 [ITS, mrSSU], 39658 [RPB2] (BG)	**KF650954**	**KF662402**	**KF662442**
M. tetramera	Finland, Uusima Prov., *C. Printzen & M. Kuusinen* s.n. (FR)	**KF650955**	**KF662403**	**KF662443**
Biatora aegrefaciens	USA, Alaska, Mitkof Isl. W, *T. Tønsberg* 30212 (BG)	**KF650956**	n/a	**KF662444**
B. alaskana	USA, Alaska, Borough of Sitka, *C. Printzen* 5229 (FR)	**KF650957**	**KF662404**	**KF662445**
B. alaskana	Japan, Hokkaido, Kitami Prov., *G. Thor* 24732 (UPS)	**KF650958**	**KF662405**	n/a
B. appalachensis	USA, North Carolina, Graham Co., *C. Printzen* 6661 (FR)	**KF650959**	n/a	n/a
B. bacidioides	Turkey, Rize Prov., *B. Kanz & C. Printzen* s.n. (FR)	n/a	**KF662406**	n/a
B. beckhausii	Norway, *H. Holien* 6744 (TRH)	AF282071	**KF662407**	n/a
B. britannica	GenBank	AY032897	n/a	n/a
B. chrysantha	Czech Rep., W-Bohemia, Šumava Mts., *Z. Palice & C. Printzen* s.n. (FR)	AJ247569	**KF662408**	n/a
B. chrysanthoides	USA, Washington, Clallam Co., *C. Printzen* 5318 (FR)	**KF650960**	**KF662409**	**KF662446**
B. cuprea	Sweden, Torne Lappmark, par. Jukkasjärvi, *B. Kanz & C. Printzen* 5437 (BG)	**KF650961**	**KF662410**	**KF662447**
B. efflorescens	Czech Rep., S-Bohemia, Šumava Mts., *Z. Palice* s.n. (FR)	AJ247555	n/a	n/a
B. ementiens	Sweden, Torne Lappmark, *B. Kanz & C. Printzen* 5440 (BG)	**KF650962**	**KF662411**	**KF662448**
B. fallax	Czech Rep., S-Bohemia, Šumava Mts., *Z. Palice* s.n. (FR)	AJ247548	**KF662412**	n/a
B. flavopunctata	USA, Washington, Clallam Co., *C. Printzen* 5327 (FR)	**KF650963**	**KF662413**	**KF662449**
B. globulosa	Sweden, *S. Ekman* 3142 (BG)	AF282073	**KF662414**	**KF662450**
B. helvola	Finland, Etelä-Savo, *M. Kuusinen* s.n. (BG)	**KF650964**	n/a	n/a
B. cf. *helvola*	GenBank	AJ247570	n/a	n/a
B. cf. *helvola*	Japan, Hokkaido, Kitami Prov., *G. Thor* 24259 (UPS)	**KF650965**	**KF662415**	n/a
B. hemipolia	USA, Washington, Kittitas Co., *T. Tønsberg* 25091 (BG)	AF282072	AF282072	**KF662451**
B. hertelii	Madeira, Rabaçal, *B. Kanz & C. Printzen* s.n. (FR)	AJ247536	**KF662416**	**KF662452**
B. hypophaea	USA, Oregon, Linn Co., *C. Printzen* s.n. (BG)	**KF650966**	n/a	n/a
B. kodiakensis	USA, Alaska, Kodiak Island Borough, *T. Tønsberg* 29371(BG)	**KF650967**	**KF662417**	**KF662453**
B. ligni-mollis	Czech Rep., S-Bohemia, Novohradské hory Mts., *J. Maliček* & *Z. Palice* 14609 (FR)	**KF650968**	**KF662418**	n/a
B. ligni-mollis	GenBank	EU669178	n/a	n/a
B. longispora	USA, Massachussetts, Berkshire Co., *P. May* 5409 (hb. May)	**KF650969**	**KF662419**	**KF662454**
B. meiocarpa	GenBank	AM292667	AM292710	AM292757
B. meiocarpa var. *tacomensis*	USA, Washington, Lewis Co., *C. Printzen* 5015 (FR)	n/a	**KF662420**	n/a

Table 1. *Continued*

Species	Origin	GenBank Accession No.		
		ITS	mrSSU	RPB2
B. nobilis	USA, Washington, *T. Tønsberg* 29057 (BG)	**KF650970**	**KF662421**	**KF662455**
B. ocelliformis	Germany, Bavaria, Niederbayern, *C. Printzen* s.n. (FR)	**KF650972**	n/a	**KF662457**
B. oligocarpa	USA, Alaska, Kodiak Island Borough, *T. Tønsberg* 29571 (BG)	**KF650973**	**KF662423**	**KF662458**
B. "orientalis"	Japan, Hokkaido, Kitami Prov., *G. Thor* 23714 (UPS)	**KF650974**	**KF662424**	n/a
B. pallens	Sweden, Lule Lappmark, Jokkmokk par., *U. Nordin* 2161 (BG)	**KF650975**	**KF662425**	n/a
B. pausiaca	USA, Washington, Clallam Co., *T. Tønsberg* 28017 *& C. Printzen* (BG)	**KF650976**	**KF662426**	**KF662459**
B. pontica	Turkey, Trabzon Prov., *C. Printzen* 6114 (BG)	**KF650977**	**KF662427**	**KF662460**
B. printzenii	USA, North Carolina, Swain Co., *C. Printzen* 6837 (BG)	**KF650978**	**KF662428**	**KF662461**
B. pycnidiata	Canada, Newfoundland, Ferryland District, *C. Printzen* 5497 (BG)	**KF650979**	**KF662429**	**KF662462**
B. "radicicola"	Czech Rep., Bohemia, Nové Mesto, *J. Halda* 4104 (hb. Halda)	**KF650980**	n/a	**KF662463**
B. rufidula	USA, Washington, Pierce Co., *C. Printzen* 5055 (FR)	**KF650981**	**KF662430**	**KF662464**
B. sphaeroidiza	Sweden, Uppland, Alsike par., *Z. Palice* s.n. (FR)	**KF650982**	n/a	n/a
B. subduplex	Sweden, Torne Lappmark, par. Jukkasjärvi, *B. Kanz & C. Printzen* 5436 (FR)	**KF650983**	**KF662431**	**KF662465**
B. cf. *subduplex* (Alps)	GenBank	AJ247540	n/a	n/a
B. "terrae-novae"	Canada, Newfoundland, Fortune Bay-Hermitage District, *C. Printzen* 5758 (BG)	**KF650971**	**KF662422**	**KF662456**
B. toensbergii	USA, Washington, Pierce Co., *C. Printzen* 5053 (FR)	**KF650984**	**KF662432**	**KF662466**
B. vacciniicola	USA, Alaska, City and Borough of Juneau, *T. Tønsberg* 27486 (BG)	**KF650985**	**KF662433**	**KF662467**
B. vernalis	GenBank	AF282070	AM292711	AM292758
B. veteranorum	Czech Rep., S-Bohemia, Novohradské hory Mts., *J. Maliček* & *Z. Palice* 14753 (FR)	**KF650986**	**KF662434**	n/a
B. species (Norway)	Norway, Nord-Trøndelag, Steinkjer, *H. Holien* 8595e (hb. Holien)	KF650987	KF662435	KF662468

TABLE 2. *Optimal partitioning scheme and substitution models for each data partition inferred by PartitionFinder, version 1.0.1, and used in the phylogenetic analyses*

Subset	Best Model	Partitions	Subset Sites
1	SYM+I+G	ITS1, ITS2	1–113, 273–367
2	K2P+I+G	5.8S	114–272
3	HKY+I+G	mrSSU, RPB2 codon 3	368–1711, 1174–2233\3
4	K2P+I+G	RPB2 codon 1	1172–2233\3
5	GTR+I+G	RPB2 codon 2	1173–2233\3

consensus tree 0·0361, indicating that branch length estimates of the MCMC tree are biologically plausible (Brown *et al.* 2010). If not stated otherwise, anatomical, chemical and geographical data for the species discussed below are taken from Printzen (1995, 2004), Printzen & Tønsberg (1999, 2003, 2004) and Printzen *et al*. (1998, 2001).

Phylogenetic relationships of *Biatora*

The overall topology of the tree agrees with the phylogeny of *Lecania* and related genera presented by Reese Næsborg *et al.* (2007), with *Mycobilimbia* s. lat. as sister to a clade formed by *Lecania* and *Bilimbia* species. With one exception (the relationship between *Bilimbia sabuletorum* and *Lecania cyrtella*), all branches in the basal part of the tree receive strong support. The position of *Mycobilimbia* and "*Lecidea*" *albohyalina* as close relatives of *Lecania* and *Bilimbia* and the exclusion of these species from *Biatora* are thus strongly supported. A close relationship between *M. pilularis* (Körb.) Hafellner & Türk (as *Biatora sphaeroides*) and *Lecania* was already inferred by Ekman (2001), based on ITS sequences and a wide taxonomic sample from the *Ramalinaceae*.

The monophyly of *Cliostomum griffithii* (Sm.) Coppins and *C. corrugatum* (Ach.) Fr. and their position as sister to *Biatora* is strongly supported in the present analysis. Such a relationship has so far not been reconstructed, although both genera are anatomically similar (Printzen 1995). Ekman (2001) found *C. griffithii* as sister to *Ramalina*, while Reese Næsborg *et al.* (2007) found a close relationship between *C. tenerum* (Nyl.) Coppins & S. Ekman and *Lecania furfuracea* Vězda. These differences are perhaps explained by different taxon sampling. *Ramalina* was excluded from the present dataset, because preliminary analyses inferred a group in which *R. fastigiata* was firmly embedded between *C. corrugatum* and *C. griffithii* (not shown). The relationship between *Ramalina* and *Cliostomum* needs further clarification, but is not the focus of this contribution. *Cliostomum tenerum*, on the other hand, differs from all other currently accepted *Cliostomum* species by having a lecanorine margin and might not belong in this genus.

The remaining 44 collections included as known or putative members of *Biatora* form a sister group to *Cliostomum* but the monophyly of this group was only supported in the ML analysis. Surprisingly, the posterior probability of a monophyletic *Biatora* was considerably higher when the three conflictory taxa *B. chrysantha, B. pontica* and *B. printzenii* were included in the analysis, while the ML bootstrap support remained unaffected (Fig. 1, support values in brackets).

Infrageneric species groups within *Biatora*

The infrageneric systematics of *Biatora* in its modern circumscription, that is excluding older and much wider interpretations such as that of Körber (1855) or "*Lecidea* subgenus *Biatora*" (Fries 1874), has not been explicitly dealt with before. Because data on crucial characters such as ascus apical structure, apothecial ontogeny or secondary metabolites were lacking, early authors often joined largely similar species into groups. Fries (1874), for example, combined *B. cuprea, B. vernalis* and *B. helvola* into a "stirps *L. vernalis*"

which also included the remotely related *Lecidea gibberosa sensu* Th. Fr. [= *Puttea exsequens* (Nyl.) Printzen & Davydov], but excluded *L. atroviridis* (Arnold) Th. Fr. (= *B. ocelliformis*). Printzen (1995) noted similarities between *B. aegrefaciens* and *B. rufidula*, as well as between *B. flavopunctata* and *B. subgilva*, but otherwise did not propose any subgeneric groupings.

Figure 1 shows that some well-supported clades can be distinguished within *Biatora*. Because the relationships among these groups are largely unsettled, no attempt is made to assign them any systematic rank. The crown group (here called the "*vernalis*-group") combines species with a typical *Biatora* anatomy: hyaline, light or reddish brown apothecial tissue and a strongly gelatinized excipulum with cylindrical cell lumina. The most common secondary metabolites in *Biatora* are found in species from this group; gyrophoric acid in *B. helvola* Hellb. and *B. chrysantha*, argopsin in *B. efflorescens* (Hedl.) Räsänen, *B. pycnidiata* Printzen & Tønsberg, *B. "terraenovae" B. toensbergii* Holien & Printzen and *B. cuprea* (Sommerf.) Fr., and both in *B. fallax* Hepp. The *vernalis* group falls into two major clades, one of which combines the corticolous *B. efflorescens, B. helvola, B. pycnidiata* and *B. toensbergii*. The other comprises all 'non-corticolous' *Biatora* species (except *L. ementiens*, see below), which either overgrow bryophytes (*B. chrysantha, B. vernalis*) or rotten bark (*B. fallax*). *Biatora cuprea* and *B. subduplex* (Nyl.) Printzen prefer detritus in (sub)arctic tundra. *Biatora alaskana* Printzen & Tønsberg and *B. longispora* (Degel.) Lendemer & Printzen are the only species in this group restricted to bark.

With the exception of *B. subduplex* and *B. vernalis*, the species within the *vernalis* group are morphologically and chemically easily distinguished. The extremely short branches within this group indicate that the species are genetically closely related and probably diverged from each other recently. In spite of these short branches, the *vernalis* group is the only one with a well-resolved and supported internal topology. The only branches with insufficient statistical support are the ones that combine *B. alaskana* and *B. subduplex,* and *B. pycnidiata* and *B. toensbergii.*

The *meiocarpa*-group is closely related to the *vernalis* group. Species of this group have more rounded excipular cell lumina and apically broadened paraphyses, but otherwise the two groups are very similar. The close relationship between both groups has a high posterior probability but slightly less than 70% bootstrap support. With the exception of *B. kodiakensis* Printzen & Tønsberg, with gyrophoric acid, species of the *meiocarpa* group do not produce secondary metabolites. The phylogenetic tree allows no conclusion whether *B. oligocarpa* Printzen & Tønsberg belongs in the group or not, but the anatomical similarities between this species and *B. meiocarpa* (Nyl.) Arnold make it likely.

Four further groups can be distinguished in the basal part of the *Biatora*-tree: the *hertelii*-group, the *ocelliformis* group, the *beckhausii* group and the *rufidula* group. Among these are spread numerous (pairs of) taxa with unclear relationships. The *hertelii* group comprises *B. britannica* Printzen *et al.* and *B. hertelii*. In the extended dataset, *B. pontica* and *B. printzenii* appear as closely related to these two. The group is mainly characterized by the production of insoluble pigments rarely found outside the group and a preference for Tertiary relict areas (Macaronesia, south-eastern North America; see e.g. Tiffney 1985). *Biatora britannica* and *B. hertelii* contain Hertelii-green (Meyer & Printzen 2000), otherwise only known from the thallus of *Lecania leprosa* Reese Næsborg & Vondrák (Reese Næsborg 2008). *Biatora hertelii* has up to now only been collected on Madeira. The ascogenous hyphae of *B. pontica* are surrounded by Pontica-blue and Pontica-red (Printzen & Tønsberg 2003), so far not known from any other lichen. *Biatora printzenii* is only known from eastern North America, while *B. pontica* is also found in East Asia and the eastern Black Sea Region.

The *ocelliformis* group consists of the closely related and anatomically very similar *B. hypophaea* and *B. ocelliformis*, and a well-supported clade formed by the Arctic *"Lecidea" ementiens* and *B. bacidioides*, which is so far only known

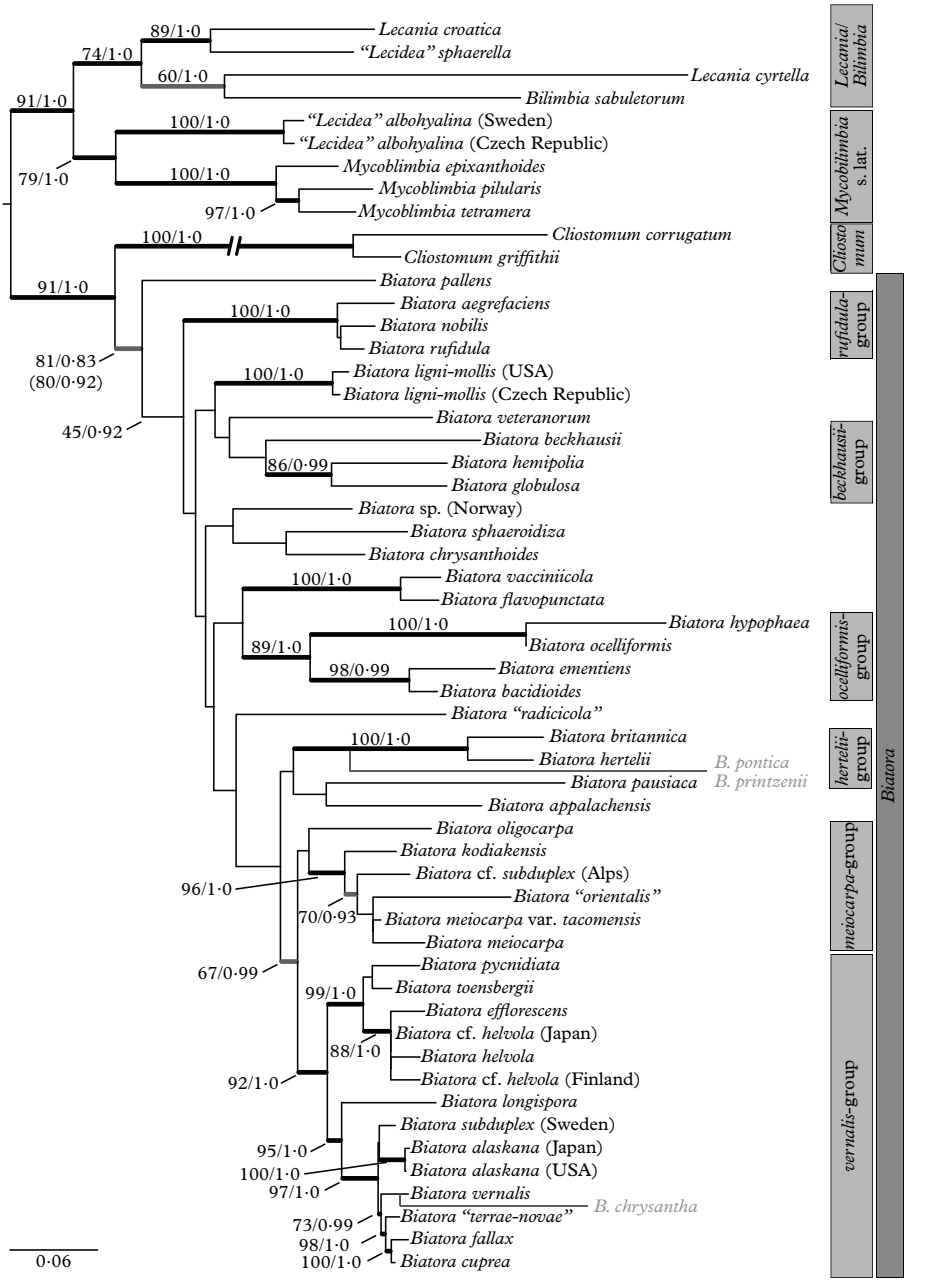

FIG. 1. Midpoint-rooted Maximum Likelihood phylogenetic tree of *Biatora*. Species of *Bilimbia*, *Cliostomum*, *Mycobilimbia* and *Lecania* were added to root the tree and to ascertain the phylogenetic position of unnamed taxa included in the analysis. Bold branches received ML bootstrap support ≥70% and posterior probability ≥0·95. Bold grey branches were only supported by one of the analyses. Exact support values above or left of branches. Support values in brackets underneath the branch leading to *Biatora* indicate those of an analysis including species with conflicting signal. The position of these species (grey) in the extended analysis is indicated to the right of the tree. The branch leading to *Cliostomum* is reduced to half its length.

https://doi.org/10.1017/S0024282913000935 Published online by Cambridge University Press

from north-eastern Turkey. This last relationship is not supported by anatomical or chemical characters. *Biatora bacidioides* has long, bacidioid ascospores and produces the pigment Sedifolia-grey, which rather suggests a relationship with *Bacidia beckhausii* as claimed by Printzen & Tønsberg (2003).

A close relationship between *Bacidia beckhausii* and *Bacidia hemipolia* was postulated by Ekman (1996). In the present analysis, both species group together with *Biatora globulosa* (Flörke) Fr. Although the *beckhausii*-group as a whole is unsupported, the relationship between *B. globulosa* and *B. hemipolia* was reconstructed with high confidence. The peculiar position of *B. bacidioides* and the occurrence of Cinereorufa-green and Sedifolia-grey in both groups might indicate a closer relationship between the *ocelliformis* group and the *beckhausii* group. Within *Biatora*, Cinereorufa-green is otherwise only produced by *B. sphaeroidiza* (Vain.) Printzen & Holien and the undescribed *B. "radicicola"*, both with an undecided phylogenetic position.

The *rufidula* group is highly supported and morphologically clearly distinguished by broadly ellipsoid, rather thick-walled, 3-septate ascospores and an exciple in which individual hyphae can be discerned. Typically, the exciple in *Biatora* consists of a gelatinous matrix, in which only the cell lumina appear as 'holes'.

The sorediate species *B. flavopunctata* (Tønsberg) Hinteregger & Printzen and *B. vacciniicola* (Tønsberg) Printzen appear as closely related taxa. Both species are typically found on twigs of shrubs in (sub)arctic-alpine situations and may grow side by side. *Biatora flavopunctata* shows the most complex chemical pattern of all *Biatora* species, including the *β*-orcinol para-depside atranorin, *β*-orcinol depsidones from the stictic acid complex, the dibenzofurans usnic and isousnic acid and an unknown terpenoid. The only other species producing usnic and isousnic acid is *B. subgilva* (Arnold) Hinteregger, which is so far only known from twigs of *Rhododendron ferrugineum* in the Eastern Alps (Hinteregger 1994). Because recent collections were lacking it was not included in the present analysis, but it is likely that it belongs in the same group.

Phylogenetic position of single species within *Biatora*

A number of taxa appeared in surprising positions on the tree. This affects already described taxa as well as collections of uncertain identity. In order to facilitate communication about these taxa, I have here given them informal names within quotation marks.

An ITS-sequence of a collection identified as *Biatora subduplex* and growing on *Rhododendron ferrugineum* in the Italian Alps appears to be a member of the *meiocarpa* group, while another specimen growing on detritus in northern Sweden is a member of the *vernalis*-group (see above). Four highly-supported branches separate the two specimens, which rules out any possibility that both can be conspecific. The question is whether there is a distinction between corticolous specimens and those growing on detritus, or whether arctic and alpine populations belong to different species. It is necessary to reinvestigate the morphology of what was until now regarded as *Biatora subduplex* to see whether unobserved phenotypic differences would support the molecular distinction.

Another interesting taxon in this respect is *Biatora* "*orientalis*", which is also assigned to the *meiocarpa* group but closely resembles *B. vernalis*. It is distinguished from this species by more narrowly elongate ascospores and has so far been found in eastern North America, East Asia and the eastern Black Sea Region, where it tends to grow directly on bark rather than over bryophytes, the typical substratum for *B. vernalis*. Its placement in the *meiocarpa* group shows that even extremely subtle morphological differences are important for species recognition in *Biatora*.

A third case concerns two collections tentatively identified as *B. helvola* but lacking gyrophoric acid. One of the collections is from Finland (named *"B. pseudohelvola"* in Printzen & Lumbsch 2000), the other from Japan. In this case the three samples are closely related to each other but the comparatively long branches between them indicate that superficially similar but distinct species might be involved. It is necessary to investigate the chemical variability of *B. helvola* in more detail to solve this question. Finally,

Biatora "terrae-novae" is similar to *B. pycnidiata*, the most common *Biatora* species on Newfoundland, but has more convex apothecia. The phylogeny shows that it is not closely related to this species but rather belongs in the vicinity of *B. fallax*. On the other hand, a collection from Japan identified as *B. alaskana* with unusually long ascospores proved to be conspecific with North American material. *Biatora alaskana* was so far regarded as endemic to north-western North America. This finding therefore extends the known range of the species considerably.

Biatora "radicicola" contains the pigment Cinereorufa-green and is somewhat similar to *B. ocelliformis*, but in contrast to this species the pigment is concentrated in the interior of the apothecium. Again, the analysis shows that it is not conspecific or closely related to *B. ocelliformis*.

The phylogenetic position of *Biatora pallens* (Kullh.) Printzen, a species with 3-septate ascospores, remains unresolved between the *rufidula* group, likewise with 3-septate ascospores, and *Cliostomum*. Interestingly, *B. pallens* was treated as a member of *Cliostomum* by Ekman (1997). *Cliostomum corrugatum* and *C. griffithii* are clearly distinguished from *Biatora* by their large, pigmented pycnidia and the anatomy of the exciple. However, *C. vitellinum* has unpigmented pycnidia and in those of *C. flavidulum* the pigmentation is restricted to a narrow zone (Ekman 1997). An analysis including additional species of *Cliostomum* is necessary to resolve the exact delimitation of both genera and the position of *B. pallens*.

Four sorediate *Biatora* species with gyrophoric acid as the only secondary metabolite are currently known: *B. appalachensis* Printzen & Tønsberg from the southern Appalachian Mountains, the circumboreal *B. chrysantha*, and *B. chrysanthoides* Printzen & Tønsberg and *B. kodiakensis*, both known from western North America and Central Norway (Spribille *et al*. 2009; Holien & Tønsberg 2012). Sterile collections of these species can be difficult to distinguish. However, the phylogenetic analysis shows that despite their morphological and chemical similarity, they have obviously not evolved from the same ancestor.

Sorediate taxa in general are scattered rather randomly across the tree, some as sister taxa to other sorediate species (*B. flavopunctata*, *B. vacciniicola*), some basal to clades of non-sorediate species (*B. kodiakensis, B. oligocarpa*), and some as sisters of esorediate taxa (*B. britannica*, *B. hertelii*). *Biatora fallax*, sister to *B. cuprea*, can be called a facultatively sorediate species, in which the squamules of the thallus are sometimes dissected to the point of becoming sorediate in appearance. With one exception, *Biatora hertelii* and *B. britannica*, there are always additional characters to distinguish these from their closest relatives. They therefore do not fit the original concept of "species pairs" as introduced by Du Rietz (1924), in which two closely related taxa are only distinguished by the presence or absence of vegetative propagules. Because several recent studies involving molecular datasets (e.g. Buschbom & Mueller 2006; Tehler *et al*. 2009) have found no evidence for the existence of species pairs, this concept now seems to be obsolete.

Conclusions

The published phylogenies of *Biatora* have so far been based on rather small datasets involving one or two gene loci and 11–22 taxa (Printzen & Lumbsch 2000; Spribille *et al*. 2009). Even with an extended three-gene dataset, the circumscription and delimitation of *Biatora* remains somewhat uncertain. As delimited here, it comprises crustose lichen species with a green-algal photobiont, biatorine apothecia, an exciple of more or less parallel, anticlinal hyphae, asci of the *Biatora*-type containing eight colourless, more or less thin-walled ascospores of variable shape and septation, strongly gelatinized apothecial tissues, and inconspicuous, immersed pycnidia with bacilliform conidia (an exception is *B. meiocarpa*, for which long, sausage-shaped conidia have been reported). The genus *Cliostomum* is very similar in most respects but has more irregularly branched excipular hyphae, and more conspicuous, often dark pigmented pycnidia containing ellipsoid to ovoid conidia (when *C. pallens* and *C. tenerum* are excluded). *Biatora* comprises several evolu-

tionary lineages that are also supported by phenotypic differences. However, the rather high number of taxa that cannot be attributed to any of these groups at present precludes attempts to split *Biatora* into smaller genera. Using the name *Biatora* in the broad circumscription suggested here, necessitates a few nomenclatural changes, as outlined below.

Several unidentified samples included in this study proved to belong to so far undescribed species. Because descriptions of new taxa are outside the scope of this study, these will be published separately. The confounding phylogenetic placement of many similar species in different clades (e.g. the two collections of *B. subduplex* or *B. orientalis* and *B. vernalis*) suggests that the diversity of *Biatora* is not yet fully explored. This is, however, not surprising considering that most *Biatora* species occur in boreal coniferous forests and that the huge Asian part of its range has not so far been monographed.

Nomenclature

Biatora beckhausii (Körb.) Tuck.

Syn. N. Amer. Lich. **2:** 46 (1888).

Bacidia beckhausii Körb., *Parerga lichenol.*: 134 (1860).—*Secoliga beckhausii* (Körb.) Stizenb., *Nova Acta Acad. Leopoldin.-Carolin.* **30:** 21 (1863).—*Patellaria beckhausii* (Körb.) Müll. Arg., *Flora* **57:** 485 (1879).—*Micarea beckhausii* (Körb.) Vězda, in Poelt, *Bestimmungsschlüssel europ. Flechten, Ergänzungsheft* **1:** 162 (1977); type: Germany, "Westphalen", *Beckhaus* (L-910.137 1363—lectotype, selected by Coppins 1983: 196).

Biatora ementiens (Nyl.) Printzen comb. nov.

MycoBank No.: MB805892

Lecidea ementiens Nyl., *Flora* **67:** 222 (1884); type: [Russia: Chukchi], "Fretum Behring, Konyambay, fjellet i öster.", [28–30 vii] 1879, *E. Almquist* (H-Nyl 20912!—lectotype, selected here).

Biatora hemipolia (Nyl.) S. Ekman & Printzen comb. nov.

MycoBankNo.: MB805810

Lecidea arceutina * *hemipolia* Nyl., *Flora* **56:** 294 (1873).—*Lecidea hemipolia* (Nyl.) Nyl., *Lich. Envir. Paris*: 84 (1896).—*Bacidia hemipolia* (Nyl.) Malme., *Bot. Notiser* **1895:** 140 (1895); type: Tavastia, Hollola, Manskivi, ad cort. alni, 1863, legit *J. P. Norrlin* 244 (H-Nyl. 17975—lectotype, selected by S. Ekman in sched. and designated here; H—isolectotype).

Note: The name "*Lecidea arceutina* f. *hemipolia* Nyl." was first published in *Flora* **52:** 413 (1869) but, as already mentioned by Fries (1874: 353), without any diagnostic characters. Arnold (1870: 472) only lists "*Bacidia arceutina* f. *hemipolia* N." without any description. The earliest diagnosis of the taxon appears to be that mentioned above.

I dedicate this article to Brian Coppins on the occasion of his 65th birthday. Our discussions on the circumscription of *Biatora* and his (and Sandy's) hospitality during a visit to Edinburgh helped me greatly when my Ph.D. thesis was still in its infancy, and a worn-out copy of his *Micarea*- monograph on my bookshelf testifies to its importance for everyone dealing with non-saxicolous lecidoid lichens.

I am indebted to the following collectors who made fresh samples available to me: Håkon Holien, Fredrik Jonsson, Mikko Kuusinen, Philip May, Ulrika Nordin, Zdeněk Palice, Toby Spribille, Göran Thor and Tor Tønsberg. I am very grateful to Stefan Ekman who allowed me to use several DNA samples and unpublished sequences for this study and to formally publish his lectotypification of *Biatora hemipolia*. Technical support by the staff of the Grunelius Möllgaard laboratory, especially Heike Kappes, is also gratefully acknowledged. Parts of this study received financial support from the Research Council of Norway through the Strategic University Programme 'Applications of molecular techniques in systematic biology'. Field trips of the author were partly financed by the Grolle Olsen fund and the Felix-Ungerer-Stiftung.

Supplementary Material

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0024282913000935

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