# Molecular systematics, taxonomy, and museomics of tropical Ramalinaceae

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# Preface

I started the studies for this PhD on January 5, 2015. Doing a PhD is rarely straightforward; thus, there have been many ups and downs for me during the last four years. I have indisputably learned a lot in that time, hardly knowing what lichens were before I started. In particular, I was not aware that there were so many new lichen species to be found in the tropical material, which I was supposed to investigate. Discovering a new species was kind of a childhood's dream for me and I am happy it came true. In the end, it was not only one new species but eleven.

I thank Mirko Dreßler, supervisor of my Diplom thesis at the University of Rostock, Germany, who first aroused my interest for taxonomy and molecular systematics. He always encouraged me to do a PhD. Admittedly, I first considered taking up this PhD position in lichen systematics, because I missed working taxonomically. Getting the chance to go on fieldwork in the tropics was a very tempting prospect as well. Lichens are indeed a special group to work with and I am very glad I decided to start this journey.

# Contents

Preface ..... III

Abbreviations ..... VI
Index Herbariorum ..... VII

Summary ..... 1
List of Papers ..... 3

1 Introduction ..... 5
1.1 The family Ramalinaceae ..... 6
1.2 The tropical genus *Phyllopsora* and its relative *Krogia* ..... 8
1.3 Historical lichen collections ..... 10
1.4 Main objectives of the thesis ..... 12

2 Material and methods ..... 13
2.1 Taxon sampling ..... 13
2.2 Morphological and anatomical investigations ..... 13
2.3 Thin-layer chromatography ..... 13
2.4 Molecular methods ..... 14
2.5 Sequence editing ..... 14
2.6 Alignment ..... 14
2.7 Phylogenetic analysis ..... 15
2.8 Ancestral state reconstruction ..... 16
2.9 Species delimitation analysis ..... 16
2.10 Statistical analysis ..... 16

3 Main findings and discussion ..... 17
3.1 The family Ramalinaceae ..... 17
3.2 The genus *Phyllopsora* ..... 19
3.3 The genus *Krogia* ..... 23
3.4 Sequencing historical lichens ..... 24
3.5 Additional comments ..... 26

4 Concluding remarks and perspectives ..... 27

Acknowledgements ..... 28
References ..... 31

Papers I–V ..... 39

# Abbreviations

| | |
|---|---|
| *BEAST | StarBeast |
| ASR | ancestral state reconstruction |
| bp | base pairs |
| BS | bootstrap |
| ch | chemotype |
| DNA | deoxyribonucleic acid |
| HTS | high-throughput sequencing |
| I | solution of iodine (Lugol's solution in 50% lactic acid) |
| ICN | International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) |
| nrITS | nuclear ribosomal internal transcribed spacer |
| K | 10% solution of potassium hydroxide |
| KOH | 10% solution of potassium hydroxide |
| nrLSU | nuclear ribosomal large subunit |
| MCMC | Markov Chain Monte Carlo |
| ML | maximum likelihood |
| mtSSU | mitochondrial ribosomal small subunit |
| NHM | Natural History Museum, University of Oslo |
| PCR | polymerase chain reaction |
| PGM | personal genome machine |
| PP | posterior probabilities |
| PTP | Poisson tree processes |
| RPB1 | largest subunit of the RNA polymerase II |
| RPB2 | second largest subunit of the RNA polymerase II |
| s. str. | *sensu stricto* ("in the narrow sense") |
| TLC | thin-layer chromatography |

# Index Herbariorum

| | |
|---|---|
| AAS | British Antarctic Survey, Cambridge, U.K. England |
| ABL | Adviesbureau voor Bryologie en Lichenologie, Soest, The Netherlands |
| ASU | Arizona State University, Tempe, U.S.A. Arizona |
| B | Botanischer Garten und Botanisches Museum Berlin-Dahlem, Zentraleinrichtung der Freien Universität Berlin, Berlin, Germany |
| BG | Universitetet i Bergen, Bergen, Norway |
| BM | The Natural History Museum, London, U.K. England |
| BORH | Universiti Malaysia Sabah, Kinabalu, Malaysia |
| BR | Botanic Garden Meise, Meise, Belgium |
| CANB | Australian National Herbarium, Canberra, Australia |
| E | Royal Botanic Garden Edinburgh, Edinburgh, U.K. Scotland |
| FR | Senckenberg Gesellschaft für Naturforschung: Senckenberg Forschungsinstitut und Naturmuseum, Frankfurt, Germany |
| G | Conservatoire et Jardin botaniques de la Ville de Genève, Genève, Switzerland |
| GZU | Karl-Franzens-Universität Graz, Graz, Austria |
| H | University of Helsinki, Helsinki, Finland |
| HO | Tasmanian Museum and Art Gallery, Hobart, Tasmania, Australia |
| ISE | Universidade Federal de Sergipe, Itabaiana, Brazil |
| KR | Staatliches Museum für Naturkunde Karlsruhe, Karlsruhe, Germany |
| LD | Lund University, Lund, Sweden |
| M | Botanische Staatssammlung München, Munich, Germany |
| MIN | University of Minnesota, St. Paul, U.S.A. Minnesota |
| NY | The New York Botanical Garden, Bronx, U.S.A. New York |
| O | Botanical Museum, University of Oslo, Oslo, Norway |
| OTA | University of Otago, Dunedin, New Zealand |
| PDA | Royal Botanic Gardens, Peradeniya, Sri Lanka |
| SBBG | Santa Barbara Botanic Garden, Santa Barbara, U.S.A. California |
| SP | Instituto de Botânica, São Paulo, Brazil |
| TNS | National Museum of Nature and Science, Tsukuba, Japan |
| TRH | Norwegian University of Science and Technology, Trondheim, Norway |
| TROM | Universitetet i Tromsø, Tromsoe, Norway |
| TSB | Department of Life Sciences, Trieste, Italy |
| UPS | Museum of Evolution, Uppsala, Sweden |
| VEN | Universidad Central de Venezuela, Caracas, Venezuela |

# Summary

The lichen family Ramalinaceae is one of the largest families of lichenized ascomycetes comprising morphologically rather heterogeneous representatives found all over the world. Since the end of the last century, DNA sequence data have been incorporated in lichen taxonomy as a valuable source of information. Molecular systematics, however, have shown that traditional taxonomy, i.e. exclusively based on morphological and anatomical characteristics as well as chemical analyses of lichen substances, may fall short in recognizing natural groups of lichenized fungi. This is also the case for several genera of the Ramalinaceae, where previous studies have indicated the need for revisionary work at genus and family levels; the tropical genera, in particular, exhibit similar morphological features. The tropical genera have not been studied in a molecular phylogenetic context at all and little is known about their evolutionary history. The genus *Phyllopsora* is the largest tropical genus in this family and *Phyllopsora* species are expected to have evolved from multiple ancestors, rendering its current classification in need of taxonomic revision as well. Species identification in this genus and its relatives is challenging when using morphology and chemistry, thus leaving a substantial proportion of specimens unidentified. While regional reports of *Phyllopsora* species have been provided for almost all continents, several Asian countries have escaped proper investigations of phyllopsoroid species.

The aims of this PhD thesis were to contribute to the systematics of the lichen family Ramalinaceae in general and the genus *Phyllopsora* in particular. By collecting additional material of *Phyllopsora* and allied genera in South America (Brazil and Venezuela) and Asia (Sri Lanka), I have added to the knowledge of the species' occurrences and diversity. Moreover, I conducted a pilot-study for overcoming some of the challenges in obtaining DNA sequence data from old herbarium material.

This thesis includes five papers, of which three are published (Papers I, IV–V) and two are in revision (II–III). **Paper I** presents the first comprehensive molecular phylogeny of the family Ramalinaceae including five genetic markers of representatives from 36 out of 42 genera previously included in the family. Based on the supported phylogenetic relationships, we revise the taxonomy of the family and trace the main clades' evolutionary history through ancestral state reconstructions. Four genera showed to be polyphyletic as circumscribed at that point. Thus, we describe two new genera and remodel several additional genera. Especially the tropical genera are found to have evolved independently and the large tropical genus *Phyllopsora* is split into segregates. The core group of *Phyllopsora* is examined in a global study in **Paper II**, where we investigate species circumscriptions within the genus based on morphological, chemical and molecular data. Additional nine species

of *Phyllopsora* are excluded from the genus and five species are described as new. We treat the investigated Asian and Melanesian collections of *Phyllopsora* s. str. in a regional study in **Paper III**, where we describe three species as new to science and report eight species as new for Asia and Melanesia. In **Paper IV**, we focus on the genus *Krogia*, which is morphologically similar to *Phyllopsora*. We describe three new species of *Krogia* from Asia and Melanesia employing morphological, chemical and DNA sequence data, and provide a key to all known species. In **Paper V**, we present a pilot-study for obtaining DNA sequence data from old lichen herbarium specimens. We investigate the applicability of an Ion Torrent protocol for sequencing specimens from four different families, which were collected up to 155 years ago. Although DNA sequence data is challenging to obtain from old specimens, we successfully generated sequence information using a two-step PCR protocol followed by Ion Torrent sequencing. For ca. 65% of specimens collected more than 100 years ago, the obtained sequence information was sufficient for identification at species level.

In summary, this work has contributed to a revised taxonomy of the family Ramalinaceae and the genus *Phyllopsora*, thereby providing a framework for further and more in-depth research. The description of new species of *Phyllospora* and *Krogia* increases the known diversity of these tropical lichen genera. In addition, our attempt to DNA sequence old herbarium specimens represents the first study that systematically investigates potential patterns of DNA degradation in lichens. Results from this thesis should therefore be of interest to lichenologists working on the family Ramalinaceae, tropical rainforest biodiversity, or DNA sequencing of degraded material.

# List of Papers

This thesis is based on the following five papers, referred to by their Roman numerals in their abbreviated form. Paper I is reprinted with the permission of the publisher; Papers IV and V are open-access articles published under the terms of the Creative Commons Attribution License. Papers II and III are submitted manuscripts and all new species names therein are provisional as they will be formally published elsewhere (ICN Art. 36.1a, F.5.1).

I. **Kistenich, S.**, Timdal, E., Bendiksby, M. & Ekman, S. (2018) Molecular systematics and character evolution in the lichen family Ramalinaceae (Ascomycota: Lecanorales). *Taxon* 67 (5): 871–904. doi:10.12705/675.1.

II. **Kistenich, S.**, Bendiksby, M., Ekman, S., Cáceres, M.E.S., Hernández M., J.E. & Timdal, E. Towards an integrative taxonomy of *Phyllopsora* (Ramalinaceae). Submitted to *The Lichenologist*, in revision.

III. **Kistenich, S.**, Bendiksby, M., Vairappan, C., Weerakoon, G., Wijesundara, S., Wolseley, P.A. & Timdal, E. A regional study of the genus *Phyllopsora* (Ramalinaceae) in Asia and Melanesia. Submitted to *MycoKeys*, in revision.

IV. **Kistenich, S.**, Rikkinen, J., Thüs, H., Vairappan, C., Wolseley, P.A. & Timdal, E. (2018) Three new species of *Krogia* (Ramalinaceae, lichenized Ascomycota) from the Paleotropics. *MycoKeys* 40: 69–88. doi:10.3897/mycokeys.40.26025.

V. **Kistenich, S.**, Halvorsen, R., Schrøder-Nielsen, A., Thorbek, L., Timdal, E. & Bendiksby, M. (2019). DNA sequencing historical lichen specimens. *Frontiers in Ecology and Evolution* 7 (5): 1–20. doi:10.3389/fevo.2019.00005.

# 1 Introduction

Lichens comprise a fascinating symbiosis formed by two main partners, a mycobiont and a photobiont (Schwendener 1869; see also Lutzoni and Miadlikowska 2009 for general information on lichens). The mycobiont, i.e. the main fungal partner, usually belongs to the Ascomycota, but also 172 species of the Basidiomycota are known to form a lichen symbiosis (Lücking et al. 2017b; c). The photobiont is either a green alga or a cyanobacterium and sometimes a combination of both. Lichens disperse by producing and spreading (asco-)spores, the sexual reproduction unit of the mycobiont. They may also form special structures for vegetative dispersal, such as isidia or soredia, which spread both the myco- and photobiont at the same time (reviewed by Seaward 2008).

The main fungal partner determines the taxonomy in lichenology. Currently, ca. 20,000 species of lichen-forming fungi are known worldwide (Hawksworth and Lücking 2017). Genus and species delimitation in lichens is traditionally based on morphological characters, such as apothecial anatomy including ascus and ascospore characteristics, thallus construction and vegetative dispersal units. In addition, many mycobionts produce secondary metabolite compounds (lichen substances). These substances are often species specific in their composition, and consequently, they can be used for identification (Elix and Stocker-Wörgötter 2008; Nylander 1866). The main lichen substances are commonly analysed by thin-layer chromatography (TLC) given reference substances, while high-performance liquid chromatography and related techniques are more suitable for identifying satellite compounds. More than 800 different lichen substances have been described (Elix 2014).

During the last decades, DNA sequences have increasingly been used for species identification (Hajibabaei et al. 2007; Hebert et al. 2003; Schoch et al. 2012). Usually, short DNA fragments, for example from the mitochondrial and nuclear ribosomal DNA or from various protein-coding genes, are targeted, amplified and sequenced to obtain information about taxonomic and evolutionary relationships on all levels in molecular phylogenetic trees (e.g., Divakar et al. 2017; Prieto and Wedin 2017; Stenroos et al. 2018; Thell et al. 2012). Such molecular studies have frequently shown that the traditional understanding (i.e., based on morphology and chemistry) of species, genus and/or family affiliations and circumscriptions are not always congruent with statistically inferred evolutionary trees based on DNA sequence data. Thus, many groups of lichens have undergone extensive taxonomic revisions based on molecular data. Several new species and genera have first been detected and/or described because of DNA sequence data (e.g., Bendiksby and Timdal 2013; Lendemer and Hodkinson 2013; Otálora et al. 2014).

Lichens are globally distributed and may grow on stones, soil, bark of trees, leaves or other living organisms. While temperate lichens have been rather well studied within the last centuries, tropical lichens are less well known and have only been studied more intensively within the last decades. Recent publications suggest that a considerable part of the lichen biodiversity in tropical rainforests is still undescribed and may yet to be discovered (e.g., Lücking et al. 2014).

## 1.1 The family Ramalinaceae

The lichen family Ramalinaceae C. Agardh (Lecanorales) is the fourth largest family of lichenized ascomycetes. The family has a worldwide distribution, i.e. occurring in both dry and wet habitats from the tropics to even Antarctica. According to the latest classification — prior to this thesis — by Lücking et al. (2017b; c), the family comprises 42 genera and 913 species, representing both macro-lichens and those with crustose growth forms (Fig. 1), most of which grow on the bark of trees or on stone, rarely on soil or on leaves. The circumscription of the family is generally based on visible morphological characters, such as the presence of a chlorococcoid photobiont, mostly biatorine or lecideine apothecia, hyaline and often transversely septate ascospores as well as asci typically with an amyloid apex and a more or less conical ocular chamber and axial body.

Miadlikowska et al. (2014) presented a molecular phylogenetic hypothesis of the Lecanoromycetes and included several taxa of the Ramalinaceae, of which many were resolved as sister to the family Psoraceae Zahlbr. The study corroborated many of the findings by Ekman (2001) showing the current family circumscription to be non-monophyletic. Generic boundaries have been left largely unexamined apart from a few DNA based studies targeting selected genera (e.g., *Bacidia* De Not.: Ekman 2001; *Biatora* Fr.: Printzen 2014; *Lecania* A. Massal.: Reese Næsborg et al. 2007). These studies included only a limited number of species, and yet, they showed the genera to be polyphyletic to various degrees. This clearly indicated the need for a taxonomic revision of the whole family that includes DNA sequence data. Tropical genera in particular (i.e., *Bacidiopsora* Kalb, *Crocynia* (Ach.) A. Massal., *Eschatogonia* Trevis., *Krogia* Timdal, *Phyllopsora* Müll. Arg. and *Physcidia* Tuck.) have yet to be studied in a molecular phylogenetic context. Species of these corticolous genera are morphologically similar possibly because they grow in the same type of habitat, moist tropical rainforests. Convergent evolution of morphological traits might therefore be expected. So far, little is known about their phylogenetic relationships internally and to each other.

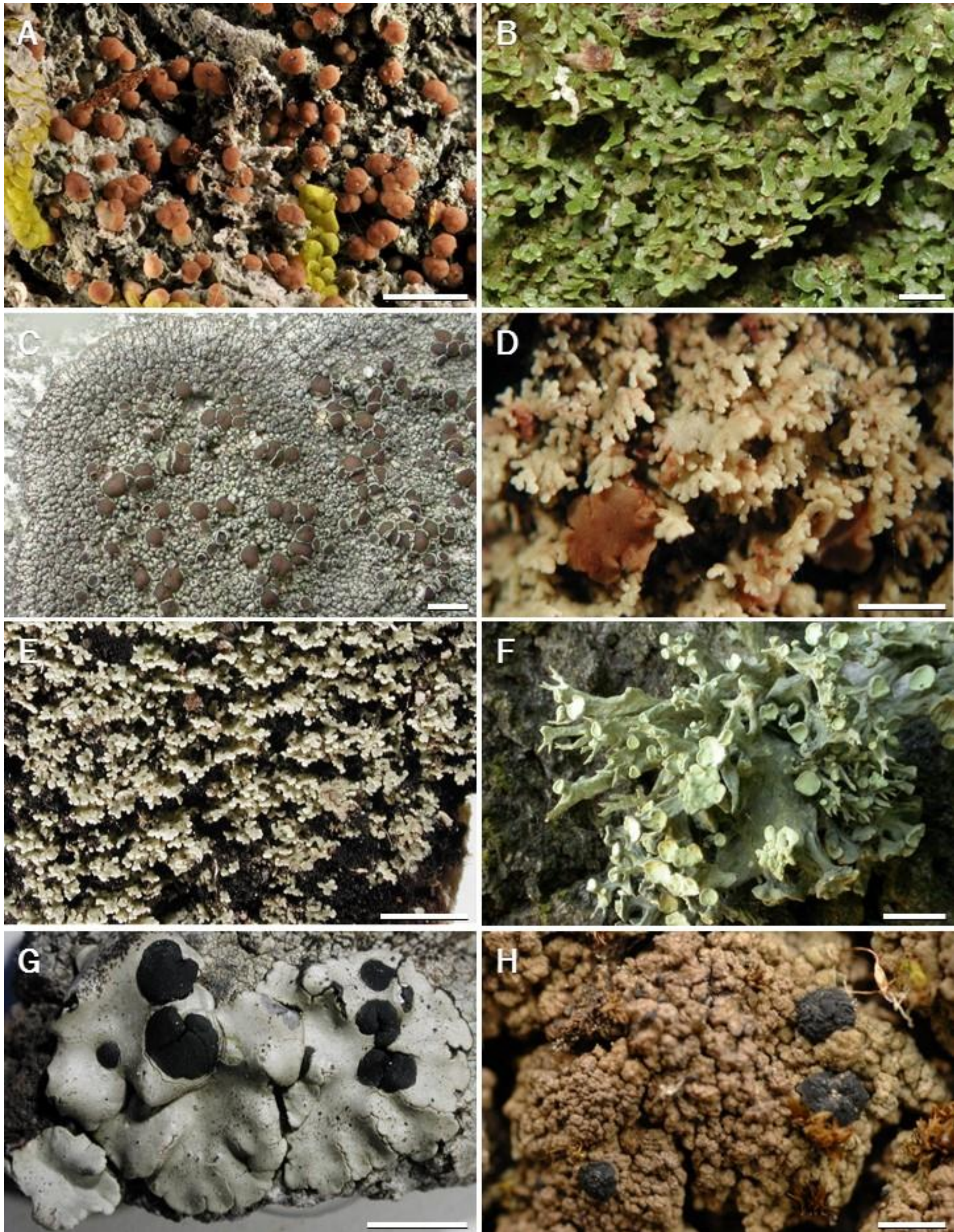



**Figure 1.** Selected species belonging to the family Ramalinaceae according to Lücking et al. (2017b; c). A: *Biatora vernalis* (L.) Fr. (O-L-164706); B: *Eschatogonia prolifera* (Mont.) R. Sant. (O-L-144572); C: *Lecania aipospila* (Wahlenb.) Th. Fr. (O-L-123172); D: *Krogia coralloides* Timdal (O-L-21909); E: *Phyllopsora chlorophaea* (Müll. Arg.) Zahlbr. (O-L-73858); F: *Ramalina fastigiata* (Pers.) Ach. (O-L-130217); G: *Rolfidium coccocarpioides* (Nyl.) Timdal (O-L-22063); H: *Toninia squalida* (Ach.) A. Massal. (O-L-149088). Scale bar in A–C and E ca. 2 mm, in D and H ca. 1 mm, in F ca. 0.5 mm. Photos by E. Timdal.

As the current taxonomy of this family does not seem to accommodate evolutionary lineages correctly, more DNA sequence data representing a comprehensive taxon sampling is likely to give new insight into circumscriptions of natural groups. Phylogenetic relationships between the genera, along with information about their evolutionary history, are necessary to revise the taxonomy of the family towards a more natural classification.

## 1.2 The tropical genus *Phyllopsora* and its relative *Krogia*

The genus *Phyllopsora* is almost exclusively tropical with a pantropical distribution. It usually grows on the bark of trees (rarely on stones) in rainforests or moist woodlands ranging from low to high altitudes (up to 3500 m; Brako 1991; Fig. 2). A typical growth form characterizes the genus, where the areoles or squamules often grow on a thick prothallus ('phyllopsoroid' growth form; Fig. 1E). It remains unclear whether this growth form is a useful taxonomic character or whether it evolved independently as a result of ecological adaptation (Lakatos et al. 2006). Prior to the taxonomic conclusions made in this thesis, the genus comprised ca. 72 accepted species (Brako 1991; Elix 2009; Kondratyuk et al. 2016; Mishra et al. 2011; Swinscow and Krog 1981; Timdal 2008; 2011; Timdal and Krog 2001).

Species of *Phyllopsora* are usually distinguished by a combination of morphological and chemical features (Elix 2009; Swinscow and Krog 1981). Prothallus and squamule/areole morphology along with ascospore size and vegetative dispersal propagules play an important role in morphological species discrimination. In addition, many species contain a characteristic composition of lichen substances, some of which are not found outside the genus, for instance, furfuraceic acid and parvifoliellin. Hence, TLC is an invaluable identification tool, which often provides more reliable results than morphological species identification (Timdal 2008). Some species are reported to form different chemotypes, such as *P. buettneri* (Müll. Arg.) Zahlbr. and *P. porphyromelaena* (Vain.) Zahlbr. It remains unclear whether these chemotypes represent different species or merely genetic or geographical variation. Despite several useful diagnostic characters from morphology and chemistry, species identification and delimitation has proven difficult when specimens are sterile, exhibit a reduced thallus and/or lack lichen substances (Timdal 2008). In such cases, the correct identification is nearly impossible and the only means to assign the specimen reliably to any known species is achieved through generating and comparing DNA sequences. Prior to this thesis, only eight sequences of *Phyllopsora* were available in public DNA sequence repositories, such as GenBank (Benson et al. 2018), of which seven are not identified to species level. Preliminary studies by Bendiksby and Timdal

**Figure 2.** A typical *Phyllopsora* habitat at ca. 2000 m elevation in Caracas, Venezuela. Photo by S. Kistenich.

prior to this PhD project revealed that the genus *Phyllopsora* was non-monophyletic and comprised a heterogeneous assemblage of species, which most likely belonged to several, not closely related genera. A thorough molecular study targeting all known species seems necessary to elucidate which species belong in *Phyllopsora* s. str. and which should be excluded from this genus. Moreover, comparative DNA sequencing might also disclose useful characters for species delimitation.

As many species of *Phyllopsora* are rather inconspicuous, they have not been comprehensively collected and studied. Even though travelling to tropical regions and returning safely was more challenging a century ago than it is today, difficult administrative processes for obtaining all necessary permissions often hamper fieldtrips to tropical rainforests nowadays. Therefore, collections are available mainly from readily accessible tropical regions and the species distribution pattern is therefore strongly biased. Fresh collections of *Phyllopsora* and other tropical genera (i.e., suitable for generating DNA sequences) from more remote regions are highly needed for a global monographic treatment of the genus. While regional monographic revisions of *Phyllopsora* have been published from East Africa (Swinscow and Krog 1981; Timdal and Krog 2001), the Neotropics (Brako 1991), Australia (Elix 2009), Peru (Timdal 2008) and the West Indies (Timdal 2011), little is known about the genus in West Africa or in Asia (except for India; Mishra et al. 2011). Consequently, little is known about the occurrence of the various potential segregates of *Phyllopsora* in these

Paleotropical areas. Several species of *Phyllopsora* are known to exhibit a pantropical distribution, such as *P. corallina* and *P. chodatinica*, but it remains unclear whether specimens from different continents indeed belong to the same species.

The tropical genus *Krogia* (Fig. 1D) is morphologically similar to *Phyllopsora* and often misidentified as such at first glance. *Krogia* is thus assumed to be closely related to *Phyllopsora* (Timdal 2002). The genus is distinguished by usually having red to purple patches on the thallus and by forming a nearly non-amyloid tholus of the asci as well as long, curved, spirally arranged ascospores (Timdal 2002). It was first described in 2002 by Timdal as a monotypic genus from Mauritius, but two additional species have been described from the Neotropics (Timdal 2009; Timdal in Lumbsch et al. 2011). Specimens of *Krogia* are not collected very often, indicating that they might occur less frequently than specimens of *Phyllopsora*. So far, it remains unknown if species of the genus *Krogia* occur at all in tropical Asia or Australia.

## 1.3 Historical lichen collections

In the Natural History Museum of Oslo, the lichen herbarium houses ca. 330,000 specimens with the earliest specimen collected in the year 1800. DNA sequence data has become essential for the identification of lichen species, but DNA fragments can often not be amplified and sequenced from old and degraded specimens. Sometimes, specimens collected a few years or even merely a few months ago seem to be too old for DNA sequencing (Gueidan et al. 2015; Kelly et al. 2011). For some tropical species in the family Graphidaceae Dumort., DNA has to be extracted within three months after collection to ensure successful DNA sequencing (Staiger et al. 2006). Some researchers, on the other hand, report successful polymerase chain reaction (PCR) amplification and Sanger sequencing of century-old specimens (Bendiksby et al. 2014). Generally, specimens collected within the last 30 years are suitable for DNA sequencing using standard protocols, which involve PCR amplification and Sanger sequencing of ca. 500–800 base pair (bp) long DNA fragments routinely used for molecular lichen systematics. Thus, a large proportion of material in lichen collections worldwide remain unused for phylogenetic studies. Ironically, collections from the 19$^{th}$ century and older may be the most important ones to place into a molecular phylogenetic context to obtain information about the application of the names in the past vs. the present. In this regard, types are extremely valuable specimens, which connect names to taxonomical concepts, laying the foundation for the following species identification. In *Phyllopsora*, several species have been described from types collected in either the 19$^{th}$ or the early 20$^{th}$ century (Fig. 3). Sometimes, these types are in such a poor condition that the application of the name is impossible to understand and it

remains unknown whether recent collections of this species exist or not. In these cases, DNA sequences are the only means to interpret the species names.

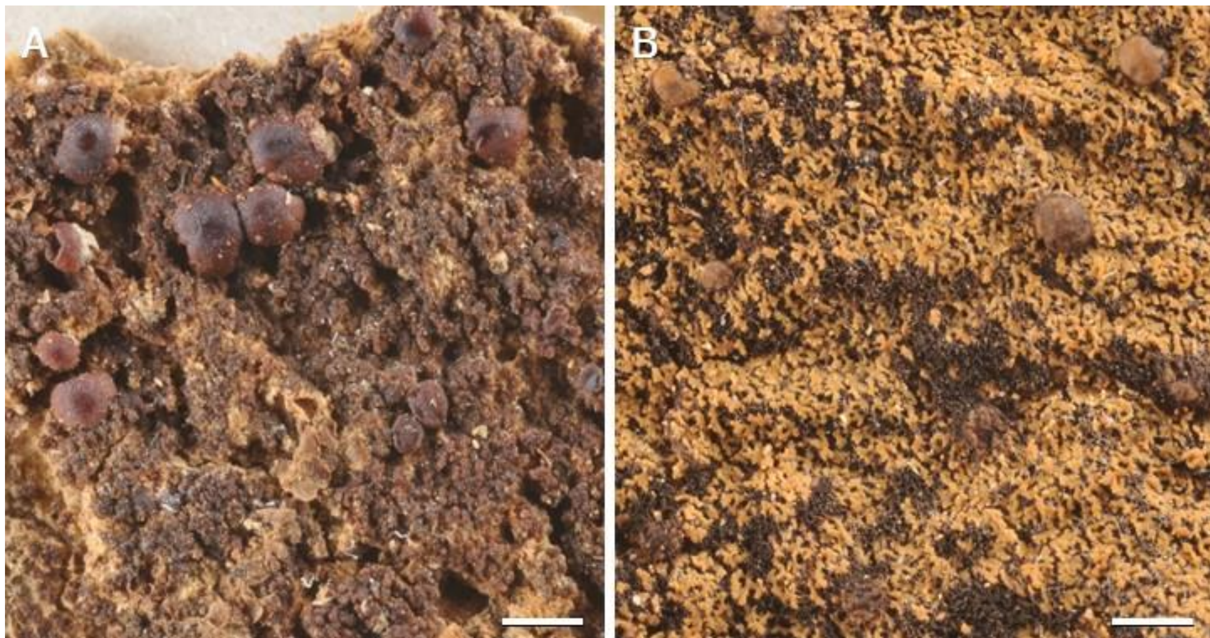



**Figure 3.** Old and poorly understood *Phyllopsora* types. A: Syntype of *P. bibula* (Taylor) Swinscow & Krog, collected in 1830 (H-NYL 20540); B: Lectotype of *P. minor* Brako, collected in 1892 (TUR-V 22612). Scale bar = 1 mm. Photos by E. Timdal.

Unfortunately, there are several challenges connected with obtaining DNA sequences from old material: DNA extracts from old specimens show typically highly fragmented DNA (ca. < 200 bp), contain low amounts of DNA (< 0.1 ng/µl) and often PCR inhibitors (Staats et al. 2011). While standard PCR amplification and Sanger sequencing usually fails under such conditions, high-throughput sequencing (HTS) technologies make use of fragmented DNA by producing millions of short reads (often < 200 bp) from the same genetic locations (Goodwin et al. 2016). In fungal, plant and insect specimens, HTS technologies have already contributed to successfully obtaining DNA sequence information from more than 100-year-old specimens (Andreasen et al. 2009; Gutaker et al. 2017; Larsson and Jacobsson 2004; Prosser et al. 2016). In lichens, however, no such study has been attempted. Moreover, no studies have systematically investigated the extent of DNA degradation in lichens with regard to age, taxonomic affinity, or ecology. In addition, some lichens from wet-tropical areas seem to be more difficult to DNA sequence than those growing in dry habitats. Any study, which facilitates routine sequencing of old lichen material, would dramatically increase the value of these collections for taxonomic research.

## 1.4 Main objectives of the thesis

The present thesis aims at revising the taxonomy of the lichen family Ramalinaceae and at increasing knowledge about the evolution and phylogenetic relationships of the tropical genera, in particular *Phyllopsora*. The thesis explores the diversity of the genus *Phyllopsora* as well as its relatives by involving an integrative approach, comprising phylogenetic analyses of DNA sequence data in combination with traditional morphological and chemical investigations. Moreover, it provides a pilot-study for sequencing old lichen specimens, serving as starting-point for further research on obtaining DNA sequences from old archived material.

**Paper I** aims at improving the knowledge of phylogenetic relationships as well as character evolution in the family Ramalinaceae and at revising the taxonomy accordingly. Special focus is on the relations between the tropical genera.

**Paper II** focuses on investigating species boundaries within the genus *Phyllopsora* s. str. (as delimited in Paper I) based on DNA sequence data complemented by morphological and chemical assessment. The taxonomy is revised accordingly.

**Paper III** aims at giving an overview of the species of *Phyllopsora* in Asia and Melanesia. Three new species are described based on morphology, chemistry and DNA sequence data accompanied by a key to the Asian and Melanesian *Phyllopsora* species.

**Paper IV** focuses on the tropical genus *Krogia*. Three new species are described from Asia and Melanesia based on morphology, chemistry and DNA sequence data accompanied by a key to the six species of the genus.

**Paper V** is a pilot-study for obtaining DNA sequence reads from old lichen herbarium material using HTS on a time series of selected species collected up to 155 years ago. Correlation between sequencing success and age, taxonomic affinity as well as ecologic preferences of the selected specimens are investigated.

# 2 Material and methods

## 2.1 Taxon sampling

More than 2000 specimens were studied for Papers I–V in this PhD thesis. In addition to specimens already held at O, material collected during my fieldwork in Brazil (Pará, Pernambuco, Rio de Janeiro, São Paulo), Sri Lanka (Central, Kalutiare, Sabaragamuwa, Western) and Venezuela (Capital District, Carabobo, Miranda) is used in Papers I–III. For Papers I–V, further material was loaned from the following herbaria: AAS, ABL, ASU, B, BG, BM, BORH, BR, CANB, E, FR, G, GZU, H, HO, HUTPL, KR, LD, M, MIN, NY, OTA, PDA, SBBG, SP, TNS, TRH, TROM, TSB, and UPS (acronyms according to Index Herbariorum). The Korean Lichen Research Institute (Suncheon, South Korea) and the private herbaria of P. Diederich, A. Frisch, H. Holien, T. Johansson, D. Killmann, P. Kirika, Mellansel, Z. Palice, S. Pérez-Ortega, C. Printzen, and P. van den Boom also lent specimens for this project. In addition, relevant DNA sequences were downloaded from GenBank (Benson et al. 2018).

## 2.2 Morphological and anatomical investigations

For morphological investigations of the thallus and apothecia including ascus analyses and ascospore measurements, selected specimens in Papers I–IV were studied using light microscopy. Microscope sections were cut on a freezing microtome at 16–20 µm thickness and mounted in water, 10% KOH (K), lactophenol cotton blue and a modified Lugol's solution, in which water was replaced by 50% lactic acid (I). Amyloid reactions were observed in the modified Lugol's solution after pre-treatment in K (KI reaction).

## 2.3 Thin-layer chromatography

Lichen substances of selected specimens from Papers I–IV were identified by applying TLC, following the standard methods of Culberson and Kristinsson (1970) and Culberson (1972), modified as suggested by Menlove (1974) and Culberson and Johnson (1982). Examinations were made in the three standard solvent systems A, B' and C. In rare cases, two-dimensional chromatography was performed (Culberson and Johnson 1976). The presence of fatty acids was generally not investigated, but when so, in system C.

## 2.4 Molecular methods

For all samples in Papers I–IV and some in Paper V, DNA was extracted of thallus and/or apothecial tissue using the E.Z.N.A. HP Plant DNA Mini Kit (OMEGA Bio-tek). For Paper V, some samples were extracted following either the protocol by Werth et al. (2016) or using the DNeasy Plant Mini Kit (QIAGEN).

For Paper I, five DNA regions were amplified by PCR and sequenced: the mitochondrial ribosomal small subunit (mtSSU), the nuclear ribosomal internal transcribed spacer region (nrITS: ITS1, 5.8S, ITS2), the nuclear ribosomal large subunit (nrLSU) as well as the protein-coding genes for the largest subunit of the RNA-polymerase II (RPB1) and the second largest subunit of the RNA-polymerase II (RPB2). For Papers II–IV, only the mtSSU and the nrITS regions were used. In Paper V, the mtSSU was amplified in a two-step, nested and multiplex PCR protocol using seven primer pairs according to Prosser et al. (2016). Detailed descriptions of the primers and PCR programmes used are found in the respective papers.

All PCR products were enzymatically purified and prepared for sequencing. Sanger sequencing was used in all papers. Samples in Paper V also underwent library preparation and sequencing on an Ion Torrent PGM (Life Technologies, Thermo Fisher Scientific Inc.).

## 2.5 Sequence editing

Raw sequences and sequence reads were edited using the software Geneious R9 (Kearse et al. 2012). In Papers I–V, the trace files generated through Sanger sequencing were assembled, trimmed and corrected to generate a consensus sequence. In Paper V, sequence reads generated by the Ion Torrent PGM were demultiplexed, duplicate reads removed and PCR primers as well as low quality reads trimmed using the BBTools package v.35.8 (Bushnell 2015) as implemented in Geneious. The reads were then mapped to reference sequences downloaded from GenBank using the Geneious Read Mapper.

## 2.6 Alignment

The Sanger sequences were subjected to various sequence alignment algorithms with all genetic markers aligned separately (Papers I–IV). In Paper I, PASTA v.1.7 (Mirarab et al. 2015) was used for the separate alignment of the ITS1 and ITS2 regions as well as for the mtSSU, the online version of MAFFT v.7.3 (Katoh and Standley 2013) was used for the 5.8S (G-INS-i) and nrLSU (E-INS-i) regions. The translation align function in Geneious (Kearse et al. 2012) was applied to RPB1 and RPB2. Introns were deleted

from the RPB1 alignment. All alignments were adjusted manually when necessary. The same algorithms as used in Paper I were run for the mtSSU and nrITS datasets in Paper IV. In Papers II–III, the mtSSU and the nrITS region were each subjected to the online version of MAFFT v.7.4 (Katoh and Standley 2013) using the E-INS-i algorithm.

## 2.7 Phylogenetic analysis

In Papers I and IV, the best-fitting substitution models and partitioning schemes were inferred using PartitionFinder 2 (Lanfear et al. 2016) and incorporated into the subsequent phylogenetic analyses. The maximum likelihood (ML) analyses were conducted with Garli v.2.0 (Zwickl 2006) using 500 standard non-parametric bootstrap (BS) replicates. In Papers II and III, the software IQ-TREE v.1.6 (Nguyen et al. 2015) was applied to find the best-fitting substitution models as well as partitioning schemes, and to conduct the ML analyses with 1000 BS replicates. In addition, Bayesian analyses were run using MrBayes v.3.2.6 (Altekar et al. 2004; Ronquist and Huelsenbeck 2003) with BEAGLE (Ayres et al. 2012) by conducting four Makov Chain Monte Carlo (MCMC) runs with six chains in Papers I–IV. Tree Graph 2 v.2.14 (Stöver and Müller 2010) was used to construct the final, extended majority-rule consensus trees.

Topological incongruences between preliminary ML trees of each marker generated either by IQ-TREE v.1.6 or RAxML v.8.2 (Stamatakis 2014) were assessed using the programme compat.py (Kauff and Lutzoni 2002). If no strongly supported incongruences, affecting the respective focal taxa, were reported, the alignments were concatenated for the final phylogenetic analyses (Papers I, III, IV).

Additional analyses were carried out for Paper I: A rogue taxa analysis was conducted to identify place-shifting taxa in the preliminary ML trees using the dropset algorithm (Pattengale et al. 2011) as implemented in RAxML v.8.2. The identified rogues were pruned from the respective dataset. Furthermore, approximately unbiased (AU) tests (Shimodaira 2002) as implemented in IQ-TREE v.1.6 were performed to test specific phylogenetic hypotheses regarding monophyly of selected taxa.

For Paper II, we constructed a species tree from the incongruent mtSSU and nrITS gene trees using StarBeast (*BEAST) v.2.0.3 (Heled and Drummond 2010) of the BEAST 2 package v.2.5.1 (Bouckaert et al. 2014). Three MCMC runs were conducted and assessed using Tracer v.1.7.1 (Rambaut et al. 2018). The runs were combined with LogCombiner v.2.5.0 (BEAST 2 package) and used as input file for generating a maximum clade credibility tree with posterior probabilities (PP) in TreeAnnotator v.2.5.0 (BEAST 2 package).

For further details about the phylogenetic analyses and chosen settings, see the respective papers.

## 2.8 Ancestral state reconstruction

In Paper I, ancestral character state reconstructions (ASR) were performed to investigate the evolution of selected morphological and ecological traits in the family Ramalinaceae. All calculations were carried out in R v.3.4 (Team 2017). Stochastic mapping of the coded character states on both phylograms and chronograms as well as character state reconstructions at selected nodes were conducted using phytools v.0.6.4 (Revell 2012). Transformation counts as well as statistics were summarized and extracted using additional functions of phytools v.0.6.4, matrixStats v.0.52.2 (Bengtsson 2017), and coda v.0.19.1 (Plummer et al. 2006). Root and node reconstructions were checked for potential influences caused by distribution assumptions using corHMM v.1.22 (Beaulieu et al. 2013).

## 2.9 Species delimitation analysis

In Paper II, species delimitation analyses were performed to investigate the correlation between species circumscriptions in *Phyllopsora* based on morphology and chemistry versus molecular data. The software mPTP v.0.2 (Kapli et al. 2017; Zhang et al. 2013) was used to infer species boundaries on the two gene trees generated by IQ-TREE. Both Bayesian and ML analyses were conducted.

## 2.10 Statistical analysis

In Paper V, additional statistical analyses were performed to investigate the three hypotheses about obtaining sequence reads based on age, taxonomic affinity and ecology. The software R v.2.3 (Team 2018), including the package vegan v.2.4 (Oksanen et al. 2016) was used to calculate generalized linear models (McCullagh and Nelder 1989) and Pearson's $r$ (Pearson 1901) to inform about correlations between selected predictor and response variables.

# 3 Main findings and discussion

## 3.1 The family Ramalinaceae

In Paper I, the heterogeneous lichen family Ramalinaceae was investigated in a molecular phylogenetic context by employing 156 accessions and five molecular markers. Most Ramalinaceae genera (*sensu* Lücking et al. 2017b; c) jointly form a strongly supported monophyletic clade (Fig. 4), while some genera and selected species fell outside this clade (Paper I: Fig. 2). Our results corroborate the family circumscription by Miadlikowska et al. (2014). Based on our molecular phylogenetic hypothesis, we named five major, well-supported clades after the largest represented genus (Fig. 4: C–G): The *Bacidia*-, *Biatora*-, *Ramalina*-, *Rolfidium*- and *Toninia*-clades. The genera *Bacidia*, *Phyllopsora*, *Physcidia* and *Toninia* A. Massal. were found to be polyphyletic and thus split into segregates. Some of the *Phyllopsora* and *Toninia* segregate species were found in clades A and B (Fig. 4), respectively, thus belonging in different families (I: Figs. 2, S1). To accommodate these segregates, 49 new combinations were made, the genera *Bibbya* J.H. Willis, *Kiliasia* Hafellner, *Sporacestra* A. Massal. and *Thalloidima* A. Massal. were resurrected and the two new genera *Bellicidia* Kistenich et al. and *Parallopsora* Kistenich et al. (I: Fig. 3) were described. With our taxonomic conclusions made in Paper I, the family Ramalinaceae comprises 39 genera.

In addition to revising the taxonomy of the family, we traced the evolution of selected character traits. We were interested in the morphological and ecological nature of the Ramalinaceae ancestor. Our results indicate that the ancestor had most likely arisen from moist, temperate forests growing on the bark of trees with a crustose growth form and reproduced mainly by forming apothecia and long, multi-septate spores (I: Table 4). The same pattern was also found for the immediate ancestors of the five major clades except for the *Ramalina*-group, where the ancestor was found to have produced single-septate spores (I: Table 4). This finding was unexpected since we anticipated that short, single-septate spores, as found in the sister family Psoraceae, were the plesiomorphic state in the Ramalinaceae. Instead, the transformation counts in the ASR analysis (I: Table 5) indicated repeated reductions in spore length and amounts of septa throughout the phylogenetic tree. The presence of vegetative dispersal units had also shifted frequently throughout the evolution of genera in the Ramalinaceae.

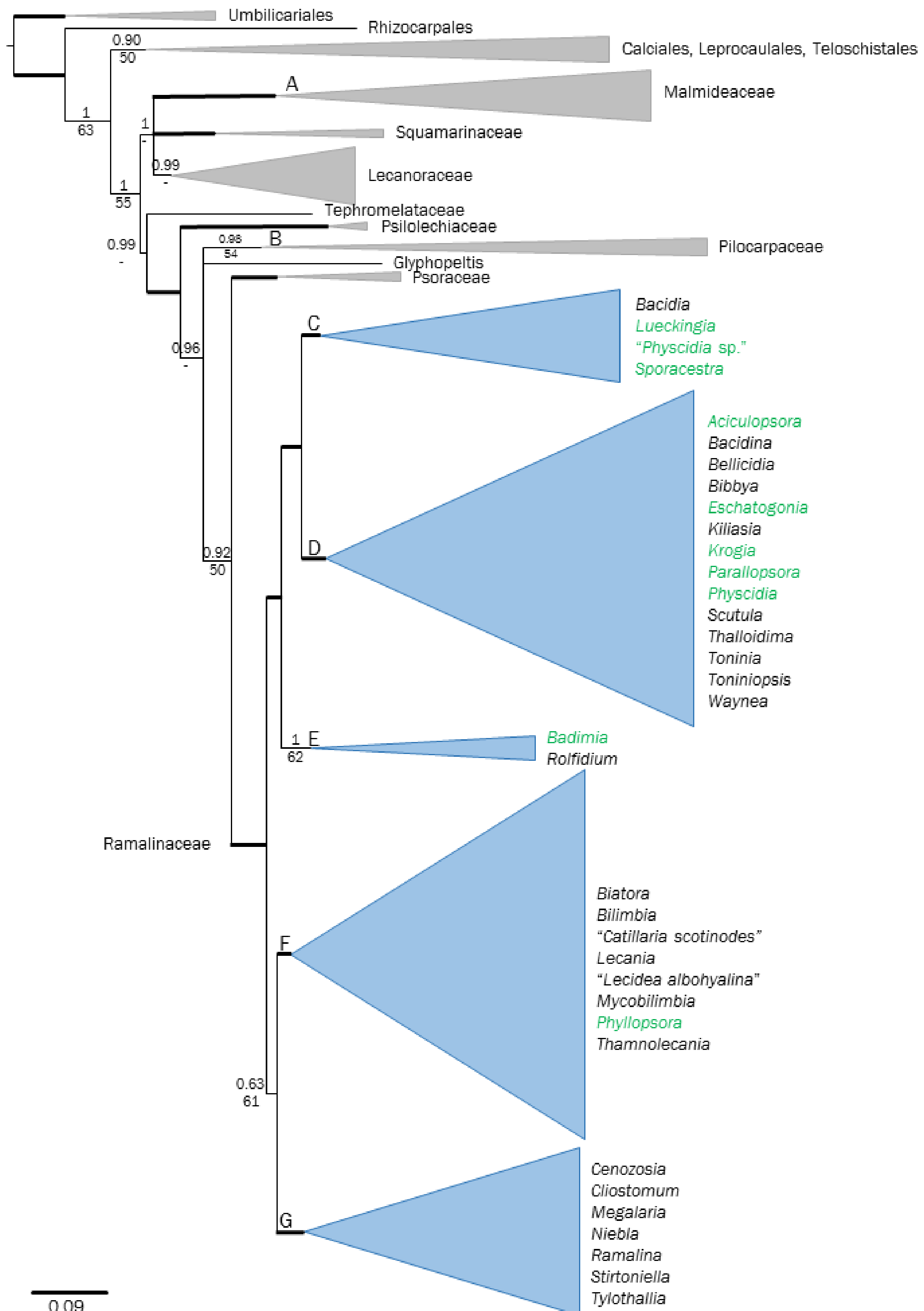



**Figure 4.** Simplified molecular phylogeny of the family Ramalinaceae including the revised genus names. Extended majority-rule consensus tree with BS ≥ 50 and PP ≥ 0.7, modified from the molecular phylogenetic hypothesis in Paper I: Fig. 2. Strongly supported branches (BS ≥ 75 and PP ≥ 95) are marked in bold; for all other branches support values are indicated (BS below and PP above branch). Seven clades are indicated to facilitate discussion (A–G). Genera occurring mainly in the wet-tropics are marked in green.

When focusing on the tropical genera of the family Ramalinaceae, we found them to occur in all of the five major groups (Fig. 4: C–G). The transformation counts indicated several state changes from temperate to tropical habitats, but only rarely vice versa (I: Table 5). Several independent transitions from the crustose to the phyllopsoroid growth form could be observed. Lakatos et al. (2006) advocated that the presence of a prothallus in lichens growing in tropical lowland-rainforests might be ecophysiologically advantageous. According to the authors, a thick prothallus may increase the possibility of continued photosynthesis by keeping the squamules free of water, while rainwater is running down the tree stems (Lakatos et al. 2006). This might explain the high degree of convergent evolution found in the corticolous tropical genera of the Ramalinaceae.

The results obtained in Paper I indicate that the growth form is subject to frequent changes and thus not a reliable character to delimit lineages in the Ramalinaceae. We investigated most of the 42 genera assigned to the Ramalinaceae by Lücking et al. (2017b; c), but could not generate sequences for the following six genera: *Auriculora* Kalb, *Echidnocymbium* Brusse, *Heppsora* D.D. Awasthi & Kr.P. Singh, *Jarmania* Kantvilas, *Pseudohepatica* P.M. Jørg., and *Tibellia* Vězda & Hafellner. For these genera, PCR amplification or Sanger sequencing failed. While most of these genera were probably too old to produce long DNA sequences, our specimens of *Jarmania* failed PCR amplification despite being more recently collected. New analytical approaches are needed to sequence old or difficult herbarium material in order to clarify the taxonomic position of these genera.

## 3.2 The genus *Phyllopsora*

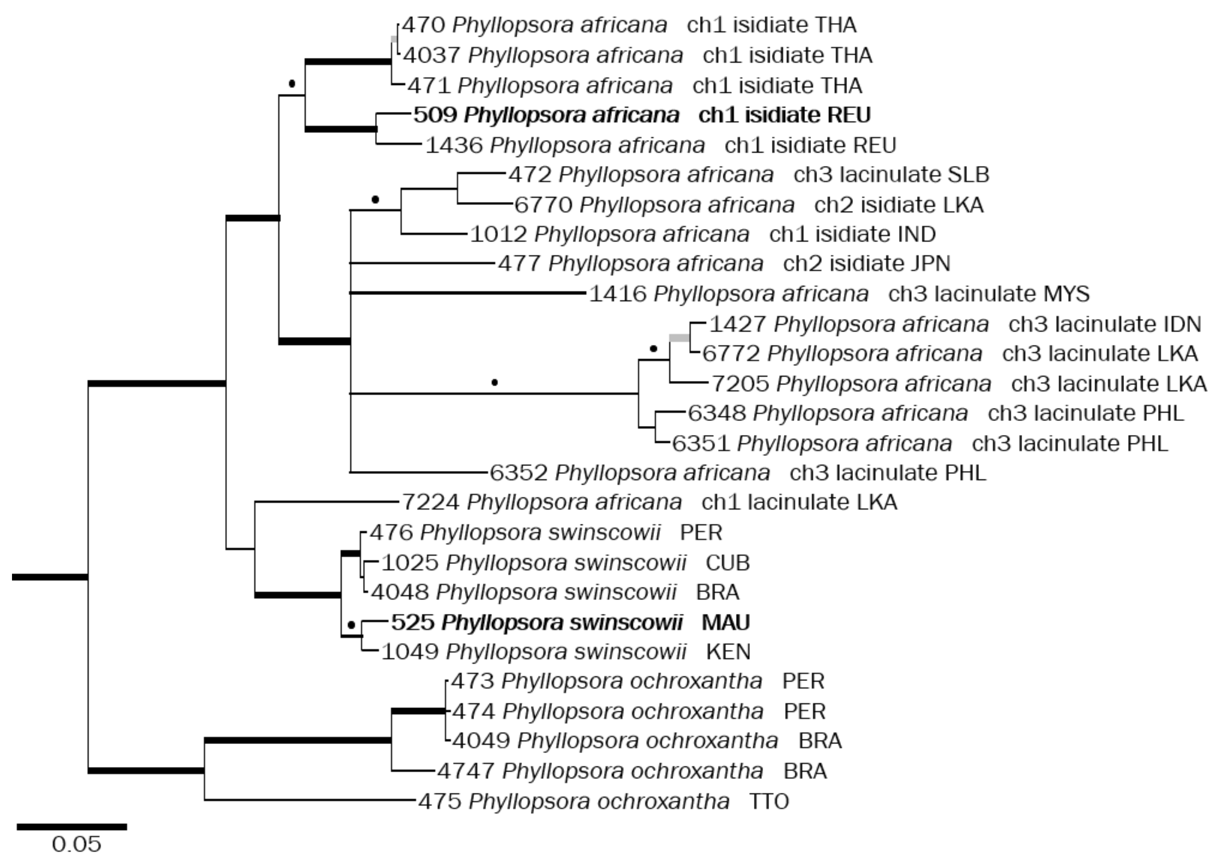
In the molecular phylogeny of the Ramalinaceae (Paper I), we showed the tropical genus *Phyllopsora* to be polyphyletic splitting into four segregates (I: Fig. 2): (1) three species outside the family Ramalinaceae (Fig. 4: A), as sister to *Crustospathula* Aptroot, and as such, most likely belonging to the family Malmideaceae Kalb et al.; (2) three species, of which one is sister to *Physcidia cylindrophora* (Taylor) Hue belonging to *Bacidia*, and two species that we placed in the resurrected genus *Sporacestra* (*Bacidia*-group, Fig. 4: C); (3) four species falling out in the *Toninia*-group (Fig. 4: D), of which one belongs to the genus *Bacidina* Vězda, while a new genus, *Parallopsora*, was established for the remaining three species; (4) the true *Phyllopsora* s. str. including the type species *P. breviuscula* (Nyl.) Müll. Arg., sister to *Biatora* (Fig. 4: F). Two species of *Crocynia*, including the type species, *C. gossypina* (Sw.) A. Massal., were nested in *Phyllopsora* s. str. and, hence, reduced to synonymy. Thus, *Phyllopsora*, which comprised 72 species prior to this thesis, was in Paper I remodelled

to comprise 64 species (II: Table S1). The Ramalinaceae phylogeny clearly showed that *Phyllopsora* s. str. is in strong need of revision and that the typical phyllopsoroid growth form does not represent a good diagnostic character at the genus level because of its seemingly parallel or convergent pattern of evolution.

In Paper II, we focused on species delimitation in *Phyllopsora* s. str. as delimited in Paper I (I: Fig. 2). We investigated archived material directly available at O as well as extensive loans from other herbaria. This, in addition to our newly collected material from Brazil, Venezuela and Sri Lanka. Based on morphological and chemical investigations, we assigned most of the fresh specimens to 48 morphospecies, from which we later on also generated DNA sequences. Unfortunately, only about 75% of the total investigated material could be identified to species level. Still, we were able to generate DNA sequences for most of the 64 accepted species of *Phyllopsora*. Based on mtSSU and nrITS sequence data, we constructed phylogenetic gene trees, which were subjected to species delimitation analyses using the software mPTP. Most of the accessions of each morphospecies grouped together in well-supported clades, indicating that morphology in combination with TLC are useable tools for species delimitation. Using morphology on its own, however, had proven to be difficult for many species and nearly impossible for certain species. We found that four morphospecies (i.e., *P. byssiseda* (Nyl.) Zahlbr., *P. chodatinica* Elix, *P. furfuracea* (Pers.) Zahlbr., and *P. parvifoliella* (Nyl.) Müll. Arg.) were split into two clades each (II: Figs. 2, 3). In these cases, only minute anatomical details or chemical differences distinguished between the two clades. These differences formed the basis for describing the separate clades as the new species *P. isidiosa* Kistenich & Timdal, *P. neotinica* Kistenich & Timdal, *P. furfurella* Kistenich & Timdal and *P. concinna* Kistenich & Timdal (II: Figs. 5, 6), respectively. In addition, we described the new species *P. amazonica* Kistenich & Timdal (II: Fig. 5) based on two specimens from Brazil, which were distinct in morphology, chemistry and DNA sequence data.

Several species of *Phyllopsora* showed intraspecific chemical variation, such as *P. buettneri* and *P. porphyromelaena*. In Paper II, we attempted to investigate as many of these chemotypes as possible. In *P. buettneri*, five chemotypes had been reported (Timdal 2011). We found that the former chemotype 3 represented a separate species (II: Figs. 2, 3) and the old species name *P. melanoglauca* Zahlbr. was resurrected for it. Unfortunately, we were unable to investigate chemotype 5 (Elix 2006). The sequences of chemotype 1, 2 and 4 grouped according to chemotype into a larger clade together with the chemotypes of *P. porphyromelaena* and P. *chodatinica* (II: Figs. 2, 3). The accessions of chemotypes 1–3 of *P. porphyromelaena*, however, did not separate equally well (II: Figs. 2, 3). Especially chemotype 3 (a new chemotype described in Paper II), might be more closely related to *P. chodatinica* and thus

represent a different species. Further new chemotypes were found in *P. africana* Timdal & Krog, now comprising three chemotypes (described in Paper II). However, chemotype 2 of *P. africana* shows the same chemical pattern as found in the morphologically identical species *P. swinscowii* Timdal & Krog. In Paper III, additional specimens of *P. africana* were sequenced, and all chemotypes and morphs were found to mix (Fig. 5). While the majority of the newly sequenced specimens grouped together with the other *P. africana* accessions, a lacinulate specimen associated more closely with the *P. swinscowii* clade (Fig. 5). Thus, only specimens of *P. ochroxantha* (Nyl.) Zahlbr. can be distinguished without DNA sequence data, while the border between *P. africana* and *P. swinscowii* in their current circumscription clearly overlaps. More in-depth analyses are necessary to evaluate the taxonomic status of the chemotypes in these species and of the species themselves (i.e., *P. africana*, *P. buettneri*, *P. porphyromelaena* and *P. swinscowii*).

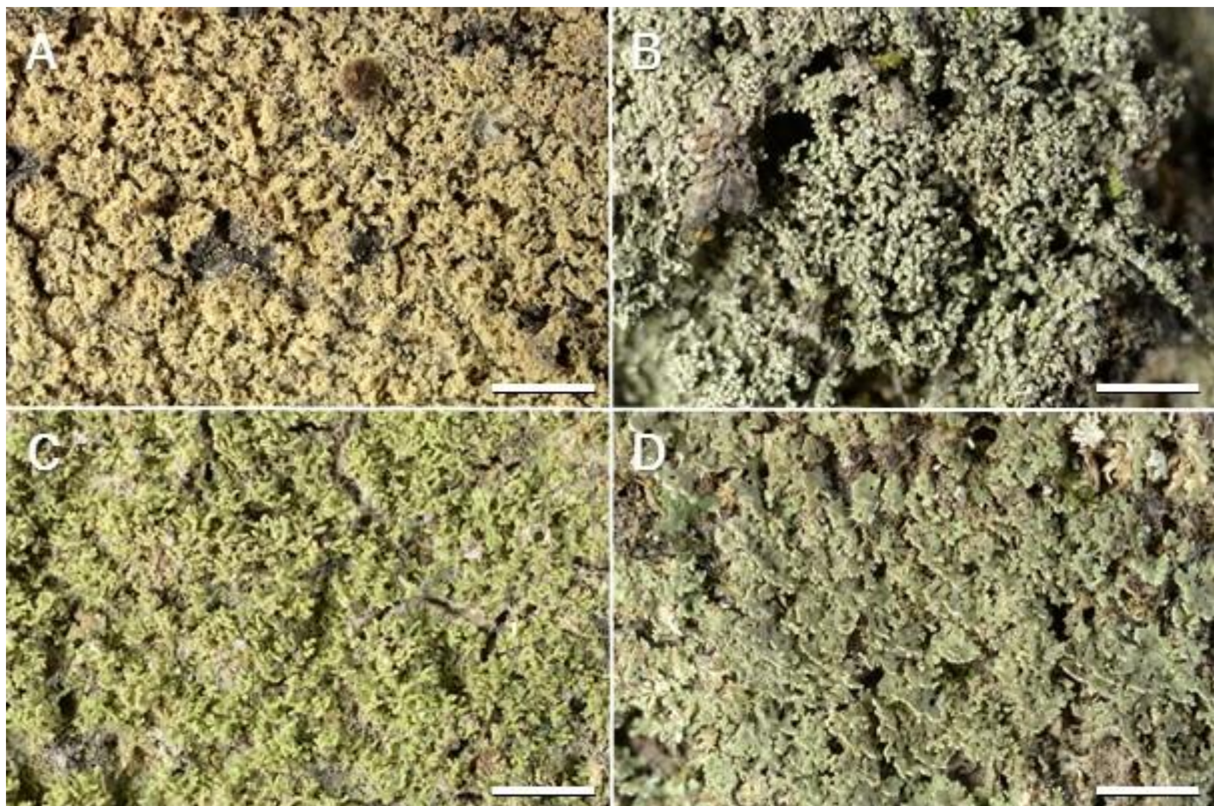



**Figur 5.** Phylogenetic relationships of *P. africana*, *P. ochroxantha* and *P. swinscowii*. Modified from the molecular phylogenetic hypothesis in Paper III: Fig. S1. Strongly supported branches (PP ≥ 0.95 and BS ≥ 75) are marked in bold; branches only supported with PP ≥ 0.7 or BS ≥ 50 are marked with a dot above the branch. Accessions in bold mark type specimens. Terminal names include extract number, species name, and the three-letter country codes according to ISO 3166-1 alpha-3. For *P. africana* specimens, chemotype (ch) and morph (isidiate/lacinulate) are also indicated.

While many accessions grouped into well-delimited clades according to species circumscriptions in Paper II, we also encountered several clades with species

complexes, which could not be fully resolved (II: Figs. 2, 3). The accessions of *P. hispaniolae* Timdal and *P. rosei* Coppins & P. James mixed in a strongly supported clade. Accessions of *P. gossypina* (Sw.) Kistenich et al. and *Crocynia molliuscula* (Nyl.) Nyl. also mixed with each other in a strongly supported clade. It is possible that neither mtSSU nor nrITS might be variable enough to distinguish between each species pair. In these two cases, the two respective species are morphologically different and a possible synonymization has to be evaluated with additional data.

As there has never been made a comprehensive revision of the genus *Phyllopsora* in Asia and Melanesia, we investigated phyllopsoroid material (ca. 900 specimens, of which 625 turned out to represent *Phyllopsora*) from 17 Asian countries including freshly collected (2017) specimens from Sri Lanka (Paper III). In total, we found 28 different species of *Phyllopsora* in the material including three new species: *P. pseudocorallina* Kistenich & Timdal (III: Fig. 2A), *P. sabahana* Kistenich & Timdal (III: Fig. 2B) and *P. siamensis* Kistenich & Timdal (III: Fig. 2C). The three species were not recognized as new species at first sight, as they are morphologically and chemically very similar to *P. corallina* (Eschw.) Müll. Arg., *P. porphyromelaena* and *P. imshaugii* Timdal, respectively. The results of the phylogenetic analyses, however, showed that the species pairs in all three cases were not sister species (III: Fig. 1: A–C). Re-




**Figur 6.** Unidentified specimens potentially representing one or more new species. A: *Phyllopsora* sp. from Malaysia (BM001104019); B–D: *Phyllopsora* spp. from Sri Lanka; B: O-L-207864; C: O-L-207854; D: O-L-207879. Scale bar = 2 mm. Photos by E. Timdal.

investigating the morphology and anatomy of the tentatively new species revealed subtle differences in squamule form and/or ascospore size, enough to distinguish the new species from their look-alikes. In addition, four unidentified specimens (one from Malaysia and three from Sri Lanka; Fig. 6) sat on long branches in the phylogenetic tree (III: Figs. 1, S1). These might represent additional new species, but more specimens are necessary to investigate their morphological and genetic variability before possibly describing them formally.

In total, eight species of *Phyllopsora* are reported as new for Asia and Melanesia in Paper III, two of which are *P. cuyabensis* (Malme) Zahlbr. and *P. parvifoliella*. About 30% of the specimens belonged to other phyllopsoroid genera, both from the Ramalinaceae and the Malmideaceae. They were generally not identified to the species level because of taxonomically unresolved problems at the genus level. Moreover, 141 *Phyllopsora* specimens could not be assigned to any known species as they usually were poorly developed and/or lacked lichen substances. This indicates that the diversity of phyllopsoroid species in Asia and Melanesia is probably much higher than described in Paper III. The material needs further attention, for example by obtaining DNA sequence data of the remaining unidentified specimens.

Following these taxonomic revisions, we acknowledge 57 species in the genus *Phyllopsora*. Several more species are considered dubious and poorly understood, including several old types (II: Taxonomy B).

## 3.3 The genus *Krogia*

In the molecular phylogeny of Paper I, the genus *Krogia* formed a supported clade together with *Physcidia striata* Aptroot et al. and accessions of the genus *Bacidina* (I: Fig. 2). Molecular sequence data of the type species, *K. coralloides* (Fig. 1) corroborates its placement in the family Ramalinaceae. Molecularly, the genus *Krogia* is clearly distinct from the morphologically similar genus *Phyllopsora* s. str., which was found in a different major subclade of the family (*Toninia*-group vs. *Biatora*-group, respectively; Fig. 4).

Before the start of this PhD project, only three species of *Krogia* were known from the Neotropics and tropical Africa. No records of the genus had been reported from Asia or Oceania. When we routinely investigated phyllopsoroid material from Malaysia and New Caledonia, we discovered three new species of *Krogia*. In Paper IV, we describe these as *K. borneensis* Kistenich & Timdal, *K. isidiata* Kistenich & Timdal and *K. macrophylla* Kistenich & Timdal. *Krogia borneensis* (IV: Fig. 2) was described from the island of Borneo (Malaysia), while *K. isidiata* (IV: Fig. 3) and *K. macrophylla* (IV: Fig. 4) were both described from the island Grand Terre (New Caledonia). The

three new species are distinguished by morphological, chemical and DNA sequence data from each other and the three previously known species (i.e., *K. antillarum* Timdal, *K. coralloides* and *K. microphylla* Timdal). We constructed a molecular phylogenetic tree using both mtSSU and nrITS sequence data of the three new species along with sequences of *K. antillarum* and *K. coralloides*. Unfortunately, we were not able to sequence specimens of *K. microphylla*. All five species formed together a well-delimited, monophyletic clade, sister to *Bacidina*.

Morphological investigation of *K. borneensis* revealed the presence of a unicellular cortex surrounding the thallus. This type of cortex is characteristic for the genus *Eschatogonia* indicating that this morphological trait may have originated at least twice in the family. In the Ramalinaceae phylogeny, *Eschatogonia* was also found in the same major group, the *Toninia*-group (I: Fig. 2: D), but in a different clade than *Krogia*. This group, however, is characterized by rather poor phylogenetic resolution and it is not unlikely that the two genera may be closely related. More sequence data of additional *Eschatagonia* species should be included in a phylogeny of the *Toninia*-group to investigate this relationship in more detail.

In Paper IV, we doubled the amount of species in this genus. Two new species were found on New Caledonia, seemingly a hotspot for discovering new lichen species (Aptroot 2014; Papong et al. 2014). Hence, additional material of *Krogia* should be collected throughout the wet-tropics and especially from islands to gain more information about distribution ranges and to find out if additional species remain to be discovered.

## 3.4 Sequencing historical lichens

In Paper V, we investigated the sequencing success along an age gradient with reference to genus affiliation and ecological preferences. To evaluate the sequencing success of historical lichens systematically, we selected four species pairs from Norway, each from the same genus from four different families: *Cladonia floerkeana* (Fr.) Flörke and *C. gracilis* (L.) Willd. (Cladoniaceae Zenker), *Nephroma laevigatum* Ach. and *N. arcticum* (L.) Torss. (Nephromataceae Wetmore), *Peltigera collina* (Ach.) Schrad. and *P. malacea* (Ach.) Funck (Peltigeraceae Dumort.), *Ramalina siliquosa* (Huds.) A.L. Sm. and *R. fraxinea* (L.) Ach. (Ramalinaceae; V: Fig. 1). The former representative in each species pair has a mainly coastal (humid) distribution, while the latter occurs in more continental (dry) habitats. Each species was sampled from herbarium vouchers collected between 2010 and 1860, compiling a time series with 25 years between each sampling point (i.e., seven periods in total).

Using a modification of the Ion Torrent approach developed by Prosser et al. (2016), we were in Paper V able to generate sequence reads of the mtSSU for 54 of the 56 selected specimens, including a full sequence of a specimen collected 127 years before DNA extraction (i.e., 2018). In addition, we obtained enough sequence information for species identification of a 150-year-old specimen. In contrast, when using Sanger sequencing, we only received useable trace files for 15 of the 56 specimens, mainly from recent collection periods, but also from 108–127-year-old specimens. Three of the specimens from the 1985 and 1960-period showed longer sequences obtained by Sanger sequencing compared with those from the Ion Torrent protocol.

Statistical analyses indicate that younger specimens perform better than older ones (V: Fig. 6). While we were able to assemble nearly 100% of the reference sequence length for the most recently collected specimens, only short sequences could be assembled for the oldest ones. Genus affiliation also showed to influence the sequencing success (V: Table 4). Specimens of *Peltigera* Willd. performed extremely well, whereas specimens of *Nephroma* Ach. showed the poorest sequencing success (V: Fig. 5). For specimens of *Ramalina* Ach., we never managed to assemble the full target sequence. In contrast, we could not observe any statistically significant difference between sequencing success of specimens with humid versus dry habitat preferences (V: Table 4). As our selected species pairs have partly overlapping distribution ranges, we assume that the difference in humidity was not large enough to give a statistically significant result. Therefore, a parallel study should ideally be tested on specimens from the wet-tropics versus arid regions. In that way, one could simultaneously assess the applicability of this approach on types of *Phyllopsora*, for example.
We also found that the DNA concentration of the initial extracts appeared to be uncorrelated with sequencing success when inspecting data of all specimens. However, when using data from specimens more than 100 years old only, we received a significant correlation between DNA concentration and sequencing success. In addition, genus affiliation explained a larger fraction of variation in DNA concentration than did age (V: Table 4). This indicates that successful DNA sequence generation of old lichen specimens largely depends on the taxonomic group for deciding whether high or low DNA concentrations are needed.

Our sample size was rather low with only one individual from each species per period. Increasing the sample size is necessary to obtain more robust results. It is possible that different sequencing platforms (e.g., Illumina platforms) may give better results and these should be explored as well. Therefore, different taxonomical groups from the humid tropics should be subjected to this approach prior to destructive sampling of old *Phyllopsora* types, such as for example *P. bibula* from 1830.

## 3.5 Additional comments

After the Parmeliaceae Zenker (Crespo et al. 2010), the Graphidaceae (Staiger et al. 2006) and the Verrucariaceae Zenker (Gueidan et al. 2007), the Ramalinaceae, as the fourth largest lichen family has now also received a new family circumscription, which includes DNA sequence data. In the Ramalinaceae, we encountered the same challenges with polyphyletic taxa and phylogenetically unresolved relationships as in the three above-mentioned studies, and we likewise resolved polyphyly with taxonomic remodeling of genera. The tropical genera in particular proved to be polyphyletic.

In recent years, lichenologist have discovered immense numbers of new tropical lichen species, for instance, placed in the Graphidaceae (Lücking et al. 2014), the Trypetheliaceae Zenker (Aptroot et al. 2016b), and the basidiomycete lichen genus *Cora* Fr. (Lücking et al. 2017a). Estimates suggest that the diversity in these groups may be even higher (see Aptroot et al. 2016a and references therein). There may be comparable numbers of new species to be discovered in the phyllopsoroid genera. In this thesis, we describe 11 new tropical species and detected several additional new candidate species (Fig. 6; Papers II–IV). Tropical lichens are generally found to diversify to a greater extent than their temperate counterparts (Singh et al. 2018). Our species delimitation analyses in the genus *Phyllopsora* suggest splitting off many species from seemingly monospecific assemblages. Additional specimens providing DNA sequence data, however, are necessary to support these inferred species entities. Species delimitation studies focusing on other genera, such as *Diploschistes* Norman (Zhao et al. 2017) and *Peltigera* (Magain et al. 2018), reveal up to 50 new lineages that may deserve species level recognition. In the genus *Usnea* Dill. ex Adans. (Gerlach et al. 2019), most delimited lineages in *U. cornuta* Körb. correspond with secondary chemistry, but these were not formally described as new species. We also discovered a similar pattern in *P. buettneri*, but we only acknowledged one chemotype as a separate species in the taxonomic treatment. The remaining chemotypes should be re-investigated with additional specimens. All species delimitation analyses, however, will greatly benefit from including types where morphologically cryptic species are split. There have not yet been conducted molecular studies, which systematically investigate the feasibility of sequencing old herbarium specimens from the tropics. Thus, we encourage the initiation of these studies to avoid redundant descriptions of new species.

# 4 Concluding remarks and perspectives

In the course of this thesis, the fourth largest lichen family, the Ramalinaceae, has received a new family circumscription based on new insights gained by molecular phylogenetic data and knowledge about character evolution. Moreover, this thesis has contributed to increase the understanding of species limits within *Phyllopsora* s. str. and led to the description of 11 new species of *Phyllopsora* and *Krogia*. Even though the taxon sampling for Papers I–IV has been balanced and rather extensive, not all targeted taxa could be sequenced. There are various reasons for not obtaining DNA sequences of these taxa: (1) We could not get in contact with the respective herbaria that hosted the taxa of interest; (2) specimens were too old to attempt DNA extraction as Sanger sequencing would most likely have failed; and/or (3) despite being collected only a few years ago, some specimens failed PCR amplification because of, for instance, inhibitors, degraded DNA, or poor primer binding. In Paper V, we made a first attempt to develop a protocol for routinely sequencing old lichen specimens, which proved successful for specimens up to 150 years old. This protocol may be applied to the missing taxa from Papers I–IV to investigate its potential to generate sequences from further Ramalinaceae species.

While this thesis has initiated taxonomic revisions of the family Ramalinaceae and the genus *Phyllopsora* in particular, some taxonomic affinities could not be resolved simply by investigating selected molecular markers. Some species complexes in the genus *Phyllopsora* remain phylogenetically unresolved despite morphological differences. In these cases, additional sequence data should be included to inform about the species status of the respective specimens. Moreover, some specimens, which could not be identified by morphology or chemistry, remain unidentifiable (i.e., unassigned to species) even when sequence data is available. Phylogenetic species boundaries may be uncertain and/or reference sequences may lack to assign questionable specimens unambiguously to a known species. It is likely that our understanding of the extent of morphological, chemical and molecular variation in several phyllopsoroid species is still incomplete. It is also probable that there are additional new species in the unidentified material of *Phyllopsora* and its related genera. There are still many tropical regions that have not yet been investigated for phyllopsoroid species, and additional collections will most likely benefit our taxonomic understanding of the genus *Phyllospora* and its relatives.

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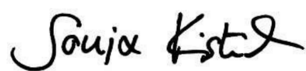

Sonja Kistenich

The pictures show merely a few of the many persons who accompanied me during the fieldwork for this thesis in Venezuela (A) and Brazil (B, C) in 2015 as well as in Sri Lanka (D) in 2017. They made the fieldwork not only possible but also contributed to collections, ensured our safety and supplied us with delicious food. They introduced me to their country with immense amounts of enthusiasm and hospitality to make each stay unforgettable! Thanks to all of them for many lovely memories!

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# Papers I–V

I

# Molecular systematics and character evolution in the lichen family Ramalinaceae (Ascomycota: Lecanorales)

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**Abstract** The Ramalinaceae is the fourth-largest family of lichenized ascomycetes with 42 genera and 913 species exhibiting considerable morphological variation. Historically, generic boundaries in the Ramalinaceae were primarily based on morphological characters. However, molecular systematic investigations of subgroups revealed that current taxonomy is at odds with evolutionary relationships. Tropical members of the family remain particularly understudied, including the large genus *Phyllopsora*. We have generated and collected multilocus sequence data (mtSSU, nrITS, nrLSU, *RPB1*, *RPB2*) for 149 species associated with the Ramalinaceae and present the first comprehensive molecular phylogeny of the family. We used ancestral state reconstructions on our molecular family phylogeny to trace the evolution of character states. Our results indicate that the Ramalinaceae have arisen from an ancestor with long, multiseptate ascospores living in humid temperate forests, and that the phyllopsoroid growth form has evolved multiple times within the family. Based on our results using integrative taxonomy, we discuss sister-relations and taxon-delimitation within five well-supported clades: The *Bacidia*, *Biatora*-, *Ramalina*-, *Rolfidium*-, and *Toninia*-groups. We reduce six genera into synonymy and make 49 new nomenclatural combinations. The genera *Bacidia*, *Phyllopsora*, *Physcidia* and *Toninia* are polyphyletic and herein split into segregates. We describe the two genera *Bellicidia* and *Parallopsora* and resurrect the genera *Bibbya*, *Kiliasia*, *Sporacestra*, and *Thalloidima*. According to our new circumscription, which also includes some additional changes, the family Ramalinaceae now comprises 39 genera.

**Keywords** ancestral state reconstruction; integrative taxonomy; multilocus phylogeny; *Phyllopsora*; *Toninia*; tropical lichens

**Supplementary Material** The Electronic Supplement (Table S1; Figs. S1 & S2) is available from https://doi.org/10.12705/675.1.S; DNA sequence alignments are available from TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S22266).

## ■ INTRODUCTION

The Ramalinaceae C.Agardh is the fourth-largest family of lichen-forming ascomycetes, with 42 genera and 913 species (Lücking & al., 2017a, b). This family contains members with a chlorococcoid photobiont, mostly biatorine or lecideine apothecia (when present) and hyaline and often transversely septate ascospores. Historically, generic boundaries were primarily based on growth form, ascospore septation and even ecological preferences. Growth form varies considerably among species. The majority of species form crustose or squamulose thalli, but fruticose or foliose species occur in a couple of genera. Members of the family inhabit a wide spectrum of habitats, including lichenicolous life forms, but corticolous and saxicolous species are the most numerous. Being globally distributed, the Ramalinaceae span all climatic zones from arctic to temperate and tropical, and occur in humid as well as dry habitats.

The Ramalinaceae, as currently circumscribed (sensu Lücking & al., 2017a, b), has been variously delimited in the past. Originally, it was described by Agardh in 1821 as "Ramalineae" and included the eight genera *Sphaerophoron* (now *Sphaerophorus*, Sphaerophoraceae), *Roccella* (Roccellaceae), *Evernia* (Parmeliaceae), *Dufourea* (Teloschistaceae), *Alectoria* (Parmeliaceae), *Ramalina* (Ramalinaceae), *Cornicularia* (Parmeliaceae) and *Usnea* (Parmeliaceae; Agardh, 1821). More than 100 years later, Watson (1929) excluded all other genera besides *Ramalina* and was the first to use the correct family name, Ramalinaceae. Zahlbruckner (1921–1940) assigned some foliose and fruticose genera to this family, while crustose species now included here were placed by him in the family Lecideaceae Chevall. He divided the genus *Ramalina* into three sections: sect. *Ecorticatae*, sect. *Desmaziera* and sect. "*Euramalina*" (not validly published; Zahlbruckner, 1921–1940). Keuck (1979) was the first to investigate morphological and anatomical features of the family's – at that point – five genera: *Cenozosia*, *Niebla*, *Ramalina*, *Ramalinopsis* and *Trichoramalina*. Crustose genera were at the time still included in other families, for example Bacidiaceae Walt.Watson, Biatoraceae A.Massal. ex Stizenb., Lecaniaceae Walt.Watson, Lecideaceae, and Phyllopsoraceae Zahlbr. In his extensive work, Hafellner (1984) reclassified the large families Lecanoraceae and Lecideaceae and erected the following additional families for some of the genera now placed in Ramalinaceae: Catillariaceae Hafellner, Catinariaceae Hale ex Hafellner, Crocyniaceae M.Choisy ex Hafellner, Megalariaceae Hafellner, Mycobilimbiaceae Hafellner, and Schadoniaceae Hafellner. Later, most of those families were

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reduced into synonymy with Bacidiaceae (see Ekman, 2001 for a thorough historical overview). Ekman (2001) investigated taxa belonging to both Bacidiaceae and Ramalinaceae in a broad molecular phylogeny and pointed out the striking morphological similarities in the ascus structure between the two families. The inclusion of the Bacidiaceae, Megalariaceae (Ekman, 2001), and later Crocyniaceae (Ekman & al., 2008) resulted in 42 component genera (Lücking & al., 2017a, b). Even so, the synonymy of Bacidiaceae with Ramalinaceae does not seem to have been broadly accepted, as the Bacidiaceae as a separate family is still being used in some modern literature (e.g., Sérusiaux & al., 2012).

The first molecular studies of the Ramalinaceae that included more than five genera (e.g., Ekman, 2001; Reese Næsborg & al., 2007; Schmull & al., 2011; Miadlikowska & al., 2014) rendered the family circumscription non-monophyletic as several genera and species were nested in other lichen families. A few Ramalinaceae genera have undergone further molecular phylogenetic investigations, for example *Bacidia* De Not. (Ekman, 2001), *Biatora* Fr. (Printzen, 2014), and *Lecania* A.Massal. (Reese Næsborg & al., 2007). None of these, however, included a family-wide taxonomic sampling, which left most generic boundaries largely unexamined, while at the same time rendering these genera polyphyletic. The recent compilation by Lücking & al. (2017a, b) reported that only 55% of the 42 Ramalinaceae genera are represented by DNA sequence data in GenBank, and less than half of those genera have ten or more sequence records. Despite recent molecular efforts to delimit the family within Lecanorales (Miadlikowska & al., 2014), comprehensive phylogenetic investigations of the family with an exhaustive genus-level sampling have so far not been conducted. Genera lacking molecular sequences to verify their placement in the Ramalinaceae include the enigmatic *Heppsora* D.D.Awasthi & Kr.P.Singh, *Physcidia* Tuck., *Pseudohepatica* P.M.Jørg., and *Tasmidella* Kantvilas & al., among others (Lücking & al., 2017a, b: their supplementary table S1).

While many morphological studies and monographs have been published on temperate species of the Ramalinaceae in North America and Europe (Howe, 1913a, b; Mayrhofer, 1988; Timdal, 1992; Printzen, 1995; Ekman, 1996a; Printzen & Tønsberg, 1999), tropical members of the family remain understudied. A set of genera occurring almost uniquely in the tropics include *Bacidiopsora* Kalb, *Crocynia* (Ach.) A.Massal., *Eschatogonia* Trevis., *Krogia* Timdal, *Phyllopsora* Müll.Arg. and *Physcidia* Tuck. Except for *Crocynia*, all are characterized by largely squamulose thalli, with the areoles or squamules often overgrowing a thick prothallus (Fig. 1). This morphology is here termed “phyllopsoroid”. Even though *Crocynia* has a more felt-like thallus, we consider this genus also associated with the phyllopsoroid growthform due to the distinct prothallus. *Phyllopsora* is the largest of these genera and has been placed in various families, for example Phyllopsoraceae (Hafellner, 1984; Elix, 2009), Bacidiaceae (Brako, 1989, 1991), and Ramalinaceae (Timdal, 2008, 2011). The circumscription of the Phyllopsoraceae was thereby mainly based on the phyllopsoroid growth form, which may have evolved independently in the family as a response to similar ecological conditions (shaded tree trunks in tropical rainforests). Furthermore, many of these species form vegetative dispersal units such as phyllidia or lacinules that are rarely found in other temperate genera of the Ramalinaceae. Apart from *Crocynia* and *Phyllopsora*, there are no published molecular studies or sequences in GenBank for the abovementioned tropical genera. Hence, the evolution of the phyllopsoroid growth form has so far not been explored in a phylogenetic context, nor has the inclusion of all these genera in the Ramalinaceae been confirmed.

**Fig. 1.** Photograph of *Phyllopsora breviuscula* (type of the genus name) illustrating the phyllopsoroid growth form with the areoles/squamules overgrowing a thick prothallus (B 60 0083819); scale bar = 2 mm.

The aim of this study was to improve the knowledge about phylogenetic relationships and character evolution in the family Ramalinaceae and to update its classification accordingly. To achieve this aim, we conducted phylogenetic analyses with multilocus DNA sequence data with a comprehensive taxon representation. We used ancestral state reconstruction to trace the evolution of morphological features through time and to explore the two following hypotheses: (1) ellipsoid, simple ascospores are plesiomorphic in the family; (2) the phyllopsoroid growth form has evolved repeatedly and independently from crustose ancestors. Our suggested taxonomic changes to the classification of the family are based on an integrative approach, abiding by molecular phylogenetic principles while at the same time thoroughly evaluating morphological characteristics.

## ■ MATERIALS AND METHODS

**Taxon selection and sampling. —** We obtained sequence data from a large sample of Ramalinaceae representatives, including nearly all types of genera included in the family by Robert & al. (2005), Eriksson & al. (2006), Lumbsch & Huhndorf (2011) and Lücking & al. (2017a, b) . In addition, we included taxa suspected by us to belong in this family based on their morphology as well as members of nominal genera treated as synonyms in Zahlbruckner (1921–1940) and MycoBank (http://www.mycobank.org). In addition, we included selected members of various families within the Lecanoromycetidae and Umbilicariomycetidae. All specimens were either taken from our own herbaria (O, UPS) or borrowed from other institutions (AAS, ABL, ASU, B, BG, BM, BR, CANB, E, FR, G, GZU, HO, KR, LD, M, MIN, NY, SBBG, SP, TRH, TROM, TSB). Additionally, we downloaded DNA sequences from GenBank. Whenever possible, several species per genus, including the type, were included. In all, we used DNA sequence data from 175 specimens representing 149 species (Appendix 1). Authorships for genera and species are provided in Appendix 1 and in the Taxonomy chapter, or, for additional taxa, at first mention in the text.

**Morphology and chemistry. —** Selected specimens were subjected to morphological investigations of the thallus and apothecia, ascus analyses and ascospore measurements using light microscopy. Microscope sections were cut on a freezing microtome at 16–20 µm thickness and mounted in water, 10% KOH (K), lactophenol cotton blue and a modified Lugol's solution, in which water was replaced by 50% lactic acid (I). Amyloid reactions were observed in the modified Lugol's solution after pretreatment in K (KI reaction). For identification of lichen substances, we applied thin-layer chromatography (TLC), using the standard methods of Culberson & Kristinsson (1970) and Culberson (1972), modified as suggested by Menlove (1974) and Culberson & Johnson (1982). Examinations were made in the three standard solvent systems A, B' and C.

**Extraction, PCR and sequencing. —** Part of the laboratory work followed the procedure described by Ekman & al. (2008) and Ekman & Blaalid (2011). Other parts used the following approach: Genomic DNA was extracted from apothecia and/or thallus tissue (ca. 1–3 mg) using the E.Z.N.A. HP Plant DNA Mini Kit (OMEGA Bio-tek, Norcross, Georgia, U.S.A.) following the manufacturer's instructions with the modifications described in Bendiksby & Timdal (2013). We selected four nuclear and one mitochondrial genetic marker: the internal transcribed spacer (ITS) region (including ITS1, 5.8S and ITS2) and the downstream large subunit (LSU) of the nuclear ribosomal DNA, the largest subunit of the RNA polymerase II gene (*RPB1*), the first part of the second-largest subunit of the RNA polymerase II gene (*RPB2*) and the small subunit (mtSSU) of the mitochondrial ribosomal DNA. Polymerase chain reactions (PCR) were performed with the primer pairs listed in Table 1. When the first round of amplification was unsuccessful, we applied a nested PCR approach or used internal PCR primers). We used half reactions of the Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, U.K.), i.e., prior to adding DNA, we transferred 12 µl of the mixture to a new PCR tube. To this, we added 0.5 µl of template DNA for all markers except *RPB2*, for which we added the double amount of both DNA template (1 µl) and each primer (2 µl; 10 µM). The PCR products were purified with the Illustra ExoProStar Clean-Up Kit (GE Healthcare) following the manufacturer's instructions, but with a 10-fold enzyme dilution. We used the ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, Massachusetts, U.S.A.) for sequencing reactions following the manufacturer's instructions except that the BigDye was diluted four to 10 times for a final 10 µl reaction volume. The respective PCR primers were also used as sequencing primers. We performed a standard ethanol precipitation with EDTA (125 mM), NaOAc (3 M) and 96% ethanol followed by two 70% ethanol washes to clean the final extension PCR products. We added 10 µl of Hi-Di Formamide (Applied Biosystems) to the cleaned extension products and subjected them to automatic Sanger sequencing on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Alternatively, we sent the purified PCR products to Macrogen Europe (Amsterdam, The Netherlands) for Sanger sequencing according to the company's instructions for sample preparation.

**Sequence alignment. —** We assembled and edited the resulting sequences using the software Geneious R9 (Kearse & al., 2012). For the separate alignment of the highly variable ITS1 and ITS2 sequences, we used PASTA v.1.7 (Mirarab & al., 2015) with OPAL as aligner and merger, the maximum subproblem set to 50%, RAxML as the tree estimator under a GTR+Γ model and a maximum of 400 iterations. We also used PASTA for the mtSSU alignment with the same settings, except that we used a GTR+I+Γ model to handle potentially invariant sites in conserved regions. As the 5.8S and LSU alignments contain more conserved regions, the online version of MAFFT v.7.313 (http://mafft.cbrc.jp/alignment/software/; Katoh & Standley, 2013) was used (G-INS-i and E-INS-i algorithms, respectively), with default settings, except that the scoring matrix was set to 20PAM. PASTA iteratively optimizes the alignment and a maximum likelihood phylogeny, which is an accurate approach for highly variable datasets, whereas MAFFT produces equally accurate alignment estimations for less variable datasets in considerably shorter run times than PASTA (Mirarab & al., 2015). For the

**Table 1.** Primers used for PCR and sequencing including respective PCR programmes. Primer sequences in 5′→3′ direction.

| PCR primers | mtSSU | ITS | LSU |
|---|---|---|---|
| Standard primers | mtSSU1<br>AGCAGTGAGGAATATTGGTC<br>Zoller & al., 1999 | ITS1-F<br>CTTGGTCATTTAGAGGAAGTAA<br>Gardes & Bruns, 1993 | LSU_RamF<br>GACCTCGGATCAGGTAGGG<br>this study |
| | mtSSU3R<br>ATGTGGCACGTCTATAGCCC<br>Zoller & al., 1999 | ITS4<br>TCCTCCGCTTATTGATATGC<br>White & al., 1990 | LR5<br>TCCTGAGGGAAACTTCG<br>Vilgalys & Hester, 1990 |
| Nested primers | mtSSU_RamF<br>AATAGCCTAACGGCTGAACC<br>this study | ITS5<br>GGAAGTAAAAGTCGTAACAAGG<br>White & al., 1990 | LSU_hypF<br>CGCTGAACTTAAGCATATC<br>Bendiksby & Timdal, 2013 |
| | mtSSU_RamR<br>AGGCCATGATGACTTGTCTT<br>this study | ITS6<br>TAAGTTCAGCGGGTATCCCTA<br>Bendiksby & Timdal, 2013 | LSU_hypR2<br>CTATCCTGAGGGAAACTTCG<br>Bendiksby & Timdal, 2013 |
| Internal primers | mtSSUF<br>ACCAGTAGTGAAGTATGTTGTT<br>this study | ITS_lichR<br>ATTCACTGAATTCTGCAATTCA<br>Bendiksby & Timdal, 2013 | — |
| | mtSSUR<br>AACAACATACTTCACTACTGGT<br>this study | ITS_lichF<br>TGAATTGCAGAATTCAGTGAAT<br>Bendiksby & Timdal, 2013 | |
| PCR programme | | | |
| Initial denaturation | 95°C 7 min | 95°C 7 min | 95°C 7 min |
| Phase 1 | 35 cycles[a]<br>95°C 30 s<br>60(48[b])°C 30 s<br>72°C 30 s | 35 cycles[a]<br>95°C 30 s<br>60°C 30 s<br>72°C 30 s | 35 cycles[a]<br>95°C 30 s<br>60°C 30 s<br>72°C 30 s |
| Phase 2 | — | — | — |
| Final extension | 72°C 7 min | 72°C 7 min | 72°C 7 min |

**Table 1.** Continued.

| PCR primers | *RPB1* | *RPB2* |
|---|---|---|
| Standard primers | gRPB1-A<br>GAKTGTCCKGGWCATTTTGG<br>Stiller & Hall, 1997 | fRPB2-5F<br>GAYGAYMGWGATCAYTTYGG<br>Liu & al., 1999 |
| | fRPB1-C<br>CNGCDATNTCRTTRTCCATRTA<br>Matheny & al., 2002 | fRPB2-7cR<br>CCCATRGCTTGYTTRCCCAT<br>Liu & al., 1999 |
| Nested primers | RPB1_RamF<br>AGGYTTCATGACRAARATCAARA<br>this study | RPB2-5Fram<br>TTCHACAARCTBACVAARGAYGT<br>this study |
| | RPB1_RamR4<br>GCTTGWGGCTGBCCAGCRATDTC<br>this study | RPB2-7Rram<br>GTCATVACDATCATDATNGTYTCYTC<br>this study |
| Internal primers | — | — |
| PCR programme | | |
| Initial denaturation | 94°C 2 min | 92°C 2 min |
| Phase 1 | 7 cycles<br>94°C 60 s<br>60°C (−1°C/cycle) 90 s<br>72°C 1 min 45 s | 8 cycles<br>94°C 60 s<br>59°C 60 s<br>72°C 2 min |
| Phase 2 | 33 cycles<br>94°C 60 s<br>56°C 90 s<br>72°C 2 min (+3 s/cycle) | 33 cycles<br>95°C 30 s<br>50°C 30 s<br>72°C 2 min |
| Final extension | 72°C 10 min | 72°C 10 min |

a, number of cycles reduced to 25 in PCRs which were followed by nested PCRs; — b, only in nested PCR

protein-coding genes *RPB1* and *RPB2*, we started with the Translation Align option in Geneious R9 (Kearse & al., 2012) combined with the G-INS-i algorithm and PAM100 scoring matrix of the MAFFT v.7 plugin (Katoh & Standley, 2013), and subsequently adjusted resulting alignments manually. Introns were excised from *RPB1*; no other data was excluded from the alignments. We trimmed the ends of all resulting alignments to minimize the amount of terminal missing data.

We generated two different alignments: (1) a concatenated alignment containing all five loci but including only terminals for which at least two loci were represented (altogether 156 terminals, from here on referred to as 5-locus dataset); and (2) a concatenated mtSSU and ITS alignment which additionally included taxa for which only one mtSSU or ITS sequence was available (171 terminals, from here on referred to as 2-locus dataset).

**Partitioning and model testing. —** We inferred the best-fitting substitution models and partitioning scheme for the concatenated 5-locus alignment with PartitionFinder2 (Lanfear & al., 2016), using the Bayesian information criterion (BIC) to select among all possible combinations of models implemented in MrBayes v.3.2.6 (1-, 2-, and 6-rate models). Subset rates were treated as proportional ("linked branch lengths"). We defined 11 potential subsets prior to the analysis: mtSSU, ITS1, 5.8S, ITS2, LSU, *RPB1* codon position (cp) 1, *RPB1* cp2, *RPB1* cp3, *RPB2* cp1, *RPB2* cp2 and *RPB2* cp3. PartitionFinder2 suggested that our data be divided into seven subsets, each with the following substitution model: a GTR+I+Γ model for (1) mtSSU, (2) ITS1 and ITS2, (3) LSU, (4) *RPB1* cp1 and *RPB2* cp1, (5) *RPB1* cp2 and *RPB2* cp2, and a SYM+I+Γ model for (6) 5.8S, and (7) *RPB1* cp3 and *RPB2* cp3. The models for the mtSSU and ITS regions as suggested by PartitionFinder2 were also used for the 2-locus dataset.

**Phylogenetic analyses. —** *Boreoplaca ultrafrigida* and *Ropalospora lugubris* were used for rooting all phylogenies. We checked for incompatibilities among gene trees by subjecting each marker to a maximum likelihood bootstrap analysis as implemented in RAxML Black Box v.8.2.10 (Stamatakis & al., 2008) on the CIPRES webserver (Miller & al., 2010) with default settings. Resulting gene trees were input to compat.py (Kauff & Lutzoni, 2002) using a 75% cut-off for supported incompatibilities.

Rogue taxa in the 2-locus alignment were identified using the dropset algorithm suggested by Pattengale & al. (2011) as implemented in RAxML v.8.2.10 (Stamatakis, 2014). We excluded three taxa identified as rogue (i.e., *Compsocladium archboldianum*, *Myelorrhiza antrea*, *Schadonia fecunda*) from the subsequent 2-locus dataset analyses.

Both 5-locus and 2-locus alignments were subjected to maximum likelihood analyses using Garli v.2.01 (Zwickl, 2006) on the CIPRES webserver (Miller & al., 2010) and on the Abel high performance computing cluster (University of Oslo, Norway) under the models and partitioning scheme suggested by PartitionFinder2. We searched for the best tree using 1000 repetitions from a random tree. The nonparametric bootstrapping analyses included 500 replicates, each on 10 search replicates from a random tree.

We also analysed the datasets phylogenetically using MrBayes v.3.2.6 (Ronquist & Huelsenbeck, 2003; Altekar & al., 2004) with BEAGLE (Ayres & al., 2012) on the CIPRES webserver (Miller & al., 2010). We used a (1, 1, 1, 1, 1, 1) Dirichlet for the rate matrix, a (1, 1, 1, 1) Dirichlet for the state frequencies, an exponential (1) distribution for the gamma shape parameter and a uniform (0, 1) distribution for the proportion of invariable sites. Subset rates were assumed proportional with the prior distribution following a (1, 1, 1, 1, 1, 1, 1) Dirichlet. We assumed a compound Dirichlet prior on branch lengths (Rannala & al., 2011; Zhang & al., 2012). For the gamma distribution component of this prior, we set $\alpha = 1$ and $\beta = 0.05$ (0.04 for the 2-locus alignment), as the expected tree length $\alpha/\beta$ (taken from the preceding maximum likelihood analysis) was approximately 21 (26). The Dirichlet component of the distribution was set to the default (1, 1). Four parallel Markov chain Monte Carlo (MCMC) runs were performed, each with six chains and the temperature increment parameter set to 0.05 (0.1; Altekar & al., 2004). The appropriate degree of heating, adjusted for swap rates in the interval 0.1–0.7, was determined by monitoring cold and hot chains in preliminary runs. We used a burnin of 50% and sampled every 10,000th tree. The runs were diagnosed for convergence every $10^6$ generations and were set to terminate either at convergence or after having reached $100 \times 10^6$ generations. Convergence was defined as an average standard deviation of split frequencies (ASDSF) smaller than 0.01. We projected the bootstrap support values (BS) from the Garli-analysis onto the MrBayes majority rule consensus tree with posterior probabilities (PP) and collapsed branches with BS < 50 and PP < 0.7. The resulting trees were edited in TreeGraph v.2 (Stöver & Müller, 2010).

While analyzing our phylogenetic results, we decided to investigate whether a series of specific phylogenetic hypotheses were within the error margin of the best tree using Shimodaira's approximately unbiased (AU) test (Shimodaira, 2002) under maximum likelihood conditions as implemented in IQ-TREE v.1.6 beta 4 (Nguyen & al., 2015). We used the 5-locus data with the same partitioned model as in previous analyses. Heuristic searches were carried out for an unconstrained tree as well as the best (constrained) tree agreeing with each of the null hypotheses. We expanded the default search criteria by starting each analysis from 1000 parsimony trees (keeping the 100 best), increasing the SPR radius to 10 nodes and checking all NNI swap configurations. The search was stopped after 500 steps without likelihood score improvement. The AU test was carried out with 10,000 multiscale bootstrap replicates with a depth of $K = 10$ (fixed by the software). Hypotheses were rejected if they were less than 5% likely to be best tree. We also checked for breakdown of asymptotic conditions (Shimodaira, 2002: app. 10) by testing the residual sums of squares against a chi-square distribution with $K - 2$ degrees of freedom.

**Analyses of character evolution. —** We investigated the evolution of selected morphological and ecological traits by performing character transformation counts as well as reconstruction of ancestral states. The aim was to test the two following hypotheses on our dataset: (1) ellipsoid, simple spores are plesiomorphic in the family; (2) the phyllopsoroid growth

form has evolved repeatedly and independently from crustose ancestors. All data manipulation and calculations were carried out in R v.3.4.2 (R Core Team, 2017). We started by coding the following seven morphological traits as discretely valued characters, either binary or with multiple states: ascospore shape (length : width ratio: 0 = ≤3, 1 = >3) and septation (0 = none or pseudoseptate, 1 = single septate, 2 = multiseptate), growth form (0 = crustose, 1 = fruticose, 2 = foliose, 3 = phyllopsoroid), climate preference (0 = arctic, 1 = temperate, 2 = tropical, and 0 = dry, 1 = moist), substrate choice (0 = soil, 1 = rock, 2 = bark/wood, 3 = living organisms) and the presence of specialised vegetative dispersal structures, i.e., isidia, lacinules, phyllidia and soredia (0 = absent, 1 = present). The coding was based on relevant literature (mostly Smith & al., 2009 for the European taxa, various monographs for the extra-European taxa, e.g., Timdal, 1992; Ekman, 1996a) and on our own observations whenever necessary. When the state was unknown for a taxon, we coded the prior probability as equally divided across all known states. In the next step, we randomly downsampled the Bayesian posterior tree sample from the 5-locus tree inference to 1000 trees. Taxa not belonging to the Ramalinaceae were excluded and so were one of the terminals (the one on the longer branch) in all cases with a species being represented by two terminals. The resulting trees, which preserved the original branch lengths in number of changes per site, were rooted and are referred to here as our sample of "phylograms".

Phylograms are desirable if it is assumed that morphological change is proportional to genetic change. However, applications of ancestral state reconstruction often assume that morphological change is proportional to time, in which case reconstructions need to be performed on trees with branch lengths proportional to time ("chronograms"). Litsios & Salamin (2012) and Cusimano & Renner (2014) demonstrated that reconstructions on phylograms and chronograms, while often similar, can sometimes give different results. However, to safeguard against results sensitive to the proportionality assumption, we carried out all reconstructions on phylograms as well as chronograms. Chronograms were generated from the phylograms using penalized likelihoods under a correlated model as described by Paradis (2013) with the *chronos()* function of ape v.5.0.

Stochastic mapping (Nielsen, 2002; Huelsenbeck & al., 2003; Bollback, 2006) was carried out on the phylograms and chronograms using the *make.simmap()* function of phytools v.0.6.44 (Revell, 2012). We simulated 100 character mappings for each of the 1000 trees. For two-state characters, we applied an asymmetric model that allowed forward and backward rates to be different. For the sake of minimizing the number of parameters estimated, however, multistate characters (characters with more than two states) were assumed to follow a symmetric model. In the symmetric model, forward and backward rates between all pairs of states are assumed equal, whereas these rates can be different between pairs of states. Simulations were set to use an instantaneous rate matrix, Q, estimated from the empirical data and a prior distribution of states on the root node estimated from the stationary distribution of Q. Character transformation counts were subsequently extracted from the sampled maps by using the *countSimmap()* function and summarized using table summary functions from *matrixStats* v.0.52.2 (Bengtsson, 2017) as well as the *HPDinterval()* function of coda v.0.19.1 (Plummer & al., 2006). In addition, we extracted and summarized inferred ancestral states for nodes present in the majority-rule consensus tree of all mapped trees using the *describe.simmap()* function on two randomly selected maps among the 100 per tree. This downsampling was necessary for reasons of computational time and memory usage.

Ancestral state reconstructions (ASR) at or near the root node can potentially be influenced by the assumptions made about the distribution of states at the root. Therefore, we wanted to check for sensitivity to those assumptions. We did this by use of the *rayDISC()* function of the corHMM v.1.22 package (Beaulieu & al., 2013). Reconstructions were made on the extended majority-rule consensus tree from the Bayesian 5-locus inference and was based on the same character information as in the stochastic mapping, including distributing unknown states equally across the known states. We performed marginal reconstructions using three different assumptions about the distribution of states at the root node: (1) equal distribution of states, (2) the same distribution as in the observed data (Yang, 2006: 124), and (3) estimated from the data and model (Maddison & al., 2007; FitzJohn & al., 2009). The latter setting most closely resembled the one used in the stochastic mapping.

## ■ RESULTS

**Molecular data. —** Sequences were successfully generated for most of our specimens except for old and/or poor-quality specimens (Appendix 1). In total, we produced 458 new sequences for this study, ranging from 79 to 106 for the various genetic markers (Appendix 1; Table 2). The amplification and sequencing success was highest for mtSSU and ITS, followed by LSU, whereas the amplification of low-copy genes, *RPB1* and *RPB2*, was more challenging. We obtained *RPB1* and *RPB2* sequences for about 50% of our samples. The 5-locus dataset consisted of 156 taxa and resulted in a 5520 bp long alignment with 2346 parsimony-informative sites and 30.2% missing data (Table 2). The 2-locus dataset of 171 accessions resulted in a 2468 bp long alignment with 974 parsimony-informative sites and 20.9% missing data. Both alignments are available from TreeBase (study no. 22266).

**Phylogenetic analyses. —** Our compat.py analyses revealed a few cases of incongruence between individual gene trees, all involving subterminal branches within clades of closely related congeneric species. This incongruence occurred between the (1) mtSSU and *RPB1* tree, (2) mtSSU and *RPB2* tree, and (3) ITS and *RPB1* tree, but affected neither genus delimitations nor deeper branches.

The Bayesian phylogenetic analyses halted automatically after $15 \times 10^6$ generations for the 5-locus alignment and after $37 \times 10^6$ generations for the 2-locus alignment, when the ASDSF in the last 50% of each run had fallen below 0.01. We used 3004 (7404 for the 2-locus analysis) trees for constructing the final majority-rule consensus tree. The phylogenetic results generated by Garli and MrBayes showed no incongruences,

**Table 2.** Overview of the numbers of included accessions and newly produced sequences, the amount of missing data, and the lengths of alignments for each genetic marker and the concatenated 5-locus alignment.

| | mtSSU | ITS | LSU | *RPB1* | *RPB2* | 5-locus dataset |
|---|---|---|---|---|---|---|
| Number of accessions | 139 | 135 | 116 | 108 | 99 | 156 |
| Newly produced sequences | 99 | 86 | 91 | 90 | 79 | 445 |
| Missing data (%) | 16.3 | 18.6 | 41.3 | 33.9 | 44.2 | 30.2 |
| Length including gaps (bp) | 1173 | 1323 | 1176 | 678 | 1170 | 5520 |

only varying resolution. The extended majority-rule consensus tree of the 5-locus alignment (Fig. 2), based on the Bayesian topology with all compatible groups (BS ≥ 50 and/or PP ≥ 0.7), showed good resolution and branch support at both genus and species levels. Overall, the 5-locus tree (Fig. 2) was better resolved than the 2-locus tree (Electr. Suppl.: Fig. S1). The vast majority of taxa traditionally classified as Ramalinaceae form a well-supported clade (Fig. 2: PP = 1, BS = 82). The resulting tree from the 2-locus alignment displays the same monophyletic group (Electr. Suppl.: Fig. S1: PP = 1, BS = 60). Among strongly supported clades, eight are considered particularly taxonomically relevant and are indicated to facilitate their discussion (Fig. 2: Ramalinaceae and clades A–G). The genera *Bacidia*, *Bacidina*, *Phyllopsora*, *Physcidia* and *Toninia*, as currently circumscribed, appear non-monophyletic in both the 5- and the 2-locus phylogenies (Fig. 2; Electr. Suppl.: Fig. S1; notice Fig. 2: clade A and Electr. Suppl.: Fig. S1: clade B). The following species of genera in the Ramalinaceae fall outside of the family: *Phyllopsora atrocarpa*, *P. lividocarpa*, *P. nigrocincta*, *Toninia squalescens*, and *T. thiopsora*. The following eight genera, currently assigned to the Ramalinaceae, fall outside of the family either based on the clade referred to as Ramalinaceae in our phylogenetic tree (Fig. 2; Electr. Suppl.: Fig. S1) or based on BLAST searches of generated sequences: *Adelolecia*, *Catinaria*, *Compsocladium*, *Crustospathula*, *Frutidella*, *Japewia*, *Schadonia* and *Tasmidella*. Multiple accessions of species grouped together, except for *Mycobilimbia tetramera*. This situation may have resulted from a misidentification, which, however, does not impair the genus delimitation.

The results of the AU tests rejected ($p < 0.05$) six of our hypotheses and confirmed ($p > 0.05$) the following four hypotheses: *Bacidina* incl. *Woessia*+*Lichingoldia* is monophyletic, *Toniniopsis* is monophyletic, *Toninia*+*Toniniopsis*+*Kiliasia*+*Bibbya*+*Thalloidima* is monophyletic, *Parallopsora* gen. nov. (i.e., *Phyllopsora brakoae*, *P. labriformis*, *P. leucophyllina*) is monophyletic (Table 3). The residual sums of squares were all within the lower 95% of a chi-square distribution with eight degrees of freedom, indicating that test conditions were valid.

**Ancestral character states. —** We reconstructed seven ancestral states for the most recent common ancestor (MRCA) of the Ramalinaceae (Fig. 2) as well as for five selected subclades (Fig. 2 clades C–G). The coding matrix for all coded taxa is provided together with the 5-locus phylogeny (Fig. 2). Median results of the simulations on both phylograms and chronograms show the highest probabilities for the ancestor of the Ramalinaceae to have originated from temperate, moist forests and to have reproduced mainly sexually with long multiseptate ascospores (state probabilities 76%–100%; Table 4). Node reconstructions for the five subclades (Fig. 2 clades C–G) are largely concordant with the Ramalinaceae MRCA apart from the *Ramalina*-group having highest probabilities for 1-septate spores (Table 4). Results of inferences on phylograms and chronograms are mostly similar and both analyses types always recovered the same most probable states. However, differences of up to 22.7 percentage points can be found within the characters "Spores", "Climate" and "Vegetative dispersal" (Table 4). The three priors on the distribution of states at the root node generated similar results (max. ±5% differences) for the root node as well as five selected subclades (Fig. 2 clades C–G; Electr. Suppl.: Table S1), except for the character "Vegetative dispersal" (±30% differences; Electr. Suppl.: Table S1). The transformation counts of state changes reveal frequent transitions from short to long spores, from crustose to phyllopsoroid growth form and from temperate to tropical climate zones (Table 5). Furthermore, the results indicate that it was more common to go from a humid ("moist") habitat to a dry one than the reverse (Table 5). Gains as well as losses of vegetative dispersal have been frequent (Table 5).

## ■ DISCUSSION

In this study, we present the first detailed multilocus phylogeny of the family Ramalinaceae, including many types (Fig. 2), and present novel results from ASR analyses on this phylogeny (Tables 4, 5). Most Ramalinaceae genera form a monophyletic clade (Fig. 2: Ramalinaceae; see section on the Ramalinaceae family circumscription). Some genera, however, exhibit varying degrees of non-monophyly, for example *Phyllopsora*, *Physcidia* and *Toninia*. In the current taxonomy, which is mainly based on morphology and apothecial characters, these genera are polyphyletic. Molecular phylogenetic studies – such as the present one – are therefore essential for revealing occurrences of convergent evolution or parallelism within these traditionally morphology-based classifications, and to guide taxonomic genus delimitation. Similar instances of polyphyly can be found throughout several other lichen families and genera (e.g., Divakar & al., 2006; Bendiksby & Timdal, 2013; Kirika & al., 2016; Zhao & al., 2016). In our study, we show that phenotypic features, such as the growth form or the presence of a prothallus, are often a result of adaptation to a

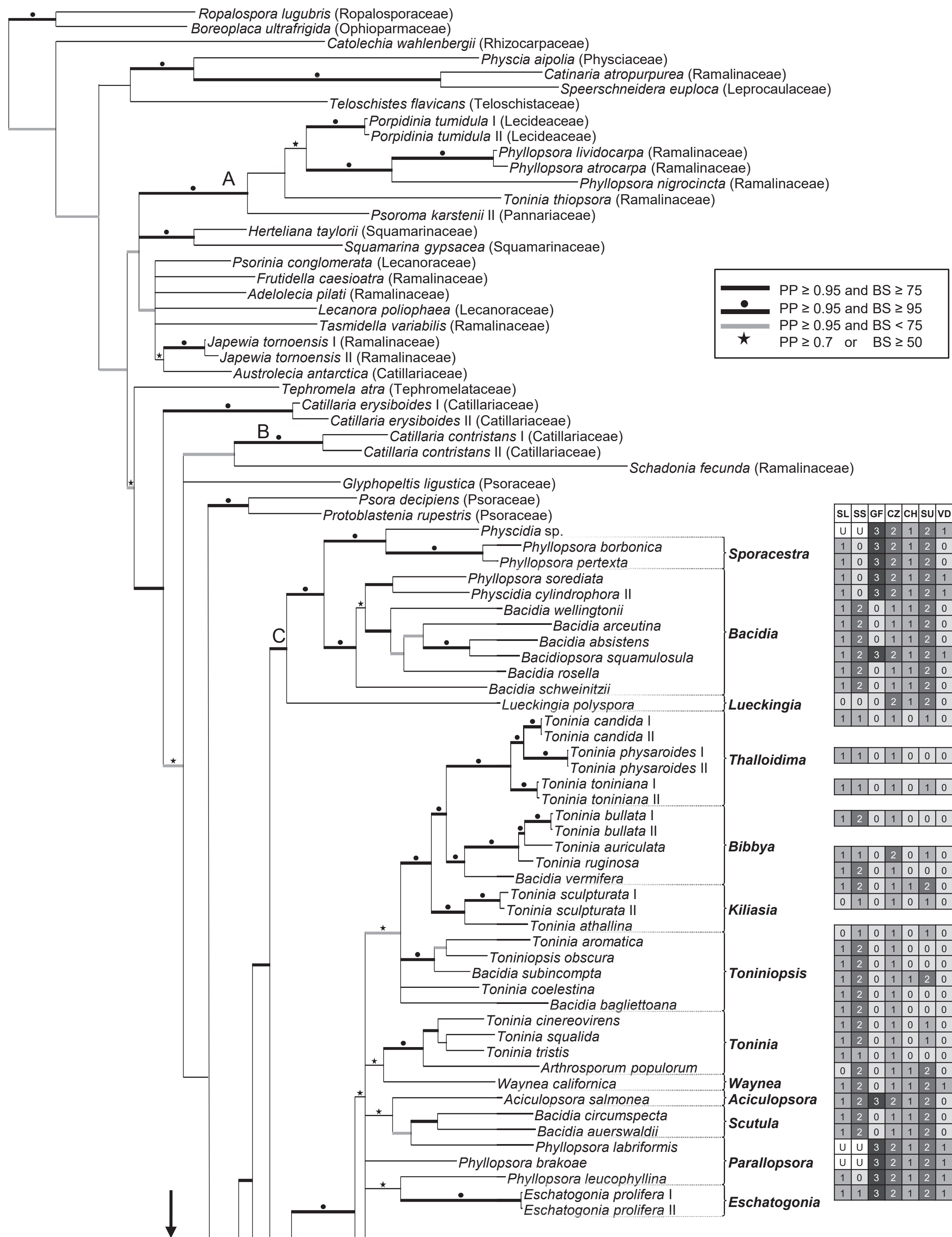
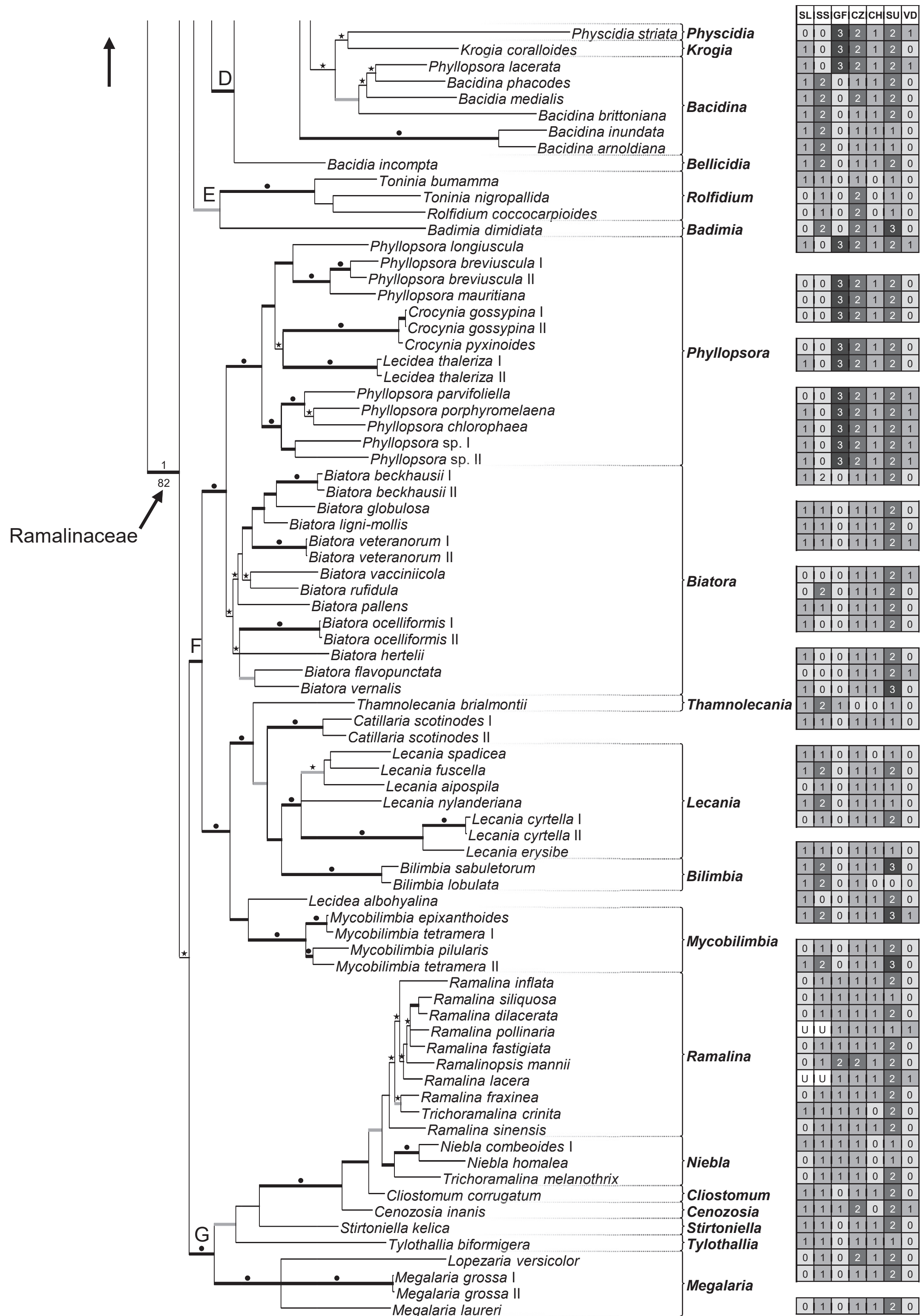



**Fig. 2.** Extended majority-rule consensus tree resulting from the Bayesian MCMC analysis of the 5-locus alignment with Bayesian PP ≥ 0.7 and/or Garli maximum likelihood BS ≥ 50 and branch lengths. Strongly supported branches (PP ≥ 0.95 and BS ≥ 75) are marked in bold; strongly supported branches with BS ≥ 95 are also marked with a dot above the branch; branches with PP ≥ 0.95 and BS < 75 are marked in bold grey; branches only supported with PP ≥ 0.7 or BS ≥ 50 are marked with an asterisk above the branch. The starting node of the Ramalinaceae is indicated with an arrow, PP (above branch) and BS (below branch). Family affiliations according to Lücking & al. (2017a, b). *Boreoplaca* ▶




▶ *ultrafrigida* and *Ropalospora lugubris* were used for rooting. Seven major clades are distinguished (A–G). Terminals are named according to the taxonomy prior to this study. Our revised genus affiliation is indicated to the right. The character state matrix for the coded taxa is provided to the far right. Abbreviations used: SL = spore length, SS = spore septation, GF = growth form, CZ = climate zone, CH = climate humidity, SU = substrate, VD = vegetative dispersal; U = unknown. See Materials and Methods for coding specifics.

specific habitat, for example to tropical rainforests, and do not necessarily represent a diagnostic character. In *Phyllopsora*, the ascospore type has proven to be a more reliable taxonomic character at the genus level than the presence and extent of prothallus. In *Toninia*, on the other hand, the pigmentation of the epithecium is of higher taxonomic value. See the respective clade sections for further discussions of each genus.

**Ancestral state reconstruction. —** In our ASR analyses, we used both phylograms and chronograms for reconstruction. Most empirical DNA sequence datasets violate the strict clock assumption (e.g., Ho, 2014) indicating that genetic change is often not proportional to time, and there is no obvious reason why morphological change would be different. Hence, we think it is more reasonable to assume that morphological change is proportional to genetic change rather than to time. However, we decided to reconstruct ancestral states on both phylograms and chronograms for comparison, both tree types providing similar transformation counts and state probabilities at nodes (Tables 4, 5). We also tested for sensitivity against assumptions about the state distribution at the root node (Electr. Suppl.: Table S1). This test indicates that characters are insensitive to these assumptions, with one distinct exception, viz. vegetative dispersal structures. For that character, assuming an empirical distribution of states at the node ("Yang") provides a relatively certain inference that vegetative dispersal structures were absent at the root, whereas other root node assumptions lead to more uncertain inferences. The effect of the varying assumptions at the root has the largest effect on root node inferences but also has some influence on nodes higher up in the tree. Clearly, inferences about the history of vegetative dispersal structures need to be interpreted with care.

According to our overall results from the ASR analyses, the MRCA of the Ramalinaceae most likely evolved in moist temperate forests and reproduced by forming apothecia with long, multiseptate spores (Table 4). Hence, our first hypothesis that simple, ellipsoid spores are plesiomorphic in the family is not supported by the ASR analysis (probability < 14%; Table 4). Extant Ramalinaceae taxa display a wide variety of ascospore types. The phylogenetic sister-family of the Ramalinaceae, the Psoraceae, is a rather small family, which forms mainly ellipsoid and simple spores. This led us to hypothesize the same character state (i.e., ellipsoid, unseptated spores) for the MRCA of the Ramalinaceae, with the different states in ascospore length and septae in extant taxa having evolved repeatedly. Not only do our ASR analyses provide fair support for the long and multiseptate spores in the MRCA of the Ramalinaceae (Table 4), the transformation counts also clearly show a repeated reduction in spore septa and length (Table 5). Apart from reproducing by ascospores, gains as well as losses of vegetative dispersal have generally been frequent (Table 5).

The phyllopsoroid growth form (Fig. 1) seems to occur exclusively in tropical genera. Both our phylogenetic trees, including mapped character states (Fig. 2) and ASRs (Tables 4, 5), suggest that this growth form developed independently and repeatedly, confirming our second hypothesis. Colonization of the tropical zone from a temperate ancestor as well as from humid ("moist") habitats to dry ones has been more common than the reverse (Table 5). Although the Ramalinaceae ancestor apparently arose in temperate forests, tropical genera occur in all major Ramalinaceae clades (Fig. 2: clades C–G). Some species displaying typical phyllopsoroid growth form, expected to belong in the tropical genus *Phyllopsora*, fell outside the family in the molecular phylogeny (e.g., *P. atrocarpa* or *P. nigrocincta*; Fig. 2: clade A). Moreover, the transformation counts reveal repeated state changes from crustose to phyllopsoroid growth form, rarely the other way around (Table 5). The evolutionarily flexible nature of this character state on the Ramalinaceae molecular phylogeny (Fig. 2) suggests careful use as a morphological taxon delimitation criterion. This growth form rather seems to be advantageous in tropical moist forests. Lakatos &

**Table 3.** Output from the approximately unbiased tests of a series of phylogenetic hypotheses (the best tree included): The –ln likelihood score of the best tree fulfilling the constraint inherent in the null hypothesis (−lnL), the difference between this score and the score of the best (unconstrained) tree (ΔlnL) and the probability of observing the data given a true null hypothesis ( *p*).

| Null hypothesis | −lnL | ΔlnL | *p* |
|---|---|---|---|
| *Bacidina* incl. *Woessia*+*Lichingoldia* is monophyletic | 129862.620 | 23.975 | **0.0861** |
| *Toniniopsis* is monophyletic | 129839.530 | 0.885 | **0.5587** |
| *Toninia*+*Toniniopsis* is monophyletic | 129871.667 | 33.022 | 0.0087 |
| *Toninia*+*Toniniopsis*+*Scutula* is monophyletic | 129913.344 | 74.699 | 0.0013 |
| *Toninia*+*Toniniopsis*+*Kiliasia* is monophyletic | 130045.464 | 206.819 | 0.0002 |
| *Toninia*+*Toniniopsis*+*Kiliasia*+*Scutula* is monophyletic | 129997.172 | 158.527 | 0.0000 |
| *Toninia*+*Toniniopsis*+*Kiliasia*+*Bibbya*+*Thalloidima* is monophyletic | 129844.584 | 5.939 | **0.4172** |
| *Toninia*+*Toniniopsis*+*Kiliasia*+*Bibbya*+*Thalloidima*+*Scutula* is monophyletic | 130045.463 | 206.818 | 0.0004 |
| *Scutula*+*Bellicidia* is monophyletic | 130020.183 | 181.538 | 0.0078 |
| *Parallopsora* gen. nov. is monophyeltic | 129859.041 | 20.396 | **0.1965** |

Hypotheses were rejected when $p < 0.05$. Values for $p > 0.05$ marked in bold.

al. (2006) investigated the growth form of typical corticolous, lowland rainforest lichens and found that the prothallus, which is characteristic for the phyllopsoroid growth form, serves to diminish the danger of suprasaturation by rain running down tree stems. A dense prothallus takes up water like a sponge and keeps the lichen's surface dry enough to ensure an active photosynthesis. Lakatos & al. (2006) claim that the squamulose growth form results in a larger relative surface area and that the lichen is hence able to capture more light. Lichens with a phyllopsoroid growth form can also grow more easily on irregular surfaces and are more competitive than crustose lichens with the same biomass (Lakatos & al., 2006). Consequently, these species may grow more easily in the understory of tropical rainforests. These ecophysiological advantages may explain the high degree of convergent evolution in the phyllopsoroid growth form of tropical genera.

**Ramalinaceae family circumscription. —** Based on our molecular phylogenetic hypotheses (Fig. 2; Electr. Suppl.: Fig. S1), the Ramalinaceae is not monophyletic as currently circumscribed (sensu Lücking & al., 2017a, b). However, most genera and species assigned to the family form a highly supported (PP = 1, BS = 82), monophyletic clade. We propose this clade

**Table 4.** Median character state probabilities from ancestral state reconstruction of seven characters on both phylograms (P) and chronograms (C) of the 5-locus Ramalinaceae phylogeny.

| | | Spores | | | | | | Climate | | | | | | Vegetative dispersal | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Length : Width | | Septation | | Growth form | | Zone | | Humidity | | Substrate | | Special structures | |
| | | 0: ≤3<br>1: >3 | | 0: none<br>1: single septate<br>2: multiseptate | | 0: crustose<br>1: fruticose<br>2: foliose<br>3: phyllopsoroid | | 0: arctic/antarctic<br>1: temperate<br>2: tropical | | 0: dry<br>1: moist | | 0: soil<br>1: rock<br>2: bark/wood<br>3: living organisms | | 0: absent<br>1: present | |
| Root node | State | P | C | P | C | P | C | P | C | P | C | P | C | P | C |
| Ramalinaceae | 0 | 1.7 | 11.9 | 2.3 | 0.5 | **100** | **100** | 0 | 0 | 0.2 | 0.1 | 0 | 0 | **76.2** | **96** |
| | 1 | **98.3** | **88.9** | 13.4 | 8.6 | 0 | 0 | **96.4** | **96.8** | **99.8** | **99.9** | 0.7 | 0.2 | 23.9 | 4.1 |
| | 2 | – | – | **84.4** | **90.9** | 0 | 0 | 3.7 | 3.3 | – | – | **99.3** | **99.8** | – | – |
| | 3 | – | – | – | – | 0 | 0 | – | – | – | – | 0 | 0 | – | – |
| *Bacidia*-group | 0 | 4 | 11.1 | 14.5 | 10.6 | **98** | **99.4** | 0 | 0 | 0.2 | 0.1 | 0 | 0 | **73** | **91.6** |
| | 1 | **96** | **88.9** | 1.9 | 0.9 | 0 | 0 | **77** | **99.7** | **99.8** | **99.9** | 0.1 | 0.1 | 27 | 8.4 |
| | 2 | – | – | **83.6** | **88.5** | 0 | 0 | 23 | 0.3 | – | – | **99.9** | **99.9** | – | – |
| | 3 | – | – | – | – | 2 | 0.6 | – | – | – | – | 0.1 | 0 | – | – |
| *Toninia*-group | 0 | 1.6 | 8.9 | 1 | 0.6 | **99.9** | **99.6** | 0 | 0 | 0.1 | 0.2 | 0 | 0 | **77.6** | **92.9** |
| | 1 | **98.4** | **91.2** | 1.9 | 1.6 | 0 | 0 | **91.3** | **94** | **99.9** | **99.8** | 0.1 | 0.1 | 22.4 | 7.2 |
| | 2 | – | – | **97.1** | **97.8** | 0 | 0 | 8.8 | 6 | – | – | **100** | **99.9** | – | – |
| | 3 | – | – | – | – | 0.1 | 0.5 | – | – | – | – | 0 | 0 | – | – |
| *Rolfidium*-group | 0 | 17.8 | 25.5 | 1.1 | 0.3 | **99.7** | **100** | 0 | 0 | 10.9 | 8.9 | 2 | 2.3 | **78.9** | **93.9** |
| | 1 | **82.2** | **74.5** | 26 | 19.6 | 0 | 0 | **76.2** | **72.2** | **89.1** | **91.1** | 23 | 23.4 | 21.1 | 6.1 |
| | 2 | – | – | **72.9** | **80.1** | 0 | 0 | 23.8 | 27.8 | – | – | **71.9** | **70.1** | – | – |
| | 3 | – | – | – | – | 0.3 | 0 | – | – | – | – | 3.1 | 4.2 | – | – |
| *Biatora*-group | 0 | 0.4 | 14.4 | 5.9 | 2.6 | **100** | **100** | 0 | 0 | 0 | 0.1 | 0 | 0 | **76.2** | **93.7** |
| | 1 | **99.7** | **85.6** | 14.4 | 5.6 | 0 | 0 | **99.6** | **99.2** | **100** | **100** | 0.4 | 0.2 | 23.9 | 6.3 |
| | 2 | – | – | **84.4** | **91.8** | 0 | 0 | 0.5 | 0.8 | – | – | **99.6** | **99.8** | – | – |
| | 3 | – | – | – | – | 0 | 0.1 | – | – | – | – | 0 | 0.1 | – | – |
| *Ramalina*-group | 0 | 6.4 | 26.1 | 0.1 | 0.1 | **100** | **100** | 0 | 0 | 0.3 | 0.3 | 0 | 0 | **79.2** | **94.3** |
| | 1 | **93.6** | **73.9** | **78.5** | **73** | 0 | 0 | **99.2** | **98.8** | **99.7** | **99.8** | 0.2 | 0.1 | 20.9 | 5.7 |
| | 2 | – | – | 21.5 | 27 | 0 | 0 | 0.9 | 1.3 | – | – | **99.9** | **99.9** | – | – |
| | 3 | – | – | – | – | 0 | 0 | – | – | – | – | 0 | 0 | – | – |

Character state probabilities are given for the MRCA of Ramalinaceae clade as well as for five selected subclades (Fig. 2 clades C–G) that we have named the *Bacidia*-, *Toninia*-, *Rolfidium*-, *Biatora*- and *Ramalina*-group, respectively. Highest probabilities are marked in bold. See Table S1 (Electr. Suppl.) for character state probabilities under three different priors.

as the revised family circumscription for the Ramalinaceae (Fig. 2: Ramalinaceae). This circumscription is congruent with molecular phylogenetic results of the Lecanoromycetes produced by Miadlikowska & al. (2014). Their extensive phylogeny corroborates previous findings by Andersen & Ekman (2005) and Arup & al. (2007) that the genera *Frutidella* and *Japewia* do not belong to the Ramalinaceae, as well as Ekman & al.'s (2008) results showing that the three genera *Adelolecia, Catinaria* and *Schadonia* also fall outside of the family. Our phylogenetic results (Fig. 2) support the exclusion of these five genera from the family and additionally provide molecular evidence for the exclusion of the genera *Compsocladium*, *Crustospathula* and *Tasmidella*. Even though we removed *Compsocladium* from the 2-locus dataset because of the outcome of the rogue taxa analysis, performing BLAST searches of its mtSSU sequence shows high similarity to taxa within the Lecanorales, mostly from the Pilocarpaceae, but anyway outside of the Ramalinaceae. Additional DNA sequencing data is necessary to phylogenetically identify its correct taxonomic placement. *Tasmidella* was, based on morphological investigations, placed in the Megalariaceae by Kantvilas & al. (1999). The Megalariaceae has since been included in the Ramalinaceae (Ekman, 2001). However, the genus *Tasmidella* clearly does not belong in the Ramalinaceae (Fig. 2). Results from BLAST searches indicate that it associates with the Lecanoraceae. Detailed phylogenetic studies of possible close relatives are necessary to determine its correct taxonomic position. The genus *Crustospathula* was recently placed in the family Malmideaceae based on morphological evidence only (Cáceres & al., 2017). For a more detailed discussion of *Crustospathula*, see section about clade A. It is

**Table 5.** Median transformation counts of state changes from the ancestral state reconstruction analyses for phylograms (P) and chronograms (C) of the 5-locus Ramalinaceae phylogeny.

| | Spores | | | | | | Climate | | | | | | Vegetative dispersal | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Length : Width | | Septation | | Growth form | | Zone | | Humidity | | Substrate | | Special structures | |
| | 0: ≤3<br>1: >3 | | 0: none<br>1: single septate<br>2: multiseptate | | 0: crustose<br>1: fruticose<br>2: foliose<br>3: phyllopsoroid | | 0: arctic/antarctic<br>1: temperate<br>2: tropical | | 0: dry<br>1: moist | | 0: soil<br>1: rock<br>2: bark/wood<br>3: living organisms | | 0: absent<br>1: present | |
| Changes | P | C | P | C | P | C | P | C | P | C | P | C | P | C |
| Total | 31 (22–41) | 49 (34–64) | 37 (28–45) | 38 (29–46) | 17 (14–22) | 17 (13–21) | 27 (20–34) | 27 (21–34) | 16 (11–22) | 15 (11–21) | 35 (28–43) | 36 (29–45) | 59 (40–78) | 41 (17–58) |
| 0→1 | 8 (3–15) | 21 (10–36) | 0 (0–2) | 0 (0–0) | 2 (2–3) | 2 (2–3) | 0 (0–0) | 0 (0–0) | 5 (2–9) | 5 (2–9) | 3 (0–6) | 3 (0–7) | 30 (18–38) | 25 (17–33) |
| 0→2 | – | – | 2 (0–6) | 2 (0–6) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | – | – | 0 (0–0) | 0 (0–0) | – | – |
| 0→3 | – | – | – | – | 12 (9–15) | 12 (8–14) | – | – | – | – | 2 (1–3) | 2 (1–4) | – | – |
| 1→0 | 23 (18–28) | 28 (18–35) | 0 (0–2) | 0 (0–2) | 1 (0–1) | 1 (0–2) | 0 (0–0) | 1 (1–1) | 11 (9–14) | 10 (8–13) | 9 (5–12) | 10 (6–13) | 29 (12–48) | 14 (0–31) |
| 1→2 | – | – | 6 (2–12) | 6 (1–12) | 1 (1–1) | 1 (1–1) | 20 (11–26) | 21 (13–26) | – | – | 6 (3–8) | 5 (3–8) | – | – |
| 1→3 | – | – | – | – | 0 (0–0) | 0 (0–0) | – | – | – | – | 0 (0–0) | 0 (0–0) | – | – |
| 2→0 | – | – | 11 (7–15) | 12 (8–15) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | – | – | 0 (0–0) | 0 (0–0) | – | – |
| 2→1 | – | – | 16 (9–21) | 17 (11–22) | 0 (0–0) | 0 (0–0) | 6 (1–15) | 5 (0–12) | – | – | 11 (8–14) | 11 (8–14) | – | – |
| 2→3 | – | – | – | – | 0 (0–0) | 0 (0–0) | – | – | – | – | 3 (1–4) | 3 (1–4) | – | – |
| 3→0 | – | – | – | – | 1 (0–5) | 1 (0–4) | – | – | – | – | 0 (0–2) | 0 (0–2) | – | – |
| 3→1 | – | – | – | – | 0 (0–0) | 0 (0–0) | – | – | – | – | 0 (0–0) | 0 (0–0) | – | – |
| 3→2 | – | – | – | – | 0 (0–0) | 0 (0–0) | – | – | – | – | 1 (0–2) | 1 (0–2) | – | – |

The HPD interval is indicated in parentheses.

beyond the scope of this study to determine the final taxonomic placement of the excluded genera.

On the other hand, we suggest including the genera *Scutula* and *Tylothallia* in the Ramalinaceae (Fig. 2; Electr. Suppl.: Fig. S1). *Scutula* was placed in the Ramalinaceae also by Jaklitsch & al. (2016: 127). The affinity of *Tylothallia* with the Ramalinaceae was revealed already by Ekman (2001) and the molecular phylogeny by Andersen & Ekman (2005) placed *Scutula* in this family.

Ekman (2001) first mentioned the overall anatomical similarities between Ramalinaceae and Bacidiaceae and suggested synonymization after studying members of both families also in a molecular context. The previous delimitation of the two families was based on growth form: fruticose and foliose in Ramalinaceae and crustose lichens in Bacidiaceae. As the two families share anatomical features such as ascus and ascospore morphology, as well as chemistry, the suggested synonymy was generally quickly accepted. Lücking & al. (2017a, b) did not mention the Bacidiaceae in their recent classification. Still, the name Bacidiaceae repeatedly appears in the literature. While Miadlikowska & al. (2014) accept a large Ramalinaceae, they still indicate the Bacidiaceae (based on the location of the type species) in their molecular phylogeny, which seemed to receive strong support. Also Sérusiaux & al. (2012), Lendemer & al. (2016) and McMullin & Lendemer (2016) still accept the Bacidiaceae as a separate family. However, the "new" understanding of the Bacidiaceae (sensu Miadlikowska & al., 2014) is not congruent with the "former" Bacidiaceae (sensu Zahlbruckner, 1921–1940), which originally included all crustose taxa, for example also *Biatora*. Five major clades receive strong and medium support in our molecular phylogeny (Fig. 2: clades C–G), one of which corresponds to the Bacidiaceae (Fig. 2: clade C) sensu Miadlikowska & al. (2014). We therefore continue to synonymize the two families due to their common ascus morphology and the lack of any consistent diagnostic features that would separate the clades at family level. Both families are strongly supported as a monophyletic group (Fig. 2) while underlying branches lack consistent morphological differences to support splitting them up in smaller families. In the following, we discuss in detail the seven major clades (Fig. 2: clades A–G) recovered in our analysis, including the two clades (A, B) falling outside the Ramalinaceae. For details of taxonomic changes, see the Taxonomy chapter.

**Clades A and B: excluded species. —** A group of species previously included in the Ramalinaceae form a strongly supported clade that falls outside of our currently suggested family delimitation (Fig. 2: clade A, PP = 1, BS = 100). Because taxon sampling is scarce outside the Ramalinaceae, it remains uncertain whether or not all of the species found in clade A are indeed closely related.

A standard BLAST search of our DNA sequence data for the Australian species *Psoroma karstenii* indicates that this species does not belong in the genus *Psoroma*. Elix (1992) excluded the species together with *P. caesium* from the genus *Psoroma* and suggested transferring them to *Phyllopsora* due to overall morphological similarities. Our initial morphological investigations suggested placing the two *P. karstenii* specimens close to *Physcidia* due to the overall larger thallus size than found in *Phyllopsora*. However, our molecular phylogeny clearly indicates that neither *Phyllopsora* nor *Physcidia* is the correct genus for this species (Fig. 2; Electr. Suppl.: Fig. S1).

The three *Phyllopsora* species, *P. atrocarpa*, *P. lividocarpa* and *P. nigrocincta*, form a strongly supported group with long branches, indicating that they may be distinct species. Timdal (2008) mentioned the morphological similarity of *P. atrocarpa* and *P. lividocarpa* as well as the disparity of *P. nigrocincta* from other *Phyllopsora* species. Both *P. atrocarpa* and *P. lividocarpa* produce long, acicular spores whereas *P. nigrocincta* has ellipsoid to fusiform spores (Timdal, 2008). However, all three species have an overlapping chemistry (Timdal, 2008) indicating that they might be closely related. The mtSSU sequence of the genus *Crustospathula* (Malmideaceae) shows it to form a strongly supported group with the three *Phyllopsora* species mentioned above (Electr. Suppl.: Fig. S1). *Crustospathula* forms stalked soredia (Aptroot, 1998), which distinguishes it from the three *Phyllopsora* species. We propose excluding these three *Phyllopsora* species from the family Ramalinaceae, but more in-depth studies are needed to reveal their correct taxonomic affiliation and degree of relatedness to each other.

*Toninia thiopsora* was excluded from *Toninia* by Timdal (1992) due to deviating paraphyses and ascus morphology. However, the species was never assigned to a new genus. Our results demonstrate that *T. thiopsora* does not belong in *Toninia* (Fig. 2: clade A), but further studies are necessary to identify its closest relatives.

It is out of the scope of this study to find the closest relatives to all species in clade A (Fig. 2). Both the close affinity to *Crustospathula* and GenBank BLAST searches revealed that the three *Phyllopsora* species, *Psoroma karstenii* and *T. thiopsora* may be members of the Malmideaceae. Our morphological investigations of the different ascus-structures of the *Phyllopsora*, *Psoroma* and *Toninia* species, show that they differ strongly from those reported to occur in *Malmidea* by Kalb & al. (2011). However, recent studies on the Malmideaceae have indicated that the ascus and ascospore structures vary between the different genera (Cáceres & al., 2017). The family Malmideaceae has lately been investigated in more detail and several new genera have been described (Cáceres & al., 2017; Muggia & al., 2017; Sodamuk & al., 2017). Further molecular studies with a dataset including sequences of all known Malmideaceae genera are necessary to find out whether all species from clade A belong into this family and whether they form distinct genera.

Another *Toninia* species falling out of the Ramalinaceae in our 2-locus phylogenetic hypothesis is *Toninia squalescens* (Electr. Suppl.: Fig. S1: clade B). Timdal (1992) excluded *T. squalescens* from *Toninia* based on the different paraphyses and asci. In our molecular phylogeny, *T. squalescens* groups together with two accessions of *Catillaria contristans* in a strongly supported clade (Electr. Suppl.: Fig. S1: clade B). BLAST searches of our mtSSU and ITS sequences of *C. contristans* and *T. squalescens* indicated a high degree of similarity with species of *Brianaria*, *Micarea*, *Psora* and *Sphaerophorus*. Despite grouping together, *C. contristans* and *T. squalescens* are morphologically distinct from each other and are not necessarily

closely related. *Toninia squalescens* clearly does not belong in the genus *Toninia* or any of its segregates, but more detailed analyses are necessary to find its taxonomic affinity. Finally, *C. contristans* is morphologically very different (e.g., in ascus structure) from members of *Catillaria* s.str. and clearly does not belong in that genus.

**Clade C: *Bacidia*-group. —** The *Bacidia*-group (Fig. 2: clade C) comprises *Bacidia* s.str. in the sense of Ekman (2001) and includes the type *Bacidia rosella*. According to our ASR analyses, the ancestor of this group had character states that were identical to the overall Ramalinaceae MRCA (Table 4). In addition to *Bacidia*, this clade also comprises the genera *Bacidiopsora* and *Lueckingia* as well as three species previously referred to *Phyllopsora* and two species previously referred to *Physcidia*.

Our phylogeny shows that *Phyllopsora* is highly polyphyletic and can be found in four different clades (Fig. 2: clades A, C, D, F). The synonymy of *Phyllopsora pertexta* with *Sporacestra prasina* and *P. borbonica* was indicated by Ekman (1996a) and Timdal (2011). As these species clearly do not belong in *Phyllopsora* s.str. (Fig. 2: clade F, *Biatora*-group), we resurrect the genus *Sporacestra* for *Phyllopsora pertexta* and related species. The *Physcidia* sp. accession is strongly supported as phylogenetic sister to the *Sporacestra* clade in our phylogeny. The thallus of the *Physcidia* sp. specimen is large and almost foliose. However, our current molecular phylogeny shows that growth form is not necessarily a good diagnostic character (Fig. 2). Unfortunately, the *Physcidia* sp. specimen is sterile and consequently provides no information about apothecium characters, which may be of higher diagnostic value. We anticipate that this specimen eventually will be placed in a new genus due to its much larger thallus size and lack of the pronounced prothallus, which contrasts to the minutely areolate *Sporacestra* with a well-developed prothallus. Additional apotheciate collections of this species should be studied prior to further evaluating its generic affiliation.

The type of the genus *Bacidiopsora*, *B. squamulosula*, is nested within *Bacidia* s.str. and should be transferred to *Bacidia*. The close relationship is supported also by the ascospore anatomy, both having thick-walled, multiseptate ascospores. Already Brako (1991) pointed out that *Bacidiopsora* was not clearly distinct from *Bacidia*. *Phyllopsora sorediata* was originally described as *Triclinum sorediatum* by Aptroot & al. (2007), but according to an ongoing study of *Phyllopsora* s.str. by Kistenich & al. (in prep.), *P. sorediata* does not belong in the genus *Triclinum*. The species contains acicular ascospores like *Bacidia*, but these are indistinctly (1–)3-septate unlike most members of *Bacidia*. Given its robust phylogenetic placement together with *Bacidia* (Fig. 2), we regard *P. sorediata* as better accommodated in *Bacidia* than in *Phyllopsora* or *Triclinum*, but refrain from formally transferring it to *Bacidia* pending additional studies at the species level. *Physcidia cylindrophora* is another species found in the strongly supported *Bacidia*-group (Fig. 2: clade C). This species forms typical lobate thalli of up to 13 cm in diameter (Kalb & Elix, 1995), and does not resemble a *Bacidia* on first sight. However, like *Bacidia* it has long, multiseptate ascospores and one of the two chemical strains contains the homosekikaic acid complex (Kalb & Elix, 1995) also found in species referred to *Bacidiopsora* as well as in *Bacidia absistens*. While *Bacidia* species typically form a crustose thallus, *Bacidiopsora* species form squamules, and *Physcidia cylindrophora* is an almost foliose species. However, the ASR analyses indicate that growth form is flexible and subject to frequent change in an evolutionary perspective (Table 5). Growth form has been shown to be distributed non-monophyletically across lichen genera and families (e.g., Arup & al., 2013; Lendemer & Hodkinson, 2013). Hence, we accept an extended *Bacidia* s.str. that includes *Bacidiopsora* and *Physcidia cylindrophora*.

The monospecific genus *Lueckingia* is for the first time shown here to belong in the Ramalinaceae by molecular data. It is the phylogenetic sister to the remaining *Bacidia*-group members and the only genus with polysporous asci in clade C (Fig. 2). Aptroot & al. (2006) proposed a possible close relationship with the genus *Physcidia*. However, the type of *Physcidia* belongs in the *Toninia*-group (Fig. 2: clade D).

**Clade D: *Toninia*-group. —** The *Toninia*-group is a well-supported clade (Fig. 2: clade D, PP = 1, BS = 87). However, resolution and branch support inside this clade is poor in many instances. Our ASR analyses of the *Toninia*-group node reveal the ancestor to have character states similar to the Ramalinaceae ancestor (Table 4). The first shift from corticolous to saxicolous life forms seems to have taken place during the diversification of *Toninia* s.l. (Tables 4, 5). The *Toninia*-group comprises the genera *Aciculopsora*, *Arthrosporum*, *Bacidia*, *Bacidina*, *Eschatogonia*, *Krogia*, *Phyllopsora*, *Physcidia*, *Scutula* (Electr. Suppl.: Fig. S1), *Toninia*, *Toniniopsis* and *Waynea*.

The tropical genera *Eschatogonia* and *Krogia* are both morphologically readily distinguishable from other genera. *Eschatagonia* is the only genus in the Ramalinaceae that forms a characteristic unicellular cortex, while *Krogia* is the only genus forming asci with a nearly non-amyloid tholus and filiform, curved ascospores that are spirally arranged in the ascus. Our representation of taxa in these two genera is scarce and it remains to be seen whether they form monophyletic groups.

The tropical genus *Physcidia* consists of eight described and one undescribed species, four of which have been studied here (Appendix 1). Two species, *P. cylindrophora* and *Physcidia* sp., are phylogenetically placed in the *Bacidia*-group (Fig. 2: clade C), the other two species, *P. wrightii* and *P. striata*, appear in the *Toninia*-group (Electr. Suppl.: Fig. S1: clade D). The type, *P. wrightii*, does not cluster together with *P. striata* (Electr. Suppl.: Fig. S1: clade D). Yet, the poor resolution in Fig. S1 (Electr. Suppl.) did not unequivocally exclude the possibility of *P. wrightii* and *P. striata* forming a monophyletic group. *Physcidia striata* differs from *P. wrightii* by having biatorine apothecia and ellipsoid ascospores (Aptroot & Cáceres, 2014). This might indicate that *P. striata* should not remain in the genus *Physcidia*. Due to the low branch support and limited taxon sampling of the genus *Physcidia*, we refrain from making taxonomic changes for *Physcidia*. Increased taxon sampling and more molecular data are needed to address monophyly of *Physcidia*, after the exclusion of the two species in the *Bacidia*-group (Fig. 2: clade C).

Three of the four *Phyllopsora* species occurring in the *Toninia*-group (Fig. 2: clade D), *P. brakoae*, *P. labriformis* and *P. leucophyllina*, are phylogenetically unresolved. Their rather diverse thallus morphology and secondary chemistry do not provide clues to their relationships. The AU test (Table 3), however, does not reject monophyly of these three species ($p = 0.2$). Hence, we propose the most parsimonious solution of describing the single new genus *Parallopsora* gen.nov. to accommodate *P. brakoeae*, *P. labriformis* and *P. leucophyllina* (Fig. 3B). A fourth *Phyllopsora* species, *P. lacerata*, falls out in a supported subclade together with *Bacidina* species (Fig. 2), and is therefore transferred to *Bacidina* (see discussion below). All four *Phyllopsora* species in the *Toninia*-group (Fig. 2: clade D) differ morphologically from the typical *Phyllopsora* s.str. in the *Biatora*-group (Fig. 2: clade F) by having longer ascospores and/or forming soredia (cfr. Timdal, 2008).

We included 13 of the 55 currently accepted species of *Toninia* in our multilocus phylogeny (Fig. 2; Electr. Suppl.: Fig. S1) and an additional 16 species in an auxiliary ITS phylogeny (Electr. Suppl.: Fig. S2). These include the types of the formerly synonymized genera *Bibbya*, *Kiliasia*, *Thalloidima,* and *Toniniopsis*, as well as *Toninia* s.str. (Figs. 2; Electr. Suppl.: Fig. S2), which conform, at least partly, with the informal infrageneric arrangement of *Toninia* proposed by Timdal (1992). Supported subclades of the *Toninia*-group in our multilocus molecular phylogeny (Fig. 2: clade D) largely correspond to the same groupings. Moreover, the monophyly of none of these genera is contradicted in the purely ITS tree (Electr. Suppl.: Fig. S2), which has increased taxon sampling. Timdal (1992) carried out a worldwide taxonomic revision of *Toninia* and excluded numerous taxa that were morphologically similar but distantly related (mostly outside our current understanding of the Ramalinaceae). Left in *Toninia* was a core of 48 relatively closely related species, all terricolous or saxicolous and with weakly conglutinated paraphyses, and many with the squamulose habit growth form used by Zahlbruckner (1921–1940) to delimit the genus. Our phylogeny (Figs. 2; Electr. Suppl.: Fig. S1) and that of Ekman (2001) indicate that numerous species previously treated in *Bacidia*, with a dominance of corticolous and non-squamulose ones, are more closely related to *Toninia* sensu Timdal (1992) than to *Bacidia* s.str. The AU tests indicate that splitting *Toninia* is acceptable and so would accepting one large *Toninia* (as long as *Scutula* is not included; Table 3). However, accepting a single *Toninia* s.l. would make it very species-rich and morphologically extremely heterogeneous. Therefore, we prefer to divide the old *Toninia*, including numerous species currently treated in *Bacidia*, into five genera, all of which are already described. In the Taxonomy chapter, an additional 15 species are transferred to the five *Toninia* segrate genera based on overall morphological and anatomical similarity with one or more of the 29 *Toninia* species in Fig. S2 (Electr. Suppl.). Altogether 11 species are left in *Toninia* s.l., however, pending further study.

The type of *Toninia*, *T. cinereovirens*, groups together with *T. squalida*, *T. tristis* and *Arthrosporum populorum* in a strongly supported clade. This core group of *Toninia* s.str. ("group 2" sensu Timdal, 1992) contains the same kind of green apothecial pigment ("Bacidiagrün", = Bagliettoana-green, in the terminology of Meyer & Printzen, 2000) that can be found in *Arthrosporum* and *Toniniopsis* (Timdal, 1992). Despite having polysporous asci and a corticolous growth form, *Arthrosporum* closely resembles *Toninia* in other morphological characteristics (Timdal, 1992). We therefore propose to include *Arthrosporum* in the genus *Toninia*. For the remainder of the investigated *Toninia* species, we suggest resurrecting the genera *Bibbya*, *Kiliasia*, and *Thalloidima*. The former genus *Bibbya* is resurrected for *T. bullata*, *T. auriculata*, *T. ruginosa*, as well as for *Bacidia vermifera*, which together form a strongly supported clade (Fig. 2; Electr. Suppl.: Fig. S2). These species are characterized by having a reddish brown pigment (Ruginosa-brown, Meyer & Printzen, 2000) and usually long, 3–7-septate ascospores (Timdal, 1992; Ekman, 1996a). The similarity in pigment composition and ascospore/thallus morphology between *T. bullata*, *T. auriculata*, and *T. ruginosa* was noted by Timdal (1992), while Ekman (1996a) described similar characters for *B. vermifera*. *Toninia sculpturata*, *T. philippea*, and *T. athallina* form a supported clade in our phylogeny that represents the genus *Kiliasia* (Fig. 2; Electr. Suppl.: Fig. S2). All three species were formerly placed in the crustose genus *Catillaria* (e.g., by Kilias, 1981, 1984) before Hafellner (1984) described *Kiliasia* and Timdal (1992) later moved them to *Toninia*. Timdal (1992) elaborated on the similarity of the crustose species *T. philippea* and the cryptothalline species *T. athallina* with "apparently no diagnostic anatomical differences between these species" (Timdal, 1992: 43). The clade consisting of the three species *T. candida*, *T. physaroides*, and *T. toniniana* (Fig. 2) corresponds partly to *Thalloidima* (sensu A.Massal.; i.e., species having 1-septate ascospores) and to *Toninia* "group 1" and "group 10" sensu Timdal (1992; i.e., species having mainly the pigment "Thalloidimagrün" in the epithecium). In the new circumscription proposed here, *Thalloidima* contains species that (with few exceptions) have the characteristic greyish pigment "Thalloidimagrün" (= Sedifolia-grey, Meyer & Printzen, 2000), a thallus partly or entirely covered by white pruina formed by calcium oxalate, and fusiform, 1-septate ascospores. The correspondence is not absolute, however, 1-septate ascospores (mainly ellipsoid) also occuring in *Kiliasia*, *Bibbya*, and *Toninia*, and "Thalloidimagrün" in *Kiliasia*. The species *T. candida*, *T. physaroides* and *T. toniniana* are transferred here to *Thalloidima*.

The genus *Toniniopsis* was originally described for the single species *T. obscura*, a synonym of *Bacidia illudens*, and was reduced to synonymy with *Bacidia* by Ekman (1996a). Lücking & al. (2017a, b), however, list it as a separate genus in the Ramalinaceae with *T. obscura* as the type, and Timdal (1992) temporarily accepted it as a separate genus from *Toninia* and *Bacidia*. In our 5-locus phylogeny (Fig. 2), *Toniniopsis obscura* forms a well-supported clade with *Toninia aromatica* and *Bacidia subincompta*, which appears in the same polytomy as *T. coelestina* and *B. bagliettoana*. An AU test is unable to reject the possibility that *Toniniopisis*, including all these species, is monophyletic (Table 3). In addition, *T. verrucarioides* belongs in this genus, as shown by our ITS phylogeny (Electr. Suppl.: Fig. S2), corroborating Ekman (2001). The members of

*Toniniopsis*, as understood here, share the presence of a blue-green pigment in the hymenium and sometimes proper exciple ("Bacidiagrün"; = Bagliettoana-green, Meyer & Printzen, 2000), a red-brown pigment throughout the proper exciple, as well as general apothecium anatomy. Close relationships between, for example, *T. aromatica*, *T. verrucarioides* and *B. bagliettoana* have previously been pointed out on morphological grounds (Timdal, 1992; Ekman, 1996a).

We included two species of the genus *Scutula*, *S. miliaris*, the type, and *S. tuberculosa* (Appendix 1; Electr. Suppl.: Fig. S1). We investigated the apothecial structure and ascus morphology of *S. miliaris* and found the ascus to be extremely similar to *Toninia*. However, the conidial trimorphism found in *Scutula*, with the macroconidial stage (Karsteniomyces) having oblong to bacilliform conidia, the mesoconidial stage (Libertiella) bacilliform to falcate conidia, and the microconidial stage bacilliform to filiform conidia (Triebel & al., 1997), seems to be a distinctive character at the genus level. *Scutula tuberculosa* and *S. miliaris* form a well-supported clade with *Bacidia circumspecta* as their supported phylogenetic sister in our 2-locus phylogeny (Electr. Suppl.: Fig. S1) and cluster together with *B. auerswaldii* in a strongly supported clade in the 5-locus phylogeny (Fig. 2). Ekman (1996a) reported three different conidial types for *B. circumspecta* (fusiform to bacilliform, filiform, falcate), whereas *B. auerswaldii* is only known to produce short-bacilliform conidia (Arvidsson & al., 1988). Upon anatomical reexamination of the aforementioned *Scutula* and *Bacidia* species, we found them to resemble each other strikingly. The two *Bacidia* species lack parasitic stages, but a mixture of parasitic (e.g., *Toninia plumbina*) and non-parasitic species is known to occur also in *Toninia*. Due to the strong phylogenetic support (Fig. 2; Electr. Suppl.: Fig. S1) and the morphological similarities, above all in conidial morphology, we transfer *B. auerswaldii* and *B. circumspecta* to the genus *Scutula*.

The *Toninia*-group (Fig. 2: clade D) also contains four *Bacidina* species, including the type *B. phacodes*. However, the four *Bacidina* species do not cluster together. The type, *B. phacodes*, forms a well-supported clade with *Bacidia medialis*, *Bacidina brittoniana* and *Phyllopsora lacerata*. Ekman (2001) suggested moving *Bacidia medialis* into *Bacidina*. *Phyllopsora lacerata* clearly does not belong to *Phyllopsora* s.str., which phylogenetically falls in the *Biatora*-group (Fig. 2: clade F). *Phyllopsora lacerata* instead shares several morphological characters with members of *Bacidina*, for example the lack of prothallus and the bacilliform to acicular ascospores (Ekman, 1996a; Timdal, 2008). Squamulose species are already known from *Bacidina* (e.g., *B. squamellosa*), despite the majority forming crustose thalli (Ekman, 1996a). Hence, we move *Phyllopsora lacerata* to *Bacidina*. The remaining two *Bacidina* species in our tree, *B. inundata* and *B. arnoldiana*, are separated from the core of *Bacidina* s.str. Despite the apparent non-monophyly on our phylogenetic tree, the AU test does not reject the possibility that these two species form a monophyletic group with *Bacidia medialis*, *Bacidina brittoniana* and *Phyllopsora lacerata* ($p = 0.09$; Table 3). This result means that our data cannot separate between the possibilities of a monophyletic and non-monophyletic *Bacidina*. Therefore, we choose the conservative option of retaining *Bacidina* in the wider sense for the time being. Older genus names for the clade encompassing *B. inundata* and *B. arnoldiana* already exist: *Bacidina sulphurella*, a taxonomic synonym of and the older name for the nomenclatural type of *Woessia*, *W. fusarioides*, is a close relative of *B. arnoldiana*; *Bacidina inundata*, on the other hand, is a taxonomic synonym of and older name for the nomenclatural type of the genus, *Lichingoldia*, *L. gyalectiformis* (Ekman, 1996b). However, *Woessia* has nomenclatural priority over *Lichingoldia*.

*Bacidia lutescens* was included in our 2-locus phylogeny (Electr. Suppl.: Fig. S1), where its position remains unresolved in the *Toninia*-group. The species has been connected with the Australian genus *Jarmania* by Palice & al. (2013), mostly because of the presence of lobaric acid in one of the species (Kantvilas, 2008). However, lobaric acid is also present in species of *Biatora* and *Phyllopsora* s.str. (Palice & al., 2013), which are phylogenetically placed in the *Biatora*-group (Fig. 2: clade F). Moreover, the type of *Jarmania*, *J. tristis*, has grayanic acid as its major substance (Kantvilas, 1996). Unfortunately, we were unable to generate sequences from our DNA extracts of *Jarmania* and consequently cannot place the genus in our phylogeny. We therefore refrain from making taxonomic changes, although *B. lutescens* clearly does not belong in the genus *Bacidia*. Future studies that include DNA sequences of *Jarmania* and further species within the "*B. lutescens* group" sensu Ekman (1996a) are necessary to draw taxonomic conclusions.

*Bacidia incompta* is supported as phylogenetic sister to the remainder of the *Toninia*-group (Fig. 2; Electr. Suppl.: Fig. S1: clade D). The difficulty of placing *B. incompta* taxonomically was discussed by Ekman (2001). The species is characterized by a dark red-brown pigment in the apothecia and pycnidia, bacilliform ascospores and ellipsoid conidia, traits that together separate it from other genera in the *Toninia*-group (Fig. 2: clade D). Consequently, we describe the new and monotypic genus *Bellicidia* gen. nov. to accommodate *B. incompta* (Fig. 3A).

**Clade E: *Rolfidium*-group. —** The *Rolfidium*-group forms a small clade comprising, in the taxonomy prior to this work, representatives of the three genera *Badimia*, *Rolfidium* and *Toninia* (Fig. 2: clade E, PP = 1, BS = 62). The inferred most probable character states for the MRCA of this group are identical to those inferred for the MRCA of the Ramalinaceae, i.e., it probably evolved from humid temperate forests dispersing by long, multiseptate spores and had a crustose growth form (Table 4).

The three species *Toninia bumamma*, *T. nigropallida* and *Rolfidium coccocarpioides* form a strongly supported clade with moderate internal support and with *Badimia dimidiata* as its phylogenetic sister (Fig. 2). Timdal (1992) excluded both *T. bumamma* and *T. nigropallida* from *Toninia* due to differences in paraphyses and asci. At the same time, he mentioned the apothecial similarities, which are also found in *Rolfidium,* between these two species. *Rolfidium coccocarpioides* has previously been placed in *Toninia* (Zahlbruckner, 1921–1940), and

Moberg (1986) pointed to the similarities in ascus morphology between *R. coccocarpioides* and both *Lobiona* (synonym of *Bibbya*) and *Kiliasia*. However, our molecular phylogeny shows that *Rolfidium* is not closely related to either *Bibbya* or *Kiliasia* (Fig. 2). We transfer *Toninia bumamma* and *T. nigropallida* to the genus *Rolfidium* because of morphological similarities and phylogenetic support. Both mtSSU and TLC data showed that the specimen of *Heppsora indica* (UPS-L-106423) investigated for this study is misidentified *Rolfidium coccocarpioides*. The two are morphologically highly similar, and Moberg (1986) remarked that the monotypic genus *Heppsora* is probably closely related to *Rolfidium*. Sequencing of type material is necessary to find out if *Heppsora* is synonymous with *Rolfidium*. If so, the name *Heppsora* has nomenclatural priority.

The foliicolous genus *Badimia* has been placed in various families, for example in the Pilocarpaceae and Ectolechiaceae due to the presence of campylidia (Lücking & al., 1994), i.e., erect, helmet-shaped conidiomata. Andersen & Ekman (2005) were the first to investigate the genus with molecular methods. However, the position of the type *B. dimidiata* in their phylogeny was not clear and the species was inferred to belong either in Ramalinaceae or Psoraceae. Our molecular phylogenetic results provide strong support for including *B. dimidiata* in the Ramalinaceae. Its sister-group relationship with *Rolfidium*, however, appears less clear.

**Clade F: *Biatora*-group. —** The *Biatora*-group (Fig. 2: clade F, PP = 1, BS = 94) almost exclusively contains crustose lichens mainly from temperate habitats, with apparently only one transition to the tropics (Fig. 2). This agrees with our ASR results of this clade, which reveal no differences in the most probable character states of the ancestor of this group compared to those of the Ramalinaceae MRCA (Table 4). The clade contains members of the tropical genera *Crocynia* and *Phyllopsora* including the species *Lecidea thaleriza*, the mainly temperate genera *Biatora*, *Bilimbia*, *Mycobilimbia* and *Lecania*, as well as the Antarctic genus *Thamnolecania*. The species *Catillaria scotinodes* and *Lecidea albohyalina* (Fig. 2; Electr. Suppl.: Fig. S1) and the genera *Ivanpisutia*, *Myelorrhiza* and *Myrionora* are also associated with this group (Electr. Suppl.: Fig. S1).

The genus *Biatora* forms a monophyletic group, albeit weakly supported (PP = 0.9, BS < 50), and is phylogenetic sister to the strongly supported *Phyllopsora*-*Crocynia* clade. Our taxon sampling followed the group delimitation by Printzen (2014). *Myrionora* (Lecanoraceae) was included in the Ramalinaceae by Palice & al. (2013). *Ivanpisutia* (Lecanorales, incertae sedis) on the other hand, has never been included in the Ramalinaceae, and both are listed as Lecanorales incertae sedis by Lücking & al. (2017a, b). In our 2-locus phylogeny, both appear in supported clades with species of *Biatora* (Electr. Suppl.: Fig. S1). The morphological similarity of *Ivanpisutia oxneri* with *Biatora pacifica* was pointed out by Printzen & al. (2016). In our phylogeny, *Ivanpisutia* forms a strongly supported clade with *Biatora ocelliformis*. *Myrionora albidula* was originally described as *Biatora albidula* and groups here together with *B. ligni-mollis* (Electr. Suppl.: Fig. S1). Consequently, we synonymize both *Ivanpisutia* and *Myrionora* with *Biatora*.

Several *Phyllopsora* species fall outside the core group of clades A, C and D (Fig. 2). The type of *Phyllopsora*, *P. breviuscula*, and the majority of *Phyllopsora* species appear to belong within the *Biatora*-group (Fig. 2: clade F), forming a strongly supported clade with *Lecidea thaleriza* and the genus *Crocynia*. *Lecidea thaleriza* was included in *Phyllopsora* by Swinscow & Krog (1981) with doubt, but was subsequently excluded by Brako (1991). She concluded that *L. thaleriza* "belongs to an undescribed genus in the Bacidiaceae" (Brako, 1991: 58). *Crocynia* has historically been used for lichens with a cobwebby, non-corticate, felt-like thallus (Plitt, 1923). This type of morphology rapidly led to the assignment of many new species to *Crocynia*: Hue (1924) listed 37 species, while 123 species are listed in GBIF and 169 in MycoBank (both accessed June, 2018). Many of these species have since been shown to belong to other genera and families, for example *Lepraria*. Lücking & al. (2017a, b) list only three species in their overview for *Crocynia*. The thallus morphology used to characterize *Crocynia* is also found in a less extreme form in some *Phyllopsora* species, such as in *P. cuyabensis*. Given the morphological agreement and the nested position of *Crocynia* inside *Phyllopsora* s.str. (Fig. 2: clade F), we synonymize *Crocynia* with *Phyllopsora*. *Triclinum* is another genus, which has been proposed to be a synonym of *Phyllopsora* by Timdal (2008). Kistenich & al. (in prep.) investigated several accessions of *P. cinchonarum*, the type of *Triclinum*, and found the species to cluster together with other *Phyllopsora* species. We therefore support the decision of Timdal (2008) to synonymize *Triclinum* with *Phyllopsora*. As both *Crocynia* (1860), its synonym *Symplocia* (1854), and *Triclinum* (1825) are older than *Phyllopsora* (1894), we will propose the latter genus name for conservation (Kistenich & al., in prep.).

The Australian genus *Myelorrhiza* is not included in the list of genera in Ramalinaceae in Lücking & al. (2017a, b). Our sequence of the type *M. antrea* was excluded from the 2-locus alignment after a rogue taxon analysis. However, BLAST searches of the mtSSU sequence from *M. antrea* indicate a close relationship with *Crocynia*. Verdon & Elix (1986) originally postulated an affinity to *Phyllopsora*, but assigned the genus to Cladoniaceae after additional morphological and anatomical investigations. The other species in the genus, *M. jenjiana*, as well as an unidentified *Myelorrhiza* species are each represented in GenBank by an 18S rDNA sequence generated in a molecular study on the Cladoniaceae by Stenroos & al. (2002). The authors show the species to cluster with *Bacidia* and *Toninia*. However, these were the only two additional species from the Ramalinaceae included in the study. Further molecular data is required to clarify whether or not a synonymization with *Phyllopsora* is necessary.

The well-supported clade comprising, among others, *Bilimbia*, *Lecania*, *Mycobilimbia*, and *Thamnolecania* (Fig. 2; Electr. Suppl.: Fig. S1) is largely concordant with the phylogeny presented by Reese Næsborg & al. (2007) and Sérusiaux & al. (2010). *Bilimbia*, *Lecania*, and *Mycobilimbia* form well-supported genera. Sometimes classified as part of a greater *Lecania*, the fruticose genus *Thamnolecania* was considered problematic by Reese Næsborg & al. (2007) because its

circumscription left some species without genus affiliation in their study. An inclusion of *Thamnolecania* in *Lecania* would require the undesired inclusion of *Bilimbia* and a morphologically heterogeneous assemblage of species in *Lecania*, which is why we choose to recognize *Thamnolecania* as a genus. This heterogeneous assemblage of species is represented by *Catillaria scotinodes* in our phylogeny and further species in the phylogeny of Reese Næsborg & al. (2007), for example, "*Lecania*" *naegelii*, "*Cliostomum*" *tenerum*, "*Lecidea*" *sphaerella* and "*Catillaria*" *croatica*. Coppins & al. (1992) excluded *C. scotinodes* from the Catillariaceae on morphological grounds, noting the similarity with *Toninia*. Reese Næsborg & al. (2007), based on DNA sequence data, confirmed the association with the Ramalinaceae and suggested a close relationship with *Bilimbia*, *Lecania* and *Thamnolecania*. Similarly, *Lecidea albohyalina* awaits proper classification. In the molecular phylogeny of Printzen (2014), *L. albohyalina* is resolved as sister to *Mycobilimbia* and hence included in *Mycobilimbia* s.l. Our phylogeny (Fig. 2) corroborates the close relationship with *Mycobilimbia*. Scarce taxon sampling and the substantial morphological difference between *L. albohyalina* and the otherwise homogeneous *Mycobilimbia* s.str., however, lead us to await further studies before suggesting a formal reclassification.

**Clade G: *Ramalina*-group. —** The *Ramalina*-group contains the typical fruticose and foliose macrolichens and additional crustose species (Fig. 2: clade G, PP = 1, BS = 96). The ancestor of the clade turned out to be similar to the Ramalinaceae MRCA, clearly evolving from a crustose ancestor (Electr. Suppl.: Table S1). However, single-septate spores seem to be plesiomorphic for this clade (Electr. Suppl.: Table S1). The *Ramalina*-group contained, in the taxonomy prior to this work, the genera *Cenozosia*, *Cliostomum*, *Lopezaria*, *Megalaria*, *Niebla*, *Ramalina*, *Ramalinopsis*, *Stirtoniella*, *Trichoramalina*, and *Tylothallia*.

We confirm the placement of *Stirtoniella* and *Cenozosia* in the Ramalinaceae and present multilocus sequence data for both genera for the first time. In concordance with the molecular phylogenetic hypothesis presented by Ekman (2001), *Cliostomum* is the sister group to the genus *Ramalina*. Lücking & al. (2017a, b) reduced the foliose genus *Ramalinopsis* into synonymy with *Ramalina* without further explanation. We support this decision due to its nested position within *Ramalina* (Fig. 2). This is in agreement with the observation that growth form is subject to frequent change in the Ramalinaceae. The genus *Trichoramalina* was reduced to synonymy with *Ramalina* by Kashiwadani & Nash (2004). The two species of *Trichoramalina*, *T. crinita* and *T. melanothrix*, were originally excluded from *Ramalina* due to their characteristic cilia (Rundel & Bowler, 1974). The authors, however, note that morphological characters were overlapping with the genera *Ramalina* and *Desmazieria* (the latter synonymous with *Niebla*). In our molecular phylogeny, the genus *Trichoramalina* turned out to be polyphyletic: While *T. crinita* is nested within *Ramalina*, *T. melanothrix* forms a strongly supported clade with *Niebla* (Fig. 2). Both species occur in the same kind of habitat, i.e., semi-arid, coastal areas with frequent fog formation: *T. crinita* is restricted to southern California and Baja California and *T. melanothrix* to South Africa. It is possible that the characteristic cilia are merely a product of convergent evolution because of adaptation to similar habitats. In the same way that a dense prothallus can prevent suprasaturation of lichens in tropical lowland rainforests (Lakatos & al., 2006), cilia might provide the advantage of condensing water from fog to increase photosynthetic activity during dry periods (Kappen, 1988: 59). Cilia should therefore be seen as an adaptation to the habitat rather than a taxonomically useful character. Another morphological character claimed to discriminate *Trichoramalina* from *Ramalina* is the presence of black pycnidia (Rundel & Bowler, 1974). However, pale pycnidia, similar to those often found in *Ramalina*, have been observed in *T. crinita*, whereas black pycnidia, similar to those in *Niebla*, have been observed in *T. melanothrix* (Keuck, 1979). The genus *Niebla* generally resembles *Ramalina* and exhibits extreme plasticity in morphological appearance (Bowler & Marsh, 2004). Hence, we accept the synonymy of *T. crinita* with *Ramalina*, but consider it best to accommodate *T. melanothrix* in *Niebla* based on both molecular and morphological data.

The genus *Megalaria* forms a strongly supported clade in our *Ramalina*-group and includes the genus *Lopezaria* (Fig. 2). Both *Lopezaria* and *Catillochroma* have been synonymized with *Megalaria* by Fryday & Lendemer (2010) because of very similar and overlapping morphological characters. However, Lücking & al. (2011) rejected this synonymization because the types of those genera are quite distinct and appear on long branches in a phylogenetic tree with limited taxon sampling (albeit forming a monophyletic group). In our phylogeny, branches within *Megalaria* s.l. are also found to be long (Fig. 2), although not longer than in other parts of the tree thought to represent infrageneric variation (e.g., *Bacidia*, *Bacidina*). Recognizing *Lopezaria* as a separate genus, however, would orphan *M. laureri* (a species never proposed to belong to either *Lopezaria* or *Catillochroma*; Fig. 2). We recognize the shortcoming that no member of *Catillochroma* was included in our study. Given the monophyly of the group and the apparent absence of morphological characters to distinguish between two or three genera, we choose to follow Fryday & Lendemer (2010) for the time being and accept a wide circumscription of *Megalaria* that includes *Catillochroma* and *Lopezaria*.

**Genera not investigated. —** Unfortunately, we were not able to sample all genera currently included in the Ramalinaceae by Lücking & al. (2017a, b). We did not investigate representatives from the following six genera: *Auriculora*, *Echidnocymbium*, *Heppsora*, *Jarmania*, *Pseudohepatica*, and *Tibellia*. The specimens were either too valuable to sample destructively for DNA (e.g., *Heppsora indica*) or did not amplify with PCR (e.g., *Jarmania tristis*, *Pseudohepatica* sp.), potentially due to fragmented DNA or development of PCR-inhibitory substances in old fungarium specimens. Clearly, there is a need for improved methods to obtain high-quality DNA from old and/or poor specimens. High-throughput sequencing methods might hold the key to successfully sequencing fragmented DNA in the future. Until further molecular or morphological evidence becomes available, we recommend treating these genera as recognized members of the Ramalinaceae.

## ■ TAXONOMIC TREATMENT

Revised taxonomy of the family Ramalinaceae including accepted names and their basionyms, names used in our figures and tables as well as important synonyms. Cited *ICN* Articles refer to the *Shenzhen Code* (Turland & al., 2018). Names are indicated as follows:

[0] Not studied molecularly
[1] Studied sequence(s) of one or more included non-type species
[2] Studied sequence(s) of the type
[3] Studied sequence(s) of type specimen of the type

***Aciculopsora*** Aptroot & Trest[3] in J. Hattori Bot. Lab. 100: 618. 2006 – Type: *Aciculopsora salmonea* Aptroot & Trest.

***Auriculora*** Kalb[0], Lichenes Neotrop. 10: 2. 1988 – Type: *Auriculora byssomorpha* (Nyl.) Kalb [≡ *Lecidea byssomorpha* Nyl.].

***Bacidia*** De Not.[2] in Giorn. Bot. Ital. 2: 189. 1846 – Type (designated by Clements & Shear, Gen. Fung., ed. 2: 319. 1931): *Bacidia rosella* (Pers.) De Not. [≡ *Lichen rosellus* Pers.].
= *Byssopsora* A.Massal.[0] in Mem. Reale Ist. Veneto Sci. 10: 89. 1861 – Type: *Byssopsora stupposa* A.Massal. [≡ *Bacidia stupposa* (A.Massal.) Zahlbr.].
= *Psorella* Müll.Arg.[2] in Bull. Herb. Boissier 2, App. 1: 11. 1894 – Type (designated by Clements & Shear, Gen. Fung., ed. 2: 319. 1931): *Psorella pannaroidea* (C.Knight) Müll.Arg. [≡ *Bacidia pannaroidea* C.Knight; = *Bacidia wellingtonii* (Stirt.) D.J.Galloway].
= *Megalopsora* Vain.[2] in Ann. Acad. Sci. Fenn., Ser. A, 15(6): 27. 1921 – Type (designated by Clements & Shear, Gen. Fung., ed. 2: 322. 1931): *Megalopsora cylindrophora* (Taylor) Vain. [≡ *Parmelia cylindrophora* Taylor ≡ *Physcidia cylindrophora* (Taylor) Hue ≡ *Bacidia cylindrophora* (Taylor) Kistenich, Timdal, Bendiksby & S.Ekman].
= *Bacidiomyces* Cif. & Tomas.[0] in Atti Ist. Bot. Lab. Crittog. Univ. Pavia 10: 39, 65. 1953, nom. illeg. (Art. 52.1, superfluous name for *Bacidia*) – Type: *Bacidiomyces rubellae* Cif. & Tomas., nom. illeg. [≡ *Bacidia rubella* (Hoffm.) A.Massal.].
= *Bacidiopsora* Kalb[2], Lichenes Neotrop. 10: 4. 1988 – Type: *Bacidiopsora squamulosula* (Nyl.) Kalb [≡ *Lecidea squamulosula* Nyl. ≡ *Bacidia squamulosula* (Nyl.) Zahlbr.].

*Notes.* – Ekman (1996a: 36) attempted a morphological circumscription of *Bacidia* that included species referred here to *Bibbya*, *Scutula*, *Toninia*, and *Toniniopsis*. In the revised circumscription, the genus includes species with acicular ascospores, pycnidia with filiform and curved conidia, and a proper exciple consisting of furcate hyphae with very thin cell lumina and thick, heavily gelatinized cell walls (terminal cells sometimes excepted). In addition, cell lumina are thinner and cell walls thicker in the old (lower) part of the exciple than in the younger (upper) part.

***Bacidia cylindrophora*** (Taylor) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824363] ≡ *Parmelia cylindrophora* Taylor in London J. Bot. 6: 165. 1847 – Lectotype (designated by Kalb & Elix in Biblioth. Lichenol. 57: 284. 1995): India. Madras, *C. Wright s.n.* (BM, n.v.).

***Bacidina*** Vězda[2] in Folia Geobot. Phytotax. 25: 431. 1991, nom. cons. – Type: *Bacidina phacodes* (Körb.) Vězda [≡ *Bacidia phacodes* Körb.].
= *Woessia* D.Hawksw. & Poelt[0] in Pl. Syst. Evol. 154: 207. 1986, nom. rej. – Type: *Woessia fusarioides* D.Hawksw., Poelt & Tscherm.-Woess [= *Bacidina sulphurella* (Samp.) M.Hauck & V.Wirth].
= *Lichingoldia* D.Hawksw. & Poelt[2] in Pl. Syst. Evol. 154: 203. 1986, nom. rej. – Type: *Lichingoldia gyalectiformis* D.Hawksw. & Poelt [= *Bacidina inundata* (Fr.) Vězda].

*Notes.* – The genus *Bacidina* is treated here in a wide sense to include *Woessia* and *Lichingoldia*, because of morphological similarities and because the AU test (Table 3) indicates that the best tree in which *Bacidina* in this sense forms a single monophyletic group is not significantly different from the overall best tree. Despite a vast increase in the amount of data, this is the exact same situation reported by Ekman (2001). *Bacidina* is represented here by the type *B. phacodes* (Körb.) Vězda as well as *B. arnoldiana* (Körb.) V.Wirth & Vězda, *B. brittoniana* (Riddle) LaGreca & S.Ekman (treated as *B. varia* S.Ekman by Ekman, 1996a), *B. inundata* (Fr.) Vězda, *B. lacerata* (Timdal) Kistenich & al., and *B. medialis* (Tuck. ex Nyl.) Kistenich & al. *Bacidina* is characterized by the wide excipular cell lumina (causing what is sometimes referred to as a paraplectenchymatous exciple) that tend to become wider with age (the gelatinized cell walls more or less maintaining their thickness). With the inclusion of *Bacidina lacerata*, overall thallus morphology in the genus is extended to include forms with up to 1 mm wide squamules. Species forming smaller squamules are known from before, for example *B. squamellosa* S.Ekman and *B. neosquamulosa* (Aptroot & Herk) S.Ekman. The "*Woessia* group", represented here by *B. inundata* and *B. arnoldiana*, differs somewhat from the type of *Bacidina* and its relatives in often having a more distinctly paraplectenchymatous exciple to the point where individual hyphae are difficult to follow from the inner part of the exciple to the edge. Furthermore, filiform, curved and non- or few-septate conidia are the rule in *Woessia*, whereas *B. phacodes* and relatives mostly have straight or moderately curved conidia with multiple septa. In the end, *Woessia* may very well turn out to constitute a separate genus from *Bacidina*, but this requires further investigation.

***Bacidina lacerata*** (Timdal) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824364] ≡ *Phyllopsora lacerata* Timdal in Lichenologist 39: 352. 2008 – Holotype: Peru. Loreto, Reserva Nacional Allpahuayo Mishana, within a 2.3 km distance from Centro de Investigaciones Allpahuayo, N of the road, site 19, 03°57.31′ S, 73°25.46′ W, alt. 120–150 m, tree trunk in rainforest, 2006, *E. Timdal 10213* (O No. L-144583!).

***Bacidina medialis*** (Tuck. ex Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824494] ≡ *Lecidea medialis* Tuck. ex Nyl. in Ann. Sci. Nat., Bot., ser. 4, 19: 346. 1863 ≡ *Bacidia medialis* (Tuck. ex Nyl.) B.de Lesd. in Bryologist 24: 68. 1921 – Lectotype (designated by Ekman in Opera Bot. 127: 86. 1996): Nicaragua, *C. Wright s.n.* (H-NYL No. 17372 specimen c!).

***Badimia*** Vězda[2] in Folia Geobot. Phytotax. 21: 206. 1986 – Type: *Badimia dimidiata* (Leight.) Vězda [≡ *Lecanora dimidiata* Leight.].

***Bellicidia*** Kistenich, Timdal, Bendiksby & S.Ekman[2], **gen. nov.** [MB 824445] – Type: *Bellicidia incompta* (Borrer) Kistenich, Timdal, Bendiksby & S.Ekman.

*Description.* – *Thallus* crustose coarsely granular with confluent granules, grey-green to grass-green to brown-green, indeterminate (Fig. 3A). Prothallus not present. Upper cortex a false cortex ("Scheinrinde") sensu Poelt (1958). Lower cortex lacking. Photobiont a unicellular green alga. *Ascomata* apothecia, biatorine, black, mostly flat but sometimes becoming convex, with distinct, shiny margin, often irregular in shape, adnate to thallus. Proper exciple and hypothecium dark red-brown (K+ purplish). Hymenium colourless or with faint red-brown pigment below, but young asci often surrounded by a gelatinous cap with red-brown pigment. Paraphyses simple or sparingly branched above; apices not or only narrowly clavate. Asci octosporous, clavate. Ascospores bacilliform, straight or only slightly curved, 15–30 µm long and 1.5–2 µm wide, with (1–)3(–5) thin septa. *Conidiomata* pycnidia, black, more or less immersed in thallus, unilocular, up to ca. 0.2 mm wide, with dark red-brown (K+ purplish) pigment. Conidiophores lining pycnidial cavity, forming conidia terminally. Conidia more or less ellipsoid, 5–9 µm long and 2–2.5 µm wide, non-septate or sometimes with a single septum.

*Chemistry.* – No secondary substances.

**Fig. 3.** Photographs of the newly described genera. **A,** *Bellicidia incompta* (O-L-175984); **B,** *Parallopsora leucophyllina* (O-L-144645). — Scale bars = 1 mm.

*Etymology.* – Based on the concatenation of *bellus* and the ending -*cidia* from its former placement in *Bacidia*. The Latin word *bellus* means "pretty" and is meant to balance the somewhat disparaging epithet given to the only known species, derived from *incomptus* and meaning "plain".

*Notes.* – *Bellicidia incompta* appears in a solitary position on a branch as sister to the rest of the *Toninia* clade (Fig. 2). The branch uniting the group has strong support and so has the sister branch to *Bellicidia*. Morphologically, *Bellicidia* stands out by its combination of dark red-brown pigment in the apothecia and pycnidial wall, bacilliform ascospores and prominent pycnidia with ellipsoid conidia.

***Bellicidia incompta*** (Borrer) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824446] ≡ *Lecidea incompta* Borrer in Smith & Sowerby, Engl. Bot., Suppl. 2: t. 2699. 1831 ≡ *Bacidia incompta* (Borrer) Anzi, Cat. Lich. Sondr.: 70. 1860 – Syntypes: United Kingdom. England, marked "E.B.S.", *W. Borrer s.n.* (BM barcode BM000974478, n.v.; UPS No. L-906205!).

***Biatora*** Fr. : Fr.[2], Lichenum Dianome Nova: 7. 1817, non Ach. 1809 – Type (designated by Clements & Shear in Gen. Fung, ed. 2: 319. 1931): *Biatora vernalis* (L.) Fr. [≡ *Lichen vernalis* L.].

= *Myrionora* R.C.Harris[2] in Evansia 5: 27. 1988 – Type: *Myrionora albidula* (Willey) R.C.Harris [≡ *Biatora albidula* Willey].

= *Ivanpisutia* S.Y.Kondr., Lőkös & Hur[2] in Acta Bot. Hung. 57: 97. 2015 – Type: *Ivanpisutia oxneri* S.Y.Kondr., Lőkös & Hur [≡ *Biatora oxneri* (S.Y.Kondr., Lőkös & Hur) Printzen & Kistenich].

***Biatora oxneri*** (S.Y.Kondr., Lőkös & Hur) Printzen & Kistenich, **comb. nov.** [MB 824365] ≡ *Ivanpisutia oxneri* S.Y.Kondr., Lőkös & Hur in Acta Bot. Hung. 57: 100. 2015 – Holotype: Russia. Far Eastern Federal District, Khasan District, along the pass to the second Golden Stream, along the Kamenisty stream, 43.115278° N, 131.518889° E, deciduous forest, on bark of deciduous tree, 2013, *S.Y. Kondratyuk & al. RU-13* (herbarium of the Korean Lichen Research Institute, Suncheon, Korea No. 020233, n.v.).

***Bibbya*** J.H.Willis[2] in Victorian Naturalist 73: 125. 1956 – Type: *Bibbya muelleri* (F.Wilson) J.H.Willis [≡ *Siphula muelleri* F.Wilson; = *Toninia bullata* (Meyen & Flot.) Zahlbr.].

= *Lobiona* H.Kilias & Gotth.Schneid.[2] in Lichenologist 10: 27. 1978 – Type: *Lobiona albomarginata* H.Kilias & Gotth. Schneider [≡ *Toninia auriculata* Timdal].

*Notes.* – This genus was included in *Toninia* by Timdal (1992) and corresponds to his species groups 4 and 8 and partly group 5. We also include a species previously placed in *Bacidia*. Morphologically, this genus is characterized by a reddish brown, K+ red pigment ("Ruginosa-brown", in the terminology of Meyer & Printzen, 2000) in the epithecium and rim of the exciple. The thallus varies from crustose to squamulose or bullate and the ascospores from ellipsoid, 1-septate to filiform, pluriseptate.

According to our molecular phylogeny (Fig. 2; Electr. Suppl.: Fig. S2), the following species belong in the genus: *Bibbya albomarginata* (H.Kilias & Gotth.Schneid.) Kistenich & al., *B. bullata* (Meyen & Flot.) Kistenich & al., *B. lutosa* (Ach.) Kistenich & al., *B. ruginosa* (Tuck.) Kistenich & al., and *B. vermifera* (Nyl.) Kistenich & al.

The following species and subspecies are included here in *Bibbya* because of morphological similarities with one or more of the species listed above, even though DNA sequences are not available: *Bibbya australis* (Timdal) Timdal, *B. austroafricana* (Timdal) Timdal, *B. glaucocarpa* (Timdal) Timdal, *B. hosseusiana* (Gyeln.) Timdal, *B. ruginosa* subsp. *pacifica* (Timdal) Timdal, and *B. subcircumspecta* (Coppins) S.Ekman.

***Bibbya albomarginata*** (H.Kilias & Gotth.Schneid.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824366] ≡ *Lobiona albomarginata* H.Kilias & Gotth.Schneid. in Lichenologist 10: 27. 1978 ≡ *Toninia auriculata* Timdal in Opera Bot. 110: 44. 1992 – Holotype: Peru. Huanuco, Stadt Huanuco, Hügel am Stadtrand, Erde, alt. 1950 m, 1973, *E. Hegewald & P. Hegewald s.n.* (GZU!).

***Bibbya australis*** (Timdal) Timdal, **comb. nov.** [MB 824371] ≡ *Toninia australis* Timdal in Opera Bot. 110: 45. 1992 – Holotype: Australia. South Australia, between Waikirie and Blanchetown, poorly developed soil crusts in opened-up mallee, 1967, *W.A. Weber & D. McVean L-47162* (COLO!).

***Bibbya austroafricana*** (Timdal) Timdal, **comb. nov.** [MB 824372] ≡ *Toninia austroafricana* Timdal in Opera Bot. 110: 47. 1992 – Holotype: Leshoto. Div. Qachas Nek, Mokhotlong, bank of a stream, on dolorite, 1963, *L. Kofler s.n.* (LD!).

***Bibbya bullata*** (Meyen & Flot.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824367] ≡ *Lecidea bullata* Meyen & Flot. in Nov. Actorum Acad. Caes. Leop.-Carol. Nat. Cur. 19, Suppl. 1: 227. 1843 ≡ *Toninia bullata* (Meyen & Flot.) Zahlbr. in Beih. Bot. Centralbl., Abt. 2, 19: 76. 1905 – Lectotype (designated by Timdal in Opera Bot. 110: 48. 1992): Chile/Peru. Peruvia, ad Tacoram, terricola, *G. Meyen s.n.* (G!).

***Bibbya glaucocarpa*** (Timdal) Timdal, **comb. nov.** [MB 824373] ≡ *Toninia glaucocarpa* Timdal in Opera Bot. 110: 63. 1992 – Holotype: Australia. Australian Capital Territory, Paddy's River, a tributary of the Cotter, W of Canberra, on limestone outcrops, 1968, *W.A. Weber & L.G. Adams L-49550* (COLO!).

***Bibbya hosseusiana*** (Gyeln.) Timdal, **comb. nov.** [MB 824374] ≡ *Toninia hosseusiana* Gyeln. in Ann. Hist.-Nat. Mus. Natl. Hung. 35: 98. 1942 – Holotype: Argentina. Córdoba, La Calera Sud, 1933, *C.C. Hosseus 213* (BP!).

***Bibbya lutosa*** (Ach.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824368] ≡ *Lecidea lutosa* Ach.,

Lichenogr. Universalis: 182. 1810 ≡ *Toninia lutosa* (Ach.) Timdal in Opera Bot 110: 69. 1992 – Holotype: Switzerland. “Helvetia”, *s.coll.* (H-ACH No. 299A!).

***Bibbya ruginosa*** (Tuck.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824369] ≡ *Lecidea ruginosa* Tuck., Lich. Calif.: 26. 1866 ≡ *Toninia ruginosa* (Tuck.) Herre in Proc. Wash. Acad. Sci. 12: 103. 1910 – Lectotype (designated by Lamb in Rhodora 56: 144. 1954): U.S.A. California, Oakland Hills, serpentine rocks, *H.N. Bolander 102* (FH!).

***Bibbya ruginosa*** subsp. ***pacifica*** (Timdal) Timdal, **comb. nov.** [MB 824375] ≡ *Toninia ruginosa* subsp. *pacifica* Timdal in Opera Bot. 110: 90. 1992 – Holotype: U.S.A. California, Santa Barbara Co., Santa Cruz Island, W end of the island, on the rim of the “Trailer Barranca”, in rock crevices, 1986, *W.A. Weber & C. Bratt s.n.* [Weber, Lich. Exs. COLO No. 663] (COLO!).

***Bibbya subcircumspecta*** (Coppins) S.Ekman, **comb. nov.** [MB 824406] ≡ *Bacidia subcircumspecta* Coppins in Lichenologist 24: 351. 1992 – Holotype: U.K. Caledonia, Mid Ebudes (V.C.103), Mull, Tobermory, Upper Druimfin, on lignum of *Pinus* trunk, 1968, *P.W. James s.n.* (BM, n.v.).

***Bibbya vermifera*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824370] ≡ *Lecidea vermifera* Nyl. in Bot. Not. 1853: 98. 1853 ≡ *Bacidia vermifera* (Nyl.) Th.Fr., Lichenogr. Scand.: 363. 1874 – Lectotype (designated by Ekman in Opera Bot. 127: 110. 1996): Sweden. Holmiae (Marieberg), ad quercus, *W. Nylander s.n.* (H-NYL No. 17507!).

***Bilimbia*** De Not.[2] in Giorn. Bot. Ital. 2: 190. 1846 ≡ *Weitenwebera* Opiz[2] in Lotos 7: 235. 1857, nom. nov. pro *Bilimbia* De Not., nom. illeg. (Art. 52.1, 53.1), non *Weitenwebera* Opiz 1839 – Type (designated by Timdal in Opera Bot. 110: 24. 1992; non Fink in Contr. U.S. Natl. Herb. 14: 85. 1910, Art. 10.5): *Bilimbia hexamera* De Not. [= *Bilimbia sabuletorum* (Schreb.) Arnold].

= *Myxobilimbia* Hafellner[2] in Stapfia 76: 154. 2001 – Type: *Myxobilimbia lobulata* (Sommerf.) Hafellner [≡ *Lecidea lobulata* Sommerf. ≡ *Bilimbia lobulata* (Sommerf.) Hafellner & Coppins].

***Cenozosia*** A.Massal.[2], Neagen. Lichenum: 4. 1854 – Type: *Cenozosia inanis* (Mont.) A.Massal. [≡ *Ramalina inanis* Mont.].

***Cliostomum*** Fr.[2], Syst. Orb. Veg. 1: 116. 1825 ≡ *Rhytismella* P.Karst.[2] in Hedwigia 23: 60. 1884, nom. illeg. (Art. 52.1, superfluous name for *Cliostomum* Fr.) – Type: *Cliostomum corrugatum* (Ach.) Fr. [≡ *Lecidea corrugata* Ach. ≡ *Rhytismella corrugata* (Ach.) P.Karst.].

= *Sporoblastia* Trevis.[1] in Nuovi Ann. Sci. Nat. Rendiconti Sess. Soc. Agrar., ser. 3, 3: 460. 1851 – Lectotype (designated by Hafellner in Beih. Nova Hedwigia 79: 268. 1984): *Sporoblastia griffithii* (Sm.) Trevis. [≡ *Lichen griffithii* Sm. ≡ *Cliostomum griffithii* (Sm.) Coppins].

***Echidnocymbium*** Brusse[0] in Mycotaxon 29: 173. 1987 – Type: *Echidnocymbium speciosum* Brusse.

***Eschatogonia*** Trevis.[2], Spighe e Paglie: 6. 1853 – Type: *Biatora prolifera* Mont. [≡ *Eschatogonia prolifera* (Mont.) R.Sant. ≡ *Eschatogonia montagnei* Trevis., nom. illeg. (Art. 52.1)].

***Heppsora*** D.D.Awasthi & Kr.P.Singh[0] in Bryologist 80: 537. 1977 – Type: *Heppsora indica* D.D.Awasthi & Kr.P.Singh.

***Jarmania*** Kantvilas[0] in Lichenologist 28: 230. 1996 – Type: *Jarmania tristis* Kantvilas.

***Kiliasia*** Hafellner[2] in Beih. Nova Hedwigia 79: 261. 1984 – Type: *Kiliasia athallina* (Hepp) Hafellner [≡ *Biatora athallina* Hepp ≡ *Toninia athallina* (Hepp) Timdal].

*Notes.* – This genus was included in *Toninia* by Timdal (1992) and includes species from his groups 1, 3, and 7. Morphologically, this genus is characterized by a more or less crustose thallus, although both non-lichenized and squamulose species occur. The ascospores vary from ellipsoid, 1-septate to shortly bacilliform, 3-septate.

According to our molecular phylogeny (Fig. 2; Electr. Suppl.: Fig. S2), the following species belong in the genus: *Kiliasia athallina* (Hepp) Hafellner, *K. nordlandica* (Th.Fr.) Kistenich & al., *K. pennina* (Schaer.) Kistenich & al., *K. philippea* (Mont.) Hafellner, *K. sculpturata* (H.Magn.) Kistenich & al.

The following species are included here in *Kiliasia* because of morphological similarities with one or more of the species listed above, even though DNA sequences are not available: *Kiliasia episema* (Nyl.) Hafellner, *K. granulosa* (Szatala) Timdal, *K. superioris* (Timdal) Timdal, *K. tristis* (Müll.Arg.) Hafellner.

***Kiliasia granulosa*** (Szatala) Timdal, **comb. nov.** [MB 824379] ≡ *Thalloidima granulosum* Szatala in Ann. Hist.-Nat. Mus. Natl. Hung., n.s., 5: 132. 1954 ≡ *Toninia weberi* Timdal in Opera Bot. 110: 118. 1992 – Holotype: Iran. Semnān, Montes Elburs orient., Firukuh, saxa calc., 1948, *K.H. Rechinger & F. Rechinger s.n.* (W!).

***Kiliasia nordlandica*** (Th.Fr.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824376] ≡ *Toninia nordlandica* Th.Fr., Lichenogr. Scand.: 339. 1874 – Holotype: Norway. Nordland, Gildeskål, ad praedium Indyr, *J.M. Norman s.n.* (UPS!).

***Kiliasia pennina*** (Schaer.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824377] ≡ *Lecidea pennina* Schaer., Lich. Helv. Spic.: 120. 1828 ≡ *Toninia pennina* (Schaer.) Gyeln. in Lilloa 3: 52. 1938 – Neotype (designated by Timdal in Opera Bot. 110: 77. 1992): Switzerland. Zürich, an Alpenfindlingen, *s.coll.* [Hepp, Flechten Eur. No. 238] (G!).

***Kiliasia sculpturata*** (H.Magn.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824378] ≡ *Catillaria sculpturata* H.Magn., Lich. Cent. Asia: 66. 1940 ≡ *Toninia sculpturata* (H.Magn.) Timdal in Opera Bot. 110: 92. 1992 – Holotype: China. Gansu, Yü-her-hung, alt. 2700–2800 m, 1932, *B. Bohlin 80* (S!).

***Kiliasia superioris*** (Timdal) Timdal, **comb. nov.** [MB 824380] ≡ *Toninia superioris* Timdal in Opera Bot. 110: 103. 1992 – Holotype: U.S.A. Michigan, Keweenaw Co., Isle Royale National Park, Edwards Island at NE end of Tobin Harbor, in balsam fir woods along rock cliffs and on upper shore at NE tip of island, 1983, *C.M. Wetmore 48492* (MIN!).

***Krogia*** Timdal[3] in Lichenologist 34: 293. 2002 – Type: *Krogia coralloides* Timdal.

***Lecania*** A.Massal.[2], Alc. Gen. Lich.: 12. 1853 – Type: *Lecania fuscella* (Schaer.) A.Massal. [≡ *Parmelia pallida* var. *fuscella* Schaer.].

= *Bayrhofferia* Trevis.[2] in Rivista Period. Lav. Regia Accad. Sci. Lett. Arti Padova 5: 69. 1857 – **Type (designated here):** *Bayrhofferia spadicea* (Flot.) Trevis. [≡ *Lecanora spadicea* Flot. ≡ *Lecania spadicea* (Flot.) Zahlbr. – Lectotype (designated by Mayrhofer in Biblioth. Lichenol. 28: 93. 1988): Italy. Apulien, Brindisi, Isola Petagne, 1847, *G.L. Rabenhorst s.n.* (L, n.v.)].

= *Dimerospora* Th.Fr.[2], Lich. Arct.: 97. 1860 – Type: *Dimerospora aipospila* (Wahlenb.) Th.Fr. [≡ *Parmelia aipospila* Wahlenb. ≡ *Lecania aipospila* (Wahlenb.) Th.Fr.].

= *Lecaniella* Jatta[2], Monogr. Lich. Ital. Merid.: 142. 1889 – Type (designated by Hafellner in Beih. Nova Hedwigia 79: 289. 1984): *Lecaniella cyrtella* (Ach.) Jatta [≡ *Lecidea cyrtella* Ach. ≡ *Lecania cyrtella* (Ach.) Th.Fr.].

= *Adermatis* Clem.[2], Gen. Fung.: 79, 175. 1909 – Type: *Adermatis nylanderiana* (A.Massal.) Clem. [≡ *Lecania nylanderiana* A.Massal.].

= *Dyslecanis* Clem.[2], Gen. Fung.: 79, 175. 1909 – Type: *Dyslecanis syringea* (Ach.) Clem. [= *Parmelia pallida* var. *fuscella* Schaer. ≡ *Lecania fuscella* (Schaer.) A.Massal.].

***Lueckingia*** Aptroot & L.Umaña[3] in J. Hattori Bot. Lab. 100: 619. 2006 – Type: *Lueckingia polyspora* Aptroot & L.Umaña.

***Megalaria*** Hafellner[2] in Beih. Nova Hedwigia 79: 302. 1984 – Type: *Megalaria grossa* (Nyl.) Hafellner [≡ *Lecidea grossa* Nyl.].

= *Lopezaria* Kalb & Hafellner[2] in Kalb, Lich. Neotrop. 11: 2. 1990 – Type: *Lopezaria versicolor* (Flot.) Kalb & Hafellner [≡ *Heterothecium versicolor* Flot. ≡ *Megalaria versicolor* (Flot.) Fryday & Lendemer].

= *Catillochroma* Kalb[0] in Biblioth. Lichenol. 95: 298. 2007 – Type: *Catillochroma endochroma* (Fée) Kalb [≡ *Lecanora endochroma* Fée ≡ *Megalaria endochroma* (Fée) Fryday & Lendemer].

***Mycobilimbia*** Rehm[2] in Rabenh. Krypt.-Fl., ed. 2, 1(3): 295, 327. 1889 ≡ *Probilimbia* Vain.[2] in Természetrajzi Füz. 22: 318. 1899, nom. nov. pro *Mycobilimbia* Rehm, nom. illeg. (Art. 52.1) – Type (designated by Clements & Shear, Gen. Fung, ed. 2: 315. 1931): *Mycobilimbia obscurata* (Sommerf.) Rehm [≡ *Lecidea spheroides* var. *obscurata* Sommerf.; = *Mycobilimbia tetramera* (De Not.) Hafellner & Türk].

***Myelorrhiza*** Verdon & Elix[3] in Brunonia 9: 194. 1986 – Type: *Myelorrhiza antrea* Verdon & Elix.

*Note.* – The genus may be close to, or a synonym of, *Phyllopsora*, see Discussion above.

***Niebla*** Rundel & Bowler[2] in Mycotaxon 6: 497. 1978, nom. nov. pro *Desmazieria* Mont. ≡ *Desmazieria* Mont.[2] in Ann. Sci. Nat., Bot., ser. 3, 18: 303. 1852, nom. illeg. (Art. 53.1), non Dumort. 1822 – Type: *Desmazieria homalea* (Ach.) Mont. [≡ *Ramalina homalea* Ach. ≡ *Niebla homalea* (Ach.) Rundel & Bowler].

= *Vermilacinia* Spjut & Hale[2] in Daniëls & al., Flechten Follmann: 345. 1995 – Type: *Vermilacinia combeoides* (Nyl.) Spjut & Hale [≡ *Ramalina combeoides* Nyl. ≡ *Niebla combeoides* (Nyl.) Rundel & Bowler].

*Notes.* – *Trichoramalina melanothrix* (Laurer) Rundel & Bowler is the sister taxon of *Niebla* in our phylogeny (Fig. 2) and seems to be better accommodated in *Niebla* than in *Ramalina*; see Discussion above. Hence, we make the new combination *Niebla melanothrix* (Laurer) Kistenich & al.

***Niebla melanothrix*** (Laurer) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824407] ≡ *Ramalina melanothrix* Laurer in Nylander, Syn. Meth. Lich. 1: 290. 1860 – Isotypes/syntypes: South Africa. Cap, *s.coll.* (O No. L-189956!), Cap B. Spei, *J.F. Drège s.n.* (H-NYL No. 37197, n.v.).

***Parallopsora*** Kistenich, Timdal & Bendiksby[2], **gen. nov.** [MB 824439] – Type: *Parallopsora leucophyllina* (Nyl.) Kistenich, Timdal & Bendiksby.

*Description.* – *Thallus* squamulose, effuse, mainly geotropically arranged or forming circular thalli with radiating marginal lobes (Fig. 3B); squamules up to 2 mm wide, adnate when young, soon ascending, more or less imbricate; upper side pale green to bluish green, glabrous to finely tomentose; soralia present or absent; prothallus absent. Upper cortex composed of thick-walled, irregularly oriented hyphae with angular to shortly cylindrical lumina, 10–35 µm thick, lacking an epinecral layer, not containing crystals. Photobiont unicellular green algae. Medulla containing lichen substances or not, KI−. Lower cortex lacking. *Ascomata* apothecia, biatorine, up to 1.0 mm diam., weakly convex, with an indistinct and more or less disappearing margin, brown, dull, epruinose, not pubescent along the margin. Proper excipulum composed of radiating, conglutinated, thick-walled hyphae with thread-like lumina; hypothecium not distinctly delimited from excipulum, pale brown to colourless, chondroid, composed of irregularly oriented, thick-walled hyphae with cylindrical to thread-like

lumina; hymenium colourless or faintly yellow, with amyloid gelatin, ca. 40 µm high; paraphyses conglutinated, straight, simple or rarely branched, ca. 2 µm wide, with a slightly swollen, colourless apical cell; ascus clavate, up to 30 × 8 µm, with a well-developed, amyloid tholus often with a small ocular chamber and with an axial mass in lower part or extending through the tholus (*Bacidia*-type), octosporous; ascospores bacilliform to acicular, simple or with indistinct pseudosepta, colourless, not halonate. *Conidiomata* not seen.

*Chemistry.* – Homosekikaic acid, sekikaic acid, methylbarbatate, or no lichen substances.

*Etymology.* – Based on the concatenation of *para*- (by the side of, near) and -*llopsora* (from the three included species' former placement in *Phyllopsora*).

*Notes.* – According to our phylogeny (Fig. 2), two more *Phyllopsora* species appear in this clade and belong in *Parallopsora*: *P. brakoae* (Timdal) Kistenich & al. and *P. labriformis* (Timdal) Kistenich & al.

***Parallopsora brakoae*** (Timdal) Kistenich, Timdal & Bendiksby, **comb. nov.** [MB 824442] ≡ *Phyllopsora brakoae* Timdal in Lichenologist 40: 343. 2008 – Holotype: Peru. Loreto, Reserva Nacional Allpahuayo Mishana, within a 2.3 km distance from Centro de Investigaciones Allpahuayo, N of the road, site 43, 03°58.48′ S, 73°25.86′ W, alt. 120–150 m, tree trunk in rainforest, "bosque de varillal seco", 2006, *E. Timdal 10253* (O No. L-144623!).

***Parallopsora labriformis*** (Timdal) Kistenich, Timdal & Bendiksby, **comb. nov.** [MB 824443] ≡ *Phyllopsora labriformis* Timdal in Lichenologist 40: 350. 2008 – Holotype: Peru. Loreto, Jenaro Herrera, within a 3.6 km distance from the Research Center, N of the road, site 112, 04°53.93′ S, 73°83.91′ W, alt. 120–150 m, tree trunk in rainforest, 2006, *E. Timdal 10419* (O No. L-144789!).

***Parallopsora leucophyllina*** (Nyl.) Kistenich, Timdal & Bendiksby, **comb. nov.** [MB 824441] ≡ *Lecidea leucophyllina* Nyl. in Ann. Sci. Nat., Bot., ser. 4, 19: 347. 1863 ≡ *Phyllopsora leucophyllina* (Nyl.) Timdal in Lichenologist 40: 352. 2008 – Holotype: Cuba. s.loc., *C. Wright s.n.* (H-NYL No. 17345c,e!).

***Phyllopsora*** Müll.Arg.[3] in Bull. Herb. Boissier 2, App. 1: 11. 1894 – Type (designated by Clements & Shear, Gen. Fung., ed. 2: 319. 1931): *Phyllopsora breviuscula* (Nyl.) Müll.Arg. [≡ *Lecidea breviuscula* Nyl.].

= *Triclinum* Fée[2], Essai Crypt. Écorc.: 147, pl. 33, fig. 4. 1825 – Type: *Triclinum cinchonarum* Fée [≡ *Phyllopsora cinchonarum* (Fée) Timdal].

= *Symplocia* A.Massal.[2], Neagen. Lich.: 4. 1854, nom. rej. (vs. *Crocynia* (Ach.) A.Massal.) – Type: *Symplocia gossypina* (Sw.) A.Massal. [≡ *Lichen gossypinus* Sw. ≡ *Phyllopsora gossypina* (Sw.) Kistenich, Timdal, Bendiksby & S.Ekman].

= *Crocynia* (Ach.) A.Massal.[2] in Atti Reale Ist. Veneto Sci. Lett. Arti, ser. 3, 5: 251. 1860, nom. cons. (vs. *Symplocia* A.Massal) ≡ *Lecidea* sect. *Crocynia* Ach., Lichenogr. Universalis: 217. 1810 – Type: *Crocynia gossypina* (Sw.) A.Massal. [≡ *Lichen gossypinus* Sw. ≡ *Phyllopsora gossypina* (Sw.) Kistenich, Timdal, Bendiksby & S.Ekman].

= *Squamacidia* Brako[2] in Mycotaxon 35: 6. 1989 – Type: *Squamacidia janeirensis* (Müll.Arg.) Brako [≡ *Thalloidima janeirensis* Müll.Arg.; = *Phyllopsora cinchonarum* (Fée) Timdal].

*Notes.* – The circumscription of *Phyllopsora* proposed here differs markedly from that proposed by Swinscow & Krog (1981) and modified by Timdal (2008). The new circumscription is based on our molecular phylogeny (Fig. 2), which places *Phyllopsora* in a sister position to *Biatora*. *Phyllopsora* differs morphologically from *Biatora* mainly in forming larger thallus elements, usually squamules, and in being attached to a generally more felty hypothallus. Printzen (1995) discussed the morphological differences between the two genera and, based on chemistry, shape of conidiospores and apothecial anatomy, assumed that the genera are closely related. All *Phyllopsora* species studied so far having soredia and/or acicular to filiform ascospores are now excluded from the genus. We exclude 10 species and place them in the genera *Bacidia*, *Bacidina*, *Parallopsora*, *Sporacestra* and an unnamed genus. We include two species that were previously placed in *Crocynia* and one in *Lecidea*, together with eight studied *Phyllopsora* species, in the newly circumscribed genus *Phyllopsora*. Still, ca. 50 species of *Phyllopsora* remain to be studied with molecular methods.

The name *Phyllopsora* is antedated by *Triclinum*, *Symplocia* and *Crocynia*, but will be proposed for conservation (Kistenich & al., in prep.).

According to our molecular phylogeny (Fig. 2), at least one more *Crocynia* species belongs in *Phyllopsora*: *Phyllopsora pyxinoides* (Nyl.) Kistenich & al.

***Phyllopsora gossypina*** (Sw.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824408] ≡ *Lichen gossypinus* Sw., Prodr.: 146. 1788 ≡ *Crocynia gossypina* (Sw.) A.Massal. in Atti Reale Ist. Veneto Sci. Lett. Arti, ser. 3, 5: 252. 1860 – Syntypes: Jamaica. 1784–1786, *O. Swartz s.n.* (UPS Nos. L-000259! & L-134473!).

***Phyllopsora pyxinoides*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824409] ≡ *Crocynia pyxinoides* Nyl., Sert. Lich. Trop: 37. 1891 – Holotype: Cuba. "In ins. Cuba", *C. Wright s.n.* [Wright, Lich. Cub. Ser. 2, No. 145] (H-NYL No. 22059, n.v.).

***Physcidia*** Tuck.[2] in Proc. Amer. Acad. Arts 5: 399. 1862 – Type (designated by Clements & Shear, Gen. Fung., ed. 2: 322. 1931): *Physcidia wrightii* (Tuck.) Tuck. [≡ *Physcia wrightii* Tuck.].

?= *Callopis* (Müll.Arg.) Gyeln.[0] in Acta Fauna Fl. Universali, Ser. 2, Bot. 1(5–6): 8. 1933 ≡ *Physcidia* sect. *Callopis* Müll. Arg. in Jahrb. Königl. Bot. Gart. Berlin 2: 314. 1883 – Type: *Physcidia callopis* (Meyen & Flot.) Müll.Arg. [≡ *Lecanora callopis* Meyen & Flot.].

– "*Psoromopsis*" Nyl.[2], Syn. Meth. Lich. 2: 56. 1863, not validly published (Art. 36.1a).

*Notes.* – Eight species are currently included in *Physcidia* (Kalb & Elix, 1995; Aptroot & Cáceres, 2014) and an additional, apparently undescribed species is included in this study as *Physcidia* sp. Four species were included in our molecular phylogeny (Fig. 2; Electr. Suppl.: Fig. S1) and they do not form a monophyletic group. The type, *P. wrightii*, and *P. striata* Aptroot & al. are placed in the *Toninia*-group (Electr. Suppl.: Fig. S1: clade D), but low branch support in this part of the tree makes it impossible to conclude whether they are congeneric or not. *Physcidia cylindrophora* and *Physcidia* sp., however, are placed in the *Bacidia*-group (Fig. 2: clade C), the former nested within *Bacidia* and the latter as the sister to *Sporacestra*. The growth form of *Physcidia*, i.e., large, almost foliose squamules on a hypothallus, has clearly evolved repeatedly in the Ramalinaceae, even in the genus *Bacidia*. *Physcidia cylindrophora* is transferred here to *Bacidia*, but *Physcidia* sp. may either be included in *Sporacestra* or in a new genus pending further studies. The two species *P. cylindrophora* and *P. striata* differ from the other species of *Physcidia* in having biatorine, not lecanorine, apothecia, and *P. striata* also differs in having ellipsoid, not bacilliform to filiform, ascospores.

***Pseudohepatica*** P.M.Jørg.[0] in Bryologist 96: 435. 1993 – Type: *Pseudohepatica pachyderma* P.M.Jørg.

***Ramalina*** Ach.[2] in Luyken, Tent. Hist. Lich.: 95. 1809 ≡ *Ramalinomyces* Cif. & Tomas.[2] in Atti Ist. Bot. Lab. Crittog. Univ. Pavia 10: 44, 70. 1953, nom. illeg. (Art. 52.1, superfluous name for *Ramalina*) – Type: *Ramalina fraxinea* (L.) Ach. (typ. cons.) [≡ *Lichen fraxineus* L. ≡ *Ramalinomyces fraxineae* Cif. & Tomas., nom. illeg.].

?= *Platysma* Hill[0], Gener. Nat. Hist., ed. 2, 2: 88. 1773, nom. illeg., non P.Browne ex Adans. 1763 – Type: not designated.

= *Chlorodictyon* J.Agardh[0] in Öfvers. Kongl. Vetensk.-Akad. Förh. 27: 433. 1870 – Type: *Chlorodictyon foliosum* J.Agardh [= *Ramalina menziesii* Taylor].

?= *Alectoriopsis* Elenkin[0] in Izv. Glavn. Bot. Sada S.S.S.R. 28: 292. 1929 – Type: not designated.

= *Dievernia* M.Choisy[0] in Bull. Soc. Bot. France 78: 455. 1931 ≡ *Ramalina* subsect. *Solidae* Du Rietz in Svensk Bot. Tidskr. 20: 298. 1926 – Type: *Ramalina evernioides* Nyl. [= *Ramalina maciformis* (Delise) Bory].

= *Ramalinopsis* (Zahlbr.) Follmann & Huneck[2] in Willdenowia 5: 211. 1969 ≡ *Ramalina* sect. *Ramalinopsis* Zahlbr. in Ark. Bot. 32A(2): 4. 1945 – Type: *Ramalina mannii* Tuck. [≡ *Ramalinopsis mannii* (Tuck.) Follmann & Huneck].

= *Trichoramalina* Rundel & Bowler[2] in Bryologist 77: 191. 1974 – Type: *Trichoramalina crinita* (Tuck.) Rundel & Bowler [≡ *Ramalina crinita* Tuck.].

= *Fistulariella* Bowler & Rundel[2] in Mycotaxon 6: 195. 1977 ≡ *Ramalina* stirps *Fistularia* Vain. in Acta Soc. Fauna Fl. Fenn. 7: 14. 1890 – Type (designated by Bowler & Rundel in Mycotaxon 6: 195. 1977): *Ramalina inflata* (Hook.f. & Taylor) Hook.f. & Taylor [≡ *Cetraria inflata* Hook.f. & Taylor].

***Rolfidium*** Moberg[2] in Lichenologist 18: 305. 1986 – Type: *Rolfidium peltatum* Moberg [= *Rolfidium coccocarpioides* (Nyl.) Timdal].

*Notes.* – According to our molecular phylogeny (Fig. 2), the following species belong in this formerly monotypic genus: *Rolfidium bumammum* (Nyl.) Kistenich & al., *R. nigropallidum* (Nyl.) Kistenich & al.

***Rolfidium bumammum*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824411] ≡ *Lecidea bumamma* Nyl. in J. Linn. Soc., Bot. 15: 177. 1876 ≡ *Toninia bumamma* (Nyl.) Zahlbr., Cat. Lich. Univ. 4: 263. 1926 – Holotype: South Africa. Western Cape, Cap. B. Spei, 1874, *A.E. Eaton s.n.* (H-NYL No. 16843!).

***Rolfidium nigropallidum*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824412] ≡ *Lecidea nigropallida* Nyl. in Nouv. Arch. Mus. Hist. Nat., ser. 3, 3: 124. 1891 ≡ *Toninia nigropallida* (Nyl.) Abbayes in Bull. Inst. Franç. Afrique Noire, A, 17: 982. 1955 – Holotype: South Africa. Western Cape, Cap. B. Spei, *J.F. Drège 9286* (H-NYL No. 19645!).

***Scutula*** Tul.[2] in Ann. Sci. Nat., Bot., ser. 3, 17: 118. 1852, nom. cons., non Lour. 1790 – Type: [illustration] "*Scutula wallrothii*" in Ann. Sci. Nat., Bot., ser. 3, 17: t. 14, fig. 14–24. 1852 (typ. cons.) [= *Scutula miliaris* (Wallr.) Trevis.].

= *Hollosia* Gyeln.[2] in Borbásia 1: 153. 1939 – Type: *Hollosia vertesensis* Gyeln. [= *Scutula epiblastematica* (Wallr.) Rehm].

= *Karsteniomyces* D.Hawksw.[2] in Trans. Brit. Mycol. Soc. 74: 371. 1980 – Type: *Karsteniomyces peltigerae* (P.Karst.) D.Hawksw. [≡ *Stagonopsis peltigerae* P.Karst.] [anamorph of *Scutula*].

*Notes.* – *Scutula* was treated by Triebel & al. (1997) and Wedin & al. (2007) to include a set of lichenicolous species. The association with Ramalinaceae was first made by Andersen & Ekman (2005) and is confirmed here (Electr. Suppl.: Fig. S1). We expand *Scutula* here to include lichen-forming species without any obvious parasitic life-cycle stages. The parasitic members of *Scutula* are known to produce two or three types of pycnidia containing either micro-, meso- or macroconidia. Similarly, *Bacidia circumspecta* produces three pycnidial types (Ekman, 1996a), whereas *B. auerswaldii* is only known to produce short-bacilliform conidia (described by Arvidsson & al., 1988) that are likely to correspond to the microconidia in *Scutula*. Two new combinations are necessary: *Scutula circumspecta* (Vain.) Kistenich & al. and *S. effusa* (Rabenh.) Kistenich & al.

***Scutula circumspecta*** (Vain.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824488] ≡ *Lecidea bacillifera* var. *circumspecta* Nyl. ex Vain. in Meddeland. Soc. Fauna Fl. Fenn. 10: 22. 1883 ≡ *Lecidea circumspecta* (Vain.) Hedl. in Bih. Kongl. Svenska Vetensk.-Akad. Handl. 18(3,3): 71. 1892 ≡ *Bacidia circumspecta* (Vain.) Malme in Bot. Not. 1895: 140. 1895 – Lectotype (designated by Ekman

in Opera. Bot. 127: 69. 1996): Finland. Tavastia australis, Lammi, Evo, 1874, *J.P. Norrlin s.n.* [Norrlin & Nylander, Herb. Lich. Fenn. No. 185] (H!).

***Scutula effusa*** (Rabenh.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824489] ≡ *Bilimbia effusa* Rabenh., Lich. Eur. Exs.: No. 32. 1855 ≡ *Bacidia effusa* (Rabenh.) Lettau in Hedwigia 52: 132. 1912, nom. illeg., non (Sm.) Trevis. 1856 ≡ *Lecidea auerswaldii* Stizenb. in Ber. Thätigk. St. Gallischen Naturwiss. Ges. 1880/81: 416. 1882 ≡ *Bacidia auerswaldii* (Stizenb.) Mig., Krypt.-Fl. Deutschl., Flecht. 2: 267. 1929 – Lectotype (designated by Ekman in Opera Bot. 127: 66. 1996): Germany. Leipzig, an einer alten Ulme in Rosenthal, 1855, *B. Auerswald s.n.* [Rabenhorst, Lich. Eur. Exs.: No. 32] (W!).

***Sporacestra*** A.Massal.[2] in Atti Reale Ist. Veneto Sci. Lett. Arti, ser. 3, 5: 264. 1860 – Type: *Biatora prasina* Mont. & Tuck. 1857, nom. illeg., non (Fr.) Fr. 1826 [= *Sporacestra pertexta* (Nyl.) Stapnes & Timdal].

*Notes.* – *Sporacestra* consists of one or two species that were previously placed in *Phyllopsora* (Timdal & Krog, 2001; Timdal, 2011).

***Sporacestra pertexta*** (Nyl.) Stapnes & Timdal, **comb. nov.** [MB 824510] ≡ *Lecidea pertexta* Nyl. in Ann. Sci. Nat., Bot., ser. 4, 19: 347. 1863 ≡ *Phyllopsora pertexta* (Nyl.) Swinscow & Krog in Lichenologist 13: 244. 1981 – Holotype: Cuba. “in ins. Cuba”, *C. Wright s.n.* (H-NYL No. 17344, left and right hand specimens!).

***Stirtoniella*** D.J.Galloway, Hafellner & Elix[2] in Lichenologist 37: 262. 2005 – Type: *Stirtoniella kelica* (Stirt.) D.J.Galloway, Hafellner & Elix [≡ *Lecidea kelica* Stirt.].

***Thalloidima*** A.Massal.[2], Ric. Auton. Lich. Crost.: 95. 1852 ≡ *Skolekites* Norman[2], Conat. Praem. Gen. Lich.: 23. 1852 ≡ *Diphloeis* Clem.[2], Gen. Fung.: 76. 1909 ≡ *Thalloedematomyces* Cif. & Tomas.[2] in Atti Ist. Bot. Lab. Crittog. Univ. Pavia 10: 39, 66. 1953, nom. illeg. (Art. 52.1, superfluous name for *Thalloidima*) – Type: (designated by Clements & Shear, Gen. Fung., ed. 2: 319. 1931): *Thalloidima candidum* (Weber) A.Massal. [≡ *Lichen candidus* Weber ≡ *Toninia candida* (Weber) Th.Fr.].

= *Bacillina* Nyl.[2], Lich. Env. Paris: 7. 1896 – Type: *Bacillina antipolitana* Nyl. [= *Thalloidima physaroides* (Opiz) Kistenich, Timdal, Bendiksby & S.Ekman].

*Notes.* – This genus was included in *Toninia* by Timdal (1992) and corresponds to his species groups 1 (with the exclusion of four species now placed in *Kiliasia*) and 10. Morphologically, the genus is characterized by the presence of a grey, K+ violet, N+ violet pigment (“Thalloidima-grün” or “Sedifolia-grey”, Meyer & Printzen, 2000) in the epithecium and rim of the exciple, with the exception of *T. toninianum* (from group 10) which has an olivaceous brown to green, K–, N+ violet pigment. The thallus is mostly flattened squamulose to bullate, but two species are non-lichenized. Most species, perhaps all, are parasitic on cyanolichens when young or remain parasitic. The ascospores are mostly ellipsoid to fusiform, 1-septate, rarely acicular, 3-septate.

According to our molecular phylogeny (Figs. 2, Electr. Suppl.: Fig. S2), the following species belong in the genus: *Thalloidima albilabrum* (Dufour) Flagey [≡ *Toninia albilabra* (Dufour) H.Olivier], *Thalloidima alutaceum* Anzi [≡ *Toninia alutacea* (Anzi) Jatta], *Thalloidima candidum* (Weber) A.Massal. [≡ *Toninia candida* (Weber) Th.Fr.], *Thalloidima diffractum* (A.Massal.) A.Massal. [≡ *Toninia diffracta* (A.Massal.) Zahlbr.], *Thalloidima massatum* (Tuck.) Kistenich & al., *Thalloidima opuntioides* (Vill.) Kistenich & al., *Thalloidima physaroides* (Opiz) Kistenich & al., *Thalloidima rosulatum* Anzi [≡ *Toninia rosulata* (Anzi) H.Olivier], *Thalloidima sedifolium* (Scop.) Kistenich & al., *Thalloidima tauricum* Szatala [≡ *Toninia taurica* (Szatala) Oxner], *Thalloidima toepfferi* Stein [≡ *Toninia toepfferi* (Stein) Návas], *Thalloidima toninianum* (A.Massal.) A.Massal. [≡ *Toninia toniniana* (A.Massal.) Zahlbr.].

The following species are included here in *Thalloidima* because of morphological similarities with one or more of the species listed above even though DNA sequences are not available: *Thalloidima arcticum* (Timdal) Timdal, *Thalloidima collematicola* (Timdal) Timdal, *Thalloidima ioen* (Herre) S.Ekman & Timdal, *Thalloidima leptogii* (Timdal) Timdal, *Thalloidima subdiffractum* (Timdal) Timdal.

***Thalloidima arcticum*** (Timdal) Timdal, **comb. nov.** [MB 824416] ≡ *Toninia arctica* Timdal in Opera Bot. 110: 37. 1992 – Holotype: Canada. Northwest Territories, Inuvik, Banks Island, 73°13′N, 119°32′W, alt. 50–55 m, *Dryas-Oxytropis* and *Carex* community types, 1979, *G.W. Scotter 30146* (CANL!).

***Thalloidima collematicola*** (Timdal) Timdal, **comb. nov.** [MB 824417] ≡ *Toninia collematicola* Timdal in Opera Bot. 110: 57. 1992 – Holotype: Italy. Lombardia, Sondrio, along the road from Bormio to Bormio, 2000, above the village S. Pietro, 46°27′N, 10°23′E, alt. 1530 m, on *Collema* on limestone, 1988, *J. Holtan-Hartwig & E. Timdal 6724* (O No. L-119!).

***Thalloidima ioen*** (Herre) S.Ekman & Timdal, **comb. nov.** [MB 824496] ≡ *Bacidia ioessa* Herre in Proc. Wash. Acad. Sci. 12: 98. 1910 – Type: U.S.A. California, Santa Clara Co., “on igneous rock on a dry hill side, Hidden Villa Cañon, at an altitude of 800 feet”, no later than 1908, *A.W.C.T. Herre s.n.* (original material expected to be deposited in UC or FH could not be located) – **Neotype (designated here):** U.S.A. California, Santa Clara Co., just SW of Los Altos Hills, on the S-facing slope of the hills on the E side of the canyon stretching from Moody Rd to Hidden Villa, 2008, *S. Ekman 3690* (UPS No. L-878230!).

= *Toninia submexicana* B.de Lesd., Lich. Mexique: 25. 1914 – **Lectotype (designated here):** Mexico. Michoacan, Morelia, lomas à l’ouest du Zapote, 1910, *A.G. Brouard s.n.* (O No. L-829!).

***Thalloidima leptogii*** (Timdal) Timdal, **comb. nov.** [MB 824418] ≡ *Toninia leptogii* Timdal in Opera Bot. 110: 68. 1992 – Holotype: Italy. Calabria, Cosenza, Valle del Caronte, Ponte Alimena (= ca. 800 m E of Mendicino), 39°16′N, 16°13′E, alt. 450 m, 1988, *J.C. Hughes & E. Timdal 6803* (O No. L-121!).

***Thalloidima massatum*** (Tuck.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824413] ≡ *Lecidea massata* Tuck, Lich. Calif.: 25. 1866 ≡ *Toninia massata* (Tuck.) Herre in Proc. Wash. Acad. Sci. 12, 2: 103. 1910 – Holotype: U.S.A. California, San Fransisco, loose gravelly soil on the west side of last hills near the ocean, *H.N. Bolander 75* (FH!).

***Thalloidima opuntioides*** (Vill.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824414] ≡ *Lichen opuntioides* Vill., Hist. Pl. Dauphiné 3: 967, t. 55. 1789 ≡ *Toninia opuntioides* (Vill.) Timdal in Opera Bot. 110: 76. 1992 – Neotype (designated by Timdal in Opera Bot. 110: 76. 1992): France. Alpes-du-Haute-Provence, Gorges du Bachelard, 3.1 km along the road S of Uvernet-Fours, 44°20′N, 06°38′E, alt. 1300 m, 1989, *E. Timdal 7261* (O No. L-122!).

***Thalloidima physaroides*** (Opiz) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824493] ≡ *Lecidea physaroides* Opiz in Lotos 6: 158. 1856 ≡ *Toninia physaroides* (Opiz) Zahlbr., Cat. Lich. Univ. 4: 275. 1926 – Holotype: Czech Republic. Středočeský, Prosik, 1854, *F.M. Opiz s.n.* (PRM No. 698368!).

***Thalloidima sedifolium*** (Scop.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824415] ≡ *Lichen sedifolius* Scop., Fl. Carniol., ed. 2, 2: 395. 1772 ≡ *Toninia sedifolia* (Scop.) Timdal in Opera Bot. 110: 93. 1992 – Neotype (designated by Timdal in Opera Bot. 110: 93. 1992): Italy. Friuli-Venezia-Giulia, Trieste, Monrupino, 45°42′N, 13°48′E, alt. 320 m, in soil-filled crevices in calcareous rock, 1988, *J.C. Hughes & E. Timdal 6808* (O No. L-123!).

***Thalloidima subdiffractum*** (Timdal) Timdal, **comb. nov.** [MB 824421] ≡ *Toninia subdiffracta* Timdal in Opera Bot. 110: 100. 1992 – Holotype: U.S.A. Utah, Grand Co., Colorado River at Dewey bridge, 38°48′N, 109°19′W, alt. 2130 m, on steep rock wall, facing north, 1988, *E. Timdal 6890* (O No. L-124!).

***Thamnolecania*** (Vain.) Gyeln.[2] in Acta Fauna Fl. Universali, Ser. 2, Bot. 1(5–6): 8. 1933 ≡ *Lecanora* subg. *Thamnolecania* Vain., Résult. Voy. Belgica, Lich.: 16. 1903 – Type (designated by Dodge in Brit. Austral. New Zeal. Antarct. Res. Exped. Sci. Rep. 7: 181. 1948): *Thamnolecania brialmontii* (Vain.) Gyeln. [≡ *Lecanora brialmontii* Vain.].

***Tibellia*** Vězda & Hafellner[0] in Nova Hedwigia 55: 186. 1992 – Type: *Tibellia dimerelloides* Vězda & Hafellner.

***Toninia*** A.Massal.[2], Ric. Auton. Lich. Crost.: 107. 1852, nom. cons. ≡ *Syncomista* Nieuwl.[2] in Amer. Midl. Naturalist 4: 386. 1916, nom. nov. pro *Toninia* A.Massal., nom. illeg. (Art. 52.1) – Type (designated by Baumgärtner, Rev. Eur. Art. Gatt. Toninia: 30. 1979): *Toninia cinereovirens* (Schaer.) A.Massal. [≡ *Lecidea cinereovirens* Schaer.].
= *Arthrosporum* A.Massal.[2], Mem. Lichenogr.: 127. 1853 – Type: *Arthrosporum populorum* A.Massal., Mem. Lichenogr.: 128. 1853 [≡ *Toninia populorum* (A.Massal.) Kistenich, Timdal, Bendiksby & S.Ekman].
?= *Leptographa* Jatta[0] in Bull. Soc. Bot. Ital. 1: 211. 1892 – Type: *Leptographa toninioides* Jatta.

*Notes.* – The genus was monographed by Timdal (1992). It is split here into five genera based on our molecular phylogeny (Fig. 2; Electr. Suppl.: Fig. S2): *Bibbya*, *Kiliasia*, *Thalloidima*, *Toninia*, and *Toniniopsis*. In the new circumscription, *Toninia* corresponds to the species groups 2, 6, 9 and partly 5 and 7 of Timdal (1992), with the inclusion of the genus *Arthrosporum* and some species of *Bacidia*. *Toninia* species have a green, K–, N+ violet ("Bacidiagrün" or "Bagliettoana-green", Meyer & Printzen, 2000) or brown, K–, N− pigment in the epithecium and exciple; ellipsoid to acicular, (0–)1-pluriseptate ascospores; and either non-lichenized or a thallus varying from flattened squamulose to bullate.

According to our molecular phylogeny (Fig. 2; Electr. Suppl.: Fig. S2), the following species belong in the genus: *Toninia cinereovirens* (Schaer.) A.Massal., *Toninia plumbina* (Anzi) Hafellner & Timdal, *Toninia populorum* (A.Massal.) Kistenich & al., *Toninia squalida* (Ach.) A.Massal., *Toninia subdispersa* (Nyl.) K.Knudsen, *Toninia tristis* (Th.Fr.) Th.Fr.

In addition, the following species are currently included in the *Toninia*, but the lack of DNA sequences and diagnostic morphological characters makes this position uncertain: *T. corallina* Timdal, *T. gobica* N.S.Golubk., *T. himalayana* Timdal, *T. nashii* Timdal, *T. poeltiana* S.Y.Kondr. & al., *T. poeltii* Timdal, *T. subfuscae* (Arnold) Timdal, *T. subtalparum* van den Boom, *T. ualae* Etayo, *T. verrucariae* (Nyl.) Timdal, *T. wetmorei* Timdal.

***Toninia populorum*** (A.Massal.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824425] ≡ *Arthrosporum populorum* A.Massal., Mem. Lichenogr.: 128. 1853 – Holotype: Italy, "Garda ad populos", *A. Massalongo s.n.* (VER!).

***Toniniopsis*** Frey[2] in Ber. Schweiz. Bot. Ges. 35: 73. 1926 – Type: *Toniniopsis obscura* Frey [= *Toniniopsis illudens* (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman].

*Notes.* – This genus consists of species previously placed in *Bacidia* and *Toninia*, and the separation of the two genera in this species complex was discussed by Timdal (1992: 23) and Ekman (1996a: 44). We unite these these species in the previously described genus *Toniniopsis.* This genus is morphologically similar to *Toninia* but differs in the generally stronger pigmentation of the exciple. The green pigment occurring in the epithecium in most species is believed to be same as in *Toninia* ("Bacidiagrün" or "Bagliettoana-green", Meyer & Printzen, 2000).

According to our molecular phylogeny (Fig. 2; Electr. Suppl.: Fig. S2), the following species belong in the genus: *Toniniopsis aromatica* (Sm.) Kistenich & al., *T. coelestina* (Anzi) Kistenich & al., *T. subincompta* (Nyl.) Kistenich & al., *T. verrucarioides* (Nyl.) Kistenich & al., and the species currently known as *Bacidia bagliettoana* (A.Massal. & De Not.) Jatta. The complicated nomenclature of this latter species makes us refrain from making any new combination at the moment.

The following species are included here in *Toniniopsis* because of morphological similarities with one or more of the species listed above, even though DNA sequences are not available: *T. cretica* (Timdal) Timdal, *T. mesoidea* (Nyl.) Timdal.

***Toniniopsis aromatica*** (Sm.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824495] ≡ *Lichen aromaticus* Sm. in Smith & Sowerby, Engl. Bot.: t. 1777. 1807 ≡ *Toninia aromatica* (Sm.) A.Massal., Framm. Lichenogr.: 24. 1855 – Lectotype (designated by Baumgärtner, Rev. Eur. Art. Gatt. Toninia: 114. 1979): U.K. England, Norfolk, near Yarmouth, *D. Turner s.n.* (BM!).

***Toniniopsis coelestina*** (Anzi) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824430] ≡ *Bacidia coelestina* Anzi in Atti Soc. Ital. Sci. Nat. 9: 251. 1866 ≡ *Toninia coelestina* (Anzi) Vězda, Cas. Slez. Mus., Ser. A, Hist. Nat. 10: 105. 1961 – Lectotype (designated by Timdal in Opera Bot. 110: 56. 1992): Italy. Lombardia, Sondrio, in rimulis rupium calcarearum, septentrionem spectantium, in valle di Fraéle, alt. 1900–2200 m, *s.coll.* [Anzi, Lich. Rar. Langob. 517] (UPS!).

***Toniniopsis cretica*** (Timdal) Timdal, **comb. nov.** [MB 824434] ≡ *Toninia cretica* Timdal in Opera Bot. 110: 59. 1992 – Holotype: Greece. Crete, Khania, at the top of the gorge from Komitades to Imbros, 35°15′N, 24°15′E, alt. 740 m, in fissures in calcareous rock, 1988, *E. Timdal 6692* (O No. L-120!).

***Toniniopsis illudens*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824426] ≡ *Lecidea illudens* Nyl. in Flora 53: 34. 1870 – **Lectotype (designated here):** Finland. Kuusamo, Kitkajoki, 19 Aug 1867, *F. Silén s.n.* (H-NYL No. 17322!; isolectotype: UPS No. L-513287!).
= *Toniniopsis obscura* Frey in Ber. Schweiz. Bot. Ges. 35: 73. 1926 – Lectotype (designated by Ekman in Opera Bot. 127: 130. 1996): Switzerland. Val Cluozza, auf Kalkfelsgesimsen im Pinetum mont., alt. 1850 m, 1923, *E. Frey 442* (BERN!).

***Toniniopsis mesoidea*** (Nyl.) Timdal, **comb. nov.** [MB824436] ≡ *Lecidea mesoidea* Nyl. in Flora 51: 475. 1868 ≡ *Toninia mesoidea* (Nyl.) Zahlbr., Cat. Lich. Univ. 4: 289. 1926 – Holotype: U.K. Channel Islands, "Ins. Sark", 1868, *C. Larbalestier s.n.* (H-NYL No. 16719!).

***Toniniopsis subincompta*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB824431] ≡ *Lecidea subincompta* Nyl. in Flora 48: 147. 1865 ≡ *Bacidia subincompta* (Nyl.) Arnold in Flora 53: 472. 1870 – Type to be proposed for conservation: Austria. An Stämmchen von *Sorbus chamaemespilus* unterhalb der Serloswände ober der Waldrast, Matrei in Tirol, alt. 5400 ft, 1872, *F. Arnold s.n.* [Arnold, Lich. Exs.: No. 505] (H-NYL No. 17400!).

***Toniniopsis verrucarioides*** (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman, **comb. nov.** [MB 824433] ≡ *Lecidea aromatica* var. *verrucarioides* Nyl. in Bot. Not. 1853: 157. 1853 ≡ *Lecidea verrucarioides* (Nyl.) Nyl. in Actes Soc. Linn. Bordeaux 21: 369. 1856 ≡ *Toninia verrucarioides* (Nyl.) Timdal in Opera Bot. 110: 116. 1992 – Lectotype (designated by Timdal in Opera Bot. 110: 116. 1992): France. Hautes-Pyrénées, Bagnières de Bigorre, *W. Nylander s.n.* (H-NYL No. 16804!).

***Tylothallia*** P.James & H.Kilias[2] in Herzogia 5: 409. 1981 – Type: *Tylothallia biformigera* (Leight.) P.James & H.Kilias [≡ *Lecidea biformigera* Leight.].

***Waynea*** Moberg[2] in Lichenologist 22: 249. 1990 – Type: *Waynea californica* Moberg.

## ■ AUTHOR CONTRIBUTIONS

All authors participated in the design of the research. MB, SE and SK generated sequences in the lab. SE and SK designed and performed the data analysis. All authors participated actively in the interpretation and discussion of the results. SK wrote the first draft of the manuscript and ET wrote the Taxonomy chapter. All authors corrected and improved the manuscript. — ORCID: SK, https://orcid.org/0000-0001-5501-3210; MB, https://orcid.org/0000-0002-7534-6466; SE, https://orcid.org/0000-0003-3021-1821

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**Appendix 1.** Taxa including authorships, voucher information, revised taxonomy and GenBank accession numbers of sequences used in this study. An asterisk (*) indicates newly generated sequences, an n-dash (–) indicates missing data.

Species (including authorships); voucher; revised taxonomy if changed; GenBank accession numbers for mtSSU, ITS, LSU, *RPB1*, *RPB2*

***Aciculopsora salmonea*** Aptroot & Trest, Costa Rica, 2004, *Lücking 17543* (BR), isotype, MG925842*, MG925948*, –, MG926137*, –. ***Adelolecia pilati*** (Hepp) Hertel & Hafellner, Austria, 1998, *Ekman 3373* (BG), AY567713, MG925949*, AY300826, AY756379, MG926227*. ***Arthrosporum populorum*** A.Massal., Austria, 1998, *Ekman 3392* (BG), [*Toninia populorum* (A.Massal.) Kistenich & al.], MG925843*, MG925950*, MG926039*, MG926138*, MG926228*. ***Austrolecia antarctica*** Hertel, Antarctica (Antarctic Peninsula), 1997, *Smith 10517* (AAS), MG925844*, MG925951*, MG926040*, –, –. ***Bacidia absistens*** (Nyl.) Arnold, Norway, 1997, *Ekman 3223* (BG), MG925845*, AF282085, –, MG926139*, MG926229*. ***Bacidia arceutina*** (Ach.) Arnold, Sweden, 1997, *Ekman 3110* (BG), MG925846*, AF282083, MG926041*, MG926140*, MG926230*. ***Bacidia auerswaldii*** (Stizenb.) Mig., Sweden, 2000, *Th. Johansson s.n.* (hb. Th. Johansson), [*Scutula effusa* (Rabenh.) Kistenich & al.], –, AF282122, –, MG926141*, MG926231*. ***Bacidia bagliettoana*** (A.Massal. & De Not.) Jatta, Sweden, 1997, *Ekman 3150* (BG), [*Toniniopsis bagliettoana* (A.Massal. & De Not.) Kistenich & al.], MG925847*, –, MG926042*, MG926142*, MG926232*. ***Bacidia circumspecta*** (Vain.) Malme, Norway, 1992, *Tønsberg 17554* (BG), [*Scutula circumspecta* (Vain.) Kistenich & al.], MG925848*, –, –, MG926143*, –. ***Bacidia incompta*** (Borrer) Anzi, Sweden, 1997, *Ekman 3144* (BG), [*Bellicidia incompta* (Borrer) Kistenich & al.], MG925849*, AF282092, MG926043*, MG926144*, MG926233*. ***Bacidia lutescens*** Malme, U.S.A., 1999, *Ekman 3655* (BG), –, MG925952*, –, –, –. ***Bacidia medialis*** (Nyl.) B.de Lesd., U.S.A., 1999, *Ekman 3659* (BG), [*Bacidina medialis* (Nyl.) Kistenich & al.], MG925850*, –, MG926044*, MG926145*, MG926234*. ***Bacidia rosella*** (Pers.) De Not., Sweden, 1997, *Ekman 3117* (BG), AY300877, AF282086, AY300829, AY756412, AM292755. ***Bacidia schweinitzii*** (E.Michener) A.Schneid., U.S.A., 1993, *Wetmore 72619* (MIN), –, AF282080, MG926045*, MG926146*, MG926235*. ***Bacidia subincompta*** (Nyl.) Arnold, Sweden, 1998, *Ekman 3413* (BG), [*Toniniopsis subincompta* (Nyl.) Kistenich & al.], MG925851*, AF282125, MG926046*, MG926147*, MG926236*. ***Bacidia vermifera*** (Nyl.) Th.Fr., Sweden, 1998, *Johansson 1619* (BG), [*Bibbya vermifera* (Nyl.) Kistenich & al.], MG925852*, AF282109, MG926047*, MG926148*, MG926237*. ***Bacidia wellingtonii*** (Stirt.) D.J.Galloway, New Zealand, 2005, *Ziviagina s.n.* (O), MG925853*, MG925953*, –, –, –. ***Bacidina arnoldiana*** (Körb.) V.Wirth & Vězda, Sweden, 1997, *Ekman 3157* (BG), MG925854*, AF282093, MG926048*, MG926149*, MG926238*. ***Bacidina brittoniana*** (Riddle) LaGreca & S.Ekman, U.S.A., 1999, *Ekman 3657* (BG), –, MG925954*, MG926050*, MG926151*, MG926241*. ***Bacidina inundata*** (Fr.) Vězda, Norway, 1998, *Ekman 3187* (BG), MG925855*, AF282094, –, MG926150*, MG926239*. ***Bacidina phacodes*** (Körb.) Vězda, Sweden, 1998, *Ekman 3414* (UPS), AY567725, AF282100, MG926049*, –, MG926240*. ***Bacidiopsora squamulosula*** (Nyl.) Kalb, Ecuador, 1987, *Kalb & Kalb in Kalb: Lich. Neotrop. 405* (O), [*Bacidia squamulosula* (Nyl.) Zahlbr.], MG925856*, MG925955*, MG926051*, MG926152*, MG926242*. ***Badimia dimidiata*** (C.Bab.) Vězda, Costa Rica, *Lücking 16013* (BG), AY567774, MG925956*, MG926052*, –, –. ***Biatora beckhausii*** (Körb.) Tuck. **I**, Norway, 2013, *Klepsland JK13-L008* (O), MG925857*, –, MG926053*, MG926153*, –. ***Biatora beckhausii* II**, Norway, 1995, *Holien 6744* (TRH), MG925858*, AF282071, MG926054*, MG926154*, MG926243*. ***Biatora flavopunctata*** (Tønsberg) Hinter. & Printzen, Norway, 2011, *Klepsland JK11-L119* (O), MG925859*, MG925957*, –, –, –. ***Biatora globulosa*** (Flörke) Fr., Sweden, 1997, *Ekman 3142* (BG), KF662414, AF282073, MG926055*, MG926155*, KF662450. ***Biatora hertelii*** Printzen & Etayo, Portugal (Madeira), *Kanz & Printzen 3069* (hb. Printzen), MG925860*, MG925958*, MG926056*, –, –. ***Biatora ligni-mollis*** T.Sprib. & Printzen, Germany, 2011, *Malícek & Z. Palice 14609* (FR), KF662418, KF650968, –, –, –. ***Biatora ocelliformis*** (Nyl.) Arnold **I**, Norway, 2013, *Klepsland JK13-L107* (O), –, MG925959*, MG926057*, MG926156*, MG926244*. ***Biatora ocelliformis* II**, U.S.A., 1999, *Printzen s.n.* (FR), –, KF650972, MG926058*, MG926157*, –. ***Biatora pallens*** (Kullh.) Zahlbr., Sweden, 2000, *Nordin 2161* (BG), KF662425, KF650975, –, –, –. ***Biatora rufidula*** (Graewe) S.Ekman & Printzen, Germany, 1999, *Printzen 5055* (FR), KF662430, KF650981, MG926059*, MG926158*, –. ***Biatora vacciniicola*** (Tønsberg) Printzen, Norway, 2013, *Klepsland JK13-L330* (O), MG925861*, MG925960*, MG926060*, MG926159*, MG926245*. ***Biatora vernalis*** (L.) Fr., Norway, 1996, *Tønsberg 23757* (BG), DQ838753, AF282070, DQ838752, –, –. ***Biatora veteranorum*** Coppins & Sérus. **I**, Czech Republic, 2011, *Malícek & Z. Palice 14753* (FR), KF662425, KF650975, –, –, –. ***Biatora veteranorum* II**, Germany, 2015, *Printzen Lok. 909* (FR), MG925862*, MG925961*, MG926061*, MG926160*, –. ***Bilimbia lobulata*** (Sommerf.) Hafellner & Coppins, Norway, 2000, *Rui & Timdal 9169* (O), AM292712, AM292668, MG926062*, MG926161*, AM292759. ***Bilimbia sabuletorum*** (Schreb.)

**Appendix 1.** Continued.

Arnold, Norway, 1997, *Ekman 3091* (BG), AY567721, AM292670, AY756346, AY756413, AM292761. ***Boreoplaca ultrafrigida*** Timdal, Russia, 1992, *Haugan & Timdal YAK03/84* (O), paratype, DQ986813, HM161512, DQ986797, DQ986855, DQ992421. ***Catillaria contristans*** (Nyl.) Zahlbr. **I**, Norway, 2010, *Timdal 11347* (O), MG925863*, MG925962*, MG926063*, MG926162*, –. ***Catillaria contristans*** **II** , Norway, 1992, *Andersen 92* (BG), AY567733, –, AY756332, AY756394, –. ***Catillaria erysiboides*** (Nyl.) Th.Fr. **I**, Ireland, 1999, *Holien 7731* (hb. Holien), AY567732, MG925963*, –, MG926163*, –. ***Catillaria erysiboides*** **II**, *Bjørk 13369* (UBC), KJ766369, –, –, KJ766844, –. ***Catillaria scotinodes*** (Nyl.) Coppins **I**, Norway, 2008, *Haugan 8599* (O), MG925864*, MG925964*, –, –, –. ***Catillaria scotinodes*** **II**, United Kingdom, 1999, *Coppins 18298 & Coppins* (E), AM292720, AM292673, MG926064*, MG926164*, AM292763. ***Catinaria atropurpurea*** (Schaer.) Vězda & Poelt, Norway, 2012, *Klepsland JK12-L366* (O), MG925865*, MG925965*, MG926065*, MG926165*, MG926246*. ***Catolechia wahlenbergii*** (Ach.) Körb., Austria, 2001, *Hafellner 04-2002* (GZU), DQ986811, HQ650649, DQ986794, KJ766824, DQ992424. ***Cenozosia inanis*** (Mont.) A.Massal., Chile, 1995, *Elvebackk 95:303* (TROM), MG925866*, –, MG926066*, –, –. ***Cliostomum corrugatum*** (Ach. : Fr.) Fr., Sweden, 1997, *Ekman 3115* (BG), AY567722, MG925966*, MG926067*, MG926166*, KF662436. ***Crocynia gossypina*** (Sw.) A.Massal. **I**, Brazil, 2015, *Kistenich & Timdal SK1-108* (O), [*Phyllopsora gossypina* (Sw.) Kistenich & al.], MG925867*, MG925967*, MG926068*, MG926167*, MG926247*. ***Crocynia gossypina*** **II**, Brazil, 2015, *Dahl & al. SK1-287* (O), *Phyllopsora gossypina*, MG925868*, MG925968*, MG926069*, MG926168*, MG926248. ***Crocynia pyxinoides*** Nyl., Costa Rica, 2002, *Lücking 16052* (DUKE), [*Phyllopsora pyxinoides* (Nyl.) Kistenich & al.], AY584615, –, AY584653, DQ883735, DQ883748. ***Crustospathula cartilaginea*** Aptroot, Papua New Guinea, 1995, *Aptroot 36411* (B), isotype, MG925869*, –, –, –, –. ***Eschatogonia prolifera*** (Mont.) R.Sant. **I**, Peru, 2006, *Timdal 10207* (O), MG925870*, MG925969*, MG926070*, MG926169*, MG926249*. ***Eschatogonia prolifera*** **II**, Peru, 2006, *Timdal 10429* (O), MG925871*, MG925970*, –, MG926170*, –. ***Frutidella caesioatra*** (Schaer.) Kalb, Norway, 2011, *Haugan 10634* (O), MG925872*, MG925971*, –, MG926171*, –. ***Glyphopeltis ligustica*** (B.de Lesd.) Timdal, South Africa, 2014, *Timdal 13524* (O), MG925873*, MG925972*, MG926071*, MG926172*, MG926250*. ***Herteliana taylorii*** (Salwey) P.James, Ireland, 1998, *Hertel 39599* (UPS) in Hertel: Lecideac. exs. 324, AY756369, –, AY756351, AY756385, –. ***Ivanpisutia oxneri*** S.Y.Kondr., Lőkös & Hur, Korea, *Davydov 12006* (FR), [*Biatora oxneri* (S.Y.Kondr., Lőkös & Hur) Printzen & Kistenich, –, MG925973*, –, –, –. ***Japewia tornoensis*** (Nyl.) Tønsberg **I**, Norway, 2011, *Klepsland JK11-L105* (O), MG925874*, MG925974*, –, –, –. ***Japewia tornoensis*** **II**, Canada, 2000, *Printzen s.n.* (BG) ,HQ660559, HQ650656, –, –, –. ***Japewiella tavaresiana*** (H.Magn.) Printzen **I**, Czech Republic, 1998, *Hertel 39505* (M), –, MG925975*, –, –, –. ***Japewiella tavaresiana*** **II**, Argentina, 2012, *Rodriguez-Flakus 3207 & Flakus* (FR), –, MG925976*, –, –, –. ***Krogia coralloides*** Timdal, Mauritius, 1991, *Krog & Timdal MAU51/83* (O), holotype, MG925875*, MG925977*, MG926072*, MG926173*, MG926251*. ***Lecania aipospila*** (Wahlenb.) Th.Fr., Norway, 2014, *Klepsland JK14-L361* (O), MG925876*, MG925978*, MG926073*, MG926174*, MG926252*. ***Lecania cyrtella*** (Ach.) Th.Fr. **I**, Poland, 2005, *Kukwa 4609* (DUKE), –, HQ650645, –, –, KJ766933. ***Lecania cyrtella*** **II**, Sweden, 1997, *Ekman 3017* (BG), AY567720, AF282067, AY300840, MG926175*, AM292767. ***Lecania erysibe*** (Ach.) Mudd, United Kingdom, 1998, *Coppins 17537* (E), AM292733, AM292682, MG926074*, MG926176*, AM292769. ***Lecania fuscella*** (Schaer.) A.Massal., Sweden, 1989, *Ekman L1351* (LD), MG925877*, AM292684, MG926075*, –, –. ***Lecania nylanderiana*** A.Massal., Sweden, 2005, *Nordin 598* (UPS), MG925878*, MG925979*, MG926076*, –, –. ***Lecania spadicea*** (Flot.) Zahlbr., Greece, 1995, *Rui & Timdal 8021* (O), –, MG925980*, MG926077*, MG926177*, MG926253*. ***Lecanora poliophaea*** (Wahlenb.) Ach., Norway, 2014, *Klepsland JK14-L362* (O), MG925879*, MG925981*, MG926078*, MG926178*, MG926254*. ***Lecidea albohyalina*** (Nyl.) Th.Fr., Sweden, *F. Jonsson 6:29* (hb. Mellansel), KF662398, KF650950, MG926079*, –, KF662438. ***Lecidea thaleriza*** Stirt. **I**, South Africa, 2014, *Burrows & Timdal 14191* (O), [*Phyllopsora thaleriza* (Stirt.) Brako], MG925880*, MG925982*, MG926080*, MG926179*, MG926255*. ***Lecidea thaleriza*** **II**, South Africa, 2015, *Rui & Timdal 13877* (O), [*Phyllopsora thaleriza*], MG925881*, MG925983*, MG926081*, MG926180*, MG926256*. ***Lopezaria versicolor*** (Flot.) Kalb & Hafellner, Costa Rica, 2002, *Sipman 480491* (DUKE), [*Megalaria versicolor* (Flot.) Fryday & Lendemer], AY584622, –, AY584651, DQ912379, DQ912401. ***Lueckingia polyspora*** Aptroot & L.Umaña, Costa Rica, 2004, *Aptroot 60206* (BR), isotype, MG925882*, MG925984*, MG926082*, –, –. ***Malcolmiella cinereovirens*** Vězda, *Malcolm 2757* (WIS), holotype, HM447626, –, –, –, –. ***Megalaria grossa*** (Pers. ex Nyl.) Hafellner **I**, Norway, 1998, *Ekman 3466* (BG), MG925883*, AF282074, MG926083*, MG926181*, MG926257*. ***Megalaria grossa*** **II**, Norway, 1998, *Tønsberg 26038* (BG), AY762095, MG925985*, AY756356, AY756419, MG926258*. ***Megalaria laureri*** (Th.Fr.) Hafellner, Sweden, 1997, *Ekman 3119* (BG), MG925884*, AF282075, –, MG926182*, MG926259*. ***Mycobilimbia epixanthoides*** (Nyl.) Vitik. & al., Finland, *Printzen & Kuusinen s.n.* (FR), KF662401, KF650953, –, –, KF662441. ***Mycobilimbia pilularis*** (Körb.) Hafellner, Norway, 2009, *Tønsberg 39658* (BG), KF662402, KF650954, –, –, KF662442. ***Mycobilimbia tetramera*** (De Not.) Hafellner & Türk **I**, U.S.A., 2005, *Lutzoni & al. 06.04.05-8a* (DUKE), KJ766439, –, KJ766600, KJ766915, KJ766957. ***Mycobilimbia tetramera*** **II**, Norway, 1996, *Anonby 856* (BG), AM292750, MG925986*, MG926084*, –, AM292779. ***Myelorrhiza antrea*** Verdon & Elix, Australia, 1984, *Elix 15809 & Streimann* (CANB), holotype, MG925885*, –, –, –, –. ***Myrionora albidula*** (Willey) R.C.Harris, Sweden, 2011, *Svensson 2383b* (UPS), *Biatora albidula* Willey, MG925886*, –, –, –, –. ***Niebla combeoides*** (Nyl.) Rundel & Bowler **I**, U.S.A., 2008, *Ertz R655* (BR), –, GU827338, GU726379, –, –. ***Niebla combeoides*** **II**, U.S.A., 1996, *Bratt 9574* (SBBG), MG925887*, –, –, –, –. ***Niebla homalea*** (Ach.) Rundel & Bowler, U.S.A., 1998, *Nash 41462B* (ASU), MG925888*, MG925987*, MG926085*, –, MG926260*. ***Phyllopsora atrocarpa*** Timdal, Peru, 2006, *Timdal 10409* (O), paratype, MG925889*, –, –, MG926183*, –. ***Phyllopsora borbonica*** Timdal & Krog, La Réunion, 1996, *Krog & Timdal RE08/12* (O), holotype, MG925890*, MG925988*, MG926086*, MG926184*, MG926261*. ***Phyllopsora brakoae*** Timdal, Peru, 2006, *Timdal 10253* (O), holotype, [*Parallopsora brakoae* (Timdal) Kistenich, Timdal & Bendiksby], MG925891*, MG925989*, –, –, –. ***Phyllopsora breviuscula*** (Nyl.) Müll.Arg. **I**, La Réunion, 1996, *Krog & Timdal RE36/18* (O), MG925892*, MG925990*, MG926087*, MG926185*, MG926262*. ***Phyllopsora breviuscula*** **II**, Brazil, 1980, *Kalb & Marcelli in Kalb: Lich. neotrop. 515* (GZU), epitype, MG925893*, MG925991*, MG926088*, MG926186*, MG926263*. ***Phyllopsora chlorophaea*** (Müll.Arg.) Zahlbr., La Réunion, 1996, *Krog & Timdal RE08/10* (O), MG925894*, MG925992*, MG926089*, MG926187*, –. ***Phyllopsora labriformis*** Timdal, Peru, 2006, *Timdal 10419* (O), holotype, [*Parallopsora labriformis* (Timdal) Kistenich & al.], MG925895*, –, –, MG926188*, –. ***Phyllopsora lacerata*** Timdal, Peru, 2006, *Timdal 10213* (O), holotype, [*Bacidina lacerata* (Timdal) Kistenich & al.], MG925896*, MG925993*, –, –, MG926264*. ***Phyllopsora leucophyllina*** (Nyl.) Timdal, Peru, 2006, *Timdal 10275* (O), [*Parallopsora leucophyllina* (Nyl.) Kistenich & al.], MG925897*, MG925994*, –, MG926189*, MG926265*. ***Phyllopsora lividocarpa*** Timdal, Peru, 2006, *Timdal 10210* (O), paratype, MG925898*, MG925995*, –, MG926190*, –. ***Phyllopsora longiuscula*** (Nyl.) Zahlbr., Peru, 2006, *Timdal 10433* (O), MG925899*, MG925996*, MG926090*, MG926191*, MG926266*. ***Phyllopsora mauritiana*** (Taylor) Gotth.Schneid., Mauritius, 1991, *Krog & Timdal MAU09/44* (O), MG925900*, MG925997*, MG926091*, MG926192*, –. ***Phyllopsora nigrocincta*** Timdal, Peru, 2006, *Timdal 10319* (O), paratype, MG925901*, MG925998*, –, –, –. ***Phyllopsora parvifoliella*** (Nyl.) Müll.Arg., Indonesia, 2000, *Wolseley s.n.* (BM), MG925902*, MG925999*, MG926092*, MG926193*, MG926267*. ***Phyllopsora pertexta*** (Nyl.) Swinscow & Krog, Cuba, 2006, *Pérez-Ortega s.n.* (hb. Pérez-Ortega), [*Sporacestra pertexta* (Nyl.) Stapnes & Timdal], MG925903*, MG926000*, MG926093*, MG926194*, MG926268*. ***Phyllopsora porphyromelaena*** (Vain.) Zahlbr., La Réunion, 1996, *Krog & Timdal RE07/17* (O), MG925904*, MG926001*, –, MG926195*, –. ***Phyllopsora sorediata*** (Aptroot & Sparrius) Timdal, Thailand, 1992, *Wolseley & Aguirre-Hudson 3948* (BM), MG925905*, MG926002*, MG926094*, MG926196*, MG926269*. ***Phyllopsora* sp. I**, Brazil, 1979, *Kalb & Plöbst in Kalb: Lich. neotrop. 343* (GZU), MG925906*, MG926003*, MG926095*, MG926197*, MG926270*. ***Phyllopsora* sp. II**, Brazil, 2007, *Lücking & Rivas Plata 23302* (SP), MG925907*, MG926004*, MG926096*, MG926198*, –. ***Physcia aipolia*** (Ehrh.) Fürnr., U.S.A., 2002, *Hills s.n.* (DUKE), DQ912290, DQ782836, DQ782904, DQ782820, DQ782862. ***Physcidia cylindrophora*** (Taylor) Hue **I**, Thailand, 1964, *Kurokawa 1692* (O), [*Bacidia cylindrophora* (Taylor) Kistenich & al.], –, MG926005*, –, –, –. ***Physcidia cylindrophora*** **II**, Taiwan, 2009, *Ohmura 7091 in Ohmura: Lich. minus cogniti Exs. 441* (GZU), [*Bacidia cylindrophora*], MG925908*, MG926006*, –, –, –. ***Physcidia* sp.**, Cuba, 2009, *Buck 55792* (NY), holotype, MG925909*, MG926007*, MG926097*, MG926199*, MG926271*. ***Physcidia striata*** Aptroot & al., Brazil, 2012, *Cáceres & Aptroot 11640* (ABL), isotype, MG925910*, –, MG926098*, –, –. ***Physcidia wrightii*** Tuck. **I**, Mauritius, 1991, *Krog & Timdal MAU14/14* (O), MG925911*, –, –, –, –. ***Physcidia wrightii*** **II**, Mauritius, 1991, *Krog & Timdal MAU13/10* (O), MG925912, –, –, –, –. ***Porpidinia tumidula*** (Sm.) Timdal **I**, Spain, 1985, *Timdal 4363* (O), –, MG926008*, MG926099*, MG926200*, MG926272*. ***Porpidinia tumidula*** **II**, Greece, 2009, *Rui & Timdal*

**Appendix 1.** Continued.

*10979* (O), –, MG926009*, MG926100*, MG926201*, MG926273*. ***Protoblastenia rupestris*** (Scop.) J.Steiner, Norway, 2001, *Johnsen s.n.* (BG), MG925913*, MG926010*, MG926101*, MG926202*, MG926274*. ***Psora decipiens*** (Hedw.) Hoffm., Norway, 1998, *Ekman 3327* (BG), AY567772, MG926011*, MG926102*, AY756396, MG926275*. ***Psorinia conglomerata*** (Ach.) Gotth.Schneid., Norway, 1986, *Holtan-Hartwig & Timdal 4874* (O), MG925914*, MG926012*, –, –, –. ***Psoroma karstenii*** Müll.Arg. **I**, New Caledonia, 2005, *Elvebakk 05:712B* (O), MG925915*, –, –, –, –. ***Psoroma karstenii*** **II**, New Caledonia, 2005, *Elvebakk 05:721* (O), MG925916*, –, MG926103*, –, –. ***Ramalina dilacerata*** (Hoffm.) Hoffm., U.S.A., 1999, *Wetmore 83868 in Wetmore: Lich. exs. 99* (BG), MG925917*, MG926013*, MG926104*, MG926203*, –. ***Ramalina fastigiata*** (Pers.) Ach., Norge, 2000, *Ekman 3616* (UPS), AY756375, –, AY756360, AY756422, MG926276*. ***Ramalina fraxinea*** (L.) Ach., Sweden, 2002, *Ekman 3686* (UPS), MG925918*, MG926014*, MG926105*, MG926204*, MG926277*. ***Ramalina inflata*** (Hook.f. & Taylor) Hook.f. & Taylor, Australia, 1989, *Elix 22744* (O), MG925919*, MG926015*, MG926106*, –, –. ***Ramalina lacera*** (With.) J.R.Laundon, Italy, 1997, *Nimis & Tretiach 26272* (TSB), –, MG926016*, MG926107*, MG926205*, –. ***Ramalina pollinaria*** (Westr.) Ach., Sweden, 2002, *Ekman 3687* (UPS), AM292752, MG926017*, MG926108*, –, MG926278*. ***Ramalina siliquosa*** (Huds.) A.L.Sm., Norway, 2000, *Ekman 3685* (UPS), MG925920*, –, MG926109*, MG926206*, MG926279*. ***Ramalina sinensis*** Jatta, U.S.A., 1998, *Nash 42001 in Nash: Lich. exs. 333* (BG), MG925921*, MG926018*, MG926110*, MG926207*, –. ***Ramalinopsis mannii*** (Tuck.) Follmann & Huneck, U.S.A., 2003, *C. Smith s.n.* (BG), [*Ramalina mannii* Tuck.], –, MG926019*, MG926111*, MG926208*, MG926280*. ***Rolfidium coccocarpioides*** (Nyl.) Timdal, Mauritius, 1991, *Krog & Timdal MAU61/08* (O), AY762096, –, AY756361, AY756423, MG926281*. ***Ropalospora lugubris*** (Sommerf.) Poelt, Iceland, 2009, *Timdal 11170* (O), MG925922*, MG926020*, –, –, –. ***Schadonia fecunda*** (Th.Fr.) Vězda & Poelt, Austria, *Hafellner 43170* (GZU), AY756376, –, AY756362, –, –. ***Scutula miliaris*** (Wallr.) Trevis., Sweden, 2002, *Wedin 6850* (UPS), AY567790, –, –, –, –. ***Scutula tuberculosa*** (Th.Fr.) Rehm, Sweden, 2000, *Wedin 6356* (UPS), AY567789, –, –, –, –. ***Solenopsora olivacea*** (Fr.) H.Kilias, Montenegro, 2009, *Rui & Timdal 10987* (O), –, KF689887, –, –, –. ***Speerschneidera euploca*** (Tuck.) Trevis., U.S.A., 1998, *Egan 14906* (F), AY300912, –, AY300862, –, –. ***Squamarina gypsacea*** (Sm.) Poelt, Portugal, 2002, *Rui & Timdal POO2/07* (O), –, HQ650647, –, DQ986853, DQ992420. ***Stirtoniella kelica*** (Stirt.) D.J.Galloway & al., New Zealand, 1992, *Tibell 19456* (UPS), MG925923*, MG926021*, –, –, –. ***Tasmidella variabilis*** Kantvilas & al., Australia, 2002, *Kantvilas 577/02* (BG), MG925924*, MG926022*, –, –, MG926282*. ***Teloschistes flavicans*** (Sw.) Norman, Costa Rica, 2003, *Lutzoni & al. 03.22.03-13A* (DUKE), JQ301520, JQ301685, JQ301578, –, –. ***Tephromela atra*** (Huds.) Hafellner, Finland, 2005, *Stenroos 5688* (DUKE), DQ986879, HQ650608, DQ986766, DQ986835, DQ992452. ***Thamnolecania brialmontii*** (Vain.) Gyeln., Antarctica (South Sandwich Islands), 1997, *Convey 121* (AAS), MG925925*, AF282066, MG926112*, MG926209*, MG926283*. ***Toninia aromatica*** (Sm.) A.Massal., Norway, 1995, *Haugan & Timdal 4819* (O), [*Toniniopsis aromatica* (Sm.) Kistenich & al.], MG925926*, AF282126, MG926113*, MG926210*, MG926284*. ***Toninia athallina*** (Hepp) Timdal, Austria, 1994, Poelt, *Pittoni & Köckinger s.n.* (GZU), [*Kiliasia athallina* (Hepp) Hafellner], –, MG926023*, MG926114*, MG926211*, MG926285*. ***Toninia auriculata*** Timdal, Peru, 2006, *Timdal 10481* (O), [*Bibbya albomarginata* (H.Kilias & Gotth.Schneid.) Kistenich & al.], MG925927*, MG926024*, MG926115*, MG926212*, MG926286*. ***Toninia bullata*** (Meyen & Flot.) Zahlbr. **I**, New Zealand, 2002, *Bannister s.n.* (O), [*Bibbya bullata* (Meyen & Flot.) Kistenich & al.], MG925928*, MG926025*, –, –, –. ***Toninia bullata*** **II**, Australia, 1994, *Elix & Streimann 40393* (O), [*Bibbya bullata*], MG925929*, MG926026*, MG926116*, –, MG926287*. ***Toninia bumamma*** (Nyl.) Zahlbr., South Africa, 2015, *Rui & Timdal 13898* (O), [*Rolfidium bumammum* (Nyl.) Kistenich & al.], MG925930*, MG926027*, MG926117*, MG926213*, MG926288*. ***Toninia candida*** (Weber) Th.Fr. **I**, Norway, 2012, *Hofton 12366* (O), [*Thalloidima candidum* (Weber) A.Massal.], MG925931*, MG926028*, –, MG926214*, MG926289*. ***Toninia candida*** **II**, Norway, 1997, *Bratli & Timdal 8733* (O), [*Thalloidima candidum*], MG925932*, AF282117*, MG926118*, MG926215*, MG926290*. ***Toninia cinereovirens*** (Schaer.) A.Massal., Norway, 1994, *Haugan & Timdal 7953* (O), AY567724, AF282104, AY756365, AY756429, AM292781. ***Toninia coelestina*** (Anzi) Vězda, Norway, 1997, *Haugan 5985* (O), [*Toniniopsis coelestina* (Anzi) Kistenich & al.], MG925933*, AF282127, MG926119*, –, MG926291*. ***Toninia nigropallida*** (Nyl.) Abbayes **I**, Zimbabwe, 1994, *Clerc & al. s.n.* (G), [*Rolfidium nigropallidum* (Nyl.) Kistenich & al.], –, MG926029*, –, –, –. ***Toninia nigropallida*** **II**, Zimbabwe, 1994, *Clerc & al. s.n.* (G), [*Rolfidium nigropallidum*], MG925934*, MG926030*, –, –, –. ***Toninia philippea*** (Mont.) Timdal, Norway, 1994, *Haugan & Timdal H3750* (O), [*Kiliasia philippea* (Mont.) Hafellner], –, AF282112, –, –, –. ***Toninia physaroides*** (Opiz) Zahlbr. **I**, Norway, 2013, *Bendiksby & al. 12969* (O), [*Thalloidima physaroides* (Opiz) Kistenich & al.], MG925935*, MG926031*, –, MG926216*, MG926292*. ***Toninia physaroides*** **II**, Norway, 1995, *Haugan & Timdal 8121* (O), [*Thalloidima physaroides*], MG925936*, MG926032*, MG926120*, MG926217*, MG926293*. ***Toninia ruginosa*** (Tuck.) Herre, Greenland, 2005, *Timdal 10087* (O), [*Bibbya ruginosa* (Tuck.) Kistenich & al.], MG925937*, MG926033*, MG926121*, MG926218*, MG926294*. ***Toninia sculpturata*** (H.Magn.) Timdal **I**, Russia, 1992, *Haugan & Timdal YAK17/30* (O), [*Kiliasia sculpturata* (H.Magn.) Kistenich & al.], MG925938*, MG926034*, MG926122*, MG926219*, MG926295*. ***Toninia sculpturata*** **II**, Norway, 1993, *Haugan & Timdal 7829* (O), [*Kiliasia sculpturata*], –, AF282110, –, –, MG926296*. ***Toninia squalescens*** (Nyl.) Th.Fr., Norway, 2000, *Rui & Timdal 9211* (O), MG925939*, –, –, –, –. ***Toninia squalida*** (Ach.) A.Massal., Norway, 1996, *Haugan 4970* (O), MG925940*, AF282103, MG926123*, MG926220*, MG926297*. ***Toninia thiopsora*** (Nyl.) H.Olivier, Canary Islands, 2002, *Sánchez-Pinto 5146* (O), MG925941*, MG926035*, –, –, –. ***Toninia toniniana*** (A.Massal.) Zahlbr. **I**, Spain, 2015, *Timdal 13773* (O), [*Thalloidima toninianum* (A.Massal.) A.Massal.], MG925942*, MG926036*, MG926124*, MG926221*, MG926298*. ***Toninia toniniana*** **II**, Austria, 1996, *Türk 20721 in Obermayer: Lichenoth. Graec. 5: 100* (O), [*Thalloidima toninianum*], –, AF282115, MG926125*, MG926222*, MG926299*. ***Toninia tristis*** (Th.Fr.) Th.Fr., Norway, 1995, *Haugan & Timdal 8109* (O), –, AF282105, –, –, MG926300*. ***Toniniopsis obscura*** Frey, Canada, 1999, *Westberg TNW2182* (UPS), MG925943*, MG926037*, MG926126*, MG926223*, MG926301*. ***Trichoramalina crinita*** (Tuck.) Rundel & Bowler, Mexico, 1997, *Nash 40171 in Nash: Lich. exs. 295* (BG), *Ramalina crinita* Tuck., MG925944*, –, MG926127*, MG926224*, MG926302*. ***Trichoramalina melanothrix*** (Laurer) Rundel & Bowler, Namibia, 2002, *Wirth & Heklau s.n.* (KR), [*Niebla melanothrix* (Laurer) Kistenich & al.], MG925945*, MG926038*, MG926128*, MG926225*, MG926303*. ***Tylothallia biformigera*** (Leight.) P. James & H.Kilias, Sweden, 1997, *Ekman 3096* (BG), MG925946*, AF282077, MG926129*, MG926226*, MG926304*. ***Waynea californica*** Moberg, U.S.A., 1995, *Ekman L1486* (UPS), MG925947*, –, MG926130*, –, MG926305*.

**Table S1.** Median character state probabilities under three different priors for the MRCA of the Ramalinaceae and five selected subclades (Fig. 2: clades C–G) that we have named the *Bacidia*-, *Toninia*-, *Rolfidium*-, *Biatora*-, and *Ramalina*-group, respectively. The three tested priors: equal, maddfitz (= Maddision & FitzJohn) and Yang.

| | | Spores | | | | | | | | | | Climate | | | | | | | | | Vegetative dispersal | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Length:Width | | Septation | | | Growth form | | | | Zone | | | Humidity | | Substrate | | | | special structures | |
| | | 0: ≤3<br>1:>3 | | 0: none<br>1: single septate,<br>2: multiseptate | | | 0: crustose<br>1: fruticose<br>2: foliose<br>3: phyllopsoroid | | | | 0: arctic/antarctic<br>1: temperate<br>2: tropical | | | 0: dry<br>1: moist | | 0: soil<br>1: rock<br>2: bark/wood<br>3: living organisms | | | | 0: absent<br>1: present | |
| Root node | pi-priors | 0 | 1 | 0 | 1 | 2 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 2 | 3 | 0 | 1 |
| Ramalinaceae | equal | 3.18 | 96.82 | 0.35 | 9.69 | 89.96 | 99.95 | 0 | 0 | 0.04 | 0 | 95.17 | 4.83 | 0.74 | 99.26 | 0 | 0.42 | 99.56 | 0.02 | 50.98 | 49.02 |
| | maddfitz | 0 | 100 | 0 | 0.12 | 99.88 | 100 | 0 | 0 | 0 | 0 | 99.99 | 0.01 | 0 | 100 | 0 | 0 | 100 | 0 | 53.72 | 46.28 |
| | Yang | 2.04 | 97.96 | 0.06 | 3.65 | 96.29 | 99.97 | 0 | 0 | 0.03 | 0 | 95.63 | 4.37 | 0.18 | 99.82 | 0 | 2.19 | 97.78 | 0.02 | 91.91 | 8.09 |
| *Bacidia*-group | equal | 3.95 | 96.05 | 14.96 | 0.95 | 84.09 | 98.19 | 0 | 0 | 1.81 | 0 | 76.75 | 23.25 | 0.28 | 99.72 | 0 | 0.10 | 99.90 | 0 | 61.87 | 38.13 |
| | maddfitz | 2.52 | 97.48 | 14.81 | 0.36 | 84.83 | 98.20 | 0 | 0 | 1.80 | 0 | 79.98 | 20.02 | 0.20 | 99.80 | 0 | 0.09 | 99.91 | 0 | 62.47 | 37.53 |
| | Yang | 4.59 | 95.41 | 14.92 | 0.70 | 84.38 | 98.07 | 0 | 0 | 1.92 | 0 | 76.92 | 23.08 | 0.31 | 99.69 | 0 | 0.13 | 99.87 | 0 | 73.60 | 26.40 |
| *Toninia*-group | equal | 1.84 | 98.16 | 0.79 | 1.28 | 97.93 | 99.82 | 0 | 0 | 0.18 | 0 | 90.06 | 9.94 | 0.24 | 99.76 | 0 | 0.11 | 99.89 | 0 | 67.37 | 32.63 |
| | maddfitz | 0.96 | 99.04 | 0.72 | 0.61 | 98.67 | 99.83 | 0 | 0 | 0.17 | 0 | 92.81 | 7.19 | 0.18 | 99.82 | 0 | 0.10 | 99.90 | 0 | 67.91 | 32.09 |
| | Yang | 2.31 | 97.69 | 0.75 | 1 | 98.25 | 99.80 | 0 | 0 | 0.20 | 0 | 90.21 | 9.79 | 0.27 | 99.73 | 0 | 0.14 | 99.86 | 0 | 77.76 | 22.24 |
| *Rolfidium*-group | equal | 18.64 | 81.36 | 0.38 | 22.43 | 77.19 | 99.80 | 0 | 0 | 0.20 | 0 | 75.97 | 24.03 | 11.60 | 88.40 | 1.62 | 22.23 | 73.20 | 2.94 | 66.29 | 33.71 |
| | maddfitz | 16.52 | 83.48 | 0.29 | 16.92 | 82.78 | 99.81 | 0 | 0 | 0.19 | 0 | 79.39 | 20.61 | 10.98 | 89.24 | 1.58 | 21.96 | 73.54 | 2.94 | 66.97 | 33.08 |
| | Yang | 18.62 | 81.38 | 0.33 | 20.12 | 79.56 | 99.78 | 0 | 0 | 0.22 | 0 | 76.15 | 23.85 | 11.90 | 88.01 | 1.21 | 18.44 | 77.95 | 2.40 | 79.30 | 20.70 |
| *Biatora*-group | equal | 0.51 | 99.49 | 2.76 | 2.45 | 94.79 | 99.98 | 0 | 0 | 0.02 | 0 | 99.53 | 0.47 | 0.04 | 99.96 | 0 | 0.22 | 99.78 | 0 | 58.36 | 41.64 |
| | maddfitz | 0.08 | 99.92 | 2.64 | 0.61 | 96.74 | 99.98 | 0 | 0 | 0.02 | 0 | 99.90 | 0.10 | 0.02 | 99.98 | 0 | 0.15 | 99.85 | 0 | 59.48 | 40.52 |
| | Yang | 0.72 | 99.28 | 2.68 | 1.66 | 95.66 | 99.98 | 0 | 0 | 0.02 | 0 | 99.55 | 0.45 | 0.04 | 99.96 | 0 | 0.27 | 99.72 | 0 | 78.14 | 21.86 |
| *Ramalina*-group | equal | 6.82 | 93.18 | 0.01 | 79.09 | 20.90 | 99.98 | 0 | 0 | 0.02 | 0 | 99.04 | 0.96 | 0.23 | 99.77 | 0 | 0.14 | 99.86 | 0 | 66.07 | 33.93 |
| | maddfitz | 4.78 | 95.22 | 0 | 77.72 | 22.28 | 99.98 | 0 | 0 | 0.02 | 0 | 99.52 | 0.48 | 0.17 | 99.83 | 0 | 0.11 | 99.89 | 0 | 66.87 | 33.13 |
| | Yang | 7.88 | 92.12 | 0.01 | 78.33 | 21.66 | 99.98 | 0 | 0 | 0.02 | 0 | 99.07 | 0.93 | 0.28 | 99.72 | 0 | 0.20 | 99.80 | 0 | 80.68 | 19.32 |

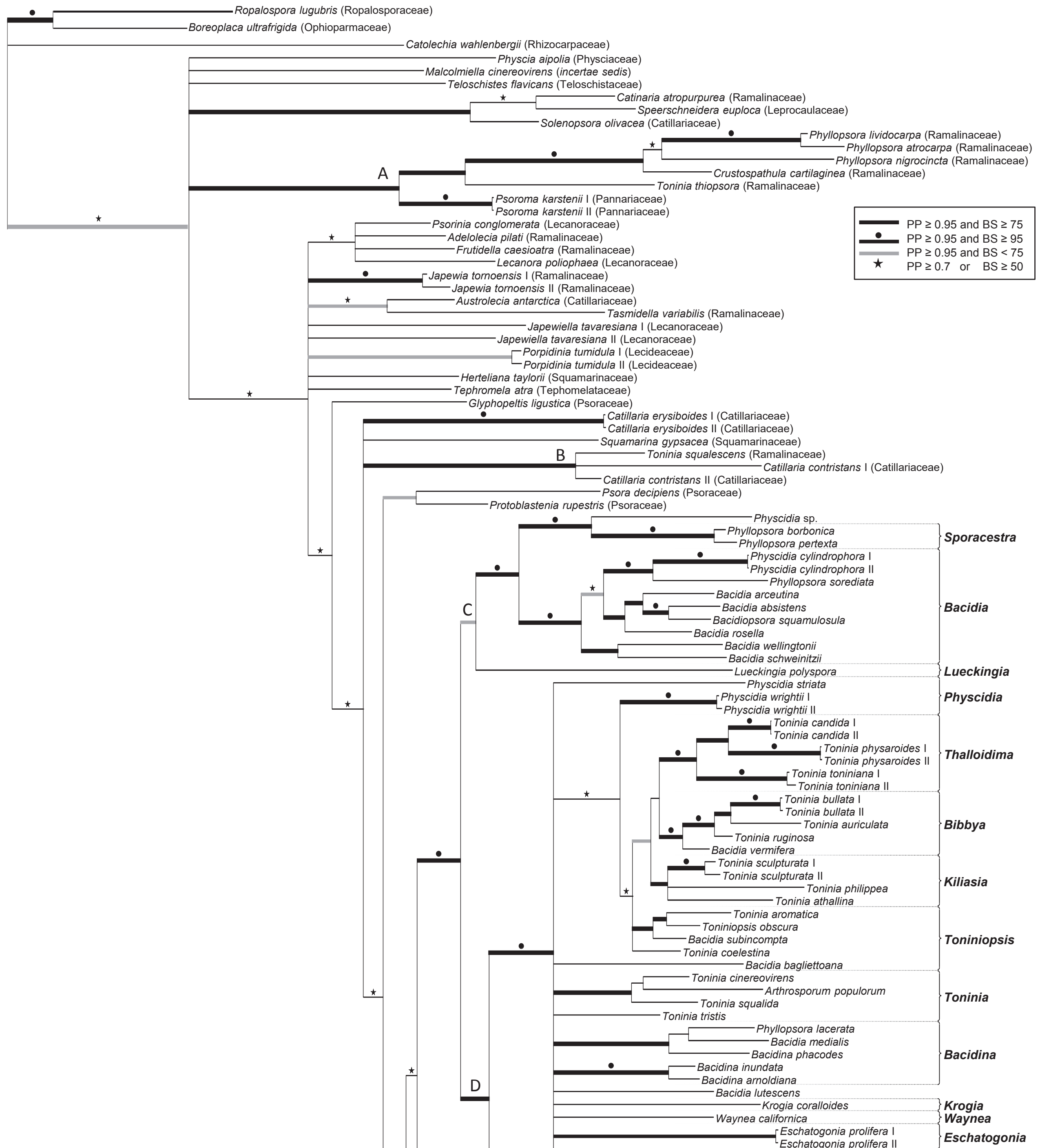


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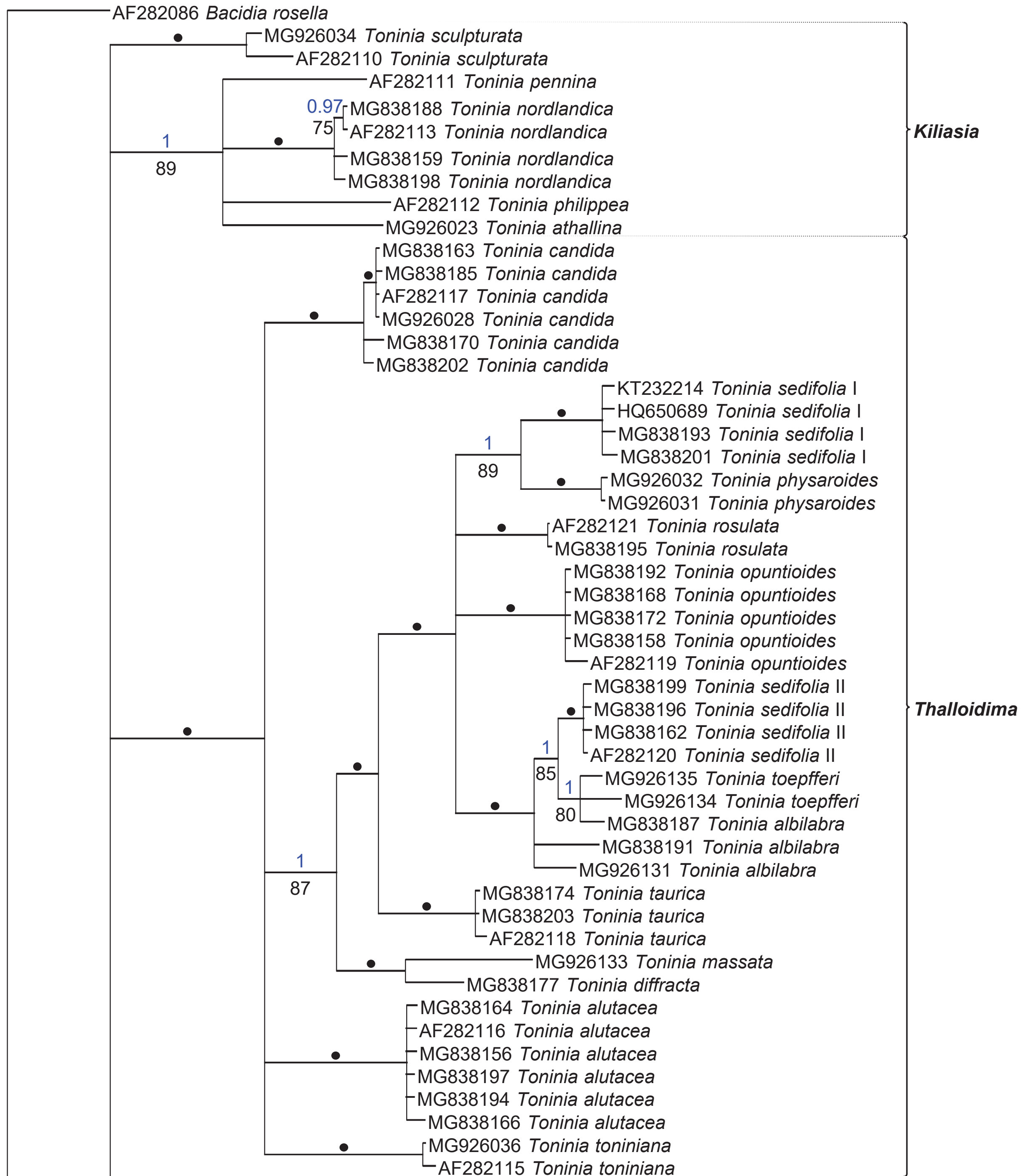
**Fig. S1.** Extended majority-rule consensus tree resulting from the Bayesian MCMC analysis on the 2-locus alignment with Bayesian PP ≥ 0.7 and/or Garli maximum likelihood BS ≥ 50 and branch lengths. Strongly supported branches (PP ≥ 0.95 and BS ≥ 75) are marked in bold; strongly supported branches with BS ≥ 95 are also marked with a dot above the branch; branches with PP ≥ 0.95 and BS < 75 are marked in bold grey; branches only supported with PP ≥ 0.7 or BS ≥ 50 are marked with an asterisk above the branch. The starting node of the Ramalinaceae is indicated with an arrow, PP (above branch) and BS (below branch). Family affiliations according to Lücking & al. (2017a, b). *Boreoplaca ultrafrigida* and *Ropalospora lugubris* were used for rooting. Seven major clades are distinguished (A–G). Terminals are named according to the taxonomy prior to this study. The revised genus affiliation is indicated on the right.

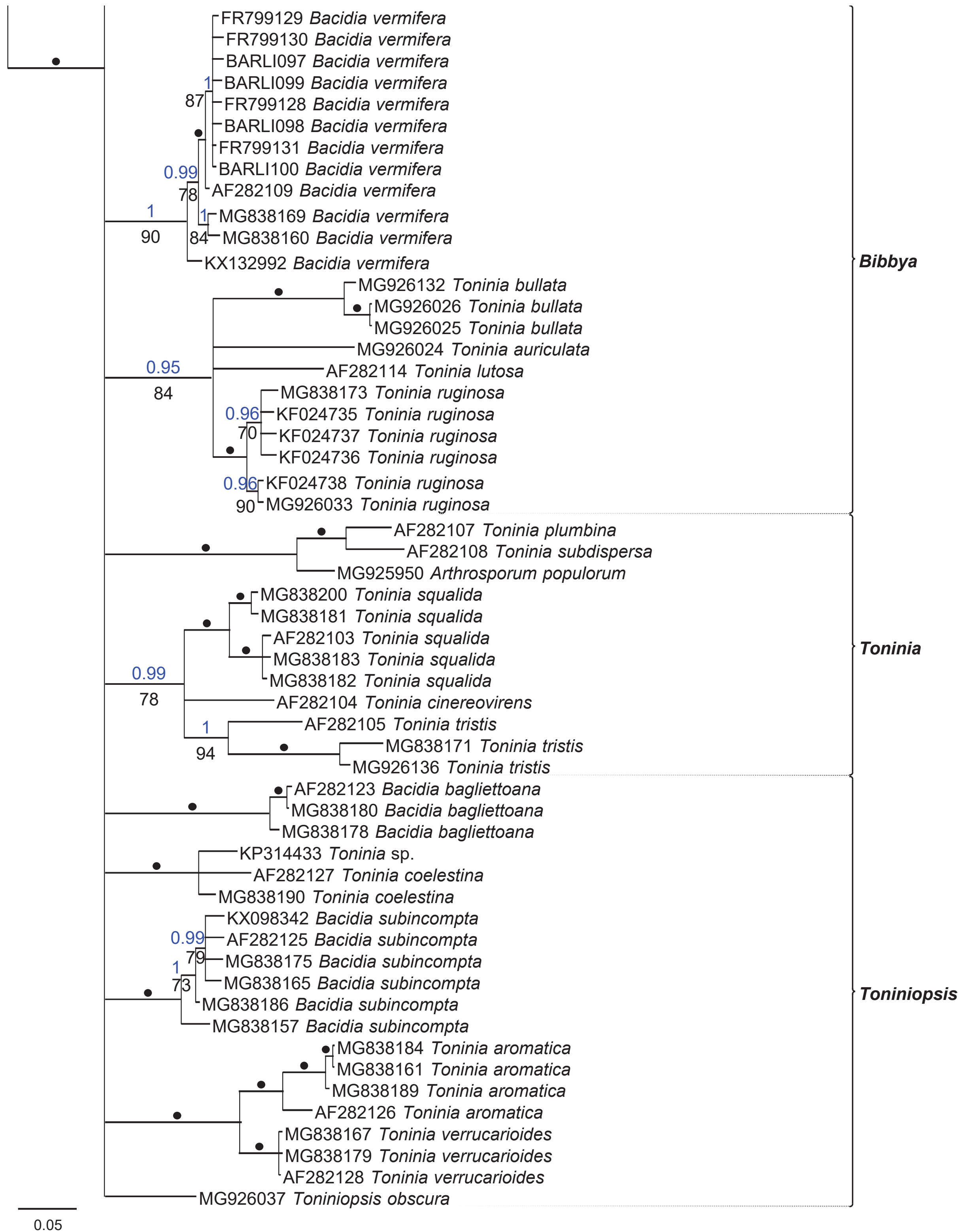
Continued from previous page
Phyllopsora leucophyllina
Phyllopsora brakoae
Phyllopsora labriformis
Aciculopsora salmonea
Scutula tuberculosa
Scutula miliaris
Bacidia circumspecta
Bacidia incompta
Toninia bumamma
Toninia nigropallida I
Toninia nigropallida II
Rolfidium coccocarpioides
Badimia dimidiata
Phyllopsora longiuscula
Phyllopsora breviuscula I
Phyllopsora breviuscula II
Phyllopsora mauritiana
Lecidea thaleriza I
Lecidea thaleriza II
Phyllopsora parvifoliella
Phyllopsora chlorophaea
Phyllopsora porphyromelaena
Phyllopsora sp. I
Phyllopsora sp. II
Crocynia gossypina I
Crocynia gossypina II
Crocynia pyxinoides
Biatora beckhausii I
Biatora beckhausii II
Biatora globulosa
Myrionora albidula
Biatora ligni-mollis
Biatora veteranorum I
Biatora veteranorum II
Biatora vacciniicola
Biatora rufidula
Biatora pallens
Biatora ocelliformis I
Biatora ocelliformis II
Ivanpisutia oxneri
Biatora hertelii
Biatora flavopunctata
Biatora vernalis
Thamnolecania brialmontii
Catillaria scotinodes I
Catillaria scotinodes II
Lecania cyrtella I
Lecania cyrtella II
Lecania erysibe
Lecania spadicea
Lecania nylanderiana
Lecania fuscella
Lecania aipospila
Bilimbia sabuletorum
Bilimbia lobulata
Lecidea albohyalina
Mycobilimbia epixanthoides
Mycobilimbia tetramera I
Mycobilimbia pilularis
Mycobilimbia tetramera II
Ramalina inflata
Ramalina fraxinea
Ramalina siliquosa
Ramalina dilacerata
Ramalina fastigiata
Ramalina pollinaria
Trichoramalina crinita
Ramalina sinensis
Ramalinopsis mannii
Ramalina lacera
Niebla combeoides I
Niebla homalea
Niebla combeoides II
Trichoramalina melanothrix
Cliostomum corrugatum
Cenozosia inanis
Stirtoniella kelica
Tylothallia biformigera
Lopezaria versicolor
Megalaria grossa I
Megalaria grossa II
Megalaria laureri
Parallopsora
Aciculopsora
Scutula
Bellicidia
Rolfidium
Badimia
Phyllopsora
Biatora
Thamnolecania
Lecania
Bilimbia
Mycobilimbia
Ramalina
Niebla
Cliostomum
Cenozosia
Stirtoniella
Tylothallia
Megalaria
E
F
G
1
60
Ramalinaceae
0.05




*Continued on next page*

**Fig. S2.** Phylogenetic hypothesis of *Toninia* species and presumed close relatives based on all available ITS sequences. The DNA sequences with GenBank accession numbers MG838156–MG838203 were produced at the Canadian Centre for DNA Barcoding (CCDB; http://www.ccdb.ca) for the OLICH project (http://nhm2.uio.no/olich) at the Norwegian Barcode of Life project (NorBOL; http://www.norbol.org). The sequences for the remaining accessions were produced as described in the Materials and Methods section. The phylogenetic analysis followed the description for the 5-locus and 2-locus alignment. *Bacidia rosella* was used for rooting. The figure shows the extended majority rule consensus tree resulting from the Bayesian MCMC analysis with Bayesian PP ≥ 0.95 (above branch) and Garli maximum likelihood BS ≥ 70 (below branch) and branch lengths. GenBank accession and BOLD record (starting with BARLI) numbers are included in the respective terminal descriptions. Our revised genus-level taxonomy is indicated on the right.

*Continued from previous page*



IV

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# Three new species of *Krogia* (Ramalinaceae, lichenised Ascomycota) from the Paleotropics

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## Abstract

*Krogia borneensis* Kistenich & Timdal, *K. isidiata* Kistenich & Timdal and *K. macrophylla* Kistenich & Timdal are described as new species, the first from Borneo and the two latter from New Caledonia. The new species are supported by morphology, secondary chemistry and DNA sequence data. *Krogia borneensis* and *K. isidiata* contain sekikaic and homosekikaic acid, both compounds reported here for the first time from the genus. *Krogia macrophylla* contains an unknown compound apparently related to boninic acid as the major compound. DNA sequences (mtSSU and nrITS) are provided for the first time for *Krogia* and a phylogeny of the genus based on 15 accessions of five of the six accepted species is presented. *Krogia antillarum* is reported as new to Brazil, Guatemala and Mexico.

## Keywords

Borneo, New Caledonia, lichens, *Phyllopsora*, phylogeny, rainforest, TLC

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## Introduction

*Krogia* Timdal is a corticolous genus occurring in tropical humid forests and rainforests. It closely resembles the much more common genus *Phyllopsora* Müll. Arg. in thallus morphology, but differs mainly in having a weak or absent amyloid reaction in the tholus of the asci and filiform, curved ascospores that are spirally arranged in the ascus (Timdal 2002). In *Phyllopsora*, the tholus shows a deeply amyloid conical structure (*Bacidia*-type) and the ascospores vary from ellipsoid to acicular, but are never spirally arranged. Nearly every examined specimen of *Krogia* has at least some scattered red or purple patches on the thallus or apothecia caused by non-crystalline, acetone-insoluble pigment(s).

Three species of *Krogia* are known: *K. antillarum* Timdal (the West Indies; Timdal 2009), *K. coralloides* Timdal (Mauritius; Timdal 2002) and *K. microphylla* Timdal (the Dominican Republic; Lumbsch et al. 2011). All species are recently discovered and known from only a few collections.

During revision of material of *Phyllopsora* from Southeast Asia and Oceania, we have come across material of three apparently undescribed *Krogia* species. There are no published sequences of *Krogia*, but we have provided sequences of the mitochondrial small subunit (mtSSU) and of the nuclear ribosomal transcribed spacer region (ITS) from the three putative new species and from two of the three previously described species. The sequences, some of which were taken from an unpublished paper on the phylogeny of the Ramalinaceae (Kistenich et al. in press), were used to infer a phylogeny.

## Material and methods

### The specimens

The specimens of the three new species were discovered during ongoing global studies of *Phyllopsora* by Kistenich and Timdal in material provided by Rikkinen (New Caledonia) and Thüs, Vairappan and Wolseley (Borneo), with additional specimens provided by A. Elvebakk (New Caledonia) and A. Paukov (Borneo). The specimens are deposited in B, BM, BORH, H, O and PC. DNA sequences of the two previously described *Krogia* species were generated from specimens in B and O and from a specimen provided by P. Diederich (hb Diederich). Additionally, we included 14 mtSSU and 12 ITS sequences (Table 1) from nine species in six genera known to be closely related to *Krogia* as well as from the holotype of the genus *Krogia*, *K. coralloides*, from a previous molecular study on the family Ramalinaceae (Kistenich et al. in press).

### Anatomy

Microscope sections were cut using a freezing microtome and mounted in water, 10% KOH (K), lactophenol cotton blue and a modified Lugol's solution, in which water

**Table 1.** Specimens used in this study with voucher information, major lichen substances and GenBank accession numbers. New sequences are indicated by accession numbers in bold.

| Species and sequence ID | Voucher | Major lichen substances | mtSSU | ITS |
|---|---|---|---|---|
| *Aciculopsora salmonea* | Costa Rica, 2004, Lücking 17543 (BR), **isotype** | – | MG925842 | MG925948 |
| *Bacidia rosella* | Sweden, 1997, Ekman 3117 (BG) | – | AY300877 | AF282086 |
| *Bacidia rubella* | Switzerland, van den Boom 41103 (LG DNA 578) | – | JQ796830 | JQ796852 |
| *Bacidia sipmanii* | Tenerife, Sérusiaux s.n. (LG DNA 361) | – | JQ796832 | JQ796853 |
| *Bacidina brittoniana* | USA, 1999, Ekman 3657 (BG) | – | – | MG925954 |
| *Bacidina delicata* | France, Sérusiaux s. n. (LG DNA 369) | – | JQ796834 | JQ796854 |
| *Bacidina neosquamulosa* | Netherlands, van den Boom 41056 (LG DNA 490) | – | JQ796837 | JQ796855 |
| *Bacidina phacodes* | Sweden, 1998, Ekman 3414 (UPS) | – | AY567725 | AF282100 |
| *Eschatogonia prolifera* I | Peru, 2006, Timdal 10207 (O) | didymic acid | MG925870 | MG925969 |
| *Eschatogonia prolifera* II | Peru, 2006, Timdal 10429 (O) | didymic acid | MG925871 | MG925970 |
| *Krogia antillarum* I | Trinidad And Tobago, 2008, Rui & Timdal 10844 (O), **paratype** | 4-O-methylcrypto-chlorophaeic acid | **MH174271** | **MH174281** |
| *Krogia antillarum* II | Guatemala, 2002, Andersohn s.n. (B) | 4-O-methylcrypto-chlorophaeic acid | **MH174272** | – |
| *Krogia antillarum* III | Mexico, 1994, Wolf & Sipman 2052 (B) | 4-O-methylcrypto-chlorophaeic acid | **MH174273** | **MH174282** |
| *Krogia antillarum* IV | Brazil, 2015, Dahl, Kistenich, Timdal & Toreskaas AM-39 (O) | 4-O-methylcrypto-chlorophaeic acid | **MH174274** | **MH174283** |
| *Krogia borneensis* I | Malaysia, 2013, Vairappan & Thüs L291 (BORH), **holotype** | sekikaic acid, homosekikaic acid | **MH174275** | – |
| *Krogia borneensis* II | Malaysia, 2012, Wolseley, Thüs & Vairappan D-3-10-2 (BM) | sekikaic acid, homosekikaic acid | **MH174276** | – |
| *Krogia borneensis* III | Malaysia, 2014, Paukov 2234 (B) | sekikaic acid, homosekikaic acid | **MH174277** | – |
| *Krogia borneensis* | Malaysia, 1997, Wolseley Q21 p.p. (BM) | sekikaic acid, homosekikaic acid | – | – |
| *Krogia borneensis* | Malaysia, 2013, Vairappan & Thüs L229 (BM) | sekikaic acid, homosekikaic acid | – | – |
| *Krogia coralloides* I | Mauritius, 1991, Krog & Timdal MAU51/83 (O), **holotype** | boninic acid, unknown | MG925875 | MG925977 |
| *Krogia coralloides* II | Mauritius, 2016, Diederich 18455 (hb. Diederich) | boninic acid, unknown | **MH174278** | **MH174284** |
| *Krogia isidiata* I | New Caledonia, 2005, Elvebakk 05:633 (O), **holotype** | sekikaic acid, homosekikaic acid | – | **MH174285** |
| *Krogia isidiata* II | New Caledonia, 2016, Rikkinen 34385 (H) | sekikaic acid, homosekikaic acid | **MH174279** | **MH174286** |
| *Krogia isidiata* | New Caledonia, 2016, Rikkinen 35034 (H) | sekikaic acid, homosekikaic acid | – | – |
| *Krogia isidiata* | New Caledonia, 2016, Rikkinen 35688 (H) | sekikaic acid, homosekikaic acid | – | – |
| *Krogia macrophylla* I | New Caledonia, 2016, Rikkinen 36047 (H) | unknown | – | **MH174287** |
| *Krogia macrophylla* II | New Caledonia, 2016, Rikkinen 36077 (H), **holotype** | unknown | – | **MH174288** |
| *Krogia macrophylla* III | New Caledonia, 2016, Rikkinen 35037 (H) | unknown | – | **MH174289** |
| *Krogia macrophylla* IV | New Caledonia, 2011, Rikkinen 38565 (H) | unknown | **MH174280** | **MH174290** |
| *Physcidia wrightii* I | Mauritius, 1991, Krog & Timdal MAU14/14 (O) | sekikaic acid, divaricatic acid | MG925911 | – |
| *Physcidia wrightii* II | Mauritius, 1991, Krog & Timdal MAU13/10 (O) | sekikaic acid, divaricatic acid | MG925912 | – |
| *Toninia cinereovirens* | Norway, 1994, Haugan & Timdal 7953 (O) | – | AY567724 | AF282104 |
| *Waynea californica* | USA, 1995, Ekman L1486 (UPS) | – | MG925947 | – |

was replaced by 50% lactic acid. Amyloid reactions were observed in the modified Lugol's solution after pretreatment in K and crystals of lichen substances were observed using polarised light.

## Secondary chemistry

Thin-layer chromatography (TLC) was performed in accordance with the methods of Culberson (1972), modified by Menlove (1974) and Culberson and Johnson (1982). Examinations were made in the three standard solvent systems A, B' and C.

## DNA extraction, PCR and sequencing

We extracted DNA from apothecia and/or thallus tissue of 14 *Krogia* specimens. The DNA extraction followed the protocol described by Bendiksby and Timdal (2013). We selected the two genetic markers mtSSU and nrITS (including ITS1, 5.8S and ITS2) for molecular analyses. Polymerase chain reactions (PCR) were performed with the primer pairs mtSSU1 and mtSSU3R (Zoller et al. 1999) for mtSSU as well as ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for ITS. In case of poor amplification success, internal primers were used: mtSSUF (5'-ACCAGTAGTGAAGTATGTTGTT-3') and mtSSUR (5'-AACAACATACTTCACTACTGGT-3') for mtSSU and ITS_lichF and ITS_lichR (Bendiksby and Timdal 2013) for ITS. We used the following cycling conditions: 95 °C for 7 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 7 min. We used Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) with half-sized reactions, i.e. prior to adding DNA, we transferred 12 µl of the mixture to a new PCR tube. To this, we added 0.5 µl of template DNA and 1 µl of each primer (10 µM). The PCR products were purified with the Illustra ExoProStar Clean-Up Kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer's instructions, but with a 10-fold enzyme dilution. We sent the purified PCR products to Macrogen Europe (Amsterdam, The Netherlands) for Sanger sequencing according to the company's instructions for sample preparation.

## DNA sequence analysis

We assembled and edited the resulting sequences using the software Geneious R9 (Kearse et al. 2012). For the separate alignment of the variable ITS1 and ITS2 sequences, we used PASTA version 1.7 (Mirarab et al. 2015) with OPAL as aligner and merger, the maximum subproblem set to 50%, RAxML as the tree estimator under a GTR+Γ model and a maximum of 500 iterations. We also used PASTA for the mtSSU alignment with the same settings except that we used a GTR+I+Γ model. As the 5.8S alignment contains mainly conserved regions, the online version of MAFFT version

7.313 (http://mafft.cbrc.jp/alignment/software/; Katoh and Standley 2013) was used (G-INS-i) with default settings except that the scoring matrix was set to 2PAM. Alignments were concatenated for subsequent analyses.

We used PartitionFinder2 (Lanfear et al. 2016) to infer the best-fitting substitution models and partitioning scheme for the concatenated alignment with the Bayesian Information Criterion (BIC) to select amongst all possible combinations of models implemented in MrBayes (1-, 2- and 6-rate models). Subset rates were treated as proportional ('linked branch lengths'). We defined four potential subsets prior to the analysis: mtSSU, ITS1, 5.8S and ITS2.

Three *Bacidia* De Not. species, *B. rosella* (Pers.) De Not., *B. rubella* (Hoffm.) A. Massal. and *B. sipmanii* M. Brand et al., were used as outgroup in all phylogenetic analyses based on the molecular phylogeny of the Ramalinaceae (Kistenich et al. in press). We checked for incompatibilities amongst gene trees by subjecting each marker to a simple maximum likelihood bootstrap analysis as implemented in RAxML Black Box 8.2.10 (Stamatakis 2014) on the CIPRES webserver (Miller et al. 2010) with default settings. Resulting gene trees were inspected manually for incompatibilities.

The alignment was subjected to maximum likelihood analyses using Garli 2.01 (Zwickl 2006) on the CIPRES webserver (Miller et al. 2010) and on the Abel high performance computing cluster (University of Oslo, Norway) under the models and partitioning scheme suggested by PartitionFinder2. We searched for the best tree using 500 repetitions from a random tree. We ran the nonparametric bootstrapping analysis with 500 replicates, each on 10 search replicates from a random tree.

We analysed the alignment phylogenetically using MrBayes 3.2.6 (Altekar et al. 2004; Ronquist and Huelsenbeck 2003) with BEAGLE (Ayres et al. 2012) on the CIPRES webserver (Miller et al. 2010). We used a (1, 1, 1, 1, 1, 1) Dirichlet for the rate matrix, a (1, 1, 1, 1) Dirichlet for the state frequencies, an exponential (1) distribution for the gamma shape parameter and a uniform (0, 1) distribution for the proportion of invariable sites. Subset rates were assumed proportional with the prior distribution following a (1, 1, 1, 1, 1, 1, 1) Dirichlet. We assumed a compound Dirichlet prior on branch lengths (Rannala et al. 2011; Zhang et al. 2012). For the gamma distribution component of this prior, we set $\alpha = 1$ and $\beta = 0.5$, as the expected tree length $\alpha/\beta$ (taken from the preceding maximum likelihood analysis) was approximately 1.9. The Dirichlet component of the distribution was set to the default (1, 1). Four parallel Markov chain Monte Carlo (MCMC) runs were performed, each with six chains and the temperature increment parameter set to 0.2 (Altekar et al. 2004). The appropriate degree of heating, adjusted for swap rates in the interval 0.1–0.7, was determined by monitoring cold and hot chains in preliminary runs. We used a burnin of 50% and sampled every 1000$^{th}$ tree. The runs were diagnosed for convergence every $10^6$ generations and were set to terminate either at convergence or after having reached $100\times10^6$ generations. Convergence was defined as an average standard deviation of split frequencies (ASDSF) smaller than 0.01. We projected the bootstrap support (BS) values from the Garli-analysis on to the MrBayes majority rule consensus tree with posterior probabilities (PP) and collapsed branches with $BS < 50$ and $PP < 0.7$. The resulting trees were edited in TreeGraph 2 (Stöver and Müller 2010).

## Results

### Secondary chemistry

The results of the TLC analyses are shown in Table 1. We identified four lichen substances: 4-O-methylcryptochlorophaeic acid (in *K. antillarum*), sekikaic acid and homosekikaic acid (in *K. borneensis* and *K. isidiata*) and boninic acid (in *K. coralloides*). An unidentified major compound, similar to boninic acid in colour and fluorescence on the developed chromatograms, occurred in *K. coralloides* and *K. macrophylla*. On the chromatograms, the two compounds were first pale brown, then after a few days turning greyish-pink, $UV_{366}$+ blue and occurred in $R_f$-classes A:5, B':5, C:6; the unknown moved just above boninic acid in all solvent systems.

### Molecular data and phylogenetic analyses

We successfully generated DNA sequences for 14 *Krogia* specimens, including 10 mtSSU and 10 ITS sequences (Table 1). The final dataset comprised 29 accessions (Table 1) and resulted in a 1424 bp long alignment counting 28% missing data and 470 parsimony-informative sites. The alignment is available at TreeBase (https://treebase.org – study no. 22518).

Initial RAxML analyses produced congruent gene trees of the mtSSU and ITS datasets; only unsupported (< 0.7) topological differences between the consensus trees were observed. We therefore continued with the subsequent phylogenetic analyses. PartitionFinder2 suggested three subsets and two different substitution models, the GTR+G model for (1) mtSSU, (2) ITS1 and ITS2 and the K80+I model for (3) 5.8S. The likelihood score of the best tree found by Garli was –8023.487881. The Bayesian analysis halted automatically after 3 million generations, when the ASDSF in the last 50% of each run had fallen below 0.01. We used 6004 trees for constructing the final majority-rule consensus tree. The phylogenetic results generated by Garli and MrBayes showed no incongruences. The extended majority-rule consensus tree of our alignment (Fig. 1), based on the Bayesian topology with all compatible groups (BS ≥ 50 and/or PP ≥ 0.7), shows that all *Krogia* accessions group together in five distinct and well-supported clades with short terminal branches. Accessions of *Bacidina* Vězda were resolved as the phylogenetic sister clade to the *Krogia* accessions, albeit only supported by PP. Not all *Bacidina* accessions formed a distinct group, but were split in two clades. Except for accessions of the same species, i.e. *Eschatogonia prolifera* (Mont.) R. Sant. and *Physcidia wrightii* (Tuck.) Tuck., there was poor resolution for the remaining accessions resulting in polytomy for the backbone of the ingroup.

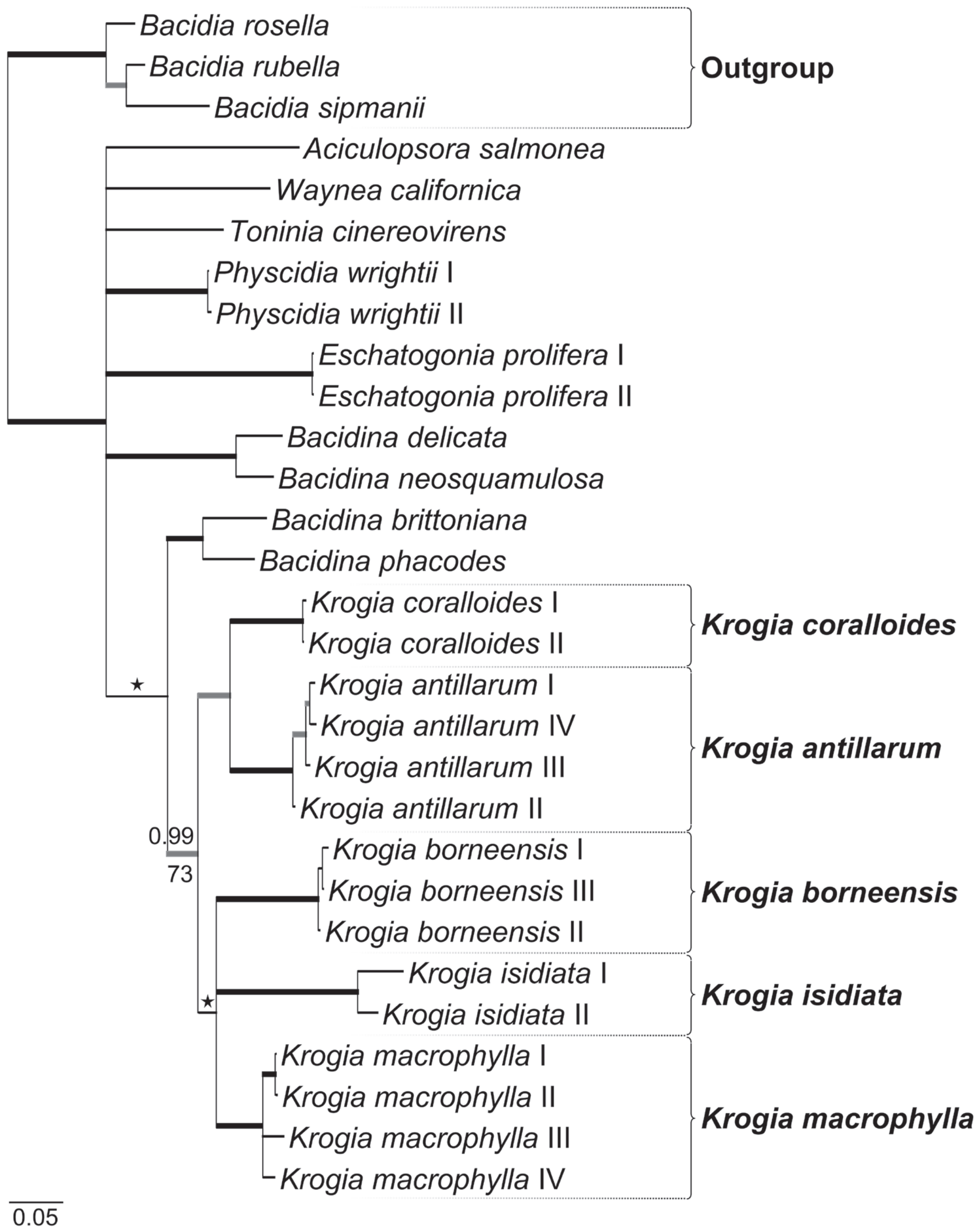


**Figure 1.** Hypothesis of the phylogenetic relationships and placement of the 15 *Krogia* accessions. It shows the extended majority-rule consensus tree resulting from the Bayesian MCMC analysis with Bayesian PP ≥ 0.7 (above branch) and/or Garli maximum likelihood BS ≥ 50 (below branch) and branch lengths. Strongly supported branches (PP ≥ 0.95 and BS ≥ 95) are marked in bold; branches with PP ≥ 0.95 and BS ≥ 70 are marked in bold grey; branches only supported by PP ≥ 0.7 are marked with an asterisk above the branch. *Bacidia rosella*, *B. rubella* and *B. sipmanii* were used as outgroup. Scale bar indicates 0.05 changes per site.

## Discussion

The genus *Krogia* was first described by Timdal in 2002 and only few reports of the genus have been published since (Lumbsch et al. 2011; Timdal 2009). Furthermore, no molecular phylogenetic studies investigating the monophyly of this genus have been conducted. In our study, we present the first multi-locus phylogenetic hypothesis of the genus *Krogia* (Fig. 1) and describe three new species from the Paleotropics based on molecular, morphological and chemical data.

All accessions of *Krogia* included in our molecular phylogeny (Table 1) form a well-supported, monophyletic group (Fig. 1). Five strongly supported clades can be distinguished within the genus. These five clades are delimited by rather long branches in comparison to the short terminal branches, indicating that the five clades correspond to five different species (Fig. 1). Two clades correspond to the two previously described species *K. coralloides* (Timdal 2002) and *K. antillarum* (Timdal 2009), while the remaining three clades correspond to the three new species *K. borneensis*, *K. isidiata* and *K. macrophylla*. The new species are morphologically distinct from one another and from the three known species, *K. antillarum*, *K. coralloides* and *K. microphylla*: *Krogia borneensis* forms more elongated and often linear squamules, *K. isidiata* forms characteristically long and sparingly branched isidia and *K. macrophylla* is a large species with wider squamules than any of the known species. We therefore describe them as new species. All *Krogia* species known contain the characteristic red or purple spots on the thallus and apothecia, consisting of one or more unknown pigments.

Our specimens of the genus *Krogia* were typically found amongst collections of undetermined tropical rainforest lichens, particularly amongst those tentatively named *Phyllopsora*. Timdal (2002) suggested a close relationship between *Krogia* and *Phyllopsora* based on overall morphological similarity. The two genera are anatomically distinct (Timdal 2002), although both form small squamules or lobes on bark. A comprehensive molecular phylogeny of the family Ramalinaceae, however, revealed the type species of the two genera to belong to different major clades within the family (Kistenich et al. in press). They are therefore not as closely related as previously anticipated.

On detailed microscopic examination of specimens of the new species *K. borneensis*, we discovered a thin, unicellular cortex on the upper and lower side of the thallus. This type of cortex, with a single layer of rounded or cuboid cells and a thick cell wall, is characteristic for the tropical genus *Eschatogonia* Trevis. (Timdal 2008). The cellular cortex surrounding the fungal tissue in *K. borneensis* has thinner cell walls and consists of somewhat longer, rather rectangular cells instead of the round and cuboid cells observed in *Eschatogonia* species. Our molecular phylogenetic hypothesis confirms that *Krogia* is not closely related to *Eschatogonia.* This indicates that the characteristic cortex in *Eschatogonia* has evolved independently.

*Krogia* is resolved as the phylogenetic sister to a clade consisting of the type species of *Bacidina*, *B. phacodes* (Körb.) Vězda and *B. brittoniana* (Riddle) LaGreca & Ekman (Fig. 1). *Krogia* differs from *Bacidina* s.str. (sensu Kistenich et al. in press) in having spirally arranged ascospores and a non- to weakly amyloid ascus tholus.

In recent years, lichenologists have increasingly focused on tropical regions and many new species have been described each year (e.g. Aptroot et al. 2018; Lücking et al. 2014; Masson et al. 2015; Naksuwankul et al. 2016; Sodamuk et al. 2017). It seems that the full diversity of tropical lichens is yet to be discovered. In our study, we report two new species of *Krogia*, *K. isidiata* and *K. macrophylla*, from but one island, the island Grande Terre belonging to New Caledonia. Therefore, further extensive collecting expeditions to remote tropical areas are necessary to explore the total diversity of the genus *Krogia*.

## Taxonomy

***Krogia borneensis* Kistenich & Timdal, sp. nov.**
Mycobank: MB825078
Fig. 2

**Diagnosis.** The species differs from *K. isidiata* in forming lacinules as vegetative dispersal units, not isidia, and from the other species in the genus in producing sekikaic and homosekikaic acid.

**Type.** Malaysia, Borneo, Sabah, Maliau conservation area, trail between Nepenthes Camp and waterfall Takob Akob, 4°43.4'N, 116°52.2'E, 900–1000 m alt., in low (few metres) and open pristine montane "Kerangas" (heath) forest with higher trees mostly along a small stream, on smooth barked tree in the vicinity of the stream, 2013-02-23, C. Vairappan & H. Thüs L291 (BORH, holotype) [TLC: sekikaic and homosekikaic acid; GenBank: MH174275 (mtSSU)].

**Description.** Thallus effuse, squamulose; squamules up to 1 mm wide, deeply divided into 0.1–0.2 wide lobes, ascending, imbricate, flattened, elongated to partly linear, often slightly laterally constricted, greyish-green with patches of red (K+ purple) spots, epruinose, glabrous; margin concolorous with upper side, not fibrillose; lower side white; lacinules formed by tips of the lobes. Upper cortex composed of a single layer of thick-walled cells with angular to shortly cylindrical lumina (resembling *Eschatogonia*-type), not containing crystals (polarised light!); algal layer 30–40 µm thick, filled with crystals dissolving in K; medulla composed of loosely interwoven hyphae, not containing crystals dissolving in K; lower cortex resembling upper cortex, both continuing over the edge of the squamule; prothallus brownish-black, often well developed. Apothecia (present in the holotype only) up to 0.6 mm diam. when simple, forming aggregates up to 1.5 mm diam., medium brown with red patches or entirely reddish-brown, more or less plane, with an indistinct, slightly paler, often flexuose margin; excipulum pale brown to colourless, composed of radiating, closely conglutinated hyphae, in inner part containing colourless crystals dissolving in K; hypothecium partly to entirely stained by a blood red pigment which dissolves in K with a purple effusion; epithecium colourless, not containing crystals. Ascospores filiform, curved, non-septate, spirally arranged in ascus, 20–31 × ca. 1.0 µm (n=10, from holotype). Conidiomata not seen.

**Figure 2.** *Krogia borneensis*. **A** Field photograph of the holotype **B** habitat at type locality **C** herbarium photograph of holotype. Scale bar: 1 mm. Photo: H. Thüs (**A, B**), E. Timdal (**C**).

**Chemistry.** Sekikaic acid (major), homosekikaic acid (major). Spot tests: all negative, except for red patches being K+ purple.

**Distribution.** The species is known from five localities in Borneo.

**Ecology**. The species occurred in rather low "Kerangas" (heath) forest vegetation or on transition vegetation between the heath and oak/conifer (particularly *Agathis*) forest at higher elevations (ca. 1000 m) on very poor soils on sandstone (Fig. 2B). The species always grew on the rather smooth barked, middle-sized trees together with various Pyrenulaceae and Graphidaceae.

**Etymology.** The specific epithet refers to its occurrence in Borneo.

**Remarks.** The medium-sized, flattened squamules make the species morphologically most similar to the neotropical *K. antillarum*. The squamules are more elongated, often linear and with more lateral constrictions in *K. borneensis* than in *K. antillarum*, which has more fan-shaped squamules. The former species has a thin, unicellular cortex on both upper and lower side, whereas the latter has a multicellular (20–30 µm thick) upper cortex and lacks a lower cortex (Timdal 2009). Chemically, the latter species differs in forming 4-O-methylcryptochlorophaeic acid.

*Krogia isidiata* shares the secondary chemistry (sekikaic and homosekikaic acid) with *K. borneensis*, but they differ in their vegetative dispersal units, the former producing cylindrical isidia, the latter flat lacinules (fragmenting squamules). The upper cortex of *K. isidiata* is multicellular (15–30 µm thick) and the lower cortex is absent.

**Additional specimens examined.** Malaysia, Borneo. *Sabah*: Danum, plot 88, dipterocarp forest logged in 1988, 4°58'N, 117°50'E, 131 m alt., 1997-04-30, P. Wolseley Q21 p.p. (BM 001104020); Danum valley, pristine lowland dipterocarp forest 4°57.96'N, 117°47.32'E, 200–400 m alt., 2012, P. Wolseley, H. Thüs & C. Vairappan D-3-10-2 (BORH); Maliau conservation area, trail between Nepenthes Camp and waterfall Takob Akob, transition between pristine montane "Kerangas" (heath forest) and montane oak-conifer (*Agathis*) forest, 4°42.6'N, 116°52.5'E, 900–1000 m alt., 2013, C. Vairappan & H. Thüs L229 (BM); Ranau district, Kinabalu park, Musang camp on the Tambuyukon trail (loc. T98), 6°12.720'N, 116°40.891' E, 1429 m alt., epiphytic, 2014-12-09, A. Paukov 2234 (B).

## *Krogia isidiata* Kistenich & Timdal, sp. nov.

Mycobank: MB825079
Fig. 3

**Diagnosis.** The species differs from *K. borneensis* in forming isidia as vegetative dispersal units, not lacinules, and from the other species in the genus in producing sekikaic and homosekikaic acid.

**Type.** New Caledonia, Province Sud, 20 km NNE of Nouméa, along dirt mountain road to Mt Dzumac, 3–400 m S of Seismic Station, ca. 22°03'S, 166°25'E, 830 m alt., on unidentified tree trunk in forest near the road, 2005-12-06, A. Elvebakk 05:633 (O L-186393, holotype; CANB, isotype [not seen]) [TLC: sekikaic and homosekikaic acid; GenBank: MH174285 (ITS)].

**Description.** Thallus effuse, squamulose; squamules up to 0.4 mm wide, rounded and adnate when young, later becoming somewhat elongated with a crenulate and slightly ascending margin, flattened, green, with scattered patches of red (K+ purple) spots, epruinose, glabrous; margin concolorous with upper side, not fibrillose; lower side white; isidia attached marginally to the squamules, simple or sparingly branched, up to 1.8 mm long and 0.1 mm wide. Upper cortex composed of a few layers of thick-walled, irregularly or mainly periclinally orientated hyphae with angular to shortly cylindrical lumina, 15–30 µm thick, lacking an epinecral layer, not containing crystals (polarised

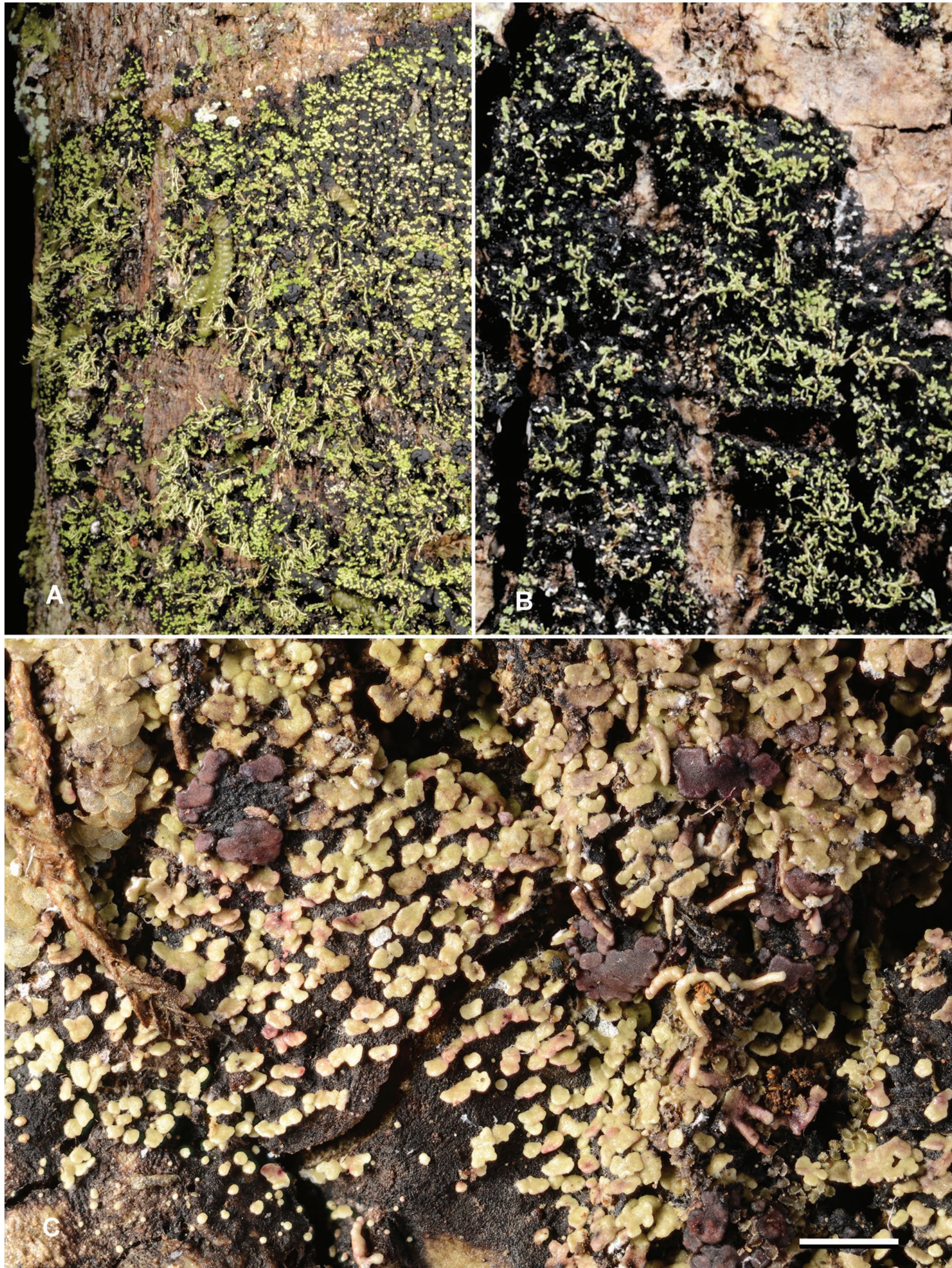


**Figure 3.** *Krogia isidiata.* **A** field photograph of JR35688 **B** field photograph of JR35034 **C** herbarium photograph of holotype. Scale bar: 1 mm. Photo: J. Rikkinen (**A, B**), E. Timdal (**C**).

light!); algal layer 30–40 µm thick, filled with crystals dissolving in K; medulla composed of loosely interwoven hyphae, containing crystals in the upper part; lower cortex lacking; prothallus brownish-black, well developed. Apothecia up to 0.8 mm diam. when simple, often forming aggregates up to 1.6 mm diam., dark reddish-brown to

brownish-black, more or less plane, with a rather distinct, concolorous or slightly darker, flexuose margin; excipulum dark reddish-brown throughout, composed of radiating, closely conglutinated, thick-walled hyphae with narrowly cylindrical lumina, inner part containing crystals dissolving in K; hypothecium dark reddish-brown, composed of closely conglutinated, thick-walled hyphae with narrowly cylindrical lumina, containing crystals dissolving in K; epithecium colourless, not containing crystals (but crystals present in hymenium below). Ascospores filiform, curved, simple, spirally arranged in ascus, ca. 20–30 × ca. 1.0 µm (estimate of curved spores). Conidiomata not seen.

**Chemistry.** Sekikaic acid (major), homosekikaic acid (major). Spot tests: all negative, except for red patches being K+ purple.

**Distribution.** The species is known from four collections at three localities in New Caledonia.

**Ecology**. The species grows on tree trunks in moist or mesic tropical forests and woodlands (Fig. 5B). All collections are from low-elevation sites and from ultramafic soils typical of the southern part of Grande Terre (main island of New Caledonia). It prefers shaded basal trunks that are otherwise mainly dominated by epiphytic bryophytes and/or leprarioid lichens.

**Etymology.** The specific epithet refers to its vegetative dispersal units, isidia.

**Remarks.** This species and *K. macrophylla* are the only isidiate species of *Krogia*. They differ morphologically mainly in the size and shape of the squamules. In *K. isidiata,* they are small (up to 0.4 mm wide), rounded and adnate to somewhat elongated and with a slightly ascending margin and, in *K. macrophylla,* large (up to 3 mm wide), elongated and ascending even when young. In *K. isidiata*, the squamules are attached to a prothallus, whereas in the latter species, a prothallus has not been observed. The former species contains sekikaic and homosekikaic acid, the latter an unknown compound resembling boninic acid.

*Krogia isidiata* shares the secondary chemistry with *K. borneensis*; see that species for discussion.

**Additional specimens examined.** New Caledonia. *Province Sud*: Yaté, dense forests along road RP 3 about 5 km west of Yaté, on tree trunk, 22°10'03.63"S, 166°54'10.15"E, 410 m alt., 2016-09-20, J. Rikkinen 34385 (H); Blue River Provincial Park, dense riparian forest near camp site on river bank, on tree trunk, 22°05'54.79"S, 166°38'20.24"E, 200 m alt. 2016-09-22, J. Rikkinen 35034 (H); Blue River Provincial Park, dense forest between camp site and road GR NC1, on tree trunk, 22°05'47.63"S, 166°38'22.54"E, 220 m alt., 2016-09-24, J. Rikkinen 35688 (H, PC).

## *Krogia macrophylla* Kistenich & Timdal, sp. nov.

Mycobank: MB825080
Fig. 4

**Diagnosis.** The species differs from all other species of the genus in forming larger (up to 3 mm wide, vs. up to 0.3–1.5 mm wide in the other species) squamules and, except for *K. coralloides*, in producing an unknown compound resembling boninic acid.

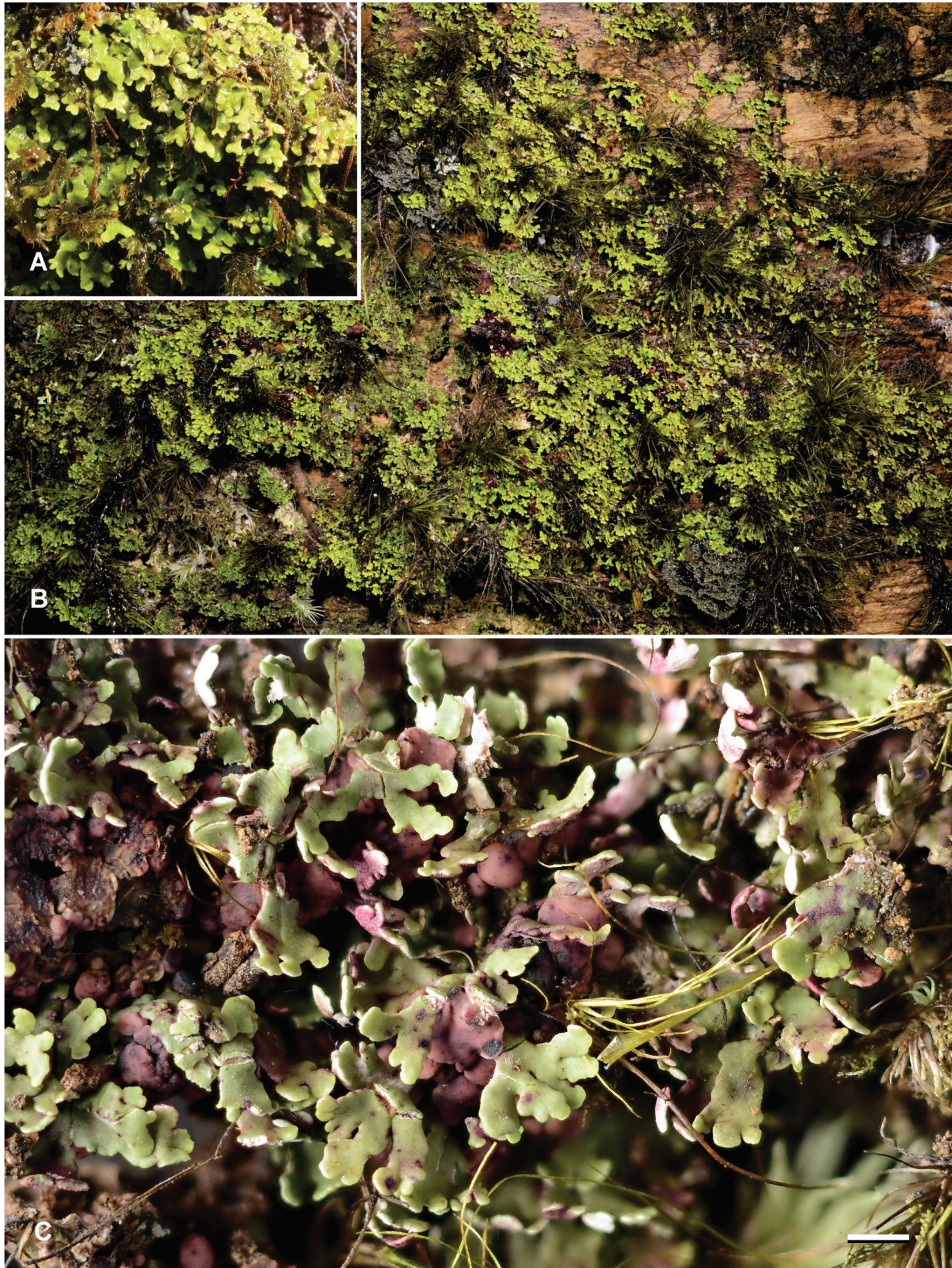


**Figure 4.** *Krogia macrophylla* **A** field photograph of JR36047 **B** field photograph of holotype **C** herbarium photograph of holotype. Scale bar: 1 mm. Photo: J. Rikkinen (**A, B**), E. Timdal (**C**).

**Type.** New Caledonia, Province Sud, Mont Mou Nature Reserve, in low dense mist forest along foot path to the mountain summit, on tree trunk, 22°03'39.66"S, 166°20'53.54"E, 1162 m alt., 2016-09-26, J. Rikkinen 36077 (H, holotype [TLC:

unknown compound resembling boninic acid; GenBank: MH174288 (ITS)]; PC, isotype).

**Description.** Thallus effuse, squamulose; squamules up to 3 mm wide, at first rounded, later becoming incised and deeply divided into up to 1 mm wide lobes, ascending even when young, often imbricate, flattened or with an up-turned tip, greyish-green, with patches of purple (K+ bluish-black) spots, epruinose, glabrous; margin concolorous with upper side, not fibrillose; lower side white; isidia (present in one specimen) attached marginally to the squamules, simple or sparingly branched, up to 1.6 mm long and 0.2 mm wide. Upper cortex composed of thick-walled, irregularly orientated hyphae with angular to cylindrical lumina, 50–80 µm thick, lacking an epinecral layer, not containing crystals (polarised light!); algal layer 25–35 µm thick, filled with crystals dissolving in K; medulla composed of loosely interwoven hyphae, upper part containing crystals dissolving in K; lower cortex lacking; prothallus lacking. Apothecia up to 1 mm diam. when simple, often forming aggregates up to 6 mm diam., pale to medium brown, with purple patches, plane to weakly convex, with an indistinct, slightly paler, often flexuose margin; excipulum pale brown to colourless, composed of radiating, closely conglutinated, thick-walled hyphae with narrowly cylindrical lumina, not containing crystals; hypothecium pale brown to colourless, composed of closely conglutinated, thick-walled hyphae with narrowly cylindrical lumina, not containing crystals; epithecium colourless, not containing crystals; purple pigment occurring patchily in exciple, hypothecium and hymenium. Ascospores filiform, curved, simple, spirally arranged in ascus, ca. 20–30 × ca. 1.0 µm (estimate of curved spores). Conidiomata not seen.

**Chemistry.** An unknown compound resembling boninic acid (major) and traces of additional compounds. Spot tests: all negative, except for purple patches being K+ deeper purple to bluish-black.

**Distribution.** The species is known from three localities in New Caledonia.

**Ecology.** The species grows on tree trunks in moist or wet tropical forests (Figs. 5A–C). Two collections are from montane mist forests and one from a low-elevation rainforest, all on ultramafic soils typical of the southern part of Grande Terre (main island of New Caledonia). It prefers shaded basal trunks that are otherwise mainly dominated by epiphytic bryophytes and/or leprarioid lichens.

**Etymology.** The specific epithet refers to the large squamules.

**Remarks.** In the examined material, one specimen (Rikkinen 38565) is isidiate, whereas the others are not. Our first assumption, that two species were involved, was not confirmed by the phylogeny (Fig. 1) and it appears that vegetative dispersal units, isidia, are produced occasionally in *K. macrophylla*. The only other isidiate species of *Krogia* is *K. isidiata*; see that species discussion.

*Krogia macrophylla* has a similar secondary chemistry to *K. coralloides* (an unknown substance resembling boninic acid as the major constituent) but differs in lacking the boninic acid that co-occurs as the major constituent in *K. coralloides* (Timdal 2002). *Krogia coralloides* forms smaller (up to 1 mm wide), more linear lobes with often down-turned tips.

**Figure 5.** Habitat images from New Caledonia **A** Mont Humboldt Nature Reserve, site of *K. macrophylla*, with *Araucaria humboldtensis* **B** Blue River Provincial Park, site of *K. isidiata* and *K. macrophylla* **C** Mont Mou Nature Reserve, holotype locality of *K. macrophylla*. Photo: J. Rikkinen.

**Additional specimens examined.** New Caledonia. *Province Sud*: Blue River Provincial Park, dense riparian forest near camp site on river bank, on tree trunk, 22°05'54.79"S, 166°38'20.24"E, 200 m alt., 2016-09- 22, J. Rikkinen 35037 (H);

locality data as for holotype, J. Rikkinen 36047 (H); Mont Humboldt Nature Reserve, close to Mont Humboldt refuge, in low dense mist forest along foot path from shelter towards the mountain summit, on tree trunk, 21°52'46.79"S, 166°24'49.17"E, 1320 m alt., 2011-11-09, J. Rikkinen 38565 (H).

## Key to the species of *Krogia*

| | |
|---|---|
| 1 | Squamules large, up to 3 mm wide and with up to 1 mm wide lobes; containing an unknown compound resembling boninic acid .......... ***K. macrophylla*** |
| – | Squamules smaller, up to 1.5 mm wide and with up to 0.4 mm wide lobes; chemistry various .......... **2** |
| 2 | Thallus with isidia; containing sekikaic and homosekikaic acid .......... ***K. isidiata*** |
| – | Thallus without isidia; chemistry various .......... **3** |
| 3 | Squamules minute, up to 0.3 wide and with up to 0.1 mm, simple lobes, forming a microphyllinous crust; not containing lichen substances .......... ***K. microphylla*** |
| – | Squamules medium sized, up to 1.5 mm wide and with up to 0.4 mm wide, coralloid elongated lobes; containing lichen substances .......... **4** |
| 4 | Thallus with brownish black hypothallus; containing sekikaic and homosekikaic acid .......... ***K. borneensis*** |
| – | Thallus without distinct hypothallus; chemistry different .......... **5** |
| 5 | Squamules mainly flattened; lobes up to 0.4 mm wide; containing 4-O-methylcryptochlorophaeic acid .......... ***K. antillarum*** |
| – | Squamules mainly convex; lobes up to 0.1 mm wide; containing boninic acid and an unknown, similar compound .......... ***K. coralloides*** |

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## Appendix

*Krogia antillarum* is reported here as new to Brazil, Guatemala and Mexico from the following examined specimens: Brazil. *Rio de Janeiro*: Parque Nacional do Itatiaia, along trail to Três Picos, 22.4358°S, 44.6118°W, 1090 m alt., on tree trunk in Atlantic rainforest, 2015-11-27, M.S. Dahl, S. Kistenich, E. Timdal & A.K. Toreskaas AM-39 (O L-202829). Guatemala. *Depto. Alta Verapaz*: NE of Cobán-Aragon, at the borders of Rio Cahabon (tierra fría), 1700 m alt., cloud forest, on *Liquidambar tyraciflua*, 2002-09-13, C. Andersohn s.n. (B 60-127330. Mexico. *Chiapas*: Municipio La Trinitaria, Parque Nacional Lagunas de Montebello, Paso del Soldado, 16°07'07"N, 91°43'09"W, 1500 m alt., bosque de *Pinus maximinoi* y *Quercus sapotifolia,* exposición N, epífita, 1994-11-29, J. Wolf & H. Sipman 2052 (B 60-110597).

V

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# DNA Sequencing Historical Lichen Specimens

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Biological specimens in natural history collections worldwide are increasingly being used in biogeographical, environmental, and taxonomic studies. For their meaningful use, correct species identification is crucial. For example, clarifying if a species is new to science requires an overview of what has already been described. This includes comparisons with existing authoritative specimens (types). Most type specimens are rather old and their DNA expected to be degraded to various extents. Comparative DNA sequence analysis is in regular use in taxonomic research of today and is essential for identifying and delimiting species. In this study, we focus on lichenized fungi (lichens), in which many species groups are highly inconspicuous and impossible to identify to species based on morphology alone. Our aim was to test the non-mutually exclusive hypotheses that DNA quality of lichens depends on (1) time since collection, (2) taxonomic affinity, and/or (3) habitat/ecology. We included two species from each of four different lichen genera (i.e., *Cladonia*, *Nephroma*, *Peltigera*, and *Ramalina*), each species pair with a different autecology. For each species, we included samples from approximately every 25 years from present to about 150 years back in time. We used a two-step PCR-based approach followed by sequencing on an Ion Torrent PGM to produce target sequences (mtSSU) of degraded DNA. We received satisfactory DNA sequence information for 54 of 56 specimens. We recovered full-length sequences for several more than 100-years-old specimens, including a 127-years-old specimen, and retrieved enough sequence information for species identification of a 150-years-old specimen. As expected, sequencing success was negatively correlated with age of the specimens. It also varied with taxonomic affinity. We found no significant correlation between sequencing success and habitat ecology of the investigated specimens. The herein tested Ion Torrent sequencing approach outperformed Sanger sequencing with regard to sequencing success and efficiency. We find the protocol used herein highly suitable for obtaining sequences from both young and old lichen specimens and discuss potential improvements to it.

**Keywords: museomics, herbarium genomics, Ion Torrent, mtSSU, lichens, natural history collections**

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## INTRODUCTION

Herbarium specimens are of immense value for biological research, for example in a wide range of spatial comparative analyses, for monitoring changes in biodiversity over time, and last, but not least, in taxonomic and systematic research (e.g., Lavoie, 2013; Greve et al., 2016; Soltis and Soltis, 2016; James et al., 2018; Meineke et al., 2018). In particular, biogeographical and environmental

research on climate change effects benefit extensively from the use of herbarium specimens (e.g., Holmes et al., 2016; Willis et al., 2017). However, a prerequisite for meaningful use of historical specimens in research is that these are correctly identified.

Taxonomic identification has traditionally been based on morphology, which has been the primary means of identification before the advance of molecular methods. Nowadays, morphology is often used in combination with DNA analyses and other data. A popular method for fast species identification of biological material is DNA barcoding (Hebert et al., 2003; Hajibabaei et al., 2007). DNA barcoding comprises the sequencing of a selected DNA region of the genome and BLAST searches against a library of named DNA barcodes (see also Kress et al., 2015), as implemented in the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert, 2007), a partner of the International Barcode of Life (iBOL, http://ibol.org) project. Of particular interest is DNA sequencing of type material, on which the barcode library ideally should be based, as these specimens link a unique scientific name to each species.

There are challenges, however, linked to obtaining DNA from long dead organic material. Weiß et al. (2016) found that DNA degrades over time, albeit contrasting earlier finding by Staats et al. (2011). Type specimens are typically rather old and the DNA is expected to have become degraded to various extents. Many types were collected during the numerous research expeditions in the nineteenth and early twentieth century and are often more than 100 years old. Moreover, type material is usually a highly limited resource (a single or few specimens, often in poor condition) and destructive sampling for DNA extraction can only be tolerated when rich specimens are available and successful output is ensured. Morphological investigations have therefore for long been the single option for identifying old specimens, and often also younger material (<10 years old) that for various reasons does not provide DNA of sufficient quality and quantity (Sohrabi et al., 2010). In addition to time since collection, also poor storage conditions, chemical treatment with mercuric chloride (mainly known to have been used on dried plants) and unfavorable drying processes may contribute to DNA degradation/inhibition in plant herbarium specimens (liverworts: Jankowiak et al., 2005; angiosperms: Staats et al., 2011; angiosperms: Lander et al., 2013), decreasing the chances for successful DNA recovery and usability in downstream processes. In recent years, increased focus on extracting and sequencing DNA from old natural history collections has led to the development of promising new approaches allowing for obtaining DNA sequences from specimens collected up to 210 years before DNA extraction (fungi: Larsson and Jacobsson, 2004; plant pathogens: Telle and Thines, 2008; angiosperms: Andreasen et al., 2009; insects: Prosser et al., 2016; algae: Suzuki et al., 2016). Such studies, however, are still few in number and restricted to a particular group of organisms. With advances in high-throughput sequencing (HTS) methods, some of the challenges (e.g., dominance of short fragments) are largely overcome (see review by Bieker and Martin, 2018). For example, Gutaker et al. (2017) managed to extract and shotgun sequence ultra-short fragments (<50 bases) of up to 180-years-old (i.e., time between collection and DNA extraction) *Arabidopsis* specimens. The problem of destructive sampling from valuable specimens has also been addressed in recent studies. For instance, Shepherd (2017) developed a non-destructive sampling technique for extracting DNA from plant specimens collected up to 73 years ago using erasers applied to the leaf surface.

In fungi, including lichens, morphological characteristics are often of limited use for taxonomic identification because of morphological similarity between genetically distinct taxa (Slepecky and Starmer, 2009). Cryptic species (sensu Struck et al., 2018) are common and represent a huge challenge for taxonomic work. The use of comparative DNA sequence analyses has therefore become crucial for inferring evolutionary history as well as identifying and delimiting fungal taxa (Lumbsch and Leavitt, 2011). DNA-sequencing of a single genetic marker, such as a DNA barcode marker, is most of the time sufficient for species identification in fungi (e.g., Seifert, 2009; Leavitt et al., 2013).

So far, only a few studies report successful DNA sequencing of selected short genetic markers from historical lichen material. Sohrabi et al. (2010) managed to PCR-amplify and Sanger-sequence 760 bases of nuclear ribosomal DNA from a 75-years-old *Aspicilia* specimen, but failed with an 80-years-old one. Bendiksby et al. (2014) successfully PCR-amplified and Sanger-sequenced several markers of two 100-years-old *Staurolemma* specimens. During routine investigations on various groups of lichenized fungi, our lichen research group has experienced that PCR-amplifying and generating Sanger sequences seems to be more difficult from some taxa (e.g., tropical rainforest lichens) than from others (e.g., boreal, saxicolous crustose lichens) of the same age. For angiosperms, Bakker et al. (2016) found herbarium material from wet-tropical regions to give lower sequence assembly success rates than material from dry regions. No studies have yet addressed similar questions for lichens and it therefore remains unclear which factors are primarily responsible for DNA degradation or failed sequencing success in lichens. We have also noticed that DNA in specimens older than 50 years often is highly fragmented (<200 bases) and extracts usually have low DNA concentrations (<0.5 ng/μl). These challenges with short fragments and low DNA concentrations were overcome by Prosser et al. (2016) who developed a simple and rather inexpensive protocol aimed at obtaining DNA barcodes from type specimens of Lepidoptera up to 120 years old. To our knowledge, no such study has so far been done on fungi, nor has the general applicability of HTS-methods for DNA-sequencing of historical lichen specimens been explored.

In the present study, we test the two-step PCR-based HTS protocol by Prosser et al. (2016) on historical lichen specimens using the Ion Torrent sequencing platform. Our aim is to acquire high quality DNA sequence data of the mtSSU, a much used DNA marker in lichen systematics. We use two lichen species from each of four different genera/families (**Figure 1**): one growing in humid coastal areas and the other in dry inland areas. We sampled specimens of each species collected in each of seven time periods from present to about 150 years back in time. The present study is a pilot-test of the following three hypotheses: (1) Sequence reads are more readily obtained from younger than older specimens, (2) sequence reads are more readily obtained from some taxa than others given the same age, and (3) sequence

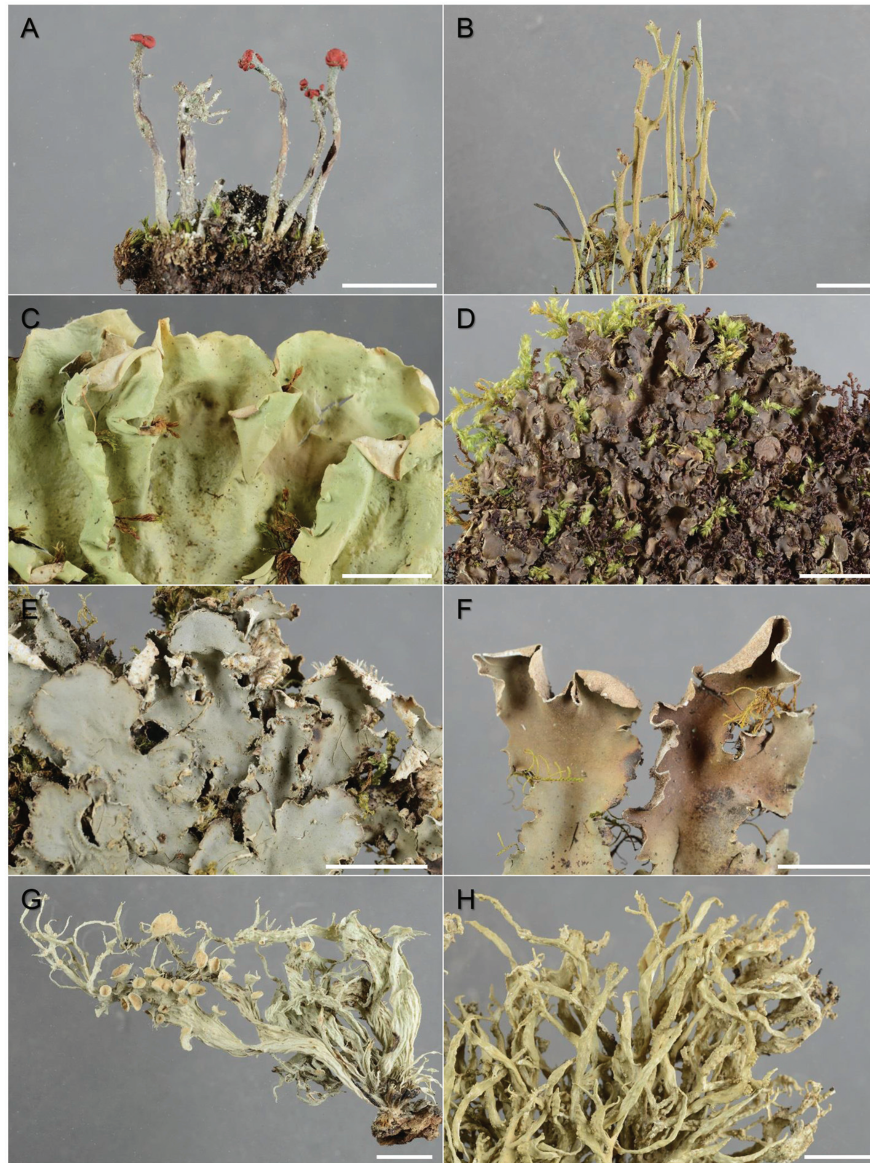


**FIGURE 1 |** Lichen species targeted in this study (specimens from the 2010-period): **(A)** *Cladonia floerkeana*, **(B)** *C. gracilis*, **(C)** *Nephroma acrticum*, **(D)** *N. laevigatum*, **(E)** *Peltigera collina*, **(F)** *P. malacea*, **(G)** *Ramalina fraxinea*, and **(H)** *R. siliquosa*. Scale bar = 1 cm.

reads are more readily obtained from species adapted to dry areas than from those adapted to humid areas.

## MATERIALS AND METHODS

### Taxon Sampling

Two species from the same genus but with different distributions (i.e., distributional point of gravity in oceanic ["humid"] vs. continental ["dry"] regions) were selected from each of four different lichen families, for which comprehensive archived collections were available at either of the herbaria O or TRH. We sampled each species from approximately every 25 years from present and to about 150 years back in time. To fulfill these criteria, we chose two representatives from the Cladoniaceae [i.e., *Cladonia floerkeana* (Fr.) Flörke and *C. gracilis* (L.) Willd.], the Nephromataceae [i.e., *Nephroma laevigatum* Ach. and *N. arcticum* (L.) Torss.], the Peltigeraceae [i.e., *Peltigera collina* (Ach.) Schrad. and *P. malaceae* (Ach.) Funck], and the Ramalinaceae [i.e., *Ramalina siliquosa* (Huds.) A.L. Sm. and *R. fraxinea* (L.) Ach.; **Figure 1**]; the first representative of each genus preferring mainly humid coastal areas while the second mainly growing in dry inland habitats. All selected species are common macrolichens in Norway and belong to the same class of lichenized ascomycetes, the Lecanoromycetes; the

genera *Cladonia* and *Ramalina* belong to the order Lecanorales, *Nephroma*, and *Peltigera* to Peltigerales. We selected a healthy and rich specimen of each species from the following seven periods: 2010 (**Figure 1**), 1985, 1960, 1935, 1910, 1885, and 1860 (±5 years if possible, in rare cases up to ±12 years; **Table 1**). Most of the selected specimens were collected in coastal and continental regions, respectively; in some cases, when no rich collections were available within the desired period, we selected "humid" specimens from inland areas and "dry" specimens from coastal areas (**Table 1**).

## Molecular Work

### DNA Extraction

We sampled 1–109 mg of thallus material from each specimen, depending on available material, for extracting DNA of the lichen mycobiont. Samples for periods 2010 and 1985 were extracted using the E.Z.N.A.® HP Plant DNA Mini Kit (OMEGA Bio-tek) following the manufacturer's instructions with modifications described by Bendiksby and Timdal (2013). Samples for time periods 1960–1885 were extracted following the protocol by Werth et al. (2016) using single silica-columns with the following modifications: We grinded the samples with two 3 mm tungsten carbide beads for 2 × 1 min at 20 Hz in a Mixer Mill 301 (Retsch GmbH & Co.). Instead of using the CTAB lysis buffer, we lysed the samples with the alternative buffer based on NaCl and SDS. For elution, we applied 60 µl elution buffer (OMEGA Bio-tek; pre-warmed to 65°C) to the column and incubated the sample at 65°C for 5 min prior to centrifugation. The elution step was repeated by applying the eluate once more on the same column to increase DNA yield resulting in 50–55 µl of DNA extract.

Samples from the 1860-period were extracted in a different lab (Clean Lab, NTNU University Museum) using the DNeasy Plant Mini Kit (QIAGEN) with the following modifications from the manual: The samples were homogenized for 2–8 min at 50 Hz on a TissueLyser LT (QIAGEN) with 1–2 steel beads. After lysis, 20 µl of 20 mg/ml proteinase K was added to each sample followed by an incubation at 45°C for 22 h. For elution, 65 µl AE buffer was added to each column and the samples incubated for 10 min at 37°C.

To reduce the risk of contamination, the DNA extractions of all samples collected prior to the 1960-period were carried out in a bleached workstation newly exposed to UV in a clean lab facility for sensitive samples with dedicated reagents, supplies and protective clothing. A Qubit 2.0 fluorometer (Invitrogen) with the High Sensitivity Kit (Invitrogen) was used for DNA quantification of all extracts. We checked the degree of DNA degradation by visualizing the DNA extracts on a standard 2% agarose gel.

### Primer Design and PCR Amplification

The setup for polymerase chain reactions (PCRs) followed the general protocol described in Prosser et al. (2016). While Prosser et al. (2016) designed their primers for a 658 bases fragment of the mitochondrial cytochrome oxidase I (COI), which is the preferred barcode marker for insects, we did not attempt to design primers for the fungal barcode marker, the internal transcribed spacer (ITS), as parts of this region are highly variable. Designing universal primers for our selected species would have been a very challenging task with low anticipated success rate. In addition, Mark et al. (2016) reported challenges with obtaining the correct ITS sequence due to several different copies within one single specimen. We therefore chose to focus our efforts on a ca. 900 bases long part of the up to ca. 2,000 bases long mitochondrial ribosomal small subunit (mtSSU). This fragment, as delimited by the primers mtSSU1 and mtSSU3R (Zoller et al., 1999), is frequently used in lichen systematics and for species delimitation (Amo de Paz et al., 2011; Leavitt et al., 2015; Zhao et al., 2017; Kistenich et al., 2018). We designed a set of seven forward and seven reverse primers (**Table 2**; **Figure 2**) covering all of the 900 bases using Primer3 v. 2.3 (Untergasser et al., 2012) based on mtSSU sequences for our selected taxa or closely related taxa available from GenBank (Benson et al., 2018). Each pair of primers used in concert, amplified fragments of ca. 110–190 bases length including overlap with the adjacent target fragments. A non-complementary 10 bases tail was added to each primer's 5′-end to decrease chimeric amplifications (**Table 2**).

We modified the two-stage, nested, multiplex PCR protocol by Prosser et al. (2016) to accommodate seven primer pairs. In the first round of PCR, we combined primers F1, F3, F5, and F7 with all seven reverse primers for PCR 1.1, and the primers F2, F4, and F6 with all reverse primers for PCR 1.2 (**Figure 2A**). In the second round of PCRs, we combined each forward primer with the three subsequent reverse primers in a separate PCR run using the PCR products from PCR 1.1 and 1.2 as template (**Figure 2B**). No second round of PCR was run for samples from the 2010-period since those amplified well enough in PCR round 1. Each PCR reaction (20 µl) contained 8.2 µl of 10% Trehalose (Merck KGaA), 0.1 µl of 10 mM dNTPs (GeneAmp), 2 µl of 25 mM $MgCl_2$ (KAPA Biosystems), 0.2 µl of each 10 µM primer (Sigma Aldrich), 0.1 µl of 5 U/µl KAPA Taq polymerase (Roche), and 2 µl of 10× polymerase Buffer B. Each reaction was filled up to 18 µl with ultra-pure DEPC-treated $H_2O$ (Invitrogen), the volume depending on the number of primers used, adding 2 µl of undiluted template. All PCRs were prepared inside a workstation for PCR set up in a clean lab for sensitive samples with dedicated reagents, supplies and protective clothing to minimize contamination. We used the same PCR programs as stated in Prosser et al. (2016), albeit with a touch-down gradient from 60 to 50°C during annealing. Products from each PCR were visualized on an agarose-gel.

To test for the presence of PCR-inhibitory substances, we performed PCR on three different dilutions (10, 100, and 1,000×) of each DNA extract from the 2010-period using the primers mtSSU1 and mtSSU3R (Zoller et al., 1999) and from the 1935-period and older using the primers F7 and R7 (for PCR set-up, see Kistenich et al., 2018).

### Library Preparation and Ion Torrent Sequencing

We pooled all PCR products from PCR sets 1 and 2 for each of the 56 individuals and purified the mixtures with the Illustra™ ExoProStar™ Clean-Up Kit (GE Healthcare) using a 10-fold enzyme dilution and incubating at 37°C for 45 min and inactivation at 80°C for 15 min. To remove any additional short molecules, we performed an AMPure®

**TABLE 1 |** Specimens used in this study including voucher information, sequencing results and GenBank accession numbers of sequences generated in this study.

| Species | Year | Country | County | Voucher | Herbarium # | Period | Input material (mg) | DNA concentration (ng/µl) | Recovered sequence | | No. of reads used | Coverage | | | | GenBank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Bases | % | | Mean | stdev | Min | Max | |
| *Cladonia floerkeana* | 2011 | Norway | Vest-Agder | J. Klepsland 11-L450 | O-L-177072 | 2010 | 1 | 0.98 | 883 | 100.0 | 23,418 | 3,620 | 4,365 | 1 | 12,660 | MH792863 |
| *Cladonia floerkeana* | 1987 | Norway | Vest-Agder | R. Haugan 674 | O-L-91628 | 1985 | 1 | 0.21 | 807 | 91.3 | 9,121 | 774 | 1,105 | 0 | 3,470 | MH792864 |
| *Cladonia floerkeana* | 1964 | Norway | Møre og Romsdal | H. Rui 13541 | O-L-90488 | 1960 | 1 | 1.94 | 883 | 100.0 | 3,491 | 473 | 443 | 3 | 1,486 | MH792865 |
| *Cladonia floerkeana* | 1935 | Norway | Møre og Romsdal | K. & G. Hygen s.n. | O-L-116164 | 1935 | 4 | 2.13 | 160 | 18.1 | 21 | 1 | 3 | 0 | 10 | S1 |
| *Cladonia floerkeana* | 1910 | Norway | Troms | B. Lynge s.n. | O-L-90542 | 1910 | 18 | 0.29 | 541 | 61.2 | 30 | 3 | 4 | 0 | 14 | MH792866 |
| *Cladonia floerkeana* | 1895 | Norway | Rogaland | S.O.F. Omang s.n. | O-L-90687 | 1885 | 15 | 0.58 | 168 | 19.0 | 5 | 0 | 1 | 0 | 3 | S1 |
| *Cladonia floerkeana* | 1863 | Norway | Oslo | N.G. Moe s.n. | O-L-90872 | 1860 | 18 | 9.26 | 43 | 4.9 | 82 | 0 | 3 | 0 | 71 | S1 |
| *Cladonia gracilis* | 2014 | Norway | Sør-Trøndelag | R. Haugan WG3-0181 | O-L-196047 | 2010 | 1 | 0.26 | 883 | 100.0 | 39,448 | 11,399 | 15,623 | 9 | 35,642 | MH792867 |
| *Cladonia gracilis* | 1985 | Norway | Sør-Trøndelag | R. Haugan 150 | O-L-91632 | 1985 | 1 | 0.33 | 763 | 86.3 | 9,609 | 1,138 | 1,116 | 0 | 3,964 | MH792868 |
| *Cladonia gracilis* | 1960 | Norway | Sør-Trøndelag | H. Rui 18920 | O-L-91400 | 1960 | 35 | 1.48 | 134 | 15.2 | 2 | 0 | 0 | 0 | 1 | S1 |
| *Cladonia gracilis* | 1934 | Norway | Akershus | P. Størmer s.n. | O-L-101109 | 1935 | 14 | 3.82 | 741 | 83.8 | 872 | 114 | 122 | 0 | 484 | MH792869 |
| *Cladonia gracilis* | 1909 | Norway | Oppland | B. Lynge s.n. | O-L-91359 | 1910 | 18 | 3.59 | 832 | 94.1 | 2,640 | 343 | 387 | 0 | 1,133 | MH792870 |
| *Cladonia gracilis* | 1892 | Norway | Sør-Trøndelag | F. Kiær s.n. | O-L-91331 | 1885 | 35 | 2.09 | 121 | 13.7 | 14 | 0 | 1 | 0 | 5 | S1 |
| *Cladonia gracilis* | 1868 | Norway | Oslo | N.G. Moe s.n. | O-L-91021 | 1860 | 83 | 4.94 | 420 | 47.5 | 20 | 2 | 3 | 0 | 11 | S1 |
| *Nephroma arcticum* | 2013 | Norway | Buskerud | S. Rui & E. Timdal 13199 | O-L-184739 | 2010 | 1 | 1.43 | 806 | 100.0 | 16,031 | 2,818 | 2,207 | 1 | 8,973 | MH792871 |
| *Nephroma arcticum* | 1984 | Norway | Vest-Agder | A. Pedersen s.n. | O-L-146106 | 1985 | 1 | 0.29 | 444 | 55.1 | 760 | 141 | 238 | 0 | 608 | MH792872 |
| *Nephroma arcticum* | 1960 | Norway | Oppland | P. Størmer s.n. | O-L-49669 | 1960 | 35 | 20.40 | 222 | 27.5 | 23 | 2 | 4 | 0 | 17 | S1 |
| *Nephroma arcticum* | 1934 | Norway | Vestfold | P. Størmer s.n. | O-L-107923 | 1935 | 55 | 19.70 | 0 | 0.0 | 0 | 0 | 0 | 0 | 0 | – |
| *Nephroma arcticum* | 1910 | Norway | Troms | B. Lynge s.n. | O-L-51534 | 1910 | 43 | 25.50 | 806 | 100.0 | 16,976 | 3,711 | 5,237 | 3 | 15,036 | MH792873 |

*(Continued)*

**TABLE 1** | Continued

| Species | Year | Country | County | Voucher | Herbarium # | Period | Input material (mg) | DNA concentration (ng/μl) | Recovered sequence | | No. of reads used | Coverage | | | | GenBank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Bases | % | | Mean | stdev | Min | Max | |
| *Nephroma arcticum* | 1886 | Norway | Vestfold | J.M. Norman 1218 | O-L-130380 | 1885 | 40 | 9.24 | 127 | 15.8 | 25 | 1 | 2 | 0 | 8 | S1 |
| *Nephroma arcticum* | 1863 | Norway | Akershus | N.G. Moe s.n. | O-L-49808 | 1860 | 109 | 3.28 | 137 | 17.0 | 3 | 0 | 1 | 0 | 2 | S1 |
| *Nephroma laevigatum* | 2014 | Norway | Sør-Trøndelag | R. Haugan 141076 | O-L-203432 | 2010 | 1 | 6.56 | 780 | 100.0 | 14,650 | 1,917 | 1,449 | 51 | 4,263 | MH792874 |
| *Nephroma laevigatum* | 1985 | Norway | Sør-Trøndelag | R. Haugan 134 | O-L-41776 | 1985 | 1 | 2.49 | 676 | 86.6 | 51,237 | 4,917 | 5,157 | 0 | 24,950 | MH792875 |
| *Nephroma laevigatum* | 1960 | Norway | Sør-Trøndelag | P. Størmer s.n. | O-L-41621 | 1960 | 13 | 18.50 | 45 | 5.8 | 2 | 0 | 0 | 0 | 1 | S1 |
| *Nephroma laevigatum* | 1934 | Norway | Hordaland | A. Røskeland s.n. | O-L-41537 | 1935 | 20 | 16.30 | 0 | 0.0 | 0 | 0 | 0 | 0 | 0 | – |
| *Nephroma laevigatum* | 1909 | Norway | Hordaland | B. Lynge s.n. | O-L-41592 | 1910 | 35 | 11.90 | 199 | 25.5 | 445 | 27 | 68 | 0 | 281 | S1 |
| *Nephroma laevigatum* | 1889 | Norway | Oslo | F. Kiær s.n. | O-L-20415 | 1885 | 10 | 10.40 | 258 | 33.0 | 4 | 0 | 1 | 0 | 2 | MH792876 |
| *Nephroma laevigatum* | 1864 | Norway | Oslo | N.G. Moe s.n. | O-L-20426 | 1860 | 26 | 11.60 | 241 | 30.9 | 39 | 3 | 6 | 0 | 34 | S1 |
| *Peltigera collina* | 2012 | Norway | Østfold | R. Haugan 12339 | O-L-190769 | 2010 | 1 | 2.48 | 835 | 100.0 | 10,379 | 1,671 | 1,488 | 3 | 6,673 | MH792877 |
| *Peltigera collina* | 1984 | Norway | Hordaland | J. Holtan-Hartwig 4222 | O-L-48488 | 1985 | 1 | 0.91 | 785 | 94.0 | 27,455 | 4,863 | 7,194 | 0 | 19,682 | MH792878 |
| *Peltigera collina* | 1965 | Norway | Møre og Romsdal | L. Malme L9 | O-L-13650 | 1960 | 2 | 28.20 | 709 | 84.9 | 11,538 | 1,966 | 2,859 | 0 | 7,805 | MH792879 |
| *Peltigera collina* | 1934 | Norway | Akershus | B. Lynge & P. Størmer s.n. | O-L-48852 | 1935 | 4 | 26.20 | 249 | 29.8 | 6 | 1 | 1 | 0 | 4 | MH792880 |
| *Peltigera collina* | 1909 | Norway | Akershus | B. Lynge s.n. | O-L-48862 | 1910 | 2 | 12.60 | 835 | 100.0 | 38,273 | 6,658 | 8,060 | 1 | 21,747 | MH792881 |
| *Peltigera collina* | 1891 | Norway | Buskerud | F. Kiær s.n. | O-L-48921 | 1885 | 2 | 31.20 | 835 | 100.0 | 63,268 | 10,721 | 13,312 | 47 | 39,260 | MH792882 |
| *Peltigera collina* | 1866 | Norway | Hordaland | C. Sommer-felt s.n. | O-L-48902 | 1860 | 52 | 1.36 | 229 | 27.4 | 6 | 1 | 1 | 0 | 4 | S1 |
| *Peltigera malacea* | 2012 | Norway | Buskerud | T.H. Hofton 12021 | O-L-205605 | 2010 | 1 | 1.07 | 831 | 100.0 | 8,663 | 1,499 | 1,052 | 3 | 3,681 | MH792883 |
| *Peltigera malacea* | 1982 | Norway | Hordaland | E. Timdal s.n. | O-L-100265 | 1985 | 1 | 0.55 | 779 | 93.7 | 19,432 | 3,123 | 3,737 | 0 | 11,503 | MH792884 |
| *Peltigera malacea* | 1958 | Norway | Buskerud | R.Y. Berg s.n. | O-L-58470 | 1960 | 4 | 52.00 | 821 | 98.8 | 4,391 | 738 | 1,095 | 0 | 3,172 | MH792885 |

*(Continued)*

**TABLE 1** | Continued

| Species | Year | Country | County | Voucher | Herbarium # | Period | Input material (mg) | DNA concentration (ng/μl) | Recovered sequence | | No. of reads used | Coverage | | | | GenBank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Bases | % | | Mean | stdev | Min | Max | |
| *Peltigera malacea* | 1937 | Norway | Oppland | H. Rui 21789 | O-L-60250 | 1935 | 4 | 22.00 | 661 | 79.5 | 144 | 18 | 18 | 0 | 56 | MH792886 |
| *Peltigera malacea* | 1910 | Norway | Troms | B. Lynge s.n. | O-L-58820 | 1910 | 3 | 21.40 | 831 | 100.0 | 36,583 | 6,896 | 8,519 | 3 | 22,911 | MH792887 |
| *Peltigera malacea* | 1897 | Norway | Troms | J.M. Norman s.n. | O-L-58828 | 1885 | 10 | 33.00 | 803 | 96.6 | 179 | 21 | 27 | 0 | 81 | MH792888 |
| *Peltigera malacea* | 1868 | Norway | Oslo | N.G. Moe s.n. | O-L-59116 | 1860 | 61 | 2.55 | 534 | 63.4 | 934 | 169 | 305 | 0 | 803 | MH792889 |
| *Ramalina fraxinea* | 2011 | Norway | Hedmark | T.H. Hofton 11360 | O-L-194358 | 2010 | 1 | 0.35 | 619 | 77.9 | 9,041 | 2,049 | 2,157 | 0 | 6,660 | MH792890 |
| *Ramalina fraxinea* | 1991 | Norway | Akershus | R. Haugan 1921 | O-L-26633 | 1985 | 1 | 0.24 | 722 | 90.8 | 36,354 | 8,273 | 10,075 | 0 | 25,622 | MH792891 |
| *Ramalina fraxinea* | 1959 | Norway | Akershus | H. Rui s.n. | O-L-25936 | 1960 | 17 | 7.64 | 674 | 84.8 | 17,759 | 4,593 | 6,537 | 0 | 14,964 | MH792892 |
| *Ramalina fraxinea* | 1933 | Norway | Østfold | A. Hagen s.n. | O-L-119012 | 1935 | 10 | 2.41 | 604 | 76.0 | 1,637 | 108 | 124 | 0 | 435 | MH792893 |
| *Ramalina fraxinea* | 1908 | Norway | Oslo | B. Lynge s.n. | O-L-25918 | 1910 | 4 | 4.57 | 733 | 92.2 | 103,248 | 37,044 | 43,935 | 0 | 92,686 | MH792894 |
| *Ramalina fraxinea* | 1880 | Norway | Vestfold | J.M. Norman s.n. | O-L-25958 | 1885 | 5 | 3.99 | 503 | 63.3 | 213 | 29 | 36 | 0 | 101 | S1 |
| *Ramalina fraxinea* | 1863 | Norway | Oslo | N.G. Moe s.n. | O-L-25911 | 1860 | 70 | 0.66 | 335 | 42.1 | 13 | 2 | 3 | 0 | 9 | MH792895 |
| *Ramalina siliquosa* | 2011 | Norway | Vest-Agder | J. Klepsland 11-L226 | O-L-176932 | 2010 | 1 | 0.76 | 745 | 93.7 | 12,908 | 2,968 | 3,070 | 0 | 9,753 | MH792896 |
| *Ramalina siliquosa* | 1986 | Norway | Sør-Trøndelag | R. Haugan 312 | O-L-58036 | 1985 | 1 | 0.20 | 708 | 89.1 | 34,552 | 9,807 | 12,148 | 0 | 27,558 | MH792897 |
| *Ramalina siliquosa* | 1960 | Norway | Møre og Romsdal | S. Ahlner s.n. | O-L-57801 | 1960 | 30 | 2.96 | 744 | 93.6 | 6,752 | 1,228 | 1,732 | 0 | 4,487 | MH792898 |
| *Ramalina siliquosa* | 1936 | Norway | Hordaland | J. Holmboe & J. Lid s.n. | O-L-58262 | 1935 | 24 | 5.81 | 688 | 86.5 | 132 | 20 | 30 | 0 | 86 | MH792899 |
| *Ramalina siliquosa* | 1912 | Norway | Hordaland | T. Lillefosse s.n. | O-L-58202 | 1910 | 22 | 7.36 | 603 | 75.8 | 2,256 | 362 | 421 | 0 | 1,431 | MH792900 |
| *Ramalina siliquosa* | 1880 | Norway | Østfold | A. Blytt s.n. | O-L-27832 | 1885 | 11 | 2.40 | 709 | 89.2 | 17,059 | 4,245 | 6,313 | 0 | 14,683 | MH792901 |
| *Ramalina siliquosa* | 1869 | Norway | Aust-Agder | R. Indebetou s.n. | TRH-L-22199 | 1860 | 1 | 0.08 | 14 | 1.8 | 2 | 0 | 0 | 0 | 1 | S1 |

*S1 indicates sequences too short or fragmented for publishing in GenBank found in* ***Supplementary File S1****; – indicates no sequence data; stdev, standard deviation.*

**TABLE 2 |** Primer sequences used for the two-stage, nested multiplex PCR protocol.

| Primer name | Code | Direction | Primer sequence 5′-3′ | Length (bases) |
|---|---|---|---|---|
| NGS-mtSSU_F1 | F1 | Forward | **CTAAGGTAAC**AGCAGTGAGGAATHTTGGTC | 30 |
| NGS-mtSSU_F2 | F2 | Forward | **CTAAGGTAAC**GAYYHWRTYRAATAAARTTCTRGGT | 35 |
| NGS-mtSSU_F3 | F3 | Forward | **CTAAGGTAAC**CCWAGACDGYDRATMAAGCC | 30 |
| NGS-mtSSU_F4 | F4 | Forward | **CTAAGGTAAC**AWGGCACNRRYMWAKGYGAA | 30 |
| NGS-mtSSU_F5 | F5 | Forward | **CTAAGGTAAC**AATKATGARTGTCATAGRTTRRAKAW | 36 |
| NGS-mtSSU_F6a | F6 | Forward | **CTAAGGTAAC**GAAACCAGTAGTGAAGTATGTYG | 33 |
| NGS-mtSSU_2F5 | F7 | Forward | **CTAAGGTAAC**GTTGCACGGCTGTCTTCA | 28 |
| NGS-mtSSU_R1a | R1 | Reverse | **CAGAAGGAAC**RGVYARRNAATGTCATYRTCAW | 32 |
| NGS-mtSSU_R2a | R2 | Reverse | **CTGCAAGTTC**RTAACTCTAGYHAAYBWGTMC | 31 |
| NGS-mtSSU_R3a | R3 | Reverse | **CTACATGCTC**TCAGTTATYACATARGRRGATGC | 33 |
| NGS-mtSSU_R4 | R4 | Reverse | **TACCAAGATC**TGGARTGCTTACACTTTCATTT | 32 |
| NGS-mtSSU_R5 | R5 | Reverse | **CAGAAGGAAC**TDYGYGKRTYATCRAATTA | 29 |
| NGS-mtSSU_2R4a | R6 | Reverse | **TACCAAGATC**GGADYTAACCWAADYCTCRCGAC | 33 |
| NGS-mtSSU_R7 | R7 | Reverse | **GACTTAGCTA**ATGTGGCACGTCTATAGCCC | 30 |

*The non-complementary 10 bases tail is marked in bold.*

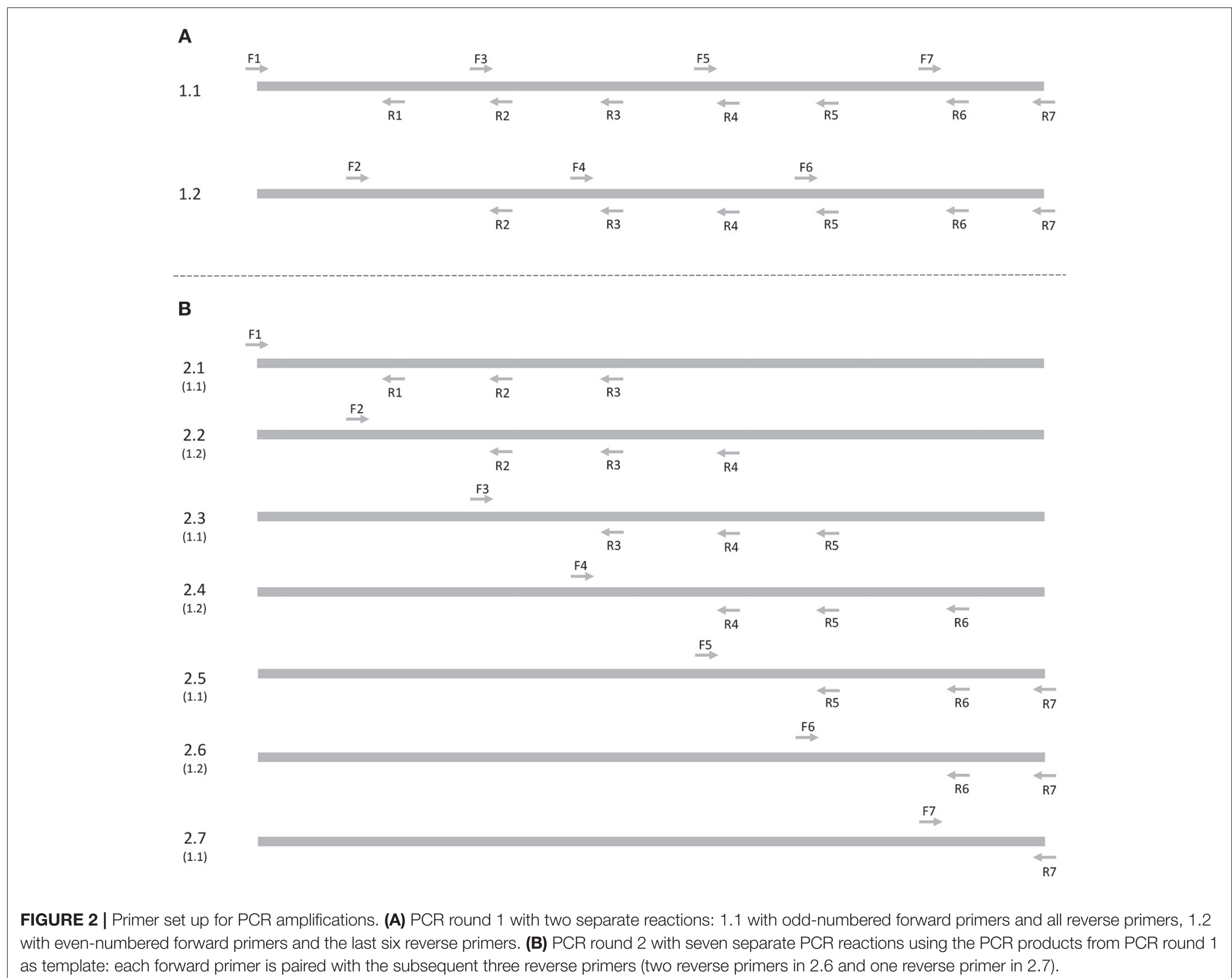


**FIGURE 2 |** Primer set up for PCR amplifications. **(A)** PCR round 1 with two separate reactions: 1.1 with odd-numbered forward primers and all reverse primers, 1.2 with even-numbered forward primers and the last six reverse primers. **(B)** PCR round 2 with seven separate PCR reactions using the PCR products from PCR round 1 as template: each forward primer is paired with the subsequent three reverse primers (two reverse primers in 2.6 and one reverse primer in 2.7).

XP (Agencourt Biosciences Corporation) paramagnetic bead purification following the manufacturer's instructions. We used a 1:1.4 volume ratio of PCR product:beads to remove fragments shorter than approximately 100 bases. The products were eluted from the beads with 50 µl 10 mM Tris-HCl buffer.

The Ion Torrent library preparation was performed using the NEBNext® E6270-kit (New England Biolabs) with these modifications to the manufacturer's instructions: Ion Xpress™ barcoded adapters (Thermo Fisher Scientific) were diluted 1:40 to better match the low DNA input amount. The size selection step 1.3 in the protocol was omitted. In step 1.4, bead cleanup was performed using a 1:1.1 volume ratio for library:beads. The library amplification was performed using 12 cycles and a final Ampure clean up using 1:0.9 volume ratio of library:beads.

We quantified DNA concentrations on a Qubit 2.0 fluorometer (Invitrogen) and visualized fragments lengths on a Fragment Analyzer™ (Advanced Analytical) using the DNF488 kit to optimize input amounts for selected samples at various steps, such as after pooling all PCR products from each individual, after library preparation and after pooling the libraries for each chip. Samples were normalized, pooled and diluted to 17.5 pM (chip 1, samples 1–30) and 15 pM (chip 2, samples 31–56). Template preparation and sequencing was performed using a Hi-Q View Chef and sequencing kit (A29902 and A30044, respectively) and two 318 v2 chips on an Ion Torrent PGM (Thermo Fisher Scientific) using 500 flows per chip.

### Sanger Sequencing

For comparison, we also analyzed our samples using standard protocols for PCR amplification and Sanger sequencing of the ca. 900 bases long mtSSU region using the primers mtSSU1 and mtSSU3R (Zoller et al., 1999) as described in Kistenich et al. (2018). All PCR-products were sequenced irrespective of showing visible bands on the gel or not.

## Sequence Assembly

For an initial overview, we investigated the raw reads from both chips with the Torrent Suite v. 5.8.0 (Thermo Fisher Scientific) and removed the adapters to check the mean sequence quality using FastQC v. 0.11.2 (Andrews, 2010). Afterwards, sequence reads were analyzed using the software Geneious R9 (Kearse et al., 2012) including various plugins. We demultiplexed the reads according to the respective indexes and removed duplicate reads to facilitate sequence analysis using Dedupe of the BBTools package v. 35.82 (Bushnell, 2015). Then, we applied several quality trimming steps, such as removal of PCR primers and low quality reads and ends using BBDuk (BBTools v. 35.82, Bushnell, 2015; minimum quality set to 5, minimum read length set to 8 bases) combined with the Trim option in Geneious (error probability limit set to 0.05). The remaining reads were mapped to species-specific reference sequences using the Geneious Read Mapper (up to five iterations, high sensitivity). In cases where we suspected a high amount of chimeric sequences, we cleaned the respective dataset using UCHIME (Edgar et al., 2011) and mapped the reads against the reference sequence once more. We carefully inspected the consensus sequence for each specimen, corrected obvious sequencing mistakes manually and removed contaminant sequence reads. Sequencing success was measured as percentage of sequence length recovered compared to the full reference sequence.

For sequences produced by Sanger sequencing, we first trimmed the primer sequences and low quality-ends and then assembled the contigs in Geneious R9 (Kearse et al., 2012).

## Statistical Analysis

The statistical analysis proceeded in two steps. In the first step, we identified two variables that served as indicators of DNA quality. Secondly, we used these variables as response variables in generalized linear models (GLM; McCullagh and Nelder, 1989) with identity link and normal errors (i.e., ANOVA and ANCOVA). Predictor variables were Age (2018—year of collection), Genus (factor variable with four levels), Species (factor variable with eight levels, nested within Genus), and Moisture (factor variable with two levels). For each response variable, models were built by forward selection of predictor variables, at each step including variables that contributed independently to explain variation in the response at the Bonferroni-corrected $\alpha = 0.05$ level (cf. Legendre and Legendre, 2012).

Basically, seven primary variables were recorded to characterize DNA quality: relative sequence length (SeqLFr); number of reads (NoReads); read coverage (Cov), for each of the 56 samples (4 genera × 2 species × 7 time-points) represented by the minimum (CovMin), the maximum (CovMax), average (CovAve) and standard deviation of read coverage; and, finally, the concentration of DNA in the analyzed tissue (DNACon). Inspection of frequency distributions revealed strong right-skewness in all primary DNA quality variables except SeqLFr. Furthermore, CovMin was omitted from further analyses because a total of 45 out of 56 recorded values were zeroes. All right-skewed variables were $\log(x + 1)$-transformed before further analyses, resulting in rather uniformly distributed variables. The three remaining lnCov variables were very strongly correlated (Pearson's $r$: $|r| > 0.98$, $p < 0.0001$, $n = 56$) and lnCovAvg was selected to represent the read coverage aspect of DNA quality in further analyses. DNA concentration was unrelated to the other three variables (Pearson's $r$: $|r| < 0.17$, $p > 0.20$, $n = 56$), while the other three variables were rather strongly correlated (Pearson's $r$: $|r| > 0.89$, $p < 0.0001$, $n = 56$). We therefore used lnDNACon as a separate response variable, while the three other DNA quality variables were concentrated into one composite DNA quality variable (referred to as PCA-axis 1) by principal component analysis (PCA; Pearson, 1901; Legendre and Legendre, 2012) of the correlation matrix, using Euclidean biplot scaling of axes (Oksanen et al., 2016) to maximize the fit between ordination scores and between-observation variation in SeqLFr, lnNoReads, and lnCovAvg. All analyses were performed using R v. 2.3.2 (R Development Core Team., 2018); package vegan v. 2.4.0 (Oksanen et al., 2016) was used for ordination analyses.

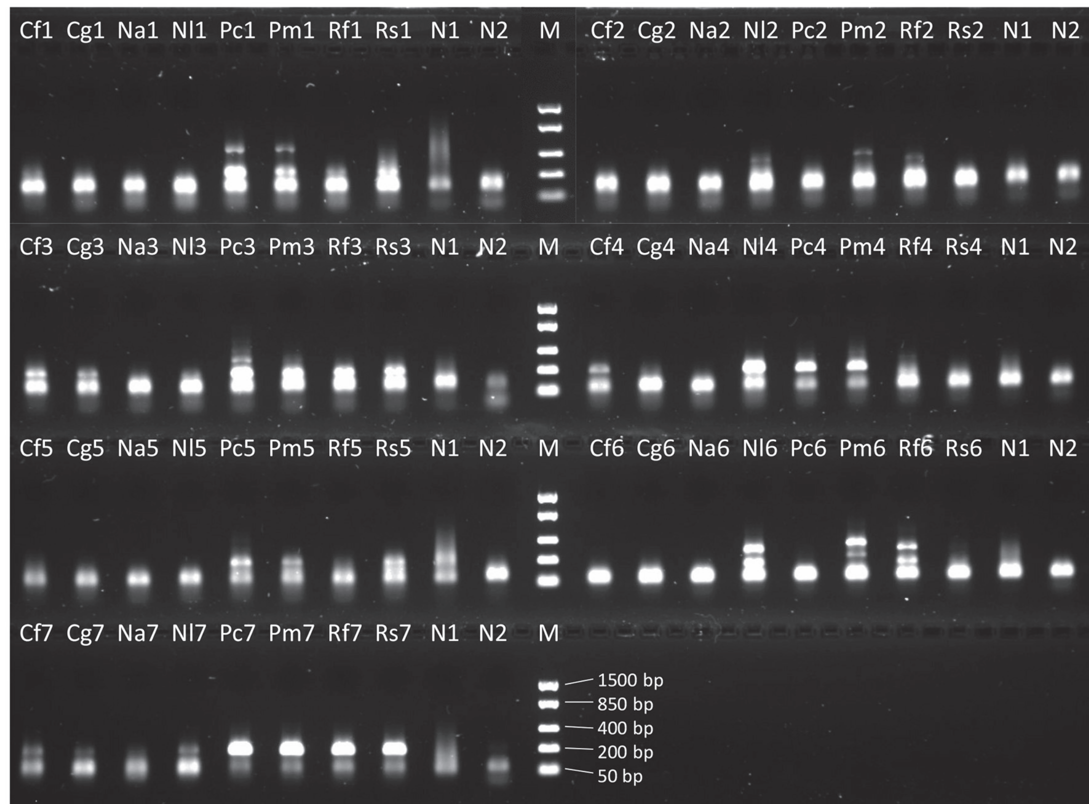


**FIGURE 3 |** Image of gel visualization of PCR products from the 1985-samples for the seven primer combinations of PCR round 2 (**Figure 2B**: 1–7). Cf, *Cladonia floerkeana*; Cg, *C. gracilis*; Na, *Nephroma arcticum*; Nl, *N. laevigatum*; Pc, *Peltigera collina*; Pm, *P. malacea*; Rf, *Ramalina fraxinea*; Rs, *R. siliquosa*; N1, negative control from PCR round 1 run with PCR round 2; N2, negative control from PCR round 2; M, marker.

# RESULTS

## Amplification Success

The concentration of the DNA extracts ranged from 0.08 ng/μl up to 52 ng/μl with relatively higher values for *Nephroma* (mean 11.3 ng/μl) and *Peltigera* (mean 16.8 ng/μl) and low values for *Cladonia* (mean 2.3 ng/μl) and *Ramalina* (mean 2.8 ng/μl; **Table 1**). DNA extracts from all periods showed a smear on the agarose gel (not shown) indicating DNA degradation. For many samples, including some from the 1860-period, the smear indicated also the presence of long fragments (>1500 bases), but from the 1960-period and older, most DNA fragments were shorter than 200 bases and often even shorter than 50 bases.

We could detect visible bands in PCR 1 and 2 mainly in samples from 2010 and 1985 (**Figure 3**). Occasionally, weak to strong bands could be observed in specimens from other periods as well, mostly in *Peltigera* specimens. The majority of PCR reactions showed no products at all. Instead, strong bands around 50–75 bases were present, also in the negative controls (**Figure 3**). These strong, short bands (presumably primer dimers) were stronger in the second PCR round (**Figure 3**).

DNA concentrations after library preparation ranged from <0.05 to 29.7 ng/μl per specimen. Visible bands during the second PCR amplification generally resulted in successful sequence recovery, but also non-visible PCR products produced correct sequence reads. Missing sequence coverage was frequently found around base pair position 120–220, corresponding to the PCR fragment amplified by primers F2 and R2, and around position 520–570(−720), corresponding to the longest PCR fragment amplified by primers F5 and R5/R6.

The gel image (not shown) of the dilution series for the samples from the 2010-period did not show any increase in PCR product with increased dilution. For the samples from the 1935-period and older, the test showed varying results: For *Peltigera* and *Ramalina* species, PCR products did not increase with dilution, while for *Cladonia* and *Nephroma* species, the intensity of bands increased in diluted extracts compared to the undiluted ones. For these species, a 100× dilution showed the best results. All specimens of each species from different periods behaved similarly in performance.

For the Sanger sequencing approach, strong PCR bands could be observed for all samples from the 2010-period apart from *Ramalina siliquosa*. Strong bands could also be detected for several *Peltigera* samples from all other periods except for the 1935-period.

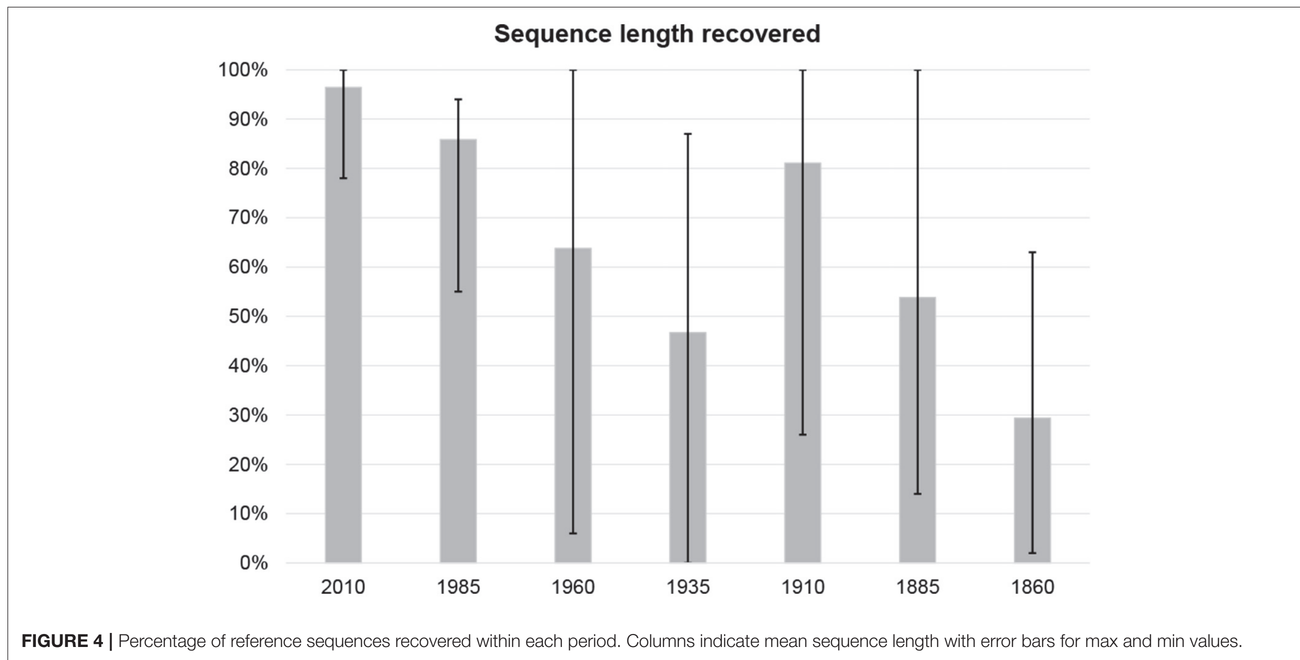


**FIGURE 4 |** Percentage of reference sequences recovered within each period. Columns indicate mean sequence length with error bars for max and min values.

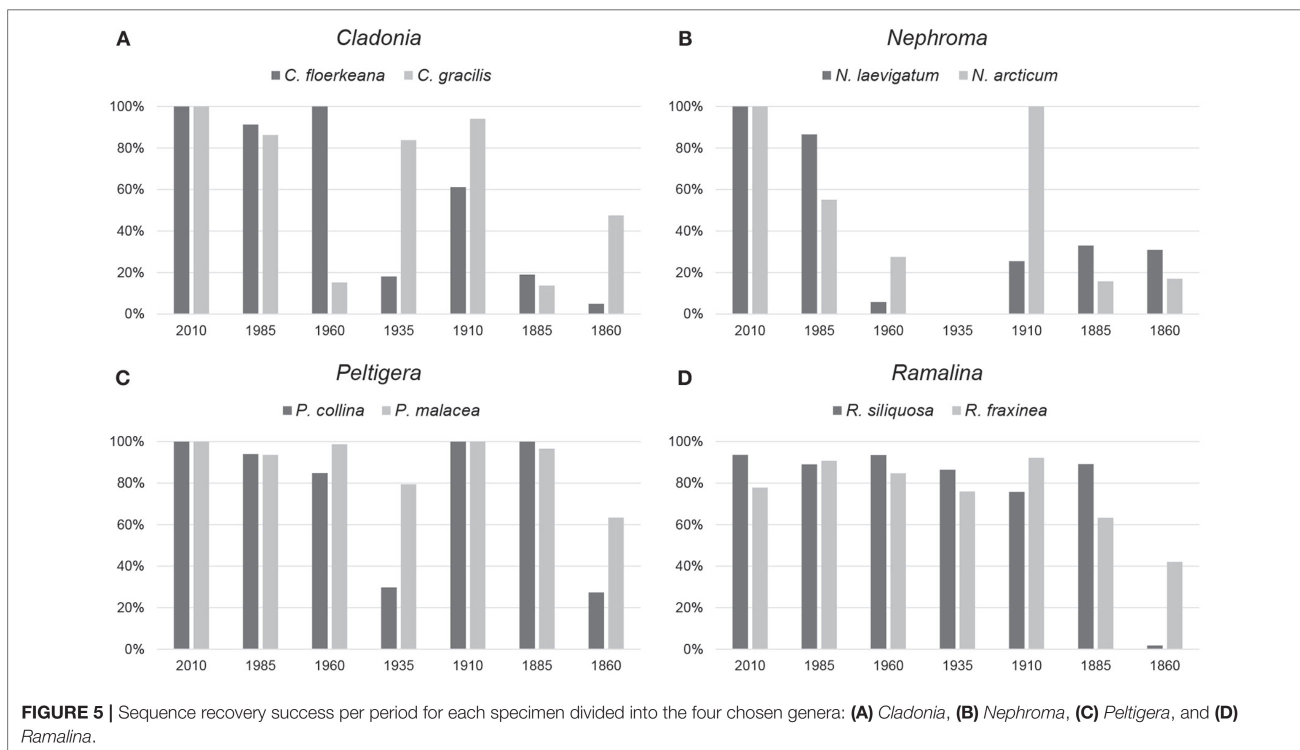


**FIGURE 5 |** Sequence recovery success per period for each specimen divided into the four chosen genera: **(A)** *Cladonia*, **(B)** *Nephroma*, **(C)** *Peltigera*, and **(D)** *Ramalina*.

## Sequencing Success

Sequencing on the Ion Torrent PGM produced 6.8 million reads (median read length 202 bases) for chip 1 and 5.2 million reads (median read length 186 bases) for chip 2. Raw reads, with the adapters trimmed, had a mean sequence quality (Phred score) of Q29. About 5.7% of the generated reads could not be assigned to any barcode. Contaminant sequence reads were largely found to belong to the lichen genera *Umbilicaria* and *Miriquidica*, rarely to other fungal genera, such as *Aspergillus*. We recovered mtSSU sequences of varying length for 54 of the 56 specimens

investigated (**Table 1**; **Figures 4**, **5**; **Supplementary File S1**). The targeted sequence length was 883 bases for each *Cladonia* species, 806 bases for *Nephroma arcticum*, 780 bases for *N. laevigatum*, 835 bases for *Peltigera collina*, 831 bases for *P. malacea*, 794 bases for *Ramalina fraxinea* and 795 bases for *R. siliquosa*. Recovery success was highest for the youngest and lowest for the oldest specimens (**Figure 4**). The number of reads used for mapping ranged from 2 to >100,000 resulting in a mean sequence coverage of up to 37,000 × per specimen (**Table 1**). For two specimens, *Nephroma arcticum* and *N. laevigatum* from the 1935-period, no good-quality sequence reads could be recovered. For an additional five specimens, *N. laevigatum* from the 1960-period, *C. gracilis* and *N. arcticum* from the 1885-period and *C. floerkeana* and *R. siliquosa* from the 1860-period, only short or strongly fragmented sequences of <130 bases length could be recovered (**Table 1**). For 11 specimens, the full sequence length was recovered, including three specimens from the 1910-period and one from the 1885-period (**Table 1**). In all, we recovered >75% of the total sequence length for 34 of the 56 specimens. The seven generated sequences from the different periods for each species showed up to six intraspecific variable sites for *C. floerkeana*, seven for *C. gracilis*, four for *N. arcticum*, two for *N. laevigatum*, one for *P. collina*, one for *P. malacea*, three for *R. fraxinea*, and six for *R. siliquosa*. Specimens of *Peltigera* gave the highest sequence recovery success (mean = 83%), followed by *Ramalina* (mean = 75%) and *Cladonia* (mean = 60%), while specimens of *Nephroma* performed the least well (mean = 43%; 5). Coastal species had a slightly lower sequencing success (62%) than inland species (68%).

Sanger sequencing of regular one-step PCR products produced 19 sequences corresponding to the samples showing visible bands on the gel. Four of those sequences, including all from the 1860-period, were contaminated with either lichenized or unlichenized fungi according to BLAST searches at NCBI. The 15 remaining sequences had a length of 506–883 bases (i.e., 61–100% of target sequence; **Table 3**; **Supplementary File S2**). Full sequences could be generated for all *Cladonia* and *Nephroma* samples plus *P. malacea* from the 2010-period, *N. laevigatum* and *P. malacea* from the 1985-period, and for *P. malacea* from the 1960 and 1910-periods (**Table 3**; **Supplementary File S2**). The oldest specimen, for which a sequence could be generated, was *P. collina* from the 1885-period with a 703 bases long sequence (**Supplementary File S2**). Sanger sequences of *N. laevigatum* (1985) and *P. malacea* (1985 and 1960) were slightly longer than sequences generated by the Ion Torrent protocol (**Table 3**). Sanger sequencing trace files showed an overall decline in quality with increased time since collection, in particular from the 1985-period and older.

**TABLE 3** | Specimens recovered with Sanger sequencing including length of sequences.

| Species | Period | Age (years) | Recovered sequence | |
|---|---|---|---|---|
| | | | Bases | % |
| *Cladonia floerkeana* | 2010 | 7 | 883 | 100 |
| *Cladonia gracilis* | 2010 | 4 | 883 | 100 |
| *Nephroma arcticum* | 2010 | 5 | 806 | 100 |
| *Nephroma laevigatum* | 2010 | 4 | 780 | 100 |
| *Peltigera collina* | 2010 | 6 | 711 | 85.1 |
| *Peltigera malacea* | 2010 | 6 | 831 | 100 |
| *Ramalina fraxinea* | 2010 | 7 | Contaminated | |
| *Nephroma laevigatum* | 1985 | 33 | **780** | **100** |
| *Peltigera collina* | 1985 | 34 | 683 | 81.8 |
| *Peltigera malacea* | 1985 | 36 | **831** | **100** |
| *Cladonia floerkeana* | 1960 | 54 | 588 | 66.5 |
| *Peltigera collina* | 1960 | 53 | 562 | 67.3 |
| *Peltigera malacea* | 1960 | 60 | **831** | **100** |
| *Peltigera collina* | 1910 | 109 | 506 | 60.6 |
| *Peltigera malacea* | 1910 | 108 | 831 | 100 |
| *Peltigera collina* | 1885 | 127 | 703 | 84.2 |
| *Cladonia gracilis* | 1860 | 150 | Contaminated | |
| *Peltigera malacea* | 1860 | 150 | Contaminated | |
| *Ramalina fraxinea* | 1860 | 155 | Contaminated | |

*Values marked in bold show higher sequencing success than with the Ion Torrent protocol.*

## Statistical Analysis

Axis 1 in the PCA ordination of relative sequence length (SeqLFr), number of reads (lnNoReads) and mean coverage (lnCovAvg) explained 95.3% of the total variation in the set of these three variables (after standardization to zero mean and unit variance), while only 4.2% was explained on axis 2 (**Figure 6**). PCA-axis 1 could therefore confidently be used as a composite variable that concentrated the three single variables into one "DNA quality variable." PCA-axis 1 was strongly related to Age (29.6% of the variation explained; $p < 0.0001$; **Table 4**). Also Genus explained significant variation along PCA-axis 1 (16.5%; $p = 0.0237$; **Table 4**), while the variations explained by Species and Moisture were not significant ($p > 0.2$; **Table 4**). The PCA ordination diagram (**Figure 6**) revealed high DNA recovery success (high DNA quality) for samples collected <50 years ago (to the left), while samples collected more than 50 years ago tended to cluster on the right-hand side in the diagram. **Figure 6** also shows that DNA quality cannot be precisely predicted from Age; old material with high DNA quality (dark dots to the left) and new material with low DNA quality (light-colored dots to the right) both occurred to the far left. Because the factorial design of the study makes Genus and Age virtually uncorrelated, Genus explained 22.3% of the variation not explained by Age ($p = 0.0038$) and the interaction between Genus and Age was insignificant. Genus affiliation explained a larger fraction of variation (30.7%; $p = 0.0002$) in DNA concentration than did Age (9.3%; $p = 0.0223$; **Table 4**). While each of the three DNA quality variables SeqLFr, lnNoReads, and lnCovAvg were uncorrelated with lnDNACon in the total material, sequence length and DNA concentration were significantly correlated in the subset of observations with age >100 years (Pearson's $r = 0.4766$, $p = 0.0185$, $n = 24$).

# DISCUSSION

In this study, we aimed at sequencing a DNA fragment of about 900 bases of the mtSSU from 56 lichen specimens of varying age

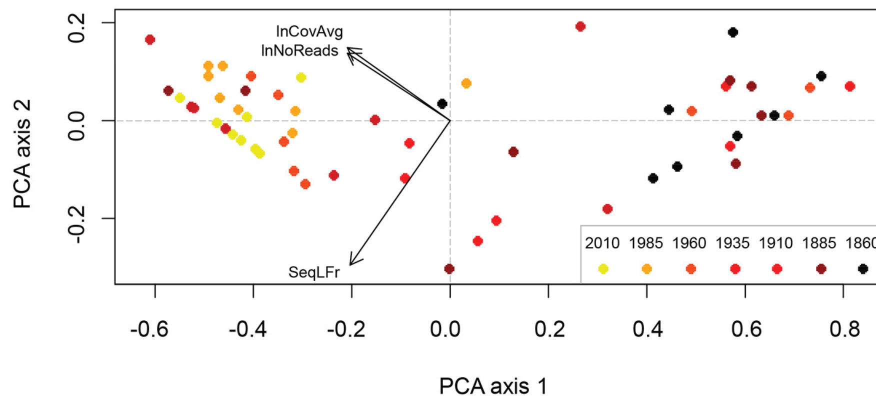


**FIGURE 6 |** PCA ordination (axes 1 and 2) of the three DNA-characteristics, relative recovered sequence length (SeqLFr), ln-transformed number of reads (lnNoReads) and ln-transformed mean read coverage (lnCovAvg) as recorded for the 56 lichen samples. The points represent samples with the time-period, in which they were collected, indicated by different colors (legend inserted lower right). The arrows indicate the direction of maximum increase of each of the three variables in the PCA ordination diagram. The axes are scaled in arbitrary units.

**TABLE 4 |** GLM modeling of DNA quality variables "PCA-axis 1" [a compositive variable that expresses relative sequence length (SeqLFr), number of reads (NoReads) and average read coverage (CovAvg)] and the logarithm of tissue DNA concentration ("lnDNACon") as a function of the predictor variables genus (Gen), species in genus (Spec), moisture (Mois) and tissue age (Age).

| Response variable | Model | Predictor(s) | Total variation | Explained variation | df | F | p |
|---|---|---|---|---|---|---|---|
| PCA-axis 1 | 1a | Factor(Gen) | 12.230 | 2.0184 | 3 | 3.424 | **0.0237** |
| | 1b | Factor(Spec) | 12.230 | 2.0599 | 7 | 1.388 | 0.2321 |
| | 1c | Factor(Mois) | 12.230 | 0.0396 | 1 | 0.175 | 0.6770 |
| | 1d | **Age** | 12.230 | 3.6164 | 1 | 22.659 | **<0.0001** |
| | 2a | [Age+]**factor(Gen)** | 8.614 | 1.9603 | 3 | 5.054 | **0.0038** |
| | 3a | [Age + factor(Gen)+] Age:factor(Gen) | 6.654 | 0.2390 | 3 | 0.601 | 0.6178 |
| lnDNACon | 1a | **Factor(Gen)** | 66.920 | 20.568 | 3 | 7.692 | **0.0002** |
| | 1b | Factor(Spec) | 66.920 | 21.112 | 7 | 3.161 | **0.0079** |
| | 1c | Factor(Mois) | 66.920 | 0.0007 | 1 | 0.000 | 0.9809 |
| | 1d | Age | 66.920 | 6.2235 | 1 | 5.537 | **0.0223** |
| | 2b | [Factor(Gen)+] factor(Spec) | 46.352 | 0.5438 | 4 | 0.143 | 0.9655 |
| | 2d | [Factor(Gen)+]**Age** | 46.352 | 6.2438 | 1 | 7.940 | **0.0069** |
| | 3d | [Factor(Gen)+Age+] factor(Gen):Age | 40.108 | 0.5276 | 3 | 0.213 | 0.8867 |

*Properties of nested models are shown for each of the two response variables. Models 1# are single-predictor models tested against the null model, Models 2# are two-variable models for individually significant predictors tested against the nested Model 1#. Model 3a is the model in which the interaction between two significant predictors is also included. For each model, the total variation (for Models 2# and 3a, the residual variation in the corresponding, simpler, nested model is given), the explained variation, the degrees of freedom for the added predictor as well as F and p statistics are given. Significant p-values ($\alpha = 0.05$) are marked in bold.*

up to 155 years since collection. We applied the two-step PCR HTS protocol of Prosser et al. (2016) to obtain DNA fragments of different lengths that were sequenced on an Ion Torrent PGM and compared the results to standard Sanger-sequenced samples. Sanger sequences could only be obtained for 15 specimens, mainly from young specimens. In contrast, we obtained Ion Torrent sequences from 54 of the 56 specimens, and for 34 of these specimens, more than 75% of the target sequence could be recovered, including specimens collected up to 138 years ago. This pilot study shows that the approach by Prosser et al. (2016) is successful in generating DNA sequences of historical lichen material when Sanger sequencing fails.

## Sequencing Success

Using the HTS protocol of Prosser et al. (2016), we obtained the entire ca. 900 bases long mtSSU target sequence from lichen specimens collected up to 127 years ago (**Table 1**; **Figure 4**). Also shorter sequences obtained from specimens up to 150 years old (**Table 1**; **Figure 4**) contained enough information for species identification. However, sequencing success for specimens from the 1860-period was greatly reduced compared with those from the 1885-period (29 vs. 54% average sequence length recovered, respectively). We found generally lower sequence length recovery from both the 1960- and 1935-periods with 64 and 47% success, respectively (**Figure 4**). In contrast, the specimens from

the 1910-period performed extremely well with 81% success (**Figure 4**).

Using our standard protocols for PCR amplification (i.e., one-step) and Sanger-sequencing of lichens, we obtained correct DNA sequences from 15 samples, mainly from the most recently collected specimens (**Table 3**; **Supplementary File S2**). Most of these sequences were shorter or equally long as the Ion Torrent sequences. Three Sanger sequences, however, resulted in longer sequence lengths than the respective Ion Torrent sequences. The fact that we were able to recover 703 bases of the 127-years-old *P. collina* specimen by standard protocols shows that also one-step PCR and Sanger sequencing can be successful even for specimens more than 100 years old. We therefore recommend testing Sanger sequencing on old samples first before using the Ion Torrent approach. Overall, however, substantially more sequence information was obtained by applying the Ion Torrent protocol.

Consensus sequences from the Ion Torrent approach showed overall better quality than those generated by Sanger sequencing. Some sequence contigs from Sanger sequencing showed missing bases at the start or end after the primer binding-site compared to the Ion Torrent and reference sequences and had to be corrected manually. Other contigs showed a high number of ambiguities due to low trace file quality and required detailed manual inspection and correction. Using the Ion Torrent protocol, these issues were reduced by increased read coverage.

There are probably several reasons for why some specimens failed to recover the full-length sequence from all seven periods. First, it has been shown that high temperatures (>42°C) and extended drying periods of freshly collected plant specimens have a strong negative impact on DNA quality and may be a more important factor causing DNA degradation than age (Taylor and Swann, 1994; Erkens et al., 2008; Staats et al., 2011; Drábková, 2014). We expect that the same factors decrease DNA quality also in lichens, but the information about how the samples were dried after collection is very limited.

Second, in old herbaria without air conditioning, fluctuations of temperature and humidity levels are common and may contribute to accelerated degradation of DNA (Adams, 2011). Unfortunately, the preservation and storage methods used are largely unknown, in particular for specimens from the 1960s and older. In addition, preserved specimens are routinely frozen for several days upon entering a new herbarium. Also the rising frequency of between herbarium-loans due to increased use of natural history collections in research may accelerate DNA degradation. However, Doyle and Dickson (1987) reported that multiple freezing-thawing cycles do not seem to affect DNA quality. We found no newer study investigating the effect of freezing-thawing cycles on DNA, despite frequent claims in the literature that this is undesirable due to DNA degradation.

A third reason for reduced sequencing success might be the presence of PCR-inhibitory substances in the extracted DNA. Many lichens contain various amounts of secondary metabolite compounds (Culberson and Culberson, 2001; Elix, 2014). These lichen substances have not been shown to inhibit PCR amplification, but polysaccharides and terpenoids may do so (Armaleo and Clerc, 1995; Ekman, 1999). The genera *Nephroma* and *Peltigera* are known to contain high amounts of terpenoids. As we received poor sequencing results for *Nephroma* in particular (43%), we tested for the presence of PCR-inhibitory substances by running a PCR on three dilutions of all DNA extracts from the 2010-samples and also of the samples from the 1935-period and older, as we expected that old herbarium samples could have accumulated PCR-inhibitory substances. Our result that amplification success was improved when extracts were diluted 100-fold indicate the presence of inhibitory substances in DNA extracts from old specimens of *Cladonia* and *Nephroma*. None of the 2010-period extracts showed an increase in PCR product with increased dilution; neither did old samples of *Peltigera* or *Ramalina*. Presence of PCR-inhibitory substances may therefore be the reason for the poor sequencing success of *Nephroma* specimens, as this test was done post-sequencing.

Fourth, the DNA extraction method strongly influences DNA yield (Rohland and Hofreiter, 2007; Särkinen et al., 2012). As expected, the specimens from the 1860-period (i.e., the oldest) showed the poorest performance (29% sequencing success). Apart from being older, the specimens from the 1860-period were extracted as part of another unpublished study using a different DNA extraction kit and protocol. This protocol was not tailored to the sensitive extraction of the mycobiont's DNA and may, at least partially, have contributed to the poorer DNA quality.

Finally, herbarium material of vascular plants has traditionally been treated with chemical preservatives (i.e., fungicides and insecticides), such as mercuric chloride (Hall, 1988), which appear to reduce the usability of the extracted DNA (Do and Drábková, 2018; own experience). We are not aware of any similar chemical treatments for the preservation of lichens in our herbaria except for the single use of gaseous insecticides in the 1990s, but such information was not recorded before the 1980s, though.

Most likely, however, the varying sequencing success was caused by a combination of the factors mentioned above; perhaps with different relative importance for different samples. Moreover, in this pilot study, we merely used a single specimen per species per period ($n = 56$). Increasing the sample size would give more robust results. Moreover, it is our experience that PCR amplification is a largely unpredictable process when the DNA extracts are of poor quality as is expected for our historical samples. Running multiple PCR reactions in parallel may result in single reactions being successful (own experience). Often, however, one does not have sufficient amounts of extracted DNA to perform this parallel-PCR test when working with old and precious samples.

We used different amounts of thallus input material for DNA extraction, varying between 1 and 109 mg, depending on the availability of material. Although one might expect the amount of input material to affect the output of DNA (and subsequently the sequencing success), we could not find a clear relationship between the amounts of input and output. For instance, we retrieved full-length sequences from specimens of the 1910-period with as little as 2 mg input material and with as much as 43 mg (**Table 1**; **Figure 5**). The fact that even small amounts of input material allow for generation of full-length sequences

from more than 100-year-old specimens is very good news for the value of precious herbarium specimens, such as type material, opening for successful analyses of DNA without the destroying much material.

We were not able to recover full-length sequences for any of the *Ramalina* specimens. Sanger sequencing failed completely, whereas a 47–48 bases long fragment always failed to assemble in the Ion Torrent approach. Even though parts of this fragment were sequenced, the middle part is always missing; a puzzling result as this region is rather conserved and with few variable sites among the selected eight species. We designed universal primers based on eight species from four distantly related lichen families. Thus, one explanation for the systematically missing sequences might be a poor annealing-property to these particular *Ramalina* species of the designed primers F5 and R5. In general, however, our degenerate primers performed well for all eight species. Studies comparing different HTS platforms have reported that the Ion Torrent PGM often performs less well in recovering the whole target sequence than other platforms (Loman et al., 2012; Quail et al., 2012). Even though the sequencing chemistry and technology has improved since 2012, runs on different sequencing platforms should be compared to explore their significance for sequencing success of historical lichen samples.

## Hypothesis Testing

As expected, we found a highly significant correlation between the age of specimens and sequencing success ($p < 0.0001$; **Figure 6**; **Table 4**), supporting our first hypothesis that quality DNA and sequence reads are more readily obtained from younger than older specimens. Erkens et al. (2008) reported a ~1% decrease in extractable amounts of DNA from plant herbarium specimens per year. We do not know about any similar estimates of yearly decrease in DNA in lichens, and our results do not support the existence of such a pattern. In our current data, degradation of DNA extracts was similar for all samples throughout all periods with the most recently collected samples showing somewhat less degradation. In samples from the 1960-period and older, most DNA fragments were only 50 bases long, but there were also some long fragments (>1500 bases) faintly visible on the agarose gel. Measured DNA concentrations was >0.1 ng/µl for almost all DNA extracts, even for the older ones (**Table 1**). When comparing all samples from every period, we could neither detect a correlation between amount of input material and DNA concentration nor between sequencing success and DNA concentration (**Table 4**). For samples from the 1910-period and older, however, we could detect a weak positive correlation between sequencing success and DNA concentration ($p = 0.0185$) indicating that sequencing success might increase with increasing DNA concentrations in old samples. Hence, special focus should be placed on the DNA extraction process when handling old material. DNA extracted from lichens usually consists of a mixture of various organisms. Therefore, the extract does not only contain the mycobiont's DNA, but most likely also contaminant DNA from the photobiont, basidiomycetes (Spribille et al., 2016) and possibly other unknown symbionts, epibionts, or endophytes. Hence, we do not know the proportion of mycobiont DNA in our DNA extracts. It is possible, therefore, that the mycobiont DNA represents only a fraction of the total DNA concentration measured and that the long fragments observed on the gel result could result from contaminants. Still, the amount and quality of mycobiont DNA in our specimens was mostly sufficient to generate reads, even though few in number for specimens from the 1860-period (**Table 1**; **Figure 4**). This fact indicates that extracting DNA of sufficient quality from even older lichen specimens should be feasible and ought to be explored further.

We found that the different genera did not perform equally well in PCR amplification and sequence recovery (**Figure 5**). The statistical analyses indicate a significant difference between sequencing success and genus affiliation ($p < 0.004$; **Table 4**), but not species affiliation (**Table 4**). The average sequence recovery for the *Nephroma* species was only 43%, about half the recovery observed for the *Peltigera* species (83%). As we were also able to produce >700 bases long Sanger sequences of various old *Peltigera* samples, DNA from this genus seemed to be in a particularly good condition with long fragments. *Peltigera* and *Nephroma* are morphologically similar with big lobes and cyanobacteria as photobionts (for *N. arcticum* only in cephalodia) and occur in similar habitats. The success rate for sequencing the ITS barcode marker of fresh *Nephroma* specimens has been high in the Norwegian Barcode of Life project (OLICH), suggesting that the low sequencing success in the present study may either be due to the low initial sample size or to the presence of PCR-inhibitory substances in old specimens (see above). The *Ramalina* species performed well with 75% recovery success followed by *Cladonia* with 60%. For ITS in the OLICH project, we experienced higher success for *Cladonia* (ca. 70%) than *Ramalina* (ca. 60%), but these figures are based on a broader specter of species from both genera, but also younger specimens than in the present study. When comparing specimens from the two most recent periods only (i.e., the 2010- and 1985-periods), average sequencing success is higher with 94% for *Cladonia*, 85% for *Nephroma*, 97% for *Peltigera* and 88% for *Ramalina*, outperforming the general OLICH success. Still, sequencing success seems to be dependent on the target genus. When using DNA concentration as response variable in our GLM analyses, we found a significant effect of genus affiliation (and in this case also of species affiliation; **Table 4**), that was even stronger than the effect of age ($p < 0.03$; **Table 4**). Thus, we cannot reject our second hypothesis that quality DNA and sequence reads are more readily obtained from some taxa than others given that age of the material is kept constant.

Our general experience when working with lichens from humid tropical regions is that they become difficult to obtain DNA sequences from shortly after collection; often, longer (>ca. 300 bases) Sanger sequences cannot be obtained after only a few months of storage. In contrast, we do not experience this difficulty with taxa adapted to the less humid boreal regions. We hypothesized that lichens adapted to more arid conditions are better equipped for keeping their DNA intact over longer periods of desiccation than species adapted to the humid tropics; the latter should not need the same mechanisms for DNA protection. The DNA of tropical lichens should degrade faster when subjected to desiccation, which is, in fact, our traditional

way of preserving lichen specimens. Specimens collected in the humid tropics need to be dried longer, but due to limited facilities and time, the drying process is often compressed by increasing the temperature. Sometimes, the process may not be fully completed for several days or weeks, facilitating enzymatic DNA degradation. Bakker et al. (2016) found the DNA of wet-tropical angiosperms to have aged faster than the DNA of angiosperms from dry habitats and attribute this difference to the more intense drying processes. This led us to formulate the third hypothesis that quality DNA and sequence reads are more readily obtained from preserved specimens of species adapted to dry habitats than from those adapted to humid conditions. This is also the reason why we chose species pairs growing in dry inland habitats vs. humid coastal areas. Our statistical analyses, however, did not support our hypothesis (**Table 4**); the average sequencing success rate for inland species was only slightly higher than for coastal species (68 vs. 62%, respectively). Due to the limited availability of relevant specimens, the sampling in this study was restricted to boreal taxa with different preferential distribution ranges. Both species of each genus exhibit distribution ranges that overlap to some extent, which may contribute to the non-significant results. Our results therefore do not preclude significant differences in sequencing success between specimens from more extreme habitats, such as (semi-) arid deserts vs. tropical rainforests.

## Applicability of the Ion Torrent Protocol for Lichen Taxonomy

Sanger sequencing is still the preferred and most commonly used method for generating sequences of the barcode marker ITS and other markers in lichen systematics (Hoffman and Lendemer, 2018). Our study shows that HTS is highly suitable for obtaining sequences from both young and old lichen specimens. We managed to recover full-length sequences from historical specimens using the two-step PCR HTS protocol by Prosser et al. (2016), specimens for which Sanger sequencing failed completely or produced substantially shorter sequences.

This study includes specimens of the same species from different time periods. Using existing DNA sequences of the same species as reference simplifies quality-checking different steps during read analysis. We therefore used available references of the same set of species for read mapping, which greatly facilitated this task including the quality-checking for mistakes. Our expectation was to receive near identical mtSSU sequences for the specimens belonging to the same species, not the least because the mtSSU marker is generally understood to be less variable than the ITS, which may vary highly within populations of the same species. Hence, we assume that read assembly might be more challenging for ITS. If there is no reference sequence available of the study species or a closely related taxon, the sequence reads need to be *de novo* assembled and proofreading will be more challenging. There are few species, for which no recently collected material exists, and which are not represented by DNA sequences in GenBank. Most challenging may be the common case in lichenology of species that are only known from an old type specimen.

In our assembled reads, we frequently encountered homopolymer-associated indel errors, especially in AT-rich regions and when compared to our Sanger-sequenced samples. This is a commonly known disadvantage of the Ion Torrent PGM sequencing method but is also a known issue for Sanger sequencing (Loman et al., 2012; Goodwin et al., 2016). In addition, the Ion Torrent PGM has a sequence read error rate of 1–1.8% (Loman et al., 2012; Quail et al., 2012), which may generate errors that are difficult to discern from the true sequence when only a few reads are recovered and no reference sequence is available. In our consensus sequences of the assembled reads, we found up to seven differing nucleotide sites between specimens of the same species. It is unclear if these differences result from Ion Torrent specific sequencing errors or if they merely represent intraspecific variation, especially when only a few reads were recovered. We found these nucleotide differences in both sequences assembled from many reads as well as those based on few reads, suggesting that the differing nucleotide sites result from intraspecific variation rather than sequencing errors. When working with long-time archived specimens, sequences should be checked and corrected for typical ancient DNA degradation patterns, especially T to C substitutions in fragment ends (Weiß et al., 2016). We expected T to C substitutions to occur in sequence reads from the older specimens compared to the reference sequences based on fresh specimens. We could not find any of these substitutions and assume that they might have been lost during read trimming steps. We discovered only three T to C substitutions in sequences from the 1960- and 1910-periods and none in the ones from the 1885- and 1860-periods. These substitutions, however, were never at the end of fragments. This is consistent with the suggestion above that the differing nucleotide sites represent true intraspecific variation.

Another challenge when assembling historical type specimens without an appropriate reference sequence is the identification of contaminant sequence reads. We discovered contaminant sequence reads in our assembled consensus sequences. When subjecting these reads to BLAST searches at NCBI or against our own DNA sequence database, we found them to often associate with *Umbilicaria* or *Miriquidica* species. Reads belonging to species of *Cladonia* or *Peltigera* could also be detected in various datasets of other species. These contaminants might result from spores of other species being attached to the fragments we chose for DNA extraction. The contamination by *Cladonia* or *Peltigera* reads might also be due to demultiplexing errors. Only rarely did some of the assembled sequences belong to other, non-lichenized, fungal species, such as *Aspergillus*. The latter genus is often encountered when sequencing lichens without conducting the preparation steps in a dedicated clean lab facility. The low amount of fungal contaminants in our sequence reads indicates that performing the DNA extractions and PCR preparations in a clean lab facility is important for eliminating such contaminants.

## Drawbacks, Potential Improvements, and Future Use of the Approach Tested in This Study

In this pilot study, we were able to recover sequences from almost all specimens investigated including several specimens collected more than 100 years ago. To our knowledge, this is the first successful attempt to recover a full taxonomic marker (i.e., the ca. 900 bases of the mtSSU regularly used in lichen

systematics) from lichen specimens that were collected up to 127 years ago. In this study, we tested specimens from two orders of lichenized ascomycetes. As the approach by Prosser et al. (2016) should be a universally applicable method, which can be tailored to any organism of interest, we expect this approach to work for lichenized basidiomycetes as well, given that adequate primers are developed. We recommend this approach to be tested on additional species from various lichen families with more representatives per species per period. Doing so, one may better understand why certain specimens fail to produce reads and further explore to what extent sequencing success depends on taxonomic affinity and/or the autecology of the species. In particular, sequencing success of specimens from moist tropical vs. dry habitats should be assessed using specimens from more extreme habitats. Future studies should make use of rich herbarium collections of species with even older specimens and a denser time-line sampling. In addition, the applicability of our Ion Torrent approach on the ITS, the fungal barcode marker, and other relevant markers ought to be explored.

We discovered that some of the old specimens (collected >80 years ago) contained PCR-inhibitory substances; a discovery we made only after we had finished sequencing our samples. Especially, *Nephroma* specimens seemed to contain strong PCR-inhibitors, which might explain their low sequencing success. Hence, we strongly recommend testing for the presence of PCR-inhibitory substances by running dilution series before the start of the Ion Torrent approach. In addition, tweaking the PCR conditions further might lead to more successful PCR amplification. Moreover, several studies have shown that different polymerases give different results (e.g., Telle and Thines, 2008; Särkinen et al., 2012), indicating several starting points for increasing sequencing success in the future.

Other HTS platforms, for example various Illumina machines, should be explored to assess if these can overcome some of the drawbacks with the Ion Torrent PGM. Using the Illumina MiSeq technology, Forin et al. (2018) managed to obtain ITS2 sequences of century-old fungal collections, indicating the potential success of this approach also for historical lichen specimens. Furthermore, single-read sequencing technologies, such as implemented in the PacBio (Pacific Biosciences) and MinION (Oxford nanopore technologies) should be tested with lichen material. Both platforms might circumvent the amplification step, thus reducing possible amplification biases. However, they also produce longer and fewer reads than the Ion Torrent or Illumina platforms (Bleidorn, 2016), which is no advantage when working with fragmented DNA, though. To our knowledge, these platforms have so far not been tested on historical herbarium material.

An alternative approach to obtaining DNA sequences from historical lichen specimens is shotgun sequencing of the entire genome. While several biogeographic studies have adopted this approach (e.g., Cao et al., 2011; Rivarola et al., 2011), research within lichen systematics and taxonomy still largely relies on Sanger sequencing of certain markers (Hoffman and Lendemer, 2018). The full genome size of some flowering plants and ferns can reach up to 147 Gb (Hidalgo et al., 2017). In contrast, the lichen mycobiont has a typical genome size of only about 35 Mb (Grube et al., 2014) and a variable mitochondrial genome size of 25–120 kb (Pogoda et al., 2018). So, the implementation and regular use of shotgun sequencing of unreduced genomic DNA seems more feasible and applicable in fungi than in plants. Surprisingly, Staats et al. (2013) found that the coverage for whole genome sequencing of fungi was lower than for other organisms, such as plants or insects. They were able, however, to recover nearly full organelle genomes. Other fungal studies can point to full genome recovery success (e.g., Van Kan et al., 2017; Armstrong et al., 2018). The amount of required input material for obtaining the complete organelle or the entire genome of an organism might be a limiting factor for historical specimens. Complete organelle genomes have been successfully sequenced from as little as 24–33 ng input DNA (Bakker et al., 2016; Zedane et al., 2016). Clearly, shotgun sequencing of unreduced genomic DNA of historical specimens should be explored further to find out if taxonomically relevant markers can be fully recovered, without increase in sample destruction and with reduced cost and effort.

The costs for using the herein described Ion Torrent approach are less than twice as high as Sanger-sequencing costs if we had attempted to Sanger-sequence all seven mtSSU fragments per specimen. Within the last decade, costs for HTS, such as Ion Torrent and Illumina, have declined rapidly and are expected to decline further in the future. Hence, it will soon become more feasible also for smaller labs to implement our Ion Torrent approach. Costs can be further reduced by multiplexing more specimens. In addition, Ion Torrent sequencing is more time-efficient than Sanger-sequencing, not necessarily for sample preparation, but concerning the actual sequencing time.

We anticipate and welcome future and more comprehensive studies on historical lichen specimens that will identify where the methodological improvements can be gained. Our results show that DNA is still present in high enough quantities and as long enough fragments in 150-years-old lichen specimens for succeeding with the Prosser et al. (2016) method. Thus, a protocol combining PCR amplification of different-length fragments and HTS seems promising for circumventing the challenges with fragmented and low-concentration DNA.

## DATA AVAILABILITY

### Datasets Are in a Publicly Accessible Repository

The sequences generated for this study can be found in GenBank under the respective accession number listed in **Table 1**. Sequences, which could not be submitted to GenBank as well as Sanger sequences, can be found in **Supplementary Files S1, S2**, respectively.

### Datasets Are Available on Request

The raw sequencing data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## AUTHOR CONTRIBUTIONS

MB and ET initiated the study, which was further developed together with SK. SK, AS-N, LT, and MB planned and designed the experiments. ET provided the specimens, which were sampled by SK. SK performed the laboratory work under guidance by AS-N, LT, and MB. SK analyzed the generated sequence data. RH performed the statistical analyses. SK wrote the first draft whereas all authors edited and completed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00005/full#supplementary-material

The file S1_HTS_sequences.fasta contains those Ion Torrent sequences generated in this study with <200 bases length or >50% ambiguous characters, which could not be submitted to GenBank. The file S2_Sanger_sequences.fasta contains all Sanger sequences generated in this study.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary file S1_HTS_sequences.txt**

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>Cladonia_gracilis_1960
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GTAGGGACTAATTCGCTAGAGTTACTTATGAGGGGGTATTAAAGTACTGCAGGTGTAGAGATGAAATTTTGTTATACCTC
TTTTCGTATGAGAAACTATGGCACAGGTATAGGTGAAAGCATCCCC??????????????????????????????????
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>Nephroma_arcticum_1960
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GCACGGGTCAATGCGAAG??????????????????????????????????????????????????????????????
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??????TTTAATTTGATAACCCGCAAAAAACCTTACCACATTTTGAATAAATATTTATATATAAATATTTATATATATAT
ATATATATTTACAAGCGTTGCACGGCTGTCTTCAGTGAAT????????????????????????????????????????
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>Nephroma_laevigatum_1960
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>Cladonia_floerkeana_1935
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????TTACAAGCGTTGCACGGCTGTCTTCAGTTAATGTCGTGAGATTTTGGTTAGTTCCATTAAATTAACGTAAACCCTT
ACTTTATTTTTATATAAAGTAGTTCCCCGCTATATTGGATATGATAACAGGGACTAAGACAAGTCATCATGGCCTGAATA
TTGT
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>Nephroma_laevigatum_1910
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GAATAAAATTCTGGGTAGACTATGATA???????TATCTATCTA??????TTGACCA?AATACGTGCCAGCAGTCGCG??
AAGACGTTAAAGACAA????????????????????????????????????????????????AGCCTACTGGGGACA?
ATTAACTAGAGTTATATGAAAGGG????????????????????????????????????????????????????????
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>Cladonia_floerkeana_1885
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TGTGTCGCGAACAGGATTAGATACCCCAGTAGTCAAAGCAGAAAATGATGAATGTCATAGATTAGATATACAATTTATTC
TATAAATGAAAGTGTAAGCA????????????????????????????????????????????????????????????
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>Cladonia_gracilis_1885
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>Nephroma_arcticum_1885
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????ATATTTACAAGCGTTGCACGGCTGTCTTCAGT????????????????????????????????????????????
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>Ramalina_fraxinea_1885
AATAGCCTAACGGCTGAACCAGCA????????????????????????????????????????????????????????

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ACTAATTTGCTAGAGTTACTTATGAGTGGGCTTTAAAGTACTAATGGTGTAGAGATAAAATTTTGTAATACCATTTTTCG
TAAAGAAAAATTATGGCACTGGTAAAGGTGAAAGCATCCCCCTATGTGATAACTGACGTTGAAGGACGAAGGCTTTGGGT
CGCGA??????????????????????????????????????????????????ATTAGATAATACAGTTTATTCTATA
AATGAAAGTGTAAGCA???????????????????????????????????????????????????????GAAACCAGT
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AGCGTTGCACGGCTGTCTTCAGTTAATGTCGTGAGACTTTGGTTAGATCCATTAAATTAACGTAAGCCCTTACTTTATTT
TCATATAAAGTAGTTCTCCACCATATTGGACAAGATAACAGGGACTAAGACAAGTCATCATGGCCTAAATATTGT
>Cladonia_floerkeana_1860
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??TAAATGAAAGTGTAAGCAT???????????????????????????????????????????????????????????
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>Cladonia_gracilis_1860
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TGATTATATCAAATAAAATTCTAGGTAGATTTTTGA?????????????????????????????????????????????
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TGTGTCGCGAACAGGATTAGATACCCCAATAGTCAAAGCAGAAAATGATGAATGTCATAGATTAGATCTGCAATTTATTC
TATAAATGAAAGTGTAAGCA????????????????????????????????????????????????????????????
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ACTTTATTTATATATAAAGTAGTTCTCCGCTATATTGGATATGATAACAGGGACTAAGACAAGTCATCATGGCCTAAATA
TTGT
>Nephroma_arcticum_1860
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ATACCCTCGTATTCTAGGCAGAAAATGATGAATGYCATAGGTTGAATATAATATTATTTATTTGGTCTAT??????????
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>Nephroma_laevigatum_1860
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ATTAACTAGAGTTATATGAAA???????????????????????????????????????????????????????????
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CCTCGTATTCTAAGCAGAAAATGATGAATGTCATAGGTTGAAGATAAATAATATTTATTTTGGTCTATAAATGAAAGTGT
AAGCATTCCA??????????????????????????????????????????????????????????????????????
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>Peltigera_collina_1860
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TTCGATACCCCAG???????????????????????????????????????????????????????????????????
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GGTTAGTTCCATTAAATTAACGAAAACCCTTACTTTATTTTAATATAAAGTTGTTCACCGTTATATTGGATATGATAATA
GGGACCAGGACAAGTCATCATGACCTAAATATTGT
>Ramalina_siliquosa_1860
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## Supplementary file S2_Sanger_sequences.txt

>Cladonia_floerkeana_2010
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GTCGCGGTAATACGTAAAAGACTAGTGTTATTCATCTTTAATTGGTTTAAAGGGTACCTAGACAGCGAATAAAGCCATAG
TAGGGACTTATTCGCTAGAGTTACTTATGAGGGGGTATTAAAGTACTGCAGGTGTAGAGATGAAATTTTGTTATACCTCT
TTTCGTTTGAGAAACTATGGCACAGGTATAGGTGAAAGCATCCCCTTATGTGATAACTGACGTTGAGGGACGAAGGCTTT
GTGTCGCGAACAGGATTAGATACCCCAGTAGTCAAAGCAGAAAATGATGAATGTCATAGATTAGATATACAATTTATTCT
ATAAATGAAAGTGTAAGCATTCCACCTCAAGAGTAATTTGGCAACATTGGAACTGAAATCATTAGACCGTTTCTGAAACC
AGTAGTGAAGTATGTTGTTTAATTTGATGATACGCAAAAAACCTTACCACAATTTGAATATTTATATATATCTTTTATTG
ATTTTTACTTACATCTATGTAAATAATTAAAATGATGCTTAAATCTCGTTTGTGAGACTGAGCATGAAAAAAGATAATAT
ATTTACAAGCGTTGCACGGCTGTCTTCAGTTAATGTCGTGAGATTTTGGTTAGTTCCATTAAATTAACGTAAACCCTTAC
TTTATTTTTATATAAAGTAGTTCCCCGCTATATTGGATATGATAACAGGGACTAAGACAAGTCATCATGGCCTGAATATT
GT
>Cladonia_gracilis_2010
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TGATTATATCAAATAAAATTCTAGGTAGATTTTTGATAATGACATTGCTTACCTAAGTCTTGACCAAATTACGTGCCAGC

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AGTCGCGGTAATACGTAAAAGACTAGTGTTATTCATCTTTAATTGGTTTAAAGGGTACCTAGACAGCGAATAAAGCCATA
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TGTGTCGCGAACAGGATTAGATACCCCAATAGTCAAAGCAGAAAATGATGAATGTCATAGATTAGATATGCAATTTATTC
TATAAATGAAAGTGTAAGCATTCCACCTCAAGAGTAATTTGGCAACATTGGAACTGAAATCATTAGACCGTTTCTGAAAC
CAGTAGTGAAGTATGTTGTTTAATTTGATGATACGCAAAAAACCTTACCACAATTTGAATATTTATATACATCTTTTATT
GATTTTTACTAACATATTTGTAGACAATTAAAATGATGCCTATGTCCCGTTTGTGAGACTAAGCATCAAAAAAGATAATA
TATTTACAAGCGTTGCACGGCTGTCTTCAGTTAATGTCGCGAGATTTTGGTTAGTTCCATTAAATTAACGTAAACCCTTA
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TGT
>Nephroma_arcticum_2010
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TAATACGTTAAAGACTAGTGTTATTCATGTTTAATCGGTTTAAAGAGTACCAAGACTGTTAATAAAGCCCATAAAGGGGA
CACATTAACTAGAGTTATATGAAAGGAGGCTCTTAGGTACTATCGAGTGTAGAGATAAAATTCTTTGATACAGATGAAGG
CACGGGTCAATGCGAAGGCATCTTCTTATGTAATAACTGACGTTGTAGGACGAAGGCTTAGCGAAGAAAAAAGGATTAGA
TACCCTCGTATTCTAGGCAGAAAATGATGAATGTCATAGGTTGAATATAATATTATTTTATTTGGTCTATAAATGAAAGT
GTAAGCATTCCACCTCAAGAGTAATTTGGCAACATTGGAACTGAAATCATTAGGCCGCTTTTGAAACCAGTAGTGAAGTA
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