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1 ABBREVIATIONS

*Beast	StarBeast
AICc	Corrected Akaike Information Criterion
BLAST	Basic Local Alignment Search Tool
BP	Base pairs
BP&P	Bayesian Phylogenetics and Phylogeography
BS	bootstrap
CADM	Congruence Among Distance Matrices
COX2	Cytochromeoxidase 2
CTAB	Cetyl-trimethyl Ammonium Bromide
dNTPs	di Nucleoside triphosphate
DNA	Deoxyribonucleic Acid.
GLM	Generalized Linear Model
GMYC	Generalized Mixed Yule Coalescent
GTR	General Time reversible model
ITS	Internal transcribed spacer, Highly variable fragment of the DNA region coding for ribosomal RNA.
LBG	Latitudinal Biodiversity Gradient
MAFFT	Multiple Alignment using Fast Fourier Transform
MCM7	Minichromosome Maintenance Complex Component 7
MCMC	Markov chain Monte Carlo
ML	Maximum Likelihood
mtSSU	mitochondrial small subunit ribosomal RNA gene
NCBI	National Centre of Biotechnology Information
NNI	Nearest Neighbor Interchangables
nuLSU	nuclear Large Sub Unit
PACo	Procrustes Application to Cophylogentic analysis
PCA	Principal Component Analysis
pPCA	Phylogenetic Principal Component Analysis
PCR	DNA Polymerase Chain Reaction.
PP	Posterior probability
RAxML-HPC	Randomized Accelerated Maximum Likelihood-High Performance Computing
RPB1	RNA polymerase II largest subunit coding gene
spedeSTEM	Species delimitation using species trees
STACEY	Species Tree And Classification Estimation, Yarely
STEM	Species Tree Estimation using Maximum Likelihood
TSR1	Ribosome biogenesis protein

2 ABSTRACT

Introduction:

The evolutionary patterns of symbiotic organisms are inferred using cophylogenetic methods. Congruent phylogenies indicate cospeciation or host-switches to closely-related hosts, whereas incongruent topologies indicate independent speciation. Recent studies suggest that coordinated speciation is a rare event, and may not occur even in the highly specialized associations. The cospeciation hypothesis was mainly tested for free-living mutualistic associations, such as plant-pollinator interactions, and host-parasitic systems but was rarely tested on obligate, mutualistic associations involving intimate physiological interactions.

Symbionts with lower partner selectivity may not experience coordinated speciation due to frequent switching of partners. On the other hand, symbionts with high partner selectivity may influence each other's evolution owing to the highly interdependent lifestyles. Symbiont association patterns are also influenced by habitat and it has been proposed that symbiotic interactions are stronger in warm regions as compared to cooler regions (also referred as latitudinal gradient of biotic specialization). This hypothesis however, has recently been challenged and it has been suggested that a gradient of biotic specialization may not exist at all.

Reliable species concepts are a prerequisite for understanding the association and evolutionary patterns of symbiotic organisms. The species concepts of many groups traditionally relied on the morphological species concept, which may not be adequate for distinguishing species due to the: i) homoplasious nature of morphological characters, and due to the inability to distinguish cryptic species. Thus phylogenetic species concept along with coalescent-based species delimitation approaches, which utilize molecular data for inferring species boundaries have been used widely for resolving taxonomic relationships.

Lichens are obligatory symbiotic associations consisting of a fungal partner (mycobiont) and one or more photosynthetic partners, algae, and/or cyanobacteria (photobionts). I used the lichen forming fungal genus *Protoparmelia* as my study system, which consists of ~25-30 previously described species inhabiting different habitats, from the arctic to the tropics. This makes *Protoparmelia* an ideal system to explore the association and evolutionary patterns across different macrohabitats.

Objectives:

The objectives of this thesis were to 1. Elucidate the phylogenetic position of *Protoparmelia* within Lecanorales, and infer the monophyly of *Protoparmelia*; 2. Understand species diversity within *Protoparmelia* s.str. using coalescent-based species delimitation approaches; and 3. To identify the *Trebouxia* species associated with *Protoparmelia* using phylogenetic and species delimitation approaches and to infer the association and cophylogenetic patterns *Protoparmelia* and *Trebouxia* in different macrohabitats.

Results and discussion:

Chapter 1: Taxonomic position of *Protoparmelia*

In the first part of this study I explored the taxonomic position of *Protoparmelia* within the order Lecanorales. Overall this study included 54 taxa from four families, sequenced at five loci (178 sequences). I found *Protoparmelia* to be polyphyletic and sister to Parmeliaceae.

Chapter 2: Multilocus phylogeny and species delimitation of *Protoparmelia* spp.

In this part of the study, I identified and delimited the *Protoparmelia* species forming a monophyletic clade sister to Parmeliaceae i.e., *Protoparmelia* sensu stricto group, based on the multilocus phylogeny and coalescent-based species delimitation approaches. I included 18 previously described and three unidentified *Protoparmelia* species, which represents ~70% of the total described species, and 73 other taxa, sequenced at six loci. I found that the sensu stricto group comprised of 25 supported clades instead of 12 previously described *Protoparmelia* species. I tested the speciation probabilities of these 25 clades using species delimitation softwares BP&P and spedeSTEM. I found nine previously unrecognized lineages in *Protoparmelia* and I propose the presence of at least 23 species for *Protoparmelia* s.str., in contrast to the 12 described species included in the study.

Chapter 3 Association and cophylogenetic patterns of *Protoparmelia* and its symbiotic partner *Trebouxia*

In this part of the study I identified and delimited species of the symbiotic partners of the *Protoparmelia* species using multilocus phylogeny and coalescent-based species delimitation approaches, BP&P and STACEY. I used 174 lichen specimens. Fungal partner was sequenced at 6-loci and the algal partner was sequenced at two loci. I found that 20 *Trebouxia* lineages are associated with 23 *Protoparmelia* species, out of which 15 are novel *Trebouxia* lineages. The present study is among the first studies employing the

coalescent-based species delimitation approaches for identifying green algal lichen symbionts.

I found that the diversity of the *Trebouxia* symbionts associated with *Protoparmelia* was comparable across different macrohabitats. This could be explained by the lifestyle of *Trebouxia* as an inhabitant of the symbiosis, which partially shields *Trebouxia* from direct influences of the external environment. As for the association patterns, symbiont interactions can be highly selective (one-to-one) or generalized (one-to-many). I found that the *Protoparmelia* selectivity is comparable across the habitats whereas *Trebouxia* selectivity is lower in the arctic/temperate regions as compared to the tropical regions. Interestingly, out of the nine specialized one-to-one associations in my study system, eight were from the tropical regions and one from the Mediterranean region. My study suggests the presence of more specialized associations in the tropical regions as compared to the arctic/temperate regions, for the *Protoparmelia-Trebouxia* system.

Cophylogenetic analyses suggested no cospeciation between the *Protoparmelia* symbionts even in the highly specialized associations, which supports the hypothesis that cospeciation is a rare event. Furthermore the evolutionary pattern of the symbionts was different in different macroclimatic regions. The main evolutionary event in arctic/temperate associations was failure to diverge, whereas the major evolutionary pattern for the Mediterranean and tropical *Protoparmelia-Trebouxia* associations was host-switch. My study suggests that different evolutionary forces shape the fungal-algal associations in different macrohabitats.

3 ZUSAMMENFASSUNG

Einleitung

Aufgrund einer Lebensweise in gegenseitiger Abhängigkeit ist zu erwarten, dass die Evolution obligater und spezialisierter Symbionten miteinander verknüpft ist. Die evolutionären Muster symbiotischer Organismen werden mittels ko-phylogenetischer Methoden abgeleitet, welche die Wirt-Symbiont Phylogenien einander gegenüberstellen und auf Kongruenz testen. Bisher wurden kongruente Phylogenien als Hinweis auf koordinierte Artbildung interpretiert, und daher können ko-phylogenetische Methoden dabei helfen zu verstehen ob die Evolution von Symbionten eine gemeinsame oder unabhängige Artbildung beinhaltet. Neuere Untersuchungen legen nahe, dass koordinierte Artbildung selten stattfindet, und die hohe Anzahl vermeintlich gemeinsamer Artbildungen auf einer Fehlinterpretation topologischer Kongruenz beruht. Abgesehen von gemeinsamer Artbildung (Kospeziation) können auch Wirtswechsel zu nahe verwandten Wirten ein kongruentes phylogenetisches Muster erzeugen. Mehrere aktuelle Studien berichten über signifikante topologische Kongruenz aber die Analysen deuten auf Wirtswechsel als treibende evolutionäre Kraft hin. Allerdings wurde die Hypothese der Kospeziation hauptsächlich an freilebenden, mutualistischen Gemeinschaften getestet wie z.B. Pflanze-Bestäuber und Wirt-Parasit Systemen; obligate, mutualistische Gemeinschaften mit engsten physiologischen Wechselbeziehung wurden hingegen kaum untersucht.

Die Art und Weise der symbiotischen Vergesellschaftung beeinflusst die Evolution eines symbiotischen Organismus ebenfalls. Beispielsweise würden Symbionten mit geringer Partner-Selektivität wohl nicht einer koordinierte Artbildung oder beiderseitiger genetischen Veränderungen unterliegen, da sie zu häufig die Partner wechseln. Andererseits können Symbionten mit hoher Partner-Selektivität, bedingt durch die starke Abhängigkeit voneinander, ihre Evolution gegenseitig beeinflussen. Auch das Habitat beeinflusst die Art der symbiotischen Vergesellschaftung und es wird vermutet, dass symbiotische Interaktionen in warmen Gebieten viel stärker ausgeprägt sind als in kalten (der sogenannte Breitengrad-Gradient biotischer Spezialisierung). Diese Hypothese wird jedoch seit Kurzem in Frage gestellt. Einaktueller Übersichtsartikel kommt zu dem Schluss, dass etwa gleichviele Studien existieren, die entweder einen solchen Breitengrad-Gradienten belegen konnten oder die keinen bzw. einen entgegengesetzten Gradienten gefunden haben.

Verlässliche Artkonzepte sind eine Grundvoraussetzung für das Verständnis der Vergesellschaftung und der evolutionären Muster symbiotischer Organismen. Während Artkonzepte makroskopischer Organismen wie Pflanzen, Vögel und Säugetiere im Wesentlichen gut etabliert sind, stecken die Artkonzepte von Mikroorganismen, wie Algen und Pilzen, noch in den Kinderschuhen. Die taxonomische Klassifikation vieler Gruppen beruhte traditionell auf dem morphologischen Artkonzept. Allerdings ist die Verwendung phänotypischer Merkmale insbesondere bei Mikroorganismen für die Artunterscheidung nur bedingt geeignet, denn: i) morphologische Merkmale neigen zur Homoplasie (bzw. Konvergenz), d.h. bestimmte Merkmale können unabhängig voneinander mehrfach entstehen oder verschwinden und geben dann nicht die wahren stammesgeschichtlichen Verhältnisse wieder, und ii) kryptische Arten mit sehr ähnlicher Morphologie können nicht erkannt werden. Aufgrund dieser Einschränkungen des morphologischen Artkonzeptes wurde in den letzten Jahrzehnten zunehmend das phylogenetische Artkonzept angewandt um taxonomische Verwandtschaftsverhältnisse zu klären. Dabei wird eine Art als Gruppe von Organismen aufgefasst, welche von einem gemeinsamen Vorfahren abstammen, also in einem Einzel- oder Multilocus-Stammbaum einen monophyletischen Ast bilden. Allerdings sind die Stammbäume der unterschiedlichen Loci eines Multilocus Datensatzes nicht zwangsläufig kongruent, da die evolutionären Entwicklungswege einzelner Gene vom Evolutionsverlauf der Art selbst abweichen können, z.B. aufgrund von unvollständiger Linientrennung und zwischenartlichem Genfluss, etc. Um den möglichen Einfluss solcher Prozesse in taxonomischen Interpretationen zu berücksichtigen, wurden, einhergehend mit Multilocus-Phylogenien, sogenannte koaleszenzbasierte Methoden der Artabgrenzung eingeführt, welche evolutionär eigenständige Entwicklungslinien erkennbar machen.

Flechten sind obligat symbiotische Organismen bestehend aus einem Pilz-Partner (Mykobiont) und einem oder mehreren photosynthetischen Partnern, Algen und/oder Cyanobakterien (Photobiont). Ich habe die flechtenbildende Pilzgattung *Protoparmelia* als Untersuchungsobjekt verwendet, welche etwa 25-30 zuvor beschriebene Arten umfasst. Die Mitglieder der Gattung *Protoparmelia* bewohnen verschiedene Habitate, von der Arktis und Antarktis bis in die Tropen. Dies macht *Protoparmelia* zum idealen Studienobjekt für die Untersuchung der Diversität, der Assoziation von Pilz- und Algenpartnern, und der evolutionären Muster von Symbionten über verschiedenste Makrohabitate hinweg. Die taxonomische Stellung und Monophylie der Mitglieder der

Gattung war zu Beginn dieser ungewiss, da die Arten eine große Variabilität in den taxonomisch relevanten Merkmalen zeigten und nur wenige DNA Sequenzen vorlagen.

Zielsetzung

Diese Dissertation hatte folgende Ziele: 1. Die Klärung der phylogenetischen Stellung von *Protoparmelia* innerhalb der Lecanorales, und Überprüfung der Monophylie von *Protoparmelia*; 2. Mittels Koaleszenzbasierter Artabgrenzungsmethoden die Artenvielfalt innerhalb von *Protoparmelia* st.str. zu ergründen; und 3. Die Identifizierung der mit *Protoparmelia* assoziierten *Trebouxia* Arten durch Methoden der phylogenetischen Artabgrenzung, sowie Kenntnisse über die ko-phylogenetischen Muster und den Grad der Partner-Selektivität assoziierter *Protoparmelia* und *Trebouxia* Arten in verschiedenen Makrohabitaten. Um diese Ziele zu erreichen, habe ich die systematische Stellung von *Protoparmelia* durch eine Multilocus-Phylogenie rekonstruiert; Koaleszenzbasierte Methoden verwendet um die Arten in *Protoparmelia* und deren symbiotischen *Trebouxia*-Grünalgen abzugrenzen; und habe die topologische Kongruenz zwischen den Phylogenien beider Symbionten analysiert, um neue Erkenntnisse über die ko-phylogenetischen Muster und den Grad der Partner-Selektivität von *Protoparmelia* und *Trebouxia* in verschiedenen Makrohabitaten zu gewinnen.

Kapitel 1: Taxonomische Stellung von *Protoparmelia*

Im ersten Teil dieser Arbeit habe ich die taxonomische Stellung von *Protoparmelia* innerhalb der Ordnung Lecanorales untersucht. Dafür habe ich fünf bereits beschriebene, phänotypisch heterogene *Protoparmelia* Arten verwendet, zusammen mit allen vermeintlich nahe Verwandten von *Protoparmelia*, welche drei verschiedenen Familien angehören. Frühere Studien deuteten eine enge Verwandtschaft von *Protoparmelia* und *Miriquidica* Arten an, daher habe ich auch drei *Miriquidica* Arten in meine Untersuchungen einbezogen. Insgesamt wurden in dieser Forschungsarbeit 54 Taxa aus den vier Familien Cladoniaceae, Gypsoplacaceae, Lecanoraceae, und Parmeliaceae s. str. bearbeitet. Bei allen Taxa wurden fünf Loci sequenziert, dies waren nuLSU, nrITS, *MCM7*, *RPB1* und *TSR1*. Der kombinierte Datensatz umfasst 178 Sequenzen. Ich habe festgestellt, dass zwei der fünf untersuchten *Protoparmelia* Arten den Parmeliaceae nahe stehen und die anderen drei eine statistisch abgesicherte monophyletische Klade mit *Miriquidica* (Lecanoraceae) bilden. Die Typusart der Gattung, *Protoparmelia badia*, steht den Parmeliaceae nahe, weswegen ich vorschlage *Protoparmelia* als Schwestergruppe der Parmeliaceae zu betrachten. Eines der wichtigsten Ergebnisse dieser Arbeit war, dass erstmals gezeigt wurde, dass *Protoparmelia* polyphyletisch ist. Dies war eine bedeutende

Erkenntnis, da Monophylie eine Grundvoraussetzung ist um die Vergesellschaftung von Symbionten und ko-phylogenetische Muster zu analysieren.

Kapitel 2: Multilocus-Phylogenie und Artabgrenzung der *Protoparmelia* spp.

In diesem Teil meiner Arbeit habe ich die Arten der *Protoparmelia* sensu stricto Gruppe, welche eine monophyletische Schwesterngruppe zu den Parmeliaceae bilden, identifiziert und gegeneinander abgegrenzt, was auf Grundlage einer Multilocus Phylogenie und einer Koaleszenzbasierten Artabgrenzungsmethodik geschah.

Diese Analyse umfasste 18 zuvor beschriebene und drei unbeschriebene *Protoparmelia*, was rund 70 % der insgesamt beschriebenen Arten entspricht, sowie 73 Taxa, welche als enge Verwandte von *Protoparmelia* gelten. Meine Arbeit zeigt, dass 12 der 18 *Protoparmelia* Arten zur sensu stricto Gruppe gehören, welche eine statistisch abgesichte monophyletische Schwestergruppe der Parmeliaceae bilden. Fünf andere *Protoparmelia* Arten bilden eine statistisch abgesicherte monophyletische Gruppe mit *Miriquidica* Arten. Die sensu stricto Gruppe, welcher 12 zuvor beschriebene *Protoparmelia* Arten angehören, besteht im kombinierten 6-Locus Maximum Likelihood Phylogramm aus 25 statistisch abgesicherten Kladen. Mit den Softwares BP&P und spedeSTEM habe ich die Wahrscheinlichkeit der Artbildung bei diesen 25 Kladen getestet. Ich schlage vor wenigstens 23 Arten in *Protoparmelia* s.str. anzuerkennen, statt lediglich 12 Arten, die vor dieser Studie, basierend auf morphologischen Merkmalen, beschrieben wurden. Ich habe neun zuvor unerkannte Abstammungslinien in *Protoparmelia* gefunden. Meine Arbeit bekräftigt die Bedeutung molekulargenetischer Phylogenien und Koaleszenzbasierter Methoden der Artabgrenzung für die Identifizierung kryptischer Arten.

Des Weiteren deuten meine Ergebnisse darauf hin, dass die kosmopolitische Arten wohl tatsächlich aus mehreren distinkten Arten in unterschiedlichen geographischen Gebieten bestehen, was zuvor bereits für mehrere andere Arten flechtenbildender Pilze nachgewiesen wurde. Die Wirtsdiversität zuverlässig zu bestimmen ist ein entscheidender Schritt um die Art der Vergesellschaftung und Ko-phylogenetische Muster abzuleiten. Durch eine zu hoch geschätzte Wirtsdiversität assoziiert sich ein Symbiont scheinbar mit mehreren Wirten, eine zu niedrig geschätzte Wirtsdiversität erzeugt fälschlicher Weise den Eindruck, dass ein Wirt sich mit mehreren Symbionten vergesellschaftet.

Kapitel 3: Assoziationsmuster der Pilz- und Algenpartner in unterschiedlichen Makrohabitaten In diesem Teil meiner Arbeit habe ich die Arten der symbiotischen Partner der *Protoparmelia* spp. identifiziert und gegeneinander abgegrenzt; dafür wurden

eine Multilocus-phylogenie, Koaleszenzbasierte Methoden der Artabgrenzung, BP&P and STACEY verwendet. Ich habe 174 Flechtenexemplare für diese Studie benutzt.

Wie in der vorangegangenen Studie, wurden 6 Loci des Pilzpartners sequenziert und beim Algenpartner wurden zwei Loci sequenziert, dies waren nrITS and *COX2*. Ich habe 20 Abstammungslinien von *Trebouxia* gefunden, die mit den 23 Protoparmelia Arten vergesellschaftet sind. Meine Arbeit bestätigt, dass die Diversität von mit Flechten assoziierten Photobionten bisher unterschätzt wurde. Dies hebt die Bedeutung der Anwendung phylogenetischer und Koaleszenzbasierter Methoden für die Identifizierung der symbiotischen Algen hervor. Diese Forschungsarbeit ist eine der ersten Studien, welche Koaleszenzbasierte Methoden der Artabgrenzung verwendet hat um die symbiotischen Grünalgen von Flechten zu identifizieren.

Nachdem ich die mit *Protoparmelia* assoziierte Symbiontendiversität bestimmt hatte, habe ich die Symbiontendiversität und Vergesellschaftungsmuster der *Protoparmelia-Trebouxia* Symbiose in verschiedenen Makrohabitaten analysiert. Interessanter Weise habe ich herausgefunden, dass die Diversität der mit *Protoparmelia* assoziierten *Trebouxia* Symbionten in den verschiedenen Makrohabitaten miteinander vergleichbar ist. Bezüglich der Vergesellschaftungsmuster zeigte sich, dass die symbiotischen Interaktionen spezialisiert (eins-zu-eins) oder generalistisch (eins-zu-vielen) sein können. Ich habe die Vergesellschaftungsmuster der Symbionten in verschiedenen Habitaten untersucht und herausgefunden, dass die Selektivität von *Protoparmelia* für Photobionten in verschiedenen Habitaten miteinander vergleichbar ist (1-3 Algenpartner in arktisch/temperaten Regionen verglichen mit 1 *Trebouxia* Partner in den Tropen), während die Selektivität von *Trebouxia* für Mykobionten in arktisch/temperaten Gebieten niedriger ist als in den Tropen (1-5 *Protoparmelia* und bis zu 65-70 andere Flechten bildende Pilzpartner in arktisch/temperaten Gebieten aber nur ein *Protoparmelia* Partner und bis zu 5 andere Flechten bildende Pilzpartner in den Tropen). Interessant ist, dass acht der neun spezialisierten eins-zu-eins Vergesellschaftungen in den Tropen vorkommen und eine im Mittelmeerraum. Meine Untersuchungen weisen darauf hin, dass die *Protoparmelia-Trebouxia* Symbiose in den Tropen spezialisiertere Gemeinschaften bildet als in arktisch/temperaten Gebieten.

Ko-phylogenetische Analysen lassen keine Kospeziation der *Protoparmelia* Symbionten erkennen, auch nicht bei stark spezialisierten Gemeinschaften. Dies unterstützt die Hypothese, dass Kospeziation selten stattfindet. Des Weiteren unterschieden sich die evolutionären Muster der Symbionten in klimatisch

unterschiedlichen Gebieten. Der bestimmende evolutionäre Vorgang in arktisch/temperaten Gemeinschaften war „failure-to-diverge“, d.h. der Pilzpartner durchlief einen Artbildungsprozess, aber der Algenpartner nicht. Als Folge daraus sind verschiedene Pilzarten mit derselben Algenart assoziiert. Wirtswechsel dagegen waren das bedeutendste evolutionäre Muster mediterraner und tropischer *Protoparmelia*-*Trebouxia*. Basierend auf meinen Analysen komme ich zu der Schlussfolgerung, dass in verschiedenen Makrohabitaten verschiedene evolutionäre Vorgänge die Pilz-Alge Assoziationen formen.

Diskussion

Meine Arbeit bekräftigt die Bedeutung molekulargenetischer und Koaleszenzbasierter Methoden für die Bestimmung der Artenvielfalt, insbesondere bei Taxa mit wenigen taxonomisch relevanten Merkmalen. Des Weiteren fanden sich Hinweise darauf, dass die angeblich kosmopolitischen Taxa wohl tatsächlich mehrere Arten umfassen, die unterschiedliche geographische Gebiete besiedeln. Die Vielfalt der Algenpartner von Flechten ist weit weniger gut erforscht als die der Pilzpartner, und die Anwendung der Koaleszenzmethodik auf die Artabgrenzung von Algen ist eher ungewöhnlich. Meine Arbeit betont die Bedeutung dieser Methodik für die Bestimmung der Vielfalt der Algenpartner, da nur fünf der 20 mit *Protoparmelia* assoziierten Abstammungslinien zuvor beschrieben waren. Meine Analysen legen nahe, dass die mit der Flechtenbildenden Pilzgattung *Protoparmelia* assoziierte Algenvielfalt über verschiedene Makroklimate hinweg vergleichbar ist. Dies steht im Widerspruch zur Erwartung einer höheren Symbiontenvielfalt in den Tropen.

Das beobachtete Muster könnte jedoch durch die Lebensweise von *Trebouxia* als einem „Bewohner“ der Symbiose erklärt werden. Ihre Lage innerhalb des Thallus schützt die *Trebouxia*-Algen zum Teil vor den direkten Einflüssen der äußeren Umwelt. Daher zeigen die *Trebouxia*-Symbionten vielleicht nicht dieselben Muster der Artenvielfalt wie Ektosymbionten oder freilebende, mutualistische Gemeinschaften. Bezüglich des Vergesellschaftungsmusters entlang eines Breitengrad-Gradienten bekräftigt meine Arbeit die traditionelle Sichtweise von Generalisten-Gemeinschaften in kühleren Gebieten und spezialisierten Gemeinschaften in warmen Regionen. Bei Flechten wurde die Vergesellschaftung einer Algenart mit mehreren Flechtenbildenden Pilzen in ähnlichen Habitaten, als Hinweise auf eine adaptive Rolle des Photobionten angesehen. Auch meine Arbeit unterstützt diese Sichtweise einer adaptiven Rolle der Algen in Flechten, da ich herausfand, dass artische/temperate *Trebouxia* Arten mit mehreren phylogenetisch nur

entfernt verwandten Flechtenbildenden Pilzen vergesellschaftet sind. Die ko-phylogenetische Analyse unterstützt die Hypothese, dass Kospeziation wohl doch nicht so verbreitet ist wie früher angenommen, selbst bei obligaten und spezialisierten eins-zu-eins Beziehungen. Daher scheinen andere evolutionäre Ereignisse wie Wirtswechsel und fehlende Divergenz (failure-to-diverge) sehr viel öfter in der Natur aufzutreten.

4 INTRODUCTION

4.1 Systematics of lichenized fungi

Lichen-forming fungi are fungi which form obligate symbiotic associations with one or more photosynthetic partners, typically green algae or cyanobacteria (Ahmadjian, 1965, 1993). The resulting coherent structures are called lichens. The name of different lichens corresponds to the species name of the fungal partner (Ahmadjian, 1965). The fungal partner is also called the mycobiont and the algal partner is the photobiont. The photobiont generates metabolic energy through photosynthesis, which is used as source of nutrition by the mycobiont, and the fungus offers a stable, supportive matrix, which serves as a protected environment for the algal partner (Honegger, 1986; Hawksworth & Honegger, 1994). Lichens represent one of the most successful examples of symbiosis, with lichens found in all habitats, ranging from the Arctic to the tropics.

About 20% of the all fungi are lichenized. Lichenized fungi are found mainly in two of seven phyla in the Kingdom fungi, phyla Ascomycota and Basidiomycota, but not in the remaining five, Chytridiomycota, Zygomycota, Microsporidia, Blastocladiomycota, and Glomerulamycota (Figure 1) (Eriksson & Hawksworth, 1989; Eriksson, 2006; Hodkinson *et al.*, 2014). More than 99% of lichenized fungi belong to phylum Ascomycota (Tehler, 1996; Feuerer & Hawksworth, 2007; Lawrey *et al.*, 2009), whereas only a few, i.e., less than 1% (comprising ~20 species) belong to phylum Basidiomycota (particularly to the orders Agaricales, Cantharellales, Corticiales and Lepidostromatales). The lichenized state has been gained and lost multiple times, creating many phylogenetic clades which contain both lichenized and non-lichenized taxa (Lutzoni *et al.*, 2004). For example, out of 10 classes in the subphylum Pezizomycotina, four classes contain both lichenized as well as non-lichenized fungi. The majority of lichen-forming fungi (75%) belong to the order Lecanorales in the class Lecanoromycetes (subphylum Pezizomycotina).

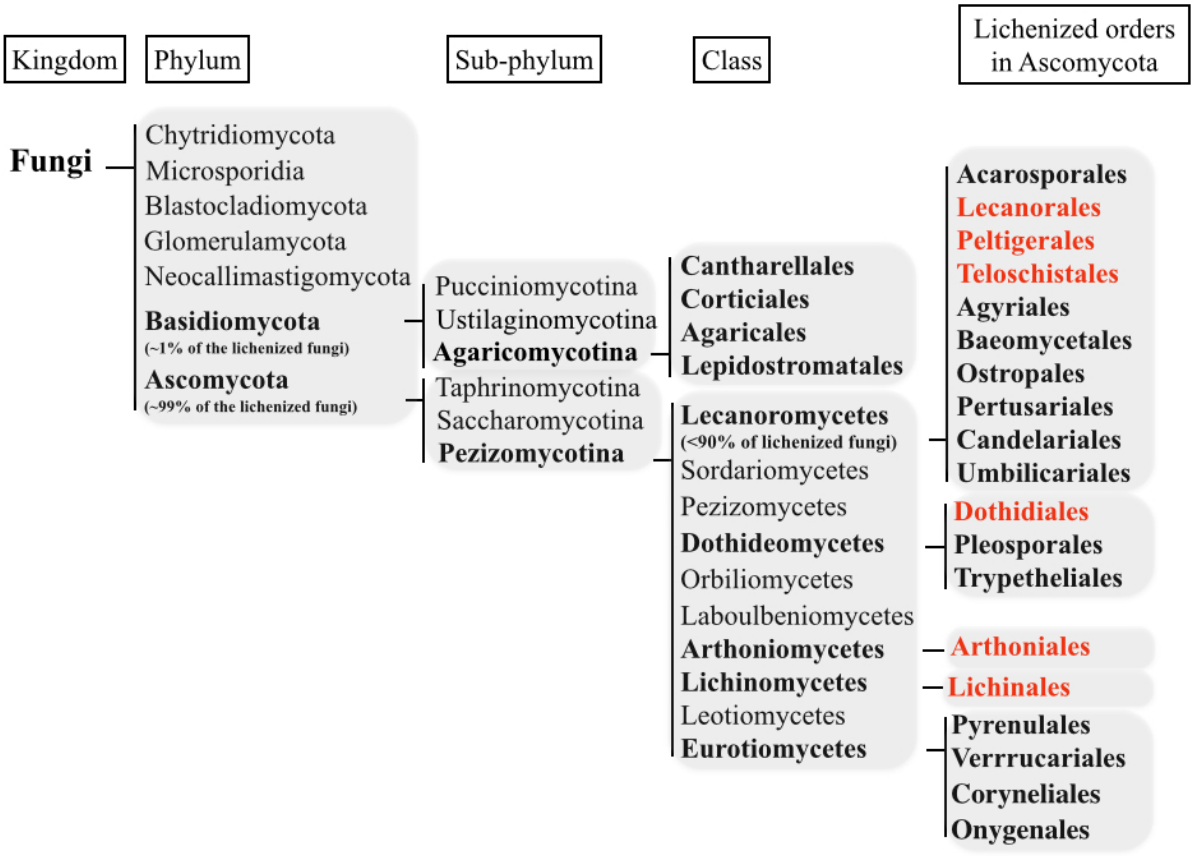

Figure 1. Classification of kingdom Fungi (Source: https://www.britannica.com/science/fungus/Annotated-classification). The figure shows seven phyla of the kingdom Fungi. The groups containing lichen-forming fungi are marked in bold. The red bold names indicate the group comprising only lichenized fungi. Only lichenized classes have been listed for the Basidiomycota. The division up to the order level is shown only for the lichenized ascomycetes.

4.1.1 Traditional approaches in fungal systematics: Morphological species concept

Early systematics of lichen-forming fungi was based on morphology-based species boundaries. Phenotypic characters, such as growth form of the thallus, structure of fruiting body, ontogeny of the fruiting body, ascus structure, shape/size of the ascospores, pycnidial and conidial characters played the predominant role in lichen systematics (Printzen, 2010; Schmitt, 2011; Thell *et al.*, 2012). Members of Ascomycota itself are characterized by the presence of a microscopic sac-like structure called an ascus (plural: asci), which is the meiotic cell containing nonmotile spores, called ascospores (Eriksson & Hawksworth, 1989; Printzen, 2010; Schmitt, 2011). Also, major groups within the Ascomycota are differentiated on the basis of the ascoma shapes: Discomycetes have cup-shaped, open apothecia (called hysterothecia when elongated), Pyrenomycetes have pear-

shaped, apically perforated perithecia, and Plectomycetes have spherical, closed cleistothecia (Schmitt, 2011).

Phenotypic characters are also used for classification of lichen-forming fungi at the family level. For instance, presence of a cupular exciple in ascoma is a typical character of Parmeliaceae (Kärnefelt & Thell, 1992; Mattsson & Wedin, 1998; Thell *et al.*, 2012). Other examples of characters which are still used for family level classification are the presence of *Lecanora*-type ascus in Lecanoraceae and *Bacidea*-type ascus in Bacideaceae.

4.1.2 Limitations of phenotype-based classification

One of the problems with a strictly morphology-based classification is the paucity and variability of morphological characters in lichenized fungi. Many of these characters are homologous and may not indicate true phylogenetic relationships (Lumbsch & Leavitt, 2011). Also, studying ascal features, the most commonly used morphological character in taxonomy of lichen-forming fungi, may not always be feasible due to the problems in identifying them. Furthermore, a classification based on ascal characters poses problems for the classification of asexual species (Printzen, 2010; Schmitt, 2011).

Another shortcoming of phenotype-based classifications is the failure to identify morphologically similar or cryptic species, and many recent molecular studies of lichen-forming fungi have reported several species hidden under a single taxon (Spribille *et al.*, 2011; Molina *et al.*, 2011; Altermann *et al.*, 2014; Lücking *et al.*, 2014). Also, morphological and chemical differences (chemotypes) may represent intraspecific variation, and are thus potentially unsuitable to distinguish evolutionary independent lineages (LaGreca, 1999; Barber *et al.*, 2006; Leavitt *et al.*, 2011b). Furthermore, some phenotypic characters might have been gained and lost multiple times during evolution and therefore could be present in several phylogenetically unrelated taxa. At the family rank certain taxa may share morphological and/or chemical characters inherited from a common ancestor, but the same character can be also found in unrelated groups where it might have appeared independently (Hafellner, 1984; Leavitt *et al.*, 2011b,a). Thus, phenotypic characters traditionally used for classification of lichen-forming fungi may not be adequate for inferring phylogenetic relatedness. In fact, it has been suggested that morphological species concept in fungi should be referred to as “morphological species recognition”, as it can be used to diagnose or identify species but do not necessarily indicate phylogenetic relatedness (Taylor *et al.*, 2000).

The biological species concept may not be applicable for species delimitation and fungal systematics as many fungi are asexual and do not involve sexual stages. These above points have led to an increased importance of molecular data and phylogenetic species circumscription in systematics of lichen-forming fungi. Currently, molecular data appear to rank among the most reliable characters for inferring evolutionary boundaries and species boundaries in lichen-forming fungi, especially in cases of cryptic species and asexual species. For instance, some fungi previously placed in Deuteromycota due to the absence of sexual stage were later moved to Ascomycota based on the molecular data (Lutzoni *et al.*, 2004).

4.2 Phylogenetic species concept and species delimitation of lichenized fungi

4.2.1 Phylogenetic species concept

Variations of the phylogenetic species criterion recognize species as group of organisms descending from a common ancestor, forming a monophyletic clade on a phylogenetic tree (Taylor *et al.*, 2000). Species delimitation based on phylogenetic data has been proposed to be more objective than morphological characters, or implementing a biological species criterion for species delimitation, as any changes in gene sequences precede the changes in phenotypic characters. In fact, in the last decades understanding of species-level diversity has vastly improved due to the use of molecular phylogenies, which facilitate the identification of phenotypically cryptic and semi-cryptic lineages previously hidden under a single taxon (Hendrixson & Bond, 2005; Gamble *et al.*, 2012; Carter, 2012; Agarwal *et al.*, 2014).

In phylogenetic approaches to species circumscription, supported monophyletic clades in phylogenetic reconstructions are considered as independent species. Phylogenetic trees are inferred by collecting sequence data from multiple loci, generally by using the best fitting model for substitution at each locus (Rokas *et al.*, 2003; Gadagkar *et al.*, 2005; Leavitt *et al.*, 2013a; Saag *et al.*, 2014). The availability of markers for amplifying phylogenetically informative loci has provided great insights into otherwise unrecognized species complexes. Some of the markers frequently used in phylogenetic studies of lichen-forming fungi are: nuITS, nuSSU, nuLSU, mtSSU rDNA, *RPB1*, *MCM7*, beta tubulin, *TSR1*, and glyceraldehyde-3-phosphate dehydrogenase (Matheny *et*

al., 2002; Crespo *et al.*, 2002; Myllys *et al.*, 2003; Raja *et al.*, 2011; Tretter *et al.*, 2013). Concatenated, multilocus data sets are now the most widespread and reliable sources for inferring species boundaries (Thell *et al.*, 2002; Šoun *et al.*, 2011; Molina *et al.*, 2011; Parnmen *et al.*, 2012; Del-Prado *et al.*, 2013; Leavitt *et al.*, 2013a).

Incongruence among gene tree topologies in a multilocus data set confounds taxonomic conclusions (Maddison, 1997; Than & Nakhleh, 2009; Liu *et al.*, 2009). The discordant divergence of different genes within a species leads to the differences in the topology among gene trees, especially in cases of recently diverged species (also referred to as deep coalescence). Processes leading to incongruent gene trees include: incomplete lineage sorting, gene duplication and loss, horizontal gene transfer and hybridization. Incomplete lineage sorting refers to retention and stochastic sorting of ancestral polymorphisms. Therefore, reliable estimates of phylogenetic inferences should consider aspects of population genetics, such as genetic drift, and selection, and also incorporate it into the phylogenetic reconstructions (Maddison & Knowles, 2006).

4.2.2 Coalescent-based species delimitation approaches

Coalescent-based species delimitation approaches quantify the probability of evolutionary independence, accommodating for the observed conflict among gene trees inferred from multiple loci (Liu *et al.*, 2009; Fujita *et al.*, 2012). These methods consider individual branches of the species tree as a separate coalescent model and use multilocus data to test the alternative hypotheses of lineage divergence that allow for gene tree discordance (Rannala & Yang, 2003; Liu *et al.*, 2009; Fujita *et al.*, 2012). These methods consider both the properties of population genetic processes and phylogenetic relatedness and thus provide a strong framework for identifying evolutionary independent lineages. These approaches assume incomplete lineage sorting as the main cause of incongruence between gene trees, although some methods also take hybridization and/or recombination into account (reviewed in Degnan & Rosenberg, 2009). The coalescent-based approaches assume free recombination between genes and absence of intra-gene recombination, absence of selection, random mating in each population, and presence of unlinked loci (Liu *et al.*, 2009; Carstens & Dewey, 2010; Jones, 2014).

Coalescent-based species delimitation approaches can be broadly classified into species discovery and species validation approaches. Species discovery approaches do not require a priori information regarding the species groups (e.g., O'Meara, 2010), and instead allocate the samples into populations and then predict species boundaries without

a priori grouping. Some common discovery approaches are Gaussian Clustering (Hausdorf & Hennig, 2010) and the General Mixed Yule Coalescent model (GMYC, Pons *et al.*, 2006). Validation approaches on the other hand require a priori assignment of samples into putative species. These approaches are applicable where either subspecific taxonomy can serve as the basis for lineage assignment (e.g., Carstens & Dewey, 2010) or where other characters can be used to formulate species scenarios. Some common softwares based on validation approaches are BP&P (Yang & Rannala, 2010, 2014) and spedeSTEM (Ence & Carstens, 2011).

Each species delimitation approach has some underlying assumptions and may not be universally suitable to all kinds of organisms/data. For instance, spedeSTEM uses user-specified gene trees to infer the maximum likelihood species tree and hence operates under the assumption that gene tree topologies are correct. Any uncertainty in gene trees may therefore compromise the estimation of number of species by spedeSTEM. Similarly, the accuracy of GMYC is affected by the imbalanced sampling across taxa and hence may not perform well when the putative species are represented by uneven sample sizes (Talavera *et al.*, 2013). Therefore, the specific empirical species delimitation analysis should be selected taking into account the data. A methodological framework of species delimitation should involve generation of sequence data from single/multiple-loci, followed by assessment of species boundaries using one or more species delimitation approaches, given the data (Fujita *et al.*, 2012; Carstens *et al.*, 2013).

4.3 Diversity of the photobionts associated with lichen-forming fungi

4.3.1 Identification of the photobionts

Traditional approach: morphological species recognition

The diversity of the photobionts associated with lichen-forming fungi is far less explored than the mycobionts, as the species circumscriptions and identification of photobionts associated with lichen-forming fungi are still in infancy (Honegger, 2009; Printzen, 2010). So far, approximately 40 genera have been recognized as typical lichen photobionts, which includes cyanobacterial as well as green algal symbionts (Ahmadjian, 1993; Friedl & Büdel, 2008). Lichenized algae have traditionally been identified based on

morphological and in-vitro culture characteristics, coupled with light or electron microscopic analyses and comparisons with reference strains (Ahmadjian, 1987a; Tschermak-Woess, 1988). Some of the characters used for the classification of algae include: the position of the chloroplast before sporogenesis, number of cells produced as a result of asexual reproduction, pyrenoids, and cell shape. However, as also previously stated, classifications based on phenotypic characters may underestimate species diversity and lead to misidentified taxa due to the homoplasious nature of certain morphological characters. Furthermore, it may be difficult to identify the characters which correspond to the taxonomic relationship. For instance, *Trebouxia* which is one of the most common green algal lichenized photobiont, was initially characterized on the basis of morphological features. Although several studies reported the heterogeneity in the phenotypical characters within the genus, the taxonomic relevance of these features were often debated (Ahmadjian, 1959, 1960, Gärtner, 1985a,b; Tschermak-Woess, 1988; Kroken & Taylor, 2000). Later, the use of molecular data revealed the genus to be paraphyletic, based on which *Trebouxia* was then split into two genera, *Trebouxia* and *Asterochloris* (Friedl, 1995; Friedl & Rokitta, 1997; Rambold *et al.*, 1998; Helms *et al.*, 2001). The monophyly of *Asterochloris* was later supported by several other studies as well (Piercey-Normore & Depriest, 2001; Friedl & Büdel, 2008). *Trebouxia* and *Asterochloris* are currently established as the most common green algal symbionts of lichens, associating with more than 50% of the lichens.

Molecular species recognition of lichen photobionts

Currently, the identification of photobionts involves amplifying one or several algal loci from the specimen of interest and comparing the sequences with the reference culture strains (Leavitt *et al.*, 2015a). The cultured strains are morphologically described algal species, many of which also have the sequence information available at one or more loci. Some of the culture collections of algae are: CPCC (Canadian Phycological Culture Centre), SAG (Sammlung von Algenkulturen der Universität Göttingen), and UTEX (Culture Collection of Algae at the University of Texas). The SAG is the culture collection of algae at Göttingen University and it consists of about 500 genera and 1400 species. The UTEX is the culture collection of algae at the University of Texas at Austin and includes approximately 3000 algal strains. The SAG includes 10 *Trebouxia* and 10 *Asterochloris* strains, whereas UTEX consists of 25 *Trebouxia* strains. For the photobiont identification, sequences of interest are compared with the sequences generated from the

cultured algal strains. The ITS rDNA sequences is the most commonly used marker and has been widely used as DNA barcode for identifying the algae associated with lichenized fungi (Muggia *et al.*, 2010; Sadowska-Deś *et al.*, 2014; Leavitt *et al.*, 2015a).

Although sequence similarity with the culture strains provides an indication of the photobiont identity, this approach remains limited as the number of cultured photobionts strains is far less than the number of lichenized algae. For instance, among the photobionts isolated from the members of the family Cladoniaceae, associating with the green algal genus *Asterochloris*, only 15% of the photobionts could be assigned to previously described species from the *Asterochloris* cultures (Skaloud & Peksa, 2010). Similarly, of the photobionts associating with the members of Parmeliaceae, only about 30% OTUs (21 OTUs out of 69) could be assigned to the previously described *Trebouxia* species from the culture collections (Leavitt *et al.*, 2015a). This clearly indicates that the phenotype-based classification does not accurately delimit evolutionarily independent lineages, especially in case of morphologically similar cryptic species (Piercey-Normore & Depriest, 2001).

Phylogenetic approaches to photobiont identification generally involve generating ML or Bayesian trees based on single- or multilocus data set (Piercey-Normore, 2006; Skaloud & Peksa, 2010; Ruprecht *et al.*, 2012; Muggia *et al.*, 2013; Dal Grande *et al.*, 2014a,b; Leliaert *et al.*, 2014; Nyati *et al.*, 2014). Some of the most commonly used markers for identifying the algae associated with lichen-forming fungi are: internal transcribed spacer region (ITS rDNA), ribulose-bis-phosphate carboxylase (*rbcL*), part of the actin gene, chloroplast intergenic spacer (*psbJ-L*), and cytochrome C oxidase II (*COX2*). In phylogenetic approaches to photobiont identification, the sequences from the culture collections are compared with the sequence of interest to identify previously described species. Multilocus phylogenies provide more resolved and better-supported topologies as compared to the ITS-based phylogeny, as many of the markers are more conserved and generate better alignments. For instance, the ITS locus cannot differentiate closely related lineages, e.g. *T. glomerata* and *T. irregularis* (Skaloud & Peksa, 2010). Studies on photobiont identification suggest several cryptic photobiont lineages hidden under a single taxon (Muggia *et al.*, 2008, 2013; Magain *et al.*, 2016). Well-supported monophyletic lineages that do not group with any previously described species are usually considered as new species. Due to the incomplete perspective of species-level diversity for the photobionts, the identified clades are commonly given provisional names, rather than formal taxonomic recognition. Therefore, although the number of studies on the

photobiont identification has increased in the last decades, the number of described species remains very low.

Studies using coalescent-based species delimitation of algae are scarce and have been so far restricted to the *Trebouxia* symbionts of the lichen-forming fungus *Lasallia pustulata* (Sadowska-Deś *et al.*, 2014). The authors used a combination of coalescent-based species delimitation approaches (GMYC, and STEM), and found *L. pustulata* to be associated with five species-level lineages of *Trebouxia.* This study highlights the importance of multilocus phylogenies and species delimitation analysis in identifying the cryptic photobiont lineages associated with lichen-forming fungi.

4.3.2 Association patterns of symbionts in lichens

Patterns of symbiont association

Symbiont interactions can be described in terms of both specialized interactions, which indicates interactions with a limited number of partners (one-to-one) or generalized interactions (one-to-many), which refers to flexible associations accepting multiple partners (Beck *et al.*, 1998; Yahr *et al.*, 2004). For example, mycobionts are photobiont specialists if they associate with only one photobiont lineage, and they are generalists if they accept more than one algal partner. Additionally, in lichens two other terms are commonly used to describe fungal-algal association patterns, namely specificity and selectivity (Beck *et al.*, 1998, 2002; Yahr *et al.*, 2004). Specificity refers to the possible range of acceptable partners for the holobiont (mycobiont and photobiont). When both partners are highly selective towards each other, the symbiosis is considered specific. Selectivity, on the other hand, indicates the preferential association with one partner when other compatible partners are available. Highly selective mycobionts associate in unequal frequencies with the available photobionts.

Association patterns of lichen symbionts have been assessed only for a limited number of taxa, mainly because of the uncertainty in species boundaries which hinders the understanding of these interactions. Different association patterns have been reported for lichens. For example, Muggia *et al*. (2014) investigated the photobiont association pattern of the lichen-forming fungus *Tephromela atra* and found that *T. atra* associates with 12 lineages of *Trebouxia*. Similarly, Yahr *et al*. (2004) studied the association pattern of eight species of the lichen-forming fungal genus *Cladonia* and found six species to be photobiont specialists and two species to be photobiont generalists. Several

studies have focused on algal selectivity and association patterns in lichens (Beck *et al.*, 1998; Yahr *et al.*, 2004, 2006; Hauck *et al.*, 2007; Muggia *et al.*, 2011, 2013, 2014; Vargas Castillo & Beck, 2012; O'Brien *et al.*, 2013; Leavitt *et al.*, 2015a). Most of these studies aimed at identifying the photobionts associated with different lichen species and in characterizing the symbiont selectivity pattern, i.e. if the lichen-forming fungi were photobiont specialists or generalists (Beck *et al.*, 1998; Yahr *et al.*, 2004; Guzow-Krzeminska, 2006; Hauck *et al.*, 2007). Although there have been several studies on fungal-algal association patterns in lichens (see above), studies on how these patterns vary across different climatic regions are lacking. A few studies have focused on the photobiont identification and symbiont association patterns in lichens with wide ecological amplitude, for example, *Tephromela atra* (alpine and Mediterranean habitats, Muggia *et al.*, 2010), *Ramalina menziesii* (subtropical, Mediterranean, and temperate climate, Werth & Sork, 2014), *Cladonia subtenuis* (coastal and dry habitats, Yahr *et al.*, 2006), *Lepraria* s.str. (sun-exposed vs. sheltered sites, Peksa & Skaloud, 2011), *Cetraria aculeata* (arctic/alpine vs. temperate habitats, Fernández-Mendoza *et al.*, 2011). In general, lichen-forming fungi occupying large ecoregions have been shown to be generalists as they tend to associate with different algae in different habitats. However, only a few of these studies used molecular markers for both partners and none of them used molecular species delimitation approach for assessing evolutionarily independent lineages. Lack of robust species concepts may lead to erroneous interpretation of symbiont associations patterns. For instance, underestimated *Trebouxia* diversity would make *Trebouxia* appear as a multi-host symbiont. Furthermore, studies encompassing different climatic zones, from arctic to the tropics to analyze symbiont association patterns are still lacking. In particular, analyzing association patterns in closely related species from different habitats could help us understand how species interactions change under different environmental conditions.

Symbiont interaction in different macrohabitats

Biotic interactions between two or more species have been proposed to be much stronger in the warm, tropical regions as compared to the cooler arctic/temperate regions (Mittelbach *et al.*, 2007; Schemske *et al.*, 2009). Variation in the strength of biotic interactions across a latitudinal gradient has been attributed to the differences in selection pressures. At higher altitudes/latitudes abiotic factors such as temperature and humidity are the major selective pressures, whereas at the lower altitudes/latitudes climate is stable

and biotic interactions are more important (Dobzhansky, 1950; Schemske, 2009). This leads to coordinated evolution and co-adaptation in the warmer regions. It is thus proposed that biotic interactions and specialization increases towards the equator (Dobzhansky, 1950; Pianka, 1966; Schemske *et al.*, 2009; Jocque *et al.*, 2010; Pellissier, 2015). Therefore one would expect more generalist species in the arctic/temperate areas and more specialist species towards the tropics. This view however has been recently challenged as several studies failed to find more specialized interactions in the tropical regions (Schleuning *et al.*, 2012; Moles & Ollerton, 2016). These results indicate that there may not be any gradient in biotic specialization.

4.4 Evolution of interacting species: Coevolution

Watson & Pollack (1999) proposed that symbiosis guides the genetic make-up of the interacting organisms in a way that would be very unlikely to occur individually as separate organisms. This process of interdependent evolution of two interacting species owing to their connected and dependent lifestyles is called coevolution (Ahmadjian, 1987b; DePriest, 2004). It involves reciprocal selection pressure leading to changes in allele frequencies between interacting species over successive generations (Fahrenholz, 1913; Klassen, 1992; Page, 2003; de Vienne *et al.*, 2013). Some familiar examples of coevolving mutualistic associations are: plants and the associated nitrogen-fixing bacteria (Jeong *et al.*, 1999), figs and fig wasps (Marussich & Machado, 2007), and Yucca and Yucca moth (Godsoe *et al.*, 2008; Althoff *et al.*, 2012).

4.4.1 Evolution of lichen symbionts

Ahmadjian (1987b) suggested that the lichen symbiosis is a highly coevolved system due to the obligatory nature of the symbiotic association, i.e. the absence of free living stages in most of the lichen-forming fungi and probably also in the green algal partners. Moreover, the increased fitness of both symbionts in the lichenized state also indicates long-term coevolution (DePriest, 2004). The symbiotic association in lichens leads to the formation of a highly integrated structure – the thallus, which is morphologically, chemically, and physiologically different from either of the symbiotic partner. The distinct phenotypic and physiological characters resulting from this symbiotic association suggest that the fungus and alga have undergone long term and reciprocal selection and adaptation. Another argument in favor of the coevolution hypothesis of lichen symbionts

is the controlled parasitism of the alga by the fungus (Ahmadjian & Jacobs, 1981; Ahmadjian, 1987b; Lücking *et al.*, 2009). Many mycobiont species have evolved hyphal structures typically found in parasites (haustoria). These specialized hyphae penetrate algal cells without killing them (Ahmadjian, 1982; Honegger, 1986), but aid the process of nutrient uptake from the photobiont.

Although several authors support the hypothesis of coevolution in lichens symbionts, it has only been tested in a few studies (Ahmadjian, 1987b; DePriest, 2004). In general, the cospeciation hypothesis has been rejected for the lichen symbionts, and, rather, algal switching has been suggested as a more common phenomenon (Kroken & Taylor, 2000; Piercey-Normore & Depriest, 2001). However, these studies were based on lichen-forming fungi with wide ecological niches, which have been shown to be flexible towards their algal partner and to associate with different algae under different environmental conditions. While algal switching may well be a common phenomenon in lichens with broad ecological amplitude, it may not be common in lichens with narrower ecological ranges. In these cases, other forces such as failure to diverge or cospeciation might be driving the fungal-algal evolution.

4.4.2 Patterns of coevolution

The most familiar pattern of coevolution is cospeciation, which indicates concomitant divergence of the symbionts. Often, coevolution is mistakenly used as a synonym of cospeciation. However, cospeciation refers to the simultaneous divergence of the species and it is one of the several processes by which the interacting species evolve. In fact, species can evolve without codiverging with their partner. The coevolution of species may involve five different events (Figure 2), i) **cospeciation**, when the symbionts diverge simultaneously (Hafner *et al.*, 1994); ii) **duplication**, when a symbiont speciates independently of the host and both the novel symbiont lineages associate with the same host; iii) **failure to diverge**, when a symbiont does not diverge with the host and the same symbiont lineage associates with both the new host lineages; iv) **host-switch**, when a symbiont switches to a closely related host; and v) **loss,** or absence of a symbiont from the host lineage, which could be due to extinction of the symbiont, or due to lineage sorting, when a symbiont associates with only one of the two host lineages (Brooks, 1988; Ronquist, 1997; de Vienne *et al.*, 2013).

4.4.3 Cophylogenetic analysis

Evolution of symbionts can be studied by simultaneously analyzing the phylogenies of interacting species and testing for the topological congruence (Peek *et al.*, 1998; Piercey-Normore & Depriest, 2001; Hosokawa *et al.*, 2006; Cuthill & Charleston, 2012; Buckley *et al.*, 2014; Rodriguez *et al.*, 2014). This approach was based on the idea that the interacting species will have congruent phylogenies if they have diversified exclusively by cospeciation. Consequently, congruence between symbiont phylogenies has been interpreted as evidence of cospeciation, whereas incongruence between host-symbiont phylogenies has been interpreted as an indication of independent evolution of the symbionts involving processes such as failure to diverge, extinction and duplication (Charleston & Robertson, 2002; de Vienne *et al.*, 2007, 2013; Giraud *et al.*, 2010; Peterson *et al.*, 2010). However, several studies suggest that multiple host-switches followed by speciation may also lead to congruent phylogenies (de Vienne *et al.*, 2007, 2013). This process is called as 'pseudocospeciation' (Hafner & Nadler, 1988).

Studies which propose strict cospeciation between interacting species solely based on the topological congruence may be misleading, as such inferences do not differentiate host-switches to closely-related hosts from cospeciation (de Vienne *et al.*, 2007, 2013). In fact, several studies have reported significant topological congruence in spite of the absence of cospeciation (Charleston & Robertson, 2002; Sorenson *et al.*, 2003; Huyse & Volckaert, 2005; Banks *et al.*, 2006; de Vienne *et al.*, 2007; Millanes *et al.*, 2014). Therefore, careful evaluation of host-symbiont topologies is important for a reliable interpretation of the cophylogenetic patterns between host and symbiont.

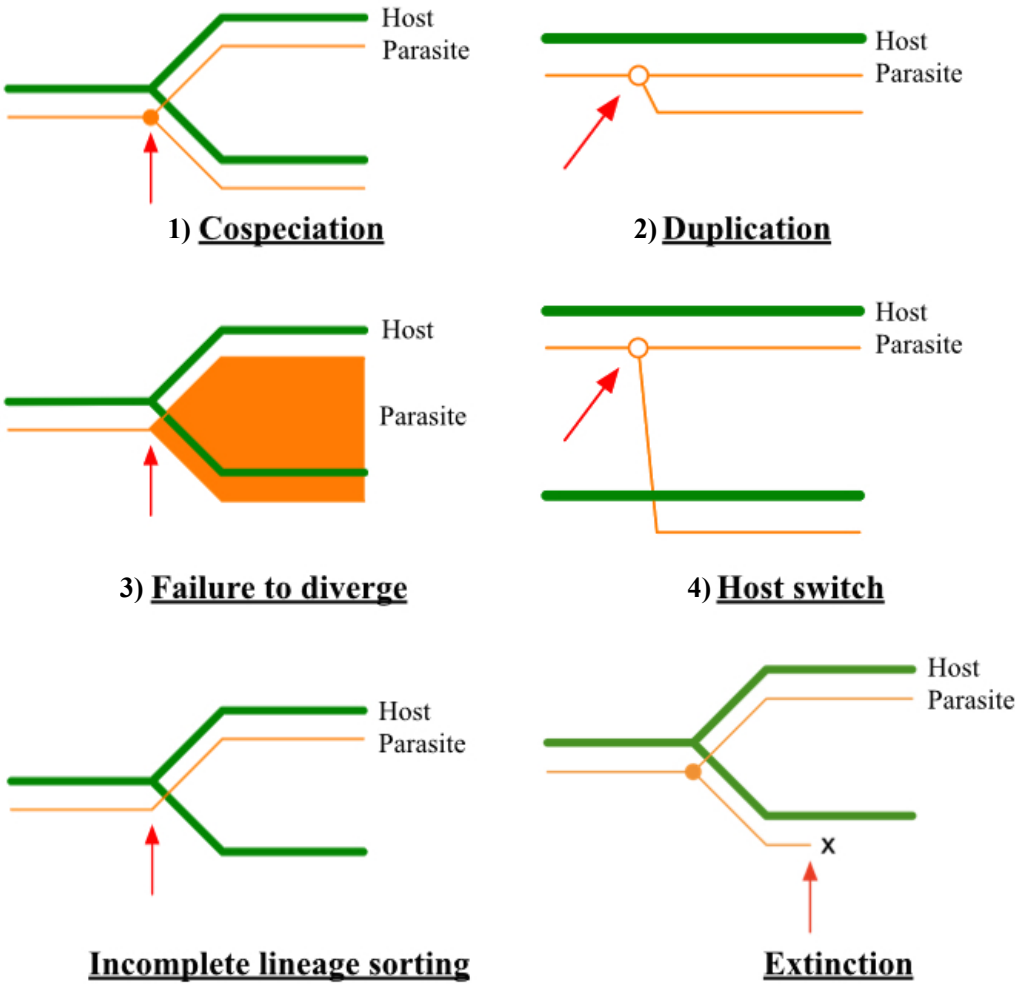

Figure 2. Five major patterns of coevolution. Green lines refer to the diversification of the host and orange lines refer to the diversification of the symbiont/parasite. Red arrows indicate the event of coevolution. The five patterns of coevolution include, 1) cospeciation- concomitant divergence of host and symbiont, 2) duplication- symbiont evolves independently of the host, 3) failure to diverge- host diverges into two lineages and both host lineages remain associated with the same symbiont, 4) host switch- symbiont is transmitted to a different host, and 5) loss- host diverges but the symbiont is missing from some of the new host lineages due to incomplete lineage sorting or extinction. Adapted from: https://sites.google.com/site/cophylogeny/glossary.

One of the consequences of inferring topological congruence as an indication of cospeciation is that it biases the major evolutionary event for interacting species towards cospeciation. As a result, cospeciation was considered as the prevalent mode of symbiont evolution, especially for specialized associations (Page, 2003; de Vienne *et al.*, 2007, 2013; Araujo *et al.*, 2015). However, with the advent of more powerful tools to

differentiate between cospeciation and host-shifts, this hypothesis is losing support. In fact, more widely accepted idea is that the cospeciation is a rare event, even in case of obligate and specialized symbionts (de Vienne *et al.*, 2007, 2013). Instead, host-shifts are emerging as the most common process shaping host-symbiont associations. For example, a recent review on cophylogenetic studies suggested that out of all the studies inferring cospeciation, only 6% constitute convincing cases of cospeciation (de Vienne *et al.*, 2013). Host-shifts have been suggested as a predominant process shaping symbiotic associations for several symbiotic systems such as: plants and their fungal parasites (Refrégier *et al.*, 2008), lichens and their fungal parasites (Millanes *et al.*, 2014), plant and their pathogens (Roy, 2001), bark beetles and nematodes (Susoy & Herrmann, 2014), and birds and their malarial parasites (Ricklefs & Fallon, 2002). With these studies supporting the predominance of host-shift events in symbiotic associations, the actual cases of cospeciation between host and symbiont are reduced to a few mutualistic associations, most often involving vertically transmitted symbionts (Hosokawa *et al.*, 2006; Desai *et al.*, 2010).

4.4.4 Evolution of symbiotic systems: The parasite paradox

Interactions in symbiotic systems are more intimate than in free-living systems owing to their physiologically interdependent life-styles. Due to interlinked lifestyles in obligate mutualistic associations, it has been proposed that the most prevalent process shaping these associations is cospeciation. The pattern of evolution has been proposed to be influenced by the strength of interaction between species (Dobzhansky, 1950; Schemske *et al.*, 2009). Highly selective interactions with only a limited number of partners may facilitate coordinated speciation or codivergence, by increasing the response of that species to the selection imposed by the partners (Ashen & Goff, 2000; Ronquist, 1997). On the other hand, symbiotic systems with flexible association between partners or generalist species may not be codiverging due to frequent switching of partners (Ronquist, 1997; de Vienne *et al.*, 2007, 2013). Some examples of specific mutualistic associations where cospeciation has been reliably reported are: bacteria associated with aphids (Jousselin *et al.*, 2009), clams (Peek *et al.*, 1998), and between plants and ants (Lo *et al.*, 2003). However, frequent host-shifts have also been reported for mutualistic associations, for example in fig trees and wasps (McLeish & van Noort, 2012) and for other specialist parasitic associations (Charleston & Robertson, 2002; Sorenson *et al.*, 2003), suggesting that even highly specific associations may not cospeciate. These studies support the idea

that even in case of highly specific symbionts cospeciation may be a rare event, and, in fact, host-switching could be the main event governing host-symbiont associations (de Vienne *et al.*, 2007, 2013; Lei & Olival, 2014; Susoy & Herrmann, 2014). This confounding observation of predominant host-switching events in specialist symbionts is called as the parasite paradox. Precisely, the specialist associations should be co-adapted and thus should alleviate host-switching; however, host switching is a common event in host-symbiont diversification (Agosta *et al.*, 2010; Araujo *et al.*, 2015).

4.4.5 Overview of the introduction: Species interactions and evolution

Evolution of species is governed by both abiotic and biotic factors. The relative importance of abiotic and biotic factors in the evolution of species has been recently reviewed by Voje *et al*. (2015). Several authors support abiotic factors as being the major evolutionary force (Barnosky, 2001; Eldredge, 2003; Benton, 2009; Lieberman, 2012), in contrast to those that argue for the overall importance of biotic interactions as the major driver of evolution (Aberhan *et al.*, 2006; Jablonski, 2008; Vermeij, 2013). However, the relative importance of abiotic and biotic factors in shaping evolution may vary across spatial and temporal scales (Dobzhansky, 1950; Thompson, 2001; Schemske *et al.*, 2009). These authors suggested abiotic factors as the major evolutionary force in unstable Arctic/temperate regions and biotic factors as the major evolutionary force in the stable tropical environments. In general, both the abiotic factors and strength of biotic interactions vary across the latitude. Consecutively, diversification patterns are expected to differ from poles to the equator. For example, a stable tropical climate is hypothesized to result in interdependent evolution and codiversification. Furthermore, warmer climates have been linked to faster evolution, potentially owing to higher mutation rates and shorter generation times of individuals as compared to temperate/arctic species (Fischer, 1960; Willig *et al.*, 2003; Araujo & Costa-Pereira, 2013; Rolland *et al.*, 2014), supporting the hypothesis that evolutionary patterns vary from poles to the equator.

4.5 Study system: *Protoparmelia*

I selected the lichen-forming fungal genus *Protoparmelia* as my study system. *Protoparmelia* includes approximately 25-30 species inhabiting diverse environments (Coppins, 1929; Poelt & Leuckert, 1991; Miyawaki, 1991; Aptroot *et al.*, 1997, 2013; Ryan *et al.*, 2004; Brodo & Aptroot, 2005; Barber *et al.*, 2006; Arup *et al.*, 2007; Pérez-Ortega & Etayo, 2008; Kantvilas *et al.*, 2010; Papong *et al.*, 2011). *Protoparmelia* species are crustose (Figure 3), have grey-brown to reddish-brown thalli, non-septate ellipsoid ascospores with thin and hyaline filamentous appendages, and a *Lecanora*-type ascus (Coppins, 1929; Ryan *et al.*, 2004). All members of *Protoparmelia* are lichenized and form symbioses with green algae of the genus *Trebouxia*. Some members of this group, such as *Protoparmelia badia* and *P. isidiata* have a broad geographic distribution and are distributed across continents whereas certain others have a limited distribution (Figure 4). For example, *P. hypotremella*, *P. oleagina*, *P. montagnei*, and some others are endemic to certain regions only, for example, *P. orientalis* and *P. corallifera* (**Table 1**). This study system therefore offers an opportunity to study how symbiont diversity, species interactions and evolution of closely related species vary across different macrohabitats.

I included 18 previously described species and two unidentified species-level lineages in my study (**Table 1**). It was not possible to include following *Protoparmelia* species either due to the unavailability of the specimens or due to the difficulty in amplifying the samples: *P. australiensis*, *P. badiola*, *P. effigurans*, *P. gesamia*, *P. hierescens*, *P. loricata*, *P. nebulosa*, *P. nitens*, *P. olivascens*, *P. placentiformis*, and *P. rogersii*.

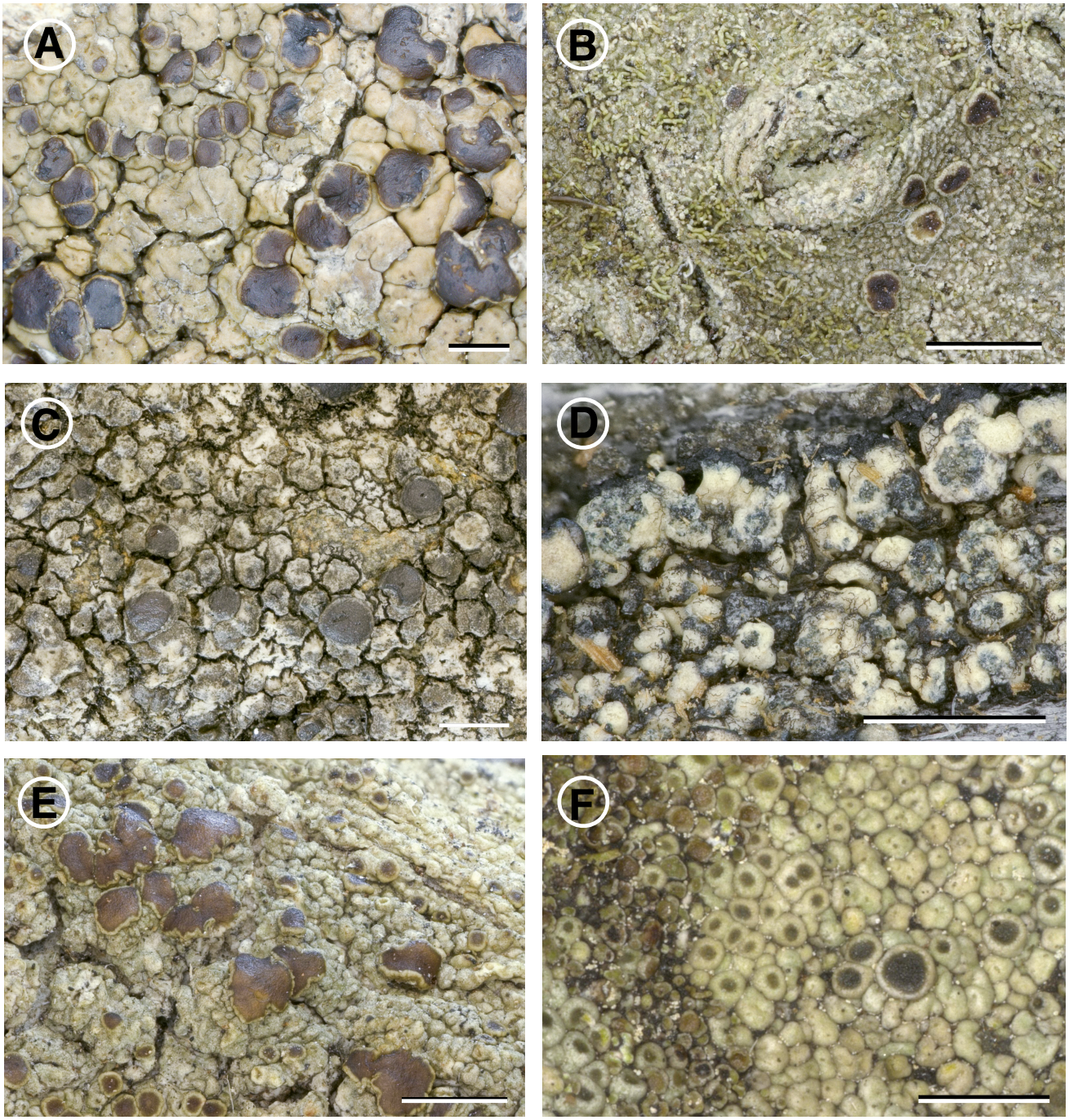

Figure 3. Different species of the crustose lichen-forming fungal genus *Protoparmelia* A) *P. badia* (*Hafellner* 68478, GZU), B*) P. corallifera* (Papong-7101, MSUT), C) *P. memnonia* (Holien-13370, TRH), D) *P. oleagina* (Holien- 10816, TRH), E) *P. orientalis* (Papong-6922, MSUT), and F) Lichenicolous *Protoparmelia* species (new species, Spribille s. n. 23.09.2012). Scale = 1mm.

Table 1. The species of *Protoparmelia* used in this study, their habitat and distribution.

Species	Habitat/ecosystem	Distribution
P. atriseda	montane/alpine	Europe, North America
P. badia	arctic/alpine	Cosmopolitan
P. capitata	subtropical	Southeastern North America
P. corallifera	tropical	Asia
P. cupreobadia	alpine	Europe, Asia, North America
P. hypotremella	temperate	Europe
P. isidiata	subtropical	Southeastern North America
P. leproloma	arctic	Northern Europe
P. memnonia	arctic/alpine	Europe
P. montagnei	Mediterranean	Southwestern Europe
P. multifera	tropical	Neotropics (Brazil, Mexico)
P. nephaea	alpine	Europe, North America
P. ochrococca	temperate	Western North America, Europe
P. oleagina	temperate	Western and northern Europe
P. orientalis	tropical	Australia, Thailand
P. phaeonesos	arctic/alpine	Europe
P. pulchra	temperate/subtropical	Australia (incl. Tasmania), Asia
P. ryaniana	Mediterranean	North America

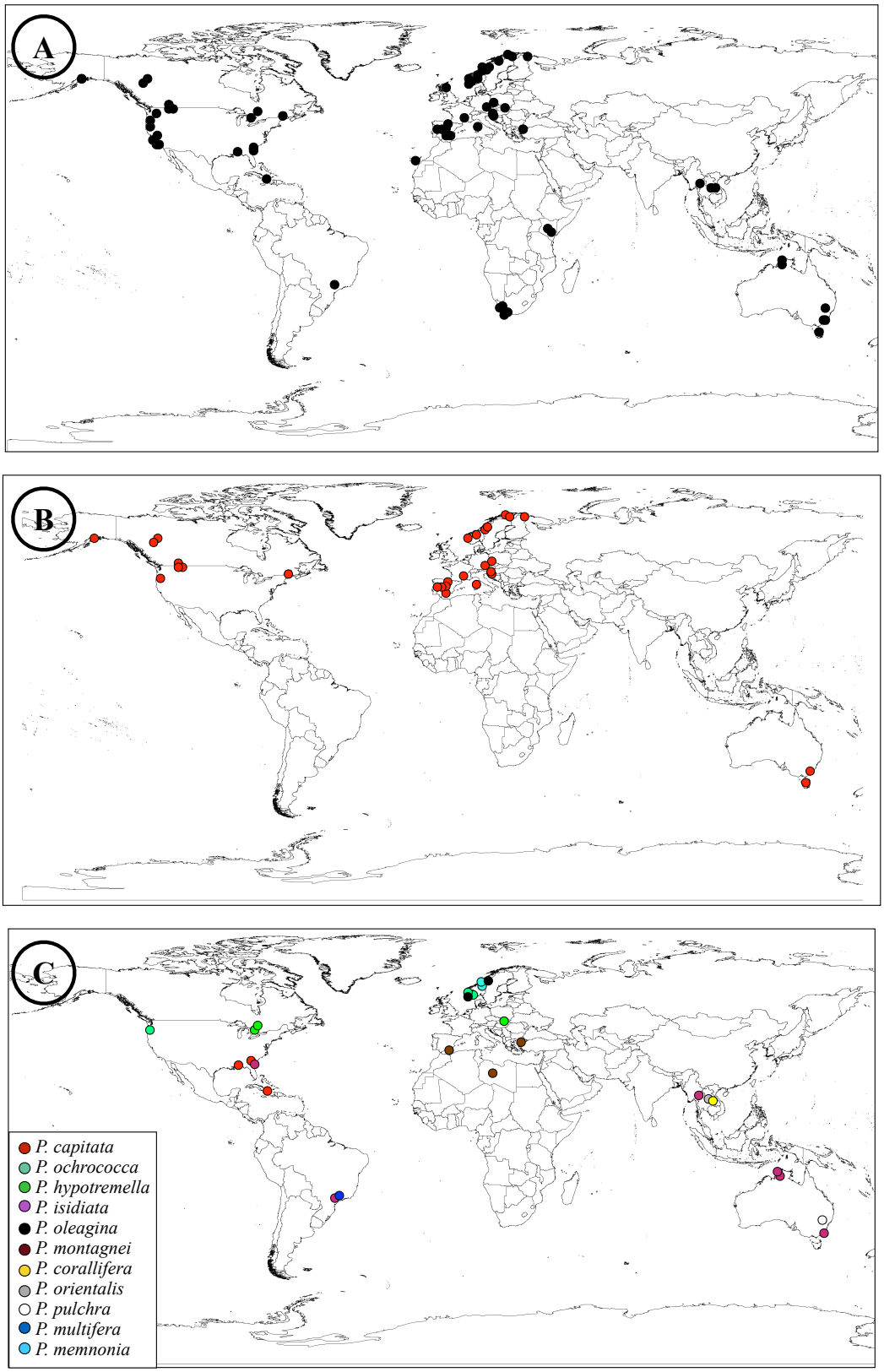

Figure 4. Sampling localities of *Protoparmelia* species included in this study: A) black dots represent the sampling localities of all the specimens included in the study, B) red dots represent sampling localities of the cosmopolitan species *P. badia*, and C) sampling localities of the *Protoparmelia* s.str. species having a limited geographic distribution, different colors indicate different species.

Protoparmelia is a member of the order Lecanorales. This order comprises 20 families, including Cladoniaceae, Gypsoplacaceae, Ramalinaceae, Lecideaceae, Lecanoraceae, Parmeliaceae, Umbilicariaceae, etc. Of these, Parmeliaceae is the largest family, consisting of over 2700 species (Thell *et al.*, 2012). The phylogenetic position of *Protoparmelia* within Lecanorales has been a matter of debate. Morphological and anatomical characters of this genus show similarity to both Lecanoraceae and Parmeliaceae. *Protoparmelia* was initially placed in the Lecanoraceae based on the presence of one-celled hyaline ascospores and *Lecanora*-type ascus. However this classification was later questioned as secondary metabolite profiles showed the presence of lobaric acid, which is typical of Parmeliaceae (Poelt & Leuckert, 1991). Moreover, ascoma ontogeny showed the presence of a cupular exciple, a cup-shaped structure below the hymenium which is a typical character of the Parmeliaceae (Poelt & Leuckert, 1991; Henssen, 1995). Most of the taxonomic affinities of *Protoparmelia* have been based on the morphological similarities (Hertel, 1984; Hertel & Rambold, 1987; Rambold G, 1990; Poelt & Leuckert, 1991; Kantvilas *et al.*, 2010) and molecular studies on *Protoparmelia* are largely scarce (Arup *et al.*, 2007; Papong *et al.*, 2011). For example, Hafellner & Rogers (1990) indicated a close relationship of *Maronina* and *Protoparmelia* on the basis of similar ascus type along with chemistry and suggested *Maronina* to be a multi-spored derivative of *Protoparmelia*. Similarly, Hertel & Rambold (1987) proposed a close affinity of *Protoparmelia cupreobadia* to *Miriquidica* (Lecanoraceae) on the basis of similar conidia and pycnidia (see also Rambold G, 1990; Ryan *et al.*, 2004).

At the beginning of this PhD work, molecular studies on *Protoparmelia* were scarce, and a comprehensive phylogeny of the genus was not available. Only a few studies so far included *Protoparmelia* (Arup *et al.*, 2007; Papong *et al.*, 2011) and the algae associated with *Protoparmelia* remain completely unexplored. The studies including *Protoparmelia* were based on a few markers only. For example, the study by Arup *et al*. (2007) was based on two-locus data set. This study showed that *Protoparmelia* is not a member of the Parmeliaceae and suggested that either *Protoparmelia* or Gypsoplacaceae could be the sister-group to Parmeliaceae. In this study, the sister relationship of *Protoparmelia* to Parmeliaceae was not supported and the alternative topology with Gypsoplacaceae as the sister to Parmeliaceae was equally probable (Figure 5). Other two studies, Kantvilas *et al*. (2010) and Papong *et al*. (2011), investigated the relationship of *Protoparmelia* to *Maronina* using molecular and morphological data. *Maronina* consists of tropical species from Australia and Thailand. Several tropical

Protoparmelia species, such as *P. orientalis*, *P. multifera*, *P. australiensis* and *P. hesperia* were previously placed in the genus *Maronina*. Later, they were included in *Protoparmelia* based on the molecular data (Kantvilas *et al.*, 2010; Papong *et al.*, 2011). *Maronina*, as previously described, contains corticolous species, characterized by crustose thallus, lecanorine apothecia, polyspored asci, hyaline, non-septate ascospores and bacilliform conidia. Apart from being corticolous and having multi-spored asci, all other features are characteristic of *Protoparmelia* as well. Molecular analyses also supported the close affinity of *Maronina* to *Protoparmelia*, and *Maronina* has been placed within *Protoparmelia* (Kantvilas *et al.*, 2010; Papong *et al.*, 2011), suggesting the former to be the multi-spore derivative of *Protoparmelia*. Before the inclusion of tropical *Maronina* species, *Protoparmelia* consisted of boreal/arctic-alpine, temperate and Mediterranean species only.

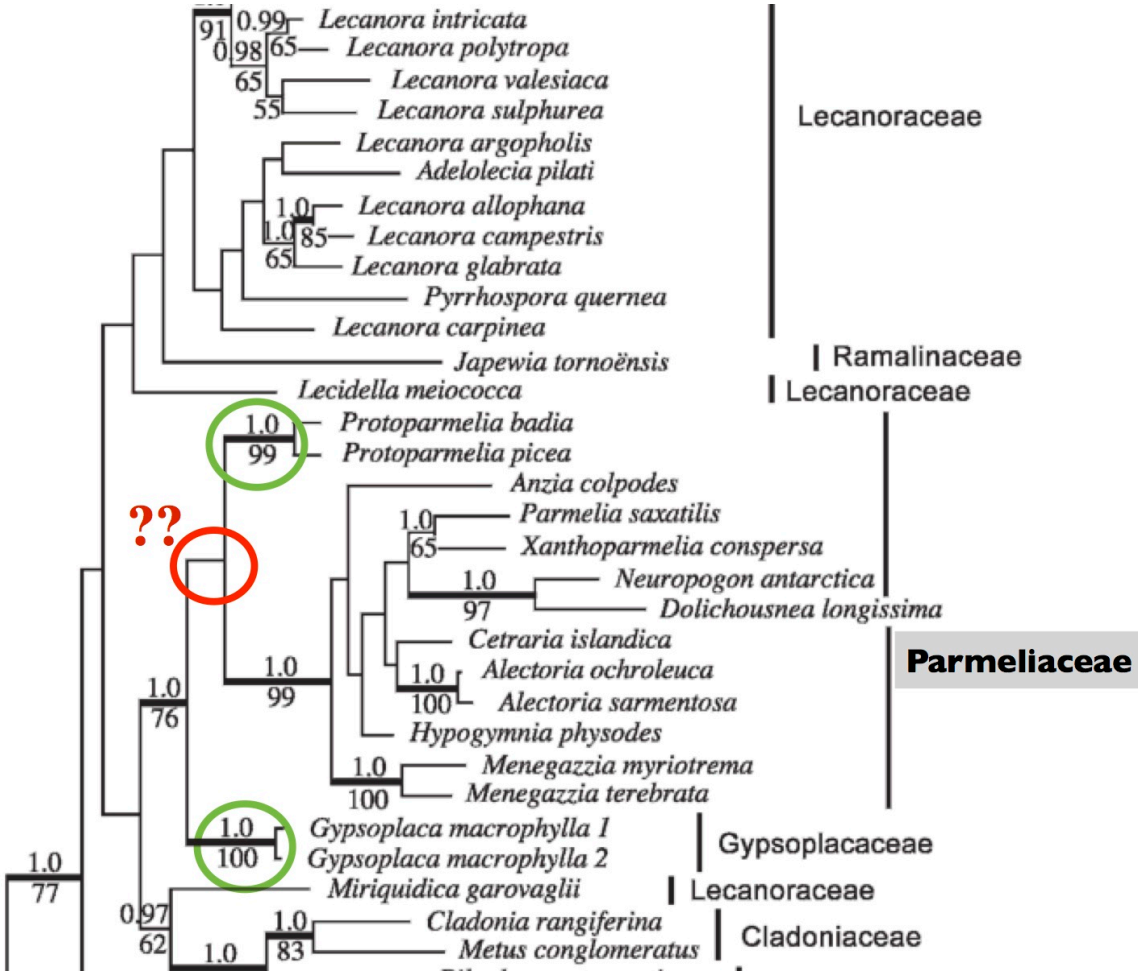

Figure 5. Phylogenetic tree of *Protoparmelia* and its allies based on RAxML analysis using a two-locus data set (mtSSU and nuLSU). The two most closely related groups to Parmeliaceae are highlighted with green circles. The red circle indicates the lack of support for the relationship between these two groups. Adapted from Arup *et al.* (2007).

5 THESIS STRUCTURE AND RESEARCH QUESTIONS

During my dissertation research, I explored the phylogenetic relationship of *Protoparmelia* within the order Lecanorales and delimited *Protoparmelia* s.str. species. I further investigated diversity of the photobionts associated with *Protoparmelia* s.str., as well as differences in fungus-alga association patterns across different macroclimatic regions. I further analyzed the cophylogenetic patterns of the members of *Protoparmelia* and their green algal partners inhabiting different macrohabitats. I attach three articles in the Appendix of this thesis. All three articles are either published (Appendix 10.1 and 10.2) or accepted (Appendix 10.3) in international, peer-reviewed journals and I am the first author in all of them. Each of these articles deals with one of the research questions stated below:

5.1 What is the phylogenetic position of *Protoparmelia* within Lecanorales? Is *Protoparmelia* monophyletic?

I investigated the phylogenetic position of *Protoparmelia* within Lecanorales and assessed the monophyly of the genus. For this, I generated a multilocus phylogeny using proposed close relatives of *Protoparmelia*, such as the members of Gypsoplacaceae, Parmeliaceae, and Lecanoraceae (Singh *et al.*, 2013). I performed maximum likelihood and Bayesian analyses on the concatenated four-locus data set. I expected *Protoparmelia* to be monophyletic. Further, I expected *Protoparmelia* to be the sister to Parmeliaceae, and Gypsoplacaceae to be the sister to *Protoparmelia* and Parmeliaceae.

5.2 How many species of *Protoparmelia* s.str. are there? Can coalescent-based species delimitation methods help to uncover species diversity in the genus?

I investigated mycobiont diversity in *Protoparmelia* using a multilocus phylogeny and species delimitation analyses. I generated a 6-locus phylogeny of *Protoparmelia* including 18 previously described species and several undescribed species of *Protoparmelia*. I performed species delimitation analyses considering current, morphologically

circumscribed species as putative lineages. In cases where a single species split up into multiple supported monophyletic clades in both maximum likelihood and Bayesian analyses, I tested the presence of cryptic species by considering all supported monophyletic clades as putative species. As morphological characters in lichen-forming fungi have been repeatedly shown to be limited in characterizing species level diversity, I expected to discover several cryptic species.

5.3 How do symbiont association patterns in *Protoparmelia* and symbiont diversity vary in different macrohabitats? Which forces drive fungus-alga evolution in the *Protoparmelia-Trebouxia* symbiosis?

In this part of my PhD, I investigated how algal diversity and association patterns in a lichen symbiosis vary across closely related species inhabiting different habitat. I first estimated the algal diversity associated with 23 *Protoparmelia* s.str. fungal species. I performed maximum likelihood search based on a 2-locus data set, followed by coalescent-based species delimitation analyses using BP&P and STACEY. I used tanglegram for representing fungus-alga association patterns in *Protoparmelia* s.str. using fungal and algal species trees. Further, I generated the fungus-alga association network using the first 100 BLAST hits of *Trebouxia* nrITS sequences with >97% threshold. This was done to check if the algal species associate with other lichen-forming fungal species. I expected algal diversity associated with the Mediterranean, tropical and sub-tropical *Protoparmelia* to be higher than their arctic-temperate counterparts. As for the association patterns, I expected symbiotic partners inhabiting warmer climates such as Mediterranean or tropical regions to be more specific in their associations as compared to cooler habitats.

I further inferred if the number of symbiotic partners in a lichen symbiosis varies with climate using phylogenetic PCA (pPCA). I then inferred the cophylogenetic pattern of the *Protoparmelia* symbionts using the fungal and algal species trees, inferred from 6-locus fungal and 2-locus algal concatenated data sets, and tested for the phylogenetic congruence using PACo (Procrustes Application to Cophylogentic analysis; Balbuena *et al.*, 2013) and ParaFit (Legendre *et al.*, 2002). Then, I used Jane (Conow *et al.*, 2010) to

ascertain the processes shaping *Protoparmelia-Trebouxia* associations. I expected cospeciation to be the major process shaping *Protoparmelia-Trebouxia* associations owing to the long-term and successful symbiotic relationship of lichen symbionts, especially in the case of highly specific or specialized symbionts.

6 RESULTS AND OVERALL DISCUSSION

6.1 Phylogenetic position of *Protoparmelia*

I inferred the phylogenetic position of *Protoparmelia* within Lecanorales based on four loci, namely ITS, two ribosomal RNA-coding genes nuLSU, and mtSSU, and one protein-coding gene *RPB*1. I included five *Protoparmelia* species and other putative closely-related species of *Protoparmelia* in this study. I conclusively showed *Protoparmelia* to be the sister-group to Parmeliaceae s.str. *Gypsoplaca* (Gypsoplacaceae) was recovered as the sister to Parmeliaceae-*Protoparmelia* s.str. group. The close affinity of *Protoparmelia* and *Gypsoplaca* to Parmeliaceae was also previously suggested (Arup *et al.*, 2007). However, the sister clade relationships were not supported, and both the topologies, with either *Gypsoplaca* or *Protoparmelia* s.str. as the sister to Parmeliaceae were shown to be equally probable. In my study, I tested the hypothesis of Gypsoplacaceae as the sister to Parmeliaceae using alternative hypothesis test. This alternative hypothesis was rejected, and thus my study suggests *Protoparmelia* as the sister to Parmeliaceae. The increased support of the sister clade relationships in my study could be attributed to the use of two additional loci, one of which is protein-coding. In fact, using a single protein-coding locus could be more efficient in resolving phylogenetic relationships of the lichenized as well as non-lichenized fungi than the combined two- and three ribosomal loci (Liu & Hall, 2004; Reeb *et al.*, 2004; James *et al.*, 2006; Hofstetter *et al.*, 2007; Truong *et al.*, 2013). My study further highlights the utility of protein-coding loci in resolving fungal phylogenetic relationships.

Species richness of clades has often been linked to certain key innovative characters which confer adaptive advantages such as the ability to colonize new habitats, leading to burst of diversification. Interestingly, in Lecanorales, Parmeliaceae is the largest family of lichen-forming fungi (~2700 species), while its two closest relatives, namely *Gypsoplaca* and *Protoparmelia*, are comparatively species poor. *Protoparmelia* consists of ~25 species and *Gypsoplaca* is the only genus, which is comprised of a single species, in the family Gypsoplacaceae. Disparities in species richness among these clades could be attributed to certain key innovations in Parmeliaceae which facilitated rapid species diversification (Sanderson & Donoghue, 1994; Rabosky & McCune, 2010). Understanding the evolution of traits in the Parmeliaceae and comparing them with its

sister-groups therefore may help in identifying the key innovations leading to the current species richness in Parmeliaceae.

In addition to affirming the sister-group relations of Parmeliaceae, I found *Protoparmelia* to be polyphyletic. The polyphyly of *Protoparmelia* was also affirmed by an alternative hypothesis test. Three of the five species included in the analysis, namely *P. atriseda*, *P. cupreobadia*, and *P. phaeonesos* grouped close to *Miriquidica* (Lecanoraceae). The genus *Miriquidica* consists of saxicolous lichens, with *Lecanora*-type asci, and miriquidic acid as the major secondary metabolite (Hertel & Rambold, 1987). The proximity of *Protoparmelia* s.lat. species to *Miriquidica* (Lecanoraceae) has been suggested previously based on similar conidia and pycnidia and lichenicolous lifestyle during early stages of life (Rambold G, 1990; Ryan *et al.*, 2004). Interestingly, the three *Protoparmelia* s.lat. species are also chemically different from *P. badia* and *P. picea*, having norstictic acid as the major secondary metabolite instead of lobaric acid which is the major secondary metabolite of *P. badia* and *P. picea.* Furthermore, all parasitic members of *Protoparmelia* were shown to belong to *Miriquidica.* Based on the above differences, these *Protoparmelia* species, along with two other species, *P. leproloma* and *P. placentiformis*, were placed in *Protoparmelia* sect. Phaeonora (Poelt & Leuckert, 1991). My study provides molecular support for the dissimilarity between *P. badia* and *P.* sect Phaeonora and the close affinity of *Protoparmelia* s.lat. to *Miriquidica*. However, further studies including more *Miriquidica* species are needed to infer the phylogenetic relationships among *Miriquidica* and *Protoparmelia* s.lat species.

6.2 Phylogeny and species delimitation of *Protoparmelia*: estimating fungal diversity

After demonstrating the polyphyly of *Protoparmelia*, I characterized species diversity in the *Protoparmelia* s.str group using phylogenetic and coalescent-based methods. For this, I performed a phylogenetic analysis based on a 6-locus data set, and including 18 previously described and several undescribed *Protoparmelia* species. Additionally, I included members of Parmeliaceae, Gypsoplacaceae and Lecanoraceae in the analysis. I recovered 23 well-supported monophyletic clades in the phylogenetic tree inferred from the 6-locus concatenated data set. Additionally, two clades were monophyletic but received low support. These 25 lineages were considered as putative species for the species delimitation analysis.

I selected BP&P and spedeSTEM for species delimitation of *Protoparmelia* and BP&P and STACEY for the species delimitation of *Trebouxia*. Another software GMYC is commonly used for species delimitation. However, I did not use GMYC because of the following reasons: i) it has been proposed to perform well when using a single locus data set and may not perform well on multilocus or concatenated data sets (Esselstyn *et al.*, 2012; Talavera *et al.*, 2013); ii) the accuracy of GMYC relies on a user-specified guide tree and uncertainty in the gene trees may bias the number suggested number of species by the program; and iii) GMYC has been proposed to be sensitive to branch length differences among taxa and may not reliably delimit species when dealing with taxa with significant differences in the branch lengths (as was in the case of *Protoparmelia*, data not shown). Similarly, I excluded the program ABGD (Automated Barcode Gap Discovery, Puillandre *et al.*, 2012) from the species delimitation of *Protoparmelia* and *Trebouxia* because it uses ITS data set for identifying species and in both of my data sets other markers provided more supported and resolved topologies as compared to the ITS. Therefore, ITS-based species delimitation may not resolve recently diverged or closely-related lineages.

I selected BP&P and spedeSTEM for the species delimitation of *Protoparmelia*. BP&P takes sequences as input and uses reversible jump MCMC to evaluate species delimitations, whereas spedeSTEM uses gene trees and calculates the maximum-likelihood species tree to select the best species scenario using information theory. SpedeSTEM is robust to phylogenetic error in species tree as it is calculates the likelihood of species tree, but being conservative approach, it may fail to recognize recently diverged lineages (Carstens *et al.*, 2013). BP&P on the other hand has been suggested to be the best approach for delimiting species when using multilocus data set, even when the lineages are recently diverged.

Both the species delimitation programs (BP&P and spedeSTEM) supported 16 species as evolutionary independent lineages, out of the proposed 25-species scenario for *Protoparmelia*. However, for eight species, i.e. *P. ochrococca A* & *B*, *P. badia A*, *B1*, *B2* & *C*, *P. montagnei A* & *B,* there was a conflict between BP&P and spedeSTEM. Conflicts among different empirical species delimitation are common and, in fact, are expected owing to the different assumptions underlying each method (Carstens *et al.*, 2013; Satler *et al.*, 2013; Giarla *et al.*, 2014). Generally, researchers apply one of the following two approaches to deal with the incongruence among analyses. First, consensus approach, in which only the species supported by all or most of the methods are proposed as

evolutionary independent lineages (Satler *et al.*, 2013). Alternatively, users may justify one method over others, given the data and underlying assumptions (Carstens & Satler, 2013; Giarla *et al.*, 2014). For my study, in case of conflicts I proposed the clades supported by BP&P as evolutionary independent lineages because of the following reasons: 1) the lineages suggested by the spedeSTEM were incongruent with the well-supported clades in the phylogenetic tree; 2) spedeSTEM has been suggested to be less accurate in identifying recently diverged lineages, while BP&P performs well even when putative species have diverged recently (Carstens & Satler, 2013; Carstens *et al.*, 2013; Giarla *et al.*, 2014); 3) BP&P has been suggested to be the best method for species delimitation using multilocus data (see introduction for details; Camargo *et al.*, 2012; Carstens *et al.*, 2013; Leavitt *et al.*, 2015b); and 4) BP&P is suggested to be conservative in delimiting species, and therefore a reliable indicator of evolutionary independence of the lineages (Yang & Rannala, 2014). In the past, BP&P has been criticized for its dependency on the user-specified guide tree, which fixes the topology of the species tree. The phylogenetic uncertainty in the guide tree might lead to miscalculated posterior probabilities and over splitting of species (Fujita *et al.*, 2012; Olave *et al.*, 2014). However, in the latest version of the software (v. 3) the developers addressed this issue by introducing the Nearest neighbor interchangeables (NNI) algorithm which allows for topological flexibility in the guide tree to avoid conflicts with the newly proposed species tree (Yang & Rannala, 2014). Therefore, I am confident that the species suggested by BP&P represent evolutionary independent lineages.

Species delimitation analysis of *Protoparmelia* s.str. revealed *Protoparmelia* to be more diverse than what the traditional taxonomy suggests. I discovered many cryptic lineages hidden within previously described species. This is in concordance with other studies where the use of molecular markers in combination with statistical tools have helped in identifying cryptic lineages (Divakar *et al.*, 2010; Gamble *et al.*, 2012; Carter, 2012; Leavitt, 2013; Satler *et al.*, 2013; Giarla *et al.*, 2014; Joly *et al.*, 2014; Lücking *et al.*, 2014). Such lineages have been proposed to be recently diverged and thus might not have had enough time for differentiating morphologically. Molecular analysis is therefore a valuable tool for recognizing such lineages. Cryptic lineages are often reported from species with broad geographical distribution (Murtagh *et al.*, 2002; Myllys *et al.*, 2003; Leavitt, 2013). Species with broad distribution usually occupy non-overlapping areas separated by geographic barriers and hence are isolated from each other such that genetic exchange is prevented for a long time possibly leading to speciation (allopatric

speciation). The cryptic diversity resulting from allopatric speciation thus is expected to correspond with the geographical regions in which the isolated lineages evolved (Parnmen *et al.*, 2012). Based on such observations, biogeography has been proposed as a supporting character for identifying cryptic taxa for species complexes with wide geographic distribution. In my study, cryptic lineages within *P. isidiata* (clades *P. isidiata A-C*) somewhat correspond to geographic regions; *P. isidiata A* occurring in the USA, *P. isidiata B* in Brazil, *P. isidiata C* in Thailand, and both *P. isidiata D* and *P. isidiata E* are found in Australia. These species might be the result of geographic isolation and allopatric speciation. However, this was not the case for another cosmopolitan species, *P. badia*, which consists of four independent evolutionary lineages. Only *P. badia A* is truly cosmopolitan, inhabiting boreal-arctic/alpine habitats in North America, Europe, New Zealand and Australia. The other three species-level lineages, *P. badia B1*, *B2*, and *C*, appear to have limited distribution (Spain and Italy only; Singh *et al.*, 2015). Thus, broad geographic distribution may not always lead to allopatric speciation as some species may be truly cosmopolitan, maintaining connectivity via gene flow among populations in spite of having intercontinental distribution. Using geography as a character for recognizing cryptic diversity is further confounded by the sympatric occurrence of cryptic species, i.e., when the species occupy overlapping geographical ranges (Crespo *et al.*, 2002). Therefore, geography may not always be a reliable character for identifying cryptic diversity.

6.3 Symbiont diversity and association patterns in *Protoparmelia*

6.3.1 Phylogeny and species delimitation of the algal partners associated with *Protoparmelia*

I assessed algal diversity associated with 23 *Protoparmelia* s.str. species using sequence data from both nuclear (ITS) and mitochondrial (*COX2*) loci. As the basis for identifying *Trebouxia* species in my sampling of *Protoparmelia* specimens, I used ITS sequence similarity in comparison with ITS sequences from previously described and publically available strains of algal species. Molecular identification of the algae associated with lichen-forming fungi majorly relies on ITS sequence similarities as ITS is the most widely sequenced locus for lichenized algae. The use of other loci, such as *COX2*, *rbcL*, SSU

rDNA, LSU rDNA, have been restricted to a limited number of studies (Dal Grande *et al.*, 2014b; Sadowska-Deś *et al.*, 2014; Werth & Sork, 2014), probably due to the difficulty in amplification owing to the lack of universal markers. Consequently, I could not use *COX2* for sequence similarity-based identification using public databases. To identify the algae associated with *Protoparmelia*, I aligned the *Trebouxia* ITS sequences representing 26 species from the SAG (algal culture collection at the University of Goettingen, Germany) and UTEX (algal culture collection at the University of Texas, USA) databases with my ITS data set and generated a ML tree with 1000 BS replicates. Five *Trebouxia* species from the SAG and UTEX databases grouped with high support with the *Trebouxia* associated with *Protoparmelia*.

In the concatenated algal data set, I found 20 supported monophyletic clades, five of which correspond to previously described species (based on the grouping in the ITS ML tree). One of the putative *Trebouxia* species corresponding to *T. jamesii* based on the ITS sequence similarity split into two supported monophyletic clades in the concatenated data set, indicating the presence of cryptic lineages in this species. These two clades were considered as separate species for the subsequent species delimitation analysis. Overall, I considered 20 supported monophyletic clades in the two-locus concatenated data set as putative species for the species delimitation analysis, of which 15 species were putative novel taxa. For the species delimitation of *Trebouxia*, I used BP&P and STACEY as both of these do not require a guide tree to infer species boundaries (Jones & Oxelman, 2014; Yang & Rannala, 2014; Jones, 2016). Both the approaches supported the proposed 20-species scenario.

Factors influencing symbiont diversity

Macrohabitat

Species diversity has been proposed to increase from poles to equator for virtually all taxonomic groups (Rohde, 1999; Willig *et al.*, 2003; Schemske, 2009; Jocque *et al.*, 2010). This pattern of increased diversity across the latitude is called latitudinal biodiversity gradient (LBG), and has been accepted as ubiquitous phenomenon with a few exceptions for free-living systems (Willig *et al.*, 2003; Hillebrand, 2004). However, the status of diversity patterns in symbiotic systems is still unsettled (Poulin, 2010; Morand, 2015), and several studies have found either no (Morand, 2000; Bordes *et al.*, 2010) or opposite gradients (Krasnov *et al.*, 2004a; Lindenfors *et al.*, 2007). The present study is in line with reports finding no diversity gradient (Morand, 2000; Bordes *et al.*, 2010) –

species diversity of algal and fungal partners was comparable in subtropical/tropical regions and arctic-alpine-boreal/temperate regions.

Lifestyle: ecto- versus endoparasitic lifestyle

Another factor influencing the symbiont diversity is suggested to be the different lifestyles of the symbionts (Rohde & Heap, 1998). For instance, ectoparasites live outside the host body and consequently are exposed to external environmental conditions in the same way as their host (Poulin, 1995; Rohde & Heap, 1998; Rohde, 2002). Thus, one would expect a similar effect of environmental conditions on both the symbionts. Conversely, endoparasites reside inside the host body, and hence they face relatively stable environmental conditions everywhere, irrespective of the external climate. Endoparasites might therefore show no variation in diversity across the latitude (Poulin, 1995; Rohde & Heap, 1998; Choudhury & Dick, 2000; Rohde, 2002; Guernier *et al.*, 2004; Bordes *et al.*, 2010; Thieltges *et al.*, 2011). Most of the studies analyzing diversity gradients of endoparasites are based on endothermic hosts with no seasonal variation in body temperature, and thus no diversity gradient would be expected.

The algal cells in the lichen symbiosis are located within the thallus, which is formed by the fungal partner, but they do not live within the fungal cells (Ahmadjian, 1965; Honegger, 1986, 2009). Therefore, the algal partners in a lichen symbiosis are "inhabitants" rather than true endosymbionts. While the algal inhabitants experience the same climatic conditions as the fungal partner, they are protected from some other direct effects of the environment by their hosts. For instance, the mycobiont shields the algae against UV radiations, partly regulates the water content within the thallus (Honegger, 2007, 2009) and can also protect the photobiont from the direct influence of substrate pH (Mollenhauer, 1997). However, lichens are poikilohydric systems, which cannot fully regulate their water content, although in general they are capable of surviving prolonged desiccation (Kappen & Valladares, 1999). It is suggested that changing environmental conditions, especially desiccation-rehydration cycles and high temperature, may cause oxidative damage in cells of the photobiont. Thus, the potential of the fungal host to shield their photobionts from the environment is limited, and the inhabitant lifestyle may not completely justify the absence of a latitudinal diversity pattern. The potential influence of the environment on photobiont diversity and distribution is also supported by the observation that lichen-forming fungi occupying wide ecological niches often associate with different photobionts (Cordeiro *et al.*, 2005; Fernández-Mendoza *et al.*,

2011; Peksa & Skaloud, 2011; Muggia *et al.*, 2013, 2014; Werth & Sork, 2014). The algal symbionts of the lichen-forming fungi are often referred to as polar, temperate or tropical lineages (Cordeiro *et al.*, 2005; Fernández-Mendoza *et al.*, 2011). Moreover, environmental factors such as rain and sun exposure have been shown to influence the small-scale occurrence of different species in the green algal genus *Asterochloris* (Peksa & Skaloud, 2011). This suggests that the environment does influence algal diversity patterns in lichens. Therefore, the inhabitant lifestyle may not entirely explain the absence of diversity gradient in the algae and other factors might also be playing a role in governing the diversity patterns of the algae associated with *Protoparmelia*.

Host distribution range

Symbiont diversity has been proposed to increase with increase in host distribution range and in general it is expected that hosts with larger geographical ranges would harbor more symbiont species (Krasnov *et al.*, 2004b; Korallo *et al.*, 2007). In my study, the boreal/arctic-alpine *Protoparmelia badia A* has a wide, cosmopolitan distribution. However, it associates with the same algal lineage everywhere. Thus, in *Protoparmelia* symbiont diversity is not higher in case of more widely distributed hosts. The geographic range of the fungus does not appear to influence algal diversity and is likely not a determinant of alga diversity for *Protoparmelia.*

Host diversity

Symbiont diversity may also be influenced by host diversity as a higher number of closely related hosts increases the chances of interspecies symbiont transmission and speciation (Nunn *et al.*, 2005; Thieltges *et al.*, 2011). In my study, I found *Trebouxia* diversity to be comparable to the *Protoparmelia* diversity in all the habitats (i.e., in boreal/arctic/alpine/-temperate regions- six *Trebouxia* species associate with eight arctic/temperate *Protoparmelia* species; in Mediterranean regions- four *Trebouxia* associate with four *Protoparmelia* species; and in the tropical regions- eleven *Trebouxia* species associate with eleven *Protoparmelia* species). Thus, fungal diversity could be a predictor of algal diversity in *Protoparmelia*-*Trebouxia* symbiosis.

Determinants of algal diversity in Protoparmelia

Algal diversity is comparable to fungal diversity in *Protoparmelia*-*Trebouxia* symbiosis in all the habitats. I did not find any effect of latitude or host geographical range on the *Trebouxia* diversity. Unlike other parasites where endosymbiotic lifestyle could explain

the absence of a latitudinal diversity gradient, in lichens the inhabitant life may not fully explain the absence of diversity gradient as lichens are poikilohydric and thus algae are not completely shielded from the effect of environment. The host diversity was comparable to the algal diversity and hence the possible influence of host diversity on algal diversity cannot be excluded. However, a reliable estimation of diversity patterns of photobionts in lichens requires insights from symbiont association patterns as they can strongly influence the diversity of algae associated with *Protoparmelia*.

6.3.2 Association patterns of *Protoparmelia* symbionts

Differences in the strength of biotic interactions along the latitude are considered to be one of the major factors causing the species diversity gradient across the latitude (Dobzhansky, 1950; Pianka, 1966; Schemske *et al.*, 2009; Jocque *et al.*, 2010; Pellissier, 2015). It has been suggested that biotic interaction strength increases with decreasing latitude, i.e. specialization increases towards the equator (Dobzhansky, 1950; Pianka, 1966; Schemske *et al.*, 2009; Jocque *et al.*, 2010; Pellissier, 2015). Therefore, one would expect more generalists species in the arctic/temperate areas and more specialists towards the tropics.

One way to measure complexity in species interactions and the degree of specialization is by calculating the connectance in the symbiotic network of interest (Jordano, 1987). Connectance refers to the proportion of the actual number of associations out of all the possible associations (Jordano, 1987; Blüthgen *et al.*, 2008). Thus arctic/temperate species would have higher connectance owing to more generalized interactions, and consecutively more "links", whereas tropical species would be expected to have a lower connectance owing to more specialized interactions (Jordano, 1987; Olesen & Jordano, 2002). In the case of *Protoparmelia-Trebouxia*, connectance was highest in the arctic/temperate regions as compared to the tropical species. My study supports the presence of more connected networks in the arctic/temperate regions as compared to tropical regions.

Fungal selectivity versus algal selectivity

Fungi and algae displayed different levels of selectivity. Fungi in general displayed higher selectivity than the alga, associating with one to three *Trebouxia* species. On the other hand, the algal symbiont accepted one to five *Protoparmelia* species and up to 65-70 other lichen-forming fungal species (Singh *et al.*, 2017). The difference between the

selectivity of fungus and alga in a lichen symbiosis could be explained by the existence of alga-mediated guilds. In a lichen guild, several lichen-forming fungi occupying similar habitats share a common, and probably a locally adapted alga (Rikkinen *et al.*, 2002; Dal Grande *et al.*, 2014b). The fungal partners in a guild are highly selective towards a certain photobiont strain whereas the photobiont partner of the guild associates with different fungal strains in the community and is considered a generalist species. The existence of photobiont-mediated guilds has been shown for the cyanolichen *Peltigera* (Rikkinen *et al.*, 2002; Rikkinen, 2013). In my study I found that the algae associated with arctic/temperate *Protoparmelia* species associate with several unrelated lichen-forming fungi occupying the same habitats. For example, the photobiont lineage *T. sp. 1* (*T. suecica*) associated with arctic/temperate *Protoparmelia* species is shared by several other lichen-forming fungi from the same environment (Singh *et al.*, 2017). My study further supports the existence of photobiont-mediated guilds in lichens, especially in colder climates where adaptation towards harsh, fluctuating climate is likely the major selective pressure (Dobzhansky, 1950; Fischer, 1960). The sharing of algae in lichen communities explains, in part, the lower selectivity of algal symbionts as compared to the fungal partners.

High selectivity of *Protoparmelia* towards its algal partner might jeopardize the relichenization (re-establishment of lichen thallus) once the symbiosis is decoupled, for example after sexual reproduction. However, as photobionts may be shared via horizontal transmission within photobiont-mediated guilds (Rikkinen *et al.*, 2002, see also Dal Grande *et al.*, 2014b), other vegetatively reproducing fungal members of the guild can serve as a source of alga. The existence of photobiont-mediated guilds could possibly explain the presence of specialist fungi but generalist arctic and temperate algae, as I found in my study.

In lichens, low specialization has been linked to wider ecological amplitude of the fungal host, which allows the fungal partner to establish symbioses with locally adapted photobionts in different habitats (Yahr *et al.*, 2006; Muggia *et al.*, 2013). Several studies suggest the possible role of alga in local adaptation, and consequent broadening of ecological range of the fungus (O'Brien *et al.*, 2005; Yahr *et al.*, 2006; Fernández-Mendoza *et al.*, 2011; Vargas Castillo & Beck, 2012). Often the same mycobiont in different habitats and climatic conditions associates with different photobionts (Blaha *et al.*, 2006; Yahr *et al.*, 2006; Muggia *et al.*, 2014). High selectivity of mycobionts towards

their photobiont partner could be the reason for the narrow ecological amplitude of *Protoparmelia* species.

Terrestrial green algae, including lichen symbionts, must have acquired a range of adaptations to cope with distinct features of terrestrial environments, such as variation in temperature and water availability, drought, high-intensity light and UV radiation (Hori *et al.*, 2014). Besides these general adaptations, it is likely that green algae also have habitat-specific adaptations, which allow them to persist in diverse ecological niches (Muggia *et al.*, 2014). This has been shown for example in plants where associating with a particular fungal endophyte provides stress tolerance (in particular heat and salt) thus allowing plants to establish in high-stress habitats (Rodriguez *et al.*, 2008; Redman *et al.*, 2011). My study suggests that green algae of the genus *Trebouxia* are generally ubiquitously distributed, and that environmental filters determine the availability of particular lineages in an environment (everything is everywhere; Baas Becking, 1931; O'Malley, 2008). Freeze tolerance is probably an important adaptation of algae in arctic-alpine and temperate environments, whereas desiccation and high-intensity light tolerance are important adaptations in Mediterranean environments.

Interactions in different macrohabitats

Species interact differently in different macrohabitats. In general, arctic/temperate species interactions are expected to be more generalized as compared to the interactions in the tropical regions (Ollerton & Cranmer, 2002; Piculell *et al.*, 2008; Schemske *et al.*, 2009). In my study, the selectivity of the fungal partner in the *Protoparmelia*-*Trebouxia* symbiosis was largely comparable across different macrohabitats. *Protoparmelia* species in the arctic/temperate regions associated with one to three *Trebouxia* species and only one *Trebouxia* species in the Mediterranean region. All tropical *Protoparmelia* species associated with only one algal partner. Thus, *Protoparmelia* species appear to be highly selective towards their photosynthetic partner in all the habitats, although one to one or otherwise highly specific associations are found only in the tropics (Singh *et al.*, 2017). For the algal partner, the selectivity was higher in the tropics as compared to the other regions. For instance, in the arctic/temperate regions *Trebouxia* associated with one to five *Protoparmelia* species and up to ~70 other lichen-forming fungal species, whereas in the tropical regions *Trebouxia* associated with only one *Protoparmelia* species and up to five other lichen-forming fungal species. In the Mediterranean regions, *Trebouxia* associated with one to two *Protoparmelia* species, but turned out to form symbiotic

associations with three to up to 30 other lichen-forming fungi. The presumably selective *Trebouxia* associated with arctic/temperate or Mediterranean *Protoparmelia* species associated with one to ~30 other lichen-forming fungi and thus have low selectivity towards their fungal partners (Figure 6). Overall, the arctic/temperate *Trebouxia* were generalists and associated with one to five *Protoparmelia* species and several other lichen-forming fungal species. Thus, the fungal hosts displayed high selectivity irrespective of the macrohabitat whereas algal partners displayed higher selectivity in the tropics as compared to the arctic/temperate regions.

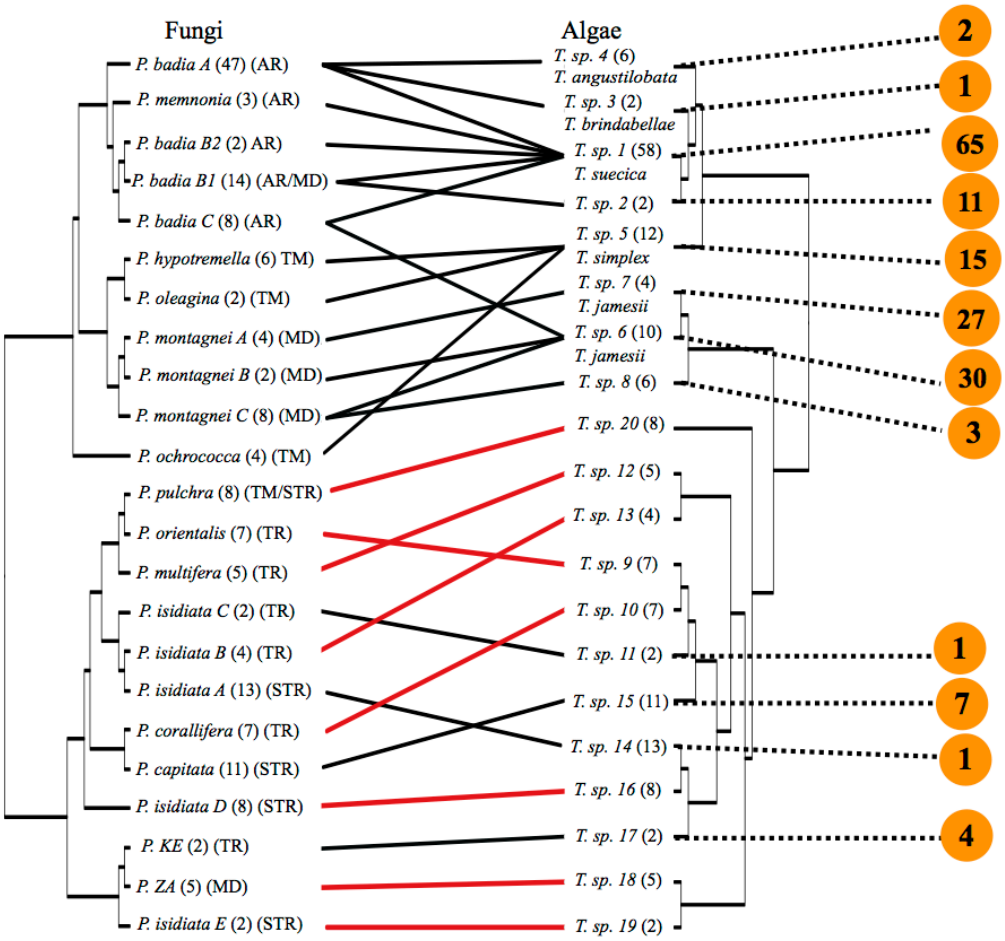

Figure 6. Adapted from Singh *et al.* (2017). Tanglegram representing the associations between lichen-forming fungal *Protoparmelia* s.str. hosts and their green algal symbionts. Trees are *BEAST species trees inferred from six fungal and two algal loci. The number of specimens for each species is given in parentheses, for a total of 138 specimens each. Habitat information is provided with the fungal species (left side; AR=arctic/alpine or boreal, TM= temperate, MD=Mediterranean, STR= sub-tropical, TR=tropical). Black lines indicate low specificity or generalist associations and red lines indicate specialized or one to one associations. Dotted lines indicate associations of *Trebouxia* species with different lichen-forming fungi (number of associations are given in the brown circles).

Specialization is higher in the tropics for Protoparmelia-Trebouxia associations

A lichen symbiosis is characterized as 'specialized' when both the partners display high reciprocal selectivity towards each other (Beck *et al.*, 2002). In my study, I found eight specialized associations, seven of which were found in the tropical regions, and one in the Mediterranean (Figure 6). None of the arctic/temperate interactions displayed strict one to one association. For instance, the photobionts associated with only one *Protoparmelia* species, i.e., *Trebouxia sp. 2*, *Trebouxia sp. 3*, and *Trebouxia sp. 4*, associated with other lichen-forming fungi species as well. Similarly, fungal partners of the highly selective *Trebouxia*, *T. sp. 3*, and *T. sp. 4*, associated with other *Trebouxia* species also. Therefore, symbiotic associations in the arctic/temperate regions could not be called specialized. In contrast, seven out of eleven *Protoparmelia* lichen associations displayed one to one relationships in tropical regions. Four *Trebouxia* species-level lineages associated with tropical *Protoparmelia* species associated with other lichen-forming fungi as well (one to seven), and therefore the associations are not specialized in these cases (Singh *et al.*, 2017).

Interestingly, recent studies on the assessment of biotic specialization do not support the idea that species interactions are stronger or more specialized in the tropics (Poore *et al.*, 2012; Schleuning *et al.*, 2012; Moles & Ollerton, 2016). In fact, a recent review on biotic interaction gradient across the altitude suggests that the biotic interactions may not show any trend across the altitude (Moles & Ollerton, 2016). Few interactions which were initially considered to be more specialized in the tropics, have been recently shown not to display any trend across the latitude (Hille Ris Lambers *et al.*, 2002; Moles & Westoby, 2003; Moles *et al.*, 2011; Poore *et al.*, 2012; Comita *et al.*, 2014). Recently, it has been suggested that the notion that biotic specialization is higher in the tropics exists due to publication bias and selective literature citation (Moles & Ollerton, 2016). In my study, however, the interactions are stronger in the tropical regions (one to seven partners) as compared to the arctic/temperate regions (one to ~70 partners). My results support the traditional idea of stronger and specialized interactions in the tropical regions and generalized interactions in the arctic/temperate regions.

Other confounding factors influencing the symbiont association patterns

Data availability in public databases

One factor which may influence my conclusion of higher selectivity of the algae in the tropics could be the potential bias in the number of studies on the tropical lichens. Most of the studies identifying photosynthetic partners of the lichen-forming fungi were done on lichens from arctic/temperate regions, and a few on lichens from the Mediterranean region. Studies on tropical lichens are rare. Thus, there is a strong bias in the data available in public databases in favor of the lichens from the arctic/temperate regions. The lack of data for the tropical algae in public databases could make the tropical algae appear more specialized than they actually are. More studies identifying photobionts associated with tropical lichen-forming fungi are required to validate if the apparently highly selective and specialized tropical *Trebouxia* are indeed specific and form one-to-one associations with *Protoparmelia*.

Age of taxa

It has been proposed that the older taxa are specialist as they have had more time for coevolution and coadaptation (Magain *et al.*, 2016). This hypothesis advocates the role of time available for adaptation as the main factor behind specialized symbionts, irrespective of the habitat. However, it is to be noted that geological age may be an important factor when comparing the specialization of various symbiotic systems from the same habitat, but such comparison may not be applied to the taxa occupying different habitats. This is because the geological and the evolutionary age of the taxa may be entirely different depending upon the habitat of the organisms. For instance, tropical taxa would have higher number of generations per year as compared to the arctic/temperate taxa owing to the stable climatic conditions and thus would be evolutionarily older given the same geological time (Rohde, 1999; Wright *et al.*, 2006; Oppold *et al.*, 2016). Additionally, the mutation rate would also be higher in the tropical taxa due to higher temperature which would lead to longer branches of the tropical species in the same amount of time (Allen *et al.*, 2006; Streicker *et al.*, 2012; Oppold *et al.*, 2016). Therefore, geological age may not entirely explain the occurrence of specialized taxa in the tropical regions for *Protoparmelia-Trebouxia* symbiosis.

6.4 Evolution of *Protoparmelia-Trebouxia* symbiosis

In my analysis, I found *Protoparmelia-Trebouxia* phylogenies to be highly congruent. Earlier, phylogenetic concordance was interpreted as an indication of cospeciation especially for host-specialized symbionts (Peek *et al.*, 1998; Jousselin *et al.*, 2009). Recent studies, however, support the idea that topological concordance does not necessarily imply cospeciation (de Vienne *et al.*, 2007, 2013). In fact, topological congruence can also be the result of repeated host shifts to closely-related hosts followed by divergence of the symbiont, giving the false impression of cospeciation (pseudo-cospeciation, Hafner & Nadler, 1988).

6.4.1 Interpretation of topological congruence in host-symbiont phylogenies: disentangling host-switches from cospeciation

Whether the topological congruence is a result of simultaneous speciation of symbionts or repeated host shifts can be verified by testing for the congruence in the speciation time of both the symbionts. Codivergence of symbionts must involve the temporal congruence in the divergence. Alternatively, in case of host shifts followed by divergence, the host divergence predates the symbiont divergence. Concomitant divergence also leads to proportional branch length and therefore for taxa in which the dating of the phylogenies is not possible, proportional branch lengths in the host-symbiont phylogenies have been used as an indication of cospeciation (Page, 1996). However, proportional branch lengths may also result from a symbiont jump to a closely related host while taking a similar time to speciate as the host (Charleston & Robertson, 2002; Wilson *et al.*, 2012). Thus branch-length-based validation of cospeciation may lead to erroneous conclusions, which leaves testing for temporal congruence between host and symbiont phylogenies the only way to reliably estimate cospeciation (Jeong *et al.*, 1999; Charleston & Robertson, 2002; Hirose *et al.*, 2005; Reed *et al.*, 2007; Mikheyev *et al.*, 2010; Badets *et al.*, 2011; de Vienne *et al.*, 2013).

Estimating the diversification time relies mainly on the availability of fossil records. Alternatively, in case of missing fossil calibration points, the information about the rates of substitution at the genus level can be used to derive diversification time (Amo de Paz *et al.*, 2011; Leavitt *et al.*, 2012, 2013b). For my study, it is possible to date *Protoparmelia* diversification using the split of Parmeliaceae and *Protoparmelia* as a calibration point (Amo de Paz *et al.*, 2011). However, the dating of lichen-associating

green algae is far less advanced and similar calibration points are not available for dating *Trebouxia*. The dating of green algae in general is a difficult task because of the sparse fossil record. As compared to the fungal hosts, dating of the green algae is restricted to a few studies only (Leliaert *et al.*, 2011, 2012). These studies demonstrated the split of the major clades at the family rank and there are no calibration points available for dating at the genus level. In addition, substitution rate information is not available for the loci used in my study, and, thus, it is not possible to estimate if the topological congruence in the *Protoparmelia-Trebouxia* phylogenies is also accompanied by temporal congruence.

Jane is an event-cost method in which each event in the symbiont phylogeny is mapped onto the host tree and the costs associated with the each of the possible cophylogenetic events (cospeciation, duplication, host switching, and loss) are inferred (Conow *et al.*, 2010). The least costly combination of events is proposed to be the evolutionary pattern history behind that association. Jane has become a popular method to investigate cophylogenetic patterns in host-symbiotic associations, especially in cases were molecular dating is not possible (Cuthill & Charleston, 2012; Rosenblueth *et al.*, 2012; du Toit *et al.*, 2013; Bellec *et al.*, 2014; Millanes *et al.*, 2014). In general, using dated phylogenies along with Jane will increase the confidence in the interpretations of the results of the cophylogenetic analysis. However, in the absence of fossil data and other calibration points, Jane has been reliably used as an alternative to infer evolutionary events behind symbiont diversification (Rosenblueth *et al.*, 2012; Cruaud *et al.*, 2012; Bellec *et al.*, 2014; Lei & Olival, 2014; Millanes *et al.*, 2014). The software potentially disentangles topological congruence resulting from cospeciation and host-shift speciation. Therefore, I used Jane to infer cophylogenetic patterns for each *Protoparmelia-Trebouxia* association. The analysis suggested topological congruence between host symbiont phylogenies. However, the least costly scenario suggested host-switching to closely-related hosts, losses or extinction, and failure to diverge as the predominant events shaping *Protoparmelia-Trebouxia* associations (Singh *et al.*, 2017). As stated previously, host-switches to closely-related hosts followed by speciation could also generate congruent phylogenetic structure and this could be the reason for the significant congruence between *Protoparmelia-Trebouxia* phylogenies. The concomitant divergence of symbionts is proposed to generate host specialist symbionts (Legendre *et al.*, 2002; Thompson, 2010; de Vienne *et al.*, 2013). However, also pseudo-cospeciation, i.e., host switches followed by speciation, could give rise to host specialist symbionts. The highly

specific associations in the tropics therefore are likely the result of host switches followed by speciation of *Trebouxia* rather than cospeciation.

6.4.2 Host switching, rather than cospeciation, produces host-specialists in the *Protoparmelia-Trebouxia* symbiosis

Cospeciation has been the predominant hypothesis to explain specialist symbiotic associations in the past (de Vienne *et al.*, 2007, 2013). In case of lichens, Ahmadjian (1987b) proposed extensive cospeciation of the symbionts due to the obligatory nature of the lichen symbiosis. However, in spite of this, cospeciation does not seem to have played a role in the diversification of *Protoparmelia-Trebouxia* associations. Even in the case of specialist interactions (one-to-one interactions), such as those found in the tropics, host switching appears to be the major event shaping *Protoparmelia-Trebouxia* associations. Recent studies suggested cospeciation to be rarer than previously thought, and instead suggested host switching to be more common (Charleston & Robertson, 2002; Lei & Olival, 2014; Susoy & Herrmann, 2014). It is suggested that the symbionts may not be passive followers of their host evolutionary history as is expected in case of cospeciation (Hoberg & Brooks, 2008; Hoberg *et al.*, 2015). Previous cophylogenetic studies on lichens also rejected the hypothesis of cospeciation, and proposed algal switching to be a rather common phenomenon (Taylor *et al.*, 2000; Piercey-Normore & Depriest, 2001). Host-switching is now emerging as a predominant hypothesis to explain host-symbiont diversification especially in case of specialist symbionts (Refrégier *et al.*, 2008; de Vienne *et al.*, 2013; Murray *et al.*, 2013; Susoy & Herrmann, 2014), as found in my study. My study supports the idea that cospeciation might be a rare event even in the case of obligate and specialized symbionts.

Ecological fitting: possible mechanism behind prevalent host switching events in specialist associations

Ecological fitting has been proposed as an alternative process (other than cospeciation) to explain the parasite paradox or the predominant host-switching in specialized associations (Janzen, 1985; Agosta *et al.*, 2010; Araujo *et al.*, 2015). It is the process by which organisms utilize the existing traits to colonize the novel environment or host, and to use resources presented by the new environment. It assumes that the traits relevant for the survival in the new conditions are already present in the organism and are not a result of shared evolutionary history. Ecological fitting thus provides the phenotypic flexibility for

rapid host switching (Agosta & Klemens, 2008). In the absence of cospeciation, as I found in my study, ecological fitting could be a possible driver for the formation of specialist *Protoparmelia-Trebouxia* associations. Ecological fitting can be achieved by (a) resource tracking, i.e., colonizing a new host species that represents similar resource as the ancestral host, or (b) via sloppy fitness space, i.e., colonizing hosts that represent new resources (Araujo *et al.*, 2015).

Host shift via ecological fitting involves an initial phase of generalization or low selectivity to colonize a new host, followed by specialization to the new host (Agosta *et al.*, 2010; Hoberg *et al.*, 2015; Araujo *et al.*, 2015). Two processes have been proposed as to be essential to promote host switching under ecological fitting. First, the opportunity for potential partners to interact is essential, i.e., the symbionts must coexist temporally and spatially to allow the switch. Secondly, the new host symbiont association must be compatible in terms of resources, and survival, not to jeopardize the species existence. It has been proposed for lichens that the symbiotic stage may be interrupted and regained in the course of their life cycle. For instance, sexual reproduction often dissociates the symbiosis and leads to independent dispersal of the symbionts (Ott, 1987a; Beck *et al.*, 1998; Dal Grande *et al.*, 2012). The non-symbiotic phase provides the opportunity for novel interactions and the formation of new symbiotic combinations. The formation of a new lichen thallus requires associating with algae from the environment, either symbiotic or free-living (Hauck *et al.*, 2007; Nelsen & Gargas, 2008). During the initial phases of re-lichenization, lichen-forming fungi may display low selectivity towards the photobiont and may thus associate with non-compatible photobionts as well (Ott, 1987a,b; Beck *et al.*, 1998). Thus the possibility of interaction with other non-compatible partners may provide the opportunity for host switching under ecological fitting.

6.4.3 Losses, and failure to diverge generate generalist species

Failure to diverge is the major event shaping boreal, arctic/alpine and temperate *Protoparmelia-Trebouxia* associations. Failure to diverge is when the host diverges and the parasite is transmitted to both new lineages and the symbionts associated with both the new host lineages remain connected via gene flow (Banks & Paterson, 2005; de Vienne *et al.*, 2013). As symbionts fail to diverge despite host diversification, several host lineages remain associated with the same symbiont lineage thus giving rise to multi-host or generalist symbionts (Banks & Paterson, 2005). Failure to diverge is also called as inertia (Paterson & Banks, 2001) or cophylogeny without cospeciation (Hugot *et al.*, 2001). In

my study, *Trebouxia sp. 1* (*T. suecica*) associates with all the five boreal, arctic/alpine *Protoparmelia* species. Similarly, *Trebouxia sp. 5* (*T. simplex*) associates with all the three temperate *Protoparmelia* species. These two *Trebouxia* species are generalists and might have resulted from the failure of *Trebouxia* to diverge with *Protoparmelia*. In fact, the BLAST hits of these *Trebouxia* species show that these two species are truly generalist as they associate with several other phylogenetically distant lichen-forming fungi. Generalist symbionts may arise due to overlapping host ranges, i.e. geographic overlap of host distribution, leading to higher opportunities of transfer among different hosts (Banks and Paterson 2005; de Vienne *et al*. 2007, 2013). The possibility of environmental transmission of symbionts may further facilitate cross-species symbiont transfer (Mikheyev *et al.*, 2010). Due to the overlapping host distribution and environmental transmission, the symbiont populations associated with several new hosts can still maintain gene flow and may not diverge with the host. In fact, several studies have reported failure to diverge as the main evolutionary event in predominantly environmentally transmitted parasites (Peek *et al.*, 1998; Longdon *et al.*, 2011; Lei & Olival, 2014; Liu *et al.*, 2014). Environmental transmission of photobionts has also been proposed for lichen-forming fungi through photobiont-mediated guilds (Rikkinen *et al.*, 2002; Dal Grande *et al.*, 2014b). The fact that *Trebouxia sp. 1*, *Trebouxia sp. 2*, and *T. sp. 5* associate with several lichen-forming fungi inhabiting similar environment indicates that these algae might be environmentally transmitted. Sympatric distribution of lichen-forming fungi and environmental transmission of algae might have resulted in generalists *Trebouxia* species due to continuous genetic exchange between symbiont populations.

On the other hand, loss or extinction occur when the symbionts are unable to survive in small populations of the diversifying host. Alternatively, incomplete lineage sorting, where the symbiont is not transmitted to a small diversifying host population, can also lead to loss of symbiont from a closely related host lineage. In my study losses were predominantly observed in the arctic/temperate and Mediterranean regions. Losses have been extensively reported in some other host-parasite associations as well (Ronquist, 1997; de Vienne *et al.*, 2013).

7 CONCLUSIONS

In my thesis I attempted to improve the understanding of variation in the symbiont diversity and symbiont interaction patterns across different macrohabitats, using the lichen association of *Protoparmelia* and *Trebouxia* as my study system. The use of multilocus phylogenies and species delimitation approaches allowed me to achieve a robust species concept for both the symbionts. I discovered several cryptic taxa for both the symbionts which were previously hidden under a single name. My study thus highlights the importance of having reliable species boundaries for an accurate estimation of the diversity and interaction patterns.

My study for the first time established *Protoparmelia* s.lat. to be polyphyletic. My findings show that the diversity of the algal partners associated with *Protoparmelia* is comparable in different macrohabitats and do not show a variation in diversity across the latitude. As for the symbiont interaction patterns, the selectivity of the fungal partner in *Protoparmelia* is generally higher than that of the algal partner in all the macrohabitats. The fungal partner displayed high selectivity towards its photosynthetic partners across all the macrohabitats whereas algae displayed variation in the interaction patterns under different macrohabitats. The selectivity of algae was higher in the tropical regions. I found eight specialized (one to one) associations in my study, out of which seven were found in the tropics, and one in the Mediterranean region. My study thus suggests that symbiont interaction patterns may be influenced by the macrohabitat of occurrence and may display variation in the strength of interaction under different macrohabitats.

In addition, I conclusively showed that the *Protoparmelia-Trebouxia* do not cospeciate in spite of the obligatory nature of the association. Even specialized associations do not cospeciate in my study system. The high specificity and overall phylogenetic congruence of *Trebouxia-Protoparmelia* association is likely the result of host-switches rather than cospeciation. In different habitats different evolutionary events shape the *Protoparmelia-Trebouxia* symbiosis.

My thesis provides a conceptual framework for analyzing the diversity and interaction patterns for other symbiotic systems, particularly in cases where the species were described based on phenotypic characters and may contain several cryptic species. In addition, my research offers an interesting perspective on the variation of the symbiont diversity, interaction patterns and evolutionary dynamics under different macrohabitats, especially for endosymbiotic systems.

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10 APPENDIX

10.1 Publication: The sister-group relationships of the largest family of lichenized fungi, Parmeliaceae (Lecanorales, Ascomycota).

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?
(1) zu Entwicklung und Planung
Garima Singh: 75%;
Pradeep K. Divakar: 10%;
H. Thosten Lumbsch: 5%;
Imke Schmitt: 15%;
(2) zur Durchführung der einzelnen Untersuchungen und Experimente
Garima Singh**:** 50% PCR and sequencing
Jürgen Otte: 50%; PCR and sequencing
(3) zur Erstellung der Datensammlung und Abbildungen
Garima Singh: 60%; Sample preparation and figures
Pradeep K. Divakar: 20%; Samples and sequences for the study
Francesco Dal Grande: 20%; Samples and sequences for the study
(4) zur Analyse und Interpretation der Daten
Garima Singh: 90%; CADM, phylogenetic analysis, interpretation of Data
5) zum Verfassen des Manuskripts
Garima Singh: 80%;
Francesco Dal Grande: 5%;
Imke Schmitt: 5%;
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The sister-group relationships of the largest family of lichenized fungi, Parmeliaceae (*Lecanorales*, *Ascomycota*)

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ABSTRACT

Parmeliaceae is the largest family of lichen-forming fungi. In spite of its importance for fungal diversity, its relationships with other families in *Lecanorales* remain poorly known. To better understand the evolutionary history of the diversification of lineages and species richness in Parmeliaceae it is important to know the phylogenetic relationships of the closest relatives of the family. A recent study based on two molecular loci suggested that either *Protoparmelia* s. str. or a group consisting of *Gypsoplaca* and *Protoparmelia* s. str. were the possible sister-group candidates of Parmeliaceae, but that study could not distinguish between these two alternatives. Here, we used a four-locus phylogeny (nuLSU, ITS, *RPB1*, *MCM7*) to reveal relationships of Parmeliaceae with other potential relatives in *Lecanorales*. Maximum likelihood and Bayesian analyses showed that *Protoparmelia* is polyphyletic, with *Protoparmelia* s. str. (including *Protoparmelia badia* and *Protoparmelia picea*) being most closely related to Parmeliaceae s. str., while the *Protoparmelia atriseda*-group formed the sister-group to *Miriquidica*. *Gypsoplaca* formed the sister-group to the Parmeliaceae s. str. + *Protoparmelia* s. str. clade. Monophyly of *Protoparmelia* as currently circumscribed, and *Gypsoplaca* as sister-group to Parmeliaceae s. str. were both significantly rejected by alternative hypothesis testing.

Introduction

Parmeliaceae is the largest family of lichen-forming fungi, consisting of over 2700 species (Thell *et al.* 2012). The family includes well-known foliose and fruticose lichens, such as beard-lichens (*Usnea*) and species that are frequently used in monitoring of air pollution, e.g. *Parmelia sulcata* and *Flavoparmelia caperata* (Nimis *et al.* 2002; Crespo *et al.* 2004). The species

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richness of a clade may be explained by the evolution of key innovative characters that confer adaptive advantages and lead to adaptive radiation (Sanderson & Donoghue 1994). One way to study key innovations is to compare evolutionary changes in sister clades. These traits affect the rate of lineage diversification and are expected to leave an imprint in the phylogeny of the affected group (Ree 2005). Thus one prerequisite for identifying such a character is elucidating the phylogenetic relationships of the group of interest (Ridley 1983; Pagel & Harvey 1988). Recent studies enhanced our knowledge of evolution and phylogenetic relationships within Parmeliaceae (Crespo *et al.* 2010, 2011; Thell *et al.* 2012). All genera currently accepted in Parmeliaceae in a wide sense (including the sometimes recognized *Anziaceae*, *Alectoriaceae*, *Hypogymniaceae*, and *Usneaceae*) form a well-supported monophyletic group in all major published analyses (Persoh & Rambold 2002; Arup *et al.* 2007; Crespo *et al.* 2007). However, the relationships of Parmeliaceae with other groups in *Lecanorales* are poorly known. The study by Arup *et al.* (2007), based on mtSSU and nuLSU rDNA sequences focused on the sister-group relations of Parmeliaceae within *Lecanorales*. This study (Arup *et al.* 2007) included all known potential relatives to Parmeliaceae (e.g. *Gypsoplaca*, *Japewia*, *Mycoblastus*, *Protoparmelia*, *Tephromela*, and *Calvitimela*), and concluded that *Protoparmelia* and *Gypsoplacaceae* were the two most closely related groups, with high support. Arup *et al.* (2007) compared the probabilities of the three possible alternative topologies and concluded that *Gyposoplacaceae* was clearly unlikely to be the closest relative to Parmeliaceae s. str. However, these authors suggested their data were not sufficient to differentiate between the scenario where *Protoparmelia* was sister to Parmeliaceae, and where (*Protoparmelia* + *Gypsoplaca*) was the sister. Crespo *et al.* (2007) likewise showed a supported relationship of *Protoparmelia badia* as a close relative to Parmeliaceae, but here *Gypsoplacaceae* was not included. Furthermore, '*Lecidea*' *rubrocastanea* was proposed to be close to *Protoparmelia* (Spribille & Printzen 2007) but there was no support for this relationship. In a recent study (Papong *et al.* 2011), which included *L. rubrocastanea*, *Protoparmelia*, *Maronina*, and other Parmeliaceae genera, the authors showed that '*Lecidea*' *rubrocastanea* neither belongs to *Protoparmelia* nor to the Parmeliaceae but falls outside of both groups. Additionally, the genus *Maronina* was considered a morphologically close relative of *Protoparmelia* (Hafellner & Rogers 1990). However this genus has recently been synonymized within *Protoparmelia*, based on molecular and morphological data (see Papong *et al.* 2011).

Protoparmelia has indeed been suggested to be better classified within the Parmeliaceae by several authors (Miyawaki 1991; Henssen 1995; Lumbsch & Huhndorf 2010). The genus was resurrected by Hafellner (1984) for species previously included in *Lecanora* but differing in details of the amyloid staining of the ascus apex, brown pigmentation, and lack of atranorin. The genus was placed in *Lecanoraceae*. Subsequently, studies on apothecial anatomy showed the presence of a cupular exciple in the type species, *P. badia*, which is typical of Parmeliaceae (Miyawaki 1991; Henssen 1995). However, *Protoparmelia* as currently circumscribed has also been shown to be heterogeneous and chemically diverse (Ryan *et al.* 2004). For example, *P. badia* and *Protoparmelia picea* have lobaric acid (Hertel 1984) whereas *Protoparmelia cupreobadia* and *Protoparmelia atriseda* contain norstictic acid as major secondary metabolites. Further, some species in *Protoparmelia* have bacilliform conidia, while others have filiform conidia, similar to the genus *Miriquidica* (Ryan *et al.* 2004). Thus, the monophyly and circumscription of *Protoparmelia* remained uncertain.

Here we use an extended taxon sampling and a data set including four loci to address the following questions: (i) Which lineage is most closely related to Parmeliaceae? (ii) Is *Protoparmelia* monophyletic, and if not, what are the phylogenetic relationships of the lineages within the genus?

Materials and methods

Sampling

A total of 54 taxa from four families were sampled: *Cladoniaceae*, *Gypsoplacaceae*, *Lecanoraceae*, and Parmeliaceae s. str. Members of the *Cladoniaceae* were selected as outgroup for the analysis because previous studies suggested that these families are closely related to Parmeliaceae (Miadlikowska *et al.* 2006; Crespo *et al.* 2007). Details of the studied material, including GenBank accession numbers are shown in Table 1.

DNA extraction and molecular methods

Genomic DNA was extracted from lichen thalli using the cetyltrimethylammonium bromide (CTAB) method (Cubero & Crespo 2002). PCR amplification was performed using general, previously published or taxon-specific primers for ITS2, *MCM7*, *RPB1*, and nuLSU (Table 2). PCR reactions were carried out in a volume of 25 µl. Each reaction mix contained 2.5 µl buffer, 0.13 µl (=0.65 U) Ex Taq polymerase, 1.0 µl dNTP mix (2.5 mM each), 1.0 µl each (10 µM) of the primer set (forward and reverse), *ca* 20 ng of template, and 16 µl H_2O. Reactions were performed with the following cycling conditions: initial denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 40 s, 72 °C for 1 min, and final elongation at 72 °C for 5 min. PCR products were checked for amplification on 1 % agarose gels. Bands of the expected size were extracted using the peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH). These fragments were then labelled for cycle sequencing using Big Dye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems) and sequenced as follows: (1) 1 min 96 °C, (2) 26 cycles of 20 s 96 °C, 5 s 50 °C, and 2 min 60 °C. Products were purified using the Big Dye XTerminator Purification Kit (Life Technologies) and then detected on ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

Phylogenetic analyses

Sequences were assembled using Geneious v. 5.4 (Drummond *et al.* 2011) followed by manual editing. Sequences from 54 species were aligned for each locus separately using MAFFT (Katoh *et al.* 2005). Gaps were treated as missing data and ambiguously aligned parts were excluded. The program Gblocks v. 0.91b (Castresana 2000; Talavera & Castresana 2007) was used to remove poorly aligned regions.

We checked for congruence of the four loci by performing congruence among distance matrices (CADM) analysis (Campbell *et al.* 2011). Data of congruent loci ($p < 0.001$)

Table 1 – Specimens used in this study including voucher information, and GenBank accession numbers.

Species	Voucher information[a]	Accession number[b]			
		nuLSU	*MCM7*	*RPB1*	ITS
Alectoria ochroleuca	–	DQ899288	n/a	n/a	DQ979997
Alectoria ochroleuca	Sweden, Härjedalen, *Wedin 6542* (UPS)	n/a	**KF562163**	DQ923677	n/a
Austroparmelina pruinata	–	EF042914	JX974675	GU994680	EF042905
Cetraria islandica	–	AY340539	JX974677	DQ923685	AF117995
Cetraria nigricans	Canada, Nunavut, *Westberg 2377* (LD)	JN000257	**KF562164**	JN000287	AF254629
Cetrariastrum andenze	–	GQ919245	GQ272429	GU994690	GQ919269
Cetrariastrum dulitens	–	GQ919246	GQ272427	GU994691	GQ919270
Cetrariella commixta	Finland, Southern Finland, *Haikonen 19093* (H)	JN000260		JN000290	AF451796
Cetrariella delisei	–	DQ923657	JX974679	n/a	DQ980005
Cetrariella delisei	Sweden, Jämtland, *Wedin 8465* (S)	n/a	n/a	**KF601228**	n/a
Cladia aggregata	–	GQ500966	HM441287	n/a	GQ500917
Cladia dumicola	–	GQ500968	HM441281	n/a	GQ500915
Cladia schizopora	Australia, *HTL 19994c* (F)	GQ500952	HM441290	**KF601229**	GQ500919
Cladonia rangiferina	–	AY300832	n/a	DQ915595	AY300881
Emodomelanelia masonii	–	GU994595	JX974681	GU994695	GU994549
Everniastrum nepalense	–	AY607783	n/a	EF092106	AY611071
Everniopsis trulla	–	EF108290	GQ272396	EF105429	EF108289
Flavoparmelia marchantii	–	GU994598	GQ272420	GU994698	DQ299905
Flavoparmelia soredians	–	AY584835	JX974684	EF092108	AY586562
Gowardia nigricans	–	DQ923649	n/a	n/a	DQ979996
Gowardia nigricans	Norway, Troms, *Wedin 7297* (UPS)	n/a	**KF562165**	DQ923676	n/a
Gypsoplaca macrophylla	–	DQ899298	n/a	n/a	n/a
Gypsoplaca macrophylla	USA, Utah, *R.W. Rosentreter 15995* (F)	n/a	n/a	**KF601230**	**KF650781**
Hypogymnia vittata	–	DQ900637	n/a	DQ923689	DQ980012
Hypogymnia vittata	Sweden, Västerbotten, *Wedin 6814* (UPS)	n/a	**KF562166**	n/a	n/a
Hypogymnia physodes	–	AY756338	n/a	AY756407	AF058036
Hypogymnia physodes	Sweden, Jämtland, *Wedin 6623* (UPS)	n/a	**KF562167**	n/a	n/a
Lecanora carpinea	–	DQ787363	n/a	n/a	AY541248
Lecanora hybocarpa	–	EF105421	n/a	EF105430	EF105417
Lecanora paramerae	–	EF105422	n/a	EF105431	EF105418
Lecanora sulphurea	–	EF105423	n/a	EF105432	AF070030
Melanelia hepatizon	–	DQ923667	JX974678	DQ923692	DQ980016
Melanelixia fuliginosa	–	AY607801	JX974686	EF092116	AY611089
Melanelixia subaurifera	–	AY607811	JX126390	EF092120	AY611095
Melanohalea elegantula	Spain, Madrid, *Crespo s.n.* (MAF-Lich 10231)	AY607806	n/a	**KF601231**	AY611094
Melanohalea exasperata	–	AY607793	n/a	EF092123	AY611081
Menegazzia terebrata	Sweden, Gästrikland, *Wedin 4392* (UPS)	DQ899304	**KF562168**	DQ923694	DQ980019
Metus conglomeratus	Australia, Tasmania, *H.T. Lumbsch 19982b* (F)	GQ500958	HM441294	**KF601232**	GQ500912
Miriquidica complanata	Poland, Karkonosze Mts, *Szczepańska 935* (herb. Szczepańska)	**KF562179**	**KF562169**	**KF601233**	**KF562187**
Miriquidica garovaglii	Slovakia, Karpaty Mts, *Szczepańska 538* (herb. Szczepańska)	**KF562180**	n/a	**KF601234**	**KF562188**
Miriquidica leucophaea	Poland, *Kossowska 448* (herb. Kossowska)	**KF562181**	**KF562170**	**KF601235**	**KF562188**
Montanelia disjuncta	Sweden, Lycksele Lappmark, *Wedin 7143* (UPS)	DQ923666	JX974699	DQ923691	DQ980015
Montanelia sorediata	–	GU994604	JX974705	GU994706	GU994556
Myelochroa irrugans	–	AY607815	JX974708	EF092128	AY611103
Nephromopsis leucostigma	Bhutan, Thimpu District, *Søchting 9151* (LD)	JN000267	**KF562172**	JN000295	AF451777
Parmelina quercina	–	AY607818	n/a	EF092136	n/a
Parmelia saxatilis	–	AY300849	JX974709	DQ923695	AF058037
Parmotrema reticulatum	–	AY584848	JX974712	GU994729	AY586577
Protoparmelia atriseda	USA, Washington, *McCune, H. Ponzetti 26046* (OSU)	**KF562182**	**KF562173**	**KF601236**	**KF562190**
Protoparmelia badia	Austria, Kärnten, *Hafellner, Muggia, Hafellner 68478* (GZU)	**KF562183**	**KF562174**	**KF601237**	**KF562191**
Protoparmelia cupreobadia	USA, Maine, *Fryday 863* (MSC)	**KF562184**	**KF562175**	**KF601238**	**KF562192**
Protoparmelia phaeonesos	Norway, Buskerud, *Rui, E. Timdal 11000* (O)	**KF562185**	**KF562176**	**KF601239**	**KF562193**
Protoparmelia picea	Norway, Sør-Trøndelag, *Haugan 9612* (O)	**KF562186**	**KF562177**	**KF601240**	**KF562194**
Pseudephebe pubescens	–	AY607839	n/a	EF092148	n/a
Relicina subnigra	–	AY785267	n/a	EF092152	n/a
Tuckermannopsis chlorophylla	Sweden, Västerbotten, Wedin 6995 (UPS)	DQ923674	**KF562178**	DQ923697	DQ980025
Vulpicida pinastri	–	DQ923675	JX974721	DQ923698	AF058039
Xanthoparmelia conspersa	–	AY578962	n/a	EF092155	n/a
Xanthoparmelia tinctina 1	Spain, Madrid, *Crespo s.n.* (MAF-Lich 6070)	AY578976	JX974720	**KF601241**	AY581108

a Herbarium acronyms follow Thiers 2012.
b New sequences are presented in bold.

Table 2 – Primers used in this study. For the genera *Protoparmelia* and *Miriquidica* we designed taxon-specific primers.

Taxon	Locus	Primer name	Sequence	Reference
Protoparmelia	*RPB1*	gRPB1Af (FOR)	GADTGTCCDGGDCATTTTGG	Stiller & Hall (1997)
		fRPB1cR (REV)	CNGGCDATNTCRTTRTCCATRTA	Matheny *et al.* (2002)
		RPB1PPspf (FOR)	GTGCTTTGCTTCAGCAGTGCTC	This study
		RPB1PPspr (REV)	AGCGACGAACATTGCCGTTCGCAC	This study
	MCM7	MCM7-709 (FOR)	ACIMGIGTITCVGAYGTHAARCC	Schmitt *et al.* (2009)
		MCM7-1348 (REV)	GAYTTDGCIACICCIGGRTCWCCCAT	Schmitt *et al.* (2009)
		MCM7PPspf (FOR)	GAICGDTGIGGITRIGARRTITTIC	This study
		MCM7PPspr (REV)	GIIARRTAITCRTACATGKIRCC	This study
	nuLSU	AL1R (FOR)	GGGTCCGAGTTGTAATTTGT	Döring *et al.* (2000); Vilgalys & Hester (1990)
		LR6 (REV)	CGCCAGTTCTGCTTACC	
		LR5 (FOR)	TCCTGAGGGAAACTTCG	Vilgalys & Hester (1990)
		LROR (FOR)	ACCCGCTGAACTTAAGC	Vilgalys & Hester (1990)
		L3 (REV)	CCGTGTTTCAAGACGGG	Vilgalys & Hester (1990)
		NULSUPPspf (FOR)	GAAACCCCTTCGACGAGTCGAG	This study
		NULSUPPspr (REV)	AGATGGTTCGATTAGTCTTTCG	This study
	ITS	ITS1-F (FOR)	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
		ITS2 (REV)	GCTGCGTTCTTCATCGATGC	White *et al.* (1990)
		ITS3 (FOR)	GCATCGATGAAGAACGCAGC	White *et al.* (1990)
		ITS4 (REV)	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
Miriquidica	*RPB1*	RPB1MIRIf (FOR)	CTACAGATGATATCAAGCTCATG	This study
		RPB1MIRIr (REV)	CATGAGCTTGATATCATCTGTAG	This study
	MCM7	MCM7MIRIf (FOR)	CAATTTACTCCAATGACTGAATGTC	This study
		MCM7MIRIr (REV)	CATGCCGTCGCCTATCTCCTTAGTC	This study
	ITS	ITSMIRIf (FOR)	TATCGAGTGGAGGGGCTTCGCTC	This study
		ITSMIRIr (REV)	TAACGTTTAGGCGGTTGTTGGC	This study

were concatenated. Maximum likelihood (ML) analysis was performed on the single-loci using RAxML v. 7.0.4 (Stamatakis 2006) using the default GTR + G model, the standard model implemented in RAxML, for all loci, with 1000 bootstrap (BS) replicates. Conflicts were considered significant if individuals group in a clade supported by >75 % ML BS support in data set from one locus, but in a different supported clade in data set from another locus. ML search was performed on the concatenated four-locus data set with RAxML-HPC BlackBox v. 7.2.8 (Stamatakis *et al.* 2008) on the Cipres Scientific gateway v. 3.3 (www.phylo.org; Miller *et al.* 2010) using the default GTR + G model with data partitioning according to the different genes (www.phylo.org). For *RPB1* and *MCM7* data were also partitioned by codon position.

We used the Corrected Akaike Information Criterion (AICc) (Sugiura 1978; Hurvich & Tsai 1989) as implemented in JModelTest v. 2.1.1 (Guindon & Gascuel 2003; Darriba *et al.* 2012) to find the appropriate model for each locus. Bayesian tree inference was carried out using the best fitting model, for both single-locus and four-locus concatenated data set as implemented in MrBayes v. 3.2.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Two parallel Metropolis-coupled Markov chain Monte Carlo (MCMCMC) runs were performed each using four chains and 5 000 000 generations, sampling trees every 100th generation. A 50 % majority rule consensus tree was generated from the combined sampled trees of both runs after discarding the first 25 % as burn-in (12 500 trees, likelihoods below stationary level).

We used alternative hypothesis testing to test whether our data are sufficient to reject (a) monophyly of *Protoparmelia*, and (b) *Gyposoplaca* forming a sister-group to Parmeliaceae s. str. The constrained and unconstrained trees were inferred using the program Tree-PUZZLE 5.2 (Schmidt *et al.* 2002) employing the GTR + I + G nucleotide substitution model. We used two methods to compare the different topologies: the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa 1999) and the expected likelihood weight (ELW) test (Strimmer & Rambaut 2002).

Results

DNA sequences

We generated 47 new sequences for this phylogeny including 14 *RPB1*, 16 *MCM7*, eight nuLSU, and eight ITS sequences. The data sets include 118 sequences from previous publications by PARSYS working group (Crespo *et al.* 2007, 2010; Divakar *et al.* 2012) and 13 downloaded from GenBank. A total of 54 taxa were analyzed including 36 representatives of the family Parmeliaceae s. str., five *Protoparmelia* species, seven *Lecanoraceae*, five *Cladoniaceae*, and the only representative of *Gypsoplacaceae*, i.e. *Gypsoplaca macrophylla*.

Phylogenetic analysis

CADM results showed no significant incongruence among loci, thus allowing concatenation. Only specimens with sequence information available for at least two loci were included in the analysis. The concatenated four-locus data set contained 54 sequences of the following lengths: 689 bp for *RPB1*, 616 bp for *MCM7*, 851 bp for nuLSU rDNA, and 534 bp for ITS rDNA. Total length of the concatenated alignment was 2693 bp. The ML tree for the concatenated data set is presented in Fig 1.

Partition finder showed that the model of sequence evolution was different for each locus. According to JModelTest v. 2.1.1, the following best fitting models were used:

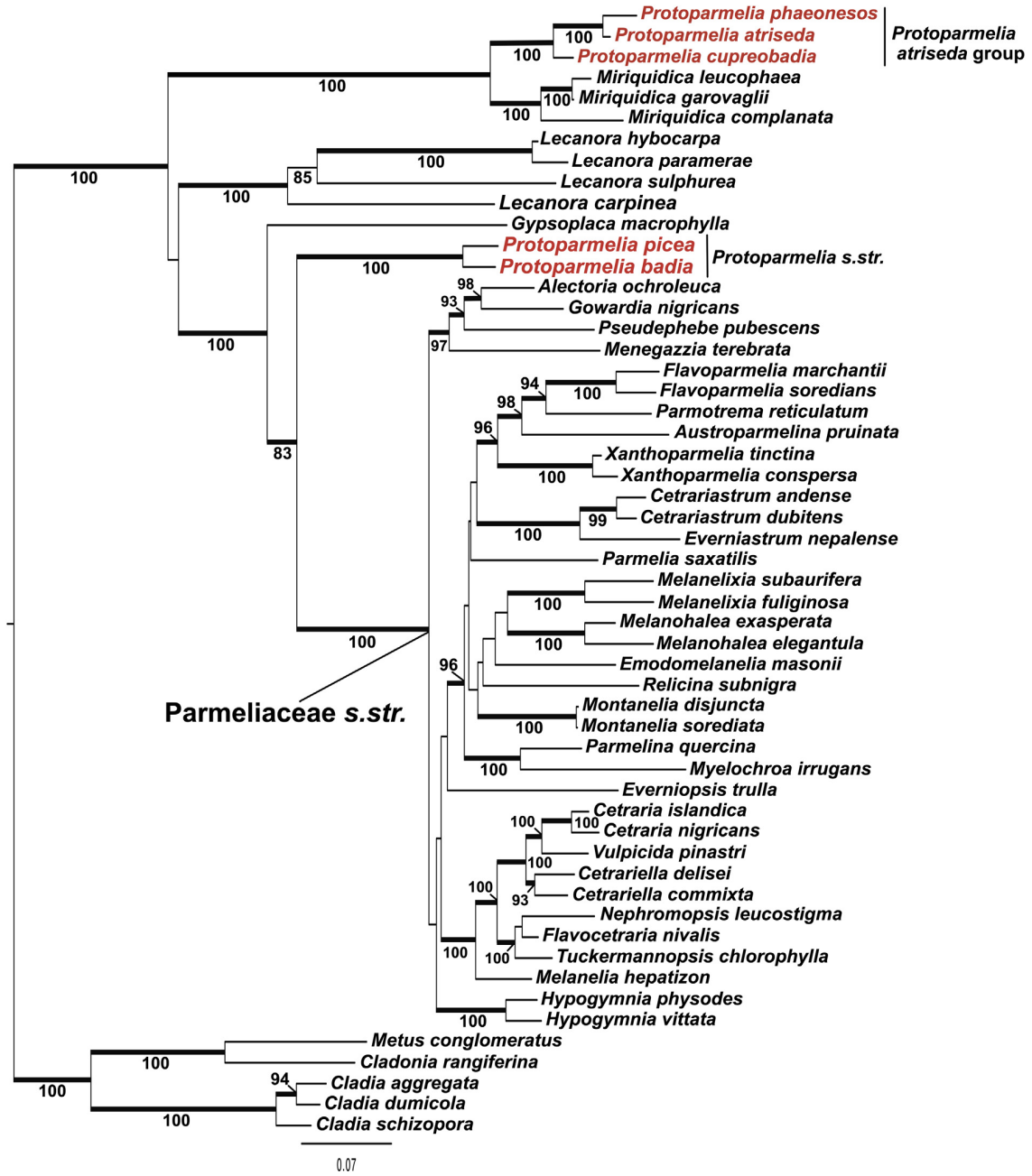

Fig 1 – Phylogeny of the Parmeliaceae and related taxa. This is a ML tree based on a concatenated alignment of nuLSU rDNA, ITS rDNA, *RPB1*, *MCM7*. ML BS support values >80 %, based on 1000 replicates, are indicated below branches. Bold branches indicate PPs >0.95 from a Bayesian analysis.

RPB1:TIM2ef + I + G; *MCM7*: HKY + G; nuLSU: GTR + I + G; ITS2: 012343 + I + G. Bayesian analysis was performed using the best fitting model for each locus in the concatenated sequence. We did not find any differences in topology between RAxML and MrBayes trees obtained from individual and concatenated data sets. Therefore only the ML tree based on the concatenated data set is presented (Fig 1). Nodes with ML BS equal to or greater than 70 % and Bayesian posterior probability (PP) greater than 0.94, were considered as strongly supported.

The family Parmeliaceae s. str. formed a well-supported monophyletic group (BS = 100 %, PP = 1.0). *Protoparmelia badia* and *Protoparmelia picea* were found to be the sister-group to Parmeliaceae s. str. with strong support from both BS (72 %) and PP (0.97). Parmeliaceae s. str., together with *Protoparmelia* s. str. and *Gypsoplaca macrophylla*, formed a well-supported group with strong support from both ML (100 %) and PP (1.0), as was previously suggested by Arup *et al.* (2007). Three species of *Protoparmelia*, *Protoparmelia atriseda*, *Protoparmelia cupreobadia*, *Protoparmelia*

phaeonesos, formed a monophyletic group sister to members of the genus *Miriquidica* (BS = 100 %, PP = 1.0).

Alternative hypothesis testing using the ELW and SH-tests, showed that the following hypotheses can be rejected: (i) *Protoparmelia* is monophyletic ($p < 0.001$ in both tests) and (ii) *Gypsoplaca* is sister to Parmeliaceae s. str. ($p < 0.001$ in both tests).

Discussion

Phylogenetic analyses were performed to infer the sister-group relation of Parmeliaceae with other potential relatives groups/families within *Lecanorales*. Our multilocus analyses focused on representatives of three lineages, *Lecanoraceae*, *Protoparmelia* spp., and *Gypsoplacaceae*.

Parmeliaceae s. str. forms a well-supported monophyletic group, confirming the results of earlier studies (Mattsson & Wedin 1999; Persoh & Rambold 2002; Arup *et al.* 2007; Crespo *et al.* 2007, 2010). Our analyses showed that two representatives of the genus *Protoparmelia*, *Protoparmelia badia*, and *Protoparmelia picea*, form the sister-group to Parmeliaceae s. str., similar to Arup *et al.* (2007) and Crespo *et al.* (2007). Furthermore, we conclusively showed that *Gypsoplacaceae* is sister to this group. Our results furthermore indicate that *Protoparmelia*, as currently circumscribed, is polyphyletic. Three species (*Protoparmelia atriseda*, *Protoparmelia cupreobadia*, and *Protoparmelia phaeonesos*) that have been placed in the section *Phaeonora* (Poelt & Leuckert 1991) based on morphological characters are found in a well-supported group sister to *Miriquidica*.

The phylogenetic relationships of the heterogeneous genus *Protoparmelia* have been matter of debate and, at present, *Protoparmelia* is placed within Parmeliaceae. Morphological and anatomical characters of this genus are difficult to interpret as they show similarity to both *Lecanoraceae* and Parmeliaceae. Interestingly, the two separate groups of *Protoparmelia* species as circumscribed in this study have different secondary metabolite profiles. The predominant compound found in *P. badia* and *P. picea* is lobaric acid (Hertel 1984), which is rare in *Lecanoraceae*. On the other hand, *P. atriseda*, *P. cupreobadia*, and *P. phaeonesos* lack lobaric acid and the major compound found in *P. atriseda* and *P. cupreobadia* is norstictic acid. The relationship of the *P. atriseda*-group and *Miriquidica* is also supported by the presence of filiform conidia in both species groups.

It is interesting to note here that the most closely related groups of the largest family of lichenized fungi are species-poor; *Protoparmelia* s. str. has few species and *Gypsoplacaceae* is monotypic (Timdal 1990). Disparity in species richness of closely related clades is often explained by the evolution of key innovative characters. We need more detailed studies on morphological characters in the genus *Protoparmelia* to understand which characters are potentially involved in adaptive radiation of Parmeliaceae. This is beyond the scope of present study and will be subject of future investigations.

In conclusion, our multilocus phylogeny indicates that (i) *Protoparmelia* s. str. forms the sister-group to Parmeliaceae s. str., (ii) Gypsoplaceae is the closest relative of the *Protoparmelia* s. str.–Parmeliaceae s. str. clade, and (iii) *Protoparmelia* is polyphyletic and the separation into two monophyletic lineages is supported by phenotypic characters.

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10.2 Publication: Coalescent-based species delimitation approach uncovers high cryptic diversity in the cosmopolitan lichen-forming fungal genus *Protoparmelia* (Lecanorales, Ascomycota).

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(1) zu Entwicklung und Planung
Garima Singh: 50%;
Francesco Dal Grande: 20%;
H. Thorsten Lumbsch: 10%;
Imke Schmitt: 20%;
(2) zur Durchführung der einzelnen Untersuchungen und Experimente
Garima Singh**:** 50% PCR and sequencing
Jürgen Otte: 50%; PCR and sequencing
(3) zur Erstellung der Datensammlung und Abbildungen
Garima Singh: 40%; Sample preparation and figures
Pradeep K. Divakar: 50%; Samples and sequences for the study
H. Thorsten Lumbsch: 10%; Samples and sequences for the study
(4) zur Analyse und Interpretation der Daten
Garima Singh: 90%; CADM, phylogenetic analysis, and species delimitation analyses, and interpretation of Data
Francesco Dal Grande: 10%; CADM
5) zum Verfassen des Manuskripts
Garima Singh: 90%;
Francesco Dal Grande: 5%;
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RESEARCH ARTICLE

Coalescent-Based Species Delimitation Approach Uncovers High Cryptic Diversity in the Cosmopolitan Lichen-Forming Fungal Genus *Protoparmelia* (Lecanorales, Ascomycota)

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Abstract

Species recognition in lichen-forming fungi has been a challenge because of unsettled species concepts, few taxonomically relevant traits, and limitations of traditionally used morphological and chemical characters for identifying closely related species. Here we analyze species diversity in the cosmopolitan genus *Protoparmelia s.l*. The ~25 described species in this group occur across diverse habitats from the boreal -arctic/alpine to the tropics, but their relationship to each other remains unexplored. In this study, we inferred the phylogeny of 18 species currently assigned to this genus based on 160 specimens and six markers: mtSSU, nuLSU, ITS, *RPB1*, *MCM7*, and *TSR1*. We assessed the circumscription of species-level lineages in *Protoparmelia s. str*. using two coalescent-based species delimitation methods – BP&P and spedeSTEM. Our results suggest the presence of a tropical and an extra-tropical lineage, and eleven previously unrecognized distinct species-level lineages in *Protoparmelia s. str*. Several cryptic lineages were discovered as compared to phenotype-based species delimitation. Many of the putative species are supported by geographic evidence.

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Introduction

Lichens are symbiotic organisms consisting of a fungal partner (mycobiont), one or more photosynthetic partners (photobionts; [1]), and diverse bacterial communities [2]. Lichens contribute to ecosystem functioning by nutrient recycling [3], weathering rocks, preventing soil erosion, and acting as pioneer species in barren areas. They inhabit diverse ecosystems from the arctic to the tropics and commonly form an integral part of terrestrial biodiversity [4]. Lichens are preferred model systems for ecological, evolutionary, phylogeographic and population genetic studies of symbiotic associations on the account of their wide, often cosmopolitan, distribution, intriguing eco-physiological interdependence and co-evolutionary and adaptive strategies [5]. Almost one fifth of all known fungi and half of all ascomycetes are lichenized, consisting of approximately 28,000 species worldwide [6,7]. However, studies suggest that the estimate of existing lichen diversity might represent only 50–60% of the real diversity [8,9], as current species recognition in lichen-forming fungi appears to vastly underestimate the true number of species. According to Galloway [8], the number of known taxa in different genera has increased from 20% (*Parmelia sensu stricto*, [10]) to 86% in the New World *Oropogon* [11]. Recent molecular studies have demonstrated the presence of many distinct lineages subsumed under a single species name (e.g., [12–15]). In the basidiolichen fungus *Dictyonema glabratum* a single taxon was found to be composed of at least 126 species [9], thus showing a tremendous amount of unexplored diversity in lichen-forming fungi.

Species recognition in lichen-forming fungi has been a challenge because of i) the few taxonomically relevant characters (reviewed by [16,17]), ii) unsettled species concepts [18,19], iii) and unexplored regions containing high levels of diversity, especially in the tropics [20,21]. Morphological and chemical characters that have commonly been used to circumscribe species may not be useful for identifying closely-related species and often fail to accurately characterize species-level diversity [2,19,22,23]. Accurate species delimitation may be obscured by cryptic speciation [24,25], incongruence between morphology and molecular data [26,27], or incongruence between gene trees and species trees [28]. Moreover, morphological and chemical variations may constitute morpho- or chemotypes of the same species with no molecular differentiation, thus blurring our understanding of species boundaries [29,30]. The implementation of molecular techniques and availability of markers for amplifying phylogenetically informative loci have provided great insights into otherwise unrecognized species complexes. Improved species recognition has important implications for understanding diversity, ecological and biogeographical patterns, factors promoting diversification, and for devising better conservation policies [31].

Different studies have utilized varied combinations of the available techniques for unraveling hidden diversity. For example, Harrington and Near [32] used STEM [33] to explore the independent evolutionary lineages within snubnose darters (*Etheostoma simoterum* species complex). Giarla et al. [15] used two coalescent-based approaches (BP&P and spedeSTEM) for delimiting species in Andean mouse opossums (*Thylamys* spp) using three nuclear loci and found three additional lineages than previously recognized. Leavitt et al. [34,35] used Bayesian population clustering, genealogical concordance, Bayesian species delimitation, and a DNA barcode approach to support the presence of five previously unrecognized species in the lichen-forming fungus *Rhizoplaca melanophthalma* species-complex (Lecanoraceae). Parnmen et al. [36] used a 4-locus phylogenetic approach, combined with GMYC [37,38] and STEM [33] and found at least 12 species in the *Cladia aggregata* complex. Mounting evidence continues to support the perspective that traditional phenotype-based species boundaries fail to adequately characterized species-level diversity in many lichen-forming fungi (reviewed in [22]).

We implemented a molecular approach for species recognition in the cosmopolitan lichen-forming genus *Protoparmelia s. str.*, combining phylogenetic trees and coalescent-based species

delimitation methods. The phylogenetic relationships of the heterogeneous genus *Protoparmelia* have been a matter of debate. Morphological and anatomical characters of this genus show similarity to both Lecanoraceae and Parmeliaceae. *Protoparmelia* was initially placed in Lecanoraceae because it includes crustose lichens, with one-celled hyaline ascospores and *Lecanora*-type ascus [39,40]. Later, secondary metabolite profiles showing the presence of lobaric acid brought into question its placement in Lecanoraceae [41]. Studies on the ascoma ontogeny [42,43] further showed the presence of a typical character of Parmeliaceae in *Protoparmelia*, i.e. cupular exciple, a cup-shaped structure below the hymenium [44]. DNA sequence-based studies suggested *Protoparmelia* to be the sister-group to Parmeliaceae [45–47]. Tropical species of *Protoparmelia* with multispored asci were previously placed in the genus *Maronina* [48]. The authors indicated a close relationship of *Protoparmelia* and *Maronina* on the basis of similar ascus types, and suggested the former to be the multi-spore derivative of *Protoparmelia*. Subsequently, Papong et al. [49] proposed the inclusion of *Maronina* in *Protoparmelia* based on molecular data. However, the tropical clade differs from other species in *Protoparmelia* in being predominantly corticolous, having alectoronic acid as a major compound, and containing many isidiate or sorediate species, whereas most species in the traditional circumscription of *Protoparmelia* are saxicolous and occur in boreal-arctic/alpine and temperate regions.

Protoparmelia s.l. offers an interesting study system for a variety of reasons. This genus is morphologically and chemically heterogeneous [43,50], and in a previous study [47], we showed that *Protoparmelia s.l.* is polyphyletic. In addition, the relationships of most taxa to each other remain largely unexplored. Members of this genus inhabit ecologically diverse habitats, such as boreal-arctic/alpine, temperate, Mediterranean, subtropical, and tropical regions and also vary greatly in their distribution range with some species being cosmopolitan (e.g., *P. badia*, *P. memnonia*), whereas other, mainly tropical species being locally restricted (e.g., *P. orientalis*, *P. multifera*). Furthermore, congeners occur on various substrates, with some species growing on bark or decorticated wood, and others on rocks. *Protoparmelia* species exhibit varied life styles. For example, some species are lichenicolous and parasitize other lichen-forming fungi during early parts of their life cycle [50]. Sexual reproduction is common in some species (*P. badia* and *P. orientalis*), whereas others propagate mainly via asexual propagules (*P. isidiata*, *P. corallifera* and *P. capitata*) with or without any sexual reproduction.

The heterogeneity of characters makes *Protoparmelia s.l.* [51] an interesting candidate for testing species delimitation scenarios using multi-locus DNA sequence data. *Protoparmelia s. str.* [47] although being a small genus, is sister to the largest family of lichen-forming fungi, i.e., Parmeliaceae [45–47], consisting of approximately 2,800 species distributed in 80 genera [52,53]. Resolving relationships of *Protoparmelia s. str.* may contribute to understanding character evolution in an important clade of lichen-forming fungi. The aims of the current study are two-fold: 1) exploring the phylogenetic relationships of *Protoparmelia s.l.* species by constructing a multi-locus phylogeny, and 2) assessing the circumscription of lineages in *Protoparmelia s. str.* based on multi-locus species-tree inference and coalescent approaches.

Materials and Methods

Taxon sampling

This study includes a total of 160 samples of *Protoparmelia s.l.* from 18 currently described species. About 70% of the total described species were included in this study. Additionally, three unidentified species, most likely new to science, were also included in the study. We selected 73 taxa from reportedly close relatives of *Protoparmelia s.l.* [45,47], namely Parmeliaceae (40 taxa), Lecanoraceae (4 taxa), Gypsoplacaceae ([54]; 2 taxa), *Miriquidica* group (12 taxa), and

Ramboldia (10 taxa) to infer the relationship of *Protoparmelia s.l.* with other taxa within related groups within Lecanorales. Cladoniaceae (5 taxa) were selected as outgroup. Details of the study material and GenBank accession numbers are given in S1 Table.

DNA amplification and sequencing

Genomic DNA was extracted from lichen thalli using the CTAB method [55]. PCR amplification was performed using general, previously published primers for *RPB1*, *TSR1*, *MCM7*, nuLSU, mtSSU and ITS (Table 1). For some species of *Protoparmelia s.l.* and *Miriquidica* group specific primers were designed (Table 1). PCR reactions were carried out in a volume of 25 μl. Each reaction mix contained 2.5 μl buffer, 0.13 μl (0.65 U) Ex Taq polymerase, 1.0 μl dNTP mix (2.5 mM each), 1.0 μl each (10 mM) of the primer set, ca. 20 ng of template, and 16 μl H_2O. Reactions were performed with the following cycling conditions: initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 1 min, and final elongation at 72°C for 5 min. PCR products were checked for amplification on 1% agarose gels. Bands of expected size were extracted using the peqGOLD Gel Extraction Kit. All PCR products were labeled with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and cycle sequenced as follows: (1) 1 min 96°C, (2) 26 cycles of 20 s 96°C, 5 s 50°C, and (3) 2 min 60°C. Products were purified using the Big Dye XTerminator Purification Kit (Life Technologies) and then detected on ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

For each locus, consensus sequences were assembled separately and aligned using MAFFT [56] as implemented in Geneious v5.4 [57], followed by manual editing. Gaps were treated as missing data and ambiguously aligned nucleotides were excluded.

Phylogenetic analyses

Model selection. Model selection was performed to find the best-fitting model for each data set. We used the Corrected Akaike Information Criterion (AICc) [58] as implemented in jModelTest v2.1.1 [59].

Congruence among loci. To test the level of congruence among loci, we used the Congruence Among Distance Matrices test (CADM, [60]), as implemented in the package ape in R. The null hypothesis assumes that all tested phylogenetic trees are completely incongruent. Incongruence here refers to phylogenetic trees with different topologies among loci, which suggests completely distinct evolutionary histories. The level of congruence ranges from 0 to 1. In addition, maximum likelihood (ML) analyses were performed individually on each locus with RAxML-HPC BlackBox v8.1.11 [61] on the Cipres Science gateway [62] using the default GTR + G model with 1,000 bootstrap (BS) replicates. Conflicts were considered significant if individuals grouped in a clade with $\geq$ 70% BS support in one data set, but in a different clade with high support in another locus.

Phylogeny of *Protoparmelia s.l.* Since no supported conflicts were observed in single locus trees and CADM analysis rejected the hypothesis of incongruence among loci, data sets were concatenated (see Results). The maximum likelihood search was performed on the concatenated 6-locus data set including all the relatives of *Protoparmelia s.l.* with RAxML-HPC BlackBox v8.1.11 [61] on the Cipres Scientific gateway [62]. Only those taxa for which the sequence information was available for at least three loci were included in the concatenated data set. The default GTR + G model was used as the substitution model and data was partitioned according to the different genes. *RPB1*, *TSR1* and *MCM7* data were also partitioned by codon position.

Table 1. Primers used in this study.

Taxa	Locus	Primer name	Sequence	Reference
Protoparmelia	*RPB1*	fRPB1cR	CNGGCDATNTCRTTRTCCATRTA	[87]
	RPB1	gRPB1Af	GADTGTCCDGGDCATTTTGG	[88]
	RPB1	RPB1PPsp FOR	GTGCTTTGCTTCAGCAGTGCTC	[47]
	RPB1	RPB1PPsp REV	AGCGACGAACATTGCCGTTCGCAC	[47]
	RPB1	PPRPB1 FOR	GATGCGGTYTGGCGGCTTTGCAAGCC	This study
	RPB1	PPRPB1 REV	GGCTTGCAAAGCCGCCARACCGCATC	This study
	TSR1	*120040PP_*TSR1*_FOR	CAGTGTTTTGCCCAGAGAAAGGCTTTCAAG	This study
	TSR1	*120082PP_*TSR1*_FOR	TAACGTCCTTGCGAAAGAACGATTAGCGAG	This study
	MCM7	MCM7 709 (f)	ACIMGIGTITCVGAYGTHAARCC	[89]
	MCM7	MCM7-1348	GAYTTDGCIACICCIGGRTCWCCCAT	[89]
	MCM7	PPspecMCM7 FOR	GAICGDTGIGGITRIGARRTITTIC	[47]
	MCM7	PPspecMCM7 REV	GIIARRTAITCRTACATGKIRCC	[47]
	MCM7	PPMCM7FOR	CTATCGACACGAGCATCCAAG	This study
	MCM7	PPMCM7REV	CATGTGACCGRAATGCTTGTATTTC	This study
	nuLSU	LR6 (r)	LR6: CGCCAGTTCTGCTTACC	[90,91]
	nuLSU	AL1R (f)	GGGTCCGAGTTGTAATTTGT	[90,91]
	nuLSU	LR5:	TCCTGAGGGAAACTTCG	[91]
	nuLSU	L3	CCGTGTTTCAAGACGGG	[91]
	nuLSU	LROR	ACCCGCTGAACTTAAGC	[91]
	nuLSU	LSUPPspFOR2	GAAACCCCTTCGACGAGTCGAG	[47]
	nuLSU	LSUPPspREV1	AGATGGTTCGATTAGTCTTTCG	[47]
	ITS	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	[92]
	ITS	ITS2	GCTGCGTTCTTCATCGATGC	[93]
	ITS	ITS3	GCATCGATGAAGAACGCAGC	[93]
	ITS	ITS4	TCCTCCGCTTATTGATATGC	[93]
	ITS	PPITSFFOR1A	GAAGGATCATTATCGAGAGAGG	This study
	ITS	PPITSFREV1A	CTTTCAAAGCGGGAGAAATTTACTAC	This Study
	ITS	PPITSFFOR1Anested	GATCATTATCGAGAGAGGGGCTTC	This Study
	ITS	PPITSFREV1Anested	GGAGAAATTTACTACGCTTAAAG	This Study
	mtSSU	mrSSU1	AGCAGTGAGGGATATTGGTC	[94]
	mtSSU	MSU7:	GTCGAGTTACAGACTACAATCC	[95]
	mtSSU	mrSSU2	CTGACGTTGAAGGACGAAGG	[94]
	mtSSU	mrSSU2R	CCTTCGTCCTTCAACGTCAG	[94]
	mtSSU	mrSSU3R	ATGTGGCACGTCTATAGCCC	[94]
	mtSSU	MSU1	GATGATGGCTCTGATTGAAC	[95]
Miriquidica	*RPB1*	RPB1MIRI FOR	CTACAGATGATATCAAGCTCATG	[47]
	RPB1	RPB1MIRI REV	CATGAGCTTGATATCATCTGTAG	[47]
	RPB1	RPB1MIRlint FOR	CATGACGAAAATCAAGAAACTGCTG	This study
	RPB1	RPB1MIRlint REV	CATGCCGTCGCCTATCTCCTTAGTC	Thus study
	RPB1	RPB1MIRIFOR1new	TAGCACAACAATCCGGCATTCAAG	This study
	RPB1	RPB1MIRIREV1new	TCATTGCTGAGTCCCATGAGCTTG	This study
	RPB1	RPB1MIRIREV2new	GCACGAATAATGTCCCCAAGCTTG	This study
	TSR1	MIRI_TSR1_FOR	CAACGTTCTGGCTAGAGAGCGTCTGGCAAG	This study
	TSR1	*MIRI_40_82_TSR1_REV	CADAGYTGMAGHGYTTGAACCARTTSAC	This study
	TSR1	*MIRI_82_TSR1_REV	CAKAGYTGCAGMGCTTTGAACCAGTTGAC	This study
	TSR1	TSRMIRIFOR1	TGAGCTGCATCCAAAYGTWCTKGC	This study
	TSR1	TSRMIRIINTREV	TAGCGRTYGAATTTGTGGACGTTG	This study

(*Continued*)

Table 1. (*Continued*)

Taxa	Locus	Primer name	Sequence	Reference
	TSR1	TSRMIRIREV1	AACATGTAGCGRAYIGTSACGAG	This study
	TSR1	GS1_22TSR1_FOR	GAKCCCATGARCCAGAAGAWTG	This study
	TSR1	GS1_22TSR1_REV	GAAGAACATGTASCGGACSGTCAC	This study
	MCM7	MCM7MIRI FOR	CAATTTACTCCAATGACTGAATGTC	[47]
	MCM7	MCM7MIRI REV	CATGCCGTCGCCTATCTCCTTAGTC	[47]
	nuLSU	NULSUMIRIINT FOR	CTCGGACCGAGGATCGCGCTTC	This study
	nuLSU	NULSUMIRIINT REV	GAAGCGCGATCCTCGGTCCGAG	This study
	nuLSU	NULSUMIRIFOR1	CAGAGACCGATAGCGCACAAGTAGAG	This study
	nuLSU	NULSUMIRIREV1	GAGCCTCCACCAGAGTTTCCTCTG	This study
	ITS	ITSfMIRI FOR	TATCGAGTGGAGGGGCTTCGCTC	[47]
	ITS	ITSfMIRI REV	TAACGTTTAGGCGGTTGTTGGC	[47]
	ITS	ITSFMIRIFOR1	GAATTCAGTGAATCATCGAATCTTTG	This study
	ITS	ITSFMIRIREV1	AGAGTGTAATGACGCTCGAACAGG	This study
Ramboldia	*RPB1*	RPB1RAMBINTFOR	GTCTGCCATAATTGYGGCAAGATC	This study
	RPB1	RPB1RAMBINTREV	GAYATTTCCACAACCRCCATGATC	This study
	RPB1	RPB1RAMFORgroup1	GTYTGCCATAATTGCGGCAAGATC	This study
	RPB1	RPB1RAMREVgroup2	ATGTGRCGAAARATRTTKAGSGCC	This study

Taxon specific primers were designed for some *Protoparmelia*, *Ramboldia* and *Miriquidica* species.

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Bayesian inference was performed using the best fitting model as inferred by jModelTest, for the single as well as concatenated data sets as implemented in MrBayes v3.2.1 [63,64] on the Cipres Scientific gateway [62]. Two parallel MCMCMC runs were performed each using four chains and 5,000,000 generations, sampling trees every 100th generation. A 50% majority rule consensus tree was generated from the combined sampled trees of both runs after discarding the first 25% as burn-in (12,500 trees, likelihoods below stationary level).

Phylogeny of *Protoparmelia s. str.* Maximum likelihood analysis was performed individually on each locus of *Protoparmelia s. str.* (excluding Lecanoraceae, Parmeliaceae, *Miriquidica* group and *Ramboldia* clades), with RAxML-HPC BlackBox v8.1.11 [61] on the Cipres Science gateway [62], using the default GTR + G model, with 1,000 BS replicates. Gypsoplacaceae was used as outgroup. Only taxa for which sequence information was available for at least three loci were included in the concatenated data set. The default GTR + G model was used as the substitution model and the data was partitioned according to the different genes. For *RPB1*, *TSR1* and *MCM7* data were also partitioned by codon position. Since no supported conflicts were observed in single locus trees and CADM analysis rejected the hypothesis of incongruence among loci, data sets were concatenated. Maximum likelihood search was then performed on the concatenated 6-locus data set using RAxML-HPC BlackBox v8.1.11 [61] on the Cipres Scientific gateway v3.3 [62].

We performed jModelTest for each locus on the reduced data set to select the best locus-specific models of evolution.

Bayesian inference was performed using the best fitting model as suggested by jModelTest, for the single and concatenated data sets separately as implemented in MrBayes v3.2.1 [63,64] on the Cipres Scientific gateway [62]. Two parallel MCMCMC runs were performed each using four chains and 5,000,000 generations, sampling trees every 100th generation. A 50% majority rule consensus tree was generated from the combined sampled trees of both runs after discarding the first 25% as burn-in (12,500 trees).

*BEAST as implemented in BEAST v2.1 [65] was used to estimate the species tree for BP&P [66]. We used a Birth-Death process and gamma-distributed population sizes for the species tree prior and a pairwise linear population size model with a constant root. *BEAST incorporates the coalescent process and the uncertainty associated with gene trees and nucleotide substitution model parameters and estimates the species tree directly from the sequence data. For each locus, the closest model to the best-suggested model from jModelTest under the AICc criterion was selected as the best substitution model for *BEAST. Two independent Markov chain Monte Carlo (MCMC) analyses were performed for a total of 100,000,000 generations, sampling every 5,000 steps. Default values were used for the remaining priors. Convergence of the runs to the same posterior distribution and the adequacy of sampling (using the Effective Sample Size [ESS] diagnostic) were assessed with Tracer v1.4 [67]. After removing the first 20% of the samples as burn-in, all runs were combined to generate posterior probabilities of nodes from the sampled trees using TreeAnnotator v1.7.4 [68]. The species tree produced by *BEAST was subsequently used for inferring speciation probabilities by BP&P [66].

Species delimitation in *Protoparmelia s. str.*

For testing the species boundaries in *Protoparmelia s. str.* [47], currently accepted taxa were taken as putative species (12 described species). In addition well-supported (BS $\geq$ 70%, PP $\geq$ 0.94) monophyletic clades from ML and Bayesian phylogenies were taken as putative species, resulting in a 25-species scenario (Figs 1 and 2).

The marginal posterior probability of 25-species scenario suggested by molecular data was estimated using the program BP&P v3 [66]. BP&P utilizes reversible-jump Bayesian Markov chain Monte Carlo (MCMC) algorithms for analyzing phylogenetic data from multiple loci to generate speciation probabilities of assigned species. It takes into account uncertainties due to unknown gene trees and ancestral coalescent processes. This method accommodates the species phylogeny as well as incomplete lineage sorting due to ancestral polymorphism. Species tree from *BEAST was used to infer the speciation probabilities by BP&P. BP&P v3 incorporates nearest-neighbor interchange (NNI) algorithm allowing changes in the species tree topology, eliminating the need for a fixed user-specified guide tree [66]. BP&P gives the posterior probability of each delimited species and the posterior probability for the number of delimited species. A gamma prior *G* (1, 10), with mean 1/10 = 0.1 (one difference per 10 bp) was used on the population size parameters (s). The age of the root in the species tree (τ_0) was assigned the gamma prior *G* (2, 2000) which means 0.1% of sequence divergence, while the other divergence time parameters were assigned the Dirichlet prior [66]. Each analysis was run twice to confirm consistency between runs.

We also used spedeSTEM for calculating probabilities of the species scenario. SpedeSTEM [69] is based on the multilocus species-tree method STEM [33]. It assumes all putative species as separate lineages and estimates gene trees in PAUP* [70]. It then calculates the likelihood for alternative species trees in various permutations and combinations of subpopulations by collapsing two or more species into a single lineage using previously estimated gene trees. Species boundaries are then compared using Akaike information criteria and gives probabilities of different species scenarios. We used $\theta = 0.05$ and each analysis included 500 replicates. We tested all 25 possible permutations for clustering within taxonomic species.

Results

DNA sequences

We generated 716 new sequences for this phylogeny, including 142 *RPB1*, 116 *TSR1*, 84 *MCM7*, 150 nuLSU, 127 mtSSU and 107 ITS sequences. The data sets included 310 sequences

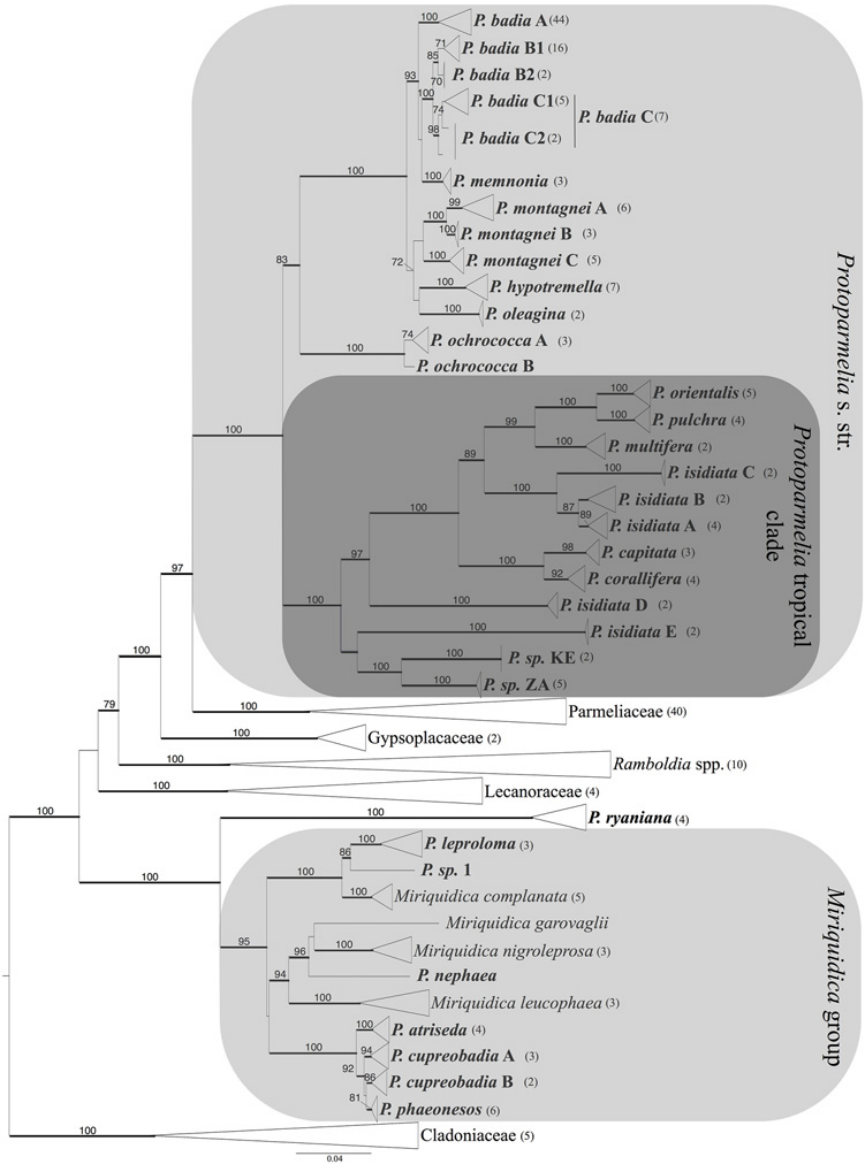

Fig 1. Phylogeny of *Protoparmelia sensu lato* and its allies based on a concatenated 6-locus data set including ITS, nuLSU, mtSSU, *MCM7*, *TSR1* and *RPB1* sequences. This is a maximum likelihood tree. Numbers above branches indicate ML BS $\geq$ 70%. Branches in bold indicate Bayesian posterior probabilities (PP) $\geq$ 0.94. Terminal clades were collapsed for clarity of presentation. The length of the triangle corresponds to branch lengths. Numbers in parentheses indicate number of specimens included in collapsed clade. Identity of each specimen in a clade is given in Supporting information S1 Table. *Protoparmelia s.l.* species are in bold.

doi:10.1371/journal.pone.0124625.g001

downloaded from NCBI. A total of 233 taxa were analyzed. The percentage of missing data for each locus was: *RPB1*- 17.17%, *TSR1*- 36.48%; *MCM7*–44.2%, nuLSU- 8.59%, mtSSU—21.45% and ITS- 26.6%.

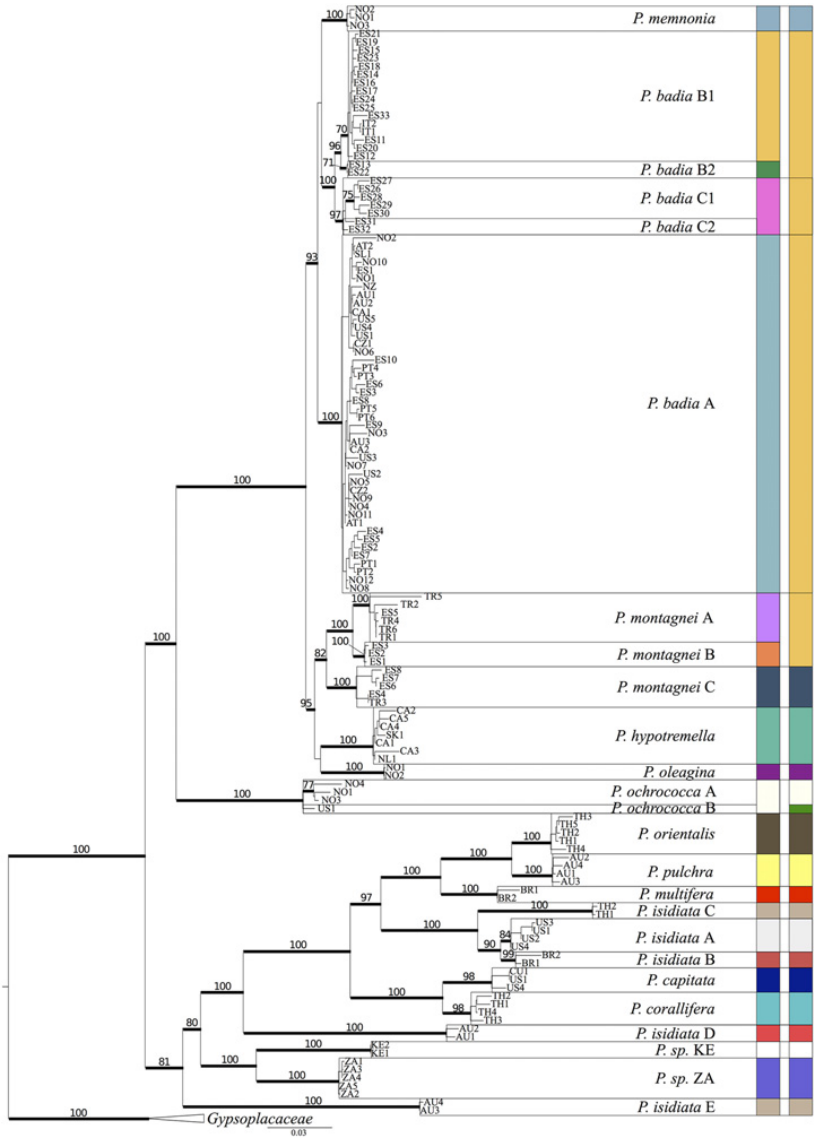

Fig 2. Phylogeny of *Protoparmelia s. str.* based on six concatenated loci. Numbers above branches indicate ML BS ≥ 70%. Branches in bold indicate Bayesian posterior probabilities (PP) ≥ 0.94. Specimen indicators include country codes (see Supporting information S1 Table). Taxon names refer to putative species supported by ML BS ≥ 70% or Bayesian Inference (PP ≥ 0.94), and tested for speciation probabilities using BP&P and spedeSTEM. Colored boxes indicate species supported by BP&P (left) and spedeSTEM (right), respectively.

doi:10.1371/journal.pone.0124625.g002

Model test

Bayesian analysis on the complete data set and the reduced *Protoparmelia s. str.* data set was performed using the best fitting model for each locus in the concatenated sequence as shown in Table 2.

Table 2. Genetic characteristics of nuclear loci used in this study.

Full data set **Locus**	**No. of seq**	**length of alignment**	**Best model**
RPB1	142	696	012232+G
TSR1	196	756	HKY+I+G
MCM7	131	655	012212+I+G+F
nuLSU	212	1064	TIM1+I+G
mtSSU	185	834	012212+I+G+F
ITS	168	807	012030+I+G
Concatenated	233	4812	NA
Protoparmelia s. str.			
Locus		**length of alignment**	**Best model**
RPB1	114	696	012232+G+F
TSR1	98	754	TPM2uf+G
MCM7	63	672	HKY+G
nuLSU	126	972	TIM1+I+G
mtSSU	93	839	: 012212+I+G+F
ITS	96	787	011230+I+G+F
Concatenated 6 loci	138	4720	NA

Genetic characteristics of nuclear loci used in this study, including the total number of sequences per locus, length of the alignment; and best model of evolution selected using the Akaike information criterion as suggested by jModelTest.

doi:10.1371/journal.pone.0124625.t002

For *BEAST, the first available best fitting model for each locus in the concatenated data set, from the models suggested by jModelTest v2.1.1 were the following: *RPB1*: GTR, *TSR1*: HKY, *MCM7*: HKY, nuLSU: GTR, mtSSU: HKY, and ITS: GTR.

Congruence among loci

CADM results showed no significant incongruence among loci, thus allowing concatenation. The null hypothesis of complete incongruence among loci was rejected for both complete ($W = 0.75$; $p<0.0001$) and reduced ($W = 0.84$; $p<0.0001$) data sets.

Phylogeny of *Protoparmelia*

Protoparmelia s.l. Nuclear and mitochondrial gene partitions supported the same overall topology. The concatenated six-locus data set contained 233 specimens. Gene partitions had the following lengths: 696 bp for *RPB1*, 756 for *TSR1*, 655 bp for *MCM7*, 1064 bp for nuLSU rDNA, 834 bp for mtSSU and 807 bp for ITS rDNA. The total length of the concatenated alignment was 4812 bp (dryad doi:10.5061/dryad.0q515). The ML tree for the concatenated data set is presented in Fig 1. Nodes with BS $\geq$ 70% and Bayesian posterior probability (PP) $\geq$0.94 were considered as supported.

The 6-locus data set yielded a resolved and well-supported topology of *Protoparmelia s.l.* (Fig 1). Members of the genus grouped either in *Protoparmelia s. str.* [47], or with representatives of the genus *Miriquidica* ("*Miriquidica*-group" in Fig 1), or as sister to the *Miriquidica*-group (*P. ryaniana*). The family Parmeliaceae *s. str.* formed a well-supported monophyletic group (BS = 100%, PP = 1; Fig 1), which was confirmed to be sister to *Protoparmelia s. str.* (BS 97% and PP = 1). Within *Protoparmelia s. str.* we found two distinct clades. One contained species with boreal-arctic/alpine, montane, temperate and Mediterranean distributions (*P. badia*, *P. memnonia*, *P. hypotremella*, *P. montagnei*, *P. oleagina*, *P. ochrococca*), the other contained

species with subtropical and tropical distributions (*P. capitata*, *P. corallifera*, *P. isidiata*, *P. multifera*, *P. orientalis*, *P. pulchra*, and two yet undescribed species from Kenya and South Africa, respectively).

Six species of *Protoparmelia* (*P. atriseda*, *P. cupreobadia*, *P. leproloma*, *P. phaeonesos*, *P. ryaniana* and *P. sp*. 1) including one yet undescribed species formed a monophyletic group together with *Miriquidica* spp.

Protoparmelia s. str. The concatenated six-locus data set contained 138 specimens, including two taxa from outgroup Gypsoplacaceae. Gene partitions had the following lengths: 696 bp for *RPB1*, 754 for *TSR1*, 672 bp for *MCM7*, 972 bp for nuLSU rDNA, 839 bp for mtSSU and 787 bp for ITS rDNA. The total length of the concatenated alignment was 4720 bp. Most species as currently circumscribed were monophyletic, except *P. isidiata*, which formed three independent lineages within the tropical clade (*P. isidiata* A-C, D and E), and the cosmopolitan species *P. badia*, which contained multiple supported lineages and formed a species complex with *P. memnonia* (Fig 2). We found evidence for cryptic species-level diversity in the nominal taxa *P. badia*, *P. montagnei*, and *P. isidiata* (clade *P. isidiata* A-E). Cryptic diversity corresponded to biogeographic patterns in *P. isidiata* (clades A-C representing North America, South America and Asia, respectively). Within *P. badia*, the largest lineage (clade *P. badia* A) was cosmopolitan, whereas the other supported lineages had a Mediterranean, or Iberian distribution (Fig 2).

Species delimitation in *Protoparmelia s. str.*

We treated terminal clades supported by $\geq$ 70% BS and $\geq$ 0.94 PP (Figs 1 and 2) as putative species for species delimitation analyses. This resulted in a 25-species scenario for *Protoparmelia s. str.*, in contrast to the current 12-species scenario for *Protoparmelia s. str.*, based on morphological and chemical characters. The 25-species scenario in *Protoparmelia s. str.* was then investigated for species delimitation using BP&P and spedeSTEM. BP&P supported the presence of 23 species with highest probability (PP = 0.41127). Posterior probability of each delimited species is given in Fig 3. *Protoparmelia ochrococca* A & B, *P. badia* C1 & C2 were not supported as separate species by BP&P. SpedeSTEM supported 19-species scenario (*P. badia* A, *P. badia* B1 & B2, *P. badia* C1 & C2, *P. montagnei* A & B collapsed as one species; Fig 3, $\theta = 0.05$, number of runs = 500), using the model that receives the highest support (100% of the model weighting; Table 3). Sixteen putative species (*P. memnonia*, *P. hypotremella*, *P. oleagina*, *P. montagnei* C, *P. orientalis*, *P. multifera*, *P. pulchra*, *P. capitata*, *P. corallifera*, *P. sp*. KE, *P. sp*. ZA and the five cryptic isidiate lineages in *P. isidiata*) were supported as separate lineages by both BP&P and spedeSTEM (Table 4), therefore we suggest these clades to be evolutionary independent. We found conflicting speciation scenarios for *P. ochrococca* A & B, *P. badia* A, B1, B2, & C and *P. montagnei* A & B by the two species delimitation approaches (Fig 2).

Discussion

The genus *Protoparmelia* is more diverse than the traditional taxonomy suggests. This diversity comprises several previously undescribed species, and cryptic lineages within currently accepted species. Most species of *Protoparmelia* belong to *Protoparmelia s. str.*, consisting of a tropical and an extra-tropical clade. The tropical clade includes several taxa having multispored asci, which were formerly classified in the genus *Maronina* [48,49,71,72]. All of its members, except the undescribed South African and Keniyan species, are corticolous. Most members of the tropical clade reproduce vegetatively, although a limited number of species propagate predominantly via sexual reproduction. All supported genetic species-level lineages in the tropical clade are congruent with biogeographic origin of the specimens. Evolutionary rates, i.e. rates of base

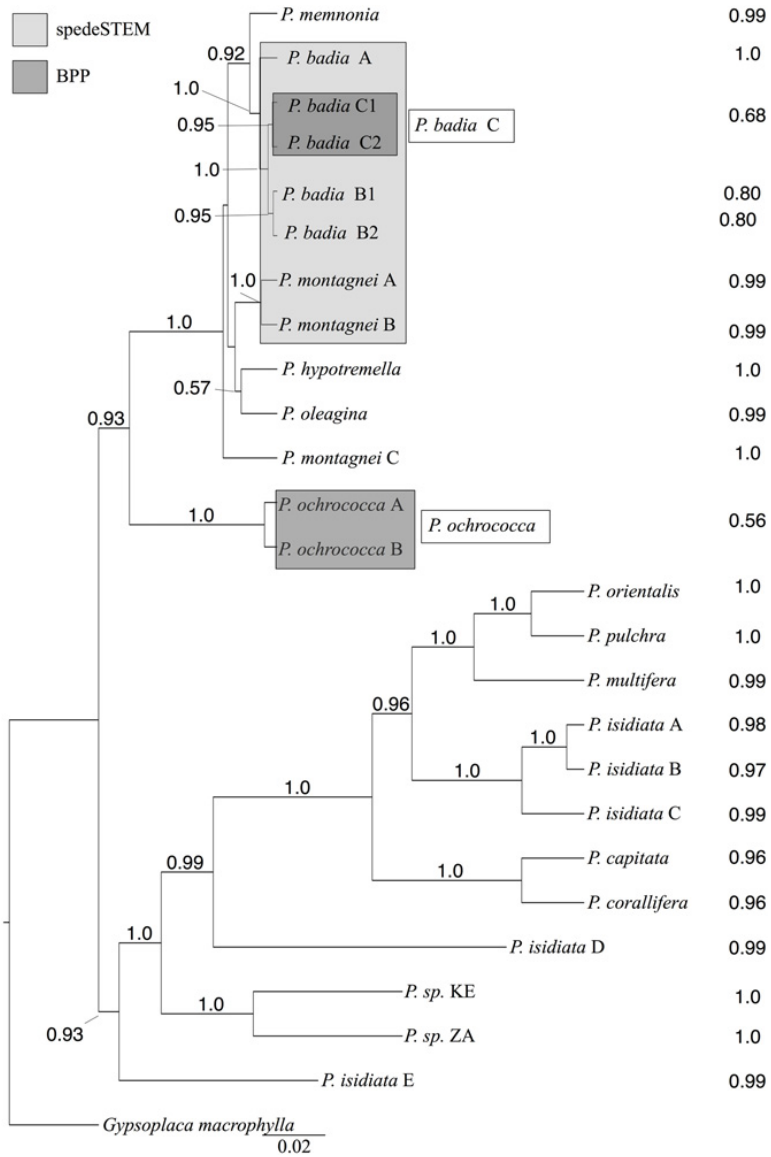

Fig 3. *BEAST species trees for *Protoparmelia s. str.* as suggested by ML (BS $\geq$ 70%) or Bayesian (PP $\geq$ 0.94). Posterior probabilities at nodes indicate support from the *BEAST analyses. The posterior probability of each delimited species calculated by BP&P are indicated in front of each putative species. Boxes in dark grey indicate clades not supported as separate taxa by BP&P. *Protoparmelia badia* B1 & B2 were supported as separate species whereas *P. badia* C1 & C2 were not supported as separate species (referred to as *P. badia* C) by BP&P. Box in light grey indicates species not supported as separate taxa by spedeSTEM.

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substitutions in an evolutionary lineage over time, appeared to be accelerated in the tropical clade. This phenomenon has also been previously observed in tropical lichens, and attributed to shorter generation times, higher metabolic rates, continuous physiological activity of a poikilohydric organism in a moist environment, and lack of sexuality [73]. The extra-tropical clade

Table 3. SpedeSTEM validation results.

Single AIC calculation					
k	**ln**	**AIC**	**delta**	**modelLik**	**wi**
1	-242465.0193	484932.0386	187997.2007	0.00	0.00
2	-230753.3543	461510.7085	164575.8707	0.00	0.00
3	-186257.4103	372520.8205	75585.98267	0.00	0.00
4	-184345.6627	368699.3255	71764.48762	0.00	0.00
5	-180035.244	360080.4881	63145.65023	0.00	0.00
6	-175225.9999	350463.9998	53529.162	0.00	0.00
7	-164198.5726	328411.1453	31476.30744	0.00	0.00
8	-160488.1749	320992.3498	24057.51199	0.00	0.00
9	-160402.6815	320823.363	23888.52519	0.00	0.00
10	-154132.577	308285.1541	11350.31624	0.00	0.00
11	-153575.6078	307173.2155	10238.37768	0.00	0.00
12	-153474.4072	306972.8143	10037.97648	0.00	0.00
13	-150731.9074	301489.8148	4554.97696	0.00	0.00
14	-149265.1449	298558.2898	1623.452	0.00	0.00
15	-149048.2275	298126.4551	1191.61724	0.00	0.00
16	-148866.247	297764.4941	829.65624	0.00	0.00
17	-148702.0018	297438.0037	503.16584	0.00	0.00
18	-148652.4701	297340.9402	406.10232	0.00	0.00
19	**-148448.4189**	**296934.8378**	**0**	**1.00**	**1.00**
20	-148536.6081	297113.2162	178.37836	0.00	0.00
21	-148526.4393	297094.8785	160.04068	0.00	0.00
22	-148522.9071	297089.8141	154.97628	0.00	0.00
23	-148515.6023	297077.2046	142.36672	0.00	0.00
24	-148515.525	297079.05	144.21212	0.00	0.00
25	-148513.4861	297076.9721	142.13428	0.00	0.00
Multiple AIC calculation					
k	**ln**	**AIC**	**delta**	**modelLik**	**wi**
1	-242465.0193	484932.0386	187997.2007	0.00	0.00
2	-230753.3543	461510.7085	164575.8707	0.00	0.00
3	-186257.4103	372520.8205	75585.98267	0.00	0.00
4	-184345.6627	368699.3255	71764.48762	0.00	0.00
5	-180035.244	360080.4881	63145.65023	0.00	0.00
6	-175225.9999	350463.9998	53529.162	0.00	0.00
7	-164198.5726	328411.1453	31476.30744	0.00	0.00
8	-160488.1749	320992.3498	24057.51199	0.00	0.00
9	-160402.6815	320823.363	23888.52519	0.00	0.00
10	-154132.577	308285.1541	11350.31624	0.00	0.00
11	-153575.6078	307173.2155	10238.37768	0.00	0.00
12	-153474.4072	306972.8143	10037.97648	0.00	0.00
13	-150731.9074	301489.8148	4554.97696	0.00	0.00
14	-149265.1449	298558.2898	1623.452	0.00	0.00
15	-149048.2275	298126.4551	1191.61724	0.00	0.00
16	-148866.247	297764.4941	829.65624	0.00	0.00
17	-148702.0018	297438.0037	503.16584	0.00	0.00
18	-148652.4701	297340.9402	406.10232	0.00	0.00
19	**-148448.4189**	**296934.8378**	**0**	**1.00**	**1.00**

(*Continued*)

Table 3. (*Continued*)

Single AIC calculation					
k	**ln**	**AIC**	**delta**	**modelLik**	**wi**
20	-148536.6081	297113.2162	178.37836	0.00	0.00
21	-148526.4393	297094.8785	160.04068	0.00	0.00
22	-148522.9071	297089.8141	154.97628	0.00	0.00
23	-148515.6023	297077.2046	142.36672	0.00	0.00
24	-148515.525	297079.05	144.21212	0.00	0.00
25	-148513.4861	297076.9721	142.13428	0.00	0.00

spedeSTEM validation results, using θ = 0.5. The absolute difference between the AICc score for the given model and the best-fitting one is listed under the column labeled "Di" and the model weighting is listed under the column labeled "wi".

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Table 4. Summary of results of ML, Bayesian and species delimitation analyses (BP&P and spedeSTEM).

Putative species	BS	BS1	PP	PP1	BP&P	spedeSTEM
Protoparmelia badia A	100		1		1.0	-
Protoparmelia badia B1	71	85	0.61	1	0.80	-
Protoparmelia badia B2	70		—		0.80	-
Protoparmelia badia C1	74	98	0.97	1	0.68	-
Protoparmelia badia C2	98		—			-
Protoparmelia capitata	98		1		0.96	+
Protoparmelia corallifera	92		1		0.96	+
Protoparmelia hypotremella	100		1		1.0	+
Protoparmelia isidiata A	89		0.96		0.98	+
Protoparmelia isidiata B	64		1		0.97	+
Protoparmelia isidiata C	100		1		0.99	+
Protoparmelia isidiata D	100		1		0.99	+
Protoparmelia isidiata E	100		1		0.99	+
Protoparmelia memnonia	100		1		0.99	+
Protoparmelia montagnei A	99		1		0.99	-
Protoparmelia montagnei B	100		1		1.0	-
Protoparmelia montagnei C	100		1		1.0	+
Protoparmelia multifera	100		1		1.0	+
Protoparmelia ochrococca A	74		0.82	1	0.56	+
Protoparmelia ochrococca B	NA		NA			+
Protoparmelia oleagina	100		1		0.99	+
Protoparmelia orientalis	100		1		1.0	+
Protoparmelia pulchra	100		1		1.0	+
Protoparmelia sp. KE	100		1		1.0	+
Protoparmelia sp. ZA	100		1		1.0	+

Clades in Column A represent putative species having ML BS support $\geq$ 70% or Bayesian PP $\geq$ 0.94, tested for speciation probabilities using BP&P and spedeSTEM. + represents supported clades;—represents clades not supported. Clades supported by BP&P were considered as separate species.
[1] represents support for 22-species scenario (*P*. *badia* B1, B2 and *P*. *badia* C1, C2, *P*. *ochrococca* A, B collapsed), i.e. three instead of five putative species within *Protoparmelia badia*.

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contains mostly saxicolous taxa, most of which reproduce sexually. Within this group, while some species show restricted distribution, some other have wide geographic distributions, such as the cosmopolitan *P.* '*badia* A' and *P. hypotremella* which occurs in Europe and North America. Five previously described species and one species putatively new to science group with members of the genus *Miriquidica*. In contrast to members of *Protoparmelia s. str*., which produce lobaric or alectoronic acids, these taxa synthesize norstictic acid as major secondary metabolite. Many of these species parasitize other lichens during at least parts of their life cycle [50], a lifestyle not known from members of *Protoparmelia s. str*. Close affiliations between *Miriquidica* and *Protoparmelia* based on shared morphological characteristics have been suggested before [74,75], and a recent molecular study confirmed the close relationship of the *P. atriseda*-group and *Miriquidica* [47]. A revision of the genus *Miriquidica* based on molecular data is currently under way by our colleagues (Timdal, pers. comm.).

Speciation analyses and cryptic diversity

We validated the 25-species scenario for *Protoparmelia s. str*., which was based on the previously defined species and a few new clades suggested by molecular data (phylogenetic species concept). Based on our sampling, this study largely supported traditionally circumscribed *Protoparmelia s. str*. species as distinct lineages. However, exceptions included *P. isidiata*, an asexual tropical species, and *P. badia*, a sexually reproducing, boreal-arctic/alpine cosmopolitan species. The former was found to be polyphyletic and separated into three distinct lineages, while the later was paraphyletic and formed a species complex with *P. memnonia* (Figs 1 and 2).

The combined use of species-tree topology and coalescent methods revealed the presence of several cryptic lineages in *Protoparmelia s. str*. This is in concordance with other studies in which molecular markers in combination with statistical tools revealed many genetically distinct lineages hidden under a single taxon [9,36,76–78]. Studies suggest that cosmopolitan species such as *P. badia* may reveal high cryptic diversity [79,80], which may or may not correlate to geography. In our study we found that the cosmopolitan *P. badia* as currently delimited consists of at least four independent evolutionary lineages. Among these newly recognized lineages only *P. badia* A turns out to be cosmopolitan, inhabiting boreal-arctic/alpine habitats in North America, Europe, New Zealand and Australia. The other lineages of *P. badia* (*P. badia* B1, B2 and C) have a more limited distribution, having been collected so far on siliceous substrates in Spain and Italy. Cryptic lineages within *P. isidiata* (clades A-C) also correspond to broad biogeographic patterns, while lineages identified within *P. montagnei* co-occur in the Mediterranean region (Fig 2). Thus, geographic evidence supports species delimitation suggested by coalescent-based speciation analyses in most cases. However, current sampling in many lineages is relatively sparse and does not allow conclusions about finer-scale biogeographic patterns, such as endemism. It remains to be seen whether sympatrically-occurring cryptic lineages identified in this study are supported by additional, previously overlooked morphological or chemical characteristics. We have preliminary evidence that the currently recognized *P. montagnei* chemotypes [81] correspond to the three molecular clades and may thus indeed represent closely related, but separate species.

Conflicts between different methodological approaches to species delimitation are common [13,15,78,82]. In general we follow the approach of adopting the speciation scenario that is supported by both the analyses, in our case 16 species [83]. For some clades, i.e. *P. ochrococca* A & B, *P. badia* A, B1, B2 & C, *P. montagnei* A & B, the most likely speciation scenario given by spedeSTEM deviates from BP&P, and contradicts supported branching patterns in the phylogeny (Figs 2 and 3). For *P. badia* A, B1, B2 & C, *P. montagnei* A & B phylogenetic tree and BP&P supported these clades to be evolutionary independent, whereas spedeSTEM suggested them to be a single species. For *P. ochrococca* A & B phylogenetic tree and spedeSTEM supported these clades to be

evolutionary independent, whereas BP&P suggested them to be a single species. Recent studies indicated that spedeSTEM may be less accurate than other species delimitation methods in cases of recent speciation events [84]. For the clades supported by BP&P and not spedeSTEM, we preferred BP&P results as BP&P has been shown to perform well even when putative species were modeled to have diverged from one another only very recently [84]. In addition, BP&P has been shown to outperform other coalescent-based species delimitation approaches especially when using multi-locus DNA sequence data and a modest number of individuals per species [69,83]. Previously the reliability of BP&P has been suggested to be dependent on the accuracy of the user-provided guide tree. However, in the latest version of BP&P the authors addressed this issue and applied the NNI algorithm, which allows flexibility in the species tree. Moreover BP&P is suggested to be conservative in delimiting species, with high probability to be a reliable indicator of evolutionary independence of the lineages [66]. Therefore in case of conflicts we considered BP&P to be more accurate and suggested the lineages supported by BP&P as distinct species.

Our analyses suggest that the sampled specimens of the tropical *Protoparmelia s. str*. group belong to five distinct species. Two sexually reproducing (apotheciate) species, *P. multifera* and *P. orientalis*, traditionally distinguished by having different minor secondary metabolites [49] were supported as different species and were not sister to each other. In fact, the sexually reproducing species *P. pulchra* was sister to *P. orientalis*. In addition, we found four distinct asexually reproducing (isidiate) species of *Protoparmelia s. str*. Two of these species (*P.* '*isidiata* D' and *P.* '*isidiata* E') occur sympatrically in Australia. Several studies have shown the occurrence of phylogenetically unrelated but morphologically similar lineages thus indicating the presence of high hidden diversity in lichen-forming fungi [25,27,34,85,86].

Conclusions

Our analyses support the presence of 23 distinct lineages in *Protoparmelia s. str*. in contrast to 12 currently delimited species, revealing much more diversity than currently suggested for this genus. Our study shows that the sister group of the largest family of lichen-forming fungi may harbor a considerable amount of cryptic lineages which can be identified using molecular data. These data highlight the presence of substantial phylogenetic diversity especially in the tropics, and the need for careful re-evaluation of morphological and chemical characters in the group.

Supporting Information

S1 Table. Specimens used in this study including voucher information and GenBank accession numbers.
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: GS IS HTL. Performed the experiments: JO GS KS. Analyzed the data: GS FDG SL. Contributed reagents/materials/analysis tools: AA AC MC VJR PKD KS. Wrote the paper: GS FDG IS HTL.

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Supporting Information

S1 Table. Specimens used in this study including voucher information and GenBank accession numbers.

	Samples	Voucher info	Sample code	MTSSU	ITS	nuLSU	RPB1	MCM7	TSR1
1	Alectoria ochroleuca	Austria: Styria, Wedin Aug. 1998 (UPS)	-	DQ899289	DQ979997	DQ899288	n/a	n/a	n/a
	Alectoria ochroleuca	Sweden: Härjedalen, Wedin 6542 (UPS)	-	n/a	n/a	n/a	DQ923677	KF562163	KP888161
2	Austroparmelina pruinata	Australia: Western Australia: E. McCrum s.n. (MAF-Lich 14270)	-	EF025481	EF042905	EF042914	GU994680	JX974675	n/a
3	Brodoa intestiniformis	Sweden: Härjedalen, Wedin 6329 (UPS)	-	DQ923624	DQ980002	DQ923653	DQ923681	KP938770	KP888171
4	Cetraria islandica	Sweden: Västerbotten, Wedin 15/05/2005 (UPS)	-	AY340486	AF117995	AY340539	DQ923685	JX974677	KP888192
5	Cetraria nigricans	Canada: Nunavut, Westberg 2377 (LD)	-	JN000236	AF254629	JN000257	JN000287	KF562164	KP888193
6	Hypotrachyna kaernefeltii	-	-	GQ919217	GQ919269	GQ919245	GU994690	GQ272429	GQ272471
7	Hypotrachyna dubitans	Peru: Ancash, Lumbsch et al. 19366 (MAF-Lich 15621)	-	GQ919218	GQ919270	GQ919246	GU994691	GQ272427	GQ919246
8	Cetrariella commixta	Finland: Southern Finland, Haikonen 19093 (H)	-	JN000237	AF451796	JN000260	JN000290	KP938771	n/a
9	Cetrariella delisei	Sweden: Västerbotten, Wedin 6351 (UPS)	-	DQ923628	DQ980005	DQ923657	n/a	JX974679	KP888195
	Cetrariella delisei	Sweden: Jamtland, Wedin 8465 (S)	-	n/a	n/a	n/a	KF601228	n/a	n/a
10	Cladia aggregata	Australia: Tasmania, HTL19994c (F)	-	GQ500940	GQ500917	GQ500966	n/a	HM441287	KP888198
11	Cladia dumicola	Australia: Tasmania, HTL19993g (F)	-	GQ500933	GQ500915	GQ500968	n/a	HM441281	KP888199
12	Cladia schizopora	Australia: Tasmania, HTL 19974c (F)	-	GQ500942	GQ500919	GQ500952	KF601229	HM441290	KP888200
13	Cladonia rangiferina	Sweden: Jämtland, Wedin 6935 (UPS)	-	AY300881	AF458306	AY300832	DQ915595	n/a	n/a
14	Emodomelanelia masonii	India: Uttaranchal, Divakar s.n. (MAF-Lich 15515, 17602)	-	GU994640	GU994549	GU994595	GU994695	JX974681	KP888208
15	Hypotrachyna nepalensis	India: Uttaranchal, Divakar (GPGC 02-000924)	-	AY611129	AY611071	AY607783	EF092106	n/a	AY607783
16	Everniopsis trulla	Peru: Ancash, Lumbsch et al. 19308c (F)	-	EF108289	EF105411	EF108290	EF105429	GQ272396	GQ272438
17	Flavocetraria nivalis	Sweden: Jämtland, Wedin 5052 (BM)	-	DQ923635	DQ980011	DQ923663	n/a	n/a	n/a
	Flavocetraria nivalis	Sweden: Västerbotten, Wedin 15/9 2003 (UPS)	-	n/a	n/a	n/a	DQ923688	JX974683	n/a
18	Flavoparmelia marchantii	Australia: Western Australia: Elix s.n. (MAF-Lich 10492)	-	GU994642	DQ299905	GU994598	GU994698	GQ272420	GQ272463
19	Flavoparmelia soredians	-	-	AY586586	AY586562	AY584835	EF092108	JX974684	n/a
	Flavoparmelia soredians	-	-	n/a	n/a	n/a	n/a	n/a	KP888217
20	Gowardia nigricans	Sweden: Dalarna, Lundqvist 8377 (UPS)	-	DQ923620	DQ979996	DQ923649	n/a	n/a	n/a
	Gowardia nigricans	Norway: Troms, Wedin 7297 (UPS)	-	n/a	n/a	n/a	DQ923676	KF562165	KP888160
21	Gypsoplaca macrophylla	Russia, Zhurbenko 92104 (UPS)	-	DQ899299	n/a	DQ899298	n/a	n/a	KP888220
	Gypsoplaca macrophylla	USA: Utah, Rosentreter 15995 (F)	-	n/a	KF650781	n/a	KF601230	n/a	n/a
22	Gypsoplaca sp.	USA: Alaska, Spribille 38752	-	**KP822511**	n/a	**KP796393**	**KP822193**	n/a	**KP823563**
23	Hypogymnia physodes	Sweden, Mattsson 4005 (UPS)	-	AY756400	AF058036	AY756338	n/a	n/a	n/a
	Hypogymnia physodes	Sweden: Jämtland, Wedin 6623 (UPS)	-	n/a	n/a	n/a	AY756407	KF562167	KP888222
24	Hypogymnia vittata	Sweden: Jämtland, Wedin 15/7/2000 (UPS)	-	DQ900629	DQ980012	DQ900637	n/a	n/a	n/a
	Hypogymnia vittata	Sweden: Västerbotten, Wedin 6814 (UPS)	-	n/a	n/a	n/a	DQ923689	KF562166	KP888223
25	Lecanora carpinea	Austria, Arup L97007 (LD)	-	DQ787364	AY541248	DQ787363	n/a	n/a	n/a
	Lecanora carpinea	Turkey: Zonguldak, Lumbsch 19611m (F)	-	n/a	n/a	n/a	n/a	GQ272400	GQ272443
26	Lecanora hybocarpa	Spain: Guadaljara, Lumbsch s.n. (F)	-	EF105417	EF105412	EF105421	EF105430	n/a	n/a
27	Lecanora paramerae	Spain: Guadaljara, Lumbsch s.n. (F)	-	EF105418	EF105413	EF105422	EF105431	n/a	n/a
28	Lecanora sulphurea	Spain: Guadaljara, Lumbsch s.n. (F)	-	EF105419	AF070030	EF105423	EF105432	n/a	n/a
29	Melanelia hepatizon	Sweden: Västerbotten, Wedin 6812 (UPS)	-	n/a	DQ980016	DQ923667	DQ923692	n/a	n/a
	Melanelia hepatizon	Sweden: Västerbotten, Wedin 6821 (UPS)	-	DQ923639	n/a	n/a	n/a	JX974678	KP888241
30	Melanelixia fuliginosa	Spain: La Rioja Blanco s.n. (MAF-Lich 10223), Crespo et al. s.n. (MAF-Lich 10219)	-	AY611146	AY611089	AY607801	EF092116	JX974686	KP888244
31	Melanelixia subaurifera	UK: England, Crespo s.n. (MAF-Lich 10215)	-	AY611156	AY611095	AY607811	EF092120	JX126390	n/a
32	Melanohalea elegantula	Spain: Madrid, Crespo s.n. (MAF-Lich 10231)	-	n/a	AY611094	AY607806	KF601231	n/a	**KP823570**
	Melanohalea elegantula	USA: California, Esslinger 18874 (F)	-	JQ813114	n/a	n/a	n/a	n/a	n/a
33	Melanohalea exasperata	Spain: Guadalajara, MAF 10214	-	AY611138	AY611081	AY607793	EF092123	n/a	**KP823571**
34	Menegazzia terebrata	Sweden: Gästrikland, Wedin 4392 (UPS)	-	DQ899305	DQ980019	DQ899304	DQ923694	KF562168	n/a
	Menegazzia terebrata	Norway: Oppland, L-51266 (TROM)	-	n/a	n/a	n/a	n/a	n/a	**KP823572**
35	*Metus conglomeratus*	Australi:, Tasmania, *Lumbsch 19982b* (F)	-	GQ500948	GQ500912	GQ500958	KF601232	HM441294	n/a
36	*Miriquidica complanata*	Poland: Karkonosze Mts, *Szczepańska 935* (herb. Szczepańska)	-	**KP822512**	KF562187	KF562179	KF601233	KF562169	n/a
37	*Miriquidica complanata*	Poland: Sudety Mts, K. Szczepańska 43 (herb. Szczepańska)		**KP940385**	n/a	**KP940386**	**KP940384**	n/a	n/a
38	*Miriquidica complanata*	Poland: Sudety Mts, M. Kossowska 520 (herb. Kossowska)	-	**KP822513**	n/a	**KP796394**	**KP822194**	**KP822386**	n/a
39	*Miriquidica garovaglii*	Slovakia: Karpaty Mts, *Szczepańska 538* (herb. Szczepańska)	-	n/a	KF562188	KF562180	KF601234	n/a	n/a
40	*Miriquidica garovaglii*	Poland: Sudety Mts, M. Kossowska 221 (herb. Kossowska)	-	**KP822514**	n/a	**KP796395**	n/a	**KP822387**	n/a
41	*Miriquidica leucophaea*	Poland: Karkonosze Mts, *Kossowska 448* (herb. Kossowska)	-	n/a	KF562188	KF562181	KF601235	KF562170	n/a
42	*Miriquidica leucophaea*	Poland: Sudety Mts, M. Kossowska 1339 (herb. Kossowska)	-	**KP822515**	**KP822310**	**KP796396**	**KP822195**	**KP822388**	**KP823564**
43	*Miriquidica leucophaea*	Poland: Sudety Mts, M. Kossowska 1354 (herb. Kossowska)	-	**KP822516**	**KP822311**	**KP796397**	**KP822196**	**KP822389**	**KP823565**
44	*Miriquidica leucophaea*	Poland: Sudety Mts, M. Kossowska 182 (herb. Kossowska)	-	**KP822517**	n/a	**KP796398**	**KP822197**	**KP822390**	**KP823566**

45	*Miriquidica nigroleprosa*	Poland: Sudety Mts, M. Kossowska 128 (herb. Kossowska)	-	**KP822518**	**KP822312**	**KP796399**	**KP822198**	**KP822391**	**KP823567**
46	*Miriquidica nigroleprosa*	Poland: Sudety Mts, M. Kossowska 154 (herb. Kossowska)	-	**KP822519**	**KP822313**	**KP796400**	**KP822199**	**KP822392**	n/a
47	*Miriquidica nigroleprosa*	Poland: West Sudety Mts, M. Kossowska 158 (hb. Kossowska)	-	**KP822520**	n/a	**KP796401**	**KP822200**	**KP822393**	n/a
48	*Montanelia disjuncta*	Sweden: Lycksele Lappmark, *Wedin 7143* (UPS)	-	DQ923638	DQ980015	DQ923666	DQ923691	JX974699	KP888258
49	*Montanelia sorediata*	India: Uttaranchal, Divakar s.n. (MAF-Lich 15512)	-	GU994645	GU994556	GU994604	GU994706	JX974704	KP888259
50	*Myelochroa irrugans*	China: Yunnan Crespo & al. s.n. (MAF-Lich 10207)	-	AY611160	AY611103	AY607815	EF092128	JX974708	n/a
51	*Nephromopsis leucostigma*	Bhutan: Thimpu District, *Søchting 9151* (LD)	-	JN000239	AF451777	JN000267	JN000295	KF562172	KP888261
52	*Parmelia serrana 2*	Spain: Madrid, Crespo & Divakar s.n. (MAF-Lich 9756)	-	AY582319	AY295109	AY578948	EF092133	JX974710	n/a
53	*Parmelia saxatilis*	Sweden: Västerbotten, Wedin 7091 (UPS)	-	AF351172	AF058037	AY300849	DQ923695	JX974709	KP888268
	Parmelina quercina	Spain: Madrid, MAF 6057	-	n/a	n/a	AY607818	EF092136	n/a	n/a
54	*Parmelina quercina*	Spain: San Quintín, Crespo et al. s.n. (MAF-Lich 13947)	-	DQ268562	n/a	n/a	n/a	n/a	KP888270
55	*Parmotrema reticulatum*	-	-	AY586599	AY586577	AY584848	GU994729	JX974712	n/a
56	Parmeliopsis hyperopta	Spain: Madrid, Blanco s.n. (MAF-Lich 10181)	-	AY611167	AY611109	AY607823	EF092142	GQ272426	GQ272468
57	*Protoparmelia atriseda*	USA: Washington, *McCune 28625* (GZU)	US1	n/a	**KP822207**	**KP796256**	**KP822066**	**KP822314**	**KP823457**
58	*Protoparmelia atriseda*	USA: Washington, McCune, Ponzetti 26046 (OSU)	US2	**KP822398**	KF562190	KF562182	KF601236	KF562173	**KP823458**
59	*Protoparmelia atriseda*	Czech Rebublic: West Bohemia, Palice 15024 (ASCR)	CZ1	**KP822399**	n/a	**KP796257**	n/a	n/a	**KP823459**
60	*Protoparmelia atriseda*	United Kingdom, Scotland, *Fryday 0108412* (MSC)	UK1	**KP822400**	**KP822208**	**KP796258**	**KP822067**	**KP822315**	n/a
61	*Protoparmelia badia A*	Austria, Hafellner, Muggia, Hafellner 68478 (GZU)	AT1	**KP822401**	KF562191	KF562183	KF601237	KF562174	n/a
62	*Protoparmelia badia A*	Slovenia, Central Alp, Kobansko, *Hafellner, 71474* (GZU)	SI1	**KP822402**	**KP822209**	**KP796259**	**KP822068**	**KP822316**	**KP823460**
63	*Protoparmelia badia A*	Austria: Steiermark, Steirisches Randgebirge,, Hafellner, 71686 (GZU)	AT2	n/a	**KP822210**	**KP796260**	**KP822069**	n/a	**KP823461**
64	*Protoparmelia badia A*	USA: Oregon, Linn County, McCune 27712 (OSU)	US1	n/a	**KP822211**	**KP796261**	**KP822070**	**KP822317**	**KP823462**
65	*Protoparmelia badia A*	Czech Rebublic: West Bohemia, Povydří, Palice 15024 (ASCR)	CZ1	**KP822404**	n/a	**KP796262**	n/a	n/a	**KP823463**
66	*Protoparmelia badia A*	Czech Rebublic: North Bohemia, Velký Kotel corrie, Malíček, Palice, Printzen, Steinová, Syrovátková 12051 (ASCR)	CZ2	**KP822405**	**KP822212**	n/a	**KP822071**	**KP822318**	**KP823464**
67	*Protoparmelia badia A*	USA: Maine, Piscataquis County, Fryday 8579, MSC0108416 (MSC)	US2	**KP822403**	n/a	**KP796263**	n/a	**KP822319**	n/a
68	*Protoparmelia badia A*	Norway: Sør-Trøndelag, Ørland, Haugan 9779, O-L168485 (O)	NO1	n/a	n/a	**KP796264**	n/a	**KP822320**	**KP823465**
69	*Protoparmelia badia A*	Norway: Oppland, Vågå, Haugan 8120, O-L160502 (O)	NO2	n/a	n/a	**KP796265**	n/a	**KP822321**	**KP823466**
70	*Protoparmelia badia A*	Norway: Østfold, Sarpsborg Løfall, Petter bpl-L7043, O-L77778 (O)	NO3	**KP822406**	**KP822213**	**KP796266**	**KP822072**	n/a	**KP823467**
71	*Protoparmelia badia A*	Australia: Betts Creek, Elix 43267, 00803551 (CANB)	AU3	n/a	**KP822214**	n/a	n/a	n/a	**KP823468**
72	*Protoparmelia badia A*	Australia: Tasmania, Kantvilas 53/09, 550225 (HO)	AU1	**KP822407**	**KP822215**	n/a	n/a	n/a	**KP823469**
73	*Protoparmelia badia A*	Australia: Tasmania, Kantvilas 7/06, 562231 (HO)	AU2	**KP822408**	**KP822216**	n/a	n/a	n/a	**KP823470**
74	*Protoparmelia badia A*	Norway: Finnmark, Nesseby, Holien 12730, L-13936 (TRH)	NO4	**KP822409**	**KP822217**	**KP796267**	**KP822073**	**KP822322**	**KP823471**
75	*Protoparmelia badia A*	Norway: Nord-Trøndelag, Namsskogan, Holien 11762, L-12476 (TRH)	NO5	**KP822410**	**KP822218**	**KP796268**	**KP822074**	**KP822323**	**KP823472**
76	*Protoparmelia badia A*	Norway: Finnmark, Vadsø, Bratli 7953, L-175593 (O)	NO6	**KP822411**	**KP822219**	**KP796269**	**KP822075**	**KP822324**	**KP823473**
77	*Protoparmelia badia A*	Norway: Finnmark, Vadsø, Bratli 7966, L-175606 (O)	NO7	**KP822412**	**KP822220**	**KP796270**	**KP822076**	**KP822325**	n/a
78	*Protoparmelia badia A*	Norway: Finnmark, Vadsø, Bratli 7959, L-175599 (O)	NO8	n/a	**KP822221**	**KP796271**	**KP822077**	n/a	n/a
79	*Protoparmelia badia A*	Norway: Nordland, Grane, Tønsberg 41335, L-92560 (BG)	NO9	**KP822413**	n/a	**KP796272**	**KP822078**	n/a	n/a
80	*Protoparmelia badia A*	Norway: Sogn og Fjordane, Vik, Tønsberg 38409, L-85832 (BG)	NO10	**KP822414**	**KP822222**	**KP796273**	**KP822079**	n/a	**KP823474**
81	*Protoparmelia badia A*	Norway: Finnmark, Loppa, Tønsberg 38628, L-92432 (BG)	NO11	**KP822415**	n/a	**KP796274**	**KP822080**	n/a	n/a
82	*Protoparmelia badia A*	Norway: Nordland, Grane, Tønsberg 41001, L-92501 (BG)	NO12	n/a	**KP822223**	**KP796275**	**KP822081**	n/a	n/a
83	*Protoparmelia badia A*	USA: Alaska, Fairbanks, Spribille 27680 (GZU)	US3	n/a	**KP822224**	**KP796276**	**KP822082**	n/a	n/a
84	*Protoparmelia badia A*	USA: Montana, Sanders County, Spribille 20996 (GZU)	US4	n/a	n/a	**KP796277**	**KP822083**	n/a	**KP823475**

85	*Protoparmelia badia A*	USA: Montana, Lincoln County, Spribille 21119 (GZU)	US5	n/a	**KP822225**	**KP796278**	**KP822084**	n/a	n/a
86	*Protoparmelia badia A*	Canada: British Columbia, Spribille *29693* (GZU)	CA1	n/a	**KP822226**	**KP796279**	n/a	n/a	n/a
87	*Protoparmelia badia A*	Canada: Yukon Territory, *Spribille 28408* (GZU)	CA2	n/a	**KP822227**	**KP796280**	n/a	n/a	n/a
88	*Protoparmelia badia A*	Spain: La Rioja, Crespo, Del-Prado 10524 (MAF)	ES1	**KP822416**	n/a	**KP796281**	**KP822085**	**KP822326**	n/a
89	*Protoparmelia badia A*	Portugal: Beira Alta, Distrito de Guarda, MAF-Lich 19441	PT1	n/a	**KP822228**	**KP796282**	**KP822086**	**KP822327**	n/a
90	*Protoparmelia badia A*	Portugal: Beira Alta, Distrito de Guarda, MAF-Lich 19442	PT2	**KP822417**	**KP822229**	**KP796283**	**KP822087**	n/a	**KP823476**
91	*Protoparmelia badia A*	Portugal: Beira Alta, Distrito de Guarda, MAF-Lich 19443	PT3	n/a	**KP822230**	**KP796284**	n/a	**KP822328**	n/a
92	*Protoparmelia badia A*	Portugal: Beira Alta, Distrito de Guarda, MAF-Lich 19444	PT4	**KP822418**	**KP822231**	**KP796285**	**KP822088**	**KP822329**	**KP823477**
93	*Protoparmelia badia A*	Portugal: Beira Alta, Distrito de Guarda, MAF-Lich 19445	PT5	n/a	**KP822232**	**KP796286**	**KP822089**	**KP822330**	**KP823478**
94	*Protoparmelia badia A*	Portugal: Beira Alta, Distrito de Guarda, MAF-Lich 19446	PT6	n/a	**KP822233**	**KP796287**	**KP822090**	**KP822331**	**KP823479**
95	*Protoparmelia badia A*	Spain: Segovia, La Granja de San Ildefonso, MAF-Lich 19449 (MAF)	ES2	**KP822419**	**KP822234**	**KP796288**	**KP822091**	**KP822332**	n/a
96	*Protoparmelia badia A*	Spain: Segovia, La Granja de San Ildefonso, MAF-Lich 19450 (MAF)	ES3	**KP822420**	**KP822235**	**KP796289**	**KP822092**	**KP822333**	**KP823480**
97	*Protoparmelia badia A*	Spain: Segovia, La Granja de San Ildefonso, MAF-Lich 19451 (MAF)	ES4	**KP822421**	**KP822236**	**KP796290**	**KP822093**	n/a	n/a
98	*Protoparmelia badia A*	Spain: Segovia, La Granja de San Ildefonso, MAF-Lich 19452 (MAF)	ES5	**KP822422**	**KP822237**	**KP796291**	**KP822094**	n/a	n/a
99	*Protoparmelia badia A*	Spain: Segovia, La Granja de San Ildefonso, MAF-Lich 19453 (MAF)	ES6	**KP822423**	n/a	**KP796292**	**KP822095**	**KP822334**	**KP823481**
100	*Protoparmelia badia A*	Spain: Segovia, La Granja de San Ildefonso, MAF-Lich 19454 (MAF)	ES7	**KP822424**	**KP822238**	**KP796293**	**KP822096**	n/a	n/a
101	*Protoparmelia badia A*	Spain: La rioja, Ezcaray, MAF-Lich 19455 (MAF)	ES8	**KP822425**	n/a	**KP796294**	**KP822097**	**KP822335**	**KP823482**
102	*Protoparmelia badia A*	Spain: La rioja, Ezcaray, MAF-Lich 19456 (MAF)	ES9	**KP822426**	**KP822239**	**KP796295**	**KP822098**	n/a	**KP823483**
103	*Protoparmelia badia A*	Spain: La rioja, Ezcaray, MAF-Lich 19457 (MAF)	ES10	n/a	**KP822240**	**KP796296**	**KP822099**	**KP822336**	**KP823484**
104	*Protoparmelia badia A*	New Zealand: South Island, Otago region, Central Otago Disttrict, Printzen FR-0217382 (FR)	NZ	**KP822427**	n/a	**KP796297**	**KP822100**	n/a	**KP823485**
105	*Protoparmelia badia B1*	Spain: Teruel, Orihuela del Tremedal, Rico, Vivas MAF-Lich 16830 (MAF)	ES12	n/a	**KP822241**	**KP796298**	**KP822101**	n/a	**KP823486**
106	*Protoparmelia badia B1*	Spain: Salamanca, Boom 46079 (herb. v.d. Boom)	ES11	**KP822428**	**KP822242**	**KP796299**	**KP822102**	**KP822337**	**KP823487**
107	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, MAF-Lich 19416	ES14	n/a	**KP822243**	**KP796300**	**KP822103**	n/a	**KP823488**
108	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, MAF-Lich 19417	ES15	**KP822429**	n/a	**KP796301**	**KP822104**	n/a	**KP823489**
109	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, MAF-Lich 19418	ES16	**KP822430**	**KP822244**	**KP796302**	**KP822105**	n/a	**KP823490**
110	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, MAF-Lich 19419	ES17	**KP822431**	**KP822245**	**KP796303**	**KP822106**	n/a	**KP823491**
111	*Protoparmelia badia B1*	Spain: Moncayo, Tarazona, Crespo, Divakar, Dal Grande MAF-Lich 19420	ES18	n/a	**KP822246**	**KP796304**	**KP822107**	n/a	**KP823492**
112	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19421	ES19	**KP822432**	**KP822247**	**KP796305**	**KP822108**	n/a	**KP823493**
113	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19422	ES20	**KP822433**	n/a	**KP796306**	**KP822109**	n/a	**KP823494**
114	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19423	ES21	**KP822434**	**KP822248**	**KP796307**	**KP822110**	n/a	**KP823495**
115	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19426	ES23	**KP822435**	n/a	**KP796308**	n/a	n/a	**KP823496**
116	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19425	ES24	**KP822436**	**KP822249**	**KP796309**	n/a	n/a	**KP823497**
117	*Protoparmelia badia B1*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19424	ES25	**KP822437**	**KP822250**	**KP796310**	n/a	n/a	**KP823498**
118	*Protoparmelia badia B1*	Italy, Sardinia, Dal Grande, Singh Mount Limbara FR-0068881	IT1	**KP822438**	**KP822251**	**KP796311**	**KP822111**	**KP822338**	**KP823499**
119	*Protoparmelia badia B1*	Italy, Sardinia, Dal Grande, Singh Mount Limbara, FR-0068882	IT2	**KP822439**	**KP822252**	**KP796312**	**KP822112**	**KP822339**	**KP823500**
120	*Protoparmelia badia B1*	Spain: Madrid, Crespo, Rico, Ruibal MAF-Lich 19435	ES33	**KP822440**	**KP822253**	**KP796313**	n/a	**KP822340**	**KP823501**
121	*Protoparmelia badia B2*	Spain: Moncayo, Crespo, Divakar, Dal Grande MAF-Lich 19415	ES13	n/a	**KP822254**	**KP796314**	**KP822113**	n/a	**KP823502**
122	*Protoparmelia badia B2*	Spain: Almería, Sierra de Los Filabres, Divakar, Dal Grande MAF-Lich 19583	ES22	**KP822441**	**KP822255**	**KP796315**	**KP822114**	n/a	n/a

123	*Protoparmelia badia C1*	Spain: Madrid, Colemenar Viejo, Crespo, Rico, Ruibal MAF-Lich 19447	ES26	n/a	**KP822256**	**KP796316**	**KP822115**	n/a	**KP823503**
124	*Protoparmelia badia C1*	Spain: Madrid, Colemenar Viejo, Crespo, Rico, Ruibal MAF-Lich 19448	ES27	n/a	n/a	**KP796317**	**KP822116**	n/a	**KP823504**
125	*Protoparmelia badia C1*	Spain: Madrid, Colemenar Viejo, Crespo, Rico, Ruibal MAF-Lich 19432	ES28	**KP822442**	**KP822257**	**KP796318**	**KP822117**	**KP822341**	**KP823505**
126	*Protoparmelia badia C1*	Spain: Madrid, Colemenar Viejo, Crespo, Rico, Ruibal MAF-Lich 19433	ES29	n/a	**KP822258**	**KP796319**	n/a	**KP822342**	**KP823506**
127	*Protoparmelia badia C1*	Spain: Madrid, Colemenar Viejo, Crespo, Rico, Ruibal MAF-Lich 19434	ES30	**KP822443**	**KP822259**	**KP796320**	**KP822118**	**KP822343**	**KP823507**
128	*Protoparmelia badia C2*	Spain: Madrid, Crespo, Rico, Ruibal, Boluda MAF-Lich 19437	ES31	**KP822444**	**KP822260**	**KP796321**	**KP822119**	**KP822344**	**KP823508**
129	*Protoparmelia badia C2*	Spain: Madrid, Crespo, Rico, Ruibal, Boluda MAF-Lich 19438	ES32	**KP822445**	**KP822261**	**KP796322**	**KP822120**	n/a	**KP823509**
130	*Protoparmelia capitata*	USA: Georgia, Candler County, Lendemer 21761, NY-1104334 (NY)	US1	**KP822446**	n/a	**KP796323**	**KP822121**	n/a	n/a
131	*Protoparmelia capitata*	Cuba, Holguín, Mayarí, *Buck 55885, NY-1149527* (NY)	CU1	**KP822447**	n/a	**KP796324**	**KP822122**	**KP822345**	n/a
132	*Protoparmelia capitata*	USA: Alabama, Escambia County, Lendemer 9164, NY-1054070 (NY)	US4	n/a	n/a	**KP796325**	**KP822123**	n/a	n/a
133	*Protoparmelia corallifera*	Thailand: Muk Dahan Province, Nhong Sung District, Papong 7022 (MSUT)	TH2	n/a	**KP822262**	**KP796326**	**KP822124**	n/a	**KP823510**
134	*Protoparmelia corallifera*	Thailand: Muk Dahan Province, Nhong Sung District, Papong 6984 (MSUT)	TH1	**KP822448**	**KP822263**	**KP796327**	**KP822125**	**KP822346**	**KP823511**
135	*Protoparmelia corallifera*	Thailand: Muk Dahan Province, Nhong Sung District, Papong 6483 (MSUT)	TH4	n/a	**KP822264**	**KP796328**	**KP822126**	n/a	**KP823512**
136	*Protoparmelia corallifera*	Thailand: Muk Dahan Province, Nhong Sung District, Papong 7102 (MSUT)	TH3	**KP822449**	n/a	**KP796329**	**KP822127**	**KP822347**	**KP823513**
137	*Protoparmelia cupreobadia B*	USA: Maine, Piscataquis County, Fryday 8579, MSC0108416 (MSC)	US1	n/a	**KP822265**	**KP796330**	**KP822128**	**KP822348**	n/a
138	*Protoparmelia cupreobadia B*	USA: Maine, Piscataquis County, Fryday 8629 MSC0108417 (MSC)	US2	n/a	**KP822266**	n/a	**KP822129**	**KP822349**	n/a
139	*Protoparmelia cupreobadia B*	USA: Maine, Piscataquis County, Fryday 8634 MSC0108420 (MSC)	US5	**KP822450**	n/a	**KP796331**	**KP822130**	**KP822350**	n/a
140	*Protoparmelia cupreobadia A*	USA: Maine, Piscataquis County, Fryday 8631 MSC0108418 (MSC)	US3	**KP822451**	**KP822267**	**KP796332**	**KP822131**	**KP822351**	**KP823514**
141	*Protoparmelia cupreobadia A*	USA: Maine, Piscataquis County, *Fryday 8633 MSC0108419* (MSC)	US4	**KP822452**	KF562192	KF562184	KF601238	KF562175	n/a
142	*Protoparmelia hypotremella*	Canada: Ontario, Bruce County, *Lendemer 14562* NY-1049774 (NY)	CA1	**KP822453**	n/a	**KP796333**	n/a	**KP822352**	n/a
143	*Protoparmelia hypotremella*	Canada: Ontario, Bruce County, *Lendemer 14305A, NY-1050828* (NY)	CA2	**KP822454**	n/a	**KP796334**	**KP822132**	**KP822353**	**KP823515**
144	*Protoparmelia hypotremella*	Canada: Ontario, Bruce County, *Lendemer 14431B, NY-1049715* (NY)	CA3	n/a	**KP822268**	**KP796335**	n/a	**KP822354**	**KP823516**
145	*Protoparmelia hypotremella*	Canada: Ontario, Bruce County, *Lendemer 14563* NY-1049772 (NY)	CA4	**KP822455**	**KP822269**	**KP796336**	**KP822133**	n/a	**KP823517**
146	*Protoparmelia hypotremella*	Canada: Ontario, Nipissing District, *Brodo, Brodo 32443, CANL 123107* (CANL)	CA5	**KP822456**	n/a	**KP796337**	**KP822134**	n/a	**KP823518**
147	*Protoparmelia hypotremella*	Slovakia: W Carpathians, Nuránska planina plateau, Bouda, Černajová, Malíček, Palice 14347 (ASCR)	SK1	**KP822457**	**KP822270**	**KP796338**	**KP822135**	n/a	n/a
148	*Protoparmelia hypotremella*	Netherlands: Prov. Utrecht Leusden, Aproot, *Aproot 72589* (ABL)	NL1	n/a	n/a	**KP796339**	**KP822136**	**KP822355**	**KP823519**
149	*Protoparmelia isidiata A*	USA: Georgia, McIntosh County, *Lendemer 20727, NY-1149936* (NY)	US1	**KP822458**	n/a	**KP796340**	**KP822137**	**KP822356**	n/a
150	*Protoparmelia isidiata A*	USA: Georgia, McIntosh County, *Lendemer 20745 NY-1149920* (NY)	US2	**KP822459**	n/a	n/a	**KP822138**	n/a	n/a
151	*Protoparmelia isidiata A*	USA: Georgia, McIntosh County, *Lendemer 20903, NY-1150773* (NY)	US3	**KP822460**	n/a	n/a	**KP822139**	n/a	n/a
152	*Protoparmelia isidiata A*	USA: Georgia, McIntosh County, *Lendemer 20992, NY-1152323* (NY)	US4	**KP822461**	n/a	**KP796341**	**KP822140**	n/a	n/a
153	*Protoparmelia isidiata B*	Brazil: Sergipe, Parque Nacional Serra de Itabaiana, Caceres, Aproot, Aproot 21684 (ISE)	BR1	**KP822462**	**KP822271**	**KP796342**	**KP822141**	**KP822357**	n/a
154	*Protoparmelia isidiata B*	Brazil: Sao Paulo, *Caceres, Aproot, Aproot 13673* (ABL)	BR2	**KP822463**	**KP822272**	**KP796343**	**KP822142**	n/a	n/a
155	*Protoparmelia isidiata D*	Australia: Solar Village, *Elix 39795, CANB-00783253* (CANB)	AU2	n/a	**KP822273**	**KP796344**	**KP822144**	**KP822358**	**KP823520**
156	*Protoparmelia isidiata D*	Australia: Solar Village, *Elix 39805, CANB-00783260* (CANB)	AU1	**KP822464**	**KP822274**	**KP796345**	**KP822145**	**KP822359**	**KP823521**
157	*Protoparmelia isidiata E*	Australia: New South Wales, *Kantvilas 228/10, HO-559228* (HO)	AU3	**KP822465**	**KP822275**	n/a	**KP822146**	n/a	**KP823522**
158	*Protoparmelia isidiata E*	Australia: Northern Territory, *Kantvilas 289/07, HO-545660* (HO)	AU4	**KP822466**	**KP822276**	n/a	**KP822147**	n/a	**KP823523**
159	*Protoparmelia isidiata C*	Thailand: Chiang Mai, *Boom 46872* (herb. v.d. Boom)	TH1	**KP822467**	**KP822277**	**KP796346**	n/a	n/a	**KP823524**

160 *Protoparmelia isidiata C*	Thailand: Chiang Mai, *Boom 46947* (herb. v.d. Boom)	TH2	**KP822468**	**KP822278**	**KP796347**	**KP822143**	n/a	**KP823525**
161 *Protoparmelia Sp KE*	Kenya: *Kirika, Lumbsch EA-3821* (EA)	KE1	**KP822469**	**KP822279**	**KP796348**	**KP822148**	n/a	**KP823526**
162 *Protoparmelia Sp KE*	Kenya: P. Kirika s.n. & H.T. Lumbsch (EA)	KE2	n/a	**KP822280**	n/a	**KP822149**	n/a	**KP823527**
163 *Protoparmelia leproloma*	USA: California, Sisikiyou County, *McCune 28138* (OSU)	US1	**KP822470**	n/a	**KP796349**	**KP822150**	**KP822360**	n/a
164 *Protoparmelia leproloma*	USA: Montana, Lake County, *Wheeler 3046* (OSU)	US2	**KP822471**	**KP822281**	**KP796350**	**KP822151**	n/a	**KP823528**
165 *Protoparmelia leproloma*	Sweden: Torne Lappmark, *Palice 7157* (ASCR)	SE1	**KP822472**	n/a	**KP796351**	**KP822152**	**KP822361**	n/a
166 *Protoparmelia memnonia*	Norway: Sør-Trøndelag, *Haugan 9612, O-L167013* (O)	NO1	**KP822473**	KF562194	KF562186	KF601240	KF562177	**KP823529**
167 *Protoparmelia memnonia*	Norway: Nord-Trøndelag, *Holien 13370, L-14269* (TRH)	NO3	**KP822474**	**KP822282**	**KP796352**	**KP822153**	**KP822362**	**KP823530**
168 *Protoparmelia memnonia*	Norway: Nord-Trøndelag, Holien 12787, L-13935 (TRH)	NO2	**KP822475**	n/a	n/a	**KP822154**	**KP822363**	**KP823531**
169 *Protoparmelia montagnei A*	Turkey: Canakale province, Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19464	TR1	**KP822476**	n/a	**KP796353**	**KP822155**	**KP822364**	n/a
170 *Protoparmelia montagnei A*	Turkey: Canakale province, *Divakar, Crespo, Candan, Lumbsch,* MAF-Lich 19465	TR2	**KP822477**	**KP822283**	**KP796354**	**KP822156**	n/a	**KP823532**
171 *Protoparmelia montagnei A*	Spain: Almeria, Crespo, Cubas, Nuñez, Divakar MAF-Lich 19463	ES5	n/a	**KP822284**	**KP796355**	**KP822157**	**KP822365**	**KP823533**
172 *Protoparmelia montagnei A*	Turkey: Canakale province, Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19466	TR4	**KP822478**		**KP796356**	**KP822158**	n/a	n/a
173 *Protoparmelia montagnei A*	Turke:, Canakale province, Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19468	TR5	**KP822479**	**KP822285**	**KP796357**	**KP822159**	n/a	**KP823534**
174 *Protoparmelia montagnei A*	Turkey: Canakale province, Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19469	TR6	**KP822480**	**KP822286**	**KP796358**	**KP822160**	**KP822366**	n/a
175 *Protoparmelia montagnei B*	Spain: Canary Islands, *Crespo, Cubas, Santo, Divakar MAF-Lich 19459*	ES1	**KP822481**	n/a	**KP796359**	**KP822161**	n/a	**KP823535**
176 *Protoparmelia montagnei B*	Spain: Canary Islands, Crespo, Cubas, Santo, Divakar, MAF-Lich 19458 Specimen 1 (MAF)	ES2	n/a	n/a	**KP796360**	**KP822162**	**KP822367**	**KP823536**
177 *Protoparmelia montagnei B*	Spian: Canary Islands, Crespo, Cubas, Santo, Divakar, MAF-Lich 19458 Specimen 2 (MAF)	ES3	n/a	n/a	**KP796361**	**KP822163**	**KP822368**	**KP823537**
178 *Protoparmelia montagnei C*	Spian: Almeria, Crespo, Cubas, Nuñez, Divakar MAF-Lich 19462	ES4	n/a	n/a	**KP796362**	**KP822164**	n/a	n/a
179 *Protoparmelia montagnei C*	Turkey: Canakale province, *Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19467*	TR3	n/a	**KP822287**	**KP796363**	**KP822165**	n/a	**KP823538**
180 *Protoparmelia montagnei C*	Spain: Almeria, Crespo, Rico, Ruibal MAF-Lich 19427	ES6	**KP822482**	**KP822288**	**KP796364**	**KP822166**	**KP822369**	**KP823539**
181 *Protoparmelia montagnei C*	Spain: Almeria, Crespo, Rico, Ruibal MAF-Lich 19428	ES7	**KP822483**	**KP822289**	n/a	**KP822167**	**KP822370**	**KP823540**
182 *Protoparmelia montagnei C*	Spain: Almeria, Crespo, Rico, Ruibal MAF-Lich 19429	ES8	**KP822484**	**KP822290**	**KP796365**	**KP822168**	**KP822371**	**KP823541**
183 *Protoparmelia multifera*	Brazil: Sao Paulo, Aproot 13667 (ABL)	BR1	**KP822485**	**KP822291**	**KP796366**	**KP822169**	n/a	n/a
184	Brazil: Sao Paulo, Aproot 13667 (ABL)	BR2	n/a	**KP822292**	**KP796367**	n/a	n/a	n/a
185 *Protoparmelia nephaea*	USA: California, *Fryday 9313, MSC0108422* (MSC)	US1	n/a	n/a	**KP796368**	**KP822170**	**KP822372**	n/a
186 *Protoparmelia ochrococca B*	USA: Oregon, *McCune 31673* (OSU)	US1	**KP822489**	**KP822293**	**KP796372**	**KP822172**	**KP822373**	**KP823542**
187 *Protoparmelia ochrococca A*	Norway: Sogn og Fjordane, Høyanger, *Klepsland JK10-L102, O L-175016* (O)	NO1	**KP822486**	n/a	**KP796369**	**KP822171**	n/a	n/a
188 *Protoparmelia ochrococca A*	Norway: Rogaland, Suldal, *Johnsen L-93143* (BG)	NO3	**KP822487**	n/a	**KP796370**	n/a	n/a	**KP823543**
189 *Protoparmelia ochrococca A*	Norway: Rogaland, Vindafjord, *Tønsberg 39290, L-87963,* (BG)	NO4	**KP822488**	n/a	**KP796371**	n/a	n/a	**KP823544**
190 *Protoparmelia oleagina*	Norway: Nord-Trøndelag, Namdalseid, *Holien 10816, L-14269* (TRH)	NO1	**KP822490**	**KP822294**	**KP796373**	n/a	**KP822374**	**KP823545**
191 *Protoparmelia oleagina*	Norway: Rogaland, Finnøy, *Johnsen L-92691* (BG)	NO2	**KP822491**	n/a	**KP796374**	n/a	**KP822375**	**KP823546**
192 *Protoparmelia orientalis*	Thailand: Muk Dahan Province, Nhong Sung District, *Papong 6922* (MSUT)	TH1	**KP822492**	**KP822295**	**KP796375**	**KP822173**	**KP822376**	**KP823547**
193 *Protoparmelia orientalis*	Thailand: Muk Dahan Province, Nhong Sung District, *Papong 6969* (MSUT)	TH2	**KP822493**	n/a	**KP796376**	**KP822174**	**KP822377**	**KP823548**
194 *Protoparmelia orientalis*	Thailand: Muk Dahan Province, Nhong Sung District, *Papong 7033* (MSUT)	TH5	**KP822494**	**KP822296**	**KP796377**	**KP822175**	n/a	**KP823549**
195 *Protoparmelia orientalis*	Thailand: Sakon Nakhon Province, Phu Phan National Park, *Papong 6488* (MSUT)	TH3	**KP822495**	n/a	**KP796378**	**KP822176**	n/a	n/a
196 *Protoparmelia orientalis*	Thailand: Sakon Nakhon Province, Phu Phan National Park, *Papong 6487* (MSUT)	TH4	**KP822496**	**KP822297**	n/a	**KP822177**	n/a	**KP823550**
197 *Protoparmelia pulchra*	Australia: Golden Highway, Elix 39560, CANB 00789446 (CANB)	AU1	n/a	**KP822298**	n/a	**KP822178**	n/a	**KP823551**
198 *Protoparmelia pulchra*	Australia: Howard Springs Road, *Elix 37097, CANB-00800711* (CANB)	AU2	**KP822497**	**KP822299**	**KP796379**	n/a	**KP822378**	n/a

200	*Protoparmelia pulchra*	Australia: Solar Village, Humpty Doo, *Elix 39806, CANB-00783261* (CANB)	AU4	n/a	**KP822301**	**KP796381**	**KP822180**	n/a	n/a
201	*Protoparmelia Sp ZA*	South Africa: Cape Region, *Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19627* (MAF)	ZA1	**KP822498**	**KP822302**	**KP796382**	**KP822181**	**KP822379**	**KP823553**
202	*Protoparmelia Sp ZA*	South Africa: Cape Region, *Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19625* (MAF)	ZA2	n/a	**KP822303**	**KP796383**	**KP822182**	**KP822380**	**KP823554**
203	*Protoparmelia Sp ZA*	South Africa: Cape Region, *Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19628* (MAF)	ZA3	**KP822499**	**KP822304**	**KP796384**	**KP822183**	n/a	**KP823555**
204	*Protoparmelia Sp ZA*	South Africa: Cape Region, *Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19584* (MAF)	ZA4	**KP822500**	n/a	**KP796385**	**KP822184**	n/a	**KP823556**
205	*Protoparmelia Sp ZA*	South Africa: Cape Region, *Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19626* (MAF)	ZA5	n/a	n/a	**KP796386**	**KP822185**	n/a	**KP823557**
206	*Protoparmelia Sp 1*	USA: Montana, *Spribille s.n., 23.09.2012* (GZU)	US3	n/a	**KP822305**	**KP796387**	**KP822186**	n/a	**KP823558**
207	*Protoparmelia phaeonesos*	Austria, *Hafellner, Hafellner 71301* (GZU)	AT1	**KP822501**	n/a	**KP796388**	**KP822187**	**KP822381**	n/a
208	*Protoparmelia phaeonesos*	Austria, *Hafellner, Muggia, Hafellner 68479* (GZU)	AT2	**KP822502**	**KP822306**	**KP796389**	n/a	n/a	n/a
209	*Protoparmelia phaeonesos*	Norway: Buskerud, *Rui, E. Timdal 11000, O-L158126* (O)	NO1	**KP822503**	KF562193	KF562185	KF601239	KF562176	**KP823559**
210	*Protoparmelia phaeonesos*	Norway: Nord-Trøndelag, Stjørdal, *Haugan, Mathiesen stjør18704h, O-L131683* (O)	NO2	**KP822504**	n/a	**KP796390**	**KP822188**	**KP822382**	n/a
211	*Protoparmelia phaeonesos*	Norway: Nord-Trøndelag, Stjørdal, *Holien 13365, Haugan, L-14268* (TRH)	NO3	**KP822505**	n/a	**KP796391**	**KP822189**	**KP822383**	**KP823560**
212	*Protoparmelia phaeonesos*	Norway: Buskerud, Sigdal, *Timdal 11781, L-163838* (O)	NO4	**KP822506**	n/a	**KP796392**	**KP822190**	**KP822384**	n/a
213	*Protoparmelia ryaniana*	USA: California, Santa Barbara County, *Knudsen 11439, Chaney, UCR-209796* (UCR)	US1	**KP822505**	n/a	n/a	n/a	n/a	**KP823561**
214	*Protoparmelia ryaniana*	USA: California, San Luis Obispo County, *Knudsen 12164, UCR-213223* (UCR)	US2	**KP822508**	n/a	n/a	n/a	n/a	n/a
215	*Protoparmelia ryaniana*	USA: California, San Luis Obispo County, *Knudsen 12146, UCR-213205* (UCR)	US3	**KP822509**	n/a	n/a	**KP822191**	**KP822385**	**KP823562**
216	*Protoparmelia ryaniana*	USA: California, Santa Barbara County, *Knudsen 12023, UCR-222111* (UCR)	US4	**KP822510**	n/a	n/a	**KP822192**	n/a	n/a
217	*Pseudephebe pubescens*	Spain: Zamora, Crespo s.n. (MAF-Lich 6774)	-	AF351180	AY611125	AY607839	EF092148	n/a	KP888283
218	*Relicina subnigra*	Australia: Molonglo Gorge Reserve, Louwhoff et al. s.n. (MAF-Lich 10184)	-	AY785281	AY785274	AY785267	EF092152	n/a	n/a
219	*Tuckermannopsis chlorophylla*	Sweden: Västerbotten, Wedin 6995 (UPS)	-	DQ923647	DQ980025	DQ923674	DQ923697	KF562178	KP888294
220	*Vulpicida pinastri*	Sweden: Uppland, Mattsson 4004 (UPS)	-	DQ923648	AF058039	DQ923675	n/a	n/a	n/a
	Vulpicida pinastri	Sweden: Västerbotten, Wedin 7620 (UPS)	-	n/a	n/a	n/a	DQ923698	JX974721	KP888307
221	*Xanthoparmelia conspersa*	Spain: Zamora, Blanco & Crespo s.n. (MAF-Lich 6793)	-	AF351186	AY581096	AY578962	EF092155	n/a	KP888311
223	Xanthoparmelia hottentota	South Africa: Cape Province, Crespo et al. s.n. (MAF-Lich 14267	-	EF025486	EF042909	EF042919	EF092153	n/a	n/a
224	*Xanthoparmelia tinctina*	Spain: Madrid, Crespo s.n. (MAF-Lich 6070)	-	AY582343	AY581108	AY578976	n/a	JX974720	n/a
225	*Pyrrhospora laeta*	Australia: Wetern Australia: Elix 31817 (F)	-	EU075530	EU075544.1	n/a	n/a	n/a	n/a
	Pyrrhospora laeta	Australia: Northern Territory, Elix 28836 (F)	-	n/a	n/a	n/a	n/a	n/a	**KP823568**
226	*Pyrrhospora russula*	Costa Rica, Luecking 17640 (F)	-	EU075533	EU075547.1	EU075524.	**KP822201**	**KP822394**	n/a
227	*Pyrrhospora sanguinolenta*	Australia: Queensland, Elix 28835 (F)	-	EU075534	EU075548.1	EU075523.1	**KP822202**	**KP822395**	n/a
228	*Pyrrhosporea Sp.*	Australia: Northern Territory, Elix 28837	-	EU075532	EU075546.1	EU075525.	**KP822203**	**KP822396**	n/a
229	*Ramboldia brunneocarpa*	-	-	EU075528	EU075542	EU075520.1	n/a	n/a	n/a
230	*Ramboldia russula*	Thailand: Papong 6507 (F)	-	**KP822521**	**KP822307**	**KP796402**	**KP822204**	n/a	n/a
231	*Ramboldia russula*	Thailand: Papong 6508 (F)	-	**KP822522**	**KP822308**	**KP796403**	**KP822205**	n/a	n/a
232	*Ramboldia stuartii*	-	-	EU075535	EU075549.1	EU075522.1	n/a	n/a	n/a
233	*Ramboldia stuartii*	Australia: Tasmania, *Elix 28664* (F)	-	**KP822523**	**KP822309**	**KP796404**	**KP822206**	**KP822397**	**KP823569**

10.3 Publication: Fungal-algal association patterns in lichen symbiosis linked to macroclimate.

Erklärung zu den Autorenanteilen an der Publikation: Fungal-algal association patterns in lichen symbiosis linked to macroclimate.
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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Garima Singh:	70%;	
Francesco Dal Grande:	15%;	
Imke Schmitt:	15%;	

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Garima Singh:	50%	PCR and sequencing
Jürgen Otte:	50%;	PCR and sequencing

(3) zur Erstellung der Datensammlung und Abbildungen

Garima Singh:	60%;	Sample preparation and figures
Imke Schmitt:	40%	

(4) zur Analyse und Interpretation der Daten

Garima Singh:	90%;	phylogenetic analysis, species delimitation analyses coevolutionary analyses, pPCA, and Interpretation of Data
Francesco Dal Grande:	10%;	PCA, GLM

5) zum Verfassen des Manuskripts

Garima Singh:	80%;
Francesco Dal Grande:	10%;
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Research

Fungal–algal association patterns in lichen symbiosis linked to macroclimate

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Summary

- Both macroclimate and evolutionary events may influence symbiont association and diversity patterns. Here we assess how climatic factors and evolutionary events shape fungal–algal association patterns in the widely distributed lichen-forming fungal genus *Protoparmelia*.
- Multilocus phylogenies of fungal and algal partners were generated using 174 specimens. Coalescent-based species delimitation analysis suggested that 23 fungal hosts are associating with 20 algal species. Principal component analysis (PCA) was performed to infer how fungal–algal association patterns varied with climate.
- Fungi associated with one to three algal partners whereas algae accepted one to five fungal partners. Both fungi and algae were more specific, associating with fewer partners, in the warmer climates. Interaction with more than one partner was more frequent in cooler climates for both the partners. Cophylogenetic analyses suggest congruent fungal–algal phylogenies. Host switch was a more common event in warm climates, whereas failure of the photobiont to diverge with its fungal host was more frequent in cooler climates.
- We conclude that both environmental factors and evolutionary events drive fungal and algal evolution in *Protoparmelia*. The processes leading to phylogenetic congruence of fungi and algae are different in different macrohabitats in our study system. Hence, closely related species inhabiting diverse habitats may follow different evolutionary pathways.

Introduction

Climate influences the evolution of species by impacting species diversity (Fischer, 1960; Vázquez & Stevens, 2004), species distribution patterns (Pianka, 1966), and species interactions (Pommier *et al.*, 2007; Jocque *et al.*, 2010). Warmer climates have often been linked to higher speciation rates and higher numbers of species compared with temperate/arctic habitats (evolutionary speed hypothesis; Fischer, 1960; Allen *et al.*, 2006; Jablonski *et al.*, 2006; but see Shaw *et al.*, 2003; Rozzi *et al.*, 2008). One factor leading to the latitudinal biodiversity gradient is suggested to be difference in biotic specialization from the poles to the equator (Jocque *et al.*, 2010; Pellissier, 2015). Biotic specialization has been suggested to increase towards the equator, and tropical species are predicted to be more specialized than polar species (Wallace, 1878; Dobzhansky, 1950; Fischer, 1960).

Apart from climate, the evolutionary history may also play an important role in determining the diversity and interactions of symbiotic organisms (the geographic mosaic theory of coevolution; Thompson, 2001; Piculell *et al.*, 2008). In general, stronger biotic interactions in the warmer climates lead to increased coadaptation and concerted evolution (Dobzhansky, 1950; Schemske, 2009; Schemske *et al.*, 2009). Therefore, analyses of diversity and association patterns across different macroclimatic regions should also take into account the evolutionary history of the symbionts. Cophylogenetic studies, which assess topological congruence of host and symbiont phylogenies, are commonly used to examine the historical mechanisms behind the host−parasite evolution (Peek *et al.*, 1998; Hosokawa *et al.*, 2006).

Evolutionary events in symbiotic systems include: (1) cospeciation: simultaneous divergence of both host and symbiont; (2) host switch: switching of the symbiotic partner, giving rise to new host–symbiont combinations; (3) duplication: independent speciation of the symbiont without host speciation, both new lineages associating with the same host. (4) loss or extinction: a symbiont is lost from the host lineage, as a result of extinction or incomplete lineage sorting; and (5) failure to diverge: the symbiont does not diverge along with the host but a single symbiont lineage associates with both new host lineages (Brooks, 1988; Ronquist, 1997; de Vienne *et al.*, 2013). Previously, topological congruence was inferred as an indication of cospeciation whereas phylogenetic incongruence was inferred as an indication of host switches, failure to diverge and losses (Peek *et al.*, 1998; Jousselin *et al.*, 2009). However, repeated host shifts to closely related hosts followed by divergence lead to congruent phylogenetic structure and give the false impression of cospeciation (Hafner & Nadler,

1988). Evidence now suggests that cospeciation is actually a rare event and host switching is the predominant event shaping symbiotic associations (de Vienne *et al.*, 2007, 2013). Careful evaluation of host and symbiont phylogenies is therefore needed to infer the most likely events that have led to congruent host and symbiont phylogenies.

Lichens are a classic example of symbiosis between a fungus and one or more photosynthetic partners (Ahmadjian, 1965, 1993). The fungal partner is heterotrophic, deriving nutrition from the extracellularly located photosynthetic partner (Honegger, 1986; Ahmadjian, 1993). Association patterns between fungus and alga in a lichen symbiosis are commonly described in terms of specificity and selectivity (Galun & Bubrick, 1984; Beck *et al.*, 1998, 2002). Specificity refers to the exclusive one-to-one interaction between fungus and alga such that the partners associate only with one another, and no other interactions are possible. Specialized lichen associations therefore refer to exclusive one-to-one interactions. Selectivity, in contrast, indicates the preferential association with one partner when more than one partner is available.

Previous studies dealing with the algal identity and association patterns in different lichen-forming fungi showed that geography and habitat can be important predictors of the symbiotic partner, and suggested ecological specialization to be important in shaping fungal–algal associations (Yahr *et al.*, 2004; Peksa & Skaloud, 2011; Muggia *et al.*, 2013). However, at certain ecogeographic scales, selectivity and specificity of mycobionts may be more important in determining fungal–algal associations than ecology (Leavitt *et al.*, 2015). Some studies rejected cospeciation between fungus and alga and suggested symbiont switches to locally adapted algae to be a rather common phenomenon (Kroken & Taylor, 2000; Piercey-Normore & Depriest, 2001; Yahr *et al.*, 2004). In this study, we aimed to analyze association patterns within a genus of lichen-forming fungi under different macroclimates at a global scale to understand how species association patterns correlate with the habitat of occurrence. For this purpose, we selected the lichen-forming fungal genus *Protoparmelia* which is a small, monophyletic, cosmopolitan genus of ~ 25–30 species inhabiting diverse macrohabitats (Table 1). The genus as a whole is cosmopolitan but different species have a rather narrow habitat range. The phylogenetic relationships and species concepts of the fungal partner of the *Protoparmelia* group were recently inferred based on a multilocus phylogeny (Singh *et al.*, 2015). In the current study we address the following questions: How does macroclimate influence symbiont diversity and association patterns in the lichen-forming fungal genus *Protoparmelia*? If so, what are the fungal and algal phylogenies congruent; what are the most likely evolutionary (and other) events that have led to the observed phylogenetic congruence?

Materials and Methods

The accuracy of both cophylogenetic analyses and association patterns depends on the reliability of the host and symbiont phylogenies and species concepts (de Vienne *et al.*, 2007). For the fungal partner, we used the species concepts of *Protoparmelia* proposed in Singh *et al.* (2015), which are based on a six-locus phylogeny and coalescent-based species delimitation analyses. For the algae, as the species concepts of the lichen photobionts are still poorly investigated, we first generated a multilocus phylogeny of the algal partners, and subsequently performed species delimitation analyses.

Table 1 The species of *Protoparmelia* and their distribution

Species	Habitat/ecosystem	Distribution
P. badia A	Boreal, arctic/alpine	Cosmopolitan
P. badia B1	Boreal, arctic/alpine	Spain, Italy
P. badia B2	Boreal, arctic/alpine	Spain
P. badia C	Boreal, arctic/alpine	Spain
P. memnonia	Arctic/alpine	Europe
P. hypotremella	Temperate	Europe, North America
P. ochrococca	Temperate	Western North America, Europe
P. oleagina	Temperate	Western and northern Europe
P. montagnei A	Mediterranean	Turkey, Spain
P. montagnei B	Mediterranean	Spain
P. montagnei C	Mediterranean	Turkey, Spain
P. ZA	Mediterranean	South Africa
P. capitata	Subtropical	Southeastern North America, Brazil
P. corallifera	Tropical	Thailand
P. isidiata A	Subtropical	USA
P. isidiata B	Tropical	Brazil
P. isidiata C	Tropical	Thailand
P. isidiata D	Subtropical	Australia
P. isidiata E	Subtropical	Australia
P. multifera	Tropical	Brazil
P. KE	Tropical	Kenya
P. pulchra	Temperate/subtropical	Australia (incl. Tasmania), Asia
P. orientalis	Tropical	Thailand

Sequencing and phylogenetic analyses

A total of 174 samples were included in the study representing 23 *Protoparmelia* s.str. species (Supporting Information Table S1). *Protoparmelia* species can be saxicolous (growing on rock) or corticolous (growing on bark). Samples were collected from rock or tree bark using a scalpel.

Total genomic DNA of the two symbionts was extracted from lichen thalli using the cetyl-trimethyl ammonium bromide (CTAB) method (Cubero & Crespo, 2002). For the algal symbiont we amplified internal transcribed spacer (ITS) ribosomal DNA (rDNA) and cytochrome c oxidase subunit II (*COX2*), using general primers or taxon-specific primers (Table S2). For the fungal phylogeny, we used the data set published in Singh *et al.* (2015) for 126 specimens (506 sequences) out of 174 specimens used in this study. Eleven *Protoparmelia* s.str. specimens from Singh *et al.* (2015) were excluded from the present study as we failed to amplify the algal symbionts. For the 48 new samples used in this study, we amplified five nuclear markers, namely the large ribosomal subunit (nuLSU), the internal transcribed spacer ribosomal DNA (ITSrDNA), the largest subunit of RNA polymerase II (RPB1), the minichromosome maintenance complex component 7 (MCM7) and the ribosome biogenesis protein (TSR1), and one mitochondrial marker (mtSSU). We

used the same primers and polymerase chain reaction (PCR) conditions as reported previously (Singh *et al.*, 2015). PCR were carried out in a volume of 25 µl. Each reaction mix contained 2.5 µl of buffer, 0.13 µl (0.65 U) of Ex Taq polymerase, 1.0 µl of dNTP mix (2.5 mM each), 1.0 µl each (10 mM) of the primer set (forward and reverse), *c.* 20 ng of template, and 16 µl of H_2O. Reactions were performed with the following cycling conditions: initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 50°C for 40 s and 72°C for 1 min, and final elongation at 72°C for 5 min. PCR products were checked for amplification on 1% agarose gels.

Bands of expected size were extracted using the peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). These fragments were then labeled for cycle sequencing using the Big Dye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and sequenced as follows: 1 min at 96°C, and 26 cycles of 20 s at 96°C, 5 s at 50°C, and 2 min at 60°C. Products were purified using the Big Dye XTerminator Purification kit (Life Technologies, Foster City, CA, USA) and then detected on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

Sequences were assembled using Geneious v.5.4 (Drummond *et al.*, 2011) followed by manual editing. Sequences were aligned separately for each locus using Mafft (Katoh *et al.*, 2005). Gaps were treated as missing data and ambiguously aligned regions were excluded. The sequences are deposited in GenBank.

We performed maximum likelihood (ML) analysis on both algal loci using RAxML-HPC BlackBox v.7.2.8 (Stamatakis *et al.*, 2008) on the CIPRES Science Gateway v.3.3 (http://www.phylo.org; Miller *et al.*, 2010). Before concatenating the data sets, the loci were checked for congruence (both algal and fungal) using Congruence Among Distance Matrices (CADM) as implemented in the package ape in R (Campbell *et al.*, 2011; R Development Core Team, 2011). ML analysis was performed on the concatenated two-locus algal and six-locus fungal data sets with RAxML-HPC BlackBox v.7.2.8 (Stamatakis *et al.*, 2008) on the CIPRES Science Gateway v.3.3 (http://www.phylo.org; Miller *et al.*, 2010) using the default GTR+G model with data partitioning according to the different genes and 1000 bootstrap (BS) replicates (http://www.phylo.org).

Bayesian inference was performed on the algal and fungal concatenated data sets, using the best fitting models of nucleotide substitutions using the corrected Akaike information criterion (AIC) as suggested by jModelTest (Darriba *et al.*, 2012) as implemented in MrBayes v.3.2.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) on the CIPRES Science Gateway v.3.3 (http://www.phylo.org). Two parallel Markov chain Monte Carlo (MCMC) runs were performed each using four chains and 20 000 000 generations, sampling trees every 1000th generation. A 50% majority rule consensus tree was generated from the combined sampled trees of both runs after discarding the first 25% as burn-in (12 500 trees).

To identify the algal species, we aligned 40 ITS sequences of *Trebouxia*, representing 26 species from the SAG (algal culture collection at the University of Goettingen, Germany) and UTEX (algal culture collection at the University of Texas, USA) databases, with our ITS data set and generated an ML tree with 1000 BS replicates using RAxML (Fig. S1). Based on highly supported phylogenetic relations, we indicated the potential names of the *Trebouxia* species in the concatenated ML tree (Fig. S2). Sequences from the reference cultures were excluded from the subsequent species delimitation analyses.

The phylogenetic trees were visualized using FigTree v.1.4.0 (Rambaut, 2008). All clades with ML ≥ 70% and posterior probabilities (PP) ≥ 0.95 were considered as supported.

Species delimitation

Species delimitation of the fungal partners followed the concept of *Protoparmelia* reported in Singh *et al.* (2015). For the species delimitation of algae, we considered the clades in the ITS RAxML tree that grouped with the cultured *Trebouxia* strains (Fig. S1) and the supported clades in the concatenated data set as putative species (Fig. S2). The resulting 20-species scenario was tested for evolutionarily independent lineages using two coalescent-based species delimitation approaches, BP&P v3 (Yang & Rannala, 2014) and Stacey (Jones, 2016). BP&P utilizes reversible-jump Bayesian MCMC algorithms to analyze phylogenetic data from multiple loci to generate the speciation probabilities of assigned species. BP&P requires users to specify the guide species tree, which was generated using a coalescent-based hierarchical Bayesian model as implemented in *Beast v.2.1 (Fig. S3; Drummond & Rambaut, 2007; Jones, 2016), with Birth Death process and gamma-distributed population sizes for the species tree prior and a pairwise linear population size model with a constant root. JModelTest was run on single gene data sets (*COX2* and ITS) to select the best locus-specific model of evolution for each gene. *Beast estimates the species tree directly from the sequence data by incorporating the coalescent process and the uncertainty associated with gene trees and nucleotide substitution model parameters.

In addition, species limits in the group were tested using Stacey as implemented in Beast v.2.2 (Jones, 2016) by searching all possible combinations among individuals in the study, using the Birth Death process and gamma-distributed population sizes for the species tree prior, and a pairwise linear population size model with a constant root, for 20 million generations and 20% burn-in. The best locus-specific model of evolution for each gene was selected according to jModelTest. Cluster analyses were performed using SpeciesDelimitationAnalyser (Jones & Oxelman, 2014, available at http://www.indriid.com).

Association pattern

Host–symbiont associations were represented using tanglegrams based on the species trees of fungi and algae in TreeMap 3.0 (Fig. 1; Hoffmann, 2004). The complexity of the interactions was measured with connectance using the package vegan in R (Oksanen *et al.*, 2013). Connectance represents the average number of links per species. High connectance indicates complex networks and more generalized interactions (Jordano, 1987; Blüthgen *et al.*, 2008). Conversely, low connectance suggests specialized and highly selective interactions.

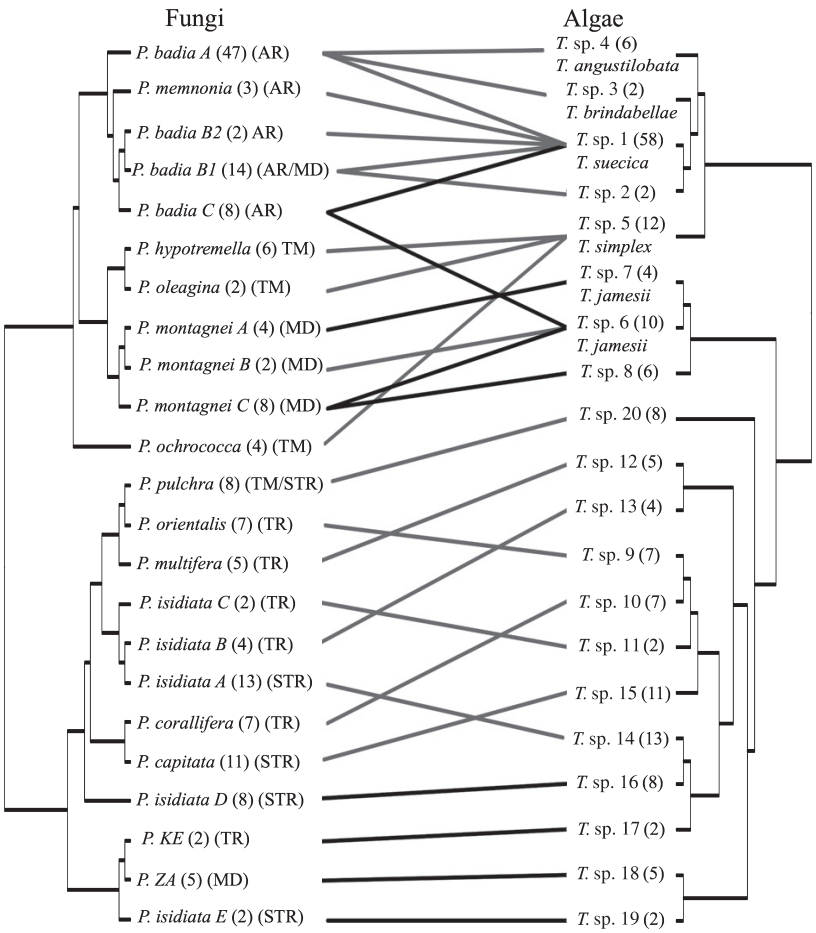

Fig. 1 Tanglegram indicating the associations between lichen-forming fungi (genus *Protoparmelia*) and their green algal symbionts. Trees are *BEAST species trees inferred from six fungal and two algal loci. The number of specimens included in each terminal branch is given in parentheses. Full phylogenetic trees contain 174 specimens each and are presented in Supporting Information Figs S2 and S6. Habitat information is provided with the fungal species (AR, arctic/alpine or boreal; TM, temperate; MD, Mediterranean; STR, subtropical; TR, tropical). Two global-fit tests, PARAFIT and PACO, rejected the hypothesis of a random association between host and symbiont. Black lines indicate links contributing to the congruent phylogenetic structure between *Trebouxia* and their hosts as indicated by PARAFIT ($P \leq 0.05$), while gray lines represent nonsignificant links.

To further explore the symbiotic range of the delimited *Trebouxia* species we performed BLAST searches against GenBank with each algal nrITS using a 97% identity threshold (Fig. S4). We created an association network of these lichen-forming fungi and associated algal nrITS haplotypes using the function *plotweb* in R (Paradis *et al.*, 2004). In addition, to infer if the *Trebouxia* species in our study have already been identified from other lichen-forming fungi, we aligned our algal ITS data set with the first 100 National Centre for Biotechnology Information (NCBI) BLAST hits of all the *Trebouxia* associated with *Protoparmelia* and generated a 1000 BS RAxML tree (Fig. S5).

Correlation between bioclimatic variables and symbiont association pattern

We extracted 19 bioclimatic variables for the localities of 168 (out of 174) *Protoparmelia* specimens (six samples were excluded from the analysis because of the absence of the spatial information) from the Worldclim database (http://www.worldclim.org) with a grid cell resolution of 2.5 min, using the software DIVA-GIS v.2 (Hijmans *et al.*, 2005). The bioclimatic variables represent annual and seasonal trends in temperature and precipitation (Hijmans *et al.*, 2005). The description of each variable is listed in the legend of Fig. 2. To examine the bioclimatic distribution of our samples and to identify the bioclimatic variables contributing the most to the total variance, we performed a principal component analysis (PCA) of the 19 bioclimatic variables, using the function PRCOMP in R 3.2.1 (R Development Core Team, 2011).

To test whether higher selectivity of tropical/subtropical fungi and algae is a phylogenetic artifact, that is, a result of closely related tropical/subtropical symbionts associating with fewer partners, we evaluated the statistical significance of the phylogenetic signal using the *K* statistic as implemented with the function *multiPhylosignal* of the R package PICANTE. The *K* statistic compares the observed signal in a trait to the signal under a Brownian motion model of trait evolution on a phylogeny. It ranges from 1, that is, strong phylogenetic signal and/or conservatism of traits, to 0, that is, random and/or convergent pattern of evolution. As no phylogenetic signal was detected (see the Results section), we evaluated the role of macroclimate in shaping fungal selectivity (i.e. number of possible photosynthetic partners) using standard (i.e. without phylogenetic control) multispecies generalized linear models (GLMs) using R. As an environmental proxy, we used both linear and quadratic values of

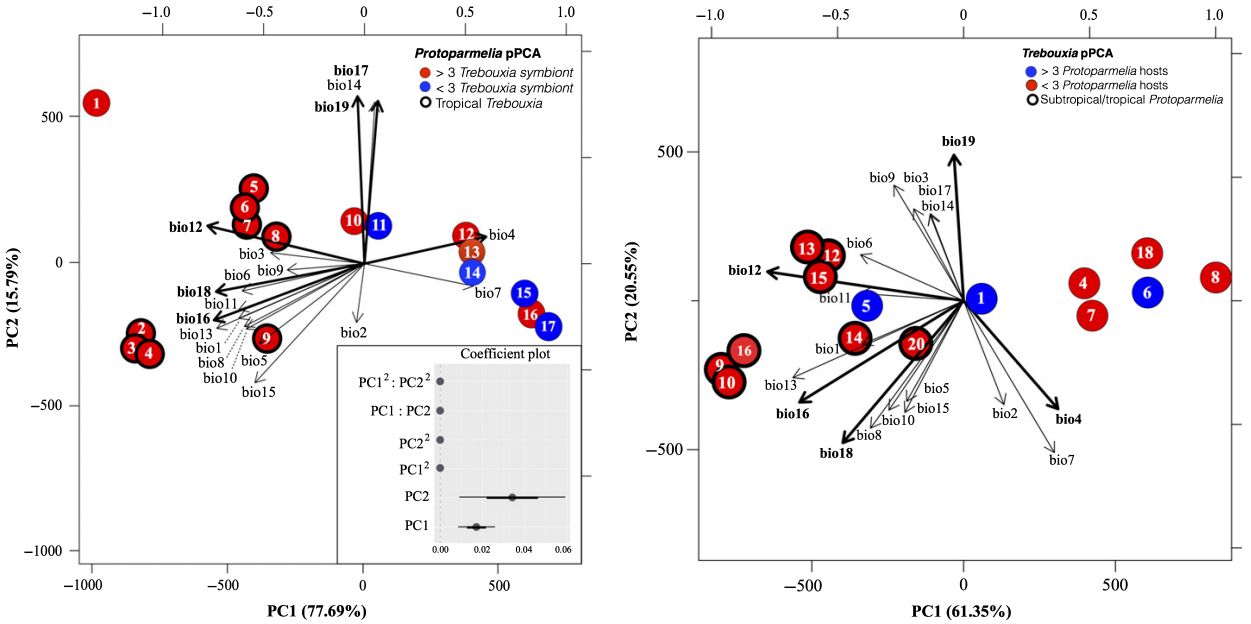

Fig. 2 Phylogenetic principal component analysis (pPCA) plot of axes 1 (horizontal) and 2 (vertical) from analysis of 19 BIOCLIM climate variables for *Protoparmelia* (left) and *Trebouxia* (right). Only species with more than three specimens were included in this analysis. On the left side is the phylogenetic PCA (pPCA) of *Protoparmelia* and on the right side is the *Trebouxia* pPCA. In the *Protoparmelia* pPCA, each circle represents a *Protoparmelia* species (1, *P. ochrococca*; 2, *P. orientalis*; 3, *P. corallifera*; 4, *P. isidiata D*; 5, *P. multifera*, 6, *P. isidiata B*; 7, *P. capitate*; 8, *P. isidiata A*; 9, *P. pulchra*; 10, *P. memnonia*; 11, *P. badia A*; 12, *P. hypotremella*; 13, *P. montagnei A*; 14, *P. badia B1*; 15, *P. badia C*; 16, *P. ZA*; 17, *P. montagnei C*). Blue circles, species associating with more than one alga; red circles, *Protoparmelia* species associating with only one alga. In the *Trebouxia* pPCA, the number in the circles represents the *Trebouxia* species. Blue circles, species associating with more than one *Protoparmelia*; red circles, *Trebouxia* species associating with only one Protoparmelia. Thick outlines of the circles represent tropical species. Bold arrows represent the variables contributing most to the total variance. Inset in the *Protoparmelia* pPCA is the coefficient plot for the relationship between number of algal symbionts and environmental proxies (i.e. PC1 and PC2, their quadratic values and their respective interaction terms) from the best fitting generalized linear model (GLM). PC2 and PC1 are strong predictors of the number of associated algal symbionts. The 19 BIOCLIM variables are: bio1, annual mean temperature; bio2, annual mean diurnal range (mean of the monthly temperature ranges (monthly maximum minus monthly minimum)); bio3, isothermality (variation in day-to-night temperatures relative to the variation in annual summer-to-winter temperatures); bio4, temperature seasonality (variation in temperature over a given year (or averaged years) based on the standard deviation (variation) of monthly temperature averages); bio5, maximum temperature of warmest month; bio6, minimum temperature of coldest month; bio7, annual temperature range (annual variation in temperature); bio8, mean temperature of wettest quarter; bio9, mean temperature of driest quarter; bio10, mean temperature of warmest quarter; bio11, mean temperature of coldest quarter; bio12, annual precipitation (sum of all total monthly precipitation values); bio13, precipitation of wettest month (total precipitation of the wettest month); bio14, precipitation of driest month (total precipitation during the wettest month); bio15, precipitation seasonality (ratio of the standard deviation of the monthly total precipitation to the mean monthly total precipitation); bio16, precipitation of wettest quarter (total precipitation of the wettest quarter); bio17, precipitation of driest quarter (total precipitation of the driest quarter); bio18, precipitation of warmest quarter (total precipitation of the warmest quarter); bio19, precipitation of coldest quarter (total precipitation of the coldest quarter).

the first two axes of the fungal phylogenetic PCA including all 19 bioclimatic variables in order to account for colinearity between covariates. To explicitly account for sampling bias, we incorporated sample counts into the models as model weights. The incorporation of the sampling bias is strongly advocated over the current practice of rarefying sample counts (McMurdie & Holmes, 2014). We tested two kinds of predictor sets, that is, one set consisting of linear principal component (PC) values and their interaction term, and one better accounting for collinearity in which we added the quadratic PC values and their interaction terms. These models were fitted using either a Poisson or a negative binomial (using the function GLM.NB in MASS, Venables & Ripley, 2002) error structure. The fit of the models was compared with AIC values. We then used ANOVA to evaluate the contribution and significance of the explanatory variables in the best performing model. Model coefficients were plotted using the R package COEFPLOT.

Analyses of phylogenetic congruence

We tested for congruence between fungal host and algal phylogenies using two global-fit methods, PARAFIT (Legendre *et al.*, 2002) and PACO (Balbuena *et al.*, 2013), both implemented in R, and an event-based method, JANE v.4 (Conow *et al.*, 2010). We selected these methods because they accept incompletely resolved phylogenies, multi-host associations, and unbalanced numbers of hosts and symbionts.

A cophylogenetic reconstruction scenario assumes that symbionts which spend part or all their life in or on their hosts track the phylogeny of their hosts (Fahrenholz, 1913). PACO

explicitly tests this hypothesis of congruence between two given topologies. Fungal host, which is the exhabitant in our case, and algal symbiont tree-based distance matrices were transformed by principal coordinates and the host–symbiont link matrix was converted into an identity matrix to account for multiple host–symbiont associations. Significance was assessed with a goodness-of-fit test based on 100 000 randomizations. The importance of each host–symbiont link was assessed by the associated squared residuals, which together with their 95% confidence intervals were estimated using a jackknife method.

PARAFIT uses the same matrices of symbiont distances as described above to test the global congruence between trees (Legendre *et al.*, 2002). In addition, PARAFIT can assess the contribution of each individual host−parasite association ('link') to this global congruence. Each fungal–algal association was tested for significance at $\alpha = 0.05$ using 9999 permutations.

Event-based methods such as implemented in JANE v.4 (Conow *et al.*, 2010) allow five host–symbiont cophylogenetic processes (cospeciation, host switch, duplication, loss or lineage sorting, and failure to diverge) to be disentangled. The analysis attributes a cost to each process or event, and aims to reconcile tree topologies of hosts and symbionts by adequately mixing events. The best reconstruction is the one that minimizes global costs. The significance of the global cost is assessed against a random distribution of costs generated using random trees. Global congruence between host and symbiont phylogenies is supported when the observed optimal cost is significantly lower than optimal costs computed from randomly generated trees. We used JANE v.4 with 23 generations and a population size of 45 for a total of *c.* 1000 iterations of the genetic algorithm. Twelve different cost models were used to find the minimum total cost (Table 2). To determine how changes to the parameter space affected the overall costs, we started from the default cost model of 0 for cospeciation, 1 for duplication of symbiont, 2 for duplication of symbiont and host switch, 1 for loss of symbiont, and 1 for failure to diverge. We then assigned the lowest cost to cospeciation while keeping the cost of host switch and duplication high (cost regimes A and D) to infer the probability of cospeciation over switch and duplication. We also (1) minimized the costs of different events while penalizing cospeciation or host switches (cost regimes B, F and G), (2) gave all events the same cost (cost regime C), and (3) rendered one of the events prohibitively expensive, giving it a cost of 10 each time (cost regimes H–L). All models were tested using random tip mapping and random parasite trees with 100 randomizations. The option 'Prevent
mid-polytomy' was selected to ensure that no evolutionary event was taking place along the short branches created to resolve eventual polytomies.

Table 2 Cost regimes used in the cophylogenetic analysis using JANE 4.0; the least costly scenario is indicated in bold

Cost regime	C-D-D+S-L-FD	C	D	D+S	L	FD	Cost
A	0-1-2-1-1	7	3	9	17	8	46
B	2-1-1-1-1	0	1	18	11	8	38
C	1-1-1-1-1	2	3	14	10	8	37
D	0-1-1-1-1	3	3	13	11	8	35
E	**1-0-0-1-1**	**0**	**1**	**18**	**11**	**8**	**19**
F	2-1-1-1-0	0	1	18	11	8	30
G*	2-1-1-0-0	0	2	17	17	8	19
H	10-1-2-1-1	0	3	16	11	8	54
I	0-1-10-1-1	9	8	2	36	8	72
J	0-10-10-1-1	9	1	9	22	8	130
K	0-1-2-10-1	2	3	14	10	8	139
L	0-1-2-1-10	7	3	9	17	8	118

*Not significant.
Events that are assigned a cost are: cospeciation (C), duplication (D), host switch (S), loss (L), and failure to diverge (FD).

Results

Sequencing and phylogenetic analyses

We generated 284 algal and 141 fungal sequences (Tables S1, S3). The sequences are deposited in GenBank (accession numbers: ITSf, KY066254–KY066279; nuLSU, KY066280–KY066323; mtSSU, KY012807–KY012840; *MCM7*, KY012796–KY012806; TSR1, KY012852-KY012867; *RPB1*, KY012841−KY012843 and KY012845−KY012851; *COX2*, KY051567–KY051726, and ITS algal, KY066324–KY066447).

We found high genealogical concordance between the nuclear ITS and mitochondrial data (Table S4). Furthermore, CADM results showed no significant incongruence between the two algal loci, and hence the data sets were concatenated ($W = 0.818$; $P = 0.0001$). The two-locus algal and six-locus fungal data sets yielded resolved and well-supported topologies (Figs S2, S6). We did not find supported topological differences between RAxML and MRBAYES trees. Therefore, only the ML tree based on the concatenated data set is presented (Figs S2, S6). The addition of extra samples to the published fungal phylogeny did not change the topology of the fungal tree and the concatenated fungal tree is concordant with Singh *et al.* (2015; Fig. S6).

Five out of 26 reference strains of *Trebouxia* from the SAG and UTEX collections grouped with high support within the *Trebouxia* associated with *Protoparmelia* s.str. (Fig. S1). All the boreal, arctic/alpine *Protoparmelia* species associated with *Trebouxia suecica*, that is, *Protoparmelia badia A*, *P. badia B1*, *P. badia B2*, *P. badia C* and *Protoparmelia memnonia*. In addition, seven distantly related boreal, arctic/alpine *Protoparmelia* s.l. species and four boreal, arctic/alpine *Miriquidica* species (Singh *et al.*, 2015), which form a monophyletic group with *Protoparmelia* s.l., were also found to be associated with *T. suecica* (Fig. S4). *Protoparmelia badia A* from Australia and Italy formed an association with *Trebouxia brindabellae* and *Trebouxia angustilobata*, respectively. All temperate *Protoparmelia* species, namely *Protoparmelia hypotremella*, *Protoparmelia ochrococca* and *Protoparmelia oleagina*, grouped with *Trebouxia simplex*. *Trebouxia* sp. *6* and *Trebouxia* sp. *7*, associated with the three Mediterranean *Protoparmelia* species (*Protoparmelia montagnei A*, *P. montagnei B* and *P. montagnei C*) and *P. badia C*, from supra-Mediterranean conditions in Spain were closely

related to *Trebouxia jamesii*. NCBI BLAST hits suggest that these clades may correspond to the *Trebouxia* clade VI from Muggia *et al.* (2008). Algae from the tropical *Protoparmelia* species did not group with any reference *Trebouxia* strain. *Trebouxia* sp. *15* associated with *P. capitata* has been previously reported as *Trebouxia usneae* (Bhattacharya *et al.*, 1996). *Trebouxia* sp. *16* associated with *P. isidiata D* is closely related to *Trebouxia* sp. OTU G04 from Leavitt *et al.* (2015). *Trebouxia* sp. *19* isolated from *P. isidiata E* is closely related to *Trebouxia* clade IV in Helms *et al.* (2001).

Species delimitation

Both BP&P and STACEY supported the 20 putative species as evolutionarily independent lineages (Table S4). Therefore, we considered 23 *Protoparmelia* species to be associated with 20 *Trebouxia* species for all the subsequent analyses.

Association patterns

We found no evidence for the presence of multiple photobiont lineages within a single fungal specimen. No ambiguous base calls were found in the algal sequence electropherograms from both the loci. *Protoparmelia* associated with one to three *Trebouxia* species whereas *Trebouxia* species accepted one to five *Protoparmelia* species. Fungi and algae displayed similar association patterns (Fig. 1). Both fungi and algae were more specific in warmer climates, associating mostly with a single partner. Interactions with more than one partner were more frequent in cooler climates for both partners. Some of the algae apparently have wide geographic distributions. For example, *T.* sp. *1* (*T. suecica*) is found in North America, Europe and Oceania, and *T.* sp. *5* (*T. simplex*) occurs in North America and Europe (Table 1; Fig. S4).

The boreal, arctic/alpine networks were most connected (connectance = 0.23), followed by the Mediterranean (connectance = 0.22) and the tropical networks (connectance = 0.1).

The NCBI BLAST hits of nrITS of algal species using a 97% threshold of pairwise identity showed *T.* sp. *1* (*T. suecica*), *T.* sp. *2*, *T.* sp. *5* (*T. simplex*), *T.* sp. *6*, *T.* sp. *7*, *T.* sp. *15* and *T.* sp. *17* to be shared by other species of lichenized fungi (at least three species). *Trebouxia* sp. *3* (*T. brindabellae*), *T.* sp. *4* (*T. angustilobata*), *T.* sp. *8*, *T.* sp. *11*, and *T.* sp. *14* were also shared but only by few (fewer than three species) other fungi. *Trebouxia* sp. *9*, *T.* sp. *10*, *T.* sp. *12*, *T.* sp. *13*, *T.* sp. *16*, *T.* sp. *18*, *T.* sp. *19* and *T.* sp. *20* were unique to *Protoparmelia* s.str. (Figs S1, S4, S5).

Correlation between bioclimatic variables and association pattern

From the fungal pPCA, we retained the first two PCs, which together explained 93.48% of the total variance in climate across the range of *Protoparmelia*–*Trebouxia* associations (Fig. 2). The first PC (horizontal axis PC1) explained 77.69% of the variance while the second PC (vertical axis PC2) explained 15.79% of the variance. Both PC1 and PC2 mainly reflected a precipitation and temperature seasonality gradient (Fig. 2).

We found no phylogenetic signal for the number of associated fungal hosts ($K = 0.061$; $P = 0.68$) and algal symbionts ($K = 0.258$; $P = 0.3$). As expected, the phylogenetic signal was strong for the environmental predictors of both algae and fungi (Table S5).

The best fitting generalized linear model of fungal selectivity weighted for disproportionate sampling was the one including both linear and quadratic PC values and their respective interaction terms using a Poisson error structure (Table S5). The ANOVA results showed significant effects of the environmental proxies on the number of associated algal symbionts (PC1: $z = 4.075$; $P < 0.001$; PC2: $z = 2.786$; $P = 0.005$; PC1 : PC2: $z = 3.474$; $P < 0.001$; PC2^2: $z = 2.532$; $P = 0.01$; see Fig. 2; Table S5). This indicates more selective *Protoparmelia* fungal hosts in warmer and wetter climates with lower seasonality.

Analyses of phylogenetic congruence

The cophylogenetic analyses were performed on a single topology as both the fungal and algal phylogenies were well supported. Global-fit tests (PARAFIT and PACO) supported concordance between fungal hosts and algal symbiont tree topologies (PARAFIT Global = 244.28; $P = 0.0002$; PACO m^2 global value = 1.01; $P < 0.0001$; Fig. S7), and rejected the null hypothesis of random association. Eighteen out of 28 (64.29%) individual fungus–alga links were significant based on both PARAFIT1 and PARAFIT2 values of $P < 0.05$ (Fig. S7). Thus, it is unlikely that correlations between fungal and algal genetic distances have arisen by chance.

Most cost scenarios tested in JANE v.4 supported significant congruence between fungal and algal phylogenies, with all random solutions being worse than the solution reconstructed by the program (Table 2), except for cost regime G (Table 2), which penalized cospeciation while it did not penalize loss and failure to diverge. Based on several cost regimes, we calculated the optimal number of each kind of event to minimize the total cost of the fungal–algal association. Among the significant reconstructions, cost regime E yielded the lowest overall cost. This cost regime assigned a lower cost to switches. Failure to diverge was inferred as the predominant event shaping the associations of the boreal, arctic/alpine, and temperate fungi and algae (Fig. 3), whereas host switch was inferred as the predominant event shaping the Mediterranean and tropical fungal algal associations. The role of these two events was so strong that, even when penalized with prohibitively high costs (cost regimes K and L), the solutions still proposed 10 to 17 losses and eight failure to speciate events.

Discussion

Genetic diversity of the algal symbionts in different climates

The algal diversity associated with subtropical, tropical, and Mediterranean *Protoparmelia* was comparable to that associated with the boreal, arctic/alpine and temperate *Protoparmelia* species (eight arctic/temperate fungal hosts associate with six *Trebouxia* species, as compared to 11 tropical fungal hosts associating with 11 *Trebouxia* species). This is in contrast to the hypothesis of

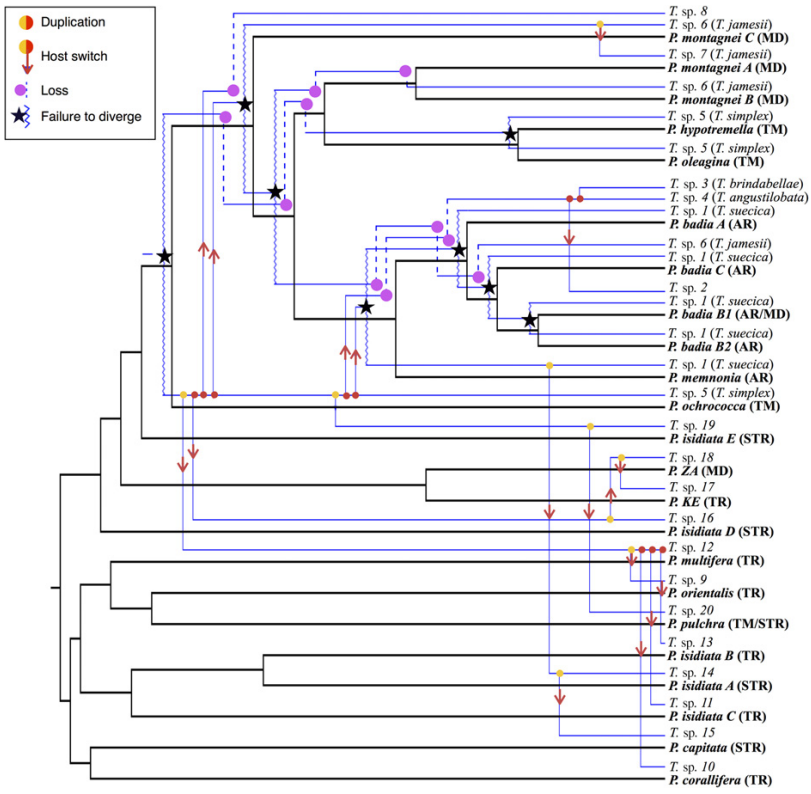

Fig. 3 Least costly cophylogenetic scenario between *Trebouxia* species and their *Protoparmelia* hosts, reconstructed using Jane 4.0. The cost regime settings were as follows: cospeciation = 1, duplication = 0, duplication with host switch = 0, losses = 1, failures to diverge = 0, corresponding to cost regime E (Table 2). Black branches, the fungal host phylogeny; blue branches, the algal symbiont phylogeny. Names of the fungal partner are indicated in bold. Yellow and red solid circles, duplications; dashed lines with purple circles, losses; dented lines with black asterisks, failures of the symbiont to diverge with its host. A yellow node indicates that there is another location of equal cost, and a red node means that all other locations it may be mapped to are of higher cost. A host switch is marked by a duplication, with a red arrow following the trajectory of the switching species. Habitat information is provided with the fungal species (AR, arctic/alpine or boreal; TM, temperate; MD, Mediterranean; STR, subtropical; TR, tropical).

higher symbiont diversity towards warmer climates which has been reported for several other symbiotic associations such as human pathogens (Guernier *et al.*, 2004), ectoparasites of marine fish (Rohde, 1978), and parasites of carnivorous mammals (Lindenfors *et al.*, 2007). Parasite diversity could also be influenced by the host geographic distribution (Dritschilo *et al.*, 1975; Price & Clancy, 1983; Gregory, 1990). However, in our study the host distribution range did not correspond with the algal symbiont diversity. For example, *P. badia A* has a cosmopolitan distribution but it associates with a single algal lineage everywhere (*T.* sp. *1*, i.e., *T. suecica*), except for the samples from Australia and Sardinia where it associates with two different algae. Similarly, *P. hypotremella* from three different countries associates with the same algal species (*T.* sp. *5*, i.e., *T. jamesii*). This has also been reported for example by Nunn *et al.* (2005) for the parasites of primates. The authors suggested latitude to be a better predictor of symbiont diversity.

Symbiont selectivity pattern in different habitats

In our study, the connectance was higher in the arctic/alpine regions as compared to the tropical regions. We found that selectivity of *Protoparmelia* is higher in the tropical regions as it associates with one to three *Trebouxia* species in the arctic/temperate regions and only one *Trebouxia* species in the tropical regions. Similarly, *Trebouxia* accepted one to five *Protoparmelia* species and several other lichen-forming fungi in the arctic/temperate regions (up to 70 other lichen-forming fungi) in contrast to only one *Protoparmelia* species and a few other lichen-forming fungi (fewer than three) in the tropical regions. However, the assessment of *Trebouxia* selectivity also relies on the data available in the public databases. As the number of studies on the arctic/temperate lichens outnumbers the studies on tropical lichens, information available on the photobionts from the tropical regions is comparatively scarce. This could make tropical *Trebouxia* species appear more specific than they actually are. Our results support lower selectivity of both *Protoparmelia* and *Trebouxia* in the arctic/temperate regions as compared to the tropical regions. More studies on the photobionts associating with the tropical lichen-forming fungi would be needed to confirm our results of high algal selectivity in the tropical regions.

The biotic diversity gradient is now established as a ubiquitous phenomenon with only a few exceptions (Hillebrand, 2004). The existence of a biotic specialization gradient across latitudes, however, is highly debated in the last decade and the number of studies reporting a biotic specialization gradient are comparable to the number of studies that found no variation in specialization across latitudes (Vázquez & Stevens, 2004; Moles & Ollerton, 2016). Several recent studies assessing biotic specialization across latitudes do not support the idea that interactions are generally stronger or more specialized in the tropics (Poore *et al.*, 2012; Schleuning *et al.*, 2012; Moles & Ollerton, 2016). Our study,

however, supports the traditional view of fewer partners and more specialized associations in the tropical regions as compared with the arctic/temperate regions.

Several studies suggested temperature differences across latitudes to be the driving force behind the variation in species diversity and interaction patterns (Wallace, 1878; Dobzhansky, 1937; Mittelbach *et al.*, 2007; Schemske, 2009). In our study, we found that climate is a strong predictor of the number of *Trebouxia* species associated with *Protoparmelia*. It has been proposed that, in the harsher and less predictable climatic conditions of the temperate regions, the primary selective pressures are abiotic factors which play a central role in adaptation and evolution (Wallace, 1878; Dobzhansky, 1937; Mittelbach *et al.*, 2007; Schemske *et al.*, 2009). Flexible partner choice and accepting locally adapted algae in alpine conditions could be considered as an adaptive strategy to survive the harsh environmental conditions (*Cetraria aculeata* (Fernández-Mendoza *et al.*, 2011) and *Xanthoparmelia* (Leavitt *et al.*, 2013); but see Blaha *et al.*, 2006; Muggia *et al.*, 2014). This could be a reason why *Protoparmelia* species are generalists in arctic/temperate regions.

Apart from the role of climate, it has also been suggested that phylogenetically older taxa might be more specialized because they have had more time to coadapt with their symbionts than generalist taxa (but see Colles *et al.*, 2009). In this regard, a recent study (Magain *et al.*, 2016) found that specialist cyanobacteria had longer branches (i.e. older taxa) as compared to generalist ones. In our study too, specialist *Protoparmelia–Trebouxia* have longer branches. Given that highly specialized symbioses are proposed to be more sensitive to environmental fluctuations (Dunn *et al.*, 2009), it is tempting to speculate that higher partner selectivity in the tropics may not only be the result of a recent adaptation to warmer climates but also an effect of longer times available for coadaptation. However, also according to this hypothesis, the role of climate in driving association patterns cannot be negated as the longer branches of tropical taxa could be a result of (1) an acceleratory effect of temperate on the mutation rate, and (2) more generations per year as a consequence of stable climatic conditions (Allen *et al.*, 2002, 2006; Schemske, 2009; Gillman & Wright, 2014; Oppold *et al.*, 2016).

Adaptive role of algae in lichens

Several studies suggest that lichen-forming fungi occupying similar habitats express their algal selectivity at the community level and share common, probably locally adapted photobionts (Rikkinen *et al.*, 2002; Dal Grande *et al.*, 2014b). In these communities, the photobiont associates with several fungi found in allopatry and is therefore a generalist species. This has been reported for lichen-forming fungi sharing green algal symbionts of the genera *Trebouxia* (Beck *et al.*, 1998; Kroken & Taylor, 2000), *Asterochloris* (Peksa & Skaloud, 2011), and *Dictyochloropsis* (Dal Grande *et al.*, 2014b). In our study, BLAST hits of the algae associated with boreal, arctic/alpine and temperate *Protoparmelia* species showed the cool-climate *Trebouxia* to be associated with several unrelated lichen-forming fungi occupying the same biomes (Fig. S4, S5). Thus, our study corroborates the hypothesis of environmental sharing of the photobionts in lichens especially in the colder boreal, arctic/alpine and temperate climates where the dry and cold, as well as fluctuating, climate is probably the major selective pressure. Several studies proposed the photobiont as an important functional trait of lichens, relevant for the response of the lichen to the environment, especially to humidity (Aptroot & van Herk, 2007; Marini *et al.*, 2011; Giordani *et al.*, 2012; Matos *et al.*, 2015). Thus, it is tempting to speculate that freeze tolerance in arctic/alpine and temperate environments, and desiccation and high-intensity light tolerance in Mediterranean environments are potentially a few such traits associated with the locally superior and adaptive algal genotypes. Supporting this hypothesis, lichen-forming fungi with wide ecological amplitude have been shown to have different photobionts in different habitats (Fernández-Mendoza *et al.*, 2011; Muggia *et al.*, 2013, 2014).

Potential events leading to fungal–algal cophylogenetic patterns

Association patterns and evolutionary events Highly selective interactions are coherent with tighter evolution, and cospeciation is more likely to occur in symbionts that are specialists rather than generalists (Giraud *et al.*, 2008; Agosta *et al.*, 2010; de Vienne *et al.*, 2013). The expectation of cospeciation is therefore higher in symbionts inhabiting warmer regions as a consequence of more selective interactions. However, in spite of this, no cospeciation was found in *Protoparmelia–Trebouxia* associations although the cophylogenetic analyses suggested significant congruence between fungal and algal phylogenies. Instead, failure to diverge, losses, and host switches to closely related hosts were found to be the main events leading to congruent fungal and algal phylogenies in the *Protoparmelia–Trebouxia* symbiosis. This confirms the reports from several recent studies showing cospeciation to be a rare event and instead failure to diverge and host switches to be more common processes shaping fungal algal associations, particularly in the case of environmentally transmitted symbionts (Longdon *et al.*, 2011; Susoy & Herrmann, 2014). Therefore, the congruence between phylogenies might simply be the result of host switches to closely related hosts (de Vienne *et al.*, 2007, 2013). This has been reported for the lichen parasite *Biatoropsis* (Millanes *et al.*, 2014), fungal parasites (Peterson *et al.*, 2010), and lice parasites of birds (Hughes & Page, 2007).

Climate and evolutionary mechanisms We found different mechanisms shaping fungus−alga associations in different macroclimatic regions. In general, for boreal, arctic/alpine and temperate *Protoparmelia–Trebouxia* species, failure to diverge was the major evolutionary driver, whereas for the tropical and Mediterranean species host switch was the main event leading to the congruent phylogenetic structure. Failure to diverge occurs when parasite populations maintain gene flow and survive despite their hosts diverging, leading to the formation of generalist symbiont species (Banks & Paterson, 2005).

Failure to diverge occurs mostly in parasite populations that occur on sympatric hosts (Banks & Paterson, 2005; de Vienne

et al., 2007, 2013), or in predominantly environmentally transmitted parasites (Peek *et al.*, 1998; Longdon *et al.*, 2011). Our study is in line with these findings, as arctic/temperate algae that failed to diverge with the fungal hosts were reported from several unrelated fungi occurring in the same biogeographic region. Furthermore, environmental transmission, where the newly dispersed germinating fungal spores take up their algal symbionts from the environment, was suggested to play a key role in *Trebouxia* dispersal in natural populations (Dal Grande *et al.*, 2014a).

Our best cophylogenetic reconstruction suggested fungal host switches by the algae as the main event shaping the Mediterranean and subtropical/tropical *Protoparmelia*–Trebouxia associations. Host switches followed by specialization have been linked to bursts of species diversification (Roy, 2001; Fordyce, 2010; de Vienne *et al.*, 2013; Millanes *et al.*, 2014). For example, Millanes *et al.* (2014) suggested host switching as the main reason for the diversification of the lichenicolous fungi *Biatoropsis* associated with *Usnea* species. The one-to-one associations found in the tropics could thus be the result of frequent host switches. We also found some symbiont loss/extinctions for *Protoparmelia*–*Trebouxia* symbiosis. This has been extensively reported in host−parasite associations, probably as a consequence of the inability of the parasites to survive in small populations of the emerging new host species (Ronquist, 1997; de Vienne *et al.*, 2013; Millanes *et al.*, 2014).

Our study highlights the importance of climate in driving the diversification of lichenized algae, thus determining fungal−algal association patterns. Furthermore, we showed that, apart from climate, other processes such as host switches and failure to diverge might also be involved in driving symbiont diversity and association patterns in this lichen group.

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Author contributions

I.S., F.D.G. and G.S. planned and designed the study; G.S. and F.D.G. analyzed the data; G.S., F.D.G. and I.S. interpreted the data; G.S. and J.O. performed the experiments; P.K.D., A.C., F.D.G. and G.S. conducted fieldwork and contributed samples; G.S. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 ITS gene tree of algae associated with *Protoparmelia* s.str. and 30 reference ITS sequences representing 26 *Trebouxia* species from the SAG and UTEX collections.

Fig. S2 Phylogeny of photobionts associated with *Protoparmelia* based on a concatenated two-locus data set including ITS and *COX2* sequences.

Fig. S3 *BEAST species trees for photobionts associated with *Protoparmelia*.

Fig. S4 Association network based on algal ITS data, given a 97% similarity BLASTN threshold.

Fig. S5 Maximum likelihood tree (1000 BS) inferred from the ITS sequences of the photobionts associated with *Protoparmelia*

and the first 100 NCBI BLAST hits of all the *Trebouxia* species associated with *Protoparmelia*.

Fig. S6 Phylogeny of *Protoparmelia* based on a concatenated six-locus data set including ITS, nuLSU, mtSSU, *MCM7*, *TSR1* and *RPB1* sequences.

Fig. S7 Boxplot of the jacknifed squared residuals with upper 95% confidence intervals (error bars) associated with each host–symbiont link from PACO.

Table S1 Specimens used in this study including voucher information and GenBank accession numbers

Table S2 Primers used in this study

Table S3 Genetic characteristics of the loci used in the study

Table S4 Genealogical concordance between nuclear ITS and mitochondrial *COX2*, and the posterior probabilities of species as suggested by BP&P

Table S5 Results of AIC model comparison analysis and summary statistics of the best fitting model

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New Phytologist Supporting Information

Article title: **Fungal-algal association patterns in lichen symbiosis linked to macroclimate.**

Authors: Garima Singh, Francesco Dal Grande, Pradeep K. Divakar, Jürgen Otte, Ana Crespo, Imke Schmitt

Article acceptance date: 19 October 2016

The following Supporting Information is available for this article:

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Table S1. Specimens used in this study including voucher information and GenBank accession numbers.

Samples	Sample code	Voucher info	*COX2*	ITSA	MTSSU	ITS	nuLSU	*RPB1*	*MCM7*	*TSR1*
Protoparmelia badia A	AT1	Austria (46.78055, 14.97083), Hafellner, Muggia, Hafellner 68478 (GZU)	**KY051567**	**KY066324**	KP822401	KF562191	KF562183	KF601237	KF562174	n/a
	SI1	Slovenia (46.652, 15.06361), Hafellner 71474 (GZU)	**KY051568**	n/a	KP822402	KP822209	KP796259	KP822068	KP822316	KP823460
	AT2	Austria (46.92916, 15.05972), Hafellner 71686 (GZU)	**KY051569**	**KY066325**	n/a	n/a	KP796260	KP822069	n/a	KP823461
	US1	USA (44.3, -122.86), McCune 27712 (OSU)	**KY051570**	n/a	n/a	n/a	KP796261	KP822070	KP822317	KP823462
	CZ1	Czech Republic (49.08472, 13.5111), Palice 15024 (ASCR)	**KY051571**	**KY066326**	KP822404	n/a	KP796262	n/a	n/a	KP823463
	CZ2	Czech Republic (50.7516, 15.53166), Malíček, Palice, Printzen, Steinová, Syrovátková 12051 (ASCR)	**KY051572**	n/a	n/a	KP822212	n/a	KP822071	KP822318	KP823464
	US6	**USA (45.928, -68.905), Fryday 8575, MSC0108415 (MSC)**	**KY051573**	n/a	**KY012807**	**KY066254**	**KY066280**	**KY012841**	n/a	**KY012852**
	US2	USA (45.92916, -68.91416), Fryday 8579, MSC0108416 (MSC)	**KY051574**	n/a	KP822403	n/a	KP796263	n/a	KP822319	n/a
	NO1	Norway (63.6511, 9.4284), Haugan 9779, O-L168485 (O)	**KY051575**	n/a	n/a	n/a	KP796264	n/a	KP822320	KP823465
	NO13	**Norway (61.542 8.66316), Haugan 8617, O-L161444 (O)**	**KY051576**	**KY066327**	**KY012808**	n/a	**KY066281**	**KY012842**	n/a	**KY012853**
	NO2	Norway (61.542 8.66316), Haugan 8120, O-L160502 (O)	**KY051577**	**KY066328**	n/a	n/a	KP796265	n/a	KP822321	KP823466
	NO14	**Norway (62.3747, 10.0312), Haugan No. ein48-2, O-L142057 (O)**	**KY051578**	**KY066329**	**KY012809**	**KY066255**	**KY066282**	n/a	n/a	**KY012854**
	NO3	Norway (59.362, 10.9853), Petter bpl-L7043, O-L77778 (O)	**KY051579**	n/a	KP822406	KP822213	KP796266	KP822072	n/a	KP823467
	AU3	Australia (-36.42472, 148.3772), Elix 43267, 00803551 (CANB)	**KY051580**	**KY066330**	n/a	KP822214	n/a	n/a	**KY012796**	KP823468
	AU1	Australia (-41.75, 146.7), Kantvilas 53/09, 550225 (HO)	**n/a**	**KY066331**	KP822407	KP822215	n/a	n/a	n/a	KP823469
	NO4	Norway (70.1252, 29.0574), Holien 12730, L-13936 (TRH)	**KY051581**	**KY066332**	KP822409	KP822217	KP796267	KP822073	KP822322	KP823471
	NO5	Norway (64.8714, 13.2265), Holien 11762, L-12476 (TRH)	**KY051582**	n/a	KP822410	KP822218	KP796268	KP822074	KP822323	KP823472
	NO6	Norway (70.1176, 29.2821), Bratli 7953, L-175593 (O)	**KY051583**	**KY066333**	KP822411	KP822219	KP796269	KP822075	KP822324	KP823473
	NO7	Norway (70.0631, 29.8239), Bratli 7966, L-175606 (O)	**KY051584**	n/a	KP822412	KP822220	KP796270	KP822076	KP822325	n/a
	NO8	Norway (70.4282, 30.728), Bratli 7959, L-175599 (O)	**KY051585**	**KY066334**	n/a	KP822221	KP796271	KP822077	n/a	n/a
	NO15	**Norway (69.97283, 23.11383), Tønsberg 38629, L-92437 (BG)**	**KY051586**	**KY066335**	n/a	**KY066256**	n/a	**KY012843**	n/a	**KY012855**
	NO9	Norway (65.1715, 13.39816), Tønsberg 41335, L-92560 (BG)	**KY051587**	**KY066336**	KP822413	n/a	KP796272	KP822078	n/a	n/a
	NO10	Norway (60.92716 6.287), Tønsberg 38409, L-85832 (BG)	**KY051588**	n/a	KP822414	KP822222	KP796273	KP822079	n/a	KP823474
	NO11	Norway (70.20816, 22.08483), Tønsberg 38628, L-92432 (BG)	**KY051589**	n/a	KP822415	n/a	KP796274	KP822080	n/a	n/a
	NO12	Norway (65.1255 13.4353), Tønsberg 41001, L-92501 (BG)	**KY051590**	**KY066337**	n/a	KP822223	KP796275	KP822081	n/a	n/a
	US3	USA (64.9604, -148.383133), Spribille 27680 (GZU)	**KY051591**	**KY066338**	n/a	KP822224	KP796276	KP822082	n/a	n/a
	US4	USA: Montana, Spribille 20996 (GZU)	**KY051592**	n/a	n/a	n/a	KP796277	KP822083	n/a	KP823475
	US5	USA: Montana, Spribille 21119 (GZU)	**KY051593**	**KY066339**	n/a	KP822225	**KP796278**	KP822084	n/a	n/a
	CA1	Canada: British Columbia, Spribille 29693 (GZU)	**KY051594**	n/a	n/a	KP822226	KP796279	n/a	**KY012797**	n/a
	CA2	Canada: Yukon Territory, Spribille 28408 (GZU)	**KY051595**	**KY066340**	n/a	KP822227	KP796280	n/a	**KY012798**	n/a
	ES1	Spain (42.254194, -2.975753), Crespo, Del-Prado 10524 (MAF)	**KY051596**	**KY066341**	KP822416	n/a	KP796281	KP822085	KP822326	n/a
	PT1	Portugal (40.325, -7.60735), Crespo, Divakar, Rico, Ruibal, Alors, MAF-Lich 19441 (MAF)	**KY051597**	**KY066342**	n/a	KP822228	KP796282	KP822086	KP822327	n/a
	PT2	Portugal (40.325, -7.60735), Crespo, Divakar, Rico, Ruibal, Alors, MAF-Lich 19442 (MAF)	**KY051598**	**KY066343**	KP822417	KP822229	KP796283	KP822087	n/a	KP823476
	PT3	Portugal (40.325, -7.60735), Crespo, Divakar, Rico, Ruibal, Alors, MAF-Lich 19443 (MAF)	**KY051599**	**KY066344**	n/a	KP822230	KP796284	n/a	KP822328	n/a
	PT4	Portugal (40.325, -7.60735), Crespo, Divakar, Rico, Ruibal, Alors, MAF-Lich 19444 (MAF)	**KY051600**	**KY066345**	KP822418	KP822231	KP796285	KP822088	KP822329	KP823477
	PT5	Portugal (40.325, -7.60735), Crespo, Divakar, Rico, Ruibal, Alors, MAF-Lich 19445 (MAF)	**KY051601**	**KY066346**	n/a	KP822232	KP796286	KP822089	KP822330	KP823478
	PT6	Portugal (40.325, -7.60735), Crespo, Divakar, Rico, Ruibal, Alors, MAF-Lich 19446 (MAF)	**KY051602**	**KY066347**	n/a	KP822233	KP796287	KP822090	KP822331	KP823479
	ES2	Spain (40.79346, -3.98703), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19449 (MAF)	**KY051603**	**KY066348**	KP822419	KP822234	KP796288	KP822091	KP822332	n/a

	ES3	Spain (40.79346, -3.98703), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19450 (MAF)	**KY051604**	**KY066349**	KP822420	KP822235	KP796289	KP822092	KP822333	KP823480
	ES4	Spain (40.79346, -3.98703), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19451 (MAF)	**KY051605**	**KY066350**	KP822421	KP822236	KP796290	KP822093	n/a	**KY012856**
	ES5	Spain (40.79346, -3.98703), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19452 (MAF)	**KY051606**	**KY066351**	KP822422	KP822237	KP796291	KP822094	n/a	n/a
	ES6	Spain (40.79346, -3.98703), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19453 (MAF)	**KY051607**	**KY066352**	KP822423	**KY066257**	KP796292	KP822095	KP822334	KP823481
	ES7	Spain (40.79346, -3.98703), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19454 (MAF)	**KY051608**	**KY066353**	KP822424	KP822238	KP796293	KP822096	n/a	**KY012857**
	ES8	Spain (42.25772, -2.99372), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19455 (MAF)	**KY051609**	**KY066354**	KP822425	n/a	KP796294	KP822097	KP822335	KP823482
	ES9	Spain (42.25772, -2.99372), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19456 (MAF)	**KY051610**	n/a	KP822426	KP822239	KP796295	KP822098	n/a	KP823483
	ES10	Spain (42.25772, -2.99372), Crespo, Rico, Ruibal, Boluda, MAF-Lich 19457 (MAF)	**KY051611**	**KY066355**	n/a	KP822240	KP796296	KP822099	KP822336	KP823484
	NZ	New Zealand (-45.34738, 3.7352), Printzen FR-0217382 (FR)	**KY051612**	**KY066356**	KP822427	**KY066258**	KP796297	n/a	n/a	KP823485
Protoparmelia badia B1	ES12	Spain (40.529464, -1.6505), Rico, Vivas, MAF-Lich 16830 (MAF)	**KY051613**	n/a	n/a	KP822241	KP796298	KP822101	n/a	KP823486
	ES1	Spain (40.5133, -6.17), Boom 46079 (herb. v.d. Boom)	**KY051614**	n/a	KP822428	KP822242	KP796299	KP822102	KP822337	KP823487
	ES14	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19416 (MAF)	**KY051615**	**KY066357**	n/a	KP822243	KP796300	KP822103	n/a	KP823488
	ES15	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19417 (MAF)	**KY051616**	**KY066358**	KP822429	n/a	KP796301	KP822104	n/a	KP823489
	ES16	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19418 (MAF)	**KY051617**	**KY066359**	KP822430	KP822244	KP796302	KP822105	n/a	KP823490
	ES17	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19419 (MAF)	**KY051618**	**KY066360**	KP822431	KP822245	KP796303	KP822106	n/a	KP823491
	ES18	Spain (41.78813, -1.83868), Crespo, Divakar, Dal Grande, MAF-Lich 19420 (MAF)	**KY051619**	**KY066361**	n/a	KP822246	KP796304	KP822107	n/a	KP823492
	ES19	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19421 (MAF)	**KY051620**	**KY066362**	KP822432	KP822247	KP796305	KP822108	n/a	KP823493
	ES21	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19423 (MAF)	**KY051621**	**KY066363**	KP822434	KP822248	KP796307	KP822110	n/a	KP823495
	ES23	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19426 (MAF)	**KY051622**	**KY066364**	KP822435	n/a	KP796308	n/a	n/a	KP823496
	ES24	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19425 (MAF)	**KY051623**	n/a	KP822436	KP822249	KP796309	n/a	n/a	KP823497
	ES25	Spain (37.2147, -2.52108), Divakar, Dal Grande, MAF-Lich 19424 (MAF)	**KY051624**	n/a	KP822437	KP822250	KP796310	n/a	n/a	KP823498
	IT1	Italy (40.8524, 9.1732), Dal Grande, Singh, Mount Limbara FR-0068881 (FR)	**KY051625**	**KY066365**	KP822438	KP822251	KP796311	KP822111	KP822338	KP823499
	IT2	Italy (40.8573, 9.1642), Dal Grande, Singh, Mount Limbara FR-0068882 (FR)	**KY051626**	**KY066366**	KP822439	KP822252	KP796312	KP822112	KP822339	KP823500
Protoparmelia badia B2	ES13	Spain (38.5337 -1.00), Crespo, Divakar, Dal Grande MAF-Lich 19415 (MAF)	**KY051627**	n/a	n/a	KP822254	**KP796314**	**KP822113**	n/a	n/a
	ES22	Spain (37.2147, -2.52108), Divakar, Dal Grande MAF-Lich 19583 (MAF)	**KY051628**	n/a	KP822441	KP822255	KP796315	KP822114	n/a	n/a
Protoparmelia badia C	ES26	Spain (40.72248, -3.7352), Crespo, Rico, Ruibal MAF-Lich 19447 (MAF)	**KY051629**	**KY066367**	n/a	KP822256	KP796316	KP822115	n/a	KP823503
	ES27	Spain (40.72248, -3.7352), Crespo, Rico, Ruibal MAF-Lich 19448 (MAF)	**KY051630**	**KY066368**	n/a	n/a	KP796317	KP822116	n/a	KP823504
	ES28	Spain (40.72248, -3.7352), Crespo, Rico, Ruibal MAF-Lich 19432 (MAF)	**KY051631**	**KY066369**	KP822442	KP822257	KP796318	n/a	KP822341	KP823505
	ES29	Spain (40.72248, -3.7352), Crespo, Rico, Ruibal MAF-Lich 19433 (MAF)	**KY051632**	**KY066370**	n/a	KP822258	KP796319	n/a	KP822342	KP823506
	ES30	Spain (40.72248, -3.7352), Crespo, Rico, Ruibal MAF-Lich 19434 (MAF)	**KY051633**	**KY066371**	KP822443	KP822259	KP796320	n/a	KP822343	KP823507
	ES34	**Spain (40.72248, -3.7352), Crespo, Rico, Ruibal MAF-Lich 19436 (MAF)**	**KY051634**	**KY066372**	**KY012810**	**KY066259**	n/a	n/a	n/a	**KY012858**
	ES31	Spain (40.86899, -3.76285), Crespo, Rico, Ruibal, Boluda MAF-Lich 19437 (MAF)	**KY051635**	n/a	KP822444	KP822260	KP796321	n/a	KP822344	KP823508
	ES32	Spain (40.72248, -3.7352), Crespo, Rico, Ruibal, Boluda MAF-Lich 19438 (MAF)	**KY051636**	n/a	KP822445	KP822261	KP796322	n/a	n/a	KP823509
Protoparmelia capitata	US1	USA (32.41694, -82.06917), Lendemer 21761, NY-1104334 (NY)	**KY051637**	**KY066373**	KP822446	n/a	KP796323	KP822121	n/a	n/a
	US5	**USA (20.4122 -75.838), Lendemer 9202, NY-1024544 (NY)**	**KY051638**	**KY066376**	**KY012811**	n/a	**KY066283**	n/a	n/a	n/a
	US4	USA (31.10277, -87.39416), Lendemer 9164, NY-1054070 (NY)	**KY051639**	**KY066377**	**KY012812**	n/a	KP796325	KP822123	**KY012799**	n/a
	US6	USA (32.4169, -82.069), S. Beeching s.n., NY-1046116 (NY)	**KY051640**	n/a	**KY012813**	n/a	**KY066284**	n/a	n/a	n/a
	CU1	Cuba (20.4122 -75.838), Buck-55885, NY-1149527 (NY)	**KY051641**	**KY066374**	KP822447	n/a	KP796324	KP822122	KP822345	n/a
	CU2	Cuba (20.463, -75.837), Buck-55895, NY-1149537 (NY)	**KY051642**	n/a	**KY012814**	n/a	**KY066285**	n/a	n/a	n/a

	US7	USA (31.024, -87.681), Lendemer-9017, NY-1024542 (NY)	**KY051643**	**KY066375**	**KY012815**	n/a	**KY066286**	n/a	n/a	n/a
	BR1	Brazil (-30.083, -51), Cáceres & Aptroot C2A 22138 (ISE)	**KY051644**	n/a	**KY012816**	n/a	**KY066287**	n/a	n/a	n/a
	BR2	Brazil (-30.083, -51), Cáceres & Aptroot C2A 22207 (ISE)	**KY051645**	n/a	**KY012817**	n/a	**KY066288**	n/a	n/a	n/a
	BR3	Brazil (-10.75, -37.37), Cáceres 7395 (ISE)	**KY051646**	n/a	**KY012818**	n/a	**KY066289**	n/a	n/a	n/a
	BR4	Brazil (-10.75, -37.37), Cáceres 7946 (ISE)	**KY051647**	**KY066378**	**KY012819**	n/a	**KY066290**	n/a	n/a	n/a
Protoparmelia corallifera	TH2	Thailand (16.716, 104.716), Papong 7022 (MSUT)	**KY051648**	n/a	n/a	KP822262	KP796326	KP822124	n/a	KP823510
	TH1	Thailand (16.716, 104.716), Papong 6984 (MSUT)	**KY051649**	**KY066380**	KP822448	KP822263	KP796327	KP822125	KP822346	KP823511
	TH4	Thailand (16.716, 104.716), Papong 6483 (MSUT)	**KY051650**	**KY066381**	n/a	KP822264	KP796328	KP822126	n/a	KP823512
	TH5	**Thailand (16.716, 104.716), Papong, Konhin & Papong-6601pp, HO 554585 (HO)**	**KY051651**	**KY066379**	**KY012820**	**KY066260**	**KY066291**	n/a	n/a	n/a
	TH7	Thailand (16.766665, 104.716667), Papong 7100, MSUT-Li-1010 (MSUT)	**KY051652**	n/a	**KY012821**	n/a	**KY066292**	n/a	n/a	n/a
	TH6	**Thailand (16.716, 104.716), Papong 7101, MSUT-Li-1011 (MSUT)**	**KY051653**	n/a	n/a	**KY066261**	n/a	**KY012845**	n/a	**KY012859**
	TH3	Thailand (16.76666, 104.7166), Papong 7102, MSUT-Li-1012 (MSUT)	**KY051654**	n/a	KP822449	n/a	KP796329	KP822127	KP822347	KP823513
Protoparmelia hypotremella	CA1	Canada (45.3038 -81.61194), Lendemer 14562, NY-1049774 (NY)	**KY051655**	n/a	KP822453	n/a	KP796333	n/a	KP822352	n/a
	CA3	Canada (45.3038 -81.61194), Lendemer 14431B, NY-1049715 (NY)	**KY051656**	**KY066384**	n/a	KP822268	KP796335	n/a	KP822354	KP823516
	CA4	Canada (45.3038 -81.61194), Lendemer 14563, NY-1049772 (NY)	**KY051657**	**KY066385**	KP822455	KP822269	KP796336	KP822133	n/a	KP823517
	CA5	Canada (47.03305, -80.0425), Brodo 32443, CANL 123107 (CANL)	**KY051658**	**KY066383**	KP822456	n/a	KP796337	KP822134	n/a	KP823518
	SK1	Slovakia (48.77472, 20.09747), Bouda, Černajová, Maliček, Palice 14347 (ASCR)	**KY051659**	**KY066382**	KP822457	KP822270	KP796338	KP822135	n/a	**KY012860**
	NL1	Netherlands: Prov. Utrecht Leusden, Den Treck, Aproot, Aproot 72589 (ABL)	**n/a**	**KY066386**	n/a	n/a	KP796339	n/a	KP822355	KP823519
Protoparmelia isidiata A	US1	USA (31.4472, -81.275), Lendemer 20727, NY-1149936 (NY)	**KY051660**	**KY066388**	KP822458	n/a	KP796340	KP822137	n/a	n/a
	US2	USA (31.4472, -81.275), Lendemer 20745, NY-1149920 (NY)	**KY051661**	**KY066389**	KP822459	n/a	**KY066293**	KP822138	n/a	n/a
	US4	USA (31.433, -81.2361), Lendemer 20992, NY-1152323 (NY)	**KY051662**	n/a	KP822461	n/a	**KY066294**	KP822140	n/a	n/a
	US5	USA (29.73, -82.8), Harris 31685, NY-1024517 (NY)	**KY051663**	n/a	**KY012822**	n/a	**KY066295**	n/a	n/a	n/a
	US6	USA (29.73, -82.76), Harris 31755 NY-1024518 (NY)	**KY051664**	n/a	**KY012823**	n/a	**KY066296**	n/a	n/a	n/a
	US7	USA (28.35, -80.93), Harris 37494, NY-1024520 (NY)	**n/a**	**KY066392**	**KY012824**	n/a	**KY066297**	n/a	n/a	n/a
	US8	USA (31.4499, -81.2638), Lendemer 20645, NY-1149867 (NY)	**KY051665**	**KY066387**	**KY012825**	n/a	**KY066298**	n/a	n/a	n/a
	US9	USA (31.4499, -81.2638), Lendemer 20688, NY-1153126 (NY)	**KY051666**	n/a	**KY012826**	n/a	**KY066299**	n/a	n/a	n/a
	US3	USA (31.506, 3 -81.24999), Lendemer 20903, NY-1150773 (NY)	**KY051667**	n/a	KP822460	n/a	**KY066300**	n/a	n/a	n/a
	US11	USA (31.449, 3 -81.2638), Lendemer 20955, NY-1152377 (NY)	**n/a**	**KY066390**	**KY012827**	n/a	**KY066301**	n/a	n/a	n/a
	US12	USA (29.4999, -82.5666), Harris 29298, NY-1024519 (NY)	**KY051668**	**KY066391**	**KY012828**	n/a	**KY066302**	n/a	n/a	n/a
	US13	USA (28.8899, -81.4616), Lendemer 15842, NY-1079560 (NY)	**KY051669**	n/a	**KY012829**	n/a	**KY066303**	n/a	n/a	n/a
	US14	USA (29.86, -83.6), Buck 31151, NY-1024516 (NY)	**KY051670**	**KY066393**	**KY012830**	n/a	**KY066304**	n/a	n/a	n/a
Protoparmelia isidiata B	BR2	Brazil (-22.8858, -48.498), Caceres, Aproot, Aproot 13673 (ABL)	**KY051671**	**KY066394**	KP822463	KP822272	KP796343	n/a	n/a	n/a
	BR3	Brazil (-22,8858 -48.498), Aproot 21684 (ISE)	**KY051672**	**KY066395**	**KY012831**	n/a	**KY066305**	n/a	n/a	n/a
	BR4	Brazil (-30.083, -51), Cáceres & Aptroot 21648 (ISE)	**KY051673**	**KY066396**	**KY012832**	**KY066262**	**KY066306**	n/a	n/a	n/a
	BR5	Brazil (-30.083, -51), Cáceres & Aptroot C2A 22137 (ISE)	**KY051674**	**KY066397**	**KY012833**	n/a	**KY066307**	n/a	n/a	n/a
Protoparmelia isidiata C	TH1	Thailand (18.9083, 98.863), Boom 46872 (herb. v.d. Boom)	**KY051675**	**KY066398**	KP822467	KP822277	KP796346	n/a	n/a	KP823524
	TH2	Thailand (18.9083, 98.863), Boom 46947 (herb. v.d. Boom)	**KY051676**	**KY066399**	KP822468	KP822278	KP796347	KP822143	n/a	KP823525
Protoparmelia isidiata D	AU1	Australia (-12.61138, 131.10083), Elix 39805, CANB-00783260 (CANB)	**KY051677**	n/a	KP822464	KP822274	KP796345	KP822145	KP822359	KP823521
	AU2	Australia (-12.61138, 131.10083), Elix 39795, CANB-00783253 (CANB)	**KY051678**	n/a	n/a	n/a	KP796344	KP822144	KP822358	n/a
	AU3	**Australia (-12.61138, 131.10083), Elix 39792, CANB 00783251 (CANB)**	**KY051679**	**KY066402**	n/a	n/a	n/a	**KY012846**	**KY012800**	**KY012861**

	AU4	**Australia (-12.61138, 131.10083), Elix 39793, CANB 00783252 (CANB)**	**KY051680**	**KY066403**	n/a	n/a	n/a	**KY012847**	**KY012801**	**KY012862**
	AU6	**Australia (-12.61138, 131.10083), Elix 39818, CANB 00783268 (CANB)**	**KY051681**	n/a	n/a	**KY066263**	**KY066308**	**KY012849**	n/a	n/a
	AU5	**Australia (-12.61138, 131.10083), Elix 39804, CANB 00783259 (CANB)**	**KY051682**	**KY066404**	n/a	**KY066264**	n/a	**KY012848**	**KY012802**	n/a
	AU7	**Australia (-13.099 130.784), Elix 38202, CANB 00800762 (CANB)**	**n/a**	**KY066400**	n/a	**KY066265**	**KY066309**	n/a	n/a	n/a
	AU8	**Australia (-13.099 130.784), Elix 38207, CANB 00800763 (CANB)**	**n/a**	**KY066401**	n/a	**KY066266**	**KY066310**	n/a	n/a	n/a
Protoparmelia isidiata E	AU3	Australia (-37.4144, 149.813), Kantvilas 228/10, HO-559228 (HO)	**KY051683**	**KY066405**	KP822465	KP822275	n/a	KP822146	n/a	KP823522
	AU4	Australia (-13.62305, 131.611), Kantvilas 289/07, HO-545660 (HO)	**KY051684**	**KY066406**	KP822466	KP822276	n/a	n/a	n/a	KP823523
Protoparmelia KE	KE1	Kenya (-1.033, 38.33), Kirika, Lumbsch EA-3821 (EA)	**n/a**	**KY066407**	KP822469	KP822279	KP796348	KP822148	n/a	KP823526
	KE2	Kenya (-1.033, 38.33), Kirika, Lumbsch s.n. (EA)	**n/a**	**KY066408**	n/a	KP822280	n/a	KP822149	n/a	KP823527
Protoparmelia memnonia	NO1	Norway (63.8011, 9.7102), Haugan 9612, O-L167013 (O)	**KY051685**	n/a	KP822473	KF562194	KF562186	KF601240	KF562177	KP823529
	NO3	Norway (63.5249, 10.8929), Holien 13370, L-14269 (TRH)	**n/a**	**KY066409**	KP822474	KP822282	KP796352	KP822153	KP822362	KP823530
	NO2	Norway (64.291, 10.9792), Holien 12787, L-13935 (TRH)	**KY051686**	**KY066410**	KP822475	n/a	n/a	KP822154	KP822363	KP823531
Protoparmelia montagnei A	TR1	Turkey (40.21667, 26.7), Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19464 (MAF)	**KY051687**	**KY066411**	n/a	n/a	KP796353	KP822155	KP822364	n/a
	TR2	Turkey (40.21667, 26.7), Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19465 (MAF)	**KY051688**	**KY066412**	n/a	KP822283	KP796354	KP822156	n/a	KP823532
	TR5	Turkey (40.21667, 26.7), Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19468 (MAF)	**KY051689**	**KY066413**	n/a	KP822285	KP796357	KP822159	n/a	KP823534
	TR6	Turkey (40.21667, 26.7), Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19469 (MAF)	**KY051690**	**KY066414**	KP822480	KP822286	KP796358	KP822160	KP822366	n/a
Protoparmelia montagnei B	ES2	Spain (28.02497, -15.58775), Crespo, Cubas, Santo, Divakar, MAF-Lich 19458, Specimen 1 (MAF)	**KY051691**	**KY066415**	n/a	n/a	KP796360	KP822162	KP822367	KP823536
	ES3	Spian (28.02497, -15.58775), Crespo, Cubas, Santo, Divakar, MAF-Lich 19458, Specimen 2 (MAF)	**KY051692**	**KY066416**	n/a	n/a	KP796361	KP822163	KP822368	KP823537
Protoparmelia montagnei C	**ES9**	Spain (36.852342, -2.046172), Crespo, Cubas, Nuñez, Divakar MAF-Lich 19461 (MAF)	**KY051693**	**KY066417**	n/a	n/a	**KY066311**	**KY012850**	**KY012803**	n/a
	ES4	Spain (36.852342, -2.046172), Crespo, Cubas, Nuñez, Divakar MAF-Lich 19462 (MAF)	**KY051694**	**KY066418**	n/a	**KY066267**	KP796362	KP822164	n/a	n/a
	TR3	Turkey (40.21667, 26.7), Divakar, Crespo, Candan, Lumbsch, MAF-Lich 19467 (MAF)	**KY051695**	**KY066419**	n/a	KP822287	KP796363	KP822165	n/a	KP823538
	ES6	Spain (36.73063, -2.17427), Crespo, Rico, Ruibal MAF-Lich 19427 (MAF)	**KY051696**	**KY066420**	KP822482	KP822288	KP796364	n/a	KP822369	KP823539
	ES7	Spain (36.73063, -2.17427), Crespo, Rico, Ruibal MAF-Lich 19428 (MAF)	**KY051697**	**KY066421**	KP822483	KP822289	n/a	n/a	KP822370	KP823540
	ES8	Spain (36.73063, -2.17427), Crespo, Rico, Ruibal MAF-Lich 19429 (MAF)	**KY051698**	**KY066422**	KP822484	KP822290	KP796365	n/a	KP822371	KP823541
	ES10	**Spain (36.73063, -2.17427), Crespo, Rico, Ruibal MAF-Lich 19430 (MAF)**	**KY051699**	**KY066423**	n/a	**KY066268**	n/a	n/a	**KY012804**	**KY012863**
	ES11	**Spain (36.73063, -2.17427), Crespo, Rico, Ruibal MAF-Lich 19431 (MAF)**	**KY051700**	**KY066424**	n/a	**KY066269**	**KY066312**	n/a	n/a	**KY012864**
Protoparmelia multifera	BR1	Brazil (-22.88583, -48.4988), Aproot 13667 (ABL)	**KY051701**	**KY066425**	KP822485	KP822291	KP796366	n/a	n/a	**KY012865**
	BR2	Brazil (-10.75 -37.37), Cáceres & Aptroot ISE 9559 (ISE)	**KY051702**	n/a	n/a	**KY066270**	**KY066313**	n/a	n/a	n/a
	BR3	Brazil (-30.083 -51), Cáceres 7933 (ISE)	**KY051703**	**KY066426**	**KY012834**	n/a	**KY066314**	n/a	n/a	n/a
	BR4	Brazil (-30.083 -51), Cáceres & Aptroot C2A 22136 (ISE)	**KY051704**	**KY066427**	**KY012835**	n/a	**KY066315**	n/a	n/a	n/a
	BR5	Brazil (-30.083 -51), Cáceres & Aptroot C2A 22119 (ISE)	**KY051705**	n/a	n/a	**KY066271**	**KY066316**	n/a	n/a	n/a
Protoparmelia ochrococca	US1	USA (44.6914, -123.3135), McCune 31673 (OSU)	**KY051706**	n/a	KP822489	KP822293	KP796372	KP822172	KP822373	KP823542
	NO1	Norway (61.106, 5.8056), Klepsland JK10-L102, OL-175016 (O)	**n/a**	**KY066428**	KP822486	n/a	KP796369	KP822171	n/a	n/a
	NO3	Norway (59.65667, 6.87133), Johnsen L-93143 (BG)	**KY051707**	**KY066429**	KP822487	n/a	KP796370	n/a	n/a	KP823543
	NO4	Norway (59.57133, 6.05867), Tønsberg 39290, L-87963 (BG)	**KY051708**	**KY066430**	KP822488	n/a	KP796371	n/a	n/a	KP823544
Protoparmelia oleagina	**NO3**	**Norway (65.17184, 13.397), Tønsberg 41328, L-92554 (BG)**	**KY051709**	**KY066431**	**KY012836**	**KY066272**	**KY066317**	n/a	**KY012805**	**KY012866**
	NO2	Norway (59.11216, 5.8123), Johnsen L-92691 (BG)	**KY051710**	**KY066432**	KP822491	**KY066273**	n/a	n/a	KP822375	KP823546
Protoparmelia orientalis	TH1	Thailand (16.7166, 104.7166), Papong 6922 (MSUT)	**KY051711**	n/a	KP822492	KP822295	KP796375	KP822173	KP822376	KP823547
	TH2	Thailand (16.7166, 104.7166), Papong 6969 (MSUT)	**KY051712**	**KY066434**	KP822493	n/a	KP796376	KP822174	KP822377	KP823548
	TH5	Thailand (16.7166, 104.7166), Papong 7033 (MSUT)	**KY051713**	**KY066435**	KP822494	KP822296	KP796377	KP822175	n/a	KP823549

	TH3	Thailand (17.05, 103.9666), Papong 6488 (MSUT)	**KY051714**	n/a	KP822495	n/a	KP796378	KP822176	n/a	n/a
	TH4	Thailand (16.76666, 104.7166), Papong 6487 (MSUT)	**KY051715**	**KY066437**	KP822496	KP822297	n/a	KP822177	n/a	KP823550
	TH6	Thailand (16.7166, 104.7166), Papong 6612, HO-554582 (HO)	**n/a**	**KY066433**	**KY012837**	**KY066274**	**KY066318**	n/a	n/a	n/a
	TH7	Thailand (16.7666, 104.716667), Papong 5631, HO-554588 (HO)	**n/a**	**KY066436**	**KY012838**	n/a	**KY066319**	n/a	n/a	n/a
Protoparmelia pulchra	AU1	Australia (-32.055, 149.28388), Elix 39560, CANB 00789446 (CANB)	**KY051716**	**KY066438**	n/a	KP822298	**KY066321**	KP822178	n/a	KP823551
	AU5	**Australia (-12.61138, 131.10083), Elix 38452, CANB 769060 (CANB)**	**KY051717**	n/a	n/a	**KY066276**	**KY066320**	n/a	**KY012806**	**KY012867**
	AU6	**Australia (-12.61138, 131.10083), Elix 39791, CANB 00783250 (CANB)**	**KY051718**	n/a	n/a	**KY066275**	**KY066322**	n/a	n/a	n/a
	AU2	Australia (-12.47694, 131.03305), Elix 37097, CANB 00800711(CANB)	**KY051719**	**KY066439**	KP822497	KP822299	KP796379	n/a	KP822378	n/a
	AU3	Australia (-12.6113, 131.1008), Elix 39787, CANB 00781897 (CANB)	**KY051720**	**KY066440**	**KY012839**	KP822300	KP796380	KP822179	n/a	n/a
	AU7	Australia (-12.6114, 131.1008), Elix 37379, CANB 00803643 (CANB)	**KY051721**	**KY066441**	**KY012840**	**KY066277**	n/a	**KY012851**	n/a	n/a
	AU8	Australia (-12.6114, 131.1008), Elix 39798, CANB 00783256 (CANB)	**n/a**	**KY066442**	n/a	**KY066278**	**KY066323**	n/a	n/a	n/a
	AU4	Australia (-12.6114, 131.1008), Elix 39806, CANB 00783261 (CANB)	**n/a**	**KY066443**	n/a	KP822301	KP796381	KP822180	n/a	n/a
Protoparmelia ZA	ZA1	South Africa (-33.74, 18.948), Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19624 (MAF)	**KY051722**	n/a	KP822498	KP822302	KP796382	n/a	n/a	n/a
	ZA2	South Africa (-33.8, 19.816), Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19625 (MAF)	**KY051723**	**KY066444**	n/a	KP822303	KP796383	KP822182	KP822380	KP823554
	ZA3	South Africa (-33.8, 20.1), Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19628 (MAF)	**KY051724**	**KY066445**	KP822499	KP822304	KP796384	KP822183	n/a	KP823555
	ZA4	South Africa (-31.7594, 18.233), Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19584 (MAF)	**KY051725**	**KY066446**	KP822500	**KY066279**	KP796385	KP822184	n/a	KP823556
	ZA5	South Africa (-31.433, 18.566), Crespo, Divakar, Hawksworth, Amo, Lumbsch MAF-Lich 19626 (MAF)	**KY051726**	**KY066447**	n/a	n/a	KP796386	KP822185	n/a	KP823557

n/a = not available

Table S2. Primers used in this study.

Primer	Sequence	Reference
ITS1T	ggaaggatcattgaatctatcgt	Kroken & Taylor (2000)
ITS4T	ggttcgctcgccgctacta	Kroken & Taylor (2000)
ITS3T	aacgatgaagaacgcagcgaa	Kroken & Taylor (2000)
ITS2T	ttcgctgcgttcttcatcgtt	Kroken & Taylor (2000)
COX2P2fw	ggcatgaaagcatggttagc	Fernández-Mendoza *et al.* (2011)
COX2P2rev	tctggatgttagcaagaactttgt	Fernández-Mendoza *et al.* (2011)
COX2FOR1new	tctttttctttatgcttgtaatc	This study
COX2REV1new	gcrtcrgttttkacacctaatg	This study
COX2REV2new	gaagtwataatcatyctaatgtgag	This study

fw= forward primer
FOR= forward primer
Rev= reverse primer

Table S3. Genetic characteristics of loci used in this study

Locus	No. of seq	length of alignment	Variable sites	Best model
Fungus				
RPB1	102	757	267	012234+I+G+F
TSR1	101	760	401	TPM2+G
MCM7	64	657	235	TrNef+G
nuLSU	156	875	280	TIM1+G
mtSSU	116	815	160	HKY+G
ITS	112	729	450	TrNef+G
Concatenated	174	4596	1793	Partitioned
Alga				
COX2	160	516	153	HKY+I
ITS	124	751	272	012340+I+G
Concatenated	174	1267	425	Partitioned

Table S4. Genealogical concordance between nuclear ITS and mitochondrial COX2, and the posterior probabilities of species as suggested by BP&P. The species supported at one locus and not at the other are highlighted in bold. Clades in bold represent the species supported at only one locus. For the clades having less than 0.90 posterior probability (PP) as calculated by BP&P, the PP is shown for the separate as well as the collapsed clades.

Putative species	COX2	ITS	BP&P	
	RAxML	RAxML	Posterior probability of the clades	Probability of the collapsed clades
***T. sp. 1* (*T. suecica*)**	**44**	**61**	**1.00**	
T. sp. 2	86	85	0.97	
T. sp. 3 (*T. brindabellae*)	NA	100	0.97	
T. sp. 4 (*T. angustilobata*)	86	100	0.96	
T. sp. 5 (*T. simplex*)	100	95	100	
T. sp. 6 (*T. jamesii*)	100	100	0.55	0.444
T. sp. 7 (*T. jamesii*)	92		0.55	
T. sp. 8	96	97	0.99	
T. sp. 9	**97**	**60**	**0.99**	
T. sp. 10	82	90	0.99	
T. sp. 11	90	NA	0.98	
T. sp. 12	91	70	0.99	
T. sp. 13	98	86	0.99	
T. sp. 14	87	100	0.99	
T. sp. 15	**85**	**64**	**0.99**	
T. sp. 16	100	100	0.99	
T. sp. 17	100	89	0.95	
T. sp. 18	100	98	1.00	
T. sp. 19	100	100	0.99	
T. sp. 20	100	97	0.99	

NA= Not applicable

Table S5. Results of AIC model comparison analysis and summary statistics of the best fitting model.

AIC		
	df	AIC
M1	**7**	**426.0003**
M2	8	428.0012
M3	4	440.0892
M4	5	442.0910

Best-fitting model (M1) summary:

Estimate	Std.	Error	z	value	Pr(>|z|)
(Intercept)	6,45E+01	2,84E+02	0.227	0.820549	
PC1	1,71E+00	4,21E-01	4.075	4.61e-05	***
PC2	3,40E+00	1,22E+00	2.786	0.005337	**
PC1^2	9,56E-05	8,93E-04	0.107	0.914804	
PC2^2	2,02E-02	7,99E-03	2.532	0.011334	*
PC1:PC2	5,14E-03	1,48E-03	3.474	0.000513	***
PC1^2:PC2^2	-1,31E-08	8,56E-09	-1.527	0.126663	

Significance codes: 0 = ***, 0.001 = **, 0.01 = *
Null deviance: 67.864 on 16 degrees of freedom
Residual deviance: 22.972 on 10 degrees of freedom
AIC: 426

Fig. S1 ITS gene tree of algae associated with *Protoparmelia s.str.* and 30 reference ITS sequences representing 26 *T.* species from the SAG and UTEX collections. This is a ML tree with 1,000BS replicates, inferred using the program RAxML. Numbers above branches indicate ML BS ≥ 70%. Identity of each specimen in a clade is given in Table S1.

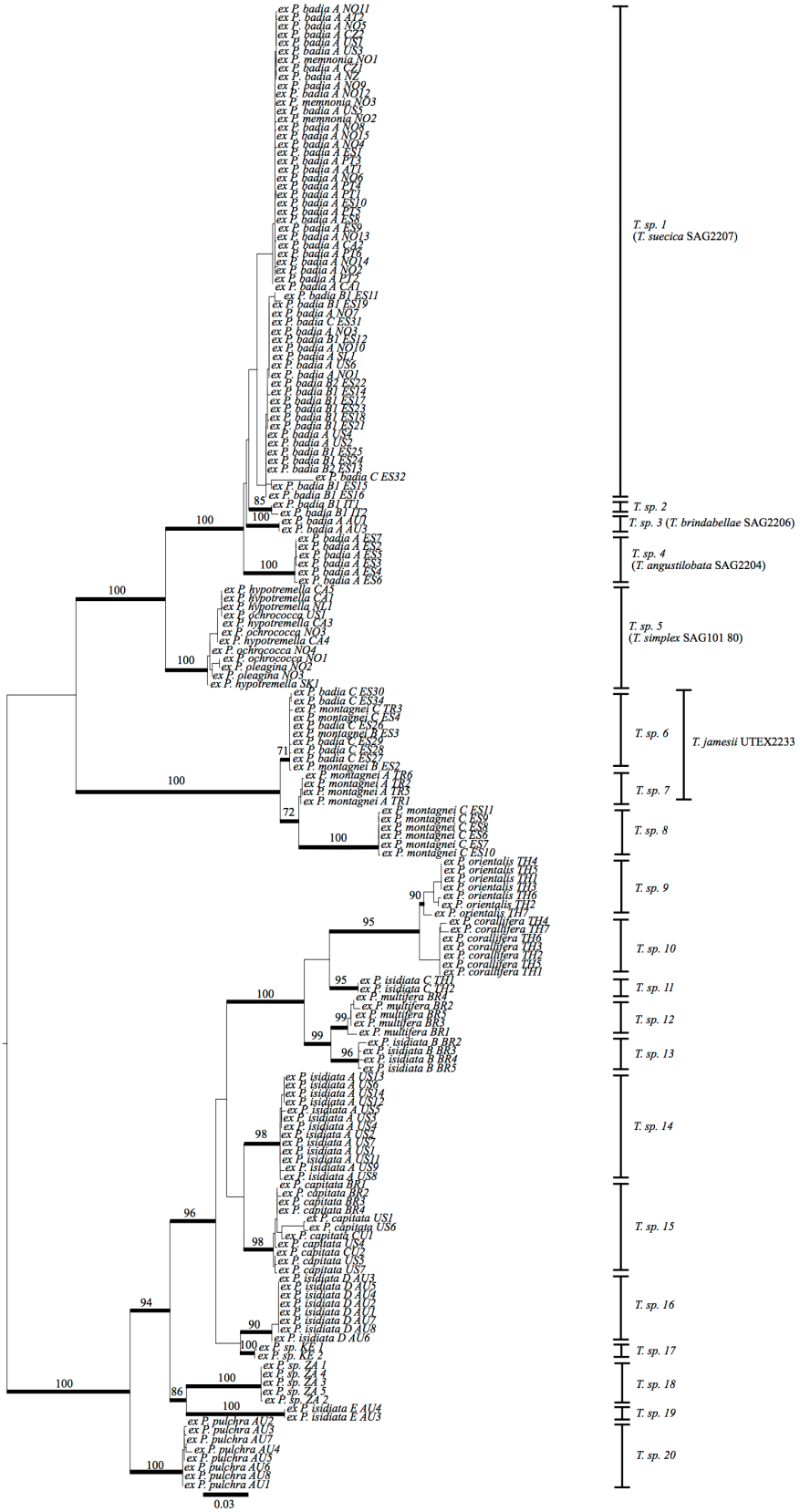

Fig. S2 Phylogeny of photobionts associated with *Protoparmelia* based on a concatenated 2-locus dataset including ITS and *COX2* sequences. Numbers above branches indicate ML BS (<70%). Branches in bold indicate Bayesian posterior probabilities (PP<0.94). Identity of each specimen in a clade is given in Supporting information Table S1.

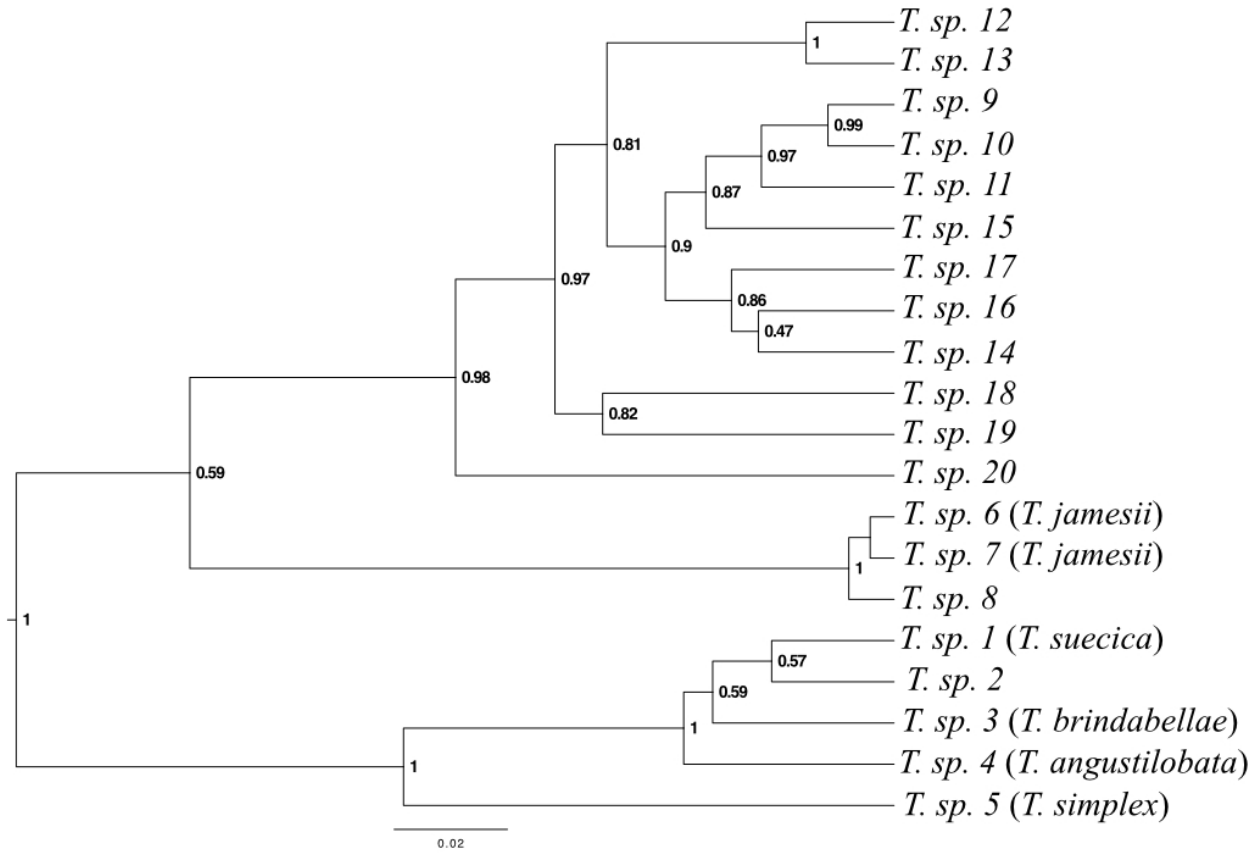

Fig. S3 *BEAST species trees for photobionts associated with *Protoparmelia*.

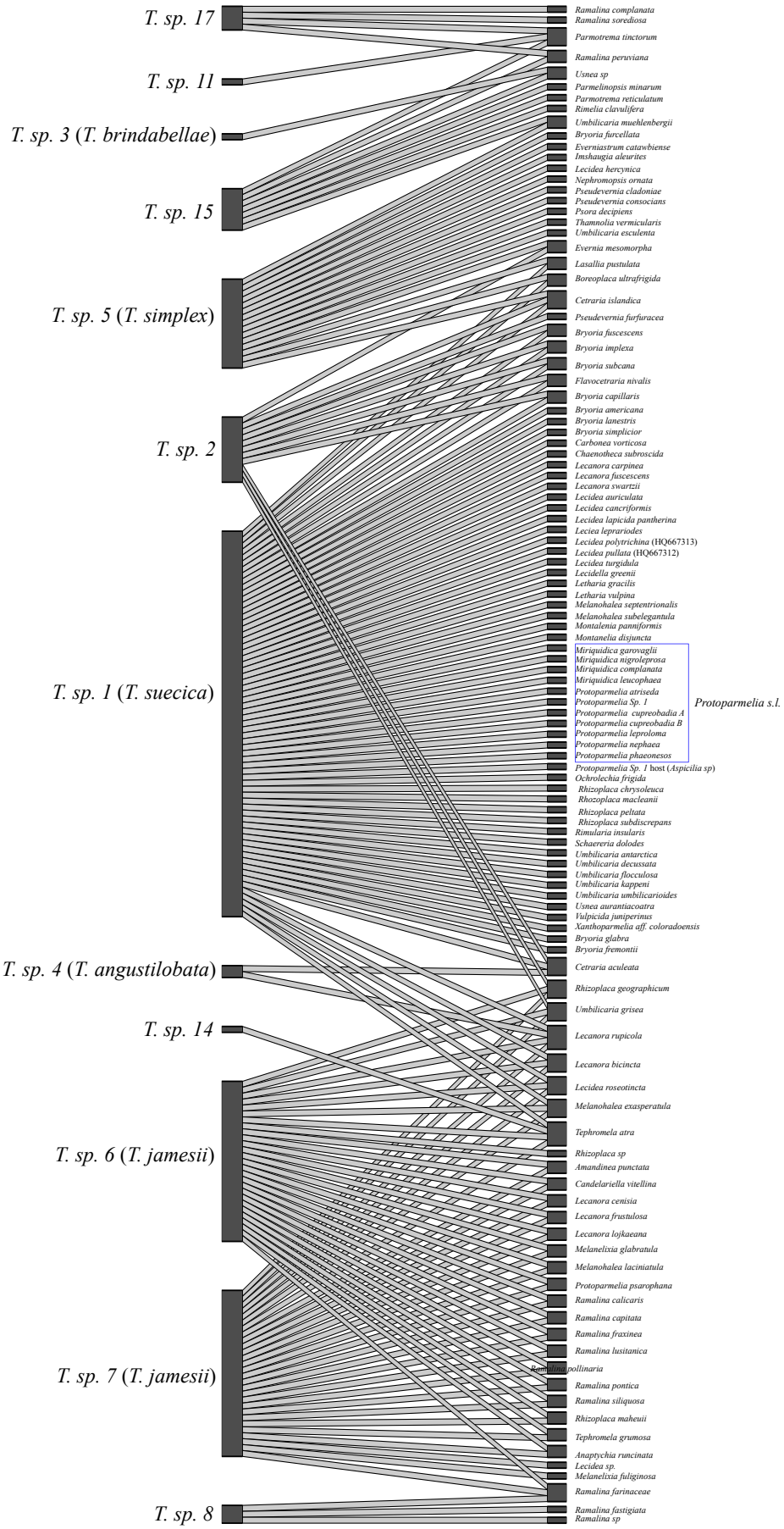

Fig. S4 Association network based on algal ITS data, given a 97% similarity BLASTn threshold.

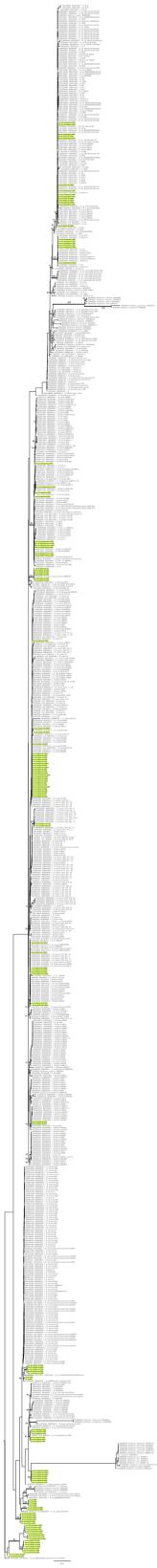

Fig. S5 Maximum likelihood tree (1000BS) inferred from the ITS sequences of the photobionts associated with *Protoparmelia* and and the first 100 NCBI BLAST hits of all the *T.* species associated with *Protoparmelia*. Numbers above branches indicate ML BS (<70%). Branches in bold indicate Bayesian posterior probabilities (PP<0.94). Identity of each specimen in a clade is given in Supporting information Table S1. *Protoparmelia* samples are highlighted in green.

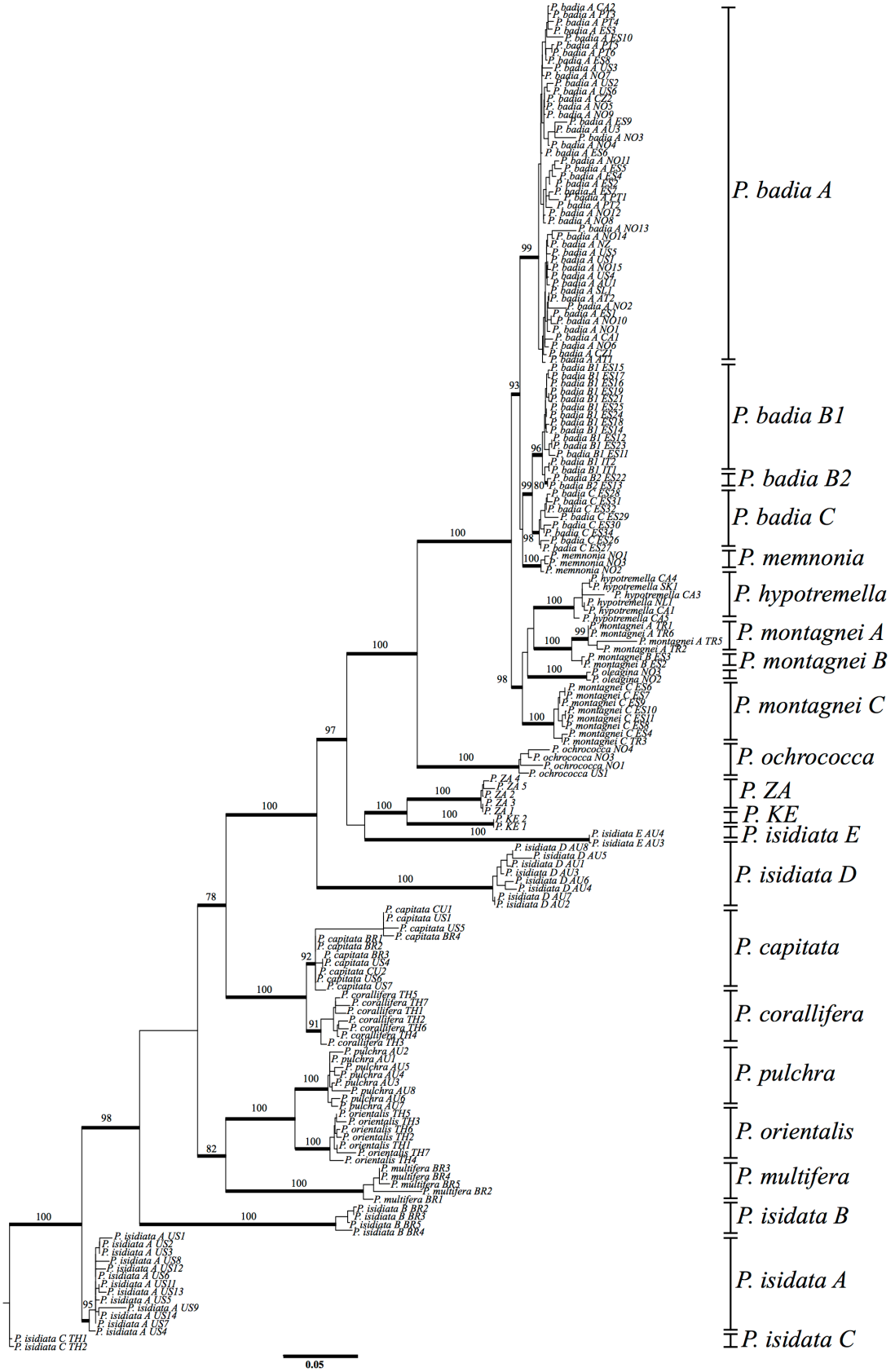

Fig. S6 Phylogeny of *Protoparmelia* based on a concatenated 6-locus dataset including ITS, nuLSU, mtSSU, *MCM7*, *TSR1* and *RPB1* sequences.

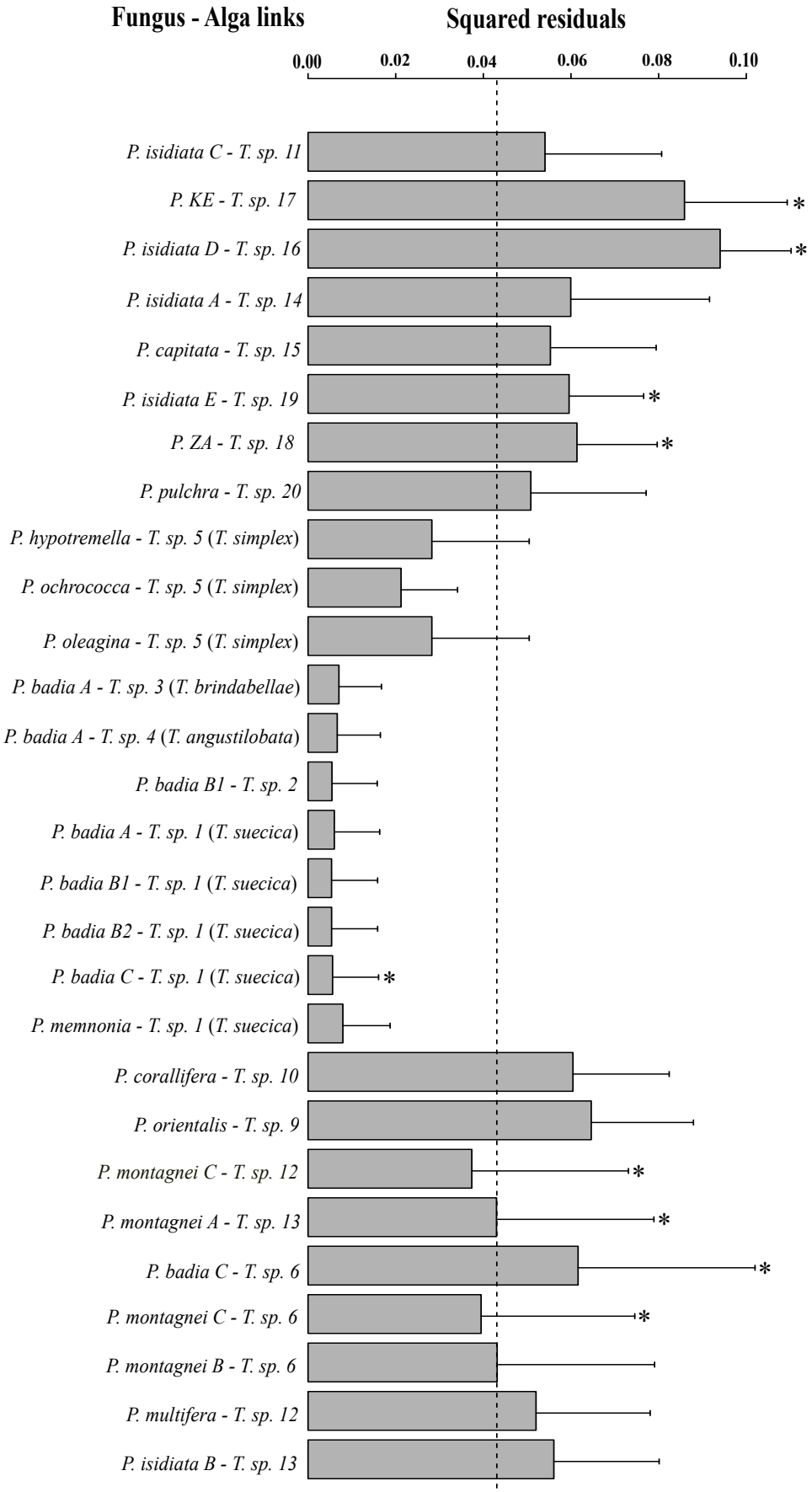

Fig. S7 Boxplot of the jacknifed squared residuals with upper 95% confidence intervals (error bars) associated to each host-symbiont link from PACo. Asterisks on the top on the top of the bars indicate significant congruence as supported by ParaFit.

References (part of New Phytologist Supporting Information)

Fernández-Mendoza F, Domaschke S, García M a, Jordan P, Martín MP, Printzen C. **2011**. Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Molecular Ecology* **20**: 1208–1232.

Kroken S, Taylor JW. **2000**. Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *The Bryologist* **103**: 645–660.

11 CURRICULUM VITAE

Personal information

Name:	Garima Singh
Date of birth:	10.07.1984
Place of birth:	Varanasi (India)
Nationality:	Indian
Office address:	Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25 60325 Frankfurt/Main Germany
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Research interests

Species diversity and interaction patterns in different macrohabitats

Phylogeny and evolution of symbiotic organisms

Population genetics and phylogeography of symbiotic organisms

Responses of symbiotic organisms to climate change

Evolution of sexual reproduction in Ascomycota

Education

2012-present	**PhD studies** Goethe University Frankfurt am Main, Germany. Title: Evolution of the lichen-forming genus *Protoparmelia.*
2009	**MSc in Zoology** from Banaras Hindu University, Varanasi, India.
2007-2009	**Masters studies in Zoology** Banaras Hindu University, Varanasi, India
2007	**BSc in Biology** from Banaras Hindu University, Varanasi, India
2004-2007	**Bachelor studies in Biology** Banaras Hindu University, Varanasi, India. Subjects: Botany, Zoology, Chemistry

2002 **Secondary High school** MASSKK, Chakia-Varanasi; Subjects: Physics, Chemistry, Biology, English, Hindi

2000 **High school** St. James School Hardoi. Subjects: English, Hindi, Science, Social Science, Mathematics

Professional appointments

2011-2012 **Research Scientist** Swiss Federal Research Institute WSL, Biodiversity and Conservation Biology (Prof. C. Scheidegger). Project title: Pyrosequencing-based analysis of MAT-loci in the threatened lichen *Lobaria pulmonaria.*

2009-2011 **Research Training** with Prof. Rajiva Raman, Dept. of Zoology, Banaras Hindu University, Varanasi, India.

Publications

1. Ludwig LR, Summerfield TC, Lord JM, **Singh G**. Characterisation of the mating type locus (*MAT*) and analysis of the mating system in *Knightiella splachnirima*. **Accepted. The Lichenologist.**
2. **Singh G**, Dal Grande F, Otte J, Divakar PK, Crespo A, Schmitt I. (2017) Fungal-algal association patterns in lichen symbiosis linked to macroclimate. **New Phytologist**. doi: 10.1111/nph.14366.
3. Divakar PK, Crespo A, Otte J, Wedin M, Leavitt SD,…….**Singh G**….. Lumbsch HT (**2015**) Evolution of complex symbiotic relationships in a morphologically derived family of lichen-forming fungi. **New Phytologist 208**: 1217-1226.
4. **Singh G**, Dal Grande F, Divakar PK, Otte J, Leavitt SD, Szczepanska K, Crespo A, Rico VJ, Aptroot A, Cáceres ME da Silva, Lumbsch HT, Schmitt I **(2015)** Coalescent-based species delimitation approach uncovers high cryptic diversity in the cosmopolitan lichen-forming fungal genus *Protoparmelia* (Lecanorales, Ascomycota). **PLoSONE**. doi**:**10.1371/journal.pone.0124625.
5. **Singh G**, Dal Grande F, Silke W, Scheidegger C **(2015)** Long term impact of different disturbances on reproductive strategies of the rare epiphytic lichen *Lobaria pulmonaria*: is clonality a gift and a curse? **FEMS Microbiology Ecology 91**: 1-11.

6. Dal Grande F, Beck A, Cornejo C, **Singh G**, Cheenacharoen S, Nelsen MP, Scheidegger C **(2014)** Molecular phylogeny and symbiotic selectivity of the green algal genus *Dictyochloropsis* s.l. (Trebouxiophyceae): a polyphyletic and widespread group forming photobiont-mediated guilds in the lichen family Lobariaceae. **New Phytologist 202**: 455-470**.**
7. **Singh G**, Divakar PK, Dal Grande F, Otte J, Parnmen S, Wedin M, Crespo A, Lumbsch HT, Schmitt I **(2013)** The sister group relationships of the largest family of lichenized fungi, Parmeliaceae (Lecanorales, Ascomycota). **Fungal Biology 117**: 715-721.
8. Dal Grande F, Beck A, **Singh G**, Schmitt I **(2013)** Microsatellite primers in the lichen symbiotic alga *Trebouxia decolorans* (Trebouxiophyceae). **Applications in Plant Sciences**. doi:10.3732/ apps.1200400.
9. **Singh G**, Dal Grande F, Cornejo C, Schmitt I, Scheidegger C (**2012**) Genetic basis of self-incompatibility in the lichen-forming fungus *Lobaria pulmonaria* and skewed frequency distribution of mating-type idiomorphs. **PLoS ONE 7**: e51402. doi:10.1371/ journal.pone.0051402.

Presentations

1. **Singh G,** Dal Grande F, Divakar PK, Otte J, Crespo A, Schmitt I (2016) Macroclimate and coevolutionary forces influence fungal-algal association patterns in *Protoparmelia***.** International Association of Lichenology-8 (IAL8), Helsinki, Finalnd (Invited talk).
2. Ludwig L, Summerfield T, Burritt D, Lord J, Knight A, **Singh G**, Kantvilas G (2016) The reproductive ecology of *Icmadophila splachnirima*. International Association of Lichenology-8 (IAL8), Helsinki, Finalnd (Poster).
3. **Singh G,** Dal Grande F, Divakar PK, Otte J, Crespo A, Schmitt I (2016) Identification, association and coevolutionary patterns of the photobionts associated with *Protoparmelia* s.str. Trieste, Italy.
4. **Singh G**, Dal Grande F, Divakar PK, Schmitt I (2015) Fungal-algal association patterns in lichen symbiosis linked to macroclimate and coevolutionary forces. National conference on cryptogam research in India: Progress and prospects, Lucknow, India (Oral presentation).

5. **Singh G**, Dal Grande F, Cornejo C, Werth S, Scheidegger C (2012) Characterization of the mating type loci in *Lobaria pulmonaria* and implications for conservation. International Association of Lichenology -7 (IAL7), Bangkok, Thailand (Poster).
6. Scheidegger C, **Singh G**, Stofer S (2012) Transplanting epiphytic lichens for conservation measures: improving population stability and mating-type balance. 3rd European congress of Conservation Biology 2012 (ECCB-2012), Glasgow, UK (Oral presentation).

Workshops

September 2016	Meeting of the *Trebouxia*-working group. University of Trieste, Trieste, Italy, 26-28.
November 2016	Managing and Curating Museum Collections III: Curation of Botanical Collections. *Goethe Graduate Academy GRADE,* Frankfurt, Germany.
September 2012	Scientific paper writing course. *Goethe Graduate Academy GRADE,* Frankfurt, Germany.

Field trips

July 2012	**Norway**- Collecting Anaptychia ciliaris and Lasallia pustulata
June 2013	**Sardinia**- Collecting *Protoparmelia* spp., *Lasallia pustulata*; placing iButtons on six different populations of *L. pustulata* along an altitudinal gradient of Mount Limbara to record temperature and humidity data.
June 2014	**Sardinia**- Collecting *Lasallia pustulata* and all lichens present in the lichen community in each of the six populations across the altitudinal gradient of Mount Limbara; downloading iButton data from iButtons placed the previous year and replacing the iButtons to record the temperature and humidity data for the next year.
June 2015	**Sardinia**- Collecting *Lasallia pustulata;* reading and replacing the iButtons.
	Corsica- Collecting *Lasallia brigantium*

Grants and Awards

2013-2016	German Academic Exchange Services (DAAD).
Dec 2009-May 2011	CSIR-NET JRF (Council of Scientific and Industrial Research-National Eligibility Test – Junior Research Fellowship).

Techniques

Molecular techniques: DNA extraction, RNA extraction, cDNA synthesis, Western blotting, , PCRs, RT-PCR, TAIL-PCR, inverse-PCR, restriction digestions, primer design, gel electrophoresis (PAGE, agarose), and RNA in situ hybridisation

Experiments involving animals: Animal (mouse) handling, setting crosses, mouse dissections, embryo dissections, organ fixation and block

Cultivation techniques: Blood cultures, mouse embryo organ cultures, bacterial culture.

Visualization techniques: Histological slide preparation, light microscopy

Sequence analysis: Sequence assembly and editing

Population genetic analysis: Softwares for population genetics: Genpop analysis; Arlequin

Phylogenetic analysis: Sequence alignment, Bayesian analysis, RAxML analysis, Alternative hypothesis testing, CADM testing, Partition finder, model testing

Species delimitation analysis: GMYC, ABGD, BP&P, spedeSTEM and STACEY

Cophylogenetic analyses: PACo, ParaFit and Jane

Languages

Hindi (mother tongue)

English (fluent)

German (familiar)

Italian (familiar)