Université de Liège

Integrating photobiont phylogenetic and geographical data in macroevolutionary studies of lichens

Case studies in the Peltigerales

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Thesis submitted in fulfillment of the requirements
for the degree of Doctor in Sciences

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Monday, October 13th 2014

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Acknowledgements

First of all, I would like to thank Emmanuël Sérusiaux, who has been a wonderful supervisor, for five years, since my Master Thesis. He has always been of great help and support, and of great advice during all the steps of this doctoral thesis. Without his trust and involvement, from the first stages, the applications to get a doctoral grant, until the last steps of the writing process, none of this work would have been possible. Moreover, I can't remember a single moment of conflict or any negative interaction during five whole years, which is remarkable. I feel that this collaboration has been much fruitful and I hope we will have the opportunity to continue in the next future. We have been found together working on this project from Belgium to Reunion Island, and later to the USA and Thailand, but it's in terms of professional and human fulfillment that I will most remember this tremendous journey that my doctorate has been.

I also want to thank very warmly François Lutzoni and Jolanta Miadlikowska, who form a fantastic and very complementary team at Duke University. They have welcomed me in their team and involved me in their project without knowing me at all, and have always been kind and trustful towards me. They were always there to help me, support me and make sure I succeeded in my adaptation to the very foreign environment that North Carolina at first represented. They have always pushed me to the next level, oriented me to new pathes, and showed me what it takes to achieve great goals. They also had the trust and goodness to embark me in multiple adventures, including amazing field trips in the Appalachians and in South America, as well as international conferences in Utah, Thailand and many more. Without these numerous stays in Durham and at Duke University, my doctoral thesis and my life in the last three years would have been completely different, and I would not be the same person as I am now. It seems that we will have the opportunity to continue this successful collaboration in the very next future and I am very happy about it.

I also thank Bernard Goffinet, who has been very helpful especially in the design, and successful implementation of this doctoral thesis project, and who always had a watchful eye on the project and useful advices, which were greatly appreciated.

I would also like to thank Orvo Vitikainen, for his kindness and patience in attempting to share with me his knowledge and wisdom about the complicated taxonomy of the genus *Peltigera*.

I also thank Alain Vanderpoorten, Damien Ertz, and Denis Baurain, who accepted to be part of my doctoral committee, and subsequently of my thesis jury, and took the time to read the present thesis and give a valuable advice. I also thank them for the advices and the help they gave me at several occasions during this doctorate. I especially thank Alain Vanderpoorten for his help during the preparation and design of the project before its submission to the FRIA.

It is not always easy to keep on tracks on such a long-term project and it has been extremely important for me to have friends and colleagues whom I could rely on, especially when constantly in transit from a country to another.

First of all, I want to thank Anthony Fratamico, who, in addition to having shared hundreds of lunches with me, has also been an amazing friend, then colleague for almost ten years, since the first months of our bachelor studies. He helped me a countless amount of times, on an extreme diversity of situations, ranging from being my personal driver when my leg was in a plaster cast, to introducing me to several programs without which the present work would not be in its present state. His help and support especially in the stressful and difficult moments have been much appreciated.

I also thank Ko Hsuan Chen, who has been the best labmate and officemate anyone could dream of. Her kindness and positivity in every moment, as well as her friendship significantly enhanced my experience at Duke University.

I also thank warmly Martin Ramirez, who has greatly helped me to integrate to this unknown and frightening environment that Duke University represented when I first arrived there, and who has always been a great friend for me. Sharing a lab bench with you during the fall of 2011 has been a very fun and especially productive experience, and made working in the evenings less painful than it appears to be at first glance.

I thank Akshay Bareja, who has been a fantastic housemate, before quickly becoming one of my best friends, and definitely brought my experience of Durham and North Carolina to a different level. Without the countless conversations, concerts, and explorations of the local and American culture, my stay in the US would not have been as rewarding. The two trips to NYC were highlights of my American experience too.

I also take the opportunity to thank all the great labmates I had, with whom the work and collaboration have been very successful, and whose friendship made the lab work very pleasant. At Duke University, I think especially about Emilie Lefevre, with whom I had a great time sharing a bench, despite a certain bossiness, which, finally, might have been helpful; Camille Truong, with whom I greatly enjoyed working on the *Peltigera* project, and especially during this super field trip in Utah; Edgar Medina, with whom I spent much of my time and had a lot of fun during several semesters; Ester Gaya, Ryoko Oono, Tami McDonald, Molly McMullen, always so kind and helpful, Michael Gajdeczka and all the other people who at some point worked with me in the lab.

In Liège, Laurent Gohy has also helped me many times with the lab work, and taught

me most of the lab skills I now have, including how to extract DNA, how to do a PCR and an agarose gel. Despite having now performed them thousands of times, I don't forget the first time he taught them to me, a cold day during the fall of 2009. Moreover, it has always been very pleasant to work with him. I also thank Ido Cremasco, who is a very interesting and fun person, whose help was also very helpful. I think in particular about an expedition to the Belgian customs to negotiate the clearance of a box full of lichens that was urgently needed. During these five years in the lab, I also enjoyed working, usually for regrettably short periods of times, with Sébastien Quennery, Martin Guissard, Julie Pisano, Virginie Hutsemeekers, Nathalie Smitz, and all the other persons who once worked in the lab with me.

A special salute to Jairo Patiño, who has been, like me, embarked in a perpetual transit from Liege to Duke and vice versa. When we happened to be on the same side of the Atlantic, which hasn't been so frequent, I appreciated it!

It has always been interesting and fun to be part of the very active graduate student and post-doc community at Duke University, and I'd like to thank all the ones who helped me feeling part of this community, among others, Dudu, Carl and Cathy, Kathryn, Jessie, Emily, Steven, Sean, Fay Wei, Tzu Dong, Lisa, and all the ones I forget!

It has also been a great chance for me to mentor several students, and try to help them in their learning process, so I thank Jade, Clémence, and from now on, Antoine, for having given me this opportunity at such an early stage of my career.

I also thank all the bryology team at UCONN: Laura Forrest, Juan Carlos Villarreal, Lily Lewis and Heather Sinclair, Yang Liu, and Jessica Budke, for their welcome and help during my short stay there.

When participating to conferences, it has also been a great pleasure, and maybe a relief, to find out that the community of young lichenologists was composed of so many nice and fun persons, with whom I had a great time. Among others, I especially think about Samantha Fernandez, Niall Higgins, Toby Spribille, Philip Resl, Theerapat Luangsuphabool, Fabio Candotto, Stefano Bertuzzi and many many more whom I met at the IAL, IMC, BLS or Evolution meetings. I also thank all the numerous colleagues who gave me feedback on my talks, advise and comments about my work during these conferences.

I also thank all the persons who have been contributing to the organization of field trips that allowed us to collect the material for this project. I especially thank Einar Timdal and Hakon Olien for their help during the field trip in Norway, and Tor Tonsberg, Mika Bendiksby, Reidar Haugan and the other participants of the workshop in Steinkjer. I thank Paul Diederich for the nice, little field trip in Luxembourg; Adriano Spielman, Luciana Canez and Marcelo Marcelli for the field trip in Brazil, Daniel Ramos and Eimy Rivas Plata for the field trip in Peru, Edier Soto and David Diaz Escandon for the field trip in Colombia, and Martin's parents, Lucila and Jorge, for making me feel like home in the center of Bogota! I also thank Renato Benesperi and Paolo Giordani

for organizing the Peltigera workshop in Genova, and Jeff Malter and Pat Wolseley for their advice in organizing my field trip to Devon.

I also thank all the persons who helped the project by sending us specimens, they would be too numerous to cite, but this work would not have been possible without them. I especially thank Trevor Goward, Robert Lücking, Bruce McCune, and the curators of all the herbaria that provided material for this study.

I also want to thank a few amazing persons whom I have the luck to be friend with, I especially think about Sébastien, Julien, Kaibeuth and Michel east of the Atlantic, and Rémi, Nicole, April and Maggie west of the Atlantic!

Most importantly, I thank very warmly all my family, who has always been there for me, my grandparents Joseph and Dola, my aunts Josiane and Fabienne, my uncle Jean-Pol, my cousins Samuel, Jonas, Charlotte, Laure, Lise and Brice (for the last ones cited, good luck with your own doctorates!), my parents, Eliane and Pierre, my brother François, Anael, and their son Tiago, born less than three months ago, thanks to whom I have now the chance to be an uncle and a godfather, and to whom I would like to dedicate this work and wish him all the luck and success in life!

I also thank sincerely all the persons who helped or contributed in a way or another to this project, and present my sincere apologies to all the ones I forgot. I could have continued for pages, but I am afraid that it would significantly increase the printing costs and turn some of the aforementioned people against me.

Overall, this adventure has been very rewarding for me, and I have probably learnt more during these four years than during the twenty-two years before. I hope that I will have the opportunity to continue to work, collaborate or hang out with all of you!

Nicolas.

Introduction

1. The lichen symbiosis

Lichens are obligatory symbioses (close and long-term relationships between at least two organisms) resulting from the mutualistic interactions (where all partners benefit from the association) between fungi (mycobionts) and one or several photosynthetic partners (photobionts), which represent green algae (phycobiont) and/or cyanobacteria (cyanobiont) (Ahmadjian, 1993a; Nash, 2008). Instances of free-living mycobionts occurring in a non-lichenized stage have been rarely encountered (in Ostropales; Wedin et al. 2004), whereas the photobionts (especially *Nostoc* spp.) are known to occur outside of lichen associations as free-living or in symbioses with other organisms (Bubrick et al., 1984; Oksanen et al., 2002; Nelsen et al., 2011). Lichen symbiosis results in a formation of a thallus, a unique morphological structure that can resemble structures of multicellular plants, which cannot be developed by any of the symbionts when growing alone. The photobiont produces (through photosynthesis) and shares carbohydrates (and nitrogen in the case of cyanobiont) with the mycobiont, which in exchange provides a habitat with a reduced level of competition, protection from predators and better access to light for the photobiont (Ahmadjian, 1993a; Honegger, 1998). This mutualistic association enables the partners to acquire a new lifestyle and survive in ecological conditions that are otherwise not accessible by the individual partners (Ahmadjian, 1993a). Lichen symbiosis has been very successful in nature and lichens have proven their ability to develop in extreme conditions, ranging from hot and dry deserts (Nash III et al., 1977) to the cold Antarctic region (Kappen, 2000), and are even capable of surviving in space (Sancho et al., 2007).

Currently about 17,500 species of lichen-forming fungi are recognized and an estimation of additional 8,500-10,500 species would remain to be discovered (Feuerer and Hawksworth, 2007; Kirk et al., 2008; Lumbsch et al., 2011; Lücking et al., 2014). A lichen name reflects the taxonomy of its mycobiont species. Most lichenized taxa are classified in 15-24 orders distributed in seven classes of Ascomycota (lichen-forming species account for 40% of all described ascomycota species; Lutzoni et al. 2001; Lumbsch and Huhndorf 2011) and in 4-7 orders of two classes of Basidiomycota (Lawrey et al., 2007). In comparison, only about 100 species of photobionts isolated from lichen

thalli have been recognized, mainly in two families of green algae, Trebouxiaceae and Trenthepohliaceae, and the cyanobacterial genus Nostoc (Friedl and Büdel, 1996). The actual number of photobiont species might be underestimated due to their complex and unclear taxonomy, and the rarity of diagnostic morphological characters available in these unicellular organisms. Other hypotheses to explain the difference in the levels of diversity between the photobionts and mycobionts remain to be explored. For example Zoller and Lutzoni (2003) demonstrated that the substitution rates in the ITS region of the mycobiont from the basidiolichen genus *Omphalina* was an order of magnitude higher than in its photobiont *Coccomyxa*. A possible explanation relies on the fact that the mycobiont reproduces sexually and the photobiont only asexually when forming a lichen symbiosis (Friedl and Büdel, 1996), which could potentially lead to a difference in rates of the evolution, and therefore results in a higher number of species and greater level of morphological adaptation for the mycobiont (Law and Lewis, 1983; Ahmadjian, 1993b; Hill, 2009). Until recently the systematic studies on lichens including molecular phylogenetics have been almost solely based on the mycobiont alone. Molecular data and associated analytical methods (building phylogenies and testing species boundaries) provided a new powerful tool in the field of lichen systematics and evolution.

2. Discrepancies between morphology and phylogeny

Molecular data highlighted the difficulty to correctly define boundaries among species (defined as "separately evolving metapopulation lineages"; De Queiroz 1998) in lichen-forming fungi in order to bridge the morphological and other commonly used species concepts (e.g., the biological species concept). The morphological species concept can be very difficult to apply in fungi, including lichens, because the absence of diagnostic traits can lead to the recognition of fewer species than phylogenetically defined (e.g., Crespo and Pérez-Ortega 2009; Miadlikowska et al. 2014b). Intraspecific plasticity can be often higher than interspecific differences, leading to the circumscription of species representing different phenotypes within a single evolutionary lineage (e.g., Pino-Bodas et al. 2011). It can also be very difficult to detect morphological convergence when characters lack distinct developmental signature, and as a consequence unrelated lineages were sometimes embedded within the same species (e.g., Lumbsch et al. 2005; Otalora and Wedin 2013; Passo et al. 2008). Moreover, cryptic species that cannot be recognized based solely on the morphology have been frequently detected in lichen-forming fungi, including well-studied taxa from well-sampled areas (e.g., Lumbsch and Leavitt 2011). Biogeographical factors shaping the systematics of lichen-forming fungi was often neglected. For example, the same species name was often applied to morphologicaly similar individuals from different continents, when they might be drastically different genetically (e.g., Leavitt et al. 2011). As a result, species based on morphological concepts (morphospecies), might not always represent biologically or phylogenetically meaningful units. Moreover, recognition and circumscription of morphological traits are sometimes arbitrary and authors may diverge on boundaries among morphologically de-

fined species. Chemotypic variation (differences in the set of secondary compounds) as an alternative tool for species delimitation was proved to be unreliable because the chemical traits often vary depending on the stage of lichen development, part of the thallus or ecological conditions (Lumbsch, 1998).

Recognizing biological species sensu Mayr (1940; "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups"), by testing the mating compatibility, is problematic in lichens because most lichenized fungi do not grow in artificial conditions. Moreover, lichens grow very slowly and their spores are tiny, difficult to observe and can be carried on very long distances, resulting in a great difficulty to monitor and test lichen reproduction (but see Zoller et al. 1999). Asexuality or homothallism in some species are also factors that complicate mating tests on lichens (Taylor et al. 2000; but see Scherrer et al. 2005).

However, the availability of molecular data has made possible to apply in lichen-forming fungi the phylogenetic species concept ("a diagnosable cluster of individuals within which there is a parental pattern of ancestry and descent, beyond which there is not, and which exhibits a pattern of phylogenetic ancestry and descent among units of like kind" ; Eldredge and Cracraft 1980) and the genealogical species concept ("basal group of organisms whose members are all more closely related to each other than they are to any organisms outside the group"; Hudson and Coyne 2002) concepts. In particular, Taylor et al. (2000) recommended the use of the Genealogical Concordance species concept or "exclusive concordance of alleles, where different gene topologies have to be congruent for interspecific relationships" (Avise and Ball, 1990) for species delimitation of fungi. Once phylogenetic or genealogical species are defined based on molecular data, it is possible to select a posteriori a set of phenotypic and chemotypic traits that are species-specific (Lumbsch and Leavitt, 2011).

3. Evolution in the context of species interactions

Molecular data provide a tool to study the systematics and evolution of fungi in a broad context of closely related taxa and interacting set of taxa. These interactions are very important in shaping the macroevolution of interacting organisms (Thompson, 1994, 2005), such as for preys and predators, interspecific competitors, host and pathogens, but especially for the interdependent mutualistic partners, such as lichen symbionts, which have been in contact for a long period of evolutionary time. The lichen symbiosis was estimated to be a very old and a stable life strategy. Fossil evidence suggests a presence of lichen-like structure 600 million years ago (Yuan et al., 2005) and fossils dating from the Ediacaran (635-542 million years ago) have been identified as lichenized fungi (Retallack, 2013). Internally stratified lichens, interpreted as members of Pezizomycotina and associating with either cyanobacteria or green algae were found in fossils from 415 millions year ago, in the Lower Devonian (Honegger et al., 2013). The origin of ascolichens was approximated to be ca. 430 million years old (Lutzoni et al., 2014)

and the diversification of major lineages of lichenized fungi was dated ca. 305 million years ago, in the Upper Carboniferous (Prieto and Wedin, 2013).

However, not every association between two specific partners results from a long-term co-occurrence; most studies on lichen mycobiont-photobiont associations did not found conclusive evidence of cospeciation between the symbionts (e.g., Elvebakk et al. 2008; Hill 2009; Lücking et al. 2009). This might be due to the absence of sexual reproduction and adaptations in the photobiont partner, as well as a consequence of photobiont switches. The latter is a fairly common phenomenon involving a mycobiont species switching to a different photosynthetic partner during its evolutionary history (Piercey-Normore and DePriest, 2001). For instance, a study on the widespread *Cetraria aculeata* (Fernández-Mendoza et al., 2011) concluded that a photobiont switch in the past enabled *C. aculeata* to colonize temperate, as well as polar habitats. The authors suggested that "rare photobiont switches may increase the geographical range and ecological niche of lichen mycobionts by associating with locally adapted photobionts in climatically different regions and, together with isolation by distance, may lead to genetic isolation between populations and thus drive the evolution of lichens". Evolution of lichen mycobionts depends on their selectivity (symbiont's preference for a particular host in a particular habitat; Thompson 1994) and specificity (range and taxonomic relatedness of acceptable partners; Rambold et al. 1998), which strongly influence mycobionts interactions with photobionts.

The reproduction and dispersal modes can shape mycobiont specificity and selectivity patterns. Sexual reproduction of the mycobiont (through ascospores) leads to higher genetic variation but results in the horizontal transmission of the photobiont, as the fungus disperses independently and has to find a new photobiont partner from the environment to reestablish the symbiosis (Budel and Scheidegger, 1996). Therefore, lower levels of selectivity and specificity are expected to be found in sexually reproducing mycobiont. On the other hand, asexual reproduction by vegetative propagules (such as isidia, phyllidia or soredia) or by thallus fragments, which contain both symbionts, allows the two partners to disperse together and to rebuild a thallus genetically identical to the one they originated from (vertical transmission of the photobiont; Budel and Scheidegger 1996). Lower diversity of photobionts and, therefore, higher levels of selectivity and specificity are expected to be found in asexually reproducing mycobionts that are vertically transmitting their photobiont to subsequent generations. A vertical transmission of symbionts result in co-dispersal and highly congruent genetic structures in the two symbionts (Werth and Scheidegger, 2012). The co-occurrence of both patterns (horizontal and vertical photobiont transmissions) was demonstrated in the species pair within the genus *Degelia*, represented by a sexual *D. plumbea* and its closely related asexual *D. atlantica*, both associating with *Nostoc* cyanobionts (Otálora et al., 2013). Higher genetic diversity for both partners was detected in sexually reproducing lichen-forming species compared to the asexual species, where the level of genetic variation was extremely low (almost clonal) and specificity of the mycobiont towards its cyanobiont was very high. Otálora et al. (2013) suggested that asexual reproduction

and clonality might affect the species capacity for exploring environmental resources and withstanding competition, limiting the species to restricted niches.

The signature of sexual versus asexual reproduction on the genetic diversity of both symbionts is such that their genetic structure can be used to determine the respective contribution of each mode of reproduction (Dal Grande et al., 2012). Reproduction and dispersion are thus key factors impacting genetic diversity, but also the partners selectivity and specificity, and thus contributing to the evolutionary fate of these lineages. However, higher genetic diversity of both symbionts does not always occur in partners reproducing sexually. For example, Wornik and Grube (2010) did not observed differences in the fertile and an asexual species of *Physconia* associating with green algae of the genus *Trebouxia*. Moreover, high variation in photobiont partners have been reported in thalli of asexually reproducing taxa (Blaha et al., 2006; Guzow-Krzeminska, 2006; Piercey-Normore, 2006), suggesting that other factors are involved in shaping diversity of partners in the lichens. Nelsen and Gargas (2008) suggested that even in asexually reproducing species where photobiont are vertically transmitted, specificity between symbionts is not strictly maintained over evolutionary time and that the ability to switch partners may provide benefits similar to genetic recombination, giving species a better chance of survival.

Although associating with the same partner should be advantageous in term of increased fitness of the symbiosis (as hypothesized by Law and Lewis 1983), one potential reason explaining why a mycobiont species associates with several partners is that different photobionts can adapt to different environments allowing the mycobiont to occupy a wider range of habitats. Therefore, a lower level of specificity of the mycobionts in selecting the photobionts, could allow them to associate with the best partner available without any loss of fitness (Nelsen and Gargas, 2008; Hill, 2009). Several studies on mycobionts associated with various photobionts (e.g., *Trebouxia* in the genus *Cladonia* by Yahr et al. 2006, in the genus *Ramalina* by Werth and Sork 2010, in the genus *Tephromela* by Muggia et al. 2008, as well as in various lichen genera associated with *Asterochloris* by Peksa and Škaloud 2011, and in the cyanobacterial genus *Rhizonema* by Lücking et al. 2009) reported that photobionts were ecologically specialized and dependent on climatic conditions (e.g., rain level or sun exposure) and that mycobionts had a low photobiont selectivity resulting in associations with various locally adapted strains in order to grow in a broader range of habitats.

Many of these hypotheses are assuming that only one photobiont is found in each lichen thallus. However, some mycobiont species can also associate with several partners within the same thallus. In the lichen forming fungus *Ramalina farinacea*, Casano et al. (2011) found the coexistence of two different taxa of *Trebouxia* within a single thallus where one photobiont strain was more efficient at high temperature and irradiance compare to the other strain which was more efficient under moderate conditions. Henskens et al. (2012) reported that several cyanolichens (with cyanobiont as the main photobiont) have in addition green algae present in the photosynthetic layer of the thallus and that both photobionts are photosynthetically active.

The ability to form symbiotic associations with multiple partners can be spectacular in the lichen order Peltigerales, where one mycobiont species can associate with both, green algae and cyanobacteria in a single thallus. In this multi-photobiont thallus, the green photobiont is present in a well-delimited algal layer within the thallus while the cyanobacterium (*Nostoc*) is confined to specific internal (e.g., in the genus *Lobaria*) or external structures (e.g., in the genus *Peltigera*) named cephalodia (James and Henssen, 1976). Sometimes, joined or independent, morphologically and chemically differentiated thalli (called photosymbiodemes, or photomorphs) containing both photobionts and/or either of the photobiont (leaf-like part with phycobiont and fruticose part with cyanobiont in the genera *Sticta* and *Lobaria*; Takahashi 2006; Högnabba et al. 2009) can develop with genetically identical mycobionts (Armaleo and Clerc, 1991). It has been shown that in photomorphs, the cyanobiont-containing and the phycobiont-containing lobes (or independent thalli) are better adapted to different environmental conditions, which stimulated their development (Green et al., 2002).

Among the 24 cyanolichens from the family Collemataceae (Peltigerales) studied by Otálora et al. (2010b), five cases of "one-to-one" mycobiont-cyanobiont specialists were found; each representing an independent transition from a generalist state (sexually reproducing mycobiont) to a strict specialist state (asexually reproducing mycobiont), resulting from the vertical photobiont transmission via specialized vegetative propagules. Other studies on cyanolichens revealed a relatively high selectivity and specificity in the studied lichen association. For instance, Myllys et al. (2007) found that several mycobiont species from the genera *Nephroma*, *Parmeliella* and *Peltigera* (Peltigerales) are highly selective towards their cyanobiont partners, and Stenroos et al. (2006) concluded that mycobionts in two other genera, *Sticta* and *Pseudocyphellaria*, seem to be able to select a specific strain, species or species group of Nostoc. Similarly, O'Brien et al. (2013) found high fungal specialization in the genus *Peltigera*, where each studied lichen-forming species was associated with either one or two of the cyanobacterial lineages, while the level of cyanobacterial specialization was variable, but generally much lower. Rikkinen et al. (2002) proposed a guild hypothesis to explain the sharing pattern of photobionts by mycobionts occupying ecologically distinct but adjacent habitats where often phylogenetically unrelated lichen-forming species from the same habitat (growing on trees) share more closely related *Nostoc* strains with each other than with their phylogenetic relatives from the adjacent but drastically different habitat (growing on the ground; epiphytic versus terricolous lichen guilds).

Several studies reported high level of specificity between mycobionts and photobionts from the genus *Trebouxia*. For instance, Yahr et al. (2004) found high specificity in the selected species of *Cladonia* and their photobiont *Trebouxia*, regardless of their habitat. Recently, Lindgren et al. (2014) reported that most mycobionts of studied *Bryoria* species were highly selective towards their *Trebouxia* photobiont. Other studies (Myllys et al., 2007; O'Brien et al., 2013) reported high specificity in sexually reproducing species, involving horizontal transmission of the photobiont, suggesting that symbionts might be co-adapted and are capable of recognizing each other via a geneti-

Figure 1: *Peltigera neopolydactyla* 2c from Eastern Russia (picture: J. Miadlikowska)

cally regulated mechanism.

4. Outline of the thesis

As part of my Doctoral Dissertation I studied associations among mycobionts and cyanobionts (*Nostoc*) in lichens that are classified in the families Peltigeraceae (the genus *Peltigera* sections *Polydactylon* and *Hydrothyriae*), Pannariaceae (the genera *Fuscopannaria*, *Kroswia*, *Physma*, *Parmeliella* and *Pannaria*, mostly), Lobariaceae (the genera *Lobaria* and *Sticta*) in the order Peltigerales, Lecanoromycetidae (comprising most known cyanolichens) and the the family Arctomiaceae (the genus *Arctomia*) in the Arctomiales (Ostropomycetidae).

I gathered multilocus molecular data and analyzed them using various software in order to reconstruct phylogenetic relationships for each, the mycobiont and photobiont partners (e.g., PAUP, Swofford 2003; BEAST, Drummond and Rambaut 2007, MrBayes, Huelsenbeck et al. 2001) and to delimit species (e.g., Structurama, Huelsenbeck et al. 2011; bGMYC, Reid and Carstens 2012; bPP, Yang and Rannala 2010). I also examined morphology and chemistry (Thin Layer Chromatography; TLC) of ca. 200 specimens collected during various field trips (Reunion Island in 2009; Norway, Canada: Québec, USA: North Carolina and Alaska in 2011; Russia, Peru and Brazil in 2012) and ca. 150 herbarium collections (from AMNH, B, BG, CGMS, CONN, DUKE, H, LG, MAF, MEXU, NSPM, NY, O, PTZ, QFA, UBC, UDBC, UGDA, UMEX, UPS). Phylogenetic

reconstructions and data on geographic distribution for studied lichens allowed me to examine patterns of specificity of mycobionts and cyanobionts as well as their impact on the evolution and diversification of lichen symbioses and morphological structure of the thallus within the studied groups. Species delimitation methods based on DNA sequences provided a base for defining species that represent meaningful evolutionary lineages and helped me to find sets of diagnostic, morphological features (including cyanobacterial identity manifested by the thallus color) circumscribing them (including many newly discovered ones) and corroborating with their geographical ranges (usually restricted to a single continent). I described a several species new to science.

I used *Peltigera* section *Polydactylon* as a case study to compare DNA-based species delimitation methods in order to identify significant species units and to assess the importance of the phylogenetic and geographic data of the cyanobiont *Nostoc* in recognizing *Peltigera* species. Most species from this section reproduce sexually, but a few members produce also vegetative propagules (mostly phyllidia). This section as a whole, has a broad, almost cosmopolitan, distribution. However, the distribution range of individual species varies greatly within the group ranging from endemism in small geographical areas such as the Azores to broad distributions covering North America, Europe and Asia (Martínez et al., 2003). It has been suspected that section *Polydactylon* contains several species complexes encompassing cryptic as well as morphologically distinct but unrecognized species. Because most species in this group have relatively uniform morphology, the implementation of a morphological species concept is likely to lead to an underestimation of the number of species. However, the recognition of geographically structured morphotypes and chemotypes within broadly distributed taxa such as for *P. neopolydactyla*, *P. scabrosa* (Holtan-Hartwig, 1993; Vitikainen, 1994), and P. dolichorhiza (Sérusiaux et al., 2009) strongly suggest the presence of multiple undescribed species.

Distinct morphological, chemical and geographical patterns detected within the section *Polydactylon* and the occurrence of *Nostoc* as the only photobiont associated with members of this section, makes it a good model system for testing:
1) the patterns of symbiotic association among mycobionts and their photobionts collected from various habitats and regions based on phylogenetic relationships reconstructed for each symbiont;
2) if cosmopolitan lichen species represent single "evolving metapopulation lineage" (De Queiroz, 1998) or assemblages of morphologically similar but evolutionary distinct lineages.

Chapter one reports patterns of associations between the mycobiont *Peltigera* and its *Nostoc* cyanobiont with section *Polydactylon*. I sequenced five loci (including three newly developed molecular markers) for the mycobionts and one locus for the cyanobionts for ca. 200 thalli representing most of the geographic and morphological variation. This is the first study attempting to determine the patterns of association among symbionts of a lichen group at a worldwide scale. I reconstructed phylogenetic

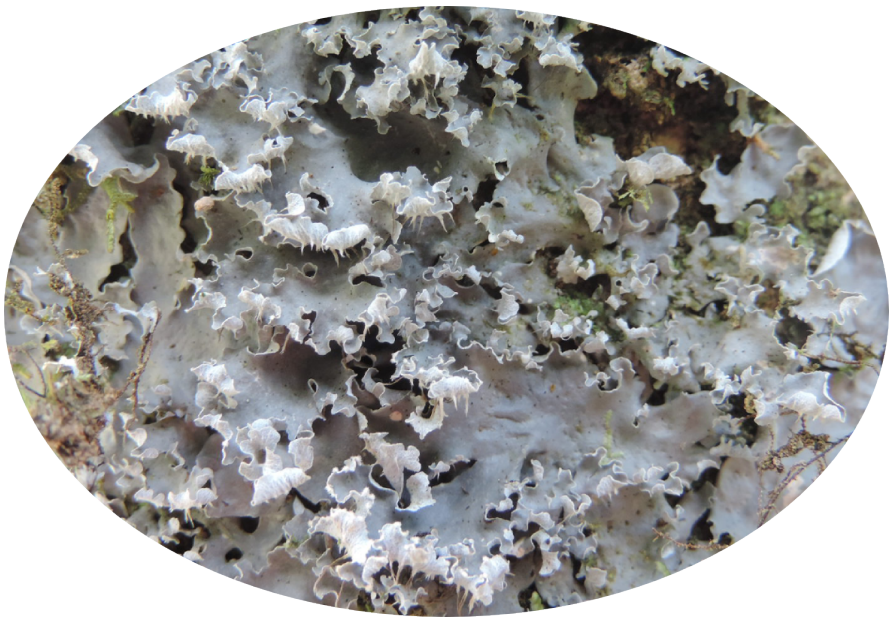

Figure 2: *Peltigera dissecta* from the Azores (picture: E. Sérusiaux)

relationships of both partners and determined the association profiles for mycobionts and cyanobionts within a phylogenetic framework.

1. I found a broad spectrum of specificity for both partners, ranging from strict specialists to generalists. The dominant trend involved mycobionts being more specialized, i.e., associating mostly with one or few *Nostoc* phylogroups (even when several other phylogroups were available in the associations with other neighboring *Peltigera* species), than cyanobionts, which were leaning more toward generalism, i.e., associating frequently with several *Peltigera* species.

2. I detected various degrees of specificity for mycobionts, ranging from strict specialists, always associating with the same *Nostoc* phylogroup despite a broad geographical range (different continents), to generalists found in associations with a variety of phylogenetically distantly related *Nostoc* groups, often within the same geographic region.

3. Mycobiont species representing recent speciation events have a potential to extend their ranges to a new biogeographical area due to switches from a specialist to a generalist selection of cyanobionts, while mycobiont specialist seems to be favored in the areas where species have been established for long periods of time.

4. I found high selectivity in the mycobiont-cyanobiont associations. Several co-existing species on different continents were often found with specific photobionts even if other photobionts were detected in association with co-occurring species. This pattern indicates the presence of a mechanism for partner recognition that lead to the establishment of the symbiosis with a suitable partner only.

5. I found a signature of local specialization, where mycobionts always associate with a certain *Nostoc* phylogroup in a certain geographical region and partner with a different phylogroup in another region. These regional switches to different cyanobiont often enable species to expand their geographical ranges latitudinally.

6. The distribution of *Nostoc* and *Peltigera* seem to be shaped by climate, as well as by the limitations on their long-distance dispersal abilities. In particular, I found that climate was a very important factor for *Nostoc* distribution as the unique sets of *Nostoc* strains were found in boreal versus tropical regions.

7. Mycobiont specialists have smaller ranges than non-specialists. In average their kilometric and latitudinal ranges were, respectively twice and three times smaller than the ranges of non-specialists. I hypothesize that this phenomenon is correlated with the ability of non-specialists to associate with cyanobacterial partners that have different non-overlapping ranges, and therefore allowed the mycobionts to expand their own ranges, whereas specialists are limited to the range of their sole partner.

8. I detected a significant increase in diversification rates in a recently diversified lineages of section *Polydactylon* encompassing many non-specialist species. Diversification rates were slower in the clades containing specialists, and overall higher for generalists. I also found that the transition rate from generalists to specialists was higher than the opposite direction, suggesting that specialization is acquired through time.

9. Specialization of the mycobiont seems to be correlated with a decrease in its genetic variation, while a switch to generalism might be one of the mechanisms leading to adaptive radiations by the mycobiont, and to the expansion of the mycobionts to new environments.

Chapter two includes the study of symbiotic associations between mycobionts and their *Nostoc* cyanobionts in the family Pannariaceae (Peltigerales). I reconstructed the phylogenies of the mycobionts and their respective photobionts and explored the revealed relationships in the context of the morphological aspects of the thalli formed by the sampled lichens.

Pannariaceae contains tripartite members associating with both, cyanobacteria and the green algae, as well as bipartite members associating only with cyanobacteria. Two types of thallus structures were defined within this family: pannarioid thallus, typical for most members of Pannariaceae (*Pannaria*, *Fuscopannaria* or *Parmeliella*) where the *Nostoc* cells are organized in a well-defined layer inside the thallus and collematoid thallus, typically found in members of the family Collemataceae where the *Nostoc* cells are spread across the thallus giving it a gelatinous consistency when wetted. Representatives of Pannariaceae with collematoid type of thallus were often erroneously classified within Collemataceae (Wedin et al., 2009; Otálora et al., 2010a).

1. Phylogenetic reconstructions showed that collematoid morphology evolved multiple times during the evolution of Pannariaceae because members with pannarioid and collematoid thalli were intermixed across the mycobiont phylogeny and were often sister to each other.

2. This study shows that collematoid morphology (e.g., in *Kroswia*) results from the association of the mycobiont with a specific strain of *Nostoc*, phylogenetically closely related to cyanobionts associated with phylogeneticaly unrelated but morphologically similar member of Collemataceae, *Leptogium lichenoides*. *Kroswia* represents a case of the "photobiont switch in progress" as its mycobiont is genetically very similar to members of Pannariaceae, *Fuscopannaria leucosticta* and *F. praetermissa*, both forming the pannarioid type of the thallus. It is very likely that other genera with the collematoid thalli in the Pannariaceae resulted from similar photobiont switches, which occurred earlier during the evolutionary history.

3. Unlike previously assumed that all tripartite Pannariaceae form a monophyletic group, the tripartite members of the genus *Psoroma* (including two recently described genera, *Xanthopsoroma* and *Psorophorus*; Elvebakk et al. 2010) are nested within several unrelated clades placed across the Pannariaceae.

4. The ancestral state reconstruction showed that in Pannariaceae most lineages of bipartite lichens (e.g., *Parmeliella mariana* and the genus *Physma*) associated with *Nostoc* only originated from the tripartite ancestors (containing both green algae and cyanobacteria) by multiple emancipation events from the cephalodia (containing *Nostoc* only) coupled with subsequent losses of green algae. This discovery was revealed by the ancestral state reconstructions and supported by the evidence that the same *Nostoc* phylogroups present in cephalodia of the tripartite thalli were also found in bipartite members of the genus *Physma*.

5. This study shows that *Nostoc* symbionts have a big impact on the morphology of the

lichen thallus and the species ecology, and therefore shapes the adaptation and evolution processes of lichen-forming lineages.

Chapter three contains a formal taxonomic transfer of the genus *Kroswia* to the genus *Fuscopannaria* based on a broad phylogeny (on 3 loci: mtSSU, nuLSU and *RPB1*) containing several representatives of the genus *Fuscopannaria*.

Chapter four consists of a phylogenetic study of section *Hydrothyriae* encompassing two aquatic members from the genus *Peltigera* (*P. hydrothyria* and *P. gowardii*). Based on a global multigene phylogeny of the genus, I confirmed the phylogenetic affiliation of section *Hydrothyriae* within *Peltigera*. However, its exact placement within the genus remains uncertain. The reconstructed phylogeny revealed three distinct, morphologically homogeneous, lineages corresponding to the two known species and a new species, which was formally described here as *P. aquatica*. All aquatic *Peltigera* are associated with a unique *Nostoc* strain, phylogenetically distinct from the cyanobionts found in other *Peltigera* sections, as revealed by a large-scale phylogenetic study of *Nostoc* using *rbcLX*.

Chapter five includes a formal description of a new species from the genus *Arctomia* (*Arctomia borbonica* Magain & Sérus.) collected on Reunion Island. Based on its morphology (non-stratified thallus), this cyanolichen was initially identified as a *Leptogium* species (Collemataceae) but phylogenetic reconstruction confirmed its placement within the genus *Arctomia* (Arctomiales, Ostropomycetidae; see also Miadlikowska et al. 2014a). This is another example of morphological convergence of the thallus structure among phylogenetically unrelated mycobionts (parts of different orders and subclasses within Lecanoromycetes) associated with closely related cyanobacteria.

Chapter six includes a report of a newly discovered photosymbiodemes from Reunion Island. I examined photosymbiodemes occurring in the genus *Sticta* and *Lobaria* in the family Lobariaceae. A single mycobiont was found to form a joint thallus composed of the lobes containing the green algae (chloromorph) and the cyanobacteria (cyanomorph) only, or both morphs were detached from each other forming independent thalli. In *Sticta dichotoma*, morphologically alike, chloro- and cyanomorphs formed a single thallus, but sometimes the chloromorphs were growing separately. In *Lobaria discolor* I observed *Dendriscocaulon*-like fruticose cyanomorph and the foliose chloromorph growing separately. Similarly to the origin of bipartite thalli in Pannariaceae, it is very likely that cyanomorphs in Lobariaceae originated from cephalodia emancipation of the typically tripartite thalli. These switches of photobionts, from green alga to cyanobacteria substantially influence the morphology of the thallus.

Chapter seven is focusing on species delimitation within section *Polydactylon* of the genus *Peltigera*. I reconstructed the phylogeny of the mycobiont based on molecular data from eight loci, including three newly designed intergenic *Peltigera*-specific markers (IGS1, IGS3 and IGS16) and applied five species delimitation methods. I focused on two major clades of the section: the Scabrosoid clade, where the lineages representing putative species are well delimited and most phylogenetic relationships among them are highly supported by bootstrap values; and the Dolichorhizoid clade, where substantially lower levels or resolution and bootstrap support was obtained and, species delimitation was more challenging.

I applied five species delimitation methods, to assess species boundaries and their overlap among methods. The methods I used rely on very different models: Structurama (Huelsenbeck et al., 2011) infers population structure based on allele distributions; bGMYC (Reid and Carstens, 2012) and bPTP (Zhang et al., 2013) search for a threshold in phylogenetic trees where branching events switch from divergence between species to coalescence within species; and spedeSTEM (Ence and Carstens, 2011) and bPP (Yang and Rannala, 2010) attempt to delimit species the way that single gene topologies best fit a species tree in a colaescent framework.

1. All methods resulted in mostly congruent species delimitations within the Scabrosoid clade. A total number of 12 species including 9 previously unrecognized species was detected. In the Dolichorhizoid clade, methods relying on different models and assumptions provided different species delimitations, highlighting the necessity to use various criteria before formal species assignments are made. The species delimitations I proposed were based on a consensus among these various methods. I concluded that the Dolichorhizoid clade comprises 29 species, for which only 7 have already been described and named. The consensus approach revealed that most "evolutionary significant" species have relatively well-defined distribution ranges (usually panboreal or restricted to a single biogeographic region).

2. In most cases the same name was applied to morphologically similar but allopatric or often sympatric species. Nevertheless, the validity of a few broadly distributed species was confirmed here. For example, *P. dolichorhiza* is present in the Neo- and Afrotropics. However, the cosmopolitan *P. neopolydactyla* and *P. scabrosa* occur only in panboreal zones and both taxa include multiple species. Morphotypes embedded within *P. neopolydactyla* represent at least nine species in the boreal zone, with two of them being endemic to the Pacific Northwest of North America. Similarly, most specimens that were identified as *P. polydactylon* in Europe and North America represent three distinct lineages, whereas specimens identified as *P. polydactylon* or *P. dolichorhiza* in Asia or Papua New Guinea contain several new species.

3. The majority of newly-delimited species showed a high specificity towards their cyanobionts (except for a group of South American species representing the most recent radiation event within the section) despite the fact that they reproduce sexually (i.e., most specimens examined had apothecia). This pattern of high specificity shown by sexually reproducing mycobionts suggests that mycobionts and cyanobionts are likely to be co-adapted and recognized by each other during the partner selection process.

4. Photobiont data, i.e. the phylogenetic identity of the cyanobiont and their phenotype (the color of the thallus when wetted or dry in some cases), as well as geographical data on the distribution of lichen thalli, i.e., geographic regions coupled with climatic factors, provide complementary information to the morphological and chemical characteristics that can be used to circumscribe and identify species, especially in the absence of molecular data.

5. Species circumscription (too broad or too narrow in comparison with evolutionary meaningful lineages) can obscure the patterns of mycobiont specificity and selectivity toward its cyanobacterial partners. For example, *P. neopolydactyla* and *P. scabrosa* as currently circumscribed are generalists, however, they consist of multiple highly specific and selective species. Therefore, it is very important to recognize biologically meaningful species before examining their patterns of symbiotic association.

6. The results fit very well the geographic mosaic of coevolution theory (Thompson, 2005) where mycobionts in different regions most often specialize on different *Nostoc* phylogroups .

7. All newly delimited species will be formally described in a separate, follow-up publication.

5. Summary

This study on the symbiotic associations in *Peltigera* section *Polydactylon* and related cyanolichens from the order Peltigerales shows that cyanobiont identity (e.g., within a phylogenetic context) shapes the ecology, evolution and speciation of mycobionts (lichen species). Photobiont switches play an important role as a mechanism impacting various aspects of lichen macroevolution, species diversification, morphological appearance and the range expansion to new regions or new ecological niches.

6. Future directions

In the future, I want to extend the study on symbiotic associations in cyanolichens to the genomic (metagenomes of mycobionts and photobionts involved in the symbiosis) and transcriptomic levels (level of gene expressions at different stages of thallus development and thallus parts for symbionts) in order to understand the mechanisms leading to the recognition of the partners, the establishment of the symbiosis, and the acquisition and maintenance of specificity. I would like to explore genetic differences between closely related bipartite and tripartite species; cosmopolitan versus locally distributed species; and specialist versus generalist species.

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Chapter 1

Evolution of specificity in cyanolichen symbioses: a case study of *Peltigera* section *Polydactylon* (lichenized Ascomycota; Peltigerales).

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1.1 Abstract

Variation in specificity among symbiotic partners is key to a comprehensive understanding of the evolution of symbiotic systems. This variation is expected to occur within species as well as within a broader inter-species phylogenetic framework. Yet, specificy of mutualistically interacting partners, based on a worldwide sampling of all known species of a monophyletic group for one of the symbiotic partners, has rarely been studied. Here we assess the level of inter-partner specificity between the cosmopolitan lichen-forming fungus (mycobiont) from the genus *Peltigera*, section *Polydactylon*, and its cyanobacterial partner *Nostoc* (cyanobiont). The phylogenies of the mycobionts and their cyanobionts, are based on five nuclear loci and the *rbcLX* region, respectively. This sequence data was obtained from 208 lichen thalli, representing ca. 40 closely related *Peltigera* species sampled worldwide. We found a broad spectrum of specificity for both partners ranging from strict specialists to broad generalists. In general, mycobionts are more specialized than cyanobionts by associating mostly with one or few *Nostoc* phylogroups, whereas cyanobionts associate most frequently with several *Peltigera* species. The relatively recent colonization of a new geographic area (South America) by members of section *Polydactylon*, seems associated with a switch to a generalist pattern of association. Our results support the hypothesis that specialization of mycobionts to one or few cyanobionts, is acquired through time and favored in geographic areas where species have been established for long periods of time. We detected a higher genetic

diversity and higher diversification rates in mycobionts with lower degrees of specialization. We also found that Peltigera species specialized on a single Nostoc phylogroup have narrower geographical distributions compared to generalist species that are associated with different cyanobionts in different geographic areas or with cosmopolitan cyanobionts. Specificity and patterns of mycobiont-cyanobiont associations seem to play a key role in various aspects of the evolution of *Peltigera*, section *Polydactylon*, and their cyanobiont *Nostoc*, including range expansion, genetic diversity and rates of diversification.

Keywords: cyanobiont; mycobiont; photobiont; lichen symbiosis; species delimitation; specificity; multigene phylogeny; mutualistic interactions

1.2 Introduction

Understanding how species interact is intrinsic to studying species evolution and ecology. For decades, species interactions were studied mostly within an ecological framework, whereas evolutionary biology was mostly restricted to the study of groups of closely related organisms without taking into account the interactions of these organisms with their environments and distantly related species present in their ecosystems (Thompson, 1994, 2005). Recent biotechnological advancements have transformed the study of complex symbiotic systems by facilitating the emergence of cross-disciplinary studies involving evolutionary biology, ecology and genomics/genetics. The resulting synergy enabled researchers to address far-reaching questions that could not be addressed previously (Thompson, 2005). Species interactions are central components of all ecosystems. Among others, they include relationships of prey-predators, competitors, parasite-host, commensalism or epiphytism (Krebs et al., 1994). Understanding species interactions is crucial to study population and ecosystem dynamics, with possible applications in agriculture or medicine. Mutualism, where all partners benefit from their interactions, is a particular case of species interactions. It evolved across kingdoms and involves all kinds of organisms, e.g. humans with their microbiome, dinoflagellae and zooxanthelle in corals, ants with fungi, Rhizobium in nodules with legumes, vascular plants with mycorrhizal fungi, and lichen-forming fungi with green alga and/or cyanobacterium (Thompson, 1994).

In the past decades, several models were developed to account for the evolution of mutualistic interactions. The simplest models, originating from prey-predator or competitor interactions and based on the Lotka-Volterra equations implied that if both partners are beneficiary, they should reach an infinite population size (see May 1982. Modifications of these models to adapt them to the specific case of mutualistic interactions have been proposed (Tainaka et al., 2003; Yoshimura et al., 2003). Other popular models are variants of the prisoners dilemma model, where both partners can get benefits by collaborating, but individually can get more profit by cheating (Trivers, 1971; Axelrod and Hamilton, 1981). One of the issues raised has been that most of these

models have ignored the difference in ecological requirements of the partners. To account for this factor, it has been suggested that the investment of a partner depends on the previous investment of the other partner, and thus could explain why an initially good investment, rather than no investment at all, could be favoured, and that the investment varies with time (Doebeli and Knowlton, 1998). Law and Lewis (1983); Law (1985) suggested that in mutualistic symbioses the endobiont (i.e., the symbiont, which is less subjected to the outside environment) should show reduced sexual reproduction because the overall selection pressure is toward maintaining genetic stasis rather than toward differences from parents. According to these authors, genetic diversity would not be promoted for a partner protected from environmental stresses (inhabitant), and therefore it can be advantageous to evolve slower, so that your partner can better adapt to you. It would result in positive selection on the most dominant haplotype, that gives predictable income and thus positively select asexuality and lower the rate of genetic change (Law, 1985). The benefits would be less virulence of the symbionts (a reduction in host fitness caused by symbionts, associated with a reduction in the group fitness of the symbionts in a host, Frank 1996) and more benefits from the symbiosis, as the partners are best adapted to each other (Law, 1985). In lichens, it has been shown for instance that the photobiont, *Coccomyxa* (inhabitant in lichen thallus) is evolving slower than the fungus (exhabitant forming lichen thallus; Zoller and Lutzoni 2003). Another model, the Red King hypothesis (contrary to the Red Queen hypothesis for competitors or parasite-host interactions, van Valen 1976) suggested that evolving slower in mutualistic relationships could be advantageous, because if the two partners give a first "selfish" investment (not investing enough to maintain a successful association), both partners will have to evolve towards generosity, investing more in the association to reach a viable equilibrium and maintain the association. Evolving slower than your partner can allow you to increase your investment slower than the partner, and therefore invest less (i.e., more selfish) when a viable equilibrium is reached (Bergstrom and Lachmann, 2003).

Recent studies (see review by Sachs et al. 2011) demonstrated that a strict delimitation between host-pathogen and mutualistic interactions might not be righteous due to a considerable degree of similarity between the two types of interactions, and higher diversity of patterns of mutualistic associations than previously assumed. Therefore, mutualism and parasitism should not be seen as binary, but rather as a continuum with many possible nuances. Mutualisms can have very different ranges of generalist/specialist patterns in terms of the number of partner species (Ollerton, 2006). Generalism allows the occupancy of more different niches and reduces pressure from a specific limited resource, while specialism allows for optimizing the benefits obtained from a specific partner (Vienne et al., 2013). According to the theory of the geographic mosaic of coevolution (Thompson, 2005), a species can adapt and specialize to different species in different geographical regions. Coevolution (in a broad sense) is thus geographically structured, with interactions evolving differently in different regions (geographic mosaics) because of the differences in various factors in different environments. As a consequence, coevolution in the long term should be seen at a global geographic scale rather than within

local populations (Thompson and Cunningham, 2002; Thompson, 2005).

Within the full spectrum of symbiotic interactions, obligate mutualism represents one of the extreme cases, because one species needs another species to be able to develop and contribute substantially to the next generation (Wolin, 1985). This is true for the great majority of multicellular organisms that depend on microbial communities to thrive in nature (plants with mycorrhizal fungi and endophytes; animals with digestive tracks that are adapted to host essential microbial communities; van der Heijden et al. 1998; Arnold and Lutzoni 2007; Turnbaugh et al. 2007). Lichen-forming fungi are obligate mutualists (mycobiont, mostly Ascomycota) associated with one or several photosynthetic partners (photobionts, mostly green algae and cyanobacteria; Honegger 1998; Nash 2008). The photobiont is usually less dependent on the mycobiont than vice versa. Several lichen photobionts are known to grow freely in nature (e.g., *Trentepohlia* spp. and *Nostoc* spp.) and are often more easily isolated in vitro than the fungal partner (Lutzoni and Miadlikowska, 2009; McDonald et al., 2013). Cases where lichen-forming fungi occur in a free-living saprotrophic stage, e.g., *Stictis* (Wedin et al., 2004), are extremely rare and are found in lineages where a lost of lichenization took place, such as in the case of the Stictidaceae within the Lecanoromycetes (Lutzoni et al. 2001, see also Chen et al. 2015). Acording to Lutzoni et al. (2014) the origin of ascolichens is estimated at ca. 430 Ma, and appears to have been very stable since then (Lutzoni et al., 2001; James et al., 2006; Honegger et al., 2013). For the great majority of lichens, the photobiont is localized inside the thallus built by the mycobiont and provides carbohydrates, as well as fixed nitrogen (by cyanobionts), to the mycobiont. The role of the mycobiont is more controversial. It has been proposed that the mycobiont reduces competition for the photobiont embedded in the thallus, increases the opportunity for the photobiont to colonize a broader range of habitats, and develops a stem-like or leaf-like thalli that increases the surface area for the photobiont to grow with appropriate exposure to light (Honegger, 1998; Nash, 2008).

Transmission of the photobiont from one generation to another occurs vertically through thallus fragments and vegetative propagules (e.g., soredia, isidia, phyllidia) containing both the mycobiont and the photobiont, or horizontally when the mycobiont is repropruducing sexually and resulting spores have to be sufficiently close to an appropriate photobiont to initiate the next generation of lichen thalli (Nash, 2008). Previous studies demonstrated that the mode of dispersion shapes the genetic structure of both partners (e.g., Dal Grande et al. 2012; Werth and Scheidegger 2012). Photobiont switches are common in lichen-forming fungi (Piercey-Normore and DePriest, 2001; O'Brien et al., 2013; Magain and Sérusiaux, 2014). The most extreme example includes photosymbiodemes where the same fungus can associate with either a green alga or a cyanobacterium, resulting in a different morphology depending on the photobionts (James and Henssen, 1976; Armaleo and Clerc, 1991; Magain et al., 2012). Lichen-forming fungi swhitching to new photobionts can also influence thallus morphology and can be an evolutionary force leading to species diversification (Magain and Sérusiaux, 2014) as well as to the colonization of new environments (Fernández-Mendoza et al.,

2011).

Lichen mycobiont-photobiont patterns of associations are difficult to study partly because of unclear species delimitations among the interacting fungi, algae and cyanobacteria. For mycobionts, problems include the presence of many cryptic species, due to the lack of diagnostic morphological characters (see Leavitt et al. 2011; Lumbsch and Leavitt 2011). Other problems include morphological convergence among distantly related taxa (e.g., Magain and Sérusiaux 2012; Bendiksby and Timdal 2013; Otálora and Wedin 2013), and morphological plasticity that can result in the description of distinct morphological species part of a single, genetically, defined species (Pino-Bodas et al., 2011). The total number of fungal species is estimated to be about 5.1 million (O'Brien et al., 2005b; Blackwell, 2011) while only ca. 100,000 of them have been described (Kirk et al., 2008; Blackwell, 2011). Approximately 16,000 of the known fungal species are lichen-forming (Kirk et al., 2008), many of which with suspiciously extensive geographic distributions. Therefore, lichen-forming fungi recognized as one species could represent multiple species. It is also believed that a large number of new species remain to be discovered in poorly sampled areas of the world and within understudied taxonomic groups (Lücking et al., 2014).

For lichen photobionts the situation is worse because most are unicellular or filamentous. Therefore, it is very difficult to distinguish species based on their morphology due to the lack of readily observable diagnostic phenotypic traits or because their morphology can change between the symbiotic (in vivo) and cultured (in vitro) stages (Vandamme et al., 1996; Beltrami, 2009; Flechtner et al., 2013; Fučíková et al., 2014). Moreover, the evolutionary histories of cyanobacteria, as for prokaryotes in general, is often obscured by well documented multiple horizontal gene transfers (Doolittle, 1999; Oren, 2004).

Associations between lichenized fungi and their cyanobacterial partners *Nostoc* have been the subject of numerous studies. The current paradigm is that a single lichen thallus hosts a single strain (single genotype) of *Nostoc* (Paulsrud and Lindblad, 1998), which usually has a wide geographic distribution, and can be found in association with a wide taxonomic range of mycobionts across continents (Paulsrud et al., 2000). However, rare cases of several photobiont genotypes within an individual lichen thallus (Casano et al., 2011) and two 'co-primary photobionts', a cyanobacterium (dominant) and a green alga forming a cryptic tri-membered symbiosis (Henskens et al., 2012), were recently reported.

It was also shown that lichen-forming *Nostoc* strains are closely related to symbiotic *Nostoc* found in associations with bryophytes and angiosperms, as well as to free-living strains (O'Brien et al., 2005a). It is not clear whether most symbiotic *Nostoc* strains can also be found free-living, even if some evidence suggests this possibility (Oksanen et al., 2002; Wirtz et al., 2003). Rikkinen et al. (2002), based on a study conducted at a small spatial scale, advocated that adjacent distinct habitats (on trees as epiphytes versus on soil as terricolous/muscicolous lichens) structure *Nostoc* symbionts in a way

that lichen-forming species in one habitat share more closely related *Nostoc* strains than with closely related lichen-forming species growing in an adjacent but drastically different habitat (epiphytic versus terricolous lichen guilds).

Otálora et al. (2010) demonstrated that closely related species of lichenized fungi from the family Collemataceae (Collemataceae; Peltigerales) have different levels of specificity, from broad generalists to strict specialists including rare cases of reciprocal one-to-one mycobiont-cyanobiont specificity at an intercontinental scale. Another study at a small spatial scale focusing on the genus *Peltigera* revealed that mycobionts display specificity in their selection of *Nostoc*, (an extreme case being *P. malacea*) and are overall more specialized than their cyanobiont partners (O'Brien et al., 2013). Most of these studies were restricted to a few lichen species or conducted at a small geographic scale, and did not confront existing hypotheses on the evolution of mutualistic systems. None of these studies attempted to include all species of a specific lichen-forming clade of fungi to assess the macroevolutionary trends of specificity of the mycobionts and photobionts across their geographical distribution.

Here we present the results of a study of the lichen-forming genus *Peltigera* (Peltigerales, Lecanoromycetes) section *Polydactylon*. *Peltigera* comprises lichen-forming fungi associated mainly with cyanobacteria from the genus *Nostoc* (to form bimembered foliose thalli) or with both *Nostoc* and a green alga *Coccomyxa* (to form trimembered foliose thalli). Section *Polydactylon* is one of the eight sections recognized in this genus (Miadlikowska and Lutzoni, 2000) and comprises only bimembered lichens. This section is cosmopolitan and its members are especially abundant in boreal old growth and tropical mountain forests (Martínez et al., 2003). Most of the fungal species in this section reproduce sexually (i.e., apothecia are commonly observed). Specialized vegetative propagules (isidia, soredia, phyllidia) that enable a codispersal of both partners (vertical transmission of the photobiont) occur only in a few members of this section (e.g., *P. pacifica*). Despite the possible occurance of simple thallus fragmentation, we are assuming that the majority of thalli from section *Polydactylon* are established through sexual reproduction of the mycobiont and, consequently, the horizontal transmission of *Nostoc*.

Currently 19 species are recognized in section *Polydactylon*. Nine species occur in the holarctic region (Martínez et al., 2003): *P. pacifica* (endemic to the Pacific Northwest); *P. neopolydactyla*, *P. scabrosa*, *P. occidentalis* and *P. scabrosella* (all panboreal except the latter one, which is restricted to Europe and North America); *P. hymenina* and *P. polydactylon* (holarctic distribution, from boreal to temperate regions); and *P. melanorrhiza* and (endemic to Azores; Vitikainen 1994). Five species are restricted to South America (Martínez et al., 2003; Vitikainen, 1998): *P. pulverulenta*, *P. microdactyla* and *P. dolichorhiza* in the Neotropics (the latter occurs also in Africa; Vitikainen 1998) and two occur in the neantarctic region: *P. truculenta* and *P. chilensis* (Martínez et al., 2003). *Peltigera nana*, *P. sumatrana*, *P. oceanica*, *P. weberi* and *P. macra* (a putative synonym of *P. nana*) are rare species reported from Eastern Asia/Australasia (Sérusiaux et al., 2009). No comprehensive phylogeny was published for section *Polydactylon* (see

Miadlikowska and Lutzoni 2000), and the monophyly of species in this section has never been investigated phylogenetically. Based on the morphological variation observed in this section of the genus *Peltigera*, at the inter- and intraspecific levels, as well as the broad geographical ranges recorded for many polydactylous species, multiple distinct species are likely to be embedded within currently accepted species, many of which are likely to be non-monophyletic (Miadlikowska et al., 2003). Due to their cosmopolitan distribution at the section level, but distinct distribution profiles at the species levels, their abundance in many parts of the world (boreal forests and mountain forests in temperate and tropical regions), as well as the presence of a single photosynthetic symbiont (*Nostoc* s. l.), the monophyletic section *Polydactylon* is a good candidate for a world-wide study of the evolution of specificity in a classic mutualistic symbiosis where the mycobiont is an obligate partner and where the *Nostoc* is believed to be capable of living (at least surviving) independently from the mycobiont. *Nostoc* associated with *Peltigera* have been isolated in axenic cultures (Drew and Smith, 1967), whereas, this was never achieved for *Peltigera* mycobionts.

The aims of this study were to: 1) confirm the delimitation of section *Polydactylon* and its phylogenetic placement within the genus *Peltigera* using multilocus data; 2) evaluate delimitations of morphospecies within this section using monophyly as a grouping criterion and species discovery methods based on multilocus data; 3) infer phylogenetic relationships among cyanobionts associated with members of section *Polydactylon* in a broad context of symbiotic and free-living *Nostoc* strains; 4) explore the biogeographic patterns, specificity and macroevolution of mycobiont-cyanobiont associations in this section and the factors shaping these trends; 5) confront these results with proposed evolutionary models for mutualistic systems (e.g., the Law and Lewis paradigm, Law and Lewis 1983; Law 1985; the Red King hypothesis, Bergstrom and Lachmann 2003; and the Geographic Mosaic of Coevolution, Thompson 2005).

1.3 Material and Methods

1.3.1 Taxon Sampling

Over 2000 specimens of *Peltigera* section *Polydactylon* (identified as such based on morphology) obtained as loans from several herbaria world-wide (AMNH, B, BG, CGMS, CONN, DUKE, H, LG, MAF, MEXU, NSPM, NY, O, PTZ, QFA, UBC, UDBC, UGDA, UMEX, UPS) and various private collections, as well as collected during numerous field trips part of this study (Reunion Island in 2009; Norway, Canada: Québec, USA: North Carolina and Alaska in 2011; Russia, Peru and Brazil in 2012) were examined to select a set of representative specimens for DNA extraction and sequencing of the mycobiont and associated cyanobiont. Species from the remaining sections of *Peltigera* (58 individuals) as well as outgroup taxa from Peltigerales suborder Peltigerinae (five individuals) were also selected for this study. The complete list of specimens included in this study

is provided in Supplementary Table S1.

1.3.2 Molecular Data Acquisition

We extracted DNA from approximately 950 well-preserved lichen specimens lacking any visible symptoms of fungal infection following two extraction protocols: Cubero et al. (1999) or modified Zolan and Pukkila (1986) using a 2% sodium dodecyl sulphate (SDS) as the extraction buffer.

We amplified the internal transcribed spacer (ITS) of the nuclear ribosomal tandem repeat of the mycobiont from about 850 lichen thalli representing a broad geographic and morphological diversity of the group, using the ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primers. We then selected 119 specimens, each represented by a unique ITS haplotype, or in some cases by identical haplotypes but collected from distant geographic regions (e.g., different continents), and we amplified four additional loci, the nuclear ribosomal large subunit (LSU) using primers LR0R and LR7 (Vilgalys and Hester, 1990), and three protein-coding genes: RNA II polymerase largest subunit (*RPB1*) using primers RPB1-AF (Stiller and Hall, 1997) and RPB1-CR (Matheny et al., 2002), elongation factor 2 region 1 (*EFT2.1*) using primers EFT2.1_1F (Miadlikowska et al., 2014) and EFT2.1_3R (primer sequence: 5'-ATCCCTGATACCAATGCATGCC-3'), and *β-tubulin* using the reverse primer BT2B (Glass and Donaldson, 1995) and the forward primer T1 (O'Donnell and Cigelnik, 1997) or alternatively bt_34F (O'Brien et al., 2009). These four loci were also sequenced for 58 specimens (representing 36 species) from the remaining seven sections of the genus *Peltigera*, as well as five representatives of the closely related genera from the suborder Peltigerineae as outgroup (Supplementary Table S1). In addition we selected a set of 208 specimens (partial overlap with the set of 119 individuals characterized by the ITS), for which we amplified the *rbcLX* region (the last 82 amino acids of the RUBISCO large subunit [*rbcL*], a putative chaperone gene [*rbcX*] and two intergenic spacers; Li and Tabita 1997) of their cyanobiont *Nostoc* using the CX and CW primers (Rudi et al., 1998).

PCR conditions are provided in Miadlikowska et al. (2014) and literature cited therein. All PCR products were cleaned with ExoSAP (Affymetrix Inc., CA, USA) following the manufacturer's protocol. Sequencing was carried out in 10 μl reactions using: 1 µl primer (10 μM), 1 μl purified PCR product, 0.75 μl Big Dye (Big Dye Terminator Cycle sequencing kit, ABIPRISM version 3.1; Perkin–Elmer, Applied Bio-systems, Foster City, CA), 3.25 μl Big Dye buffer, and 4 μl double-distilled water. Automated reaction clean up and visualization was performed at the Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies (for details see Gaya et al. 2012).

1.3.3 Single Locus and Concatenated Datasets

Sequences were edited using Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan) and subjected to BLAST searches (Wheeler et al., 2007) to confirm the fungal or cyanobacterial origin of each sequence fragment. Sequences were aligned manually using MacClade version 4.08 (Maddison and Maddison, 2005). Ambiguously aligned regions (*sensu* Lutzoni et al. 2000) were delimited manually and excluded from phylogenetic analyses.

Prior to data concatenation, single-locus phylogenies were generated for all five fungal loci (ITS, LSU, *RPB1*, β-tubulin, and *EFT2*.1) using RAxML-HPC2 version 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) as implemented on the CIPRES portal (Miller et al., 2010). Optimal tree and bootstrap searches were conducted with the rapid hill-climbing algorithm for 1000 replicates with GTRGAMMA substitution model (Rodriguez et al., 1990). Protein-coding genes were partitioned following their codon positions and introns, whereas a partition with two subsets was defined for the ITS (5.8S and ITS1+ITS2). To detect topological incongruence among single-locus datasets, a reciprocal 70% ML bootstrap support criterion was implemented (Mason-Gamer and Kellogg, 1996; Reeb et al., 2004). The single-locus topologies were congruent, i.e., no significant conflict was detected among the single locus datasets, except for the following three cases: 1) the placement of the *P. scabrosella*/*P.* sp.7 group (*EFT2*.1 versus all other loci), 2) the placement of *P. neopolydactyla* 5 and *P. scabrosa* 3 (*EFT2*.1 versus ITS), and 3) the placement of *P. neopolydactyla* 3 (ITS versus LSU and *RPB1*) (see Results and Discussion section). Because conflicting relationships did not involve major topological rearrangements, we combined the single locus datasets into three concatenated fungal matrices: Matrix 1, consisting of four loci (LSU, *RPB1*, *EFT2*.1 and *β-tubulin*) for 106 representatives (42 from section *Polydactylon*, 59 from the remaining sections of *Peltigera*, and five outgroup species), which was used to confirm the monophyly and species composition of section *Polydactylon* (Fig. 1); Matrix 2, consisting of five loci (ITS, LSU, *RPB1*, *EFT2*.1 and β-tubulin) for 119 representatives of *Peltigera* section *Polydactylon*; and Matrix 6, consisting of Matrix 2 with the addition of recoded characters (ambiguous regions of the ITS, LSU and selected introns of three protein-coding genes) using PICS-ORD (Lücking et al., 2011). In addition, four single-locus matrices were generated: Matrix 3, consisting of *rbcLX* sequences for 526 representatives of cyanobacteria (208 found in thalli of members of *Peltigera* section *Polydactylon*, 26 associated with members from other *Peltigera* sections, and 292 associated with various *Peltigera* species, other lichen genera, or plants, as well as free-living strains obtained from GenBank), that were collapsed to 417 sequences using a 100% similarity criterion in Sequencher; Matrix 4, consisting of the ITS sequences from 206 representatives of *Peltigera* section *Polydactylon* for which we sequenced the *rbcLX* region of the co-living *Nostoc*; Matrix 5, consisting of 209 *rbcLX* sequences (206 from *Peltigera* section *Polydactylon* and three outgroup sequences); and Matrix 7 containing one representative of all ITS haplotypes detected in section *Polydactylon* (Table 1).

Table 1: List of matrices used in the analyses, with the biont and the taxonomic breadth of the taxa it contains, the loci, number of taxa, number of character included, total number of sites, number of variable characters and of parsimony-informative characters. The numbers inside parentheses represent the proportion of the total.

Matrix No.	Biont	Taxonomic breadth	Loci	No. taxa	No. char. incl. / Total No. of sites	No. variable char.	No. parsimony-inf. char.
1	Mycobiont	Genus *Peltigera* (+outgroup)	Concatenated : 4	105	3135/3736 (0.84)	975 (0.31)	776 (0.25)
			LSU	84 (0.8)	1218/1392 (0.875)	244 (0.2)	163 (0.13)
			RPB1	98 (0.93)	638/716 (0.89)	276 (0.43)	235 (0.35)
			EFT2.1	78 (0.74)	784/942 (0.83)	261 (0.33)	216 (0.28)
			ß-tubulin	80 (0.76)	495/686 (0.72)	194 (0.39)	162 (0.33)
2	Mycobiont	Section *Polydactylon*	Concatenated: 5	119	3803/4621 (0.82)	566 (0.15)	410 (0.11)
			ITS	119 (1)	505/907 (0.56)	150 (0.3)	123 (0.24)
			LSU	118 (0.99)	1252/1374 (0.94)	78 (0.06)	61 (0.05)
			RPB1	109 (0.92)	676/716 (0.94)	87 (0.13)	61 (0.09)
			EFT2.1	84 (0.71)	818/942 (0.87)	120 (0.15)	81 (0.1)
			ß-tubulin	104 (0.87)	552/682 (0.81)	131 (0.24)	84 (0.15)
3	Cyanobiont	Genus *Nostoc* (+outgroup)	*rbcLX*	417	636/1162 (0.55)	319 (0.5)	277 (0.44)
4	Mycobiont	Section *Polydactylon*	ITS	206	861/907 (0.95)	313 (0.36)	280 (0.33)
5	Cyanobiont	Genus *Nostoc*	*rbcLX*	209	636/1162 (0.55)	237 (0.37)	176 (0.28)
6	Mycobiont	Section *Polydactylon*	Concatenated: 6	119	3907/4769 (0.82)	690 (0.18)	526 (0.13)
			ITS	119 (1)	475/907 (0.52)	135 (0.28)	108 (0.23)
			LSU	84 (0.8)	1231/1374 (0.9)	69 (0.06)	53 (0.04)
			RPB1	98 (0.93)	676/716 (0.94)	89 (0.13)	61 (0.09)
			EFT2.1	78 (0.74)	817/942 (0.87)	120 (0.15)	81 (0.1)
			ß-tubulin	80 (0.76)	560/682 (0.82)	129 (0.23)	82 (0.15)
			PICS-ORD char.	119 (1)	148	148 (1)	141 (0.95)
7	Mycobiont	Section *Polydactylon*	ITS	173	563/907 (0.62)	233 (0.41)	195 (0.35)

1.3.4 Phylogenetic Analyses

For maximum likelihood (ML) and Bayesian analyses on Matrices 1 and 2, data partitions were established using PartitionFinder (Lanfear et al., 2012). Thirteen initial subsets within Matrix 1 (LSU, *β-tubulin* 1st, 2nd, 3rd codon positions and introns, *RPB1* 1st, 2nd, 3rd codon positions and intron, *EFT2*.1 1st, 2nd, 3rd codon positions and intron) and 16 subsets within Matrix 2 (LSU, ITS1, ITS2, 5.8S, β-tubulin 1st, 2nd, 3rd codon positions and introns, *RPB1* 1st, 2nd, 3rd codon positions and intron, *EFT2*.1 1st, 2nd, 3rd codon positions and intron) were considered to estimate the optimal partition for subsequent phylogenetic analyses. We used the greedy algorithm to explore the nucleotide substitution models available in RAxML and MrBayes under different selection criteria (AIC, AICc and BIC) as implemented in PartitionFinder. RAxML searches for the optimal trees and bootstrap analyses (1000 replicates; GTRGAMMA substitution model) were performed on matrices 1 and 2 partitioned according to the best schemes generated using PartitionFinder and three additional arbitrary chosen partitions (commonly used in phylogenetic analyses). We compared likelihood scores, average bootstrap support values and the number of nodes that received bootstrap support values $\geq$ to 50%, 70%, 90%, 95%, and 100%. Results for Matrix 2 are presented in Table 2. Matrices 3 and 5 (*Nostoc rbcLX*) were divided into three partitions according to the codon positions. Partitions and substitution models selected for all matrices analyzed phylogenetically are included in Supplementary Table S2. These optimal partitions were found with PartitionFinder using all substitution models available despite the fact that only the GTR model could be implemented with RAxML.

The final RAxML searches for optimal trees and bootstrap analyses on matrices 1, 2, 3 and 5, were implemented using the rapid hill-climbing algorithm for 1000 replicates with the GTRGAMMA substitution model. RAxML analyses were performed on Matrix 6 using the same data partition as for Matrix 2 with the addition of one subset to accomodate recoded characters (PICS-ORD) used to capture phylogenetic signal in ambiguously aligned regions (Table 1).

We performed Bayesian analyses on Matrices 1-3 using MrBayes version 3.1.2 (Huelsenbeck et al., 2001) as implemented on the CIPRES portal with the partition schemes described in Supplementary Table S2. For Matrix 1 we used the default priors and completed 50 million generations, sampling every 500th generation. Matrix 2 was partitioned according to Arbitrary 2 partition scheme (Table 1 and Supplementary Table S2) and the best models for each partition was estimated using MrModelTest (Nylander, 2004). We used the default priors and completed 40 millions generations, sampling every 1000th generation. For Matrix 3, the subsets were defined according to codon positions and the GTR+I+G model was implemented for all subsets. We ran the program for 29 million generations, using default settings, sampling every 1000th generation. Two independent runs, each composed of four chains, were performed for each matrix. We assessed the convergence of chains using Tracer version 1.5 (Rambaut and Drummond, 2007) and Are We There Yet (AWTY, Nylander et al. 2008) as implemented on the

website http://king2.scs.fsu.edu/CEBProjects/awty/awty_start.php.

Matrix 2 was also analyzed with BEAST version 1.7.4 (Drummond and Rambaut, 2007) in order to generate a chronogram (relative time). The optimal nucleotide substitution model was selected using MrModelTest independently for all exons across all loci, and for all introns (see Supplementary Table S2). We ran BEAST with default priors, unlinking substitution models, but linking clock models (a lognormal relaxed clock) and tree models, for 50 million generations, sampling every 1000th generation. For the protein-coding genes, each codon position was treated as a separate partition and a lognormal distributed prior on the relative rate of the different codon positions with lognormal priors on relative rate parameters for codon positions were applied. We assessed the convergence of the analysis using Tracer and AWTY.

1.3.5 Species Discovery Methods

Species delimitation of *Peltigera* (mycobiont) was assessed with Structurama (Huelsenbeck et al., 2011) and bGMYC (Pons et al., 2006; Reid and Carstens, 2012). We analyzed three taxon subsets derived from Matrix 2, representing three major clades: the Polydactyloid clade with 25 individuals, the Dolichorhizoid clade with 70 individuals, and the Scabrosoid clade with 24 individuals. We coded alleles for six loci: ITS1, ITS2, *β-tubulin*, *EFT2*.1, LSU and *RPB1*. For the Polydactyloid clade dataset we also coded alleles of the 5.8S. We applied different priors (see Supplementary Figs. S1 and S2) to detect their effect on species delimitation. Analyses were run for 1 million generations, sampling every 1000th generation. Priors with the best fit to the data were selected. The gamma hyperprior shape parameter was set to vary from 1 to 50 for the Dolichorhizoid clade, and from 1 to 20 for the Polydactyloid and Scabrosoid clades. For the Dolichorhizoid clade (the most specious group within section Polydactylon) we tested various fixed hyperpriors (ranging from 5 to 35) on the expected numbers of populations, and completed analyses of 20 million generations for three different priors (gamma shape = 5, 20 and 40) to detect if the results would converge, regardless of the priors, by performing more MCMC generations. For each clade, we selected the gamma shape that gave the most coherent results (favouring monophyletic species, and fitting morphological and geographical data; see Results and Discussion section) and completed the final analyses with these priors for 20 million generations.

For the bGMYC analyses we determined the best substitution model using MrModelTest and analyzed Matrix 7 (containing all unique ITS haplotypes recovered in section Polydactylon; Table 1) using BEAST. Because of the low level of resolution obtained for the Dolichorhizoid clade resulting from section-wide analyses (due to a high proportion of characters that were excluded because they could not be unambiguously aligned), we performed another analysis on a subset of Matrix 7 restricted to the haplotypes from the Dolichorhizoid clade. For the analysis across section *Polydactylon*, we ran BEAST with a strict molecular clock, for 50 million generations, sampling one tree every 500 000th generation for a final set of 100 trees. For the analysis restricted to the Dolichorhizoid

clade, we run BEAST for 30 millions generations, sampling one tree every 2000th generation; excluded the first 10% of trees as burn-in, and then randomly selected 100 trees from the posterior distribution (13 500 trees). bGMYC analyses were executed on these two sets of 100 trees for 50 000 generations with a 40 000 generations burn-in, with threshold values of 2 and 100 and a starting point of (1,1,25). Samples were assigned to a species if the posterior probability of their haplotypes to represent the same species was higher then the probability of one of these haplotypes to belong to a distinct species (defined by a single haplotype).

We defined our species units according to a consensus based on both Structurama and bGMYC results. Discrepancies between the two methods were resolved following the most inclusive (broadly defined) species circumscriptions using monophyly as a deciding criterion. For example, if the "less splitting" method defined a monophyletic species, we accepted it and did not split according to the "more splitting" method; if the "less splitting" method defined a paraphyletic species, we chose more narrowly define species according to the "more splitting" method.

1.3.6 Haplotype Network Reconstruction

We generated haplotype networks using TCS version 1.21 (Clement et al., 2000) based on 206 mycobiont ITS haplotypes for which we sequenced the *rbcLX* of their co-living *Nostoc* symbiont (Matrix 4, Table 1). Haplotypes were connected using a parsimony criterion with the 0.95 threshold value, and gaps considered as a 5th state. When different paths of equal number of changes occurred, the path favouring indels rather than substitutions was selected because of their high frequency in the ITS alignment.

1.3.7 Phylogenetic Similarity analyses among *Nostoc* and *Peltigera* species using UniFrac

We performed three analyses with UniFrac (Lozupone and Knight, 2005; Lozupone et al., 2006) as implemented on the website http://bmf2.colorado.edu/UniFrac: (1) We compared the phylogenetic breath of *Nostoc* associated with mycobiont species based on the *Nostoc* tree resulting from the RAxML analysis of Matrix 5, where *Nostoc* communities were defined according to the putative *Peltigera* species as delimited with Structurama and bGMYC (as described above and as displayed on Fig. 2); (2) we compared *Nostoc* compositions in different biogeographic regions (as illustrated by the map in Fig. 2) using the same *Nostoc* tree as above (i.e., derived from Matrix 5); and (3) we compared *Peltigera* species composition in different biogeographic regions (map, Fig. 2) using the ML tree of *Peltigera* section *Polydactlon* based on Matrix 2 (Fig. 2).

Analyses 2 and 3 were performed twice, once with the biogeographic regions defined as depicted in Fig. 2, and a second time with finer geographic divisions: (1) the three northern regions (NA, WP and EP) were further split into arctic-boreal and temperate

subregions; (2) the neotropic (NT) region was divided into South and Central America; and 3) Australasia was subdivided by considering New Zealand, and Papua New Guinea as distinct subregions. Mycobiont species represented by a single thallus or a single photobiont sequence (*P. melanorrhiza*, *P. "hawaiensis"*, *P.* sp. 5, *P. pulverulenta* 3, *P. nana* 2, *P. macra*) were excluded from the analyses and the Oriental region was omitted from the coding scheme due to the limited number of specimens available from this region.

1.3.8 Ancestral State inferences

We inferred ancestral states for pools of *Nostoc* phylogroups shared by *Peltigera* species (i.e., cyanobiont pools, corresponding to networks formed when different *Nostoc* phylogroups are associated with the same *Peltigera* species). This compilation unveiled three cyanobiont pools: *occidentalis*, *dolichorhiza*, and *scabrosella*. *Peltigera* species associated with *Nostoc* phylogroups of the *occidentalis* pool, for example, were never found associated with *Nostoc* phylogroups of the *dolichorhiza* or *scabrosella* pools.

To infer ancestral states we used SIMMAP version 1.5.2 (Bollback, 2006), with default settings, on eight chronograms obtained from the phylogenetic Bayesian analysis of Matrix 2 using BEAST. We also implemented a maximum likelihood approach in Mesquite version 2.75 (Maddison and Maddison, 2006, 2011) using a single parameter MK1 model (because gain and loss of characters are both, here, switching from a pool to the other, we assume that the rate of gaining or losing a character is the same) and default settings (likelihood threshold T=2) to calculate the average likelihood probability of the ancestral states based on 20 chronograms, and the percentage of trees where an ancestral state was assigned to a specific node based on 3020 chronograms from the BEAST analysis of Matrix 2. We also used BEAST directly to infer ancestral states by completing 10 million generations and sampling every 1000th generation when analyzing Matrix 2. Finally, we used BayesTraits version 1.0 (Pagel et al., 2004) on a subset of 22 trees derived from the BEAST analysis of Matrix 2. We constrained selected branches (ancestors) on certain states, and compared the harmonic mean of the iterations by calculating Bayes Factors to verify which state of the ancestor leads to the best likelihood of the model (Pagel and Meade, 2004). We used the reversible jump function and a gamma hyperprior of mean and variance varying from 0 to 10 and completed 50 million iterations for each constrained state.

1.3.9 Diversification Analyses

We first conducted a phylogenetic analysis using *BEAST (Heled and Drummond, 2010) of a modified Matrix 2 with a single representative per species. For each species, we selected a representative with the highest number of loci available. We used a strict molecular clock and ran the analysis for 50 million generations, sampling every 1000th

generation. We discarded 10% of the trees (burn-in) and generated a majority rule consensus species tree based on the remaining 45 000 trees.

We performed BiSSE analyses (Binary State Speciation and Extinction; Maddison et al. 2007) as implemented in the R package diversitree (FitzJohn, 2012) on the mycobiont species tree obtained with *BEAST, testing whether being a specialist versus non-specialist (i.e., a *Peltigera* species found in association with a single versus several *Nostoc* phylogroups) plays a key role in the diversification process within the section *Polydactylon*. To determine the effect of missing data on our results, we performed two analyses: 1) with the complete species tree obtained with *BEAST and missing data coded as such for the trait tested (i.e., for *Peltigera* species for which the level of specificity to *Nostoc* is unknown); and 2) on a modified species tree, where *Peltigera* species with missing data (?) about their specificity to *Nostoc* were excluded.

To determine if major shifts in diversification rates occurred in the section *Polydactylon*, we used MEDUSA (Alfaro et al., 2009) with default settings and an AIC criterion as implemented in the R package geiger (Harmon et al., 2008) based on the species tree generated with *BEAST derived from the modified Matrix 2, where each species was represented by a single specimen.

1.3.10 Defining Biogeographic Regions

We delimited the geographic zones depicted in Fig. 2 based on the commonly recognized major biogeographic regions (Wallace, 1876): Neotropics (South and Central America, including Mexico were grouped together based on a preliminary correlation in species distribution between these two regions detected with ITS data), Nearctic (North America, without Mexico), Afrotropics, West Palearctic, East Palearctic, Oriental (represented by a single specimen from Vietnam) and Australasia. We considered the Pacific Northwest as a separate region because of its high degree of endemism for both, the mycobiont (exclusive occurrence of three *Peltigera* species: *P. pacifica*, *P. neopolydactyla* 5, and *P. neopolydactyla* 6) and two *Nostoc* phylogroups (XIb, XVII).

Based on preliminary geographical distributions of mycobionts and cyanobionts, we noticed that taxon compositions among arcto-boreal and temperate regions divided as described above were more similar within these biomes than across those biomes. For example, *Nostoc* phylogroups IV, VIIa, XIa, XIII, as well as identical haplotypes of *P. neopolydactyla* 1, 2, 4, *P. scabrosa* 1, 2, 3 and *P. occidentalis* were found in the arcto-boreal zone crossing three continents (North America, Asia and Europe). We therefore decided to further split the Nearctic, West Palearctic and East Palearctic into their temperate and arcto-boreal elements (we didn't split boreal and arctic zones because we have few data from the arctic zones, and the species found in this region are also present in the boreal zone), when comparing mycobiont species and cyanobiont phylogroup compositions among these regions using UniFrac (Supplementary Figure S3). Because the three arcto-boreal regions grouped together, we decided to treat

them as a Holarctic arcto-boreal region, and to keep the temperate regions divided into Temperate Nearctic, Temperate West Palearctic and Temperate East Palearctic.

1.4 Results and Discussion

1.4.1 Alignments and Data Matrices

The summary of the contribution of each marker to the assembled datasets is provided in Table 1. In Matrix 2 all five loci were available for 74 of the 119 taxa, whereas sequences from four, three and two loci were available for 31, 12 and 2 taxa, respectively. At the genus and section levels, *EFT2*.1 was the most difficult locus to amplify, with a 70-75% success rate, compare to 90-100% for the ITS, LSU, *RPB1* and *rbcLX*. *β-tubulin* was somewhat intermediary, with a success rate of 75-85%, due to clade specific amplification problems. For Matrices 1 and 2, LSU was the locus with the lowest proportion of variable characters and the lowest contribution to species delimitation within section *Polydactylon*. The ITS was the most variable marker at the section level, but this locus was too variable (positional homology was ambiguous for most parts of the ITS1 and 2) to be included in the phylogenetic analysis of *Peltigera* as a whole (i.e., excluded from Matrix 1). *RPB1* was the most variable protein-coding gene at the genus level (Matrix 1), but the least variable within section *Polydactylon* (Matrix 2). For the latter, *β-tubulin* delivered the greatest number of variable characters among the three protein-coding genes. It resolved the highest number of internodes with the highest level of internodal support, compared to all remaining loci (based on single locus phylogenetic analyses; trees not shown), even if the ITS contained the highest number of variable characters (150 vs. 131; Table 1). The ITS included the largest proportion of ambiguous sites that had to be excluded from the analyses (44% excluded at the section level, Matrix 2), followed by *β-tubulin* (28% excluded at the genus level, Matrix 1; 19% excluded at the section level, Matrix 2), and the remaining three loci (17-11% at the genus level, Matrix 1; and 13-6% at the section level, Matrix 2). For the *rbcLX* datasets (Matrices 3 and 5, Table 1) the two spacers were not alignable, and for this reason they were excluded from phylogenetic analyses.

1.4.2 Determining Partition Subsets for Phylogenetic Analyses

ML analyses of Matrix 2 were first designed to choose the optimal number and best types of data partitions (Supplementary Table 2). The initially defined 16 subsets (see Materials and Methods) were reduced to 11-12 based on the best likelihood scores of the resulting trees and overall bootstrap support (Table 2). Phylogenetic analyses performed on Matrix 2 divided into fewer subsets (2-7) resulted in larger negative likelihood scores, as when a partition of 1-7 arbitrarily defined subsets were selected (e.g., exons partitioned by coding positions, introns and non-protein coding loci). The best likeli-

hood tree score (-lnL = -10,970) was obtained when Matrix 2 was partitioned into 12 subsets, using all models available and chosen under the AIC criterion. Not subdividing the entire Matrix 2 (one partition for all sites) resulted in the worse tree score (-lnL = -11,352; see Table 2).

The most robust phylogeny approximated by bootstrap support was generated using a partition of 11 subsets selected with the AICc criterion and all substitution models considered (mean bootstrap support = 75.66%). This also provided the greatest number of internodes supported by bootstrap values above 50%, 70% and equal to 100% and one of the highest numbers of internodes supported above 90% and 95%. Interestingly, the mean bootstrap support values generated from the ML analyses of Matrix 2 subdivided into partitions of six and seven arbitrarily defined subsets were often higher than when partitions were defined using PartitionFinder. However, the likelihood scores of the trees derived from the arbitrarily defined partitions were among the worst across all analyses (Table 2).

Based on likelihood scores, the best criterion to select the optimal partition seems to be the AIC on all models, because it subdivided the data into finer subdivisions, allowing a better fit of the nucleotide substitution models. However, the strongest results as measured by bootstrap support (and a relatively good likelihood score) was obtained with the AICc criterion (chosen for the final analyses of Matrix 2; Fig. 2), which selected slightly less subsets (11 instead of 12 for all models, and 6 instead of 7 for RAxML models), and therefore potentially less likely to lead to cases of over-parameterization. The BIC criterion appeared to impose a far stronger constraint on the number of subsets, as only four subsets were defined in the all models/MrBayes cases and two subsets in the RAxML models case, leading to a worsening of the likelihood score of the resulting trees. However, with different datasets, e.g., Matrix 1 (Fig. 1), the best BIC scheme with fever subsets led to better results in terms of bootstrap support than when using the best AICc scheme. The best method to select the optimal number of subsets seems to be dependent on the data matrix at hand. Therefore, it is recommended to compare results obtained with different optimization criteria before settling for a specific partition.

1.4.3 Phylogeny of the mycobiont at the genus and section levels

The genus Peltigera and its eight sections as defined in Miadlikowska and Lutzoni (2000) are monophyletic and highly supported (Fig. 1). As in Miadlikowska et al. (2014), we recognize three well-supported major clades within the genus *Peltigera* (Clades I-III). It seems that section *Hydrothyriae* (clade I) shares a most recent common ancestor with Clade II, forming a larger monophyletic group where the aquatic *Hydrothyriae* clade would have evolved from the first evolutionary split, but this relationship remains weakly supported (see Miadlikowska et al. 2014). All other relationships across the genus *Peltigera* presented in our Fig. 1 are congruent with the results presented by Miadlikowska et al. (2014).

Table 2: Delimitation of data partitions within Matrix 2 using arbitrary criteria (three top rows) and PartitionFinder (the remaining rows) in comparison with the resulted ML topology and its branch robustness (bootstrap support; BS) estimated with RAxML. Arbitrary 1: single partition; Arbitrary 2: 1st codon position across all loci, 2nd codon position across all loci, 3rd codon position across all loci, introns across all loci, ITS1+ITS2, 5.8S, and LSU; Arbitrary 3: as Arbitrary 2, except 1st + 2nd codon positions across all loci. The remaining rows include the best partitioning schemes returned by PartitionFinder, for each criterion tested. ALL: models implemented in MrBayes and RAxML; RAxML: models implemented in RAxML only; MrBayes; models implemented in MrBayes only; AIC: Akaike information criterion; AICc: AIC with a correction for finite sample size; and BIC: Bayesian information criterion. We compared the number of subsets retrieved, the score of the best partition (given by PartitionFinder), the likelihood of the ML tree, the average bootstrap support (the mean of the BS of all the branches), and the number of branches with BS above 50%, 70%, 90%, 95% and equal to 100, respectively. The best value for each criterion used is shown in bold. When two models or criterions returned the same partition scheme, they are presented in a single line.

Model and criterion	No. of subsets	Scheme score	ML score = -ln L	Mean BS	No. of nodes ≥50	No. of nodes	No. of nodes ≥90	No. of nodes ≥95	No. of nodes =100
Arbitrary 1	1		-11,352	74.34	95	69	55	47	24
Arbitrary 2	7		-11,013	75.24	93	72	**58**	48	26
Arbitrary 3	6		-11,013	75.34	93	72	**58**	48	26
ALL, AIC	12	**-10,190**	-10,97	74.50	96	71	57	49	25
ALL, AICc	11		**-10,978**	**75.66**	**97**	**74**	57	49	**29**
ALL, BIC ; MrBayes, BIC	4	-10,282	-11,055	74.53	95	72	56	46	25
Mrbayes, AIC, AICc	12	-10,199	-10,974	74.40	95	73	56	50	27
RAxML, AIC	7	-10,210	-11,008	75.11	93	71	57	49	26
RAxML, AICc	6	-10,221	-11,017	74.82	93	72	**58**	**51**	26
RAxML, BIC	2	-10,312	-11,106	74.79	96	72	55	50	24

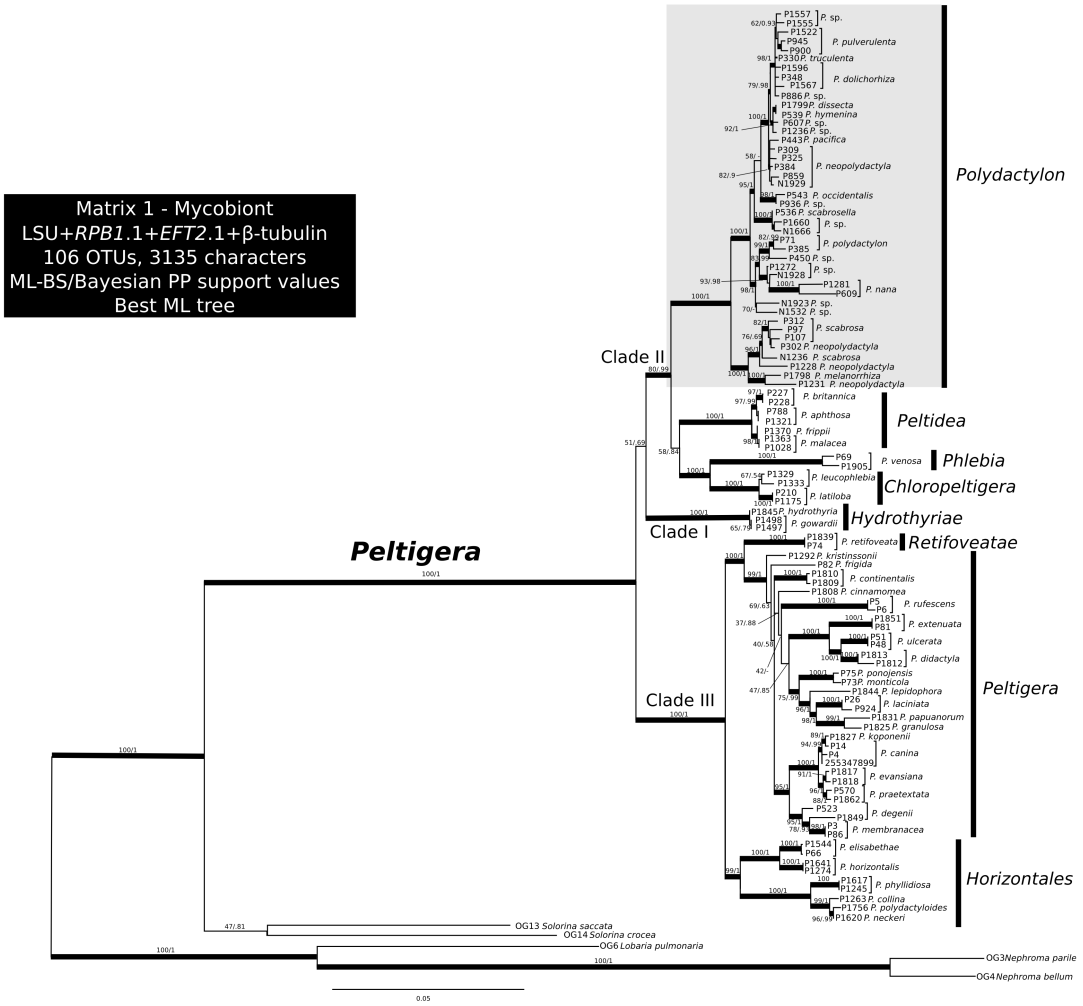
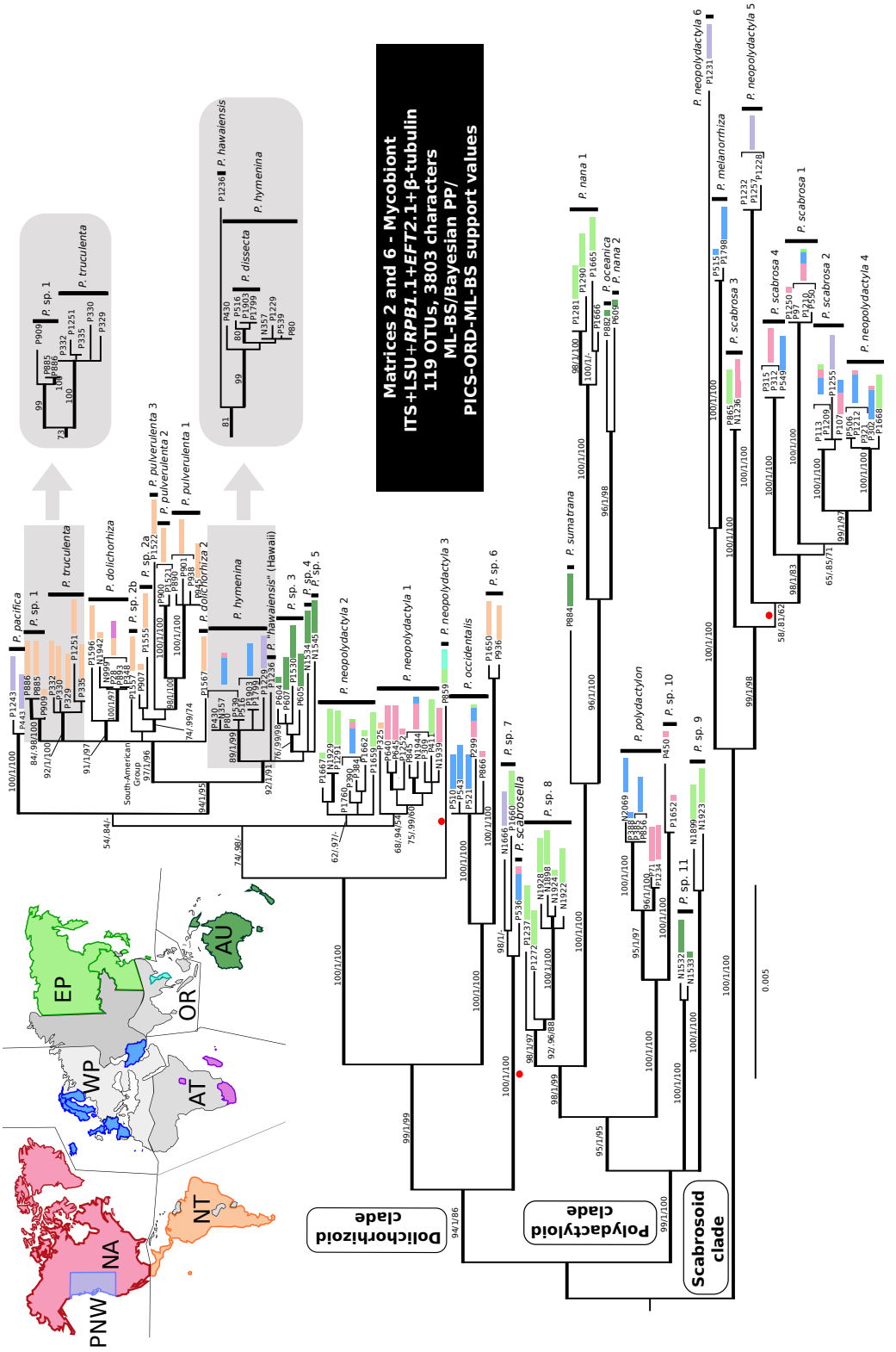


Figure 1: Phylogeny of the lichen-forming genus *Peltigera* (mycobiont, i.e., fungal partner). Most likely tree derived from an ML search using Matrix 1 (Table 1), consisting of 106 OTUs representing 64 species from all (eight) sections of *Peltigera* and five outgroup species selected from the genera *Solorina* (Peltigeraceae), *Lobaria* (Lobariaceae) and *Nephroma* (Nephromataceae). The tree was rooted according to Miadlikowska and Lutzoni (2004). Values associated with each internode represent ML bootstrap support values (ML-BS; before slash) and Bayesian posterior probabilities (PP; after slash). Thick internal branches represent internodes with ML-BS $\geq$ 70 and PP $\geq$ 0.95. Vertical bars delimit sections of the genus *Peltigera* as circumscribed by Miadlikowska and Lutzoni (2000). A grey box highlights the focus section – *Polydactylon*.

Figure 2: Phylogeny of the lichen-forming genus *Peltigera* section *Polydactylon* (mycobiont, i.e., fungal partner). Most likely tree found with an ML search using Matrix 2 (Table 1), consisting of 119 OTUs representing 39 putative monophyletic species. The tree was rooted according to the topology presented in Fig. 1. Values associated with each internode represent ML bootstrap support (ML-BS; first value), Bayesian posterior probability (PP; middle value) and ML bootstrap support derived from the analyses on Matrix 6, which incorporated recoded ambiguously aligned characters (using PICS-ORD) excluded from the alignments (POML-BS; last value). Thick internal branches represent internodes with ML-BS $\geq 70\%$, POML-BS $\geq 70\%$ and PP ≥ 0.95. Colored horizontal bars (color scheme according to the map on the top left corner) following each OTU or group of OTUs (delimited with square brackets) indicate the geographic origin of specimens included in the phylogenetic analysis, as well as other available specimens (not included in Matrix 2) that have identical ITS sequences with OTUs included in the phylogenetic analysis. The proportion of each color inside these bars corresponds to the relative number of specimens from each region. Unique ITS haplotypes have a square instead of an horizontal bar. Vertical black bars delimit recognized or putative species. Two grey boxes in the Dolichorhizoid clade indicate polytomies that were resolved when recoded characters (PICS-ORD) were added to Matrix 2 to form Matrix 6. The corresponding parts of the phylogeny resulting from the ML search based on Matrix 6 are shown in grey boxes with round corners. Abbreviations used in the map in the top left corner refer to the following regions: Afrotropics (AT), Australasia (AU), East Palearctic (EP), Nearctic (NA), Neotropics (NT), Oriental (OR), Pacific North West (PNW), West Palearctic (WP). Red dots refer to the placement of the minor conflicts detected in the single-locus topologies.

Within section *Polydactylon*, we recognize here three main and highly supported lineages within this section, hereafter named Scabrosoid, Polydactyloid, and Dolichorhizoid clades (Fig. 2). The latter two clades share a most recent common ancestor (Figs. 1 and 2). Overall, relationships across section *Polydactylon* are highly supported. However, the Dolichorhizoid clade includes a few species complexes that are not fully resolved. As expected, some "well-established" species, such as *P. neopolydactyla* and *P. scabrosa* are polyphyletic. Others seem to be conspecific (e.g., *P. dissecta* nested within *P. hymenina*), whereas many individuals form monophyletic entities (*P.* spp. 1-11) outside all currently recognized species (Fig. 2). Overall, our phylogenetic study of section *Polydactylon* suggests that the total number of species might be more than twice the number of currently known species for this section. This is remarkable given that *Peltigera* species form large foliose thalli with synapomorphies (such as veins on their undersurface) that make them easily detectable in nature and identifiable at the genus level.

With a few minor exceptions (i.e., within species), no significant conflict (as defined in Materials and Methods) was detected between the topologies generated by the phylogenetic analyses of Matrices 2 and 6 (i.e., without and with PICS-ORD characters, respectively; Table 1). Because our main goals were to assess relationships among species to infer macroevolutionary trends, these few conflicts did not affect the conclusions of this study. Most relationships were highly supported by both analyses and a few poorly supported internodes received complementary high support from one or the other analysis. The average bootstrap support for the topology without PICS-ORD characters was 74.55% (73 branches supported above 70%) versus 82.15% support (81 branches supported above 70%) obtained with the addition of PICS-ORD characters.


Matrices 2 and 6 - Mycobiont
ITS+LSU+RPB1.1+EFT2.1+β-tubulin
119 OTUs, 3803 characters
ML-BS/Bayesian PP/
PICS-ORD-ML-BS support values
P. pacifica
P. sp. 1
P. truculenta
P. dolichorhiza
P. sp. 2b
P. sp. 2a
P. pulverulenta 3
P. pulverulenta 2
P. pulverulenta 1
P. hymenina
P. "hawaiensis" (Hawaii)
P. sp. 3
P. sp. 4
P. sp. 5
P. neopolydactyla 2
P. neopolydactyla 1
P. neopolydactyla 3
P. occidentalis
P. sp. 6
P. sp. 7
P. scabrosella
P. sp. 8
P. sumatrana
P. nana 1
P. oceanica
P. nana 2
P. polydactylon
P. sp. 10
P. sp. 11
P. sp. 9
P. neopolydactyla 6
P. melanorrhiza
P. neopolydactyla 5
P. scabrosa 3
P. scabrosa 4
P. scabrosa 1
P. scabrosa 2
P. neopolydactyla 4
P. hawaiensis
P. dissecta
P. hymenina
Dolichorhizoid clade
Polydactyloid clade
Scabrosoid clade
PNW
NA
NT
EP
WP
AT
OR
AU
0.005

In general PICS-ORD provided more resolution and higher bootstrap support toward the tip of the tree, i.e., for some of the most recent speciation events (Fig. 2). For example, the sister relationship between *P. hymenina* and *P. hawaiensis*, as well as *P. truculenta* + *P.* sp. 1 were highly supported in the analysis with PICS-ORD (Matrix 6) while these four taxa were part of polytomies in the ML and Bayesian analyses of Matrix 2, i.e., without PICS-ORD characters to account for signal from ambiguously aligned sites. However, we also noticed a decrease in ML-BS values caused by the addition of PICS-ORD characters, especially at some of the deeper nodes.

This differential variation in bootstrap support values, at the tip versus the bottom of phylogenetic trees, is similar to what was observed in previous studies when ambiguously aligned regions were recoded with INAASE (Lutzoni et al., 2000; Miadlikowska et al., 2003). Ambiguously aligned regions of the ITS region, ribosomal RNA genes, and spliceosomal introns are gap rich and usually fast evolving. Therefore, these regions often include most of the phylogenetic signal within species and among species part of species complexes, and often greatly contribute to the resolution and confidence levels for these shallow relationships. INAASE and PICS-ORD can capture this signal accurately, however these methods are error prone when accommodating signal among sequences that have diverged for an extensive amount of time, which explains their poor contribution at resolving deep relationships and estimating phylogenetic confidence for these deeper nodes.

1.4.4 Comparison of species discovery methods

When implemented on the entire section *Polydactylon*, bGMYC analyses performed well on the Polydactyloid and Scabrosoid clades (in terms of the following three criteria: monophyly, as well as morphological and geographical homogeneity of circumscribed putative species), but not on the Dolichorizoid clade. A bGMYC analysis restricted to the Dolichorhizoid clade, which enabled the inclusion of more sites (that were ambiguously aligned and excluded from the analysis of the entire section), somewhat improved the results but was still not satisfactory with regard to the three criteria mentioned above. A poor performance by bGMYC within the Dolichorhizoid clade can be explained by a recent (ongoing) rapid radiation depicted by many short branches in the inferred phylogeny where expected differences in genetic distances within versus among species are not always obvious (Fig. 2).

Structurama performed best in "fast evolving" groups, where members of different putative species are characterized by different alleles in all or most molecular markers. bGMYC analyses are based on a single locus, however, unlike Structurama it takes tree topologies, including monophyly, into consideration. Whereas Structurama is not a tree-based method, and consequently it often delimits paraphyletic species, especially if sampled loci show a low level of variability, coupled with a high level of ancestral polymorphisms and shared alleles among species.

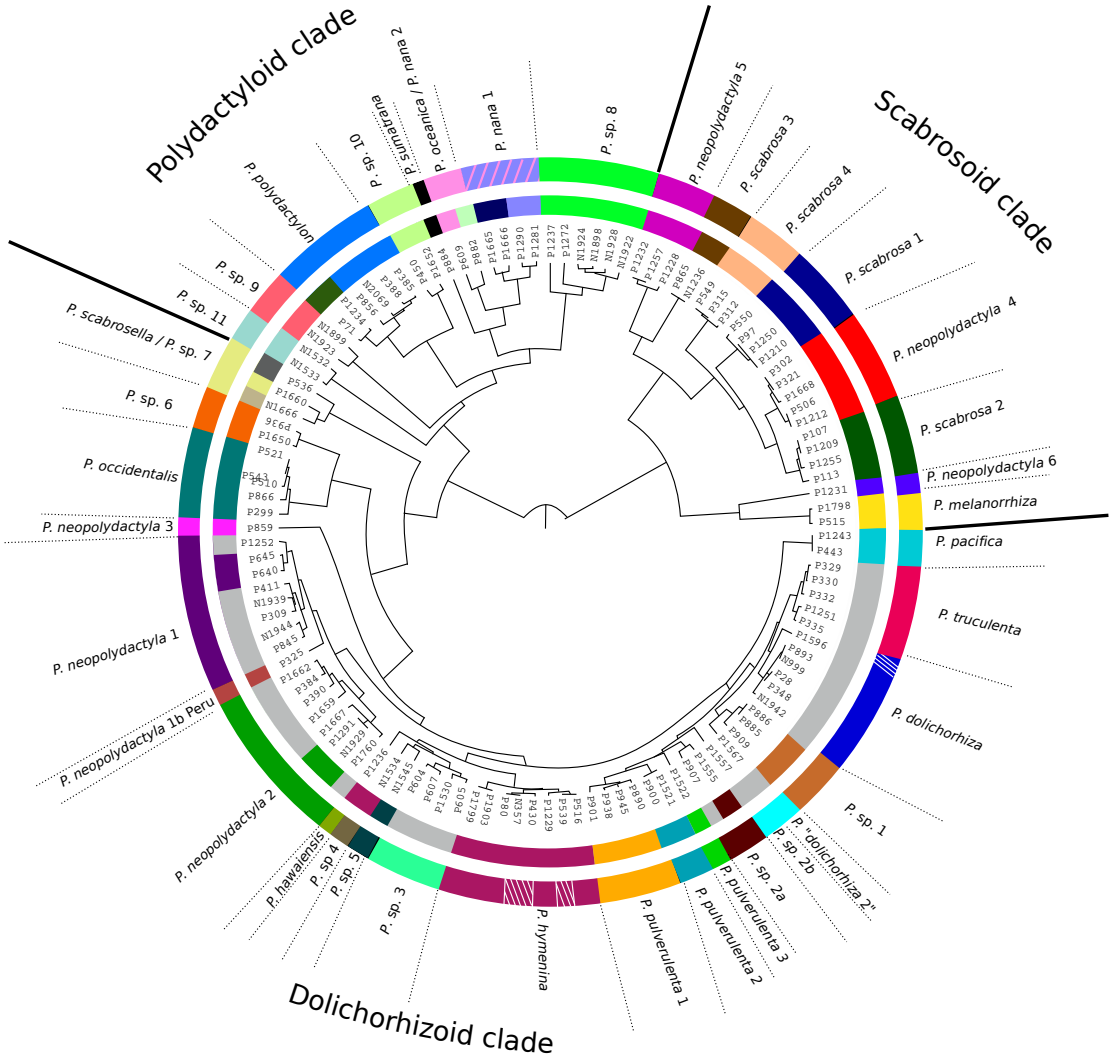


Figure 3: Species delimitation within section *Polydactylon* of the lichen-forming genus *Peltigera* (mycobiont, i.e., fungal partner). Center: chronogram derived from BEAST analysis of Matrix 2 (five loci, 119 OTUs, Table 1). Inner colored circle: species delimited using Structurama. Outer colored circle: species delimited using bGMYC. Each delimited species is represented by a different color. Alternative species delimitations by the same method are represented by stripes. Species names outside of the outer circle reflect the consensus species delimitation based on the results from Structurama and bGMYC, monophyly and branch lengths (Fig.2), as well as morphological and biogeographical homogeneity

In previous studies (Carstens et al., 2013; Satler et al., 2013) results based on the GMYC model were questioned because a substantially higher number of species was detected compared to other methods. But these studies were based on small groups of closely related taxa restricted to narrow geographical areas, and where a relatively small number of unrecognized species was expected. bGMYC seems to perform better for large clades with worldwide representatives. For section *Polydactylon* we expected a high number of undiscovered species. We also expected species delimitation to be challenging because many potential taxa were represented by only a few individuals.

Structurama analyses were conducted on each of the three main clades of section *Polydactylon* separately. Based on our test runs (Supplementary Figs. S1 and S2), we selected priors with the lowest impact on the results and that corroborated observed morphological and geographical patterns. The final estimations for the Dolichorhizoid clade were 18 species (gamma shape = 19); 11 species (gamma shape = 3) for the Polydactyloid clade; and 8 species (gamma shape = 5) for the Scabrosoid clade (Supplementary Figs. S1 and S2; Fig. 3). bGMYC delimited 21 species within the Dolichorhizoid clade, and 8 species in each of the remaining two clades (Fig. 3).

Although both methods agreed in the total number of fungal species in section *Polydactylon* (37-38 species assigned by Structurama, and 37 species by bGMYC) and the overall assignment of individuals to delimited species, several patterns of discrepancies between the two approaches were encountered, especially in the Dolichorhizoid clade, which contains the highest number of potential species resulting from a radiation that was not fully resolved with the concatenated five-locus dataset (Fig. 2). These discrepancies involved mostly splitting versus lumping closely related species that otherwise were monophyletic. For example, *P. scabrosella*/*P.* sp. 7a/*P.* sp.7b were distinguished as three separate species by Structurama but considered as one species by bGMYC (Fig. 3). There were also rare cases of paraphyletic and polyphyletic species delimitations. For instance, *P. truculenta*, *P. dolichorhiza*, *P. dolichorhiza* 2, *P.* sp. 2b, *P.* sp. 3, part of *P. neopolydactyla* 1, part of *P. neopolydactyla* 2, and part of *P.* sp. 2a were grouped into a large paraphyletic species by Structurama (grey part of the inner circle in Fig. 3), but assigned to multiple monophyletic species by bGMYC. Our species assignments within the Dolichorhizoid clade (22 putative species), the Polydactyloid (eight species) and Scabrosoid clades (eight species; outermost doted line delimitations in Fig. 3) was based on the consensus of both methods, monophyly, morphological traits (including from type specimens), geographical distributions of the studied taxa, as well as the sampling bias (many putative species being underrepresented in our datasets). Final circumscriptions, including formal descriptions, of the newly discovered species of section *Polydactylon* will be provided in a follow-up publication based on comprehensive analyses, incorporating chimeric approaches (sensu Satler et al. 2013) i.e., species discovery, validation, and combined methods.

1.4.5 Newly Delimited Fungal (Mycobiont) Species from section *Polydactylon*

As expected, several species in section *Polydactylon* that were delimited based on phenotypic traits alone, were not monophyletic and consisted of multiple, often cryptic but geographically distinct, entities that represent previously unrecognized species (Figs. 2 and 3). In most cases, delimited species based on monophyly as a grouping criterion corroborated species assignment resulting from the two discovery methods. However, a certain degree of discrepancy between the two approaches requires further investigation and better sampling before the formal recognition of these problematic species can be made. A good example is *Peltigera neopolydactyla*, which was recognized as a morphologically and chemically diverse (multiple morpho- and chemotypes were reported) species complex with a widespread panboreal Holarctic distribution (see Holtan-Hartwig 1993; Vitikainen 1994). Here we report that *P. neopolydactyla* represents an assemblage of at least six species spread over two clades (Dolichorhizoid and Scabrosoid clades); two of the species are endemic to the Pacific Northwest, one in Asia, and the remaining three species are spread throughout the Holarctic, (Western and Eastern Palearctic, and Nearctic regions). A specimen from Peru (P325), sister to *P. neopolydactyla* 1 (Fig. 2) was recognized as an additional putative species (*P. neopolydactyla* 1b) by both species discovery methods (Fig. 3).

Another widely distributed species, *Peltigera polydactylon*, appears to be restricted to Nearctic and West Palearctic zones, while specimens identified as *P. polydactylon* from other regions were part of newly delimited monophyletic species (Fig. 2): *P.* sp. 1, *P.* sp. 6 (from Neotropics), *P.* sp. 8 and *P.* sp. 9 (from Asia), and *P.* sp. 3 (from Australasia). Species discovery methods disagree on whether specimens of *P. polydactylon* from Europe and North America are conspecific or represent two different species (Fig. 3). Previously anticipated divisions of *P. dolichorhiz*a into several taxa (Sérusiaux et al., 2009) is confirmed here. *Peltigera dolichorhiza* s. str. occurs in the Neotropics and Afrotropics, while specimens identified as *P. dolichorhiza* from New Zealand and Australia belong to *P.* sp. 3, and from Papua New Guinea represent *P.* sp. 4, *P.* sp. 5 and *P.* sp. 11. The monophyletic *Peltigera pulverulenta* seems to be composed of three distinct species according to both discovery methods (Figs. 2 and 3); all morphologically similar and distributed in the Neotropics. Our results strongly suggest that *P. scabrosa*, a species that was thought to be easily recognized based on the pronounced scabrosity of its upper thallus surface, represents four species, all part of the Scabrosoid clade, but sharing a most recent common ancestor with non-scabrous *P. neopolydactyla* 4 and 5 (Fig. 2). Within the Dolichorhizoid clade each of the following species form well-supported monophyletic groups: *P. scabrosella*, *P. pacifica*, *P. occidentalis* (sometimes considered as a morphotype of *P. neopolydactyla*, see Vitikainen 1994), *P. hymenina* (including *P. dissecta*) and *P. truculenta* (including *P. chilensis*). The taxonomic status of Australasian members of the *nana* group in the Polydactyloid clade (*P. sumatrana*, *P. nana 2*, *P. macra*, *P. oceanica*, *P. weberi*) could not be addressed in this study because of insufficient sampling.

Overall, *Peltigera* section *Polydactylon* is composed of at least 38 monophyletic putative species with only ten names currently available. The remaining 28 newly delimited species (awaiting formal description) represent predominantly cryptic entities of allopatric or sympatric populations sharing similar morphology and collected mostly from poorly explored regions of the world. It is possible that *P.* sp. 9 corresponds to *P. dolichospora*, but further taxonomic work is needed to verify this possibility. Another scabrid species, *P. lyngei* may also belong to section *Polydactylon*, however, fresh collections of this species were not available.

1.4.6 Geographical Ranges of Newly Delimited Species and Clades

The actual geographic ranges of species in section *Polydactylon* are more restricted than previously reported based on phenotypic traits alone (Martínez et al., 2003). For example, "*P. hawaiensis*" is known only from Hawaii, *P.* sp. 4, *P.* sp. 5 and *P.* sp. 11 (previously recognized under *P. dolichorhiza* s. lat. A-D, Sérusiaux et al. 2009) occur exclusively in Papua New Guinea, whereas *P.* sp. 9 was collected only from the Yunnan province in China. However, the sampling in the adjacent regions is poor and these narrow distribution patterns might be artificial due to poor sampling. The geographically restricted ranges of *P. melanorrhiza*, which is known only from Azores, as well as *P. pacifica*, *P. neopolydactyla* 5 and *P. neopolydactyla* 6 endemic to the Pacific Northwest are well documented. Similarly, *P.* sp. 10 has only been found in Pennsylvania and Nova Scotia, thus being possibly an endemic of eastern North America.

Nevertheless, several *Peltigera* species remain relatively widely distributed but limited to a single biogeographic zone. *P.* sp. 8. and *P nana* 1 have only been found in the East Palearctic zone, *P.* sp. 3 in Australasia, and *P. pulverulenta* 1, 2, 3, P. dolichorhiza 2, *P.* spp. 1, 2a, 2b and 6 in the Neotropics (including Mexico and Central America) and *P. truculenta/chilensis* in the Neantarctic zone (Fig. 2). Some species are present in both Nearctic and West Palearctic regions (e.g., *P. polydactylon*, *P. hymenina*, *P. scabrosella*, *P. scabrosa* 4), Nearctic and East Palearctic regions (e.g., *P. scabrosa* 3), or all three regions (e.g., *P. neopolydactyla* 1, 2, 4, *P. scabrosa* 1, 2, and *P. occidentalis*). Within these broad geographic regions, selected species are restricted to the boreal zone (e.g., *P. scabrosa* 1, 2, 3, 4, *P. scabrosella*, and *P.* sp. 7) while others extend their ranges to temperate regions (e.g., *P. occidentalis* and *P. neopolydactyla* 1 in temperate Nearctic, *P. neopolydactyla* 2 in temperate West and East Palearctic, *P. hymenina* and *P. polydactylon* in temperate Nearctic and West Palearctic). *Peltigera neopolydactyla* 1 has also been detected in Neotropics (Peru) but this individual may represent a distinct species. *Peltigera dolichorhiza* is present in the Neotropic and Afrotropic regions and it is the only member of the *Polydactylon* section found in the latter region. Species from the *nana* group, including *P. sumatrana*, *P. macra*, *P. nana* 1, 2, *P. oceanica* and *P. weberi* seem to be restricted to the Oriental/Australasian regions, with the range of *P. nana* 1 extending to East Palearctic. However, this complex of species needs to be

sampled more intensively.

The Scabrosoid clade comprises predominantly boreal species, with only *P. melanorrhiza* present in temperate regions (Azores) while the remaining species occur mainly in the boreal zone, where they are either widespread (*P. scabrosa* 1-4 and *P. neopolydactyla* 4) or restricted to the Pacific Northwest (*P. neopolydactyla* 5, 6). Although phylogenetically closely related, *P. melanorrhiza* and *P. neopolydactyla* 6 are allopatric and occur in two geographically distant but highly humide areas, Azores and Pacific Northwest, respectively. This disjunct distribution pattern and relatively long branches separating both species (Fig. 2) may suggest that this clade diversified a long time ago and included more species in the past, but currently only two extant paleoendemic species exist. The Polydactyloid clade is especially diverse and abundant in the Asiatic/Australasian zones of the Pacific Ocean in Asia and Australasia, with *P. nana* 1, *P.* sp. 8, and *P.* sp. 9 in Asia, *P.* sp. 11 in Papua New Guinea, and species from the *nana* group detected in India, Philippines, Indonesia and New Zealand (Fig. 2). *P. polydactylon* and *P.* sp. 10, are the only species from this clade that are present in temperate/sub-boreal zones of the Nearctic and West Palearctic zone. In the Dolichorhizoid clade as well as its largest cosmopolitan *neopolydactyla/dolichorhiza* complex of species, the early diverged species are boreal (*P. scabrosella*, *P.* sp. 7 and *P. occidentalis*) or boreal/temperate (*P. neopolydactyla* 1-3) whereas the remaining lineages are found in the Neotropics (*P.* sp. 6 and South-American group), Australasia (*P.* sp. 3-5), and oceanic regions of the Palearctic (*P. hymenina*) (Fig. 2). The phylogenetic placement of boreal species suggests a boreal origin for the entire *Polydactylon* group (Fig. 2).

1.4.7 Phylogeny of the *Nostoc* cyanobiont

Our *rbcLX* phylogeny (Fig. 4) revealed *Nostoc* as a non-monophyletic assembly similar to previous studies (Svenning et al., 2005; O'Brien et al., 2005a; Otálora et al., 2010). In agreement with Otálora et al. (2010), *Nostoc* clades I and II, as well as the three subclades (1-3) of clade II, are monophyletic. However, significant support was obtained only for *Nostoc* clade I and subclade 2 of clade II (Fig. 4). Subclade 3 is composed of a huge polytomy of several small, often well supported and internally resolved subgroups.

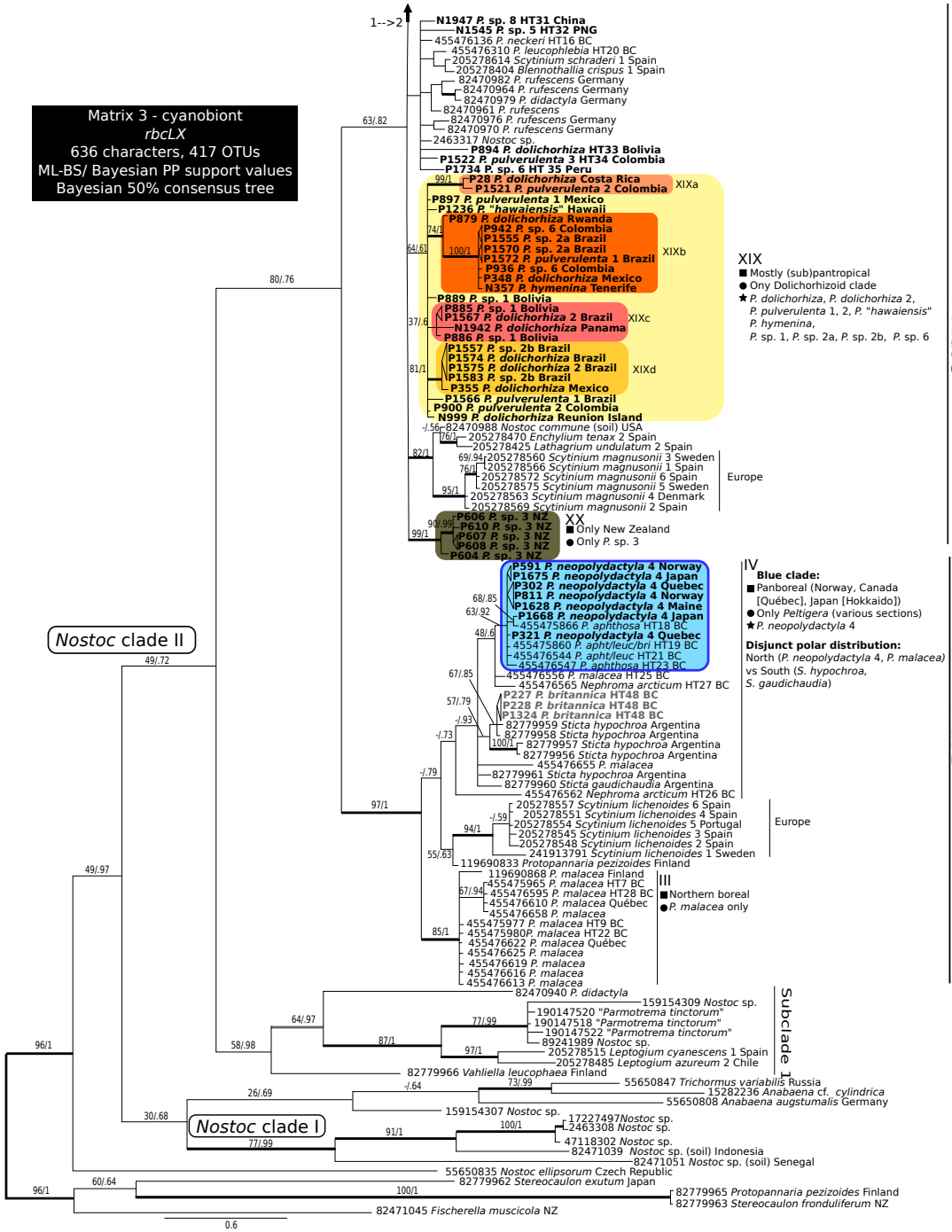
In addition to the initial six clusters (I-VI) previously recognized by O'Brien et al. (2013), we defined fourteen new *Nostoc* phylogroups (VII-XX; Fig. 4) representing well-supported clades containing *Nostoc* from *Peltigera* section *Polydactylon*. In our phylogeny, cluster I from O'Brien et al. (2013) was not retrieved as monophyletic, and is therefore not represented in the figure. To the set of 30 unique *Nostoc* haplotypes (HT 1-30) defined by O'Brien et al. (2013) we added 17 (HT31-47) newly recovered unique haplotypes from section *Polydactylon* and 15 haplotypes (HT48-62) from other sections of *Peltigera* (Fig. 4).

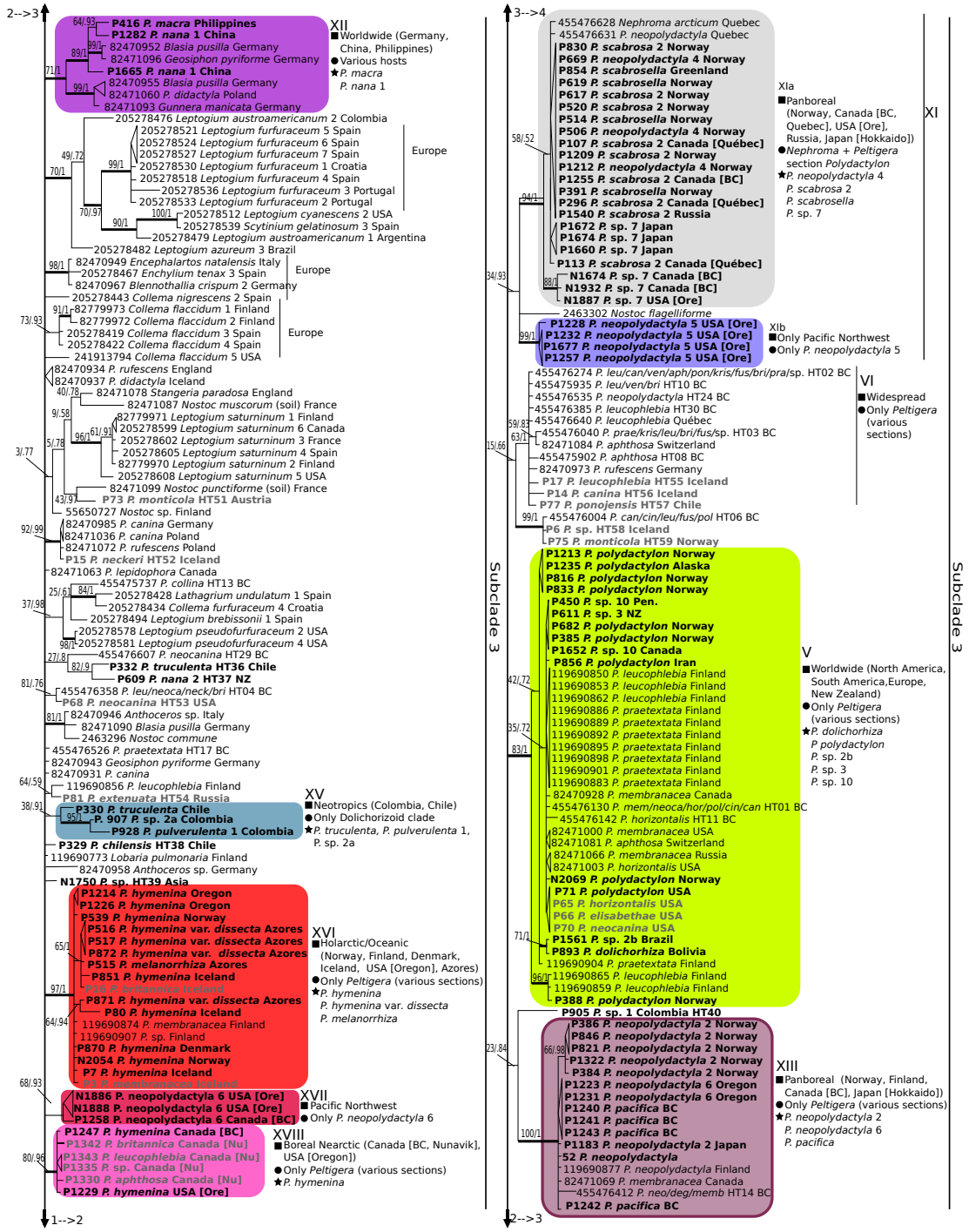
Nearly all photobionts sampled from *Peltigera* belong to the broadly defined genus *Nostoc*, clade II, subclade 3. Only of few *Nostoc* strains found in *Peltigera* thalli belong

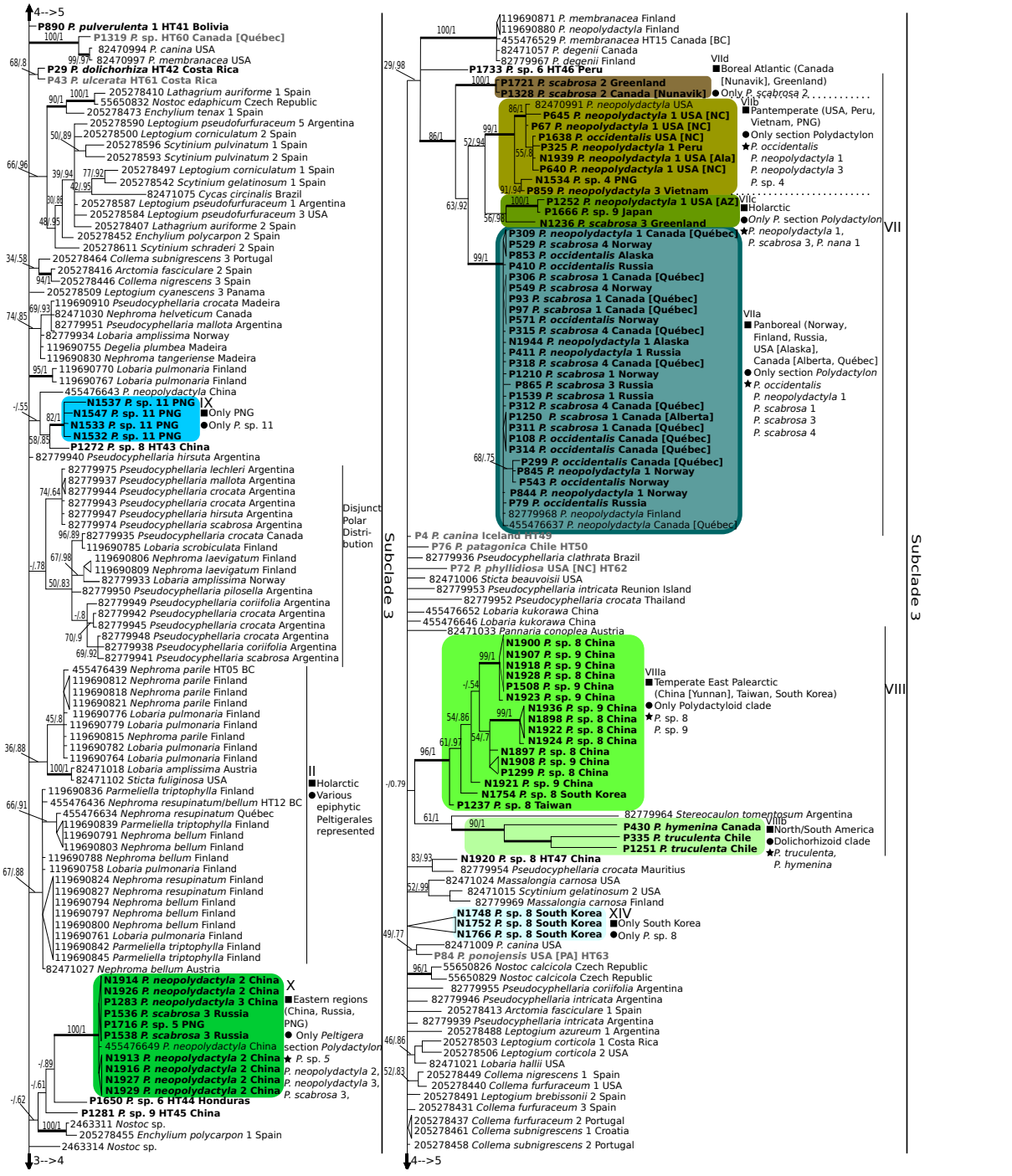
Figure 4: Phylogeny of *Nostoc* (cyanobiont). This is a 50% consensus tree of 26,100 trees that resulted from a Bayesian analysis of Matrix 3 (*rbcLX* dataset) representing 417 unique *rbcLX* haplotypes. The tree was rooted according to Otálora et al. (2010). Values associated with each internode represent ML bootstrap support (ML-BS; before slash) and Bayesian posterior probability support (PP; after slash). Thick internodes received ML-BS≥70 and PP≥0.95. Clades and subclades of *Nostoc* were defined according to Otálora et al. (2010). Newly sequenced *Nostoc* cyanobionts associated with mycobionts from section *Polydactylon* are shown in bold, whereas those from other sections of *Peltigera* are in grey bold. Geographic origin is provided after the name of each terminal OTU and for published sequences downloaded from GenBank (indicated by GB number), whenever possible. Recognized phylogroups of *Nostoc* are represented by Roman numbers; phylogroups II-VI refer to O'Brien et al. (2013), whereas phylogroups VII - XX (defined here) represent significantly or moderately supported monophyletic groups encompassing *Nostoc* associated with representatives of section *Polydactylon*. Colored boxes were attributed to each phylogroup (and four subclades within phylogroup XIX) except the ones defined by O'Brien et al. (2013), which don't contain any of the newly added *Nostoc* sequences from section *Polydactylon*. Geographic range (full squares), mycobiont affinity within within the genus *Peltigera* (full circles) and associated mycobiont species within section *Polydactylon* (full stars) are provided for each *Nostoc* phylogroup and selected clades. When associated with identical *rbcLX* haplotype, a terminal branch representing different mycobionts was replaced by a cone (horizontally oriented) comprising all collapsed individuals. Abbreviations for *Peltigera* species are: apht = P. *aphthosa*, bri = *P. britannica*, leuc = *P. leucophlebia*, neoca = *P. neocanina*, neck = *P. neckeri*, can = *P. canina*, ven = *P. venosa*, pon = *P. ponojensis*, kri = *P. kristinsonii*, fus = *P. fuscopraetextata*, pra = *P. praetextata*, cin = *P. cinnamomea*, pol = *P. polydactylon*, mem = *P. membranacea*, hor = *P. horizontalis*, neo = *P. neopolydactyla*. Abbreviations for geographic regions are: BC= British Columbia, Ala=Alabama, Ore=Oregon, NZ=New Zealand, Pen=Pennsylvania, NC=North Carolina, AZ=Arizona, PNG= Papua New Guinea, Nu=Nunavik.

to subclade 2 (Fig. 4). No *Nostoc* strains from clade I and clade II subclade 1 (sensu Otálora et al. 2010) were found associated with *Peltigera*. Cyanobionts from *Peltigera* thalli clustered with cyanobionts from various sections of the genus *Peltigera*, sometimes with cyanobionts from closely related genera from the suborder Peltigerineae e.g., *Nephroma* (phylogroups, IV and XIa) and Sticta (IV), but never with *Nostoc* from Pannariaceae or Collemataceae (suborder Collematineae). Exceptionally, representatives of phylogroup XII, clustered with symbiotic *Nostoc* (GenBank accessions) from plants such as *Gunnera* or *Blasia* and fungi such as *Geosiphon pyriforme* (Glomeromycota). The revealed phylogenetic structure in subclade 3 supports the recently proposed hypothesis that homoiomerous "collematoid" species don't share the same *Nostoc* photobiont as heteromerous "pannarioid" species (Magain and Sérusiaux, 2014)

As for the genus *Peltigera* in general, the majority of *Nostoc* strains found in thalli of *Peltigera* section *Polydactylon* are nested within subclade 3. Within this subclade, *Nostoc* strains are often grouped according to their geographic origin, e.g. Pacific North West (phylogroups XVII and XIb), Neotropics (phylogroup XV), Asia (phylogroups VIIIa and XIV), and Australasia (phylogroup XX); as well as climatic zones, e.g., boreal (phylogroups XIII, XIa, IV, and VIIa), temperate (phylogroups VIIb, and VIIc) and (sub)tropical (phylogroups XIXa-d); or a combination of both factors, e.g. boreal Nearctic (phylogroups VIId and XVIII). Only a few *Nostoc* phylogroups (e.g., V and XII) seem to have a broad cosmopolitan, or subcosmopolitan distribution, however, ad-


Matrix 3 - cyanobiont
rbcLX
636 characters, 417 OTUs
ML-BS/ Bayesian PP support values
Bayesian 50% consensus tree
1-->2
N1947 P. sp. 8 HT31 China
N1545 P. sp. 5 HT32 PNG
455476136 P. neckeri HT16 BC
455476310 P. leucophlebia HT20 BC
205278614 Scytinium schraderi 1 Spain
205278404 Blennothallia crispus 1 Spain
82470982 P. rufescens Germany
82470964 P. rufescens Germany
82470979 P. didactyla Germany
82470961 P. rufescens
82470976 P. rufescens Germany
82470970 P. rufescens Germany
2463317 Nostoc sp.
P894 P. dolichorhiza HT33 Bolivia
P1522 P. pulverulenta 3 HT34 Colombia
P1734 P. sp. 6 HT 35 Peru
P28 P. dolichorhiza Costa Rica
P1521 P. pulverulenta 2 Colombia
XIXa
P897 P. pulverulenta 1 Mexico
P1236 P. "hawaiensis" Hawaii
P879 P. dolichorhiza Rwanda
P942 P. sp. 6 Colombia
P1555 P. sp. 2a Brazil
P1570 P. sp. 2a Brazil
P1572 P. pulverulenta 1 Brazil
P936 P. sp. 6 Colombia
P348 P. dolichorhiza Mexico
N357 P. hymenina Tenerife
XIXb
P889 P. sp. 1 Bolivia
P885 P. sp. 1 Bolivia
P1567 P. dolichorhiza 2 Brazil
N1942 P. dolichorhiza Panama
P886 P. sp. 1 Bolivia
XIXc
P1557 P. sp. 2b Brazil
P1574 P. dolichorhiza Brazil
P1575 P. dolichorhiza 2 Brazil
P1583 P. sp. 2b Brazil
P355 P. dolichorhiza Mexico
P1566 P. pulverulenta 1 Brazil
XIXd
P900 P. pulverulenta 2 Colombia
N999 P. dolichorhiza Reunion Island
XIX
Mostly (sub)pantropical
Ony Dolichorhizoid clade
P. dolichorhiza, P. dolichorhiza 2, P. pulverulenta 1, 2, P. "hawaiensis" P. hymenina, P. sp. 1, P. sp. 2a, P. sp. 2b, P. sp. 6
82470988 Nostoc commune (soil) USA
205278470 Enchylium tenax 2 Spain
205278425 Lathagrium undulatum 2 Spain
205278560 Scytinium magnusonii 3 Sweden
205278566 Scytinium magnusonii 1 Spain
205278572 Scytinium magnusonii 6 Spain
205278575 Scytinium magnusonii 5 Sweden
205278563 Scytinium magnusonii 4 Denmark
205278569 Scytinium magnusonii 2 Spain
Europe
P606 P. sp. 3 NZ
P610 P. sp. 3 NZ
P607 P. sp. 3 NZ
P608 P. sp. 3 NZ
P604 P. sp. 3 NZ
XX
Only New Zealand
Only P. sp. 3
Subclade 3
P591 P. neopolydactyla 4 Norway
P1675 P. neopolydactyla 4 Japan
P302 P. neopolydactyla 4 Quebec
P811 P. neopolydactyla 4 Norway
P1628 P. neopolydactyla 4 Maine
P1668 P. neopolydactyla 4 Japan
455475866 P. aphthosa HT18 BC
P321 P. neopolydactyla 4 Quebec
455475860 P. apht/leuc/bri HT19 BC
455476544 P. apht/leuc HT21 BC
455476547 P. aphthosa HT23 BC
IV
Blue clade:
Panboreal (Norway, Canada [Québec], Japan [Hokkaido])
Only Peltigera (various sections)
P. neopolydactyla 4
Disjunct polar distribution:
North (P. neopolydactyla 4, P. malacea) vs South (S. hypochroa, S. gaudichaudia)
455476556 P. malacea HT25 BC
455476565 Nephroma arcticum HT27 BC
P227 P. britannica HT48 BC
P228 P. britannica HT48 BC
P1324 P. britannica HT48 BC
82779959 Sticta hypochroa Argentina
82779958 Sticta hypochroa Argentina
82779957 Sticta hypochroa Argentina
82779956 Sticta hypochroa Argentina
455476655 P. malacea
82779961 Sticta hypochroa Argentina
82779960 Sticta gaudichaudia Argentina
455476562 Nephroma arcticum HT26 BC
205278557 Scytinium lichenoides 6 Spain
205278551 Scytinium lichenoides 4 Spain
205278554 Scytinium lichenoides 5 Portugal
205278545 Scytinium lichenoides 3 Spain
205278548 Scytinium lichenoides 2 Spain
241913791 Scytinium lichenoides 1 Sweden
Europe
119690833 Protopannaria pezizoides Finland
119690868 P. malacea Finland
455475965 P. malacea HT7 BC
455476595 P. malacea HT28 BC
455476610 P. malacea Québec
455476658 P. malacea
455475977 P. malacea HT9 BC
455475980 P. malacea HT22 BC
455476622 P. malacea Québec
455476625 P. malacea
455476619 P. malacea
455476616 P. malacea
455476613 P. malacea
III
Northern boreal
P. malacea only
Subclade 2
Nostoc clade II
82470940 P. didactyla
159154309 Nostoc sp.
190147520 "Parmotrema tinctorum"
190147518 "Parmotrema tinctorum"
190147522 "Parmotrema tinctorum"
89241989 Nostoc sp.
205278515 Leptogium cyanescens 1 Spain
205278485 Leptogium azureum 2 Chile
82779966 Vahlella leucophaea Finland
Subclade 1
55650847 Trichormus variabilis Russia
15282236 Anabaena cf. cylindrica
55650808 Anabaena augstumalis Germany
159154307 Nostoc sp.
Nostoc clade I
17227497 Nostoc sp.
2463308 Nostoc sp.
47118302 Nostoc sp.
82471039 Nostoc sp. (soil) Indonesia
82471051 Nostoc sp. (soil) Senegal
55650835 Nostoc ellipsorum Czech Republic
82779962 Stereocaulon exutum Japan
82779965 Protopannaria pezizoides Finland
82779963 Stereocaulon fronduliferum NZ
82471045 Fischerella muscicola NZ
0.6


2-->3
1-->2
3-->4
2-->3
Subclade 3
Subclade 3
XII
■Worldwide (Germany, China, Philippines)
●Various hosts
★P. macra
P. nana 1
P416 P. macra Philippines
P1282 P. nana 1 China
82470952 Blasia pusilla Germany
82471096 Geosiphon pyriforme Germany
P1665 P. nana 1 China
82470955 Blasia pusilla Germany
82471060 P. didactyla Poland
82471093 Gunnera manicata Germany
205278476 Leptogium austroamericanum 2 Colombia
205278521 Leptogium furfuraceum 5 Spain
205278524 Leptogium furfuraceum 6 Spain
205278527 Leptogium furfuraceum 7 Spain
205278530 Leptogium furfuraceum 1 Croatia
205278518 Leptogium furfuraceum 4 Spain
205278536 Leptogium furfuraceum 3 Portugal
205278533 Leptogium furfuraceum 2 Portugal
205278512 Leptogium cyanescens 2 USA
205278539 Scytinium gelatinosum 3 Spain
205278479 Leptogium austroamericanum 1 Argentina
205278482 Leptogium azureum 3 Brazil
Europe
82470949 Encephalartos natalensis Italy
205278467 Enchylium tenax 3 Spain
82470967 Blennothallia crispum 2 Germany
Europe
205278443 Collema nigrescens 2 Spain
82779973 Collema flaccidum 1 Finland
82779972 Collema flaccidum 2 Finland
205278419 Collema flaccidum 3 Spain
205278422 Collema flaccidum 4 Spain
Europe
241913794 Collema flaccidum 5 USA
82470934 P. rufescens England
82470937 P. didactyla Iceland
82471078 Stangeria paradosa England
82471087 Nostoc muscorum (soil) France
82779971 Leptogium saturninum 1 Finland
205278599 Leptogium saturninum 6 Canada
205278602 Leptogium saturninum 3 France
205278605 Leptogium saturninum 4 Spain
82779970 Leptogium saturninum 2 Finland
205278608 Leptogium saturninum 5 USA
82471099 Nostoc punctiforme (soil) France
P73 P. monticola HT51 Austria
55650727 Nostoc sp. Finland
82470985 P. canina Germany
82471036 P. canina Poland
82471072 P. rufescens Poland
P15 P. neckeri HT52 Iceland
82471063 P. lepidophora Canada
455475737 P. collina HT13 BC
205278428 Lathagrium undulatum 1 Spain
205278434 Collema furfuraceum 4 Croatia
205278494 Leptogium brebissonii 1 Spain
205278578 Leptogium pseudofurfuraceum 2 USA
205278581 Leptogium pseudofurfuraceum 4 USA
455476607 P. neocanina HT29 BC
P332 P. truculenta HT36 Chile
P609 P. nana 2 HT37 NZ
455476358 P. leu/neoca/neck/bri HT04 BC
P68 P. neocanina HT53 USA
82470946 Anthoceros sp. Italy
82471090 Blasia pusilla Germany
2463296 Nostoc commune
455476526 P. praetextata HT17 BC
82470943 Geosiphon pyriforme Germany
82470931 P. canina
119690856 P. leucophlebia Finland
P81 P. extenuata HT54 Russia
XV
■Neotropics (Colombia, Chile)
●Only Dolichorizoid clade
★P. truculenta, P. pulverulenta 1, P. sp. 2a
P330 P. truculenta Chile
P. 907 P. sp. 2a Colombia
P928 P. pulverulenta 1 Colombia
P329 P. chilensis HT38 Chile
119690773 Lobaria pulmonaria Finland
82470958 Anthoceros sp. Germany
N1750 P. sp. HT39 Asia
XVI
■Holarctic/Oceanic (Norway, Finland, Denmark, Iceland, USA [Oregon], Azores)
●Only Peltigera (various sections)
★P. hymenina
P. hymenina var. dissecta
P. melanorrhiza
P1214 P. hymenina Oregon
P1226 P. hymenina Oregon
P539 P. hymenina Norway
P516 P. hymenina var. dissecta Azores
P517 P. hymenina var. dissecta Azores
P872 P. hymenina var. dissecta Azores
P515 P. melanorrhiza Azores
P851 P. hymenina Iceland
P871 P. hymenina var. dissecta Azores
P80 P. hymenina Iceland
119690874 P. membranacea Finland
119690907 P. sp. Finland
P870 P. hymenina Denmark
N2054 P. hymenina Norway
P7 P. hymenina Iceland
XVII
■Pacific Northwest
●Only P. neopolydactyla 6
N1886 P. neopolydactyla 6 USA [Ore]
N1888 P. neopolydactyla 6 USA [Ore]
P1258 P. neopolydactyla 6 Canada [BC]
XVIII
■Boreal Nearctic (Canada [BC, Nunavik], USA [Oregon])
●Only Peltigera (various sections)
★P. hymenina
P1247 P. hymenina Canada [BC]
P1342 P. britannica Canada [Nu]
P1343 P. leucophlebia Canada [Nu]
P1335 P. sp. Canada [Nu]
P1330 P. aphthosa Canada [Nu]
P1229 P. hymenina USA [Ore]
455476628 Nephroma arcticum Quebec
455476631 P. neopolydactyla Quebec
P830 P. scabrosa 2 Norway
P669 P. neopolydactyla 4 Norway
P854 P. scabrosella Greenland
P619 P. scabrosella Norway
P617 P. scabrosa 2 Norway
P520 P. scabrosa 2 Norway
P514 P. scabrosella Norway
P506 P. neopolydactyla 4 Norway
P107 P. scabrosa 2 Canada [Québec]
P1209 P. scabrosa 2 Norway
P1212 P. neopolydactyla 4 Norway
P1255 P. scabrosa 2 Canada [BC]
P391 P. scabrosella Norway
P296 P. scabrosa 2 Canada [Québec]
P1540 P. scabrosa 2 Russia
P1672 P. sp. 7 Japan
P1674 P. sp. 7 Japan
P1660 P. sp. 7 Japan
P113 P. scabrosa 2 Canada [Québec]
N1674 P. sp. 7 Canada [BC]
N1932 P. sp. 7 Canada [BC]
N1887 P. sp. 7 USA [Ore]
XIa
■Panboreal (Norway, Canada [BC, Quebec], USA [Ore], Russia, Japan [Hokkaido])
●Nephroma + Peltigera section Polydactylon
★P. neopolydactyla 4
P. scabrosa 2
P. scabrosella
P. sp. 7
XI
2463302 Nostoc flagelliforme
P1228 P. neopolydactyla 5 USA [Ore]
P1232 P. neopolydactyla 5 USA [Ore]
P1677 P. neopolydactyla 5 USA [Ore]
P1257 P. neopolydactyla 5 USA [Ore]
XIb
■Only Pacific Northwest
●Only P. neopolydactyla 5
455476274 P. leu/can/ven/aph/pon/kris/fus/bri/pra/sp. HT02 BC
455475935 P. leu/ven/bri HT10 BC
455476535 P. neopolydactyla HT24 BC
455476385 P. leucophlebia HT30 BC
455476640 P. leucophlebia Québec
455476040 P. prae/kris/leu/bri/fus/sp. HT03 BC
82471084 P. aphthosa Switzerland
455475902 P. aphthosa HT08 BC
82470973 P. rufescens Germany
P17 P. leucophlebia HT55 Iceland
P14 P. canina HT56 Iceland
P77 P. ponojensis HT57 Chile
455476004 P. can/cin/leu/fus/pol HT06 BC
P6 P. sp. HT58 Iceland
P75 P. monticola HT59 Norway
VI
■Widespread
●Only Peltigera (various sections)
P1213 P. polydactylon Norway
P1235 P. polydactylon Alaska
P816 P. polydactylon Norway
P833 P. polydactylon Norway
P450 P. sp. 10 Pen.
P611 P. sp. 3 NZ
P682 P. polydactylon Norway
P385 P. polydactylon Norway
P1652 P. sp. 10 Canada
P856 P. polydactylon Iran
119690850 P. leucophlebia Finland
119690853 P. leucophlebia Finland
119690862 P. leucophlebia Finland
119690886 P. praetextata Finland
119690889 P. praetextata Finland
119690892 P. praetextata Finland
119690895 P. praetextata Finland
119690898 P. praetextata Finland
119690901 P. praetextata Finland
119690883 P. praetextata Finland
82470928 P. membranacea Canada
455476130 P. mem/neoca/hor/pol/cin/can HT01 BC
455476142 P. horizontalis HT11 BC
82471000 P. membranacea USA
82471081 P. aphthosa Switzerland
82471066 P. membranacea Russia
82471003 P. horizontalis USA
N2069 P. polydactylon Norway
P71 P. polydactylon USA
P65 P. horizontalis USA
P66 P. elisabethae USA
P70 P. neocanina USA
P1561 P. sp. 2b Brazil
P893 P. dolichorhiza Bolivia
119690904 P. praetextata Finland
119690865 P. leucophlebia Finland
119690859 P. leucophlebia Finland
P388 P. polydactylon Norway
V
■Worldwide (North America, South America,Europe, New Zealand)
●Only Peltigera (various sections)
★P. dolichorhiza
P polydactylon
P. sp. 2b
P. sp. 3
P. sp. 10
P905 P. sp. 1 Colombia HT40
P386 P. neopolydactyla 2 Norway
P846 P. neopolydactyla 2 Norway
P821 P. neopolydactyla 2 Norway
P1322 P. neopolydactyla 2 Norway
P384 P. neopolydactyla 2 Norway
P1223 P. neopolydactyla 6 Oregon
P1231 P. neopolydactyla 6 Oregon
P1240 P. pacifica BC
P1241 P. pacifica BC
P1243 P. pacifica BC
P1183 P. neopolydactyla 2 Japan
52 P. neopolydactyla
119690877 P. neopolydactyla Finland
82471069 P. membranacea Canada
455476412 P. neo/deg/memb HT14 BC
P1242 P. pacifica BC
XIII
■Panboreal (Norway, Finland, Canada [BC], Japan [Hokkaido])
●Only Peltigera (various sections)
★P. neopolydactyla 2
P. neopolydactyla 6
P. pacifica


4-->5
P890 P. pulverulenta 1 HT41 Bolivia
P1319 P. sp. HT60 Canada [Québec]
82470994 P. canina USA
82470997 P. membranacea USA
P29 P. dolichorhiza HT42 Costa Rica
P43 P. ulcerata HT61 Costa Rica
205278410 Lathagrium auriforme 1 Spain
55650832 Nostoc edaphicum Czech Republic
205278473 Enchylium tenax 1 Spain
205278590 Leptogium pseudofurfuraceum 5 Argentina
205278500 Leptogium corniculatum 2 Spain
205278596 Scytinium pulvinatum 1 Spain
205278593 Scytinium pulvinatum 2 Spain
205278497 Leptogium corniculatum 1 Spain
205278542 Scytinium gelatinosum 1 Spain
82471075 Cycas circinalis Brazil
205278587 Leptogium pseudofurfuraceum 1 Argentina
205278584 Leptogium pseudofurfuraceum 3 USA
205278407 Lathagrium auriforme 2 Spain
205278452 Enchylium polycarpon 2 Spain
205278611 Scytinium schraderi 2 Spain
205278464 Collema subnigrescens 3 Portugal
205278416 Arctomia fasciculare 2 Spain
205278446 Collema nigrescens 3 Spain
205278509 Leptogium cyanescens 3 Panama
119690910 Pseudocyphellaria crocata Madeira
82471030 Nephroma helveticum Canada
82779951 Pseudocyphellaria mallota Argentina
82779934 Lobaria amplissima Norway
119690755 Degelia plumbea Madeira
119690830 Nephroma tangeriense Madeira
119690770 Lobaria pulmonaria Finland
119690767 Lobaria pulmonaria Finland
455476643 P. neopolydactyla China
N1537 P. sp. 11 PNG
N1547 P. sp. 11 PNG
N1533 P. sp. 11 PNG
N1532 P. sp. 11 PNG
IX
Only PNG
Only P. sp. 11
P1272 P. sp. 8 HT43 China
82779940 Pseudocyphellaria hirsuta Argentina
82779975 Pseudocyphellaria lechleri Argentina
82779937 Pseudocyphellaria mallota Argentina
82779944 Pseudocyphellaria crocata Argentina
82779943 Pseudocyphellaria crocata Argentina
82779947 Pseudocyphellaria hirsuta Argentina
82779974 Pseudocyphellaria scabrosa Argentina
82779935 Pseudocyphellaria crocata Canada
119690785 Lobaria scrobiculata Finland
119690806 Nephroma laevigatum Finland
119690809 Nephroma laevigatum Finland
82779933 Lobaria amplissima Norway
82779950 Pseudocyphellaria pilosella Argentina
82779949 Pseudocyphellaria corilifolia Argentina
82779942 Pseudocyphellaria crocata Argentina
82779945 Pseudocyphellaria crocata Argentina
82779948 Pseudocyphellaria crocata Argentina
82779938 Pseudocyphellaria coriifolia Argentina
82779941 Pseudocyphellaria scabrosa Argentina
Disjunct Polar Distri-bution
455476439 Nephroma parile HT05 BC
119690812 Nephroma parile Finland
119690818 Nephroma parile Finland
119690821 Nephroma parile Finland
119690776 Lobaria pulmonaria Finland
119690779 Lobaria pulmonaria Finland
119690815 Nephroma parile Finland
119690782 Lobaria pulmonaria Finland
119690764 Lobaria pulmonaria Finland
82471018 Lobaria amplissima Austria
82471102 Sticta fuliginosa USA
II
Holarctic
Various epiphytic Peltigerales represented
119690836 Parmeliella triptophylla Finland
455476436 Nephroma resupinatum/bellum HT12 BC
455476634 Nephroma resupinatum Québec
119690839 Parmeliella triptophylla Finland
119690791 Nephroma bellum Finland
119690803 Nephroma bellum Finland
119690788 Nephroma bellum Finland
119690758 Lobaria pulmonaria Finland
119690824 Nephroma resupinatum Finland
119690827 Nephroma resupinatum Finland
119690794 Nephroma bellum Finland
119690797 Nephroma bellum Finland
119690800 Nephroma bellum Finland
119690761 Lobaria pulmonaria Finland
119690842 Parmeliella triptophylla Finland
119690845 Parmeliella triptophylla Finland
82471027 Nephroma bellum Austria
N1914 P. neopolydactyla 2 China
N1926 P. neopolydactyla 2 China
P1283 P. neopolydactyla 3 China
P1536 P. scabrosa 3 Russia
P1716 P. sp. 5 PNG
P1538 P. scabrosa 3 Russia
455476649 P. neopolydactyla China
N1913 P. neopolydactyla 2 China
N1916 P. neopolydactyla 2 China
N1927 P. neopolydactyla 2 China
N1929 P. neopolydactyla 2 China
X
Eastern regions (China, Russia, PNG)
Only Peltigera section Polydactylon
P. sp. 5
P. neopolydactyla 2, P. neopolydactyla 3, P. scabrosa 3,
P1650 P. sp. 6 HT44 Honduras
P1281 P. sp. 9 HT45 China
2463311 Nostoc sp.
205278455 Enchylium polycarpon 1 Spain
2463314 Nostoc sp.
3-->4
119690871 P. membranacea Finland
119690880 P. neopolydactyla Finland
455476529 P. membranacea HT15 Canada [BC]
82471057 P. degenii Canada
82779967 P. degenii Finland
P1733 P. sp. 6 HT46 Peru
P1721 P. scabrosa 2 Greenland
P1328 P. scabrosa 2 Canada [Nunavik]
VIId
Boreal Atlantic (Canada [Nunavik], Greenland)
Only P. scabrosa 2
82470991 P. neopolydactyla USA
P645 P. neopolydactyla 1 USA [NC]
P67 P. neopolydactyla 1 USA [NC]
P1638 P. occidentalis USA [NC]
P325 P. neopolydactyla 1 Peru
N1939 P. neopolydactyla 1 USA [Ala]
P640 P. neopolydactyla 1 USA [NC]
N1534 P. sp. 4 PNG
P859 P. neopolydactyla 3 Vietnam
VIIb
Pantemperate (USA, Peru, Vietnam, PNG)
Only section Polydactylon
P. occidentalis
P. neopolydactyla 1
P. neopolydactyla 3
P. sp. 4
P1252 P. neopolydactyla 1 USA [AZ]
P1666 P. sp. 9 Japan
N1236 P. scabrosa 3 Greenland
VIIc
Holarctic
Only P. section Polydactylon
P. neopolydactyla 1, P. scabrosa 3, P. nana 1
VII
P309 P. neopolydactyla 1 Canada [Québec]
P529 P. scabrosa 4 Norway
P853 P. occidentalis Alaska
P410 P. occidentalis Russia
P306 P. scabrosa 1 Canada [Québec]
P549 P. scabrosa 4 Norway
P93 P. scabrosa 1 Canada [Québec]
P97 P. scabrosa 1 Canada [Québec]
P571 P. occidentalis Norway
P315 P. scabrosa 4 Canada [Québec]
N1944 P. neopolydactyla 1 Alaska
P411 P. neopolydactyla 1 Russia
P318 P. scabrosa 4 Canada [Québec]
P1210 P. scabrosa 1 Norway
P865 P. scabrosa 3 Russia
P1539 P. scabrosa 1 Russia
P312 P. scabrosa 4 Canada [Québec]
P1250 P. scabrosa 1 Canada [Alberta]
P311 P. scabrosa 1 Canada [Québec]
P108 P. occidentalis Canada [Québec]
P314 P. occidentalis Canada [Québec]
P299 P. occidentalis Canada [Québec]
P845 P. neopolydactyla 1 Norway
P543 P. occidentalis Norway
P844 P. neopolydactyla 1 Norway
P79 P. occidentalis Russia
82779968 P. neopolydactyla Finland
455476637 P. neopolydactyla Canada [Québec]
VIIa
Panboreal (Norway, Finland, Russia, USA [Alaska], Canada [Alberta, Québec]
Only section Polydactylon
P. occidentalis
P. neopolydactyla 1
P. scabrosa 1
P. scabrosa 3
P. scabrosa 4
Subclade 3
P4 P. canina Iceland HT49
P76 P. patagonica Chile HT50
82779936 Pseudocyphellaria clathrata Brazil
P72 P. phyllidiosa USA [NC] HT62
82471006 Sticta beauvoisii USA
82779953 Pseudocyphellaria intricata Reunion Island
82779952 Pseudocyphellaria crocata Thailand
455476652 Lobaria kukorawa China
455476646 Lobaria kukorawa China
82471033 Pannaria conoplea Austria
N1900 P. sp. 8 China
N1907 P. sp. 9 China
N1918 P. sp. 9 China
N1928 P. sp. 8 China
P1508 P. sp. 9 China
N1923 P. sp. 9 China
N1936 P. sp. 9 China
N1898 P. sp. 8 China
N1922 P. sp. 8 China
N1924 P. sp. 8 China
N1897 P. sp. 8 China
N1908 P. sp. 9 China
P1299 P. sp. 8 China
N1921 P. sp. 9 China
N1754 P. sp. 8 South Korea
P1237 P. sp. 8 Taiwan
VIIIa
Temperate East Palearctic (China [Yunnan], Taiwan, South Korea)
Only Polydactyloid clade
P. sp. 8
P. sp. 9
82779964 Stereocaulon tomentosum Argentina
P430 P. hymenina Canada
P335 P. truculenta Chile
P1251 P. truculenta Chile
VIIIb
North/South America
Dolichorhizoid clade
P. truculenta, P. hymenina
VIII
N1920 P. sp. 8 HT47 China
82779954 Pseudocyphellaria crocata Mauritius
82471024 Massalongia carnosa USA
82471015 Scytinium gelatinosum 2 USA
82779969 Massalongia carnosa Finland
N1748 P. sp. 8 South Korea
N1752 P. sp. 8 South Korea
N1766 P. sp. 8 South Korea
XIV
Only South Korea
Only P. sp. 8
82471009 P. canina USA
P84 P. ponojensis USA [PA] HT63
55650826 Nostoc calcicola Czech Republic
55650829 Nostoc calcicola Czech Republic
82779955 Pseudocyphellaria coriifolia Argentina
82779946 Pseudocyphellaria intricata Argentina
205278413 Arctomia fasciculare 1 Spain
82779939 Pseudocyphellaria intricata Argentina
205278488 Leptogium azureum 1 Argentina
205278503 Leptogium corticola 1 Costa Rica
205278506 Leptogium corticola 2 USA
82471021 Lobaria hallii USA
205278449 Collema nigrescens 1 Spain
205278440 Collema furfuraceum 1 USA
205278491 Leptogium brebissonii 2 Spain
205278431 Collema furfuraceum 3 Spain
205278437 Collema furfuraceum 2 Portugal
205278461 Collema subnigrescens 1 Croatia
205278458 Collema subnigrescens 2 Portugal
4-->5
Subclade 3

ditional sampling may change the recovered patterns. *Nostoc* from the same *Peltigera* species are frequently grouped in the same clade, however, we did not detect any evidence of strict cospeciation between the mycobiont and cyanobiont at any taxonomic level in section *Polydactylon* (Figs. 2 and 4).

1.4.8 Patterns of association and specificity between *Nostoc* and *Peltigera*

One third of *Peltigera* species were associated with only one *Nostoc* phylogroup, being thus potentially strict specialists (Fig. 5). Two of them exhibited a reciprocal one-to-one specificity with their cyanobiont, which is an extremely rare phenomenon in lichen-forming fungi where the photobiont is transmitted horizontally when the fungus is reproducing sexually. Such high level of reciprocal specificity was previously reported for the genera *Collema* and *Leptogium* (Collemataceae, Peltigerales; Otálora et al. 2010). For section *Polydactylon*, all mycobionts of *Peltigera neopolydactyla* 5 (four specimens collected in the Pacific Northwest) and *P.* sp. 11 (four specimens collected in Papua New Guinea) were associated only with *Nostoc* phylogroup XIb and phylogroup IX, respectively (and vice versa). The following *Peltigera* species were also defined as specialist: *P.* sp. 10 (four specimens in one biogeographic zone – NA) and *P. polydactylon* (ten specimens in another biogeographic zone – BO), which were always found in association with the cosmopolitan *Nostoc* phylogroup V (Fig. 4). However, O'Brien et al. (2013) reported *Nostoc* phylogroup VI associated with *P. polydactylon* in British Columbia; *P.* sp. 9 (seven specimens in one biogeographic zone – EP) always associated with phylogroup VIIIa; *P. scabrosa* 1 (seven specimens in one biogeographic zone – BO, found on three continents) and *P. scabrosa* 4 (five specimens in one biogeographic zone – BO, found on two continents) always associated with phylogroup VIIa; *P. pacifica* (four specimens in one biogeographic zone – PNW) always found associated with phylogroup XIII; and *P. scabrosella* (four specimens in one biogeographic zone – BO, found on two continents) and *P.* sp. 7a/b (six specimens in two biogeographic zones – BO and PNW) always associated with phylogroup XIa.

Several other specialists were found to be associated with two *Nostoc* phylogroups, such as *P. neopolydactyla* 6 (five specimens in one biogeographic zone – PNW) with phylogroup XIII and the rare phylogroup XVII, but never with any of the remaining numerous phylogroups occurring in the Pacific Northwest. Similarly, *P. neopolydactyla* 4 (ten specimens in BO, but located on three continents) was found with phylogroup XIa and phylogroup IV, but never with other (various) phylogroups locally available. The same type of dual association by one fungal species was observed for *P. scabrosa* 2 and *P.* sp. 3.

The association of some *Peltigera* species with multiple *Nostoc* phylogroups can be driven by natural selection resulting in adaptations to different bioclimatic zones. For example, *P. neopolydactyla* 1 (ten specimens) and *P. occidentalis* (eight specimens) partner with phylogroup VIIa in the boreal regions across all continents but with phy-

logroup VIIb in temperate USA or the very rare phylogroup VIIc in Arizona (Fig. 5). Interestingly, these three *Nostoc* strains share a most recent common ancestor, i.e., they are closely related, and their phylogenetic structure (Fig. 4) reflects their adaptation to different bioclimatic zones. Therefore, this close affiliation among these *Nostoc* strains facilitated the association of one fungal species with multiple *Nostoc* phylogroups, resulting in a *Peltigera* species with a broader geographical and bioclimatic range than its *Nostoc* phylogroups, each restricted to a specific bioclimatic zone, Fernández-Mendoza et al. (2011) showed that the lichen-forming species *Cetraria aculeata* was very specific in the sense that it always associate with one lineage of the green algal species *Trebouxia jamesii*, but associates with different lineages of this algal species in temperate versus boreal zones. However, a close relationship among photobiont strains does not seem to be required for an adaptation by differential association to occur. For example, *P. neopolydactyla* 2 (11 specimens) associates with phylogroup XIII throughout the boreal zone but is found with phylogroup X in temperate Asia. Adaptations to different environments resulting from switches to a different *Nostoc* partner has also been reported for three species of the lichen-forming genus *Pannaria* by Elvebakk et al. (2008).

Among the generalists associated with several *Nostoc* phylogroups, *P. scabrosa* 3, *P. neopolydactyla* 3, *P. hymenina*, *P.* sp. 8 and *P. nana* 1 show some degree of specificity. *Peltigera* sp. 8 in Yunnan was always found with phylogroup VIIIa, although phylogroups X and XII were present in the same locality. *Peltigera hymenina* associates exclusively with phylogroup XVI in continental Europe, while it has been found with several phylogroups (e.g., XVI, XVIII, VIIIb, and XIXb) across sampled areas.

All South American species (the South American group and *P.* sp. 6; Fig. 5), for which we had more than one specimen, seem to be true generalists. For instance, P. *dolichorhiza* (13 specimens) was associated with six phylogroups and two haplotypes, *P. truculenta* (five specimens) with two phylogroups and two haplotypes, *P. pulverulenta* 1 (five specimens) with three phylogroups and one haplotype, and *P.* sp. 6 (five specimens) with one phylogroup and three haplotypes. These species share the same subset of *Nostoc* phylogroups, from the broadly defined phylogroup group XIX to various unrelated phylogroups (phylogroup XV in P. *truculenta*, *P. pulverulenta* 1 and *P.* sp. 2a, phylogroup V in *P. dolichorhiza* and *P.* sp. 2b, phylogroup VIIIb in *P. truculenta*) that represent the diversity of *Nostoc* detected in the sampled areas of South America. Most of these *Nostoc* phylogroups seem to occur from Mexico to Chile and we did not detect a phylogenetic or geographic pattern for these mycobiont-cyanobiont associations (differences are probably due to unequal sampling). For instance in a single locality in Minas Gerais, Brazil, we found *P. dolichorhiza* associated with phylogroups V and XIXd, *P. dolichorhiza* 2 with phylogroup XIXc and *P. pulverulenta* 1 and 2 with phylogroup XIXb, while in Mexico, *P. dolichorhiza* was associated with phylogroup XIXb and in Panama with phylogroup XIXc. For these South American *Peltigera* species we also found them associated with a greater number of unique *Nostoc* haplotypes (i.e., haplotypes outside of delimited phylogroups; Fig. 4) that were frequently singletons (HT 33-36, 38, 40-42, 44, and 46 representing 10 of the 15 unique *Nostoc* haplotypes

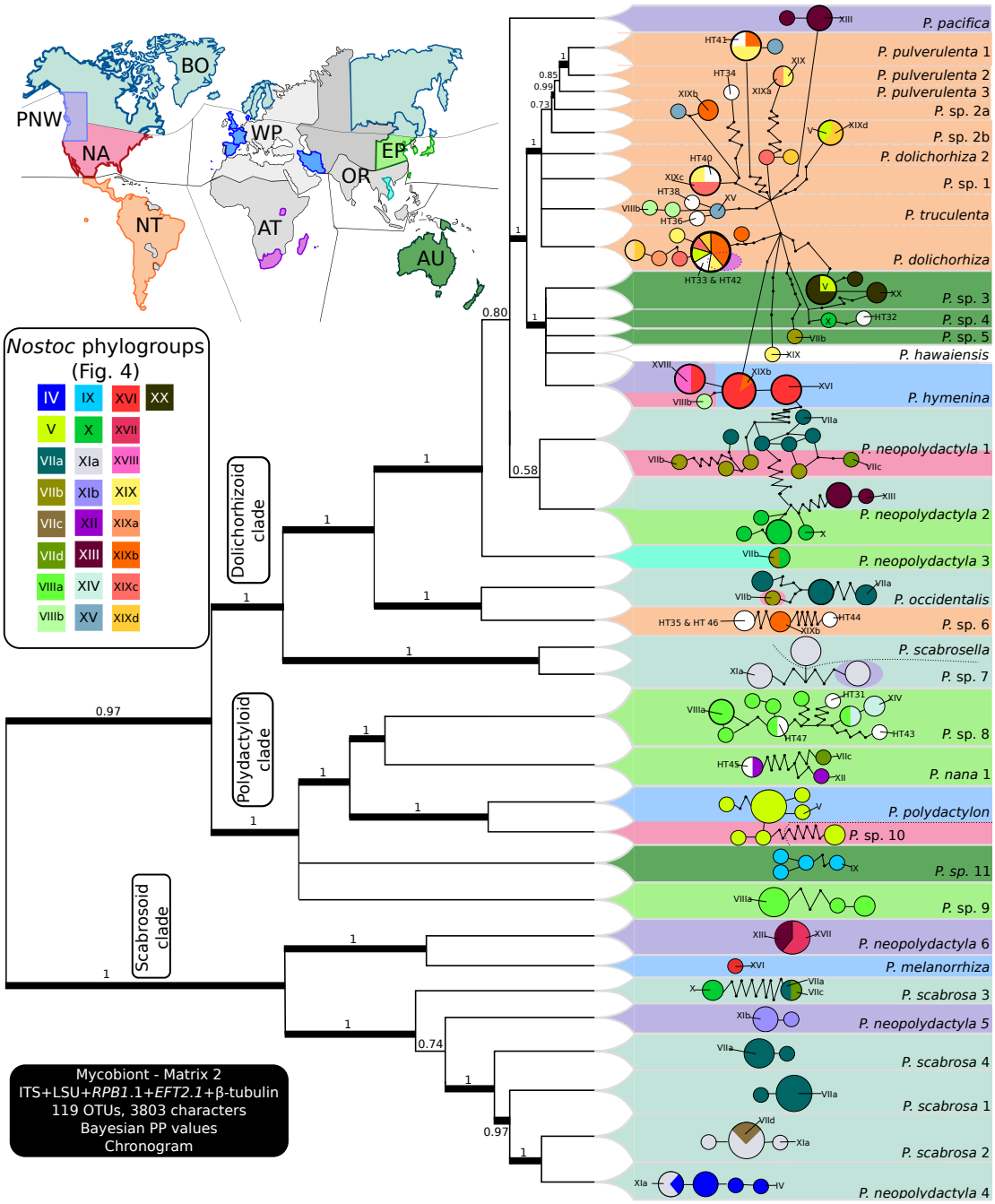

BO
PNW
NA
WP
ER
OR
NT
AT
AU
Nostoc phylogroups
(Fig. 4)
IV
IX
XVI
XX
V
X
XVII
VIIa
XIa
XVIII
VIIb
XIb
XIX
VIIc
XII
XIXa
VIId
XIII
XIXb
VIIIa
XIV
XIXc
VIIIb
XV
XIXd
Dolichorhizoid clade
Polydactyloid clade
Scabrosoid clade
0.97
1
0.80
0.58
0.74
0.97
0.85
0.99
0.73
P. pacifica
P. pulverulenta 1
P. pulverulenta 2
P. pulverulenta 3
P. sp. 2a
P. sp. 2b
P. dolichorhiza 2
P. sp. 1
P. truculenta
P. dolichorhiza
P. sp. 3
P. sp. 4
P. sp. 5
P. hawaiensis
P. hymenina
P. neopolydactyla 1
P. neopolydactyla 2
P. neopolydactyla 3
P. occidentalis
P. sp. 6
P. scabrosella
P. sp. 7
P. sp. 8
P. nana 1
P. polydactylon
P. sp. 10
P. sp. 11
P. sp. 9
P. neopolydactyla 6
P. melanorrhiza
P. scabrosa 3
P. neopolydactyla 5
P. scabrosa 4
P. scabrosa 1
P. scabrosa 2
P. neopolydactyla 4
HT41
HT34
HT40
HT38
HT36
HT33 & HT42
HT32
HT35 & HT 46
HT44
HT31
HT47
HT43
HT45
Mycobiont - Matrix 2
ITS+LSU+RPB1.1+EFT2.1+β-tubulin
119 OTUs, 3803 characters
Bayesian PP values
Chronogram

Figure 5: Associations of *Nostoc* phylogroups with *Peltigera* species within a geographic and evolutionary context. The main tree represents a simplified chronogram (BEAST analysis of Matrix 2), where each putative *Peltigera* species from section *Polydactylon* (36 of 38 species) as shown in Fig. 3 are each represented by one terminal branch. OTUs for which *Nostoc* data where not available were collapsed together with their closest relatives for which *rbcLX* data was available. Bayesian posterior probability support values from BEAST analysis are provided above branches. Thicker internodes indicate PP $\geq$ 0.95. Background colors extending from the terminal branches represent geographic regions (as shown on the map on the top left corner; abbreviations refer to the following regions: Arcto-Boreal (BO), Afrotropics (AT), Australasia (AU), Temperate East Palearctic (EP), Temperate Nearctic (NA), Neotropics (NT), Oriental (OR), Pacific North West (PNW), Temperate West Palearctic (WP).) where thalli were sampled. Haplotype networks resulted from TCS analyses based on ITS sequences from all sampled individuals of the 36 putative Peltigera species. Haplotypes were not connected at the P value below 0.95. Circles in the haplotype network represent the sampled mycobiont haplotypes, while small black dots represent putative (unsampled) haplotypes with a difference of one mutation (including indels) compared to the next adjacent haplotype(s). The size of the circles is proportional to the number of *Peltigera* mycobiont with identical ITS sequences. The colors within circles of the fungal haplotype networks correspond to phylogroups of *Nostoc* (as defined in Figure 4 and represented in the legend provided here), in association with each individual mycobiont (i.e., sampled for the same thallus). White circles, or fractions of circles, indicate *Nostoc* haplotypes that were placed outside of all defined phylogroups (IV-XX; Fig. 4); their haplotype numbers are provided (preceded by HT).

for the entire section *Polydactylon*). The only other unique haplotypes were collected from the Asiatic *P.* sp. 8 and *P. nana* 1 and the Papuan *P.* sp. 4.

Except for the two cases of reciprocal one-to-one specificity involving phylogroups IX and XIb (Fig. 5), *Nostoc* specialists are very rare in symbiotic associations within *Peltigera* species of section *Polydactylon*. By default, cases of high specificity are favored by low sampling, which means that overall we are overestimating high specificity at the cost of low specificity. For example, phylogroup XX was only found in association with *P.* sp. 3, but this is probably due to the very few samples we have from New Zealand. However, the two other cases, which involve *Nostoc* phylogroup VIId that was found associated with only *P. scabrosa* 2 and phylogroup XVII associated exclusively with *P. neopolydactyla* 6 (Fig. 5), are from relatively well sampled boreal regions.

Typically, the same *Nostoc* phylogroup can be found associated with several *Peltigera* species within section *Polydactylon*, as well as with species from different sections (Fig. 6). In a few cases a *Nostoc* phylogroup associated with *Peltigera* species can also be associated with lichen-forming species representing different lichen genera (e.g., phylogroup XIa and phylogroup IV with *Nephroma* and *Sticta*) or even be associated with plants (e.g., phylogroup XII with *Gunnera* and *Blasia*) (Fig. 4). Of the 25 *Nostoc* phylogroups detected in section *Polydactylon*, 22 represent *Nostoc* involved exclusively in associations with the genus *Peltigera*, and 18 are restricted to section *Polydactylon*. However, for many parts of the world (PNG, NZ or South America for instance) most available sequences were generated as part of this study. Therefore, future sampling of other cyanolichens and other hosts in these areas might reveal a broader spectrum of association for several *Nostoc* phylogroups as well as many new (unknown) phylogroups.

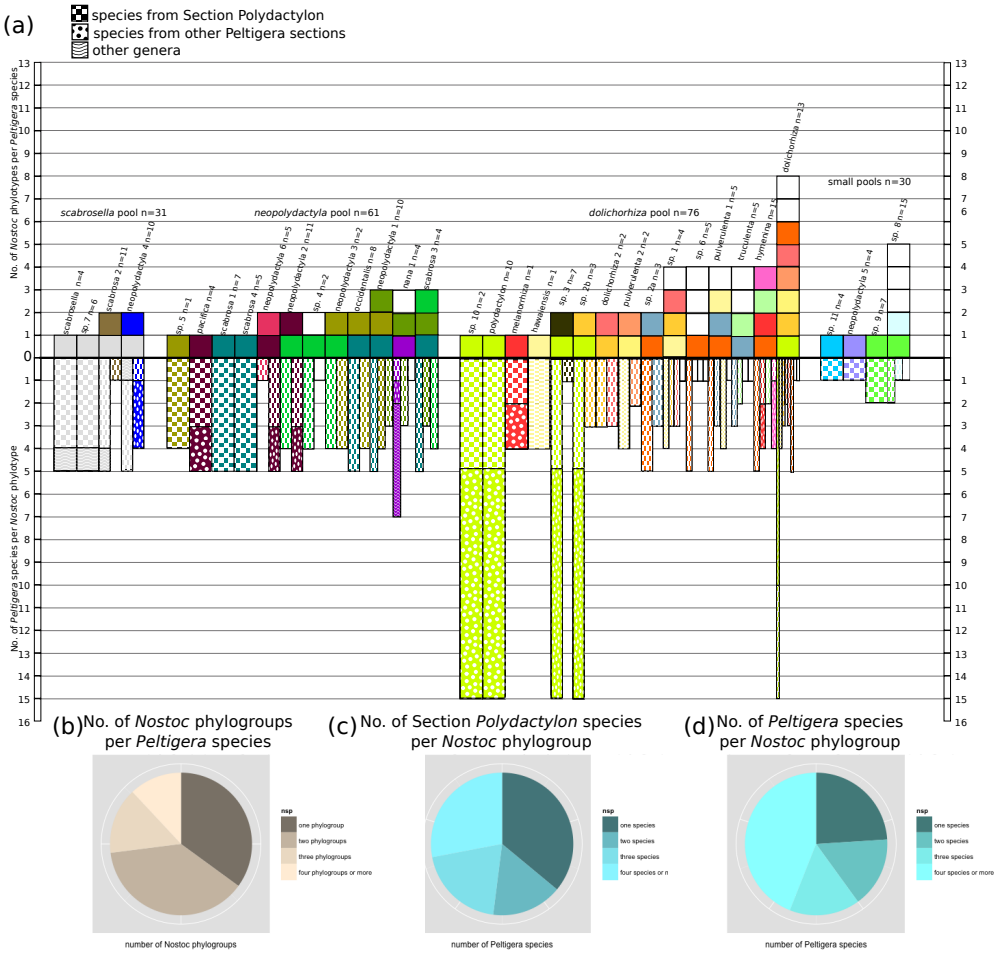


Figure 6: Comparison of levels of specificity for *Peltigera* species and *Nostoc* phylogroups. (a) Upper scale: number of *Nostoc* phylogroups associated with each *Peltigera* species (section *Polydactylon*). N indicates the number of lichen thalli sampled for each *Nostoc* association pool (as defined in Fig. 7) and each *Peltigera* species. Lower scale: number of *Peltigera* species (section *Polydactylon*) associated with *Nostoc* phylogroups. Colors correspond to *Nostoc* phylogroups defined in Fig. 4 and as shown on Fig. 5. Pie charts summarize the level of specificity for the mycobiont and cyanobiont: (b) proportion of *Peltigera* species in section *Polydactylon* associated with one to four or more *Nostoc* phylogroups, (c) proportion of *Nostoc* phylogroups associated with one to four or more *Peltigera* species from section *Polydactylon*. (d) proportion of *Nostoc* phylogroups associated with one to four or more *Peltigera* species globally.

1.4.9 Patterns of Associations among partners

Three main types of mycobiont-cyanobionts associations were found within section *Polydactylon* in terms of their reciprocal specificity (specialists interacting with specialists, generalists with generalists, and specialist *Peltigera* species with generalist *Nostoc* phylogroups (Fig. 6a). We don't have convincing cases of specialist cyanobionts associated with generalist *Peltigera* species because they involve *Nostoc* haplotypes that have been sampled only once. Further sampling is likely to show that these *Nostoc* strains are not specialists. Specialization by *Nostoc* phylogroups toward one *Peltigera* species exists, but these *Peltigera* species are also specialists. Therefore, it seems that the specialization of *Nostoc* phylogroups to one *Peltigera* species is dependent on the specialization of the *Peltigera* species. In other word is seems disadvantageous for a *Nostoc* phylogroup to associate uniquely with one species of *Peltigera* if this species can associate with several *Nostoc* phylogroups. Under such circumstances, especially where the cyanobiont is transmitted horizontally, it is likely that natural selection would lean toward the *Peltigera* species associating with generalist *Nostoc* phylogroups, which should be more abundant in nature. *Peltigera* was never found in nature without their cyanobiont, and they were never isolated in pure culture, whereas *Nostoc* cyanobionts of *Peltigera* have been isolated in culture and they can be free-living in nature (O'Brien et al., 2005a). The fact that the fungus is far more dependent on *Nostoc* than vice versa could explain this overall trend toward specialization by the fungus but not by *Nostoc*.

For cases involving reciprocal one-to-one specialization, it is assumed that partners are well adapted to each other, but geographically restricted to areas where both of them co-occur. It is also assumed that vertical transmission (through vegetative propagules of the lichen thallus) would facilitate this type of association, and promote coevolution and/or cospeciation of the partners. Otálora et al. (2010) reported five species of *Collema* and *Leptogium* (Collemataceae, Peltigerales) exhibiting a one-to-one specificity with their cyanobacterial partners (whereas most species in this family are generalists). These fungal specialist species reproduce mainly by vegetative propagules, and grow exclusively on old trees in very humid conditions, which is concordant with the expectation of vertical transmission and narrow ecological amplitudes for strict specialists. Similarly, when comparing the asexually reproducing species, *Degelia atlantica* with the sexually reproducing *Degelia plumbea*, Otálora et al. (2013) reported that the genetic diversity was lower in both partners in the asexual species (this species exhbits a narrow ecological niche) compared to the sexually reproducing species. Only two cases of such extreme specialization of both partners were detected in section *Polydactylon*: *P. neopolydactyla* 5 and *P.* sp. 11 with phylogroup XIb and IX, respectively (Figs. 5 and 6). However, none of these species seem to reproduce mainly by vegetative propagules or by thallus fragmentation. Therefore, this high reciprocal specificity detected here is likely to be genetically determined as reported for *P. malacea* and its *Nostoc* phylogroup by O'Brien et al. (2013).

Generalists interacting with generalists involves an extremely wide spectrum of as-

sociations for both partners and, therefore, should promote large geographic ranges and broad niche spectra. This trait would be advantageous for lichen-forming species and *Nostoc* phylogroups colonizing new geographic areas or habitats where competition is weak. Under such circumstances it is assumed that fungal ascospores are more likely to find a compatible *Nostoc* strain. *Peltigera* species from the South-American group and *P.* sp. 6 (Figs. 5 and 6) seem to associate with a high number of cyanobionts, and these cyanobionts are found in many, often phylogenetically unrelated species of *Peltigera*. This pattern of low specificity for both partners was suggested for several fungal-*Nostoc* associations in Antarctica (Wirtz et al., 2003) possibly due to the extreme ecological conditions and relatively recent colonization of most of these deglaciated areas.

The third pattern of association (specialist fungus associated with a generalist cyanobiont) may represent a good equilibrium between optimization of the symbiosis and adaptation to various environments. Mycobionts/photobionts associating with several partners could have broader distributions and environmental spectra than when involved in specialist-specialist interactions. In turn, this provides a larger spectrum of genetic pairs and environmental pressures for natural selection, which would lead more quickly to adaptations to different environments and potentially to an increase in speciation rate. Based on our data, most *Nostoc* phylogroups are relative generalists, while *Peltigera* species are more specialized on one or two phylogroups. In *Peltigera*, this trend was already detected to some extent, in previous studies (O'Brien et al., 2005a, 2013; Myllys et al., 2007). What seems to be emerging from our results, is that there is a time component to these three main types of associations, starting with a generalist-generalist pattern of association in newly invaded geographical area followed by differential specialization where the mycobiont specializes faster to its *Nostoc* partner than vice versa, which eventually can lead to an extreme case of one-to-one specificity. This progression would be accompanied by a burst of speciation for the partner that is specializing most quickly.

Of the 35 potential species delimited in *Peltigera* section *Polydactylon*, 35% associate with only one *Nostoc* phylogroup, 38% with two phylogroups, 15% with three phylogroups and 12% with higher numbers of cyanobionts (Fig. 6b). Of the 25 phylogroups of *Nostoc* that form symbioses with at least one *Peltigera* species from section *Polydactylon*, 36% associate with only one *Petigera* species, 16% with two species, 20% with three, and 28% with higher numbers of species (Fig. 6c). However, if we consider all *Peltigera* species (from all sections of this genus), the percentages goes down to 24% for *Nostoc* phylogroups associated with only one *Peltigera* species, 16% with two species, 16% with three species, and 44% associated with higher numbers of *Peltigera* species (Fig. 6d). The average number of species from section *Polydactylon* per *Nostoc* phylogroup is 2.6, but this number goes up to 3.4 species if we consider all species from the genus *Peltigera*. The average continues to go up if we include species from other genera within the order Peltigerales, and this number is also under-estimated because we did not sequence *Nostoc* associating with *Peltigera* species from other sections in many localities where we sampled *Peltigera* from section *Polydactylon*. The average

number of phylogroups of *Nostoc* per *Peltigera* species from section *Polydactylon* is 2 (Fig. 6).

We observed a high degree of selectivity toward certain *Nostoc* phylogroups in several *Peltigera* species across the boreal belt. We sampled *P. neopolydactyla* 1, *P. neopolydactyla* 2, *P. neopolydactyla* 4, *P. occidentalis*, *P. scabrosa* 1, *P. scabrosa* 2, and the pair of *P. scabrosella/P.* sp. 7 in four boreal biogeographic zones: Boreal West Palearctic (BWP; Norway), Boreal East Palearctic (BEP; Russia and Japan), Boreal Nearctic (BN; Québec, Alaska) and Pacific Northwest (PNW; British Columbia, Oregon), from the same localities (most of the time). In each zone, *P. neopolydactyla* 1, *P. occidentalis* and *P. scabrosa* 1 were constantly associated with *Nostoc* phylogroup VIIa, *P. scabrosa* 2 and *P. scabrosella/P.* sp. 7 with phylogroup XIa, and *P. neopolydactyla* 2 with phylogroup XIII. A similar geographic uniformity was found for two other species found only in BN and BWP: *P. scabrosa* 4 was always found with phylogroup VIIa whereas *P. neopolydactyla* 4 was associated with either phylogroup XIa or phylogroup IV. These *Peltigera* species selected specific cyanobionts from diverse *Nostoc* phylogroup communities as revealed by co-occuring *Peltigera* species in the same localities (see also O'Brien et al. 2013).

1.4.10 How are *Nostoc* phylogroups shared among *Peltigera* species?

Most *Nostoc* phylogroups are shared by at least two *Peltigera* species. Because *Peltigera* species are more specialized than cyanobionts to their respective partners, and manifest some degree of selectivity toward *Nostoc*, we attempted to build networks of *Nostoc* phylogroups (*Nostoc* pools) that are shared by *Peltigera* species. Three main *Nostoc* pools were revealed (*scabrosella*, *occidentalis* and *dolichorhiza* pool; Fig. 7a-c). Each *Peltigera* species shown on Fig. 7a was found associated with *Nostoc* phylogroups part of only one of the *Nostoc* pools shown on Fig. 7b. Based on phylogenetic relationships of *Nostoc* associating with *Peltigera* species of section *Polydactylon* (Fig. 4), UniFrac clustered *Peltigera* species into five groups corresponding to *Nostoc* pools and their internal cores: one subset associated with *Nostoc* from the *scabrosella* pool (except *P. neopolydactyla* 5, which is associated with a unique *Nostoc* phylogroup), two subsets associated with the *occidentalis* pool, core 1 and core 2, and two subsets associated with the *dolichorhiza* pool, *dolichorhiza* core and *polydactylon* core (Fig. 7a-c). *Nostoc* phylogroups found with *P.* spp. 8 and 9 (two Asian species, Fig. 5), could not been assigned to any pool, most likely due to our low sampling from Asia.

All four *Peltigera* species assigned to the *scabrosella* pool were collected in boreal regions, and at least some individuals of each species were found in association with *Nostoc* phylogroup XIa (Figs. 5 and 7). Eleven *Peltigera* species were found in association with *Nostoc* from the *occidentalis* pool. Four of them (*P. neopolydactyla* 1, *P. occidentalis*, and *P. scabrosa* 1 and 4) were found in association with phylogroups from core 1 (phylogroups VIIa-c) exclusively. *P. pacifica* and *P. neopolydactyla* 6 were associated exclusively with *Nostoc* phylogroups from core 2 (sharing phylogroup XIII),

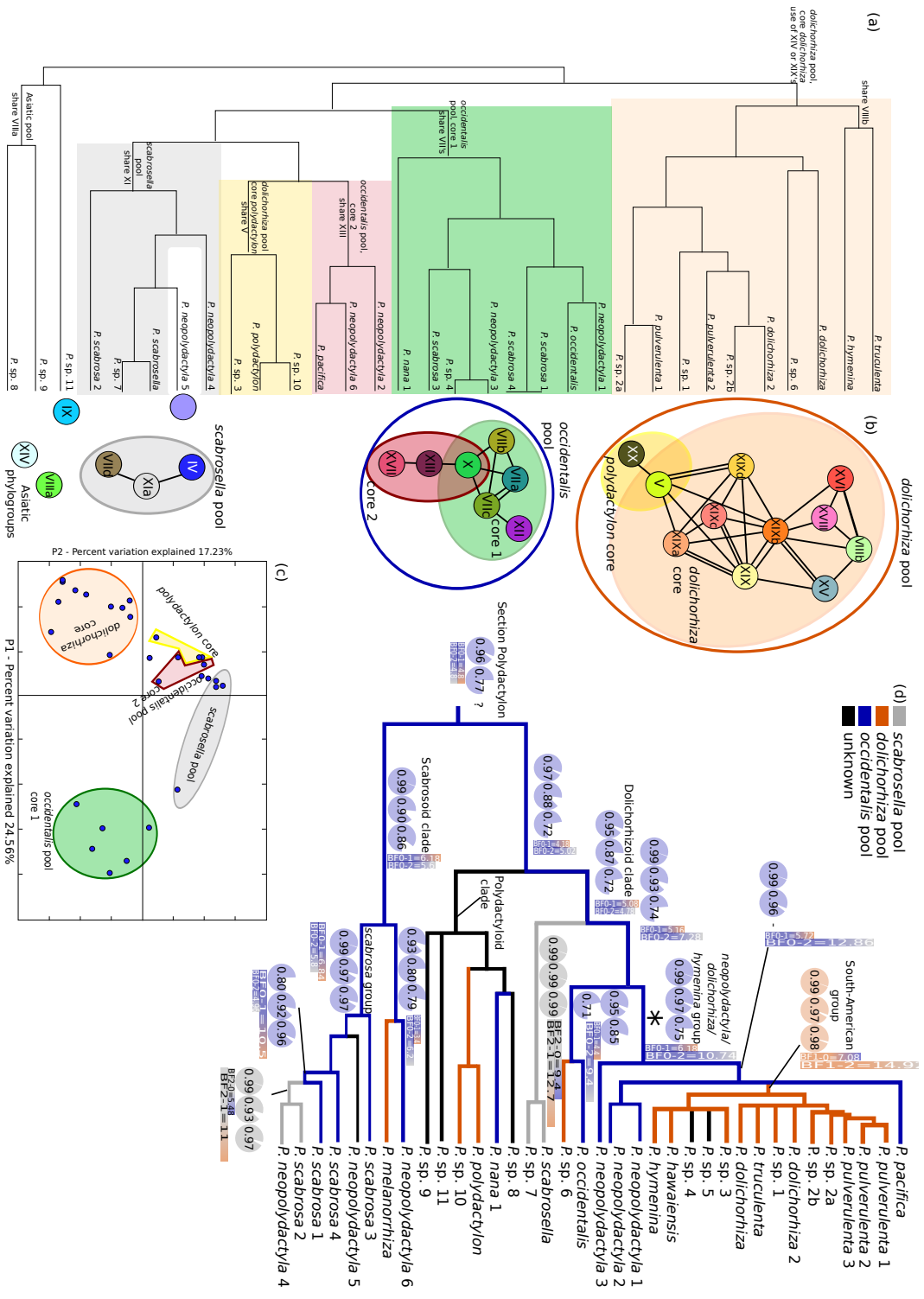

(a)
(b)
(c)
(d)
scabrosella pool
dolichorhiza pool
occidentalis pool
unknown
P. pacifica
P. pulverulenta 1
P. pulverulenta 2
P. pulverulenta 3
P. sp. 2a
P. sp. 2b
P. dolichorhiza 2
P. sp. 1
P. truculenta
P. dolichorhiza
P. sp. 3
P. sp. 5
P. sp. 4
P. hawaiensis
P. hymenina
P. neopolydactyla 1
P. neopolydactyla 2
P. neopolydactyla 3
P. occidentalis
P. sp. 6
P. scabrosella
P. sp. 7
P. sp. 8
P. nana 1
P. polydactylon
P. sp. 10
P. sp. 11
P. sp. 9
P. neopolydactyla 6
P. melanorrhiza
P. scabrosa 3
P. neopolydactyla 5
P. scabrosa 4
P. scabrosa 1
P. scabrosa 2
P. neopolydactyla 4
South-American group
neopolydactyla/ dolichorhiza/ hymenina group
Dolichorhizoid clade
Polydactyloid clade
Scabrosoid clade
scabrosa group
Section Polydactylon
dolichorhiza core
polydactylon core
occidentalis pool core 2
occidentalis pool core 1
scabrosella Pool
P1 - Percent variation explained 24.56%
P2 - Percent variation explained 17.23%
scabrosella pool
occidentalis pool core 1
occidentalis pool core 2
dolichorhiza pool
dolichorhiza core
polydactylon core
Asiatic phylogroups

Figure 7: (a) UniFrac clustering of *Peltigera* species based on their respective set of *Nostoc* phylogroups with which they associate. Color shades on the tree define clusters of *Peltigera* species sharing at least one *Nostoc* phylogroups as shown in panel b. (b) Delimitation of *Nostoc* pools within clusters of *Peltigera* species. Colored circles represent *Nostoc* phylogroups (as defined in Fig. 5) and connecting lines indicate their sharing patterns among different *Peltigera* species. Each line represents a *Peltigera* species that was found associated with the two connected *Nostoc* phylogroups. Colored background inside the pools indicate the sets of *Nostoc* phylogroups associated with *Peltigera* clusters (cores) as defined and colored in panel a. Unique *Nostoc* phylogroups are shown outside of the four delimited *Nostoc* pools. (c) PCA from the UniFrac analysis. Each dot represents *Peltigera* species and their proximity reflects similarity in their respective set of *Nostoc* phylogroups with which they associate. Colored circles correspond to *Nostoc* pools and their cores as defined in panels a and b. (d) Reconstruction of the ancestral pool of *Nostoc* associated with *Peltigera* species depicted in the chronogram presented in Fig. 5. Three main pools of *Nostoc*, as delimited in panel b, were coded. Branches are colored according to the pool of *Nostoc* reconstructed as the ancestral state. Pie charts associated with nodes summarize the results from three different analyses. The first circles represent posterior probabilities generated with SIMMAP; the second circles represent average probabilities generated with Mesquite; and the third circles represent posterior probabilities generated with BEAST. The bars represent values of the Bayes Factor resulting from the BayesTraits analysis. BFx-y is the value of the Bayes Factor for state x rather than state y. The colors correspond to the state (*Nostoc* pool) that was reconstructed as ancestral with significant value. The asterisk shows the branch where the shift in speciation rates occurred according to MEDUSA and BAMM.

whereas three other species (*P. neopolydactyla* 2 and 3, and *P. scabrosa* 3) associated with *Nostoc* phylogroups from both cores (Figs. 5 and 7a-c). Most *Peltigera* species associated with *Nostoc* from the *scabrosella* and *occidentalis* pools are sympatric, yet none of the *Nostoc* phylogroups was shared between the two *Peltigera* groups despite the boreal regions being our most intensively sampled bioclimatic biome.

The *dolichorhiza* pool involved thirteen *Peltigera* species including *P. polydactylon*, *P. hymenina*, all members of the South American group (Fig. 2) and *P.* sp. 6 also from South America (Fig 7a and b). Whithin this pool, all temperate species were found in association with *Nostoc* phylogroups from the *polydactylon* core (*P. polydactylon*, *P.* sp. 3, *P.* sp. 10) which all shared phylogroup V. This subset of species were assigned to the *dolichorhiza* pool because some South-American species (*P. dolichorhiza* and *P.* sp. 2b; Figs. 5 and 7a-c) are also associated with phylogroup V. Among various sharing patterns detected within the *Peltigera* group associated with the largest *dolichorhiza* pool, two common species, *P. hymenina* and *P. polydactylon* have never been found with the same *Nostoc* phylogroup, despite both species having overlapping geographic ranges. There is thus clear specificity even inside these *Nostoc* pools, which are structured both spatially and phylogenetically (Figs. 4 and 5). In other words several *Peltigera* species are more likely to associate with multiple closely related *Nostoc* phylogroups that are locally present than with distantly related phylogroups as exemplified by *Nostoc* phylogroups VII (*occidentalis* core) and XIX (*dolichorhiza* pool core 1). The fungal phylogenetic signal poorly explained UniFrac clusters because species from unrelated *Peltigera* clades (Fig. 5) are intermixed in the *occidentalis* and *scabrosella* pools, as well as in the *polydactylon* core group. Moreover, *P.* sp. 6 and *P. hymenina*, which are phylogenetically distant from the South American *Peltigera* clade, are included in the

dolichorhiza core (Fig. 7a).

1.4.11 Evolutionary History of section *Polydactylon* with their *Nostoc* pools

All three ancestral state reconstruction methods converged on the same ancestral states at all nodes tested. However, the degree of confidence varied considerably (Fig. 7d). In most cases, the greatest level of confidence resulted from SIMMAP analyses, followed by Mesquite, and BEAST. The Bayes factors obtained with BayesTraits cannot be directly compared to the other three results, but when testing the character state reconstructed as ancestral by other methods against the other states, BayesTraits always generated a significant positive value favoring the reconstructed state. Based on SIMMAP and Mesquite analyses, the *Nostoc* pool associated with the ancestor of section *Polydactylon* was the *occidentalis* pool (Fig. 7d), currently widespread and common in *Peltigera* from boreal regions. This pool was also reconstructed by all methods as the ancestral group of cyanobionts associated with the mycobionts at the origin of the Scabrosoid and Dolichorhizoid clades, the *neopolydactyla/dolichorhiza/hymenina* complex, and *scabrosa* clade. The origin and radiation of the South American group was subsequent to a switch from being associated with the *occidentalis Nostoc* pool to the *dolichorhiza* pool. Due to a lack of data for the Polydactyloid clade (*P.* sp. 8, *P.* sp 9 and *P.* sp. 11), the inferred ancestral states were not significant.

Based on the mycobiont phylogeny (Figs. 2, 5 and 7d) the deepest splits often involves boreal species, The Scabrosoid clade is the result of the first diversification within section *Polydactylon*, and the dominance of boreal species in this clade strongly suggests that they diversified in in boreal forests in association with *Nostoc* from the *occidentalis* pool. South American group and *P.* sp. 6 independently shifted to the *dolichorhiza* pool when they colonized South America. A switch from the *occidentalis* to the *dolichorhiza* pool also occurred along the branch leading to *P. melanorrhiza*, a species restricted to Azores. Similarly, when the Polydactyloid clade and *hymenina* group colonized Asiatic and Australasian tropical and temperate zones they switched to *dolichorhiza* pool. *P. polydactylon* (together with its sister species *P.* sp. 10) and *P. hymenina* are associated with *Nostoc* phylogroups from the *dolichorhiza* pool (Fig. 5, 7a), which likely enabled them to colonize and become widespread in more temperate habitats (both species are less frequent in boreal regions) contrary to species associated with the *occidentalis* pool from the *scabrosa* group and other species (e.g., *P. neopolydactyla* 1, 2 and *P. occidentalis*), which are rare in temperate regions and restricted there to humid high elevation mountains (Appalachian mountains in North America, Pyrenees and Alps in Europe).

In the *scabrosa* group, a relatively recent switch from the *occidentalis* to the *scabrosella Nostoc* pool (Fig. 7d) might have led to the origin of a new lineage comprising *P. scabrosa* 2 and *P. neopolydactyla* 4. Both species have been found with two different *Nostoc* phylogroups, while other species from this clade (*P. scabrosa* 1, 4, *P. neopolydactyla* 5) are consistently associated with a single cyanobiont. *P. scabrosa* 3 is some-

what different because it is composed of two very distinct mycobiont haplotypes associated with two different *Nostoc* phylogroups that might represent two species, or distinct populations in the process of speciating (see haplotype network in Figure 5).

The majority of phylogroups from the *occidentalis* pool were found exclusively with species from section *Polydactylon* (phylogroups VIIa, VIIb, VIIc, X, XVII; Fig. 4), with the exception of phylogroup XIII, a cyanobiont of two putative generalists, *P. membranacea*, and *P. degenii* (sister species in section *Peltigera*); the latter species shares a similar morphology (glossy upper surface with little or non-tomentous upper surface) and ecology (forested and humid habitats) with members of section *Polydactylon*. This result further support the orgin of section *Polydactylon* being linked to their symbiotic association with *Nostoc* phylogroups from the *occidentalis* pool. Later in their evolution, *Polydactylon* species switched to other *Nostoc* pools (*dolichorhiza* and *scabrosella*) associated with species from other sections or genera, possibly in the process of colonizing new habitats and geographic areas where these *Nostoc* phylogroups were better adapted. Contrary to the *occidentalis* group, several phylogroups from the *dolichorhiza* pool are associated with mycobionts from other sections of *Peltigera* (e.g., phylogroups V, XVI and XVIII; Fig. 4). The same is true for *Nostoc* from the *scabrosella* pool (e.g., phylogroups IV and XIa).

1.4.12 Biogeographic and climatic factors shaping *Peltigera-Nostoc* associations

Because switches to different *Nostoc* phylogroups and *Nostoc* pools seem to be linked to expansions of geographical ranges by *Peltigera* species, we explored the distributions of *Nostoc* phylogroups across the world (Fig. 8). As for most organisms, climate is an important factor shaping *Nostoc* distribution as several phylogroups are exclusively boreal (IV, VIIa, VIId, XIa, XIII, XVIII), temperate (VIIb, VIIc, VIIIa) or (sub)tropical (XV, XIXa-d). *Nostoc* phylogroups are also restricted to specific biogeographic areas as exemplified by phylogroup IX in Papua New Guinea, XX in Australia and New Zealand, and XIb and XVII in the Pacific Northwest. Phylogroup V is unusual by being present across a wide range of latitudes and longitudes (Fig. 8).

As expected, most *Peltigera* species restricted to the boreal areas were only found with boreal *Nostoc* phylogroups (*P. scabrosa* 1, 2, 4, *P. neopolydactyla* 4, *P. scabrosella-P.* sp. 7 associated with phylogroups VIIa, XIa, IV, VIId), while boreal species with distributions extending to the temperate zone (*P. neopolydactyla* 1, 2, *P. occidentalis*) switch to a temperate *Nostoc* phylogroup (VIIb, VIIc, X) in the warmer portion of their distribution (Figs. 5 and 8). Other broadly distributed species such as *P. hymenina* and *P. polydactylon*, are associated with *Nostoc* phylogroups that have ranges covering more than one bioclimatic zones (phylogroup XVI for *P. hymenina*, phylogroup V for *P. polydactylon*). However, the bioclimatic range of the *Nostoc* phylogroup, and consequently the availability of an appropriate *Nostoc* partner, is not the only factor controlling the distribution of *Peltigera* species. For examples, species associated with the cosmopolitan

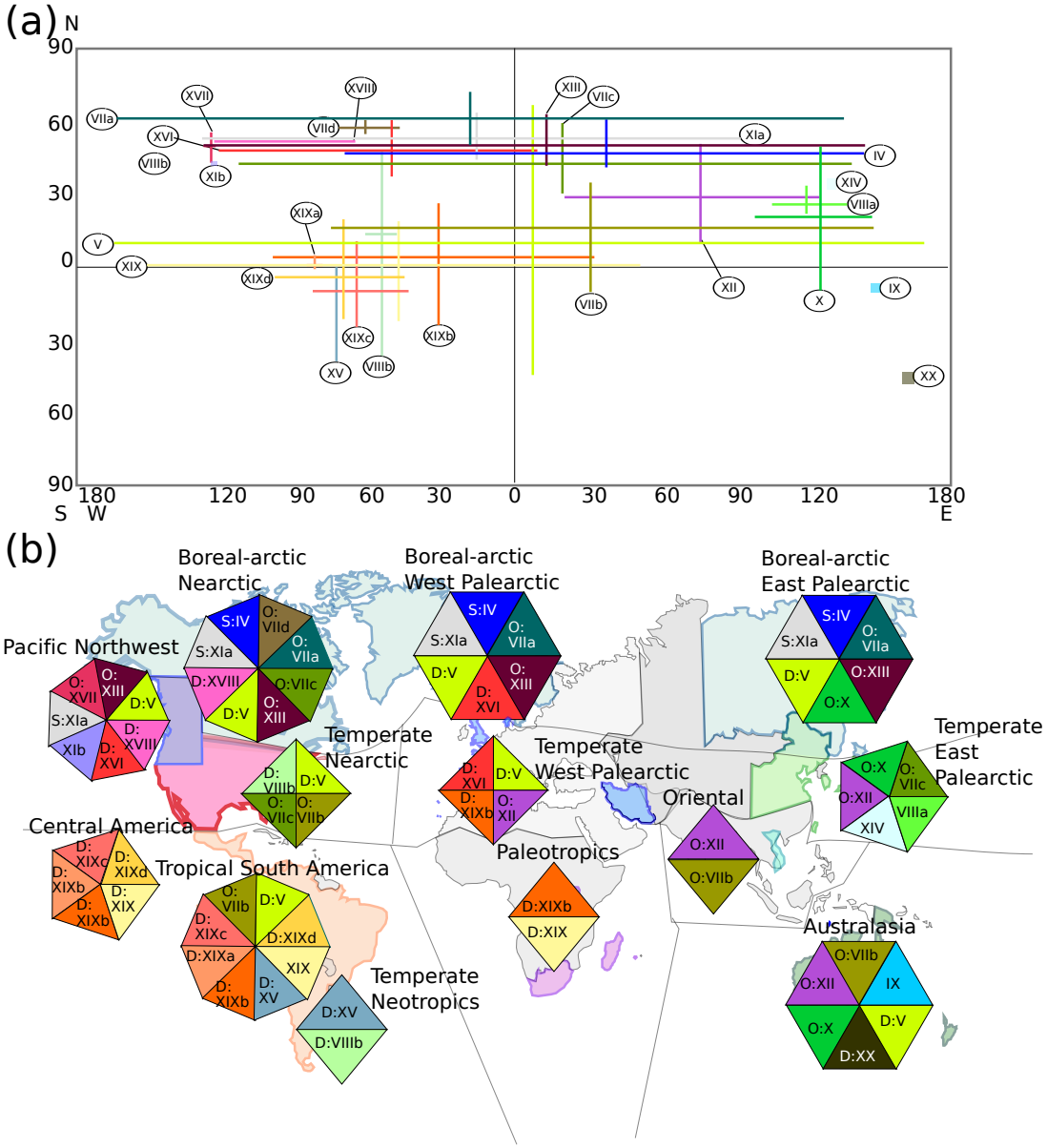


Figure 8: Worldwide repartition of the *Nostoc* phylogroups found in thalli of *Peltigera* species of section *Polydactylon*. (a) Longitude (X axis) and latitude (Y axis) range of *Nostoc* phylogroups (colors refer to the delimitation of phylogroups in Figure 4). (b) *Nostoc* phylogroup composition per biogeographic regions of the world. D, O and S refer to the *dolichorhiza*, *occidentalis* and *scabrosella* pools, respectively; roman numbers refer to the phylogroups, as defined in Figure 4.

phylogroup V (*P. polydactylon*, *P.* sp. 10, *P. dolichorhiza*, *P. pulverulenta*, *P.* sp. 3) or XII (*P.* sp. 9) have more limited distribution than their *Nostoc* partners. This pattern, where the fungal partner has a narrower ecological amplitude than it cyanobiont, has been observed for other species within section *Polydactylon*. The presence of arid zones in Northern Africa, Mexico and southwestern parts of the USA could also be barriers to the expansion of hygrophilic *Peltigera* species from South America and Africa to Europe and North America, respectively. Such geographic barriers are not encountered in the Eurasian zone, where for example a connection between tropical mountains and boreal regions along the Himalayan chain occurs.

Overall, many *Nostoc* phylogroups are restricted to a single bioclimatic zone, confirming that climate plays a major role in shaping the distribution of this bacterium (Figs. 4, 5 and 8). Phylogroup VIIIb is particularly interesting by being found only in temperate nearctic and temperate neantarctic. Most *Nostoc* phylogroups have extensive longitudinal ranges, but rather narrow latitudinal spectra (Fig. 8a). This predominance of climate in shaping photobiont distribution was previously reported by Peksa and Škaloud (2011); Fernández-Mendoza et al. (2011). Furthermore, the boreal zones clustering together both in terms of *Peltigera* species and *Nostoc* phylogroup compositions is yet another manisfestation of the importance of climate in shaping the distributions of both lichen partners.

The level of specialization of *Nostoc*, in terms of the number of *Peltigera* species with which they can associate, can also have an impact on *Nostoc* phylogroup distributions. The few *Nostoc* phylogroups that are specialists, i.e., known to associate with only one *Peltigera* species, have very narrow distributions (Figs. 5, 6 and 8) compared to the geographical ranges of generalists. Phylogroup VIId (associated with *P. scabrosa* 2) was only found in northern parts of the boreal zone of North America (Nunavik and Greenland). Phylogroup XX (associated with *P.* sp. 3) was only found in New Zealand. Similarly, phylogroups XIb and XVII (associated with *P. neopolydactyla* 5 and 6, respectively) are found only in the Pacific Northwest, and phylogroup IX (associated with *P.* sp. 11) in Papua New Guinea. *Nostoc* generalists that can associate with a high number of species have much larger geographical distributions: phylogroup V (associated with 15 *Peltigera* species, Fig. 6) is nearly cosmopolitan; phylogroup XIX (associated with 10 species of the *Polydactylon* group) was found in Hawaii, South America and Africa, and phylogroup XII (associated with seven species from various genera of cyanolichens) occurs from Papua New Guinea to Siberia.

If the propagation of *Nostoc* is dependent on cyanolichen specialized vegetative propagules or thallus fragments containing both partners, the global distribution of *Nostoc* phylogroups (i.e., including outside the lichen thallus, which might be the case for several *Nostoc* phylogroups) should be nearly identical to the geographical range of the *Nostoc* phylogroups found in lichen thalli. Otherwise, the global distribution of *Nostoc* phylogroups should be much broader than their lichen-forming fungal partners, which cannot grow without their *Nostoc* partner. A few species in section *Polydactylon* develop specialized vegetative propagules. However, all *Peltigera* species in this section

are known to reproduce sexually (based on the presence of apothecia) which requires the fungal ascospores to come to close proximity with the appropriate *Nostoc* partner for a thallus to develop. Therefore, the vertical transmission of *Nostoc* can be achieved mostly via thallus fragmentation, which is expected to have a rather limited dispersal capability.

Long-distance dispersal seems common for boreal *Peltigera* species, because the same ITS haplotypes were found on different continents for species with broad boreal distributions (*P. neopolydactyla* 1, 2, 4, *P. scabrosa* 1, 2, 4, *P. scabrosella-P.* sp. 7). More temperate species, like *P. polydactylon*, are represented by distinct haplotypes in Europe and North America suggesting that long distance dispersal along the northern boreal belt is more frequent than in more temperate areas, where the ocean gap between the continents is much wider and continents drifted apart for a longer period of time. This pattern was confirmed by UniFrac analyses when comparing biogeographic regions based on their *Peltigera* species composition. All boreal regions clustered together, whereas temperate regions were more distant from each other (see the PCA in Supplementary Figure S3c). Therefore, species with temperate/tropical distributions are often limited to a single continent. But this geographic restriction may also be due to the presence of different *Nostoc* in temperate Europe, Asia, and North America while the same *Nostoc* strains are spread across the boreal zones of various continents. It is very difficult to detect a clear pattern in *Nostoc* composition, except the separation between southern regions (Neotropics, Afrotropics and Australasia) and northern areas (Nearctic, West Palearctic, East Palearctic, and Pacific Northwest) (Supplementary Figure S3). Limited dispersal abilities of *Nostoc* might also be a factor, although we detected the presence of the same *Nostoc* in South America and Africa. These observations and our suspicion that the genus *Peltigera* is quite old, supports vicariance to be also at play here.

1.4.13 *Nostoc* distribution as a factor shaping geographic ranges of *Peltigera* species

As mentioned earlier, the distribution of *Nostoc* phylogroups is linked in part to their level of specialization, where *Nostoc* associated with many *Peltigera* species have a tendency to have more extensive geographical distributions than *Nostoc* associated with only one or few *Peltigera* species. Such pattern was also detected for the fungal partner of section *Polydactylon* where non-specialists (associated with more than one *Nostoc* phylogroup) have broader distribution (distance and latitudinal span) compared to specialists (associated with only one phylogroup; Fig. 9 a and b). The average distance between the farthest apart localities where two specimens from the same species were sampled is 7854 km for a non-specialist, and 3473 km for a specialist, whereas the average latitudinal range for a non-specialist is 25.14° for a non-specialist and 8.7° for a specialist (Fig. 9).

Of the eight *Peltigera* species showing a latitudinal range $< 10°$, six are specialized on a single phylogroup (*P.* sp. 9, *P. neopolydactyla* 5, *P. pacifica*, *P.* sp. 11, *P. scabrosella*,

and *P. scabrosa* 4). The remaining two species (*P.* sp. 3 and *P.* nana 1 from Australasia) were undersampled in this study (only material from New Zealand was included for the former, and the sampling in the Asian/Australasian pacific region is low for the latter) and might have a broader latitudinal distribution. Similarly, species distributed within less than 2000 km distance were found with one *Nostoc* phylogroup only (*P.* sp. 9, *P. pacifica*, *P. neopolydactyla* 5, *P.* sp. 11, *P. scabrosella*) or rarely with two, such as *P. neopolydactyla* 6, which is restricted to the Pacific Northwest. The latter is associated with two *Nostoc* phylogroups restricted to Pacific Northwest. However, two of the three species endemic to the Pacific Northwest show a strong specificity towards phylogroup XIII, which is a widespread phylogroup along the boreal belt.

All species with latitudinal ranges > 20° (*P.* sp.*P. occidentalis*, *P. hymenina*, *P. dolichorhiza*, *P. pulverulenta* 1, 2, *P.* sp. 2a, *P.* sp. 2b, and *P. neopolydactyla* 1) have been found with at least two different cyanobionts, except *P. polydactylon*, which is specialized on a cosmopolitan *Nostoc* (phylogroup V, but possibly also VI; see O'Brien et al. 2013). Similarly, all species covering a distance > 10 000 km (*P. neopolydactyla* 1, 2, *P. occidentalis*, *P. scabrosa* 2, *P. dolichorhiza*) were found with at least two *Nostoc* phylogroups. *Peltigera* species specialized on a widespread panboreal phylogroup, such as *P. scabrosa* 1, can also span broad geographical distances. Species that specialized on different phylogroup(s) in a different bioclimatic zone are distributed across wider latitudinal range (e.g., 77° for *P. neopolydactyla* 1 associated with phylogroups VIIa/VIIb/VIIc; 33° for *P. occidentalis* associated with phylogroups VIIa/VIIb; and 23° for *P. neopolydactyla* 2 associated with phylogroups X/XIII). Conversely, taxa that use only *Nostoc* phylogroups, even if they are generalists have restricted latitudinal ranges (2° for *P. pacifica*, 9.5° for *P. scabrosa* 4; and 14.4° for *P. scabrosa* 1). *Peltigera hymenina* is a great example of a species that has a broad geographical and latitudinal range (36°), by associating with various *Nostoc* phylogroups (phylogroup XVI in continental Europe and Azores, phylogroup XIXb in Tenerife, and phylogroup VIIIb in Canada; Fig. 5). Therefore, being either a generalist, a local specialist in multiple biogeographical regions, or strict specialist on a widespread phylogroup are three viable strategies that can result in broad geographical ranges for *Peltigera* species. Nevertheless, overall, the distribution of *Nostoc* phylogroups exceeds the distribution of *Peltigera* species in the section *Polydactylon* both in term of geographic distance and latitudinal range. This is in spite that we underestimated the distribution of *Nostoc* phylogroups, by restricting our study to species from section *Polydactylon*. Six *Nostoc* haplotypes associated with species from this section are also associated with species from other sections of the genus *Peltigera* and/or to other genera within the order *Peltigerales* (Fig. 6).

1.4.14 Genetic diversity, specificity, and age of Peltigera species

In general, twice as many ITS haplotypes were detected for non-specialist (7.4) compared to specialist *Peltigera* species (3.2; Fig. 9c). Higher genetic diversity may enable

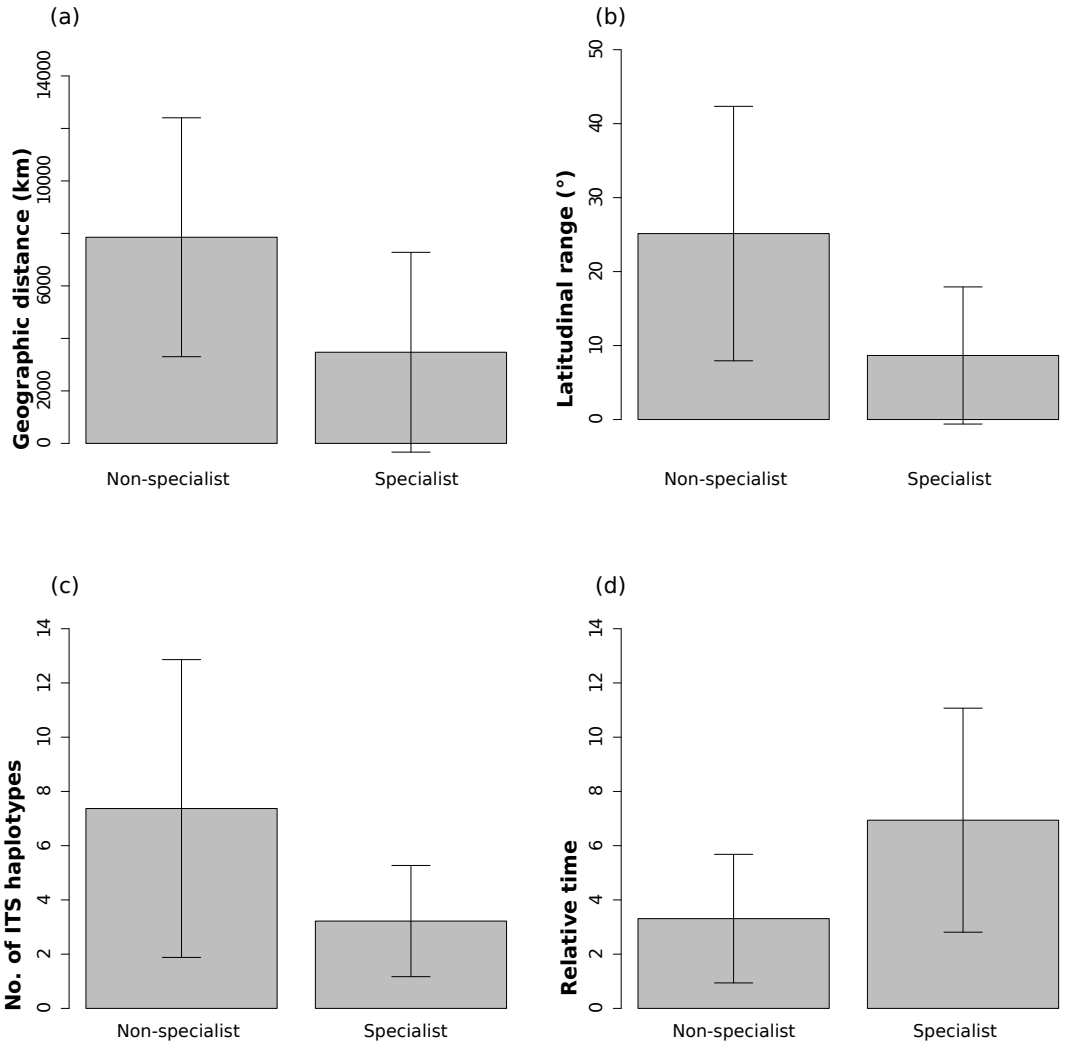


Figure 9: Comparisons of the geographic spectra of specialists versus non-specialists *Peltigera* species defined by distance in kilometers (a) and latitudinal range (b) as well as of their respective genetic diversity defined by the number of ITS haplotypes (c), and relative age of species, based on the branch length in the chronogram of Fig.5 (d). Specialists were defined as *Peltigera* species (from section *Polydactylon*) known to associate with only one phylogroup of *Nostoc* , and non-specialist as *Peltigera* species from this section known to associate with more than one *Nostoc* phylogroups

generalists to associate with multiple partners (no adaptation to a specific *Nostoc* phylogroup), while specialists might be less genetically diverse as a result of being well adapted to a single partner (see Law and Lewis hypothesis, Law and Lewis 1983; and the Red King hypothesis, Bergstrom and Lachmann 2003). The age of *Peltigera* species (i.e., the amount of time since they diverged from their most recent common ancestor) seems to also be a factor. In our study, specialist species are on average twice as old as non-specialist (Fig. 9d). The average length of terminal branches (see the chronogram in Fig. 5) of non-specialist species is 3.3 relative units compared to 6.9 units for specialists (however, these differences were not significant; Fig. 9d). This suggests that a generalist would become gradually specialized on a single cyanobiont through time. Results from BiSSE analyses supported this hypothesis as the transition rate from non-specialist to specialist was much higher than the reverse rate. Therefore, as part of the specialization process, genetic diversity and geographic ranges of species eventually decrease, but become better adapted to the selected cyanobiont resulting in higher fitness for the specialized *Peltigera* species. Future studies should compare the fitness of specialists versus recent non-specialist species.

1.4.15 Impact of Horizontal Transmission on *Peltigera* Specificity

A strict vertical transmsmission of Nostoc would favor high and reciprocal specificity, while horizontal transmission where fungal spores need to re-associate with a compatible *Nostoc* strain at each generation would favor lower specificity for *Peltigera* species (Douglas, 1998). Relatively few species from section *Polydactylon* produce deferentiated vegetative propagules (isidia, phyllidia, and squamules; e.g., *P. pacifica* and *P.* sp. 1) indicative of vertical transmission of *Nostoc*, although thallus fragmentation might be contributing to future generations for all species involved here. However, all studied species regardless of the degree of specialization toward cyanobionts, develop apothecia (at various frequencies) suggesting at least the ability for some sexual reproduction by the fungus and horizontal transmission of *Nostoc*. Apothecia were more often present in species associated with several *Nostoc* phylogroups. For instance, in a specialist such as *P. pacifica*, only two specimens out of eight examined had apothecia, but marginal phyllidia were consistently present in all collections. Less than 40% of the examined collections of specialists such as *P. scabrosa* 1, *P. scabrosa* 4, *P. scabrosella* and *P.* sp. 7 contained apothecia while apothecia were present in more than 60% (up to 100%) of thalli of generalist species (e.g., *P. dolichorhiza*, *P. hymenina*, *P. neopolydactyla* 1, *P. neopolydactyla* 2, *P. neopolydactyla* 4, *P. occidentalis*, *P. truculenta*, and *P.* sp. 6). Blaha et al. (2006) reported that lichen-forming species from the genus *Lecanora*, which are frequently found with many apothecia were associated with a large variety of photobionts (generalist pattern) and colonized a wide spectrum of habitats.

However, this trend was not consistent across section *Polydactylon*, as the majority of individuals from several specialists (associated with one *Nostoc* phylogroup) have apothecia (e.g., *P. neopolydactyla* 5, *P. polydactylon*, *P.* sp. 9 and *P.* sp. 11), whereas

apothecia were not seen in any of the five sampled specimens of a generalist *P.* sp. 1.

Peltigera species with low genetic diversity (e.g., *P. neopolydactyla* 5) that frequently produce apothecia can be explained by genetically regulated specificity. Selection against new fungal genotypes that are less adapted to the sole *Nostoc* partner known to associate with that particular *Peltigera* species, could greatly diminish genetic diversity of this species even if it is reproducing mostly sexually. Even if horizontal transmission is the prevelant mode of dispersal, specialized species may reassociate with the same *Nostoc* phylogroup through various mechanisms of recognition and signaling between the partners at the early stages of lichenization. Joneson et al. (2011) reported that extracellular communication between lichen symbionts can occur without cellular contact, and the authors identified a variety of fungal genes that are involved in self and non-self recognition. It has also been demonstrated that lectins secreated by the fungal partner can play an important role in recognizing compatible photobiont cells (Galun and Kardish, 1995), and in their communications with both, green algae (Legaz et al., 2004) and cyanobacteria (Vivas et al., 2010).

1.4.16 Contribution of specialization to diversification of *Peltigera*

BiSSE analyses revealed a similar extinction rate for specialists and non-specialists, but the speciation rate was much higher for non-specialists compared to specialists (Table 3). Similarly, we detected a higher rate of transition from non-specialist to specialist. In both analyses, a model with constrained equal rates was significantly rejected by AIC. This positive correlation between generalism and rate of speciation might be linked to a reduced genetic diversity in specialists in order to enhance their adaptation to one *Nostoc* phylogroup (Law and Lewis, 1983). The fact that specialist are restricted to a narrower niche, while non-specialist can spread to a wider range of habitats within a broader geographic range, might also enhance genetic diversification and speciation. The analyses also revealed that the rates of transition from a specialist to a generalist is close to zero while the rate of transition from a generalist to a specialist is much higher, which would suggest that specialisation is acquired through time, from a generalist ancestor. These findings support the idea that specialisation results from long-term interactions between fungal and photobiontic partners. Otálora et al. (2010) concluded that in the family Collemataceae, extreme cases of one-to-one reciprocal specialisation between *Nostoc* and these lichen-forming fungi, was also a derived state, which evolved independantly several times from various generalist groups. However, reversals from specialists to generalists do occur. A good example is *P. scabrosa* 2 and *P. neopolydactyla* 4, two relatively recent non-specialists (nested in a clade comprising mostly specialists; Fig. 5) that most likely originated as a consequence of a switch from *Nostoc* phylogroup VIIa to XIa followed by the association of *P. scabrosa* 2 and *P. neopolydactyla* 4 to an additional *Nostoc* phylogroup – VIId and IV, respectively.

Supplementary figure S4 presents the rates determined by BAMM on the species tree. The best model selected by MEDUSA and by BAMM (pp=0.53 with BAMM)

Table 3: Result of the BiSSE analyses. Comparison of extinction rates, speciation rates and transition rates of specialist and non-specialist *Peltigera* species. A1= analysis 1, A2= analysis 2.

Parameter	Specialist		Non-specialist	
	A1	A2	A1	A2
extinction rate m(x)	2.96×10^{2}	2.62×10^{2}	1.44×10^{2}	7.07
speciation rate l(x)	6.36×10^{-10}	2.66×10^{-5}	4.2×10^{2}	4.16×10^{2}
transition rate	9.39×10^{-9}	4×10^{-6}	1.72×10^{2}	1.8×10^{2}

had one significant change in diversification rate, located on the branch supporting the *neopolydactyla/dolichorhiza/hymenina* group, including *P. neopolydactyla* 3 (see the asterisk on Figure 7d) reflecting a rapid radiation in that clade of species found almost exclusively in the Southern Hemisphere. Whether this radiation is linked to the generalist profile of species in this group, or to the colonization of new geographical areas by this group, or to both, is not clear yet (Fig. 5). The expansion of species from this group to South America seems not to be the only factor. The recency of this radiation, could mean that natural selection is still acting as these species species spread to South America and are constantly exposed to new habitats and *Nostoc* phylogroups. The only member in this group that seems to be specialized on a single phylogroup, *P. pacifica*, has a very narrow distribution (Pacific Northwest) perhaps due to the fact that the species rarely produce apothecia and disperse mainly by vegetative propagules (marginal phyllidia, involving a vertical transmission of *Nostoc*). The other two species resulting from an early split within this clade, *P. neopolydactyla* 1 and 2, seem to have already acquired specificity towards a few photobionts, while species in the South American group are true generalists (Fig. 5)

1.4.17 *Peltigera-Nostoc* associations in light of mutualistic theories

Law and Lewis (1983) suggested that the "inhabitant" (corresponding to the cyanobiont and photobiont for most lichens), should be under selective pressure to reproduce asexualy, and have a lower rate of evolution compared to the exhabitant (corresponding to the mycobiont in lichen symbiosis) or closely related free-living taxa. It is generally assumed that only the fungal partner reproduces in lichens (Budel and Scheidegger, 1996). We found common instances of a low level of genetic variation or no variation at all within a single *Nostoc* phylogroup associated with a single *Peltigera* species, or occasionally with closely related species (for instance *P. scabrosa* 4 and *P. scabrosa* 1), which can share the same *Nostoc* haplotypes (Figs. 4, 5). Identical *Nostoc* haplotypes were identified across a large geographic scale: identical haplotype detected in phylogroup VIIa in Québec, Norway and Alaska; in phylogroup XIa in Norway, Québec, British Columbia, Greenland and Russia; in phylogroup X in Yunnan and Siberia; in phylogroup XIXb in Colombia and Brazil; in phylogroup IV in Norway and Japan; and in phylogroup IV in

Norway, Québec and Maine (Fig. 4). This broad distribution of highly similar *Nostoc* strains (based on *rbcLX* sequences) can be a signature of low recombination resulting in low evolutionary rates in *Nostoc* involved in lichen symbioses, as well as low rates of nucleotide substitutions due to purifying selection for an optimal association with a specific lichen-forming species. However, this pattern of highly similar *Nostoc* strains covering large areas could also be explained by efficient long-dispersal mechanisms for certain *Nostoc* phylogroups. Correspondingly, because cyanobionts are predominatly transmitted horizontally in lichens, the presence of the same *Nostoc* haplotype within and among different *Peltigera* species can be explained by a parallel acquisition of the same cyanobiont, rather than coevolution of a fast evolving exhabitant with a slow evolving *Nostoc* partner. Recent studies (see Sachs et al. 2011) on a large variety of microbial symbionts demonstrated that Law and Lewis' paradigm was too simplistic. Indeed, the mutualistic framework set by Law and Lewis (1983) involving a positive frequency-dependent selection, evolutionary stasis and high asexuality of one symbiont is consistent in some symbiotic associations, but highly incoherent in others (Sachs et al., 2011), and therefore, there is probably a continuum of different stages between arm-race in parasites and the Law and Lewis paradigm.

The Red King hypothesis (Bergstrom and Lachmann, 2003) states that in mutualistic interactions, while both partners need to find a viable equilibrium to maintain the symbiosis, the slower partner wins the race because, by reaching the equilibrium more slowly, it can benefit more from the symbiosis. In early diverged species from *Polydactylon* clade (*P.* sp. 11, *P.* sp. 9, *P. scabrosa* 1, *P. scabrosa* 4, *P. neopolydactyla* 5, *P. neopolydactyla* 6) where both partners are highly stable (a single or very few similar ITS haplotypes per *Peltigera* species associated with one or a few *Nostoc* haplotypes), the diversification rates were substantially lower than in more recently evolved and non-specialized species (Table 3, Supplementary Figure S4). Therefore, our results support both, the Law and Lewis' and the Red King hypotheses by demonstrating that both symbionts can be advantaged when involved in a specialized relationship for a long time. This long-term interaction results in a low rate of evolution leading to the reduced genetic diversity and slow diversification of both partners, so that the most frequent or best-adapted haplotype can be positively selected (Law and Lewis, 1983) to maintain the best benefits in the symbiosis.

If we consider that specialization is acquired through time (as suggested by the results of diversification analyses; Table 3), the fact that specialized species are genetically less diverse than generalists might indicate that through time, once in the process of specialisation, the mycobionts will experience slower evolution supporting the Red King Hypothesis (Bergstrom and Lachmann, 2003).

Because we don't see a high level of specialization for the photobiont, it is very likely that in the process of lichenization, the mycobiont is capturing the photobiont (shared by other species), rather than the photobiont infecting the mycobiont. The fact that the lichen-forming fungus (*Peltigera*) is highly dependent on *Nostoc* but not vice-versa, also supports the fungal capture of *Nostoc*. As a consequence, the mycobiont

evolves in adaptation to the photobiont. Slow evolution of the cyanobiont (embedded in the thallus) can be explained by a reduced selective pressure from the environment and a high selection from the mycobiont to maintain the relationship with the optimal cyanobacterial partner. A strong specialization of the mycobiont toward a single cyanobiont may limit its ablility of switching to a different *Nostoc* partner and might explain why strict *Peltigera* specialists cannot expand to new regions (have narrower geographic ranges compared to generalists). The lack of specialization of *Nostoc* toward a certain mycobiont and the fact that a single strain is used alternatively by several mycobionts may indicate that descendents of *Nostoc* lineages spend some time outside of lichen thallus.

We detected two categories of species regarding their symbiotic status: 1) "suboptimal" where partners that associated recently and/or experiencing new environmental conditions, will be evolving faster, driven by positive selection leading to an improved allelic combinations, closer to any optimum, and will be associating with many different cyanobiont or species because no specific pair is drastically better than another
2) "optimal" which are symbioses where the mycobiont is specialized to interact with one *Nostoc* partner (phylogroup) and have reached an optimal equilibrium under specific environmental conditions. This is a case where a *Peltigera* species-*Nostoc* phylogroup pair is drastically more successful in a given area and now dominates the allelic combinations of both partner (i.e. allelic fixation in both partners). At this stage, evolution is slower and genetic diversity is reduced, because this optimal allelic combination will be maintained by purifying selection.

Local specialists might become with time strict specialists. For example, *P. neopolydactyla* 1 (Figure 5) could speciate to form two species, one in a temperate zone in North America (USA) specializing on phylogroup VIIb, and the second one in boreal region, specializing on phylogroup VIIa. Similarly, populations of *P. neopolydactyla* 2 might split in two taxa, one in temperate region of Asia, in association with phylogroup X, and another in boreal region in association with phylogroup XIII (Fig. 5). In the case of *P. neopolydactyla* 2, it seems that specimens from Yunnan associating with *Nostoc* phylogroup X are already genetically differentiated from the boreal populations associating with phylogroup XIII (they don't share ITS haplotypes, and form two monophyletic lineages; Fig. 5) whereas in *P. neopolydactyla* 1, there is no such clear distinction among populations as the same haplotype was found in the association with *Nostoc* phylogroup VIIa and VIIb. *P. scabrosa* 3 might also represent a taxon undergoing speciation judged by the ten nucleotide differences (SNPs) between the haplotype associated with *Nostoc* phylogroup X and the haplotype associated with phylogroup VIIa/VIIc (Fig. 5). The ability of switching cyanobionts can facilitate the expansion of mycobiont to new environments where the former cyanobiont is not available, to avoid competition for cyanobionts from the co-existing mycobionts, or to choose better adapted cyanobiont in changing environment. These observations are in agreement with the geographic mosaic of coevolution theory where a species may adapt and become specialized on another species differentially upon different geographic regions (Thompson, 2005).

Geography has a great importance in the process of diversification in *Peltigera* mostly trough the climatic factors that shape *Nostoc* distributions, and therefore influence their availability and patterns of association with the mycobionts. Limited long distance dispersal may also influence the process.

1.5 Conclusions

Nostoc distributions are highly dependent on climate and geography. Most species of *Peltigera* exhibit a strong specificity toward their *Nostoc* partner (specialists associated with a single or few *Nostoc* phylogroups), whereas the majority of cyanobionts are generalists, infecting multiple *Peltigera* species. There is however a wide spectrum of patterns of associations in *Peltigera* species, from strict specialists to broad generalists. Some species of *Peltigera* are capable to extend their ranges in different biogeographic zones, by switching to a local *Nostoc* partner, or use a cosmopolitan *Nostoc* strain. Patterns of selection of the cyanobionts by the mycobionts, revealed multiple unique pools of *Nostoc* phylogroups shared by groups of *Peltigera* species. Mycobiont specialists seem to have originated earlier during the evolutionary history of section *Polydactylon* whereas the generalists have speciated more recently. Mycobiont generalists seem to be subjected to selective pressures leading to specialization to a more restricted set of *Nostoc* strains through time, associated with a decrease in genetic diversity and a narrower geographic and bioclimatic range. Expansion to new habitats or maintenance of *Peltigera* species in multiple habitats can occur through photobiont switches and associations with local or widespread partners.

1.6 Acknowledgements

We are extremely thankful to many people that assisted us during various *Peltigera* collecting trips: Einar Timdal, Hakon Holien (Norway), Eimy Rivas Plata, Adriano Spielmann, Luciana Canez, Marcelo Marcelli, Edier Soto, David Diaz Escandon, Martin Ramirez Mejia, Edgar Mauricio Medina Tovar, Daniel Ramos, David Sanin, Luis Coca (South America); Rimma Andronova and staff from the Bol'shekhetzkhirskii State Reserve and Durminskoye forest-hunting area (Far East Russia, Khabarovsk Territory); Anastasia Knorre, Altyna Dutbayeva and staff from "Stolby" National Wildlife Nature Reserve (Krasnoyarsk, Russia); Emilie Lefevre, Bernie Ball, Kayla Arendt, Air Saguenay pilot Jacques Berube and the team at Lac Margane (northern Québec, Canada), as well as the Société des établissements de plein air du Québec (SEPAQ) Fjord du Saguenay, especially Yana Desautels and Nathaöel Bergeron and the team at Baie Sainte Marguerite (southern Québec). A special appreciation is directed to Betsy Arnold and Mikhail Zhurbenko for their generous help in various collecting expeditions in North America and Russia. The authors are thankful to all collaborators provided material for this study: Trevor Goward, Robert Lücking, Bruce McCune, Santosh Joshi,

Thør Tønsberg, Paul Diederich, Dan Blanchon, Maria de los Angeles Herrera, Orvo Vitikainen, Soili Stenroos, Tuevo Ahti, Harry Sipman, Jason Hollinger, Richard Harris, James Lendemer, Ester Gaya, Peter Nelson, Tim Wheeler, Pradeep K. Divakar, Starri Heiðmarsson, Daphne Stone, Svetlana Chabanenko and curators of several herbaria (B, BG, CONN, DUKE, H, LG, MEXU, NY, O, UGDA, UMEX, UPS)for providing material for the study. The authors are greatful to Orvo Vitikainen for sharing his expertise on the taxonomy of Peltigera. We thank labmates Ko Hsuan Chen, Michael Gajdeczka, Camille Truong, Ester Gaya and Ryoko Oono for helpful discussion and comments at various steps during the project. We would like to acknowledge Laurent Gohy, Ido Cremasco, Molly McMullen for technical help during the study, This study was supported by the National Science Fundation REVSYS award on the genus Peltigera DEB-1025930 to J.M. and F.L.; NSF Dimensions of Biodiversity award DEB-1046065 to F.L.; and Belgium Science National Fundation (FRIA fellowship) to N.M.

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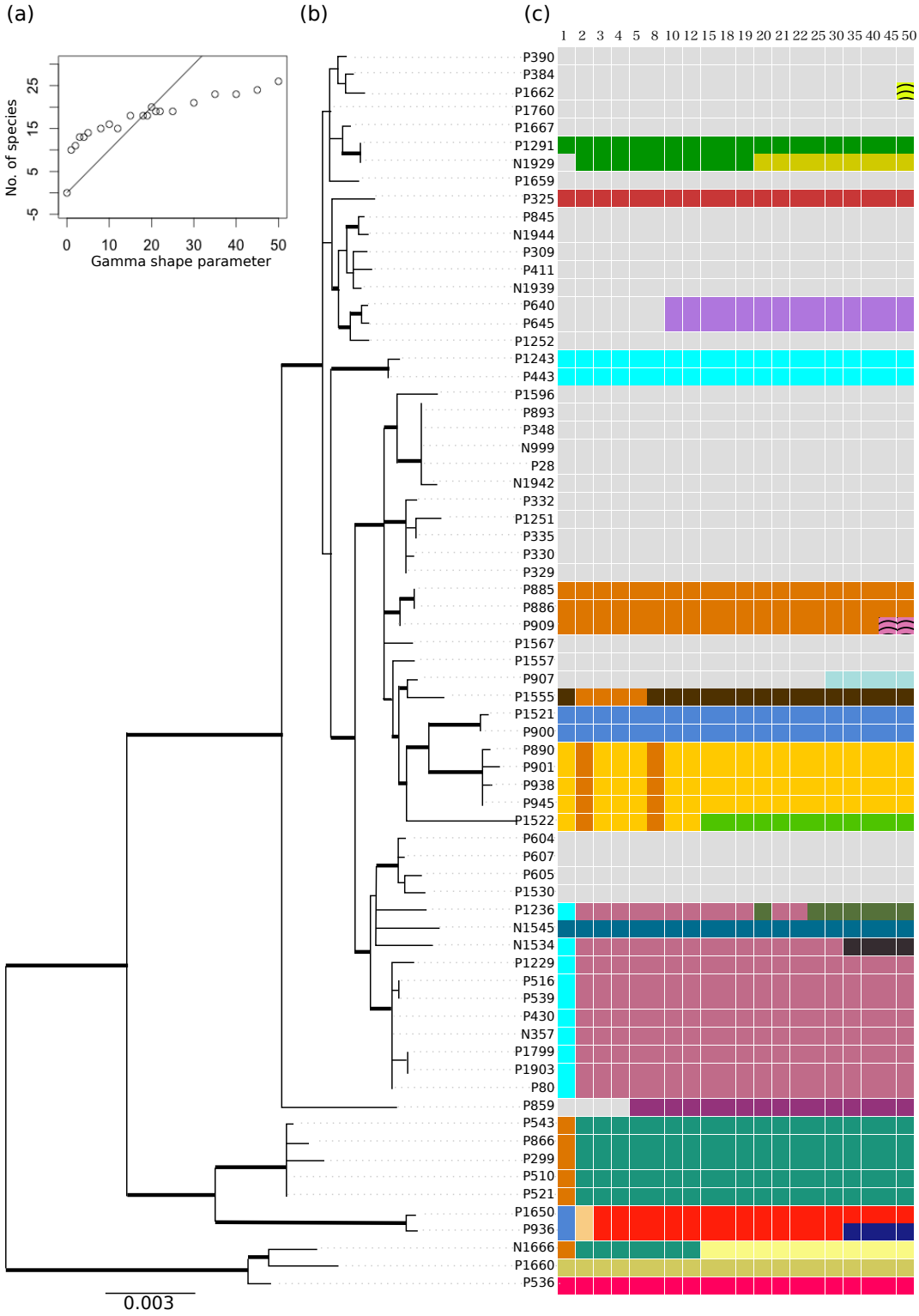
1.8 Supplementary Material

Figure S1: Effect of priors on species delimitation in Dolichorhizoid clade. (a) Number of species delimited as function of the gamma shape (with a constant gamma scale of 1) hyperprior. The straight line of equation x=y represents the evolution of the data if only driven by the priors. (b) Phylogram of the Dolichorhizoid clade based on the ITS phylogeny of individuals represented in Matrix 2 (c) Species assignments (colors) for each fungal individual as a function of the gamma shape hyperprior (with a constant gamma scale of 1). Individuals were assigned to the same species if they share the same color.


(a)
(b)
(c)
No. of species
Gamma shape parameter
0.003
1 2 3 4 5 8 10 12 15 18 19 20 21 22 25 30 35 40 45 50
P390
P384
P1662
P1760
P1667
P1291
N1929
P1659
P325
P845
N1944
P309
P411
N1939
P640
P645
P1252
P1243
P443
P1596
P893
P348
N999
P28
N1942
P332
P1251
P335
P330
P329
P885
P886
P909
P1567
P1557
P907
P1555
P1521
P900
P890
P901
P938
P945
P1522
P604
P607
P605
P1530
P1236
N1545
N1534
P1229
P516
P539
P430
N357
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P521
P1650
P936
N1666
P1660
P536

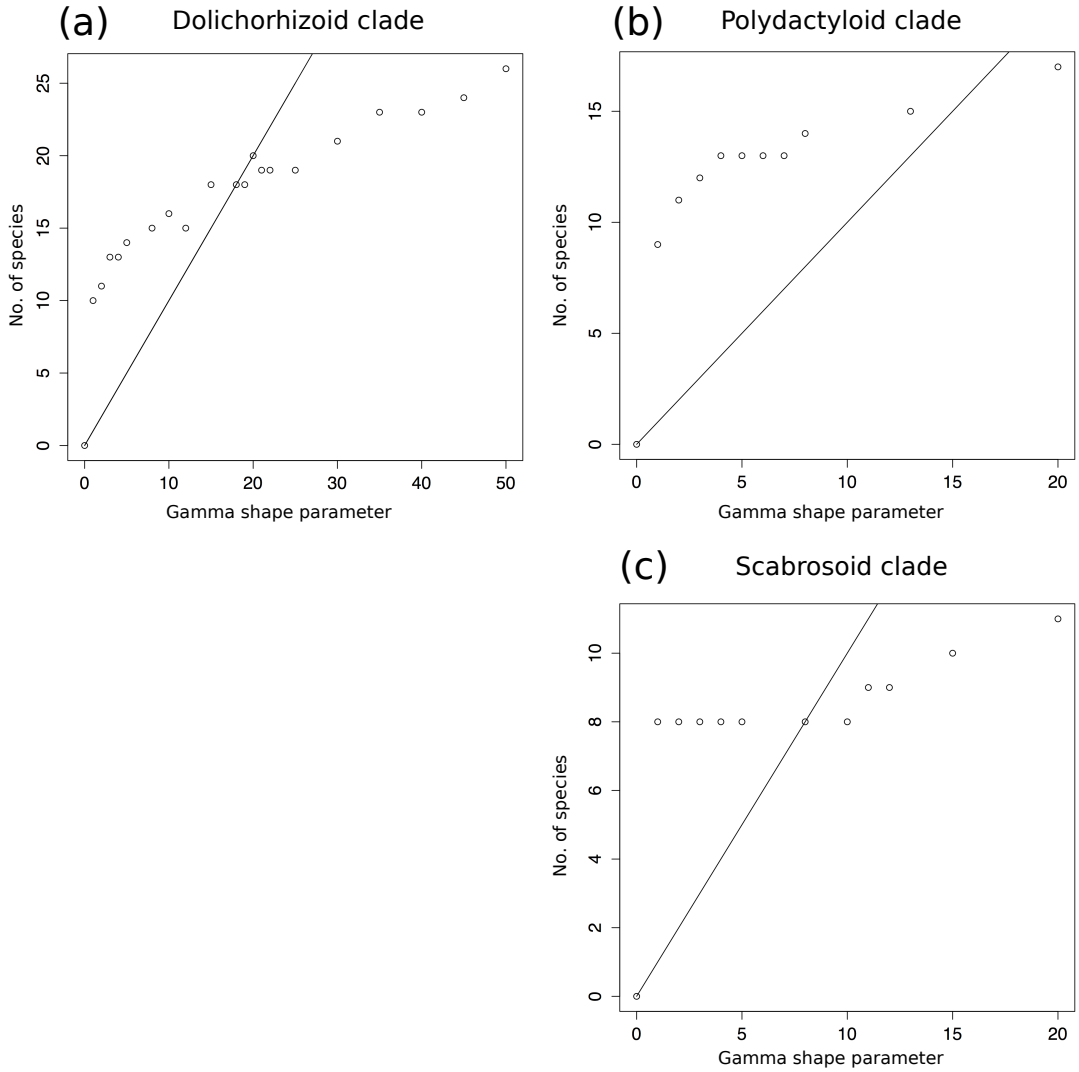

Figure S2: Variations in the number of species delimited in the Dolichorhizoid (a), Polydactyloid (b), and Scabrosoid (c) clades by Structurama when the gamma shape of the hyperprior increases (with a constant gamma scale of 1), after one million generations. The straight line of equation x=y represents the evolution of the data if only driven by the priors.

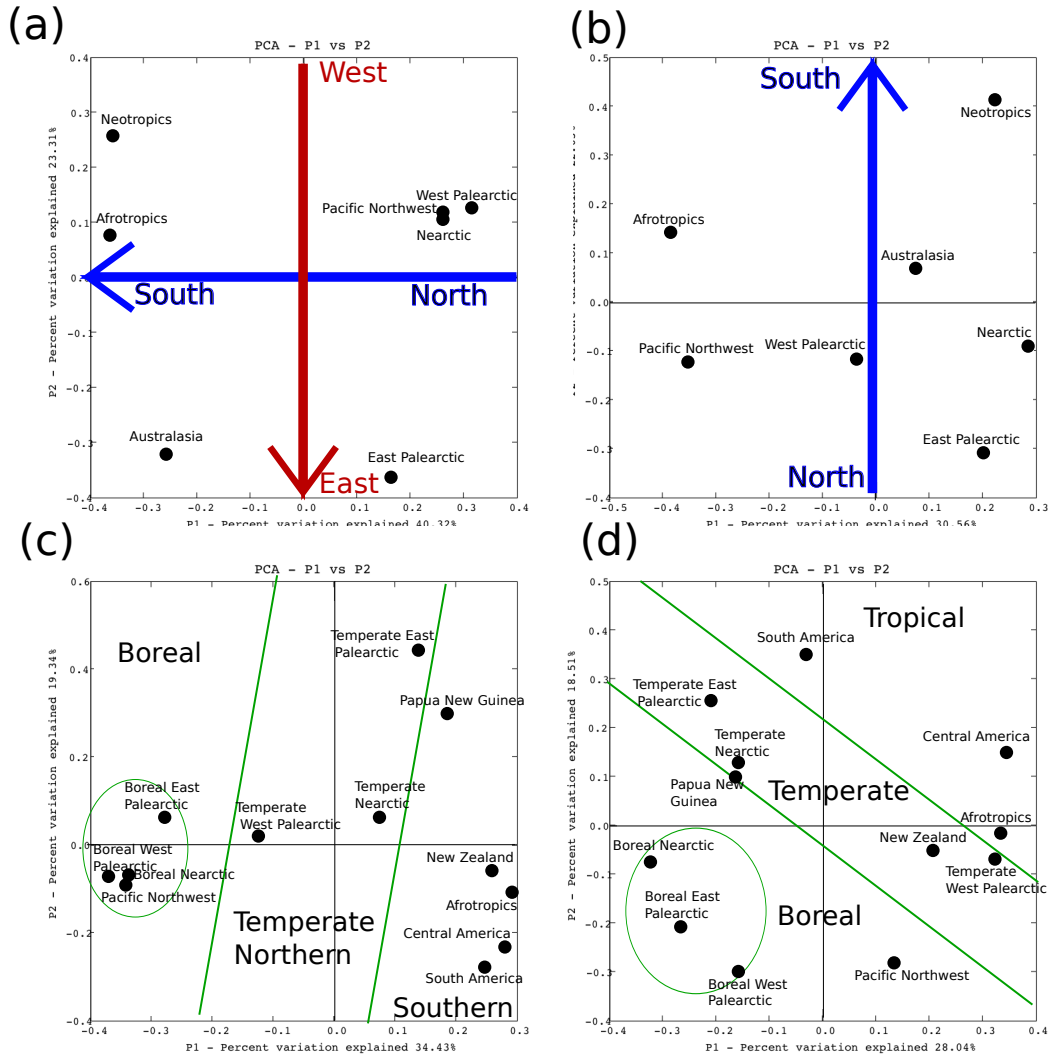

Figure S3: PCA from the UniFrac analyses, showing (a) the similarity of the biogeographic zones in terms of *Peltigera* species, (b) the similarity of the biogeographic zones in terms of *Nostoc* phylogroups, (c) the similarity of the smaller subdivisions of zones in terms of *Peltigera* species, (d) the similarity of the smaller subdivisions of zones in terms of *Nostoc* phylogroups .

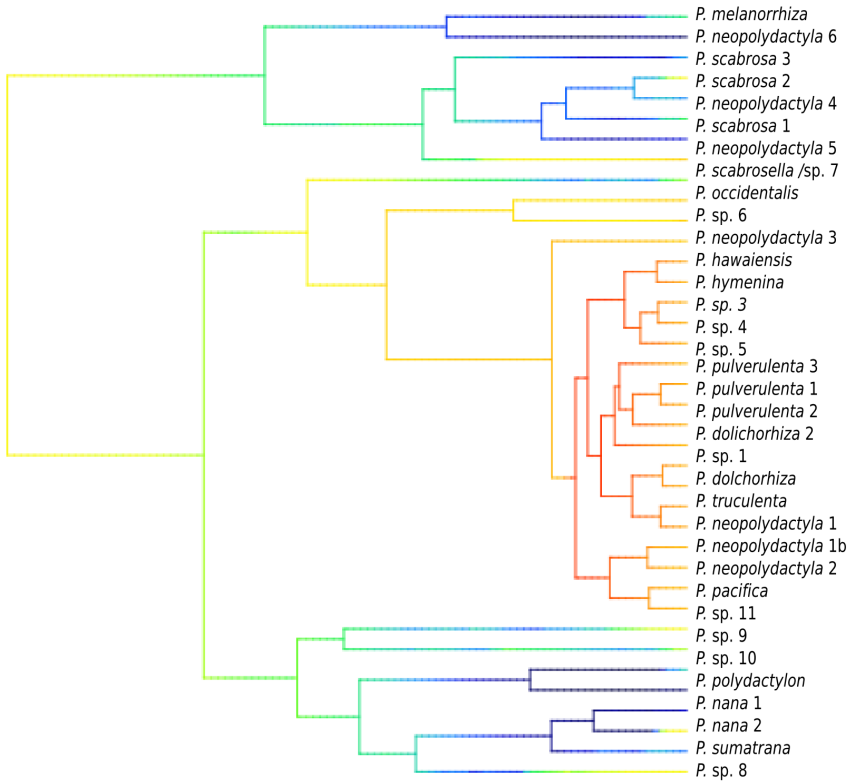

Figure S4: Rates of diversification estimated by the BAMM analysis on the species tree of *Peltigera* section *Polydactylon*. A blue color represents low rates whereas a red color represents high rates.

Table S1: Taxon sampling (A. *Peltigera* section *Polydactylon*; B. *Peltigera*, other sections; C. other genera) with associated voucher information or published source; and corresponding sequences used in this study, and, when applicable, the cyanobiont *rbcLX* haplotype or phylogroup that the specified mycobiont sample associates with. XXXX indicate newly generated sequences, whereas GB identification numbers refer to published sequences. For *rbcLX* sequences of cyanobionts, the genus and species names of the mycobiont, which it associates with, are provided, when available. When free living or of unknown source, the name of the cyanobiont is provided. Asterisks refer to sequences resulting from collapsing of Nostoc sequences associating with several species in O'Brien et al. (2013). In theses cases, we provide the identity of the species of the sequence we used, but not all the other species that associate with this haplotype. For the complete set of species, see Fig. 4 and O'Brien et al. (2013).

Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
A. *Peltigera* section *Polydactylon*									
P. dolichorhiza	P894	Bolivia; M. Kukwa 9232; UGDA-17709	XXXX	–	–	–	–	XXXX	HT33
P. dolichorhiza	P29	Costa Rica; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	HT42
P. dolichorhiza	P893	Bolivia; M. Kukwa 9740; UGDA-17730	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	V
P. dolichorhiza	N999	Reunion Island; N. Magain & E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIX
P. dolichorhiza	P28	Costa Rica; J. Miadlikowska et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXa
P. dolichorhiza	P348	Mexico; M. A. Herrera-Campos 13382; MEXU	XXXX	666666866	666667205	666666969	666667081	XXXX	XIXb
P. dolichorhiza	P879	Rwanda E. Sérusiaux PA7; LG	XXXX	–	–	–	–	XXXX	XIXb
P. dolichorhiza	N1942	Panama; B. Goffinet 753; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXc
P. dolichorhiza	P1574	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34450	XXXX	–	–	–	–	XXXX	XIXd
P. dolichorhiza	P355	Mexico; M. A. Herrera-Campos 135; MEXU	XXXX	–	–	–	–	XXXX	XIXd
P. dolichorhiza	P1596	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34453	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. dolichorhiza 2	P1567	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34530	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXc
P. dolichorhiza 2	P1575	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34456	XXXX	–	–	–	–	XXXX	XIXd
P. "hawaiensis"	P1236	USA, Hawaii; B. McCune 22196; McCune Private Coll.	XXXX	XXXX	–	XXXX	–	XXXX	XIX
P. hymenina	P430	Canada, Newfoundland; J. Lendemer 10397; H	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIb
P. hymenina	N357	Spain, Tenerife; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXb
P. hymenina	N2054	Norway; H. Bratli 4604; O-65885	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P1214	USA, Oregon; D. Kofranek 3729; McCune Private Coll.	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P1226	USA, Oregon; B. McCune 26281; McCune Private Coll.	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P516	Portugal, Azores; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	–	XXXX	XXXX	XVI
P. hymenina	P517	Portugal, Azores; E. Sérusiaux s.n.; LG	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P539	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XVI
P. hymenina	P7	Iceland; J. Miadlikowska et al. s.n. 12540; DUKE	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P80	Iceland; T. Ahti 69347; H	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XVI
P. hymenina	P851	Iceland; H. G. Kristinsson 48978; AMNH-28943	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P870	Denmark; V. Alstrup s.n.; H	XXXX	–	–	–	–	XXXX	XVI

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Table S1 – continued from previous page

Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. hymenina	P871	Portugal, Azores; F. Rodriguez 0106/2010-66; B-600173049	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P872	Portugal, Azores; F. Rodriguez 0106/2010-67; B-600173186	XXXX	–	–	–	–	XXXX	XVI
P. hymenina	P1229	USA, Oregon; B. McCune 30448; McCune Private Coll.	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XVII
P. hymenina	P1247	Canada, British Columbia; C. Björk 17053; UBC	XXXX	–	–	–	–	XXXX	XVII
P. hymenina	P1799	Portugal, Azores; D. Upreti 6915a; MAF	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. hymenina	P1903	Portugal, Azores; D. Upreti 6916s; MAF	XXXX	XXXX	XXXX	XXXX	–	–	N/A
P. macra	P416	Philippines; L. A. Ejem; H	XXXX	–	–	–	–	XXXX	XII
P. melanorrhiza	P515	Portugal, Azores; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	–	XXXX	XXXX	XVI
P. melanorrhiza	P1798	Portugal, Azores; D. Upreti 6918b; MAF	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. nana 1	P1281	China, Yunnan; J. Miadlikowska s.n.; DUKE	XXXX	XXXX	XXXX	–	–	XXXX	HT45
P. nana 1	P1666	Japan, Kochi; G. Thor 21255; UPS-383534	XXXX	XXXX	XXXX	–	–	XXXX	VIIc
P. nana 1	P1282	China, Yunnan; J. Miadlikowska s.n.; DUKE	XXXX	–	–	–	–	XXXX	XII
P. nana 1	P1665	China, Sichuan; N. Gustavsson 10; UPS-509502	XXXX	XXXX	XXXX	–	–	XXXX	XII
P. nana 1	P1290	China, Yunnan; J. Miadlikowska s.n.; DUKE	XXXX	XXXX	XXXX	–	–	–	N/A
P. nana 2	P609	New Zealand; S. Stenroos 6037; H	XXXX	XXXX	XXXX	–	XXXX	XXXX	HT37
P. neopolydactyla	GB	Canada, British Columbia; O'Brien et al. 2013						455476535	HT24

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. neopolydactyla	GB	USA; O'Brien et al. 2005						82470991	VIIa
P. neopolydactyla	GB	Finland; Stenroos et al. 2006						82779968	VIIa
P. neopolydactyla	GB	Canada, Québec; O'Brien et al. 2013						455476637	VIIa
P. neopolydactyla	GB	Canada, Québec; O'Brien et al. 2013						455476631	XIa
P. neopolydactyla	GB	Finland; Myllys et al. 2007						119690877	XIII
P. neopolydactyla	GB	China; O'Brien et al. 2013						455476643	N/A
P. neopolydactyla	GB	China; O'Brien et al. 2013						455476649	X
P. neopolydactyla	GB	Finland; Myllys et al. 2007						119690880	N/A
P. neopolydactyla 1	P67	USA, North Carolina; J. Hollinger 2732; DUKE	XXXX	–	–	–	–	XXXX	VIIb
P. neopolydactyla 1	N1944	USA, Alaska; B. Goffinet 9424; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. neopolydactyla 1	P309	Canada, Québec; J. Miadlikowska et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. neopolydactyla 1	P411	Russia, Yakutia; T. Ahti 65064; H	XXXX	XXXX	XXXX	XXXX	–	XXXX	VIIa
P. neopolydactyla 1	P844	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	VIIa
P. neopolydactyla 1	P845	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. neopolydactyla 1	N1939	USA, Alabama; B. Goffinet 5209; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIb
P. neopolydactyla 1	P640	USA, North Carolina; J. Miadlikowska et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIb
P. neopolydactyla 1	P645	USA, North Carolina; J. Miadlikowska et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIb

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. neopolydactyla 1	P1252	USA, Arizona; J. Hollinger 1781; UBC	XXXX	XXXX	XXXX	XXXX	–	XXXX	VIIc
P. neopolydactyla 1b	P325	Peru; E. Gaya 07.14.10-19; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIb
P. neopolydactyla 2	N1913	China, Yunnan; B. Goffinet 10122; CONN	XXXX	–	–	–	–	XXXX	X
P. neopolydactyla 2	N1914	China, Yunnan; B. Goffinet 10125; CONN	XXXX	–	–	–	–	XXXX	X
P. neopolydactyla 2	N1916	China, Yunnan; B. Goffinet 10126; CONN	XXXX	–	–	–	–	XXXX	X
P. neopolydactyla 2	N1926	China, Yunnan; B. Goffinet 10128; CONN	XXXX	–	–	–	–	XXXX	X
P. neopolydactyla 2	N1927	China, Yunnan; B. Goffinet 10127; CONN	XXXX	–	–	–	–	XXXX	X
P. neopolydactyla 2	N1929	China, Yunnan; B. Goffinet 10120; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	X
P. neopolydactyla 2	P1183	Japan, Hokkaido; G. Thor 25759; UPS-552780	XXXX	–	–	–	–	XXXX	XIII
P. neopolydactyla 2	P1322	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIII
P. neopolydactyla 2	P384	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIII
P. neopolydactyla 2	P386	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIII
P. neopolydactyla 2	P821	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIII
P. neopolydactyla 2	P846	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIII
P. neopolydactyla 2	P1291	China, Yunnan; J. Miadlikowska et al. s.n.; DUKE	XXXX	XXXX	–	XXXX	–	–	N/A
P. neopolydactyla 2	P1659	Japan, Hokkaido; G. Thor 25720; UPS-519479	XXXX	XXXX	XXXX	XXXX	–	–	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. neopolydactyla 2	P1662	Japan, Hokkaido; A. Frisch 10/Jp410; UPS-522008	XXXX	XXXX	XXXX	XXXX	–	–	N/A
P. neopolydactyla 2	P1667	Japan, Kochi; G. Thor 2127x; UPS-164500	XXXX	XXXX	XXXX	XXXX	–	–	N/A
P. neopolydactyla 2	P1760	Japan, Hokkaido; G. Thor 14656; UPS-166427	XXXX	XXXX	–	XXXX	–	–	N/A
P. neopolydactyla 2	P390	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	N/A
P. neopolydactyla 3	P859	Vietnam; Vo T. P. G. G06-NL86B; B-600144665	XXXX	XXXX	XXXX	XXXX	–	XXXX	VIIb
P. neopolydactyla 3	P1283	China, Yunnan; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	X
P. neopolydactyla 4	P1628	USA, Maine; W. R. Buck 54962; NY-01105128	XXXX	–	–	–	–	XXXX	IV
P. neopolydactyla 4	P1668	Japan, Hokkaido; G. Thor 24306; UPS-519471	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	IV
P. neopolydactyla 4	P1675	Japan, Hokkaido; G. Thor 24336; UPS-519493	XXXX	–	–	–	–	XXXX	IV
P. neopolydactyla 4	P302	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	IV
P. neopolydactyla 4	P321	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	IV
P. neopolydactyla 4	P591	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	IV
P. neopolydactyla 4	P811	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	IV
P. neopolydactyla 4	P1212	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	–	XXXX	XXXX	XIa
P. neopolydactyla 4	P506	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIa
P. neopolydactyla 4	P669	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. neopolydactyla 5	P1228	USA, Oregon; B. McCune 30018; McCune Private Coll.	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIb
P. neopolydactyla 5	P1232	USA, Oregon; B. McCune 26873; McCune Private Coll.	XXXX	XXXX	–	XXXX	–	XXXX	XIb
P. neopolydactyla 5	P1257	USA, Oregon; J. Hollinger 1385; UBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIb
P. neopolydactyla 5	P1677	USA, Oregon; B. McCune 26977; McCune Private Coll.	XXXX	–	–	–	–	XXXX	XIb
P. neopolydactyla 6	P1223	USA, Oregon; McCune Private Coll.	XXXX	–	–	–	–	XXXX	XIII
P. neopolydactyla 6	P1231	USA, Oregon; B. McCune 24160; McCune Private Coll.	XXXX	XXXX	XXXX	XXXX	–	XXXX	XIII
P. neopolydactyla 6	N1886	USA, Oregon; B. Goffinet 8056; CONN	XXXX	–	–	–	–	XXXX	XVII
P. neopolydactyla 6	N1888	USA, Oregon; B. Goffinet 8049; CONN	XXXX	–	–	–	–	XXXX	XVII
P. neopolydactyla 6	P1258	Canada, British Columbia; C. Björk 17289; UBC	XXXX	–	–	–	–	XXXX	XVII
P. occidentalis	P108	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. occidentalis	P299	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. occidentalis	P314	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. occidentalis	P410	Russia, Siberia; T. N. Otnyukova s.n.; H	XXXX	–	–	–	–	XXXX	VIIa
P. occidentalis	P543	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. occidentalis	P571	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	VIIa

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. occidentalis	P853	USA, Alaska; T. Ahti 63310; H	XXXX	–	–	–	–	XXXX	VIIa
P. occidentalis	P1638	USA, North Carolina; Richard C. Harris 52559; NY	XXXX	–	–	–	–	XXXX	VIIb
P. occidentalis	P510	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	–	XXXX	–	N/A
P. occidentalis	P521	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	–	XXXX	–	N/A
P. occidentalis	P866	USA, Alaska; T. Ahti 63231; H	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. oceanica	P882	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	XXXX	–	–	–	–	N/A
P. pacifica	P1240	Canada, British Columbia; T. Goward 06-23; UBC	XXXX	–	–	–	–	XXXX	XIII
P. pacifica	P1241	Canada, British Columbia; C. Björk 17500; UBC	XXXX	–	–	–	–	XXXX	XIII
P. pacifica	P1242	Canada, British Columbia; T. Goward 06-1133; UBC	XXXX	–	–	–	–	XXXX	XIII
P. pacifica	P1243	Canada, British Columbia; T. Goward 06-1522; UBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIII
P. pacifica	P443	Canada, British Columbia; O. Vitikainen 13080; H	XXXX	666666883	666667207	666666975	666667085	–	N/A
P. polydactylon	N2069	Norway; J. Holtan-Hartwig 528; O	XXXX	XXXX	XXXX	XXXX	–	XXXX	V
P. polydactylon	P1213	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	V
P. polydactylon	P1235	USA, Alaska; B. McCune 26434; McCune Private Coll.	XXXX	–	–	–	–	XXXX	V
P. polydactylon	P385	Norway; N. Magain s.n.; LG	XXXX	666666886	666667319	666666971	666667083	XXXX	V
P. polydactylon	P388	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	V

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. polydactylon	P682	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	V
P. polydactylon	P71	USA, New Mexico; J. Hollinger 2462; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	V
P. polydactylon	P816	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	V
P. polydactylon	P833	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	V
P. polydactylon	P856	Iran; A. A. Maassoumi 573; B-600114374	XXXX	XXXX	–	XXXX	–	XXXX	V
P. polydactylon	P1234	USA, Montana; B. McCune 29108; McCune Private Coll.	XXXX	XXXX	XXXX	XXXX	–	–	N/A
P. pulverulenta 1	P890	Bolivia; M. Kukwa 8536; UGDA-17702	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	HT41
P. pulverulenta 1	P897	Mexico; M. A. Herrera-Campos 122; MEXU	XXXX	–	–	–	–	XXXX	XIX
P. pulverulenta 1	P1566	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34566	XXXX	–	–	–	–	XXXX	XIXb
P. pulverulenta 1	P1572	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34445	XXXX	–	–	–	–	XXXX	XIXb
P. pulverulenta 1	P928	Colombia; R. Lücking MPNNC214; UDBC	XXXX	–	–	–	–	XXXX	XV
P. pulverulenta 1	P901	Colombia; R. Lücking 33383; UDBC	XXXX	XXXX	XXXX	XXXX	–	–	N/A
P. pulverulenta 1	P938	Colombia; R. Lücking 33321; UDBC	XXXX	XXXX	XXXX	XXXX	–	–	N/A
P. pulverulenta 1	P945	Colombia; R. Lücking 33691; UDBC	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. pulverulenta 2	P1521	Colombia; R. Lücking 34028; UDBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXa
P. pulverulenta 2	P900	Colombia; R. Lücking 33367; UDBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXa

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. pulverulenta 3	P1522	Colombia; R. Lücking 34033; UDBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	HT34
P. scabrosa 1	P1210	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	–	XXXX	VIIa
P. scabrosa 1	P1250	Canada, Alberta; J. Hollinger 1066; UBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. scabrosa 1	P1539	Russia, Siberia; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. scabrosa 1	P306	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. scabrosa 1	P311	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. scabrosa 1	P93	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. scabrosa 1	P97	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. scabrosa 1	P550	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. scabrosa 2	P1328	Canada, Nunavik; J. Gagnon; QFA-0594938	XXXX	–	–	–	–	XXXX	VIId
P. scabrosa 2	P1721	Greenland; E. Sérusiaux s.n.; LG	XXXX	–	–	–	–	XXXX	VIId
P. scabrosa 2	P107	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	666666890	666667197	666666913	666667075	XXXX	XIa
P. scabrosa 2	P113	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIa
P. scabrosa 2	P1209	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIa
P. scabrosa 2	P1255	Canada, BC; C. Björk 16230; UBC	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIa
P. scabrosa 2	P1540	Russia, Siberia; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	XIa
P. scabrosa 2	P296	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	XIa

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. scabrosa 2	P520	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa
P. scabrosa 2	P617	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa
P. scabrosa 2	P830	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa
P. scabrosa 3	P865	Russia, Yakutia; T. Ahti 65068; H	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. scabrosa 3	N1236	Greenland; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIc
P. scabrosa 3	P1536	Russia, Siberia; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	X
P. scabrosa 3	P1538	Russia, Siberia; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	X
P. scabrosa 4	P312	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. scabrosa 4	P315	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	–	XXXX	VIIa
P. scabrosa 4	P318	Canada, Québec; F. Lutzoni et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIa
P. scabrosa 4	P529	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	VIIa
P. scabrosa 4	P549	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIa
P. scabrosella	P391	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa
P. scabrosella	P514	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa
P. scabrosella	P619	Norway; N. Magain s.n.; LG	XXXX	–	–	–	–	XXXX	XIa
P. scabrosella	P854	Greenland; E. S. Hansen; H-548	XXXX	–	–	–	–	XXXX	XIa
P. scabrosella	P536	Norway; N. Magain s.n.; LG	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. sp.	N1750	Unknown; J. Santosh s.n.; J. Santosh Private Coll.	–	–	–	–	–	XXXX	HT39
P. sp. 1	P905	Colombia; R. Lücking 33334; UDBC	XXXX	–	–	–	–	XXXX	HT40
P. sp. 1	P889	Bolivia; M. Kukwa 9440; UGDA-17715	XXXX	–	–	–	–	XXXX	XIX
P. sp. 1	P885	Bolivia; M. Kukwa 9276; UGDA-17710	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXc
P. sp. 1	P886	Bolivia; M. Kukwa 9327; UGDA-17713	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXc
P. sp. 1	P909	Colombia; R. Lücking 33339; UDBC	XXXX	XXXX	–	XXXX	–	–	N/A
P. sp. 2a	P1555	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34544	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXb
P. sp. 2a	P1570	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34526	XXXX	–	–	–	–	XXXX	XIXb
P. sp. 2a	P907	Colombia; R. Lücking 33361; UDBC	XXXX	XXXX	XXXX	XXXX	–	XXXX	XV
P. sp. 2b	P1561	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34399	XXXX	–	–	–	–	XXXX	V
P. sp. 2b	P1557	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34562	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XIXd
P. sp. 2b	P1583	Brazil, Minas Gerais; J. Miadlikowska et al. s.n.; CGMS-34428	XXXX	–	–	–	–	XXXX	XIXd
P. sp. 3	P611	New Zealand; F. Högnabba 1746; H	XXXX	–	–	–	–	XXXX	V
P. sp. 3	P612	New Zealand; F. Högnabba 1471; H	XXXX	–	–	–	–	XXXX	X
P. sp. 3	P604	New Zealand; F. Högnabba 1538; H	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XX

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. sp. 3	P606	New Zealand; S. Stenroos 5942; H	XXXX	–	–	–	–	XXXX	XX
P. sp. 3	P607	New Zealand; S. Stenroos 5820; H	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XX
P. sp. 3	P608	New Zealand; S. Stenroos 5830; H	XXXX	–	–	–	–	XXXX	XX
P. sp. 3	P610	New Zealand; F. Högnabba 1575; H	XXXX	–	–	–	–	XXXX	XX
P. sp. 3	P1530	New Zealand; D. Blanchon s.n.; DUKE	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. sp. 3	P605	New Zealand; S. Stenroos 5815; H	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. sp. 4	N1534	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	XXXX	–	XXXX	VIIb
P. sp. 5	N1545	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	–	XXXX	XXXX	HT32
P. sp. 5	P1716	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	–	–	–	–	XXXX	X
P. sp. 6	P1734	Peru; N. Magain s.n.; DUKE	XXXX	–	–	–	–	XXXX	HT35
P. sp. 6	P1650	Honduras; B. Allen 17790; NY	XXXX	XXXX	XXXX	XXXX	–	XXXX	HT44
P. sp. 6	P1733	Peru; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	HT46
P. sp. 6	P936	Colombia; R. Lücking 33659; UDBC	XXXX	XXXX	XXXX	XXXX	–	XXXX	XIXb
P. sp. 6	P942	Colombia; R. Lücking 33658; UDBC	XXXX	–	–	–	–	XXXX	XIXb
P. sp. 7a	N1674	Canada, British Columbia; T. Tönsberg 20742; BG-34877	XXXX	–	–	–	–	XXXX	XIa
P. sp. 7a	N1887	USA, Oregon; B. Goffinet 7966; CONN	XXXX	–	–	–	–	XXXX	XIa

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. sp. 7a	N1932	Canada, British Columbia; B. Goffinet 3167; CONN	XXXX	–	–	–	–	XXXX	XIa
P. sp. 7a	N1666	Canada, BC; T. Tönsberg 20741; BG-34876	XXXX	XXXX	XXXX	XXXX	XXXX	–	N/A
P. sp. 7b	P1660	Japan, Hokkaido; G. Thor 25408; UPS-519475	XXXX	XXXX	XXXX	XXXX	–	XXXX	XIa
P. sp. 7b	P1672	Japan, Hokkaido; G. Thor; UPS	XXXX	–	–	–	–	XXXX	XIa
P. sp. 7b	P1674	Japan, Hokkaido; G. Thor 25401; UPS-519496	XXXX	–	–	–	–	XXXX	XIa
P. sp. 8	N1947	China, Yunnan; B. Goffinet 9978; CONN	XXXX	–	–	–	–	XXXX	HT31
P. sp. 8	P1272	China, Jilin; M. Sohrabi 16572; M. Sohrabi Private Coll.	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	HT43
P. sp. 8	N1920	China, Yunnan; B. Goffinet 9983; CONN	XXXX	–	–	–	–	XXXX	HT47
P. sp. 8	N1754	South Korea; J. Santosh 090187; J. Santosh Private Coll.	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 8	N1897	China, Yunnan; B. Goffinet 10060; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 8	N1898	China, Yunnan; B. Goffinet 10063; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIa
P. sp. 8	N1900	China, Yunnan; B. Goffinet 10102; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 8	N1922	China, Yunnan; B. Goffinet 10072; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIa
P. sp. 8	N1924	China, Yunnan; B. Goffinet 10095; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIa
P. sp. 8	N1928	China, Yunnan; B. Goffinet 10107; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIa
P. sp. 8	P1237	Taiwan; A. Mikulin T71; A. Mikulin Private Coll.	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIa

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. sp. 8	P1299	China, Yunnan; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 8	N1748	South Korea; J. Santosh 080042; J. Santosh Private Coll.	XXXX	–	–	–	–	XXXX	XIV
P. sp. 8	N1752	South Korea; J. Santosh 090132; J. Santosh Private Coll.	XXXX	–	–	–	–	XXXX	XIV
P. sp. 8	N1766	South Korea; J. Santosh 040808; J. Santosh Private Coll.	XXXX	–	–	–	–	XXXX	XIV
P. sp. 9	N1907	China, Yunnan; B. Goffinet 10100; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 9	N1908	China, Yunnan; B. Goffinet 10040; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 9	N1918	China, Yunnan; B. Goffinet 10101; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 9	N1921	China, Yunnan; B. Goffinet 10064; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 9	N1923	China, Yunnan; B. Goffinet 10073; CONN	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIa
P. sp. 9	N1936	China, Yunnan; B. Goffinet 10108; CONN	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 9	P1508	China, Yunnan; J. Miadlikowska et al. s.n.; DUKE	XXXX	–	–	–	–	XXXX	VIIIa
P. sp. 9	N1899	China, Yunnan; B. Goffinet 10014; CONN	XXXX	XXXX	XXXX	–	XXXX	–	N/A
P. sp. 10	P1652	Canada, Nova Scotia; NY	XXXX	XXXX	XXXX	–	–	XXXX	V
P. sp. 10	P450	USA, Pennsylvania; J. Lendemer 16792; NY	XXXX	XXXX	XXXX	–	XXXX	XXXX	V
P. sp. 11	N1532	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	XXXX	XXXX	XXXX	–	XXXX	IX
P. sp. 11	N1533	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	XXXX	–	XXXX	–	XXXX	IX

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. sp. 11	N1537	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	–	–	–	–	XXXX	IX
P. sp. 11	N1547	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	–	–	–	–	XXXX	IX
P. sumatrana	P884	Papua New Guinea; E. Sérusiaux s.n.; LG	XXXX	–	–	XXXX	–	–	N/A
P. truculenta	P332	Chile ; T. Wheeler 89; T. Wheeler Private Coll.	XXXX	XXXX	XXXX	XXXX	–	XXXX	HT36
P. truculenta	P329	Chile; P. Nelson 4362; T. Wheeler Private Coll.	XXXX	XXXX	XXXX	–	XXXX	XXXX	HT38
P. truculenta	P1251	Chile; J. Hollinger 1925; UBC	XXXX	XXXX	–	XXXX	XXXX	XXXX	VIIIb
P. truculenta	P335	Chile; T. Wheeler 1093; T. Wheeler Private Coll.	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	VIIIb
P. truculenta	P330	Chile; T. Wheeler 3826; T. Wheeler Private Coll.	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XV
B. *Peltigera* other sections									
P. aphthosa	GB	Canada, British Columbia; O'Brien et al. 2013						455475902	HT08
P. aphthosa	GB	Canada, British Columbia; O'Brien et al. 2013						455475866	HT18, IV
P. aphthosa	GB	Canada, British Columbia; O'Brien et al. 2013						455476547	HT23, IV
P. aphthosa	GB	Switzerland; O'Brien et al. 2005						82471081	V
P. aphthosa	P1330	Canada, Nunavik; L. Couillard 191; QFA-0594935	–	–	–	–	–	XXXX	XVIII
P. aphthosa	P1321	Norway; N. Magain s.n.; LG	–	–	666667227	666667049	–	–	N/A
P. aphthosa	P788	Norway; N. Magain s.n.; LG	–	14422959	666667213	666666983	666667091	–	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. aphthosa	GB	Switzerland; O'Brien et al. 2005						82471084	N/A
*P. aphthosa**	GB	Canada, British Columbia; O'Brien et al. 2013						455475860	HT19, IV
P. britannica	P1324	Canada, British Columbia; J. Lendemer 22342; NY	–	–	–	–	–	XXXX	HT48, IV
P. britannica	P227	Canada, British Columbia; T. Goward 09-436; UBC	–	–	666667201	666666965	–	XXXX	HT48, IV
P. britannica	P228	Canada, British Columbia; T. Goward 09-120; UBC	–	666666860	666667203	666666967	666667079	XXXX	HT48, IV
P. britannica	P16	Iceland; H. G. Kristinsson LA30299; AMNH-30229	–	–	–	–	–	XXXX	XVI
P. britannica	P1342	Canada, Nunavik; J. Gagnon GPS 099; QFA-595029	–	–	–	–	–	XXXX	XVIII
*P. britannica**	GB	Canada, British Columbia; O'Brien et al. 2013						455475935	HT10
P. canina	P4	Iceland; J. Miadlikowska & F. Lutzoni 08.08.10-3; DUKE	–	666666861	666667179	666666973	666667053	XXXX	HT49
P. canina	P14	Iceland; J. Miadlikowska & F. Lutzoni 08.09.10-3; DUKE	–	666666862	666667181	666666937	666667055	XXXX	N/A
P. canina	GB	Canada, British Columbia; O'Brien et al. 2009				255347899			N/A
P. canina	GB	Germany; O'Brien et al. 2005						82470985	N/A
P. canina	GB	Poland; O'Brien et al. 2005						82471036	N/A
P. canina	GB	Unknown; O'Brien et al. 2005						82470931	N/A
P. canina	GB	USA; O'Brien et al. 2005						82470994	N/A
P. canina	GB	USA; O'Brien et al. 2005						82471009	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
*P. canina**	GB	Canada, British Columbia; O'Brien et al. 2013						455476004	HT06
P. cinnamomea	P1808	Canada; T. Goward SN 27/09/3003; UBC	–	XXXX	XXXX	XXXX	XXXX	–	N/A
P. collina	GB	Canada, British Columbia; O'Brien et al. 2013						455475737	HT13
P. collina	P1263	Chile; J. Hollinger 1929; UBC	–	666666863	666667221	666666919	666667099	–	N/A
P. continentalis	P1809	China; T. Koponen 45091; H	–	–	XXXX	XXXX	XXXX	–	N/A
P. continentalis	P1810	Russia; J. Miadlikowska et al. s.n.; DUKE	–	666666864	666667245	666666945	666667113	–	N/A
P. degenii	P1849	China; M. Sohrabi 16583; H	–	XXXX	XXXX	–	–	–	N/A
P. degenii	P523	Norway; N. Magain s.n.; LG	–	XXXX	XXXX	–	XXXX	–	N/A
P. degenii	GB	Canada; O'Brien et al. 2005						82471057	N/A
P. degenii	GB	Finland; Stenroos et al. 2006						82779967	N/A
P. didactyla	P1812	Belgium; N. Magain s.n.; LG	–	–	XXXX	–	XXXX	–	N/A
P. didactyla	P1813	Norway; N. Magain s.n.; LG	–	XXXX	XXXX	–	XXXX	–	N/A
P. didactyla	GB	Unknown; O'Brien et al. 2005						82470940	N/A
P. didactyla	GB	Iceland, O'Brien et al. 2005						82470937	N/A
P. didactyla	GB	Poland; O'Brien et al. 2005						82471060	N/A
P. didactyla	GB	Germany; O'Brien et al. 2005						82470979	N/A
P. elisabethae	P66	USA, New Mexico; J. Hollinger 2397; DUKE	–	666666867	666667189	–	666667065	XXXX	V

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. elisabethae	P1544	Russia, Siberia; J. Miadlikowska et al. s.n.; DUKE	–	666666868	666667237	666666935	666667105	–	N/A
P. evansiana	P1817	USA, Pennsylvania; J. Lendemer 17422; NY-1105603	–	666666869	666667247	666666947	666667115	–	N/A
P. evansiana	P1818	USA, Pennsylvania; J. Lendemer 17422; NY-1103610	–	666666858	666667249	666666949	666667117	–	N/A
P. extenuata	P1851	Norway; N. Magain s.n.; LG	–	XXXX	XXXX	–	–	–	N/A
P. extenuata	P81	Russia, Kamchaktka; D. E. Himelbrandt; H	–	–	XXXX	XXXX	–	–	HT54
P. frigida	P82	Chile; C. Rubis 4064; H	–	–	XXXX	XXXX	–	–	N/A
P. fripii	P1370	Russia, Siberia; M. P. Andreev 91762; H	–	14422955	666667235	666666931	666667103	–	N/A
*P. fuscopraetextata**	GB	Canada, British Columbia; O'Brien et al. 2013						455476040	HT03
P. granulosa	P1825	Papua New Guinea; E. Sérusiaux s.n.; LG	–	–	–	XXXX	XXXX	–	N/A
P. horizontalis	GB	Canada, British Columbia; O'Brien et al. 2013						455476142	HT11, V
P. horizontalis	P65	USA, North Carolina; J. Hollinger 2691; DUKE	–	–	–	–	–	XXXX	V
P. horizontalis	GB	USA; O'Brien et al. 2005						82471003	V
P. horizontalis	P1274	China, Jilin; M. Sohrabi 16639; M. Sohrabi Private Coll.	–	666666870	666667223	666666921	666667101	–	N/A
P. horizontalis	P1641	USA, North Carolina; J. Lendemer 8136; NY	–	666666871	666667243	666666943	666667111	–	N/A
P. hydrothyria	P1497	Canada, Nova Scotia; F. Anderson 151675; NSPM	–	–	666667297	666666933	666667165	–	N/A
P. hydrothyria	P1498	Canada, Nova Scotia; F. Anderson 151055; NSPM	–	666666874	666667299	666667047	666667167	–	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. hydrothyria	P1845	Canada, Nova Scotia; F. Anderson 159031; NSPM	–	666666873	666667301	666666953	666667169	–	N/A
P. koponenii	P1827	Papua New Guinea; E. Sérusiaux s.n.; LG	–	XXXX	–	XXXX	XXXX	–	N/A
P. kristinssonii	P1292	Canada, Québec; J. Gagnon 594989; QFA-0594989	–	666666875	666667225	666666923	–	–	N/A
P. laciniata	P26	Costa Rica; J. Miadlikowska et al. s.n.; DUKE	–	666666877	666667183	666666961	666667057	–	N/A
P. laciniata	P924	Colombia; R. Lücking 33692; UDBC	–	666666876	–	666666985	666667059	–	N/A
P. latiloba	P1175	Russia, Sakha Republic; T. Ahti 64755; H	–	–	666667217	666666915	666667095	–	N/A
P. latiloba	P210	Canada, British Columbia; P. Nelson 11-034; UBC	–	–	666667199	666666963	666667077	–	N/A
P. lepidophora	P1844	Canada, British Columbia; T. Goward s.n.; UBC	–	666666859	666667251	666666951	666667119	–	N/A
P. lepidophora	GB	Canada; O'Brien et al. 2005						82471063	N/A
P. leucophlebia	GB	Canada, British Columbia; O'Brien et al. 2013						455476310	HT20
P. leucophlebia	GB	Canada, British Columbia; O'Brien et al. 2013						455476385	HT30
P. leucophlebia	P17	Iceland; S. Heidmarsson 1983; AMNH-29884	–	–	–	–	–	XXXX	HT55
P. leucophlebia	GB	Finland; Myllys et al. 2007						119690850	V
P. leucophlebia	GB	Finland; Myllys et al. 2007						119690853	V
P. leucophlebia	GB	Finland; Myllys et al. 2007						119690862	V
P. leucophlebia	GB	Finland; Myllys et al. 2007						119690865	V

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. leucophlebia	GB	Finland; Myllys et al. 2007						119690859	V
P. leucophlebia	P1343	Canada, Nunavik; J. Gagnon GPS 100; QFA-594931	–	–	–	–	–	XXXX	XVIII
P. leucophlebia	P1329	Canada, Nunavik; J. Gagnon; QFA-594936	–	–	666667229	666666925	–	–	N/A
P. leucophlebia	P1333	Canada, Nunavik; J. Gagnon; QFA-594851	–	–	666667231	666666927	–	–	N/A
P. leucophlebia	GB	Finland; Myllys et al. 2007						119690856	N/A
P. leucophlebia	GB	Canada, Québec; O'Brien et al. 2013						455476640	N/A
*P. leucophlebia**	GB	Canada, British Columbia; O'Brien et al. 2013						455476274	HT02
*P. leucophlebia**	GB	Canada, British Columbia; O'Brien et al. 2013						455476544	HT21, IV
*P. leucophlebia**	GB	Canada, British Columbia; O'Brien et al. 2013						455476358	HT04
P. malacea	GB	Canada, British Columbia; O'Brien et al. 2013						455475965	HT07, III
P. malacea	GB	Canada, British Columbia; O'Brien et al. 2013						455475977	HT09, III
P. malacea	GB	Unknown						455476625	III
P. malacea	GB	Unknown						455476613	III
P. malacea	GB	Unknown						455476658	III
P. malacea	GB	Unknown						455476616	III
P. malacea	GB	Unknown						455476619	III
P. malacea	GB	Finland; Myllys et al. 2007						119690868	III
P. malacea	GB	Canada, Québec; O'Brien et al. 2013						455476610	III

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. malacea	GB	Canada, British Columbia; O'Brien et al. 2013						455476556	HT25
P. malacea	GB	Canada, Québec; O'Brien et al. 2013						455476622	III
P. malacea	OV16029	Unknown						455476655	IV
P. malacea	P1028	Norway; N. Magain s.n.; LG	–	666666878	666667215	666666911	666667093	–	N/A
P. malacea	GB	Canada, British Columbia; O'Brien et al. 2013						455475980	HT22
P. malacea	GB	Canada, British Columbia; O'Brien et al. 2013						455476595	HT28
P. malacea	P1363	Russia, Karelia; P. Uotila 44104; H	–	–	666667233	666666929	–	–	N/A
P. membranacea	GB	Canada; O'Brien et al. 2005						82470928	V
P. membranacea	GB	USA; O'Brien et al. 2005						82471000	V
P. membranacea	GB	Russia; O'Brien et al. 2005						82471066	V
P. membranacea	GB	Canada; O'Brien et al. 2005						82471069	XIII
P. membranacea	P3	Iceland; J. Miadlikowska & F. Lutzoni 08.08.10-2; DUKE	–	666666879	666667177	666666959	666667051	XXXX	XVI
P. membranacea	P86	Madeira; H. Vare L1807; H	–	–	XXXX	–	XXXX	–	N/A
P. membranacea	GB	Finland; Myllys et al. 2007						119690874	N/A
P. membranacea	GB	USA; O'Brien et al. 2005						82470997	N/A
P. membranacea	GB	Finland; Myllys et al. 2007						119690871	N/A
P. membranacea	GB	Canada, British Columbia; O'Brien et al. 2013						455476529	HT15
*P. membranacea**	GB	Canada, British Columbia; O'Brien et al. 2013						455476412	HT14, XIII

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. monticola	P73	Austria; R. Türk 37593; H	–	666666880	–	666666979	666667069	XXXX	HT51
P. monticola	P75	Norway; T. Ahti 65831; H	–	666666881	666667195	666666981	666667073	XXXX	HT59
P. neckeri	GB	Canada, British Columbia; O'Brien et al. 2013						455476136	HT16
P. neckeri	P15	Iceland; J. Miadlikowska 08.09.10-2; DUKE	–	–	–	–	–	XXXX	HT52
P. neckeri	P1620	USA, Arkansas; R. C. Harris 45472; NY	–	666666882	666667241	666666941	666667109	–	N/A
P. neocanina	GB	Canada, British Columbia; O'Brien et al. 2013						455476607	HT29
P. neocanina	P68	USA, New Mexico; J. Hollinger 2401; DUKE	–	–	–	–	–	XXXX	HT53
P. neocanina	P70	USA, New Mexico : J. Hollinger 2460; DUKE	–	–	–	–	–	XXXX	V
*P. neocanina**	GB	Canada, British Columbia; O'Brien et al. 2013						455476130	HT01, V
P. papuanorum	P1831	Papua New Guinea; E. Sérusiaux s.n.; LG	–	–	XXXX	–	XXXX	–	N/A
P. patagonica	P76	Chile; C. Rubio 7077; H	–	–	–	–	–	XXXX	HT50
P. phyllidiosa	P72	USA, North Carolina; J. Hollinger 2708; DUKE	–	–	–	–	–	XXXX	HT62
P. phyllidiosa	P1245	Unknown; J. Hollinger 1097; UBC	–	666666884	666667219	666666917	666667097	–	N/A
P. phyllidiosa	P1617	USA, Tennessee; W. R. Buck 56402; NY	–	666666885	666667239	666666939	666667107	–	N/A
P. polydactyloides	P1756	Rwanda; E. Sérusiaux s.n.; LG	–	XXXX	XXXX	XXXX	–	–	N/A
P. ponojensis	P77	Chile; B. Goffinet 6837; CONN	–	–	–	–	–	XXXX	HT57
P. ponojensis	P84	USA, Pennsylvania; J. Lendemer 13556; H	–	–	–	–	–	XXXX	HT63
P. praetextata	GB	Canada, British Columbia; O'Brien et al. 2013						455476526	HT17

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. praetextata	GB	Finland; Myllys et al. 2007						119690886	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690889	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690892	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690895	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690898	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690901	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690883	V
P. praetextata	GB	Finland; Myllys et al. 2007						119690904	V
P. praetextata	P1862	France, Reunion Island; K. Kalb s.n.; DUKE	–	–	666667253	666666957	666667123	–	N/A
P. praetextata	P570	Norway; N. Magain s.n.; LG	–	666666887	666667211	666666989	666667089	–	N/A
P. retifoveata	P1839	Alaska; J. Miadlikowska et al. s.n.; DUKE	–	–	XXXX	–	XXXX	–	N/A
P. retifoveata	P74	Russia, Sakha Republic; T. Ahti 61821; H	–	666666888	666667193	–	666667071	–	N/A
P. rufescens	P5	Iceland; J. Miadlikowska & F. Lutzoni 08.08.10-4; DUKE	–	–	–	XXXX	–	–	N/A
P. rufescens	GB	Germany; O'Brien et al. 2005						82470982	N/A
P. rufescens	GB	Germany; O'Brien et al. 2005						82470964	N/A
P. rufescens	GB	Unknown; O'Brien et al. 2005						82470961	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. rufescens	GB	Germany; O'Brien et al. 2005						82470976	N/A
P. rufescens	GB	Germany; O'Brien et al. 2005						82470970	N/A
P. rufescens	GB	England; O'Brien et al. 2005						82470934	N/A
P. rufescens	GB	Poland; O'Brien et al. 2005						82471072	N/A
P. rufescens	GB	Germany; O'Brien et al. 2005						82470973	N/A
P. rufescens	P6	Iceland; J. Miadlikowska & F. Lutzoni 08.08.10-5; DUKE	–	666666889	–	666666977	–	XXXX	HT58
P. sp.	P1319	Canada, Québec; J. Miadlikowska & F. Lutzoni s.n.; DUKE	–	–	–	–	–	XXXX	HT60
P. sp.	P1335	Canada, Nunavik; J. Gagnon GPS 106; QFA 595025	–	–	–	–	–	XXXX	XVIII
P. sp.	GB	Finland; Myllys et al. 2007						119690907	N/A
P. ulcerata	P43	Costa Rica; J. Miadlikowska et al. s.n.; DUKE	–	–	–	–	–	XXXX	HT61
P. ulcerata	P48	Costa Rica; J. Miadlikowska et al. s.n.; DUKE	–	666666891	666667185	–	666667061	–	N/A
P. ulcerata	P51	Costa Rica; J. Miadlikowska et al. s.n.; DUKE	–	666666892	666667187	–	666667063	–	N/A
P. venosa	P1905	Norway; N. Magain s.n.; LG	–	666666893	666667255	–	666667125	–	N/A
P. venosa	P69	USA, New Mexico; J. Hollinger 2475; DUKE	–	666666894	666667191	–	666667067	–	N/A
C. Other genera									

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
Arctomia fasciculare 1	GB	Spain; Otálora et al. 2010						205278413	N/A
A. fasciculare 2	GB	Spain; Otálora et al. 2010						205278416	N/A
Anabaena augstumalis	GB	Germany; Rajaniemi et al. 2005						55650808	N/A
A. cf. *cylindrica*	GB	Unknown; Gugger et al. 2002						15282236	N/A
Anthoceros sp.	GB	Italy; O'Brien et al. 2005						82470946	N/A
Anthoceros sp.	GB	Germany; O'Brien et al. 2005						82470958	N/A
Blasia pusilla	GB	Germany; O'Brien et al. 2005						82470952	N/A
B. pusilla	GB	Germany; O'Brien et al. 2005						82470955	N/A
B. pusilla	GB	Germany; O'Brien et al. 2005						82471090	N/A
Blennothallia crispus 1	GB	Spain; Otálora et al. 2010						205278404	N/A
B. crispus 2	GB	Germany; O'Brien et al. 2005						82470967	N/A
Collema flaccidum 1	GB	Finland; Stenroos et al. 2006						82779973	N/A
C. flaccidum 2	GB	Finland; Stenroos et al. 2006						82779972	N/A
C. flaccidum 3	GB	Spain; Otálora et al. 2010						205278419	N/A
C. flaccidum 4	GB	Spain; Otálora et al. 2010						205278422	N/A
C. flaccidum 5	GB	USA; Otálora et al. 2010						241913794	N/A
C. furfuraceum 1	GB	USA; Otálora et al. 2010						205278440	N/A
C. furfuraceum 2	GB	Portugal; Otálora et al. 2010						205278437	N/A
C. furfuraceum 3	GB	Spain; Otálora et al. 2010						205278431	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
C. furfuraceum 4	GB	Croatia; Otálora et al. 2010						205278434	N/A
C. nigrescens 1	GB	Spain; Otálora et al. 2010						205278449	N/A
C. nigrescens 2	GB	Spain; Otálora et al. 2010						205278443	N/A
C. nigrescens 3	GB	Spain; Otálora et al. 2010						205278446	N/A
C. subnigrescens 1	GB	Croatia; Otálora et al. 2010						205278461	N/A
C. subnigrescens 2	GB	Portugal; Otálora et al. 2010						205278458	N/A
C. subnigrescens 3	GB	Portugal; Otálora et al. 2010						205278464	N/A
Cycas circinalis	GB	Brazil; O'Brien et al. 2005						82471075	N/A
Degelia plumbea	GB	Madeira; Myllys et al. 2007						119690755	N/A
Encephalartos natalensis	GB	Italy; O'Brien et al. 2005						82470949	N/A
Enchylium polycarpon 1	GB	Spain; Otálora et al. 2010						205278455	N/A
E. polycarpon 2	GB	Spain; Otálora et al. 2010						205278452	N/A
E. tenax 1	GB	Spain; Otálora et al. 2010						205278473	N/A
E. tenax 2	GB	Spain; Otálora et al. 2010						205278470	N/A
E. tenax 3	GB	Spain; Otálora et al. 2010						205278467	N/A
Fischerella muscicola	GB	New Zealand; O'Brien et al. 2005						82471045	N/A
Geosiphon pyriforme	GB	Germany; O'Brien et al. 2005						82471096	N/A
G. pyriforme	GB	Germany; O'Brien et al. 2005						82470943	N/A
Gunnera manicata	GB	Germany; O'Brien et al. 2005						82471093	N/A

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Table S1 – continued from previous page

Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
Lathagrium auriforme 1	GB	Spain; Otálora et al. 2010						205278410	N/A
L. auriforme 2	GB	Spain; Otálora et al. 2010						205278407	N/A
L. undulatum 1	GB	Spain; Otálora et al. 2010						205278428	N/A
L. undulatum 2	GB	Spain; Otálora et al. 2010						205278425	N/A
Leptogium austroamericanum 1	GB	Argentina; Otálora et al. 2010						205278479	N/A
L. austroamericanum 2	GB	Colombia; Otálora et al. 2010						205278476	N/A
L. azureum 1	GB	Argentina; Otálora et al. 2010						205278488	N/A
L. azureum 2	GB	Chile; Otálora et al. 2010						205278485	N/A
L. azureum 3	GB	Brazil; Otálora et al. 2010						205278482	N/A
L. brebissonii 1	GB	Spain; Otálora et al. 2010						205278494	N/A
L. brebissonii 2	GB	Spain; Otálora et al. 2010						205278491	N/A
L. corniculatum 1	GB	Spain; Otálora et al. 2010						205278497	N/A
L. corniculatum 2	GB	Spain; Otálora et al. 2010						205278500	N/A
L. corticola 1	GB	Costa Rica; Otálora et al. 2010						205278503	N/A
L. corticola 2	GB	USA; Otálora et al. 2010						205278506	N/A
L. cyanescens 1	GB	Spain; Otálora et al. 2010						205278515	N/A
L. cyanescens 2	GB	USA; Otálora et al. 2010						205278512	N/A
L. cyanescens 3	GB	Panama; Otálora et al. 2010						205278509	N/A
L. furfuraceum 1	GB	Croatia; Otálora et al. 2010						205278530	N/A
L. furfuraceum 2	GB	Portugal; Otálora et al. 2010						205278533	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
L. furfuraceum 3	GB	Portugal; Otálora et al. 2010						205278536	N/A
L. furfuraceum 4	GB	Spain; Otálora et al. 2010						205278518	N/A
L. furfuraceum 5	GB	Spain; Otálora et al. 2010						205278521	N/A
L. furfuraceum 6	GB	Spain; Otálora et al. 2010						205278524	N/A
L. furfuraceum 7	GB	Spain; Otálora et al. 2010						205278527	N/A
L. pseudofurfuraceum 1	GB	Argentina; Otálora et al. 2010						205278587	N/A
L. pseudofurfuraceum 2	GB	USA; Otálora et al. 2010						205278578	N/A
L. pseudofurfuraceum 3	GB	USA; Otálora et al. 2010						205278584	N/A
L. pseudofurfuraceum 4	GB	USA; Otálora et al. 2010						205278581	N/A
L. pseudofurfuraceum 5	GB	Argentina; Otálora et al. 2010						205278590	N/A
L. saturninum 1	GB	Finland; Stenroos et al. 2006						82779971	N/A
L. saturninum 2	GB	Finland; Stenroos et al. 2006						82779970	N/A
L. saturninum 3	GB	France; Otálora et al. 2010						205278602	N/A
L. saturninum 4	GB	Spain; Otálora et al. 2010						205278605	N/A
L. saturninum 5	GB	USA; Otálora et al. 2010						205278608	N/A
L. saturninum 6	GB	Canada; Otálora et al. 2010						205278599	N/A
Lobaria amplissima	GB	Norway; Stenroos et al. 2006						82779934	N/A
L. amplissima	GB	Norway; Stenroos et al. 2006						82779933	N/A
L. amplissima	GB	Austria; O'Brien et al. 2005						82471018	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
L. hallii	GB	USA; O'Brien et al. 2005						82471021	N/A
L. kukorawa	GB	China; O'Brien et al. 2013						455476652	N/A
L. kukorawa	GB	China; O'Brien et al. 2013						455476646	N/A
L. pulmonaria	OG6	USA, North Carolina, N. Magain s.n., DUKE	–	XXXX	XXXX	–	–	–	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690773	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690770	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690767	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690776	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690779	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690782	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690764	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690758	N/A
L. pulmonaria	GB	Finland; Myllys et al. 2007						119690761	N/A
L. scrobiculata	GB	Finland; Myllys et al. 2007						119690785	N/A
Massalongia carnosa	GB	USA; O'Brien et al. 2005						82471024	N/A
M. carnosa	GB	Finland; Stenroos et al. 2006						82779969	N/A
Nephroma arcticum	GB	Canada, British Columbia; O'Brien et al. 2013						455476562	HT26, IV
N. arcticum	GB	Canada, British Columbia; O'Brien et al. 2013						455476565	HT27, IV

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
N. arcticum	GB	Canada, Québec; O'Brien et al. 2013						455476628	XIa
N. bellum	OG4	Norway; N. Magain s.n.; LG	–	XXXX	XXXX	–	–	–	N/A
N. bellum	GB	Finland; Myllys et al. 2007						119690791	N/A
N. bellum	GB	Finland; Myllys et al. 2007						119690803	N/A
N. bellum	GB	Finland; Myllys et al. 2007						119690788	N/A
N. bellum	GB	Finland; Myllys et al. 2007						119690794	N/A
N. bellum	GB	Finland; Myllys et al. 2007						119690797	N/A
N. bellum	GB	Finland; Myllys et al. 2007						119690800	N/A
N. bellum	GB	Austria; O'Brien et al. 2005						82471027	N/A
*N. bellum**	GB	Canada, British Columbia; O'Brien et al. 2013						455476436	HT12
N. helveticum	GB	Canada; O'Brien et al. 2005						82471030	N/A
N. laevigatum	GB	Finland; Myllys et al. 2007						119690806	N/A
N. laevigatum	GB	Finland; Myllys et al. 2007						119690809	N/A
N. parile	GB	Canada, British Columbia; O'Brien et al. 2013						455476439	HT05
N. parile	OG3	Norway; N. Magain s.n.; LG	–	XXXX	XXXX	–	–	–	N/A
N. parile	GB	Finland; Myllys et al. 2007						119690812	N/A
N. parile	GB	Finland; Myllys et al. 2007						119690818	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
N. parile	GB	Finland; Myllys et al. 2007						119690821	N/A
N. parile	GB	Finland; Myllys et al. 2007						119690815	N/A
N. resupinatum	GB	Canada, Québec; O'Brien et al. 2013						455476634	N/A
N. resupinatum	GB	Finland; Myllys et al. 2007						119690824	N/A
N. resupinatum	GB	Finland; Myllys et al. 2007						119690827	N/A
N. tangeriense	GB	Madeira; Myllys et al. 2007						119690830	N/A
Nostoc calcicola	GB	Czech Republic; Rajaniemi et al. 2005						55650826	N/A
N. calcicola	GB	Czech Republic; Rajaniemi et al. 2005						55650829	N/A
N. commune	GB	USA; O'Brien et al. 2005						82470988	N/A
N. commune	GB	China; Rudi et al. 1998						2463296	N/A
N. edaphicum	GB	Czech Republic; Rajaniemi et al. 2005						55650832	N/A
N. ellipsorum	GB	Czech Republic; Rajaniemi et al. 2005						55650835	N/A
N. flagelliforme	GB	China; Rudi et al. 1998						2463302	N/A
N. muscorum	GB	France; O'Brien et al. 2005						82471087	N/A
N. punctiforme	GB	France; O'Brien et al. 2005						82471099	N/A
Nostoc sp.	GB	Unknown; Fewer et al. 2007						159154307	N/A
Nostoc sp.	GB	Unknown; Kaneko et al. 2001						47118302	N/A
Nostoc sp.	GB	Unknown; Tomitani et al. 2006						89241989	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
Nostoc sp.	GB	Svalbard, Norway; Rudi et al. 1998						2463317	N/A
Nostoc sp.	GB	Unknown; Fewer et al. 2007						159154309	N/A
Nostoc sp.	GB	USA, Rudi et al. 1998						2463308	N/A
Nostoc sp.	GB	Indonesia; O'Brien et al. 2005						82471039	N/A
Nostoc sp.	GB	Senegal; O'Brien et al. 2005						82471051	N/A
Nostoc sp.	GB	Finland; Rajaniemi et al. 2005						55650727	N/A
Nostoc sp.	GB	Unknown; Kaneko et al. 2001						17227497	N/A
Nostoc sp.	GB	Norway; Rudi et al. 1998						2463311	N/A
Nostoc sp.	GB	Antarctica; Rudi et al. 1998						2463314	N/A
Pannaria conoplea	GB	Austria; O'Brien et al. 2005						82471033	N/A
Parmeliella triptophylla	GB	Finland; Myllys et al. 2007						119690836	N/A
P. triptophylla	GB	Finland; Myllys et al. 2007						119690839	N/A
P. triptophylla	GB	Finland; Myllys et al. 2007						119690842	N/A
P. triptophylla	GB	Finland; Myllys et al. 2007						119690845	N/A
Protopannaria pezizoides	GB	Finland; Myllys et al. 2007						119690833	N/A
P. pezizoides	GB	Finland; Stenroos et al. 2006						82779965	N/A
Parmotrema tinctorum	GB	Unknown; Lei et al. 2008						190147520	N/A
P. tinctorum	GB	Unknown; Lei et al. 2008						190147518	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2.1*	*rbcLX*	*rbcLX* phylogroup or haplotype
P. tinctorum	GB	Unknown; Lei et al. 2008						190147522	N/A
Pseudocyphellaria clathrata	GB	Brazil; Stenroos et al. 2006						82779936	N/A
P. coriifolia	GB	Argentina; Stenroos et al. 2006						82779949	N/A
P. coriifolia	GB	Argentina; Stenroos et al. 2006						82779938	N/A
P. coriifolia	GB	Argentina; Stenroos et al. 2006						82779955	N/A
P. crocata	GB	Madeira; Myllys et al. 2007						119690910	N/A
P. crocata	GB	Argentina; Stenroos et al. 2006						82779944	N/A
P. crocata	GB	Argentina; Stenroos et al. 2006						82779943	N/A
P. crocata	GB	Canada; Stenroos et al. 2006						82779935	N/A
P. crocata	GB	Argentina; Stenroos et al. 2006						82779942	N/A
P. crocata	GB	Argentina; Stenroos et al. 2006						82779945	N/A
P. crocata	GB	Argentina; Stenroos et al. 2006						82779948	N/A
P. crocata	GB	Thailand; Stenroos et al. 2006						82779952	N/A
P. crocata	GB	Mauritius; Stenroos et al. 2006						82779954	N/A
P. hirsuta	GB	Argentina; Stenroos et al. 2006						82779940	N/A
P. hirsuta	GB	Argentina; Stenroos et al. 2006						82779975	N/A
P. hirsuta	GB	Argentina; Stenroos et al. 2006						82779947	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
P. intricata	GB	Reunion Island; Stenroos et al. 2006						82779953	N/A
P. intricata	GB	Argentina; Stenroos et al. 2006						82779946	N/A
P. intricata	GB	Argentina; Stenroos et al. 2006						82779939	N/A
P. mallota	GB	Argentina; Stenroos et al. 2006						82779951	N/A
P. mallota	GB	Argentina; Stenroos et al. 2006						82779937	N/A
P. pilosella	GB	Argentina; Stenroos et al. 2006						82779950	N/A
P. scabrosa	GB	Argentina; Stenroos et al. 2006						82779974	N/A
P. scabrosa	GB	Argentina; Stenroos et al. 2006						82779941	N/A
Scytinium gelatinosum 1	GB	Spain; Otálora et al. 2010						205278542	N/A
S. gelatinosum 2	GB	USA; O'Brien et al. 2005						82471015	N/A
S. gelatinosum 3	GB	Spain; Otálora et al. 2010						205278539	N/A
S. lichenoides 1	GB	Sweden; Otálora et al. 2010						241913791	N/A
S. lichenoides 2	GB	Spain; Otálora et al. 2010						205278548	N/A
S. lichenoides 3	GB	Spain; Otálora et al. 2010						205278545	N/A
S. lichenoides 4	GB	Spain; Otálora et al. 2010						205278551	N/A
S. lichenoides 5	GB	Portugal; Otálora et al. 2010						205278554	N/A
S. lichenoides 6	GB	Spain; Otálora et al. 2010						205278557	N/A
S. magnusonii 1	GB	Spain; Otálora et al. 2010						205278566	N/A
S. magnusonii 2	GB	Spain; Otálora et al. 2010						205278569	N/A

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
S. magnusonii 3	GB	Sweden; Otálora et al. 2010						205278560	N/A
S. magnusonii 4	GB	Denmark; Otálora et al. 2010						205278563	N/A
S. magnusonii 5	GB	Sweden; Otálora et al. 2010						205278575	N/A
S. magnusonii 6	GB	Spain; Otálora et al. 2010						205278572	N/A
S. pulvinatum 1	GB	Spain; Otálora et al. 2010						205278596	N/A
S. pulvinatum 2	GB	Spain; Otálora et al. 2010						205278593	N/A
S. schraderi 1	GB	Spain; Otálora et al. 2010						205278614	N/A
S. schraderi 2	GB	Spain; Otálora et al. 2010						205278611	N/A
Solorina crocea	OG14	Russia, Karelia; T. Ahti s.n.; PTZ	–	123979314	123979355	–	–	–	N/A
S. saccata	OG13	Russia, Karelia; T. Ahti s.n.; PTZ	–	XXXX	XXXX	–	–	–	N/A
Stangeria paradosa	GB	England; O'Brien et al. 2005						82471078	N/A
Stereocaulon exutum	GB	Japan; Stenroos et al. 2006						82779962	N/A
S. fronduliferum	GB	New Zealand; Stenroos et al. 2006						82779963	N/A
S. tomentosum	GB	Argentina; Stenroos et al. 2006						82779964	N/A
Sticta beauvoisii	GB	USA; O'Brien et al. 2005						82471006	N/A
S. fuliginosa	GB	USA; O'Brien et al. 2005						82471102	N/A
S. gaudichaudia	GB	Argentina; Stenroos et al. 2006						82779960	IV
S. hypochroa	GB	Argentina; Stenroos et al. 2006						82779959	IV
S. hypochroa	GB	Argentina; Stenroos et al. 2006						82779958	IV

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Taxon	DNA Number	Voucher/Published source	ITS	LSU	*RPB1*	ß-tubulin	*EFT2*.1	*rbcLX*	*rbcLX* phylogroup or haplotype
S. hypochroa	GB	Argentina; Stenroos et al. 2006						82779957	IV
S. hypochroa	GB	Argentina; Stenroos et al. 2006						82779956	IV
S. hypochroa	GB	Argentina; Stenroos et al. 2006						82779961	IV
Trichormus variabilis	GB	Russia; Rajaniemi et al. 2005						55650847	N/A
Vahliella leucophaea	GB	Finland; Stenroos et al. 2006						82779966	N/A

Table S2: Phylogenetic analyses realized on the matrices, with the partitioning method chosen, the number and list of selected subsets, and the models applied to them.

Analysis: RaxML Matrix 1	List of subsets	Model
Partitioning method:	• 3rd codons ; EFT2.1, ß-tubulin introns	GTR+G
PartitionFinder (BIC, All)	• 1st codons ; LSU	GTR+G
No. subsets: 4	• 2nd codons	GTR+G
	• RPB1 intron	GTR+G
Analysis: MrBayes Matrix 1	List of subsets	Model
Partitioning method:	• 3rd codons, EFT2.1, ß-tubulin introns	HKY+I+G
PartitionFinder (BIC, All)	• 1st codons, LSU	GTR+I+G
No. subsets: 4	• 2nd codons	HKY+I+G
	• RPB1 intron	K80
Analysis : RaxML Matrix 2	List of subsets	Model
Partitioning method:	• ITS1, ITS2, ß-tubulin and EFT2.1 introns	GTR+G
PartitionFinder (AICc, ALL)	• ß-tubulin 1st codon	GTR+G
No. subsets: 11	• ß-tubulin and RPB1 2nd codons	GTR+G
	• ß-tubulin and EFT2.1 3rd codons	GTR+G
	• EFT2.1 1st codon	GTR+G
	• EFT2.1 2nd codon	GTR+G
	• RPB1 1st codon	GTR+G
	• RPB1 3rd codon	GTR+G
	• RPB1 intron	GTR+G
	• 5.8S	GTR+G
	• LSU	GTR+G
Analysis: MrBayes Matrix 2	List of subsets	Model
Partitioning method:	• 1st codons	HKY+I
Arbitrary	• 2nd codons	HKY+I+G
No. subsets : 7	• 3rd codons	HKY+I
	• introns	HKY+G
	• LSU	GTR+I+G
	• ITS1, ITS2	K80+G
	• 5.8S	constant, excluded
Analysis: BEAST Matrix 2	List of subsets	Model
Partitioning method:	• ß-tubulin coding	HKY+I+G
Arbitrary	• EFT2.1 coding	HKY+G
No. subsets: 6	• RPB1 coding	GTR+G
	• introns	HKY+G
	• LSU	GTR+I+G
	• ITS	HKY+G
Analysis: RaxML : Mr Bayes, Matrix 3	List of subsets	Model
Partitioning method:	• 1st codon	GTR+G
Arbitrary	• 2nd codon	GTR+G
No. subsets: 3	• 3rd codon	GTR+G

Chapter 2

Do Photobiont Switch and Cephalodia Emancipation Act as Evolutionary Drivers in the Lichen Symbiosis? A Case Study in the Pannariaceae (Peltigerales)

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Published in PLoS One 9(2):e89876. 2014.

Abstract

Lichen symbioses in the Pannariaceae associate an ascomycete and either cyanobacteria alone (usually *Nostoc*; bipartite thalli) or green algae and cyanobacteria (cyanobacteria being located in dedicated structures called cephalodia; tripartite thalli) as photosynthetic partners (photobionts). In bipartite thalli, cyanobacteria can either be restricted to a well-delimited layer within the thallus ('pannarioid' thalli) or spread over the thallus that becomes gelatinous when wet ('collematoid' thalli). We studied the collematoid genera *Kroswia* and *Physma* and an undescribed tripartite species along with representatives of the pannarioid genera *Fuscopannaria*, *Pannaria* and *Parmeliella*. Molecular inferences from 4 loci for the fungus and 1 locus for the photobiont and statistical analyses within a phylogenetic framework support the following: (a) several switches from pannarioid to collematoid thalli occured and are correlated with photobiont switches; the collematoid genus *Kroswia* is nested within the pannarioid genus *Fuscopannaria* and the collematoid genus *Physma* is sister to the pannarioid *Parmeliella mariana* group; (b) *Nostoc* associated with collematoid thalli in the Pannariaceae are related to that of the Collemataceae (which contains only collematoid thalli), and never associated with pannarioid thalli; *Nostoc* associated with pannarioid thalli also associate in other families with similar morphology; (c) ancestors of several lineages in the Pannariaceae

developed tripartite thalli, bipartite thalli probably resulting from cephalodia emancipation from tripartite thalli which eventually evolved and diverged, as suggested by the same *Nostoc* present in the collematoid genus *Physma* and in the cephalodia of a closely related tripartite species; Photobiont switches and cephalodia emancipation followed by divergence are thus suspected to act as evolutionary drivers in the family Pannariaceae.

2.1 Introduction

Several spectacular aspects of the lichen symbiosis have come to light recently, the most surprizing for the general public and the most promising for evolutionary studies being the multiple variations of the association between the mycobiont and photobiont partners. The lichen as the icon of consensual and stable symbiosis between two very different partners "for better and for worse" is not the model that molecular studies have produced in recent years. Indeed, some mycobionts can incorporate several algal genotypes in their thallus (Bačkor et al., 2010; Guzow-Krzeminska, 2006; Piercey-Normore, 2006), or even different algal species (Casano et al., 2011; del Campo et al., 2013). Several phylogenetic studies have demonstrated that photobiont switching is rather widespread (Fernández-Mendoza et al., 2011), even in obligatory sterile taxa where both partners are dispersed together, and may occur repeatedly over evolutionary timescales (Nelsen and Gargas, 2008). Studies of the genetic diversity of both partners within a geographical context revealed that mycobionts can recruit several lineages of photobionts, allowing for ecotypic differentiation and thus for colonization of different ecological niches and distribution (Fernández-Mendoza et al., 2011; Yahr et al., 2006). Those multiple variations in the association between the partners involved in the lichen symbiosis may take part in their evolutionary trajectory and we here address that matter for a lichen family (the Pannariaceae) in which several very different types of thalli occur together with variation in the number of photobionts involved in their construction.

The Peltigerales, a strongly supported lineage within the Lecanoromycetes, contains many well-known lichen genera, such as *Lobaria*, *Peltigera* and *Sticta*, within 10 families (Lumbsch and Huhndorf, 2011; Spribille and Muggia, 2013; Wedin et al., 2011, 2007), including the Collemataceae and the Pannariaceae, two families that will be mentioned in this paper. Within the Peltigerales, symbiosis includes two different lineages of photobionts (Lumbsch and Huhndorf, 2011) (a) cyanobacteria mostly belonging to the genus *Nostoc*, or to *Scytonema*, *Hyphomorpha* and other taxa in the Scytonemataceae and Rivulariaceae; (b) green algae, mainly assigned to the genera *Coccomyxa*, *Dictyochloropsis*, *Myrmecia*, all belonging to the Trebouxiophyceae. The number of photobionts associated with the mycobiont provides the ground for the distinction of bi- and tripartite lichens, the latter case being much more diverse in the way of allocating space for the cyanobacteria (Lohtander et al., 2003; Magain et al., 2012; Miadlikowska and Lutzoni, 2004):

a. association with a single photobiont partner, either a cyanobacteria or a green algae; these thalli are bipartite and are referable to the cyanolichens or the chlorolichens, respectively Henskens et al. 2012;

b. association with two partners, a cyanobacteria and a green algae and corresponding thalli referred to as tripartite thalli (Elvebakk et al., 2008); the topological organization of the partners can vary : (b1) both photobionts can be present in a dedicated layer within the thallus (chloro-cyanolichen; see Henskens et al. 2012); (b2) the green photobiont is present in a dedicated layer within the thallus whilst cyanobacteria are confined to dedicated and morphologically recognizable organs, named cephalodia (Cornejo and Scheidegger, 2013); (b3) production of two different thallus types, either living independently from one another or being closely associated, one with the cyanobacteria and the other one with the green algae; these structures are referred to as "photosymbiodemes", "photopairs" or "photomorphs" and can be morphologically rather similar or very much different one from the other – in the latter case the cyanomorph has a Dendriscocaulon-like morphology (Magain et al., 2012).

Further two different types of cyanobacterial bipartite thallus can be distinguished on the basis of their response to changes in water availability (Wedin et al., 2009). A first type is characterized by thalli that swell considerably and become very much gelatinous when wet, and return to a rather brittle and crumpled condition when dry, while the second type has thalli that do not radically change when water availability varies, albeit strong changes in color can occur. The first type is associated with a homoiomerous thallus anatomy, that is absence of a specialized photobiont layer, with chains of *Nostoc* with thick mucilaginous walls being easily recognized and present throughout the thallus thickness, an upper cortex being absent or present; it will be hereafter referred to as the collematoid thallus type. The second type of thallus is heteromerous, that is with a usually very distinct photobiont layer present under the upper cortex (which is always present) and *Nostoc* (or other genera) or green algal cells compacted and assembled in clusters. Within the second group, several morphotypes can be distinguished, ranging from nearly crustose to large foliose and dendroid-fruticose; the pannarioid type refers to a squamulose to foliose thallus developed over a black prothallus. Within the Peltigerales, a thallus associated with cyanobacteria can either belong to the collematoid or to other types, incl. the pannarioid type; on the other hand, thalli associated with green algae never belong to the collematoid type.

The assignment of collematoid taxa to a single family (Collemataceae) has been the rule for a long time (Henssen, 1965, 1979, 1999, 2007; Jørgensen, 2007a; Jørgensen and Henssen, 1999). Several exceptions are worth mentioning as they anticipate the more recent resolution of several genera outside the family: the collematoid genera *Kroswia* and *Lepidocollema* and the species *Pannaria santessonii* have been assigned to the Pannariaceae (Henssen et al., 1974; Jørgensen, 2002, 2003a; Krog, 2000; Swinscow and Krog, 1986) while the genus *Hydrothyria* was recognized as close to *Peltigera* (Henssen et al., 1974; Keuck, 1977).

Access to molecular data and their optimization with modern statistical methods

caused many relocation of collematoid taxa: to the genus *Peltigera* for both species of *Hydrothyria* (Miadlikowska and Lutzoni, 2000; Lendemer and O'Brien, 2011); to another family within the Peltigerales, the Massalongiaceae for the genera *Leptochidium* and *Massalongia* (Wedin et al., 2007); to the Pannariaceae for several genera (*Leciophysma*, *Leptogidium*, *Physma*, *Ramalodium*, *Staurolemma*, *Steineropsis*) and a species of *Santessoniella* (*S. saximontana*) (Wedin et al., 2009; Otálora et al., 2010a; Muggia et al., 2011; Spribille et al., 2010); and to an unrelated family, the Arctomiaceae (Otalora and Wedin, 2013) for *Collema fasciculare* and related species.

In summary, the lichen family Pannariaceae includes genera with very different thalli, easily recognized by their morphology and anatomy and behavior to water availability, the collematoid and pannarioid thalli. We here wish :

1. to examine the phylogenetic relationships of the collematoid genera *Kroswia* and *Physma*, and to examine the phylogenetic relationships of the photobiont of these two taxa (both being lichenized with *Nostoc*);

2. to examine the phylogenetic relationships of the collematoid, pannarioid and tripartite thalli all across the family Pannariaceae, and to establish whether a photobiont switch can be associated with the transition towards from pannarioid thalli to collematoid thalli and vice versa;

3. to examine the phylogenetical position of an undescribed species with tripartite thallus, belonging to *Pannaria* s. l. (foliose species with a green algae in the thallus and developing squamulose cephalodia with *Nostoc* over its surface) and to assess the evolutionary significance of a thallus combining a green algae and a cyanobacteria.

2.2 Material and Methods

2.2.1 Taxon Sampling

We assembled material belonging to the Pannariaceae from recent field trips in Madagascar (2008), Reunion Island (2008, 2009) and Thailand (2012). The 36 specimens used for molecular analysis are listed in Table 1. Identification of these collections is based on Jørgensen (1994, 2000, 2001, 2002, 2003a,b, 2004, 2007b, 2009); Jørgensen and Schumm (2010); Jørgensen and Sipman (2007); Upreti et al. (2005); Swinscow and Krog (1988); Verdon and Elix (1995).

Table 1: Voucher table of the specimens used in the study, with the species names for the mycobiont, and the species names of the host for the photobiont, when available; the country of origin and the voucher information; GenBank accessions of the sequences.

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Degelia durietzii Arv. & D.J. Galloway	19	New Zealand		GQ259022	GQ258992	GQ259051	
Degelia plumbea (Lightf.) P.M. Jørg. & P. James	93 (ITS), 19	Norway (ITS), Portugal	AF429265	AY340491	AY340543	GQ259052	
Erioderma verruculosum Vain.	117	?		DQ972990	DQ973041	DQ973062	
Fuscoderma applanatum (D.J. Galloway & P. M. Jørg.) P.M. Jørg & D.J. Galloway	19	New Zealand		GQ259024	GQ258994	GQ259053	
Fuscopannaria ahlneri (P.M. Jørg.) P.M. Jørg.	118 (ITS), 19	Norway (ITS), South Korea	GU570097	GQ259025	GQ258995	GQ259054	
Fuscopannaria confusa (P.M. Jørg.) P.M. Jørg	118	Norway	GU570133	GU570043			
Fuscopannaria ignobilis (Anzi) P.M. Jørg.	119 (ITS), 117	?	HQ650673	DQ917416	DQ917417	DQ986839	
Fuscopannaria leucosticta (Tuck.) P.M. Jørg.	NEW	Reunion Island, R1123 (LG)	KF704257	JX494238	JX494264	JX494284	KF704325
Fuscopannaria leucosticta (Tuck.) P.M. Jørg.	93 (ITS), 19	USA	AF429277	DQ900630	DQ900640	GQ259055	
Fuscopannaria mediterranea (Tav.) P.M. Jørg.	118(ITS), 117	Norway (ITS)	GU570131	DQ917418	DQ917419		
Fuscopannaria praetermissa (Nyl.) P.M. Jørg.	118 (ITS), 19	Norway (ITS), Sweden	GU570108	GQ259026	GQ258996	GQ259056	
Fuscopannaria praetermissa (Nyl.) P.M. Jørg.	NEW	Reunion Island, R1060 (LG)	KF704258	JX494239		JX494285	KF704346
Fuscopannaria sampaiana (Tav.) P.M. Jørg.	118	Norway		GU570030			
Joergensenia cephalodina (Zahlbr.) Passo, S. Stenroos & Calvelo	96	Argentina	EU885308	EU885329			
Kroswia crystallifera P.M. Jørg.	NEW	Madagascar, M788 (LG)	KF704254	JX494235	JX494261	JX494281	KF704343

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Table 1 – continued from previous page

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Kroswia crystallifera P.M. Jørg.	NEW	Reunion Island, R1055 (LG)	KF704255	JX494236	JX494262	JX494282	KF704345
Kroswia crystallifera P.M. Jørg.	NEW	Reunion Island, R1679 (LG)	KF704256	JX494237	JX494263	JX494283	KF704344
Leciophysma furfurascens (Nyl.) Gyeln.	19	Sweden		GQ259028	GQ258998	GQ259058	
Leptogidium contortum (Henssen) T. Sprib. & Muggia	34	Chile		JF938195			
Leptogidium dendriscum (Nyl.) Nyl.	34	USA, Alaska		JF938202	JF938143		
Leptogium lichenoides (L.) Zahlbr.	119 (ITS), 114	?	HQ650672	DQ923120	DQ917412	DQ917414	
Pannaria athroophylla (Stirt.) Elvebakk & Galloway	96	Argentina	EU885303	EU885325			
Pannaria calophylla (Müll. Arg.) Passo & Calvelo	96	Argentina	EU885296	EU885318			
Pannaria conoplea (Ach.) Bory	93 (ITS), 15	Norway (ITS)	AF429281		AY424209		
Pannaria implexa (Stirt.) Passo, Calvelo & Stenroos	95	Argentina	EU885311	EU885333			
Pannaria lurida (Mont.) Nyl.	NEW	Madagascar, M786 (LG)	KF704248	JX494240	JX494265	KF704307	
Pannaria lurida (Mont.) Nyl.	NEW	Reunion Island, R1033 (LG)	KF704252	JX494247	JX494272	KF704311	
Pannaria lurida (Mont.) Nyl.	NEW	Reunion Island, R1012 (LG)	KF704253	JX494246	JX494271	KF704312	
Pannaria microphyllizans (Nyl.) P.M. Jørg.	93 (ITS), 96	Australia (ITS), Argentina	AF429279	EU885322			
Pannaria multifida P.M. Jørg.	NEW	Reunion Island, R942 (LG)	KF704249	JX494241	JX494266	KF704308	
Pannaria multifida P.M. Jørg.	NEW	Reunion Island, R960 (LG)	KF704251	JX494242	JX494267	KF704309	
Pannaria multifida P.M. Jørg.	NEW	Reunion Island, R961 (LG)	KF704250	JX494243	JX494268	KF704310	
Pannaria pallida (Nyl.) Hue	96 (ITS, mtSSU), 87 (LSU)	Argentina	EU885301	EU885323	GQ927270		

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Table 1 – continued from previous page

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Pannaria rubiginella P.M. Jørg.	19		GQ927269	GQ259037	GQ259007	GQ259074	
Pannaria rubiginosa (Thunb. ex. Ach.) Delise	19	Portugal	GQ927267	AY340513	AY340558	GQ259073	
Pannaria rubiginosa (Thunb. ex. Ach.) Delise	NEW	Reunion Island, R1008 (LG)	KF704259	JX494244	JX494269	KF704313	KF704321
Pannaria rubiginosa (Thunb. ex. Ach.) Delise	NEW	Reunion Island, R1126 (LG)	KF704260	JX494249	JX494274	KF704315	
Pannaria rubiginosa (Thunb. ex. Ach.) Delise	NEW	Reunion Island, R1011 (LG)	KF704261	JX494245	JX494270	KF704314	KF704323
Pannaria sp.	NEW	Thailand, T4 (LG)	KF704247	KF704289	KF704290	KF704306	KF704333
Pannaria sphinctrina (Mont.) Hue	96 (ITS, mtSSU), 87 (LSU)	Argentina	EU885302	EU885324	GQ927271		
Pannaria tavaresii P.M. Jørg.	96	Argentina	EU885294	EU885316			
Pannaria sp. (tripartite thallus)	NEW	Reunion Island, R969 (LG)	KF704268	KF704286		KF704299	KF704341
Parmeliella appalachensis P.M. Jørg.	117	?		DQ972992		DQ973064	
Parmeliella borbonica P.M. Jørg. & Schumm	NEW	Reunion Island, R1122 (LG)	KF704271	JX494259			KF704320
Parmeliella brisbanensis (C. Knight) P.M. Jørg. & D.J. Galloway	NEW	Thailand, T1 (LG)	KF704246	KF704280		KF704292	
Parmeliella brisbanensis (C. Knight) P.M. Jørg. & D.J. Galloway	NEW	Thailand, T3 (LG)	KF704277	KF704281		KF704294	KF704351
Parmeliella brisbanensis (C. Knight) P.M. Jørg. & D.J. Galloway	NEW	Thailand, T7 (LG)	KF704276	KF704282		KF704295	KF704352
Parmeliella brisbanensis (C. Knight) P.M. Jørg. & D.J. Galloway	NEW	Reunion Island, R1019 (LG)	KF704278	JX494255		KF704296	KF704350
Parmeliella brisbanensis (C. Knight) P.M. Jørg. & D.J. Galloway	NEW	Reunion Island, R1247 (LG)	KF704262	JX494258		KF704297	KF704347
Parmeliella mariana (Fr.) P.M. Jørg. & D.J. Galloway	NEW	Reunion Island, R974 (LG)	KF704275	JX494256		KF704301	KF704330

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Table 1 – continued from previous page

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Parmeliella miradorensis Vain.	12	Spain, La Gomera		HQ268592		HQ268591	
Parmeliella parvula P.M. Jørg.	118	Norway	GU570099	GU570031			
Parmeliella polyphyllina P.M. Jørg.	NEW	Reunion Island, R1021 (LG)	KF704265	JX494251	JX494276	KF704317	KF704327
Parmeliella polyphyllina P.M. Jørg.	NEW	Reunion Island, R1058 (LG)	KF704267	JX494252	JX494277	KF704319	KF704326
Parmeliella polyphyllina P.M. Jørg.	NEW	Reunion Island, R1120 (LG)	KF704266	JX494250	JX494275	KF704318	
Parmeliella sp. (mariana gr.)	NEW	Thailand, T2 (LG)		KF704283		KF704293	KF704348
Parmeliella sp. (mariana gr.)	NEW	Thailand, T6 (LG)	KF704279	KF704284		KF704304	KF704349
Parmeliella stylophora (Vain.) P.M. Jørg.	NEW	Reunion Island, R979 (LG)	KF704274	JX494257		KF704300	KF704331
Parmeliella triptophylla (Ach.) Müll. Arg.	120(ITS), 19	Finland (ITS), Sweden	HM448807	AY652623	GQ259008	GQ259075	
Parmeliella triptophylloides P.M. Jørg.	NEW	Reunion Island, R965 (LG)	KF704264	JX494253	JX494278	KF704316	KF704324
Peltigera aphthosa (L.) Willd.	121, 122	Sweden (*RPB1*)	KC437624	AY340515	AF286759	DQ915598	
Physma byrsaeum (Ach.) Tuck.	19	Tahiti		GQ259039	GQ259010	GQ259077	
Physma byrsaeum (Ach.) Tuck.	NEW	Reunion Island, R2847 (LG)	KF704272	JX494260		KF704303	KF704338
Physma byrsaeum (Ach.) Tuck.	NEW	Reunion Island, R2 (LG)	KF704273	KF704287		KF704302	KF704340
Physma byrsaeum (Ach.) Tuck.	NEW	Reunion Island, R1121 (LG)	KF704269	JX494254		KF704298	KF704337
Physma pseudoisidiatum Aptroot & Sipman	19	USA		GQ259041	GQ259012		
Physma radians Vain.	19	Japan		GQ259040	GQ259011	GQ259078	
Physma radians Vain.	NEW	Thailand, T5 (LG)	KF704270	KF704285		KF704305	KF704336

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Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Placynthium nigrum (Huds.) Gray	119 (ITS), 19	Sweden	HQ650699	AY340518	AF356674	GQ259079	
Protopannaria pezizoides (Weber ex. F.H. Wigg.) P.M. Jørg. & S. Ekman	93 (ITS), 19	Sweden	AF429271	AY340519	AY340561	GQ259081	
Psoroma hypnorum (Vahl.) Gray	93 (ITS), 19	Sweden	AF429272	AY340523	AY340565	GQ259085	
Psoroma palaceum (Fr.) Nyl.	96 (mtSSU), 87	Argentina	GQ927304	EU885327	GQ927305		
Psorophorus pholidotus Elvebakk & S.G. Hong	96 (mtSSU), 87	Argentina	EU885314	EU885336	GQ927289		
Ramalodium succulentum Nyl.	19	Australia		GQ259043	GQ259013	GQ259086	
Staurolemma omphalarioides (Anzi) P.M. Jørg. & Henssen	19	Norway		GQ259044	GQ259014		
Staurolemma sp.	NEW	Reunion Island, R982 (LG)	KF704263	KF704288	KF704291		KF704329
Vahliella californica (Tuck.) P.M. Jørg.	12	Canada, British Columbia		HQ268594		HQ268593	
Vahliella leucophaea (Vahl.) P.M. Jørg.	94 (ITS), 19	Sweden	AF429266	AY652621	DQ900642	GQ259090	
Vahliella saubinetii (Mont.) P.M. Jørg.	12	Croatia		HQ268602		HQ268601	
Xanthopsoroma contextum (Stirt.) Elvebakk	97	Argentina	EU885313	EU885335			
Xanthopsoroma soccatum (R. Br. ex Cromb.) Elvebakk	96, 87 (LSU)	Argentina	EU885315	EU885337	GQ927283		
Cyanobacterial species (or host when applicable)							
Anabaena flos-aquae Brébisson ex Bornet & Flauhault	Choi & Oh unpublished						DQ234825
Anabaena oryzae F.E. Fritsch	Mishra et al. unpublished	India					HM573456
Anabaena vaginicola F.E. Fristsch & Rich	Aghashariatmadari et al. unpublished	Iran					JN873351

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Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Blasia pusilla 1 L.	Liaimer et al. unpublished	Norway					EU022724
Blasia pusilla 2 L.	Liaimer et al. unpublished	Norway					EU022708
Blasia pusilla 3 L.	Liaimer et al. unpublished	Norway					EU022728
Blasia pusilla 4 L.	Liaimer et al. unpublished	Norway					EU022717
Chroococcus sp.	123	Italy					FR798931
Collema flaccidum (Ach.) Ach.	124	Finland					DQ265959
Collema nigrescens (Huds.) DC.	125	USA California					JN847352
Cycas revoluta Thunb.	126	Italy					AM711533
Fischerella muscicola (Thuret) Gomont	127	strain PCC 7414					AF132788
Fuscopannaria leucosticta (Tuck.) P.M. Jørg.	NEW	Reunion Island, R1009 (LG)					KF704322
Fuscopannaria leucosticta (Tuck.) P.M. Jørg.	NEW	Reunion Island, R1124 (LG)					KF704353
Gloeocapsa sp.	128	strain PCC 73106					AB039000
Gunnera prorepens Hook. f.	126	New Zealand					AM711541
Leptogium furfuraceum 1 (Harm.) Sierk	125	USA California					JN847353
Leptogium furfuraceum 2 (Harm.) Sierk	129	USA, California					JQ007761
Leptogium gelatinosum (With.) J.R. Laundon	130	USA					DQ185232
Leptogium lichenoides 1 (L.) Zahlbr.	129	Scotland					JQ007765
Leptogium lichenoides 2 (L.) Zahlbr.	129	Scotland					JQ007766
Leptogium palmatum (Huds.) Mont.	125	USA Oregon					JN847344
Leptogium pseudofurfuraceum P.M. Jørg. & A.K. Wallace	125	USA California					JN847347

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Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Leptogium saturninum (Dicks.) Nyl.	124	Finland					DQ265957
Leptogium sp.	NEW	Reunion Island, R2848 (LG)					KF704328
Leptogium sp.	NEW	Reunion Island, R2849 (LG)					KF704335
Leptogium sp.	NEW	Reunion Island, R2850 (LG)					KF704334
Lobaria pulmonaria 1 (L.) Hoffm.	125	USA Oregon					JN847345
Lobaria pulmonaria 2 (L.) Hoffm.	125	Norway					JN847357
Lobaria scrobiculata (Scop.) P. Gaertn.	129	Scotland					JQ007744
Massalongia carnosa (Dicks.) Körb.	130	USA					DQ185235
Microcoleus chthonoplastes Thur.	131						DQ460700
Nephroma arcticum (L.) Torss.	129	Finland					JQ007764
Nephroma bellum 1 (Spreng.) Tuck.	120	Finland					HQ591510
Nephroma bellum 2 (Spreng.) Tuck.	120	Finland					HQ591518
Nephroma laevigatum Ach.	125	Norway					JN847359
Nephroma parile (Ach.) Ach.	120	Finland					HQ591521
Nephroma resupinatum (L.) Ach.	120	Finland					HQ591528
Nephroma washingtoniense Gyeln.	125	USA Oregon					JN847341
Nodularia spumigena Mertens	Beer et al. unpublished	USA Utah					FJ546713
Nostoc commune 1 Vaucher	132						AB088405
Nostoc commune 2 Vaucher	Gachon et al. unpublished	South Africa					HE974995
Nostoc entophytum Bornet & Flahault	Seo & Yokota unpublished						AB093490
Nostoc linckia (Roth) Bornet ex Bornet & Flahault	Seo & Yokota unpublished						AB074503

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Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Nostoc linckia var. *arvense* C.B. Rhao	132						AB325907
Nostoc muscorum 1 C. Agardh ex Bornet & Flahault	133	Czech Republic					AJ630451
Nostoc muscorum 2 C. Agardh ex Bornet & Flahault	126	Czech Republic					AM711524
Nostoc muscorum 3 C. Agardh ex Bornet & Flahault	Mishra et al. unpublished	India					HM573462
Nostoc muscorum 4 C. Agardh ex Bornet & Flahault	126	Czech Republic					AM711523
Nostoc muscorum 5 C. Agardh ex Bornet & Flahault (soil)	130	France					DQ185254
Nostoc punctiforme (Kützing) Hariot Gunnera manicata	130	Germany					DQ185256
Nostoc sp. 1	Liaimer et al. unpublished	Norway					EU022737
Nostoc sp. 2	Suzuki et al. unpublished						GU062468
Nostoc sp. 3	Suzuki et al. unpublished						GU062469
Nostoc sp. 4(root of plant)	126	Italy					AM711532
Nostoc sp. 5	134	South Africa					AJ344563
Nostoc sp. 6	Liaimer et al. unpublished	Norway					EU022709
Nostoc sp. 7	Liaimer et al. unpublished	Norway					EU022729
Nostoc sp. 8	Mishra et al. unpublished	strain PCC 7120					HM573458
Nostoc sp. 9	132	strain PCC 7906					AB325908
Nostoc sp. 10	Liaimer et al. unpublished	Norway					EU022713
Nostoc sp. 11	126	Italy					AM711549

Continued on next page

Table 1 – continued from previous page

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Nostoc sp. 12	135	Spain					HM623782
Pannaria aff. *leproloma* 1	17	Chile					EF174208
Pannaria aff. *leproloma* 2	17	Chile					EF174213
Pannaria andina 1 P.M. Jørg. & Sipman	17	Peru					EF174233
Pannaria andina 2 P.M. Jørg. & Sipman	17	Chile					EF536022
Pannaria araneosa (C. Bab.) Hue	17	New Zealand					EF174222
Pannaria athroophylla (Stirt.) Elvebakk & Galloway	17	Chile					EF174202
Pannaria cf. *allorhiza*	17	New Zealand					EF174206
Pannaria conoplea (Ach.) Bory	17	Norway					EF174221
Pannaria durietzii (P. James & Henssen) Elvebakk & D.J. Galloway	17	New Zealand					EF174227
Pannaria elixii P.M. Jørg. & D.J. Galloway	17	New Zealand					EF174230
Pannaria fulvescens (Mont.) Nyl.	17	New Zealand					EF174231
Pannaria isabellina 1 (Vain.) Elvebakk & Bjerke	17	Chile					EF174226
Pannaria isabellina 2 (Vain.) Elvebakk & Bjerke	17	Chile					EF174223
Pannaria obscura Müll. Arg.	17	Australia					EF174232
Pannaria patagonica (Malme) Elvebakk & D.J. Galloway	17	Chile					EF174204
Pannaria rubiginella P.M. Jørg.	17	Chile					EF536024
Pannaria rubiginosa (Thunb. ex. Ach.) Delise	17	Norway					EF174220
Pannaria sphinctrina Zahlbr.	17	Chile					EF174205
Parmeliella triptophylla (Ach.) Müll. Arg.	125	Norway					JN847361

Continued on next page

Table 1 – continued from previous page

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Peltigera aphthosa (L.) Willd.	130	Switzerland					DQ185253
Peltigera canina 1 (L.) Willd.	130	USA					DQ185230
Peltigera canina 2 (L.) Willd.	Liaimer et al. unpublished	Norway					EU022726
Peltigera didactyla (With.) J.R. Laundon	130	Poland					DQ185245
Peltigera evansiana Gyeln.	129	USA, Oregon					JQ007784
Peltigera leucophlebia 1 (Nyl.) Gyeln.	136	Finland					FJ815321
Peltigera leucophlebia 2 (Nyl.) Gyeln.	129	Svalbard					JQ007783
Peltigera malacea (Ach.) Funch	137	Finland					EF102280
Peltigera rufescens 1 (Weiss) Humb.	130	Germany					DQ185219
Peltigera rufescens 2 (Weiss) Humb.	130	Germany					DQ185215
Peltigera scabrosa Th. Fr.	Liaimer et al. unpublished	Norway					EU022727
Peltigera sp.	129	Argentina					JQ007785
Physma byrsaeum (Ach.) Tuck.	NEW	Reunion Island, R1 (LG)					KF704342
Physma byrsaeum (Ach.) Tuck.	NEW	Reunion Island, R2846 (LG)					KF704339
Protopannaria pezizoides (Weber ex. F.H. Wigg.) P.M. Jørg. & S. Ekman	124	Finland					DQ265953
Pseudocyphellaria gilva (Ach.) Malme	17	Chile					EF536023
Pseudocyphellaria sp.	125	USA California					JN847355
Pseudocyphellaria sp.	NEW	Reunion Island, R2332 (LG)					KF704332
Scytonema cf. *fritschii*	138	New Zealand					JN565281
Scytonema hyalinum 1 Gardner	139	USA					AF334698

Continued on next page

Table 1 – continued from previous page

Mycobiont species	Reference	Country of origin and voucher information	ITS	mtSSU	LSU	*RPB1*	cyanobacterial 16S
Scytonema hyalinum 2 Gardner	139	USA					AF334700
Scytonema sp. 1	140	Mexico					EU818953
Scytonema sp. 2	140	Costa Rica					EU818954
Scytonema sp.3	140	Costa Rica					EU818950
Stereocaulon fronduliferum I.M. Lamb.	124	New Zealand					DQ265951
Stereocaulon ramulosum Raeusch.	124	Hawaii					DQ265949
Sticta limbata (Sm.) Ach.	125	USA California					JN847351

2.2.2 Molecular Data

Well-preserved lichen specimens lacking any visible symptoms of fungal infection were selected for DNA isolation. Extraction of DNA followed the protocol of (Cubero et al., 1999). We sequenced the ribosomal nuclear loci ITS, using primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), and LSU with primers LR0R (Vilgalys and Hester, 1990) and either LR7 (Vilgalys and Hester, 1990) or LIC2044 (Kauff and Lutzoni, 2002), the mitochondrial ribosomal locus mtSSU, using primers SSU1 and SSU3R (Zoller et al., 1999), and part of the protein-coding gene *RPB1* with RPB1AF (Stiller and Hall, 1997) and RPB1CR (Matheny et al., 2002). We sequenced the 16S ribosomal region of the *Nostoc* symbiont of 25 of this set of Pannariaceae as well as 2 additional *Fuscopannaria leucosticta*, 2 additional *Physma* and 4 from two other genera (*Leptogium* and *Pseudocyphellaria*) belonging to the Peltigerales, using the two primer pairs fD1 (Weisburg et al., 1991) –revAL (Elvebakk et al., 2008) and f712 (Svenning et al., 2005)–rD1 (Weisburg et al., 1991). Amplicons were sequenced by Macrogen® or by the GIGA technology platform of the University of Liège.

2.2.3 Sequences Editing and Alignment

Sequence fragments were assembled with Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were subjected to megaBLAST searches (Wheeler et al., 2007) to detect potential contaminations. Sequences were aligned manually using MacClade version 4.08 (Maddison and Maddison, 2002). Ambiguous regions were delimited manually and excluded from the analyses. Substitutions and indels in ITS1 and ITS2 were so numerous that no unambiguous alignment could be realized; therefore ITS sequences were reduced to the less variable 5.8S portion.

2.2.4 Concatenation and Partitioning

Congruence of the four fungal loci was assessed by the comparison of single-locus phylogenetic trees produced with RAxML HPC2 version 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) as implemented on the CIPRES portal (Miller et al., 2010), looking for the best ML tree and boot- strapping with 1000 pseudoreplicates in the same run, using GTRCAT model and the default settings. No significant conflict with bootstrap values (BS)$\geq$ 70 was detected and we therefore concatenated the different loci. As several species are represented by sequences obtained from specimens collected in the different parts of the world, mostly with ITS, we further assembled a 3 loci dataset excluding ITS. We thus produced three matrices, two for a large sampling of the Pannariaceae including our target taxa (*Kroswia*, *Physma* and the undescribed species with a tripartite thallus), including the four loci 5.8S, mtSSU, LSU and *RPB1* or including only the latter three, and one with the *Nostoc* 16S data.

For the concatenated analysis of the four loci, we partitioned the data in different subsets to optimize likelikood. We used PartitionFinder (Lanfear et al., 2012) to choose the best partition and determine the best models for the different subsets. We used BIC as the criterion to define the best partition, and compared all models implementable in MrBayes (Ronquist and Huelsenbeck, 2003). The partition tested for the analysis on the four loci was composed of 6 subsets: *RPB1*, 1st codon position, *RPB1*, 2nd codon position, *RPB1* 3rd codon position, mtSSU, LSU, 5.8S. For the 16S analysis on *Nostoc*, we used MrModelTest version 2.3 (Nylander, 2004) to determine the best model.

2.2.5 Maximum Likelihood and Bayesian Phylogenetical Analyses

For each matrix, we produced the best likelihood tree and bootstrapped for 1000 pseudoreplicates in the same run using RAxML version 7.4.2 (Stamatakis, 2006; Stamatakis et al., 2008) with the default settings and the GTRCAT model. We further ran a Bayesian analysis using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Each analysis consisted of 2 runs of 3 heated chains and 1 cold one. We assessed the convergence using Tracer version 1.5 (Rambaut and Drummond, 2007) and stopped the runs after checking with AWTY (Nylander et al., 2008) that convergence was reached for each run and that tree topologies have been sampled in proportion of their true posterior probability distribution. The analysis for the family Pannariaceae was stopped after 15×10^6 generations, the analysis on *Nostoc* 16S after 37×10^6 generations.

2.2.6 Ancestral State Reconstruction

We reconstructed ancestral character states using SIMMAP version 1.5.2 (Bollback, 2006), with default settings, on the consensus Bayesian tree produced by the MrBayes analysis on the Pannariaceae 4 loci concatenated dataset, as well as on a subset of 20 trees (10 from each run of the Bayesian analysis) and with Mesquite version 2.75 (Maddison and Maddison, 2006, 2011) using the likelihood parameters and the default settings, calculating the average probabilities of the ancestral states based on the same subset of 20 trees. We also used BayesTraits version 1.0 (Pagel et al., 2004) on a set of 2 trees: the best tree produced by the ML analysis on the Pannariaceae 4 loci concatenated dataset and on the best tree of the concatenated analysis without 5.8S, as they were slightly different, to constrain some branches (ancestors) to be to a certain state. We compared the harmonic mean of the iterations, which is an approximation of the marginal likelihood of the model, calculating the Bayes Factor, which is twice the difference of likelihood between the models, with each state of ancestor, to see which state of the ancestor leads to the best likelihood of the model. A positive Bayes Factor suggests that the first character state tested has a better likelihood than the second one, and a Bayes Factor above 2 is considered significant (Bayestraits Manual, available at http://www.evolution.rdg.ac.uk/BayesTraits.html). We used reversible jump and a gamma hyperprior whose mean and variance vary between 0 and 10. We ran the

program for 50×10^6 iterations for each constrained state. The character reconstructed was the type of thallus, and the character states considered were tripartite, pannarioid bipartite and collematoid bipartite.

2.2.7 Topological Tests

We tested different tree topologies on the concatenated dataset of 4 loci for the Pannariaceae. We generated 8 constrained best trees with RAxML, with the same settings as above, and using the following constraints: (1) the 3 accessions of *Kroswia* forming a monophyletic group; (2) *Kroswia* as a monophyletic group basal to a group formed by *Fuscopannaria ahlneri*, *F. confusa*, *F. leucosticta* and *F. praetermissa*; (3) *Kroswia* as a monophyletic group basal to all accessions of *Fuscopannaria* except *F. sampaiana*; (4) all accessions of *Fuscopannaria* except *F. sampaiana* as basal to the *Physma* clade (which includes *Parmeliella borbonica*, the *Parmeliella mariana* group and the tripartite R969 in addition to all accessions of *Physma*) and the *Pannaria* clade (all *Pannaria* except the tripartite R969), to compare our results with the topology retrieved in Wedin et al. (2009) and Spribille and Muggia (2013); (5) the tripartite species annotated as the tripartite R969 as basal to a group formed by all accessions of *Parmeliella mariana* group and *Physma* resolved in the same clade; (6) all accessions of *Physma* as basal to all accessions of *Parmeliella mariana* group and the tripartite R969 in the same clade; (7) *Parmeliella borbonica* basal to all accessions of *Physma*; (8) all accessions of *Physma* basal to all accessions of *Parmeliella mariana* group including *Parmeliella borbonica* in the same clade.

We computed the likelihood of 100 trees (the best constrained tree, the best unconstrained tree and a random sample of 98 bootstrap replicate trees from the unconstrained analysis), estimating parameters on a NJ tree, using an HKY model with a gamma rate of heterogeneity and 4 gamma categories (parameters choice and methodology suggested by Schmidt 2009). We performed the 1sKH test (Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999; Goldman et al., 2000), the SH test (Shimodaira and Hasegawa, 1999) and the ELW test (Strimmer and Rambaut, 2002) on the constrained tree using TreePuzzle v. 5.2. (Schmidt et al., 2002). Due to its very low power (see for instance Pagel et al. 2004), we did not consider the results of the SH test.

2.3 Results

2.3.1 Molecular Data

We amplified ITS, mtSSU and *RPB1* for all 36 selected specimens, except one for *RPB1*. We amplified LSU for 21 specimens, all 15 negative results being resolved in a single clade comprising all accessions of *Physma*, the *Parmeliella mariana* gr. (*P. brisbanensis*,

P. mariana and *P. stylophora*), *Parmeliella borbonica* and the undescribed tripartite *'Pannaria'* R969 (here annotated the tripartite R969). Wedin et al. (2009) could amplify the LSU loci for three species of *Physma*, but, for unknown reasons, all our attempts to amplify LSU for this clade failed.

2.3.2 Matrix Assemblage and Concatenation

For the analysis on the Pannariaceae mycobiont, we could include the following newly sequenced specimens: 21 specimens with all 4 loci, 14 with 3 loci (lacking LSU) and 1 specimen with 2 loci (lacking LSU and RPB1). We added 46 taxa retrieved from GenBank to complete our sampling, 39 members of the Pannariaceae, and 7 outgroup taxa all belonging to the Peltigerales (3 Vahliellaceae, 1 Collemataceae, 1 Placynthiaceae, 1 Peltigeraceae). Those included either the 4 loci or a subset of them. Detailed information can be found in Table 1. For the 16S dataset on *Nostoc*, we produced 36 new sequences; we added 93 *Nostoc* sequences retrieved from GenBank, chosen either on the phylogenetic position of their fungal partner or their nucleotide similarity to our sequences, based on megaBLAST searches (Wheeler et al., 2007), and 14 outgroup sequences, belonging to other genera, to complete our sampling.

2.3.3 Partitioning and Model Selection

For the analysis on the Pannariaceae mycobiont, PartitionFinder divided the partition in 4 subsets: one composed of *RPB1* 1st and 2nd codon positions with LSU, one with mtSSU only, one with 5.8S only and one with *RPB1* 3rd codon position only. For the first subset, the model selected was GTR+I+G, as well as for mtSSU and *RPB1* 3rd codon position; for 5.8S, the model selected was K80+I+G. For the analysis on the *Nostoc* 16S dataset, the model selected was GTR+I+G.

2.3.4 Phylogenetic Analyses

The 50% Bayesian consensus tree of the analysis of the Pannariaceae mycobiont dataset comprizing 4 loci is presented in Figure 1, with the bootstrap values of the ML analysis and the Bayesian PP values written above the branches. The same consensus tree obtained with the 3 loci dataset is available in the Supplementary Material (Figure S1). The 50% Bayesian consensus tree of the analysis of the *Nostoc* 16S dataset is presented in Figure 2, with the bootstrap values of the ML analysis and the Bayesian PP values written above the branches.

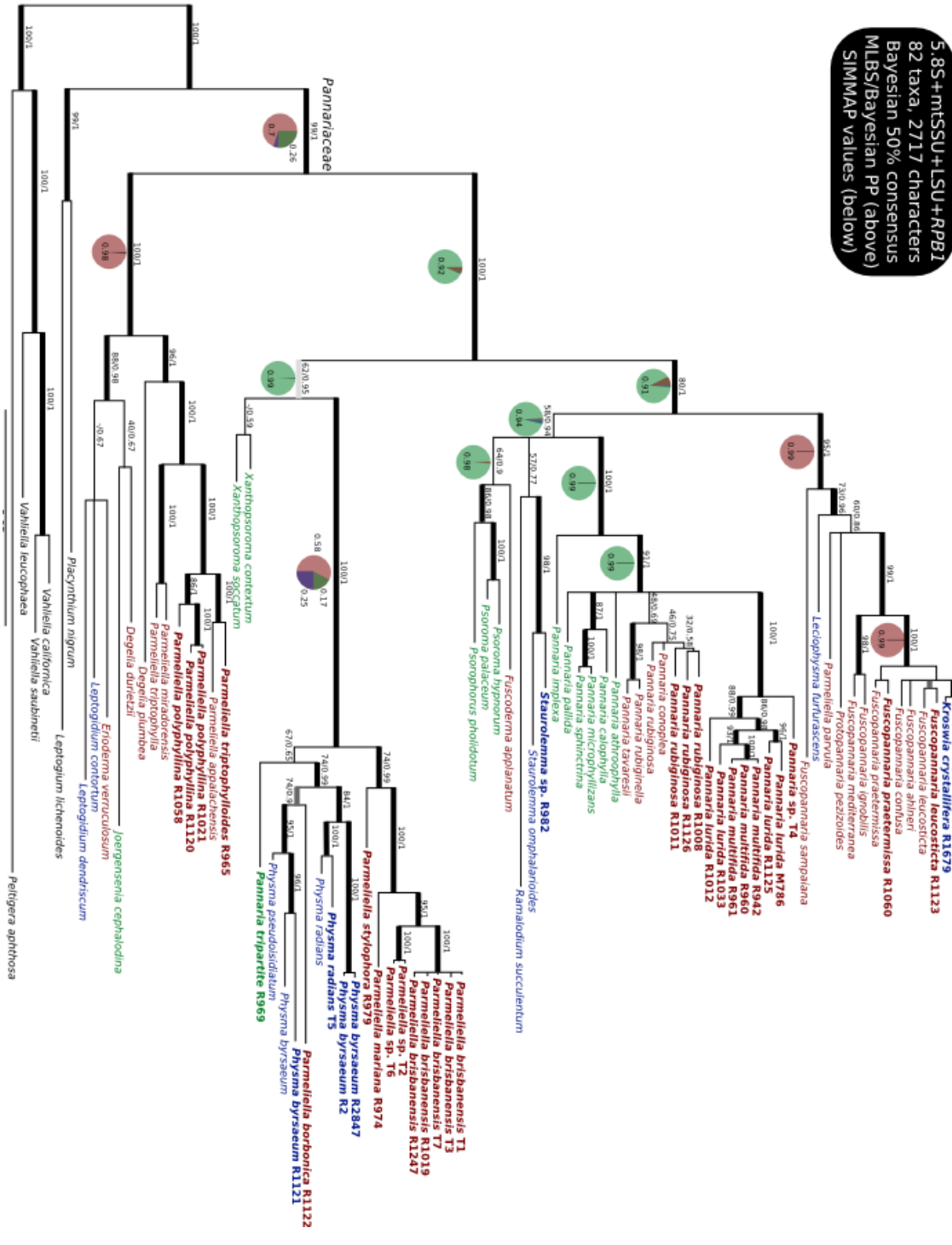

5.8S+mtSSU+LSU+RPB1
82 taxa, 2717 characters
Bayesian 50% consensus
MLBS/Bayesian PP (above)
SIMMAP values (below)
Pannariaceae
Kroswia crystallifera M788
Kroswia crystallifera R1055
Kroswia crystallifera R1679
Fuscopannaria leucosticta R1123
Fuscopannaria leucosticta
Fuscopannaria ahlneri
Fuscopannaria confusa
Fuscopannaria praetermissa R1060
Fuscopannaria praetermissa
Fuscopannaria pleurostigmella
Fuscopannaria ignobilis
Fuscopannaria mediterranea
Protopannaria pezizoides
Parmeliella parvula
Lecidiophysma furfuraceum
Fuscopannaria sampaiana
Pannaria sp. T4
Pannaria lurida M786
Pannaria lurida R1125
Pannaria multifida R942
Pannaria multifida R960
Pannaria multifida R961
Pannaria lurida R1033
Pannaria lurida R1012
Pannaria rubiginosa R1008
Pannaria rubiginosa R1126
Pannaria rubiginosa R1011
Pannaria conopoea
Pannaria rubiginosa
Pannaria rubiginella
Pannaria tavaresii
Pannaria athroophylla
Pannaria calophylla
Pannaria microphyllizans
Pannaria sphinctrina
Pannaria pallida
Pannaria implexa
Staurolemma sp. R982
Staurolemma omphalarioides
Ramalodium succulentum
Fuscoderma applanatum
Psoroma hypnorum
Psoroma palaceum
Psorophorus pholidotum
Parmeliella brisbanensis T1
Parmeliella brisbanensis T3
Parmeliella brisbanensis T7
Parmeliella brisbanensis R1019
Parmeliella brisbanensis R1247
Parmeliella sp. T2
Parmeliella sp. T6
Parmeliella mariana R974
Parmeliella stylophora R979
Physma byrsaeum R2847
Physma byrsaeum R2
Physma radians T5
Physma radians
Physma pseudoisidiatum
Physma byrsaeum
Parmeliella borbonica R1122
Physma byrsaeum R1121
Parmeliella tripartita R969
Xanthopsoroma contextum
Xanthopsoroma spoliatum
Parmeliella triptophylloides R965
Parmeliella appalachensis
Parmeliella polyphyllina R1021
Parmeliella polyphyllina R1120
Parmeliella polyphyllina R1058
Parmeliella miradorensis
Parmeliella triptophylla
Degelia plumbea
Degelia durietzii
Enoderma verruculosum
Joergensenia cephalodina
Leptogidium contortum
Leptogidium dendriscum
Placynthium nigrum
Leptogium lichenoides
Vahliella californica
Vahliella saubinetii
Vahliella leucophaea
Peltigera aphthosa

Figure 1: Phylogenetic relationships in the family Pannariaceae, based on the 50% Bayesian consensus tree of the analysis on 4 loci (5.8S, LSU, mtSSU, *RPB1*). Values above branches represent ML bootstrap and Bayesian PP values, respectively. Colors in the taxa names and pie charts represent the type of the thallus: in green tripartite thalli, in red pannarioid thalli and in blue collematoid thalli. Pie charts refer to the SIMMAP analysis on this tree. Names in bold are those for which DNA sequences were produced for this study. Thick black branches have MLBS≥70 and Bayesian pp≥0.95, dark grey branches have MLBS ≥70 but pp<0.95 and light grey branches have pp≥0.95 but MLBS<70.

2.3.5 Phylogeny of the Family Pannariaceae (Fig. 1)

Topology of the family. The analysis of the 3 and 4 loci datasets yielded the same topology, albeit with less support for some branches for the former; as expected the 5.8S loci provides an interesting resolution power to discriminate branches at the generic and infrageneric level. We retrieved the Pannariaceae as a monophyletic group, divided into two strongly supported clades: the first one includes all *Parmeliella* accessions, incl. the genus type *P. triptophylla*, except for the *P. mariana* group and *P. borbonica* which are resolved with strong support in the other clade. The so-called *Parmeliella* s. str. clade further includes *Degelia* (here resolved as polyphyletic, as already detected by Wedin et al. (2009)), *Erioderma*, *Leptogidium* and the monotypic *Joergensenia* which represents the only tripartite species in this clade. The second clade can be divided into three groups: (1) the first one is not supported in ML optimization but gets a PP = 0.95 in the Bayesian analysis; it is composed of *Xanthopsoroma*, *Physma*, the *Parmeliella mariana* group, *Parmeliella borbonica* and the tripartite species R969, and will be referred to as the *Physma* group; (2) a group not supported in ML optimization but getting a PP = 0.94 in Bayesian analysis, composed of *Pannaria*, *Staurolemma*, *Ramalodium*, *Fuscoderma*, *Psoroma* and *Psorophorus*, that will be referred to as the *Pannaria* group; and finally (3) a group composed of *Fuscopannaria*, *Kroswia*, *Protopannaria*, *Leciophysma* and *Parmeliella parvula*, that will be referred to as the *Fuscopannaria* group.

Wedin et al. (2009) and Spribille and Muggia (2013) retrieved the *Parmeliella* s. str. group, the *Pannaria* group and the *Fuscopannaria* group with similar topology as ours. However, in their studies, their single or multiple accessions of *Physma* is or are nested within the *Pannaria* group. With our dataset, which includes a larger sampling of *Physma* and representatives of the closely related *Parmeliella mariana* gr., *P. borbonic*a and the tripartite R969, the hypothesis of the whole *Physma* group nested in the *Pannaria* group and the *Fuscopannaria* group as basal is strongly rejected by two topological tests (ELW and 1sKH tests; see table 2).

2.3.6 Monophyly of Several Genera

Our accessions of *Kroswia crystallifera* (the type species of the genus; Jørgensen 2002 gathered in Madagascar and Reunion are not resolved as a monophyletic group: they are nested within *Fuscopannaria*, and closely related to its type species (Jørgensen,

Figure 2: Phylogenetic relationships in the genus *Nostoc*, based on the best ML tree of the analysis on the 16S dataset. Values above branches represent ML bootstrap and Bayesian PP values, respectively. Names in bold are those for which DNA sequences were produced for this study. Color boxes represent phylotypes containing our sequences and defined by well-supported monophyletic groups. Colors in the taxa names represent the type of the thallus containing the *Nostoc*: in green tripartite thalli, in red pannarioid thalli and in blue collematoid thalli. Taxa names refer to the host of the *Nostoc* symbionts, when available. Thick black branches have MLBS $\geq$70 and Bayesian pp$\geq$0.95, dark grey branches have MLBS $\geq$70 but pp$<$0.95 and light grey branches have pp$\geq$0.95 but MLBS$<$70.

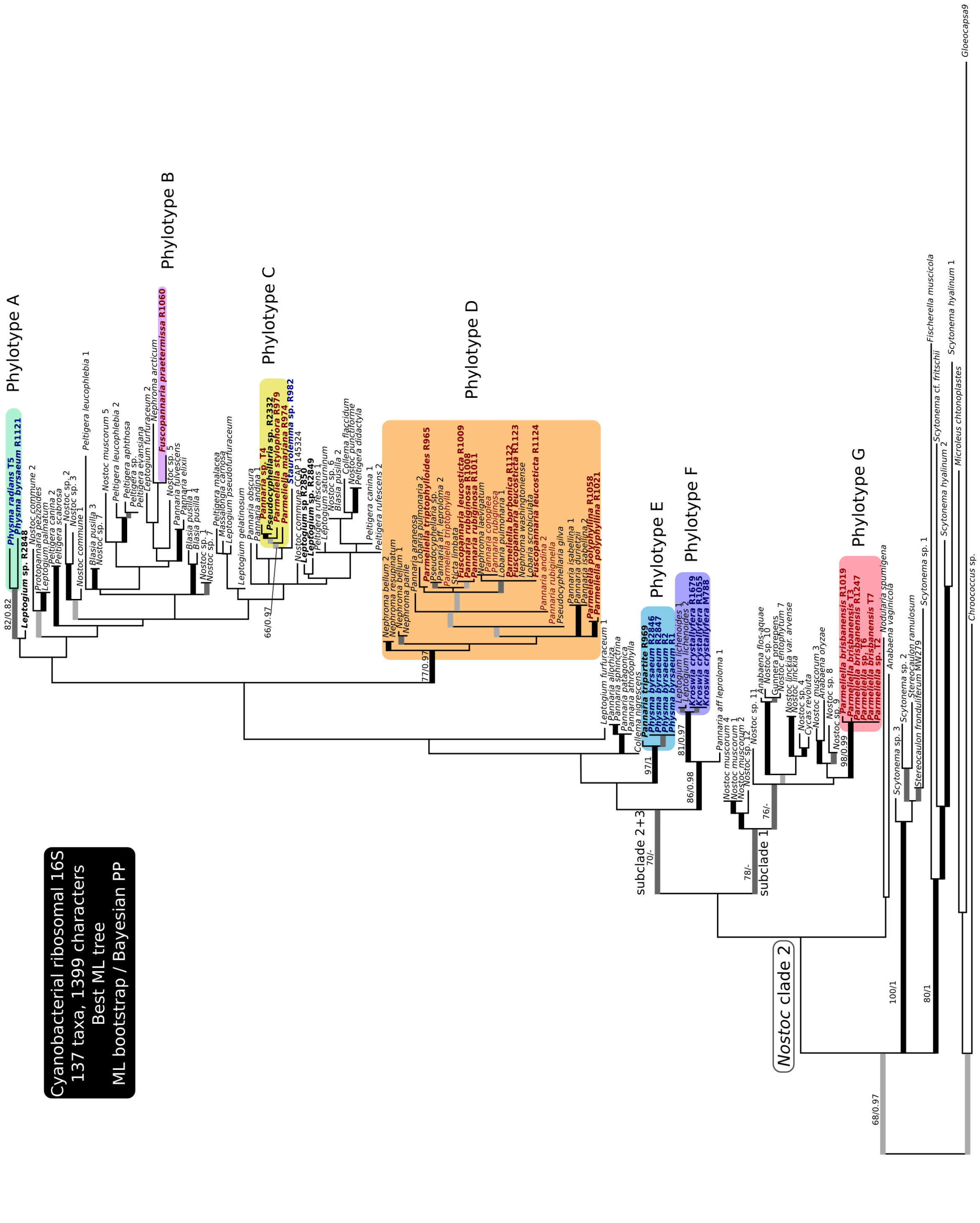
1994). Even with the exclusion of species now referred to Vahliella (Jørgensen, 2008; Wedin et al., 2011), the genus *Fuscopannaria* is not resolved as monophyletic, unless *F. sampaiana* is excluded and *Kroswia crystallifera* included. Two strongly supported clades can be distinguished if the genus is so recircumscribed: one with *F. ignobilis* and *F. mediterranea* and the other with the type species and *Kroswia crystallifera*.
Pannaria is resolved as a diverse but nevertheless well-supported genus, including several tripartite species formally placed in the genus *Psoroma* and which were transferred to *Pannaria* following the detailed studies by Elvebakk (2007, 2012a,b, 2013); Elvebakk and Bjerke (2005); Elvebakk and Galloway (2003); Elvebakk et al. (2010). Interestingly, our single accession of the tripartite *Pannaria*-like R969 is not resolved amongst other tripartite *Pannaria* but within the *Physma* clade with strong support. It therefore appears that the tripartite *Pannaria*-like species are more diverse than expected and that the tripartite habit is widespread amongst the *Pannariaceae*, being absent only in the *Fuscopannaria* group. Two recently described and tripartite genera *Xanthopsoroma* and *Psorophorus*, segregated from *Psoroma* (Elvebakk et al., 2010), are retrieved as a part of the *Physma* gr. with support only in the Bayesian analysis for the former, and as sister to *Psoroma* s. str. in the *Pannaria* group for the latter.

Parmeliella (type species: *P. triptophylla*) is a well-supported monophyletic group if the *Parmeliella mariana* gr., *Parmeliella borbonica* and *P. parvula* are excluded. The latter is resolved with strong support within the *Fuscopannaria* gr. whilst the others are resolved within the *Physma* group, on a long and strongly supported branch. Further, *P. borbonica* appears nested inside *Physma*, which is therefore paraphyletic.

2.3.7 *Nostoc* Phylogeny (Fig. 2)

We defined phylotypes (A to G) on the *Nostoc* tree based on well-supported monophyletic groups containing sequences from our representatives of the Pannariaceae family. All our sequences are part of *Nostoc* clade 2 (sensu Svenning et al. 2005; Otálora et al. 2010b) except phylotype G, which seems related to *Nostoc* clade 3 sensu Svenning et al. (2005).

There is no evidence suggesting coevolution or cospeciation events between the mycobiont and the photobiont. The phylogeny of *Nostoc* involved in the lichen symbiosis does not match the phylogeny of the Pannariaceae.


Cyanobacterial ribosomal 16S
137 taxa, 1399 characters
Best ML tree
ML bootstrap / Bayesian PP
Phylotype A
Phylotype B
Phylotype C
Phylotype D
Phylotype E
Phylotype F
Phylotype G
subclade 2+3
subclade 1
Nostoc clade 2

2.3.8 Topological Uncertainties (Table 2)

The tests do not reject the monophyly of *Kroswia*, either its position outside of the polytomy including i.a. *Fuscopannaria leucosticta* and *F. praetermissa*, although the difference of likelihood with the best unconstrained tree is relatively high (13.68). However, the position of *Kroswia* outside of *Fuscopannaria* s. str. (including *F. mediterranea* and *F. ignobilis*) is significantly rejected by the ELW and 1sKH tests. Therefore *Kroswia crystallifera* should be considered as part of *Fuscopannaria*.

Concerning the position of the tripartite R969, the topological tests do not reject its position at the base of the *Physma* group as a whole. However, its position at the base of the *Parmeliella mariana* gr., with *Physma* basal to both of them, is significantly rejected by the ELW and 1sKH tests.

Concerning the position of *Parmeliella borbonica*, the topological tests do not reject its position neither as basal to *Physma*, nor as basal to the *Parmeliella mariana* gr., with *Physma* basal to both of them, although the difference of likelihood for the latter case is relatively high (10.29). We consider that the weak resolution of the test regarding the position of *Parmeliella borbonica* might be due to a large amount of missing data as only 2 loci are available for this accession, reducing its impact on the likelihood of the trees. More material should therefore be studied before the taxonomic status of *P. borbonica* can be revised.

As commented above, we also tested the topology proposed by Wedin et al. (2009); Spribille and Muggia (2013) where their accessions of *Physma* are resolved within the *Pannaria* gr. Such a topology is rejected on our dataset by the ELW and 1sKH tests.

2.3.9 Reconstruction of Ancestral States (Fig. 1, Table 3)

Results of the SIMMAP reconstructions on the Bayesian consensus tree are shown in pie charts on Figure 1. Results of the BayesTraits and Mesquite reconstructions, as well as the SIMMAP reconstruction on 20 trees are shown in table 3.

Even though the probability values can vary quite widely from a reconstruction method to the other, the same ancestral character state is recovered for most branches.

For the *Fuscopannaria* group, a pannarioid ancestor is strongly supported, incl. for the *Fuscopannaria* s. str. clade (all *Fuscopannaria* except for *F. sampaiana*). Within the Pannaria group, two deep nodes are recovered with a tripartite ancestor (the unresolved clade with all accessions of *Pannaria*, and the clade including *Fuscoderma*, *Psoroma* and *Psorophorus*) as well as the node supporting the whole group. The node supporting both groups (the *Fuscopannaria* and the *Pannaria* gr.) also has tripartite thallus as the most likely ancestral type. For the clade comprizing *Physma*, the *Parmeliella mariana* gr., *P. borbonica* and the tripartite R969, reconstructions favor a pannarioid ancestor without much support, except the Bayes Factor that slightly favors

Table 2: Topology tests. Likelihood values of the best trees and results of the 1sKH test and ELW test on the different constraints on the topology of the tree. Results in bold significantly reject the concerned topologies.

Constraint	**logL best tree**	**diff. with unconstrained**	**1sKH test**	**ELW test**
Kroswia monophyletic	-19700.43	2.77	0.312	0.0816
Kroswia out of *F. leucosticta* group	-19711.34	13.68	0.145	0.0239
Kroswia out of *Fuscopannaria* s. str.	-19741.75	44.09	**0.002**	**0**
Physma group in *Pannaria* group, *Fuscopannaria* group basal	-19730.55	32.89	**0.019**	**0.011**
R969 basal out of *Physma*/*Parmeliella mariana* group	-19701	3.34	0.299	0.0816
Physma basal to R969/*Parmeliella mariana* group	-19731.4	33.75	**0.007**	**0**
R1122 basal to *Physma*	-19703.25	5.59	0.165	0.041
R122 basal to P. *mariana* group; *Physma* outside	-19707.95	10.29	0.094	0.018

a tripartite ancestor. However, for the whole group and thus including both accessions of *Xanthopsoroma*, reconstructions recover a tripartite ancestor with strong support. The node supporting the three groups (*Fuscopannaria*-, *Pannaria*-, and *Physma*-group) has most likely a tripartite thallus, as recovered by all four methods. The *Parmeliella* s. str. group most probably had a pannarioid ancestor, as well as the family Pannariaceae.

2.4 Discussion

2.4.1 *Nostoc* from Collematoid and Pannarioid Thalli (Fig. 2)

Thalli belonging to the collematoid or pannarioid types never share the same *Nostoc* phylotype. Phylotypes A, E and F only contain symbionts from collematoid thalli. Moreover phylotype F also contains symbionts associated with the lichen genus *Leptogium*, a typical representative of the collematoid type, these accessions being resolved in a strongly supported clade together with the *Kroswia* symbionts. Phylotype E includes the photobiont of several *Physma* accessions together with that of the cephalodia of the tripartite R969, and these cephalodia have the same homoiomerous structure as the thallus of *Physma byrsaeum* (Fig. 3a, c).

Phylotypes B, C, D and G only contain symbionts from pannarioid thalli. Phylo-

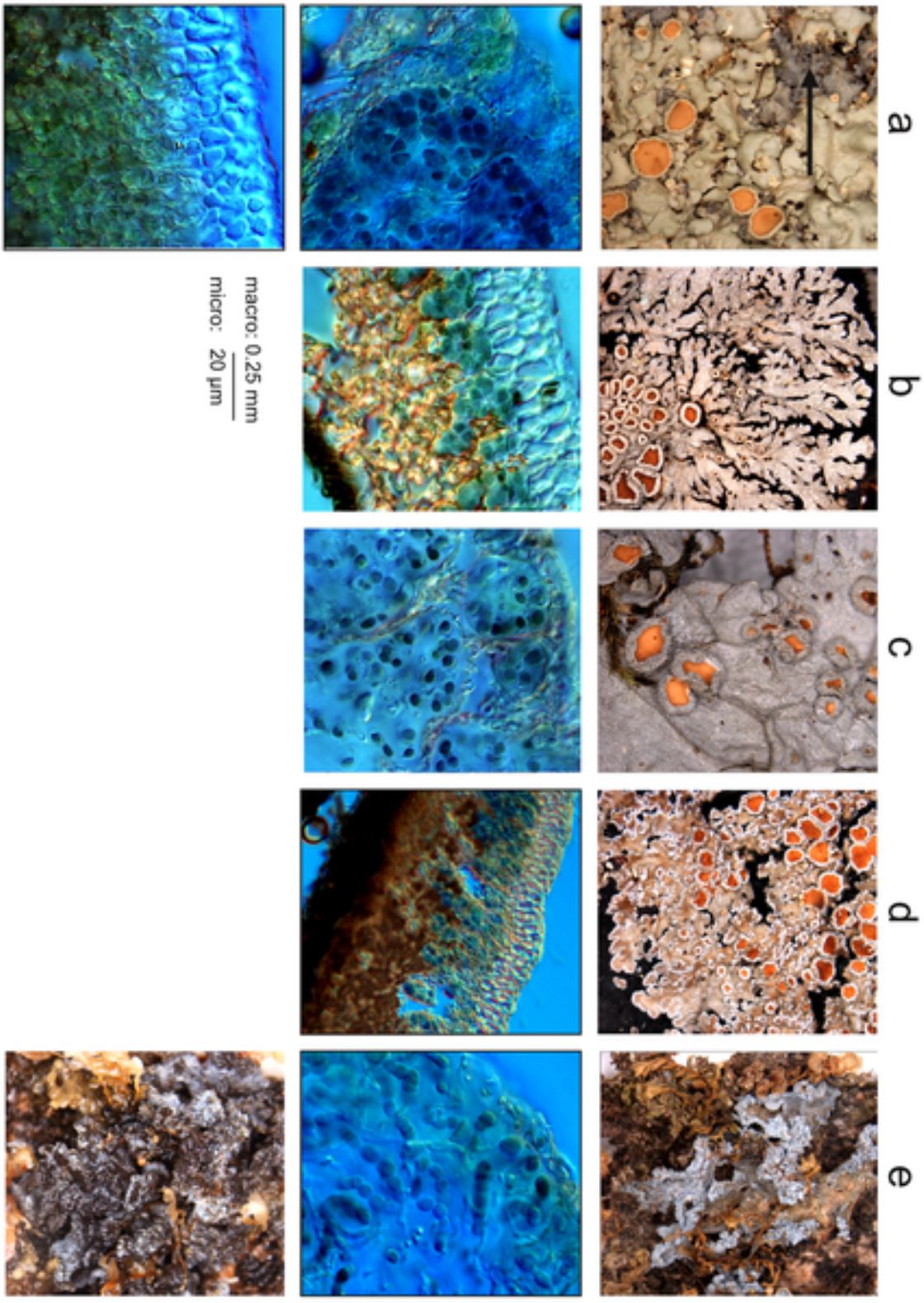

a
b
c
d
e
macro: 0.25 mm
micro: 20 µm

Figure 3: Selected pictures of studied Pannariaceae. Column, from left to right: a: tripartite R969, b: pannarioid *Parmeliella mariana*, c: collematoid *Physma byrsaeum*, d: pannarioid *Fuscopannaria leucosticta*, e: collematoid *Kroswia crystallifera*. Top row: macroscopic pictures showing the general aspect of the thallus; arrow point to cephalodia. Middle row: microscopic pictures showing the position of the *Nostoc* cells inside the thallus. Bottom row, left: Microscopic picture showing the position of the green algal cells in the thallus; right: macroscopic picture showing the aspect of *Kroswia* when wet.

Table 3: Reconstruction of ancestral states. T=tripartite, P=pannarioid, C=collematoid. SB=SIMMAP results on the 50% consensus Bayesian tree, S20 = SIMMAP results on the subset of 20 trees, M= Mesquite results, BF= Bayes Factor of the BayesTraits analysis, T>P= Tripartite rather than pannarioid ancestor, T>C=Tripartite rather than collematoid ancestor

Node	SB	S20	M	BF[T>P]	BF[T>C]
F. leucosticta + *F. praetermissa*	P=0.99	P=0.99	P=0.99		
Fuscopannaria s. str. (incl. *F. ignobilis*, wo *F. sampaiana*)	P=0.99	P=0.99	P=0.99		
Fuscopannaria group (incl. *F. sampaiana*)	P=0.99	P=0.97	P=0.73		
genus *Pannaria*	T=0.99	T=0.98	T=0.91	9.66	
genus *Pannaria* wo *P. implexum*	T=0.99	T=0.8	T=0.84		
Psoroma + *Psorophorus* + *Fuscoderma*	T=0.98	T=0.93	T=0.83		
Pannaria group (incl. *Psoroma*, *Staurolemma* etc.)	T=0.94	T=0.86	T=0.81		
Fuscopannaria + *Pannaria*	T=0.91	T=0.84	T=0.77	1.4	
Physma + *P. mariana*	P=0.58	P=0.5	P=0.39	0.32	3.94
Physma + *P. mariana* + *Xanthopsoroma*	T=0.99	T=0.99	T=0.91	11.7	8.7
Fuscopannaria + *Pannaria* + *Physma*	T=0.92	T=0.89	T=0.815	1.06	
Parmeliella s. str. Group (incl. *Erioderma* etc.)	P=0,98	P=0.99	P=0.87		
Family Pannariaceae	P=0.7	P=0.71	P=0.46		

type B which contains the photobiont of our accession of the terricolous *Fuscopannaria praetermissa* is closely related to sequences from terricolous-muscicolous *Nephroma arcticum* photobionts whereas phylotypes C and D contain Nostoc sequences from epiphytic *Lobaria*, *Nephroma* and *Pseudocyphellaria*, along with our accessions of epiphytic Pannariaceae with pannarioid thalli. This confirms that *Nostoc* from epiphytic heteroimerous thalli cluster together, although they group in a polyphyletic assemblage of different phylotypes (Elvebakk et al., 2008; Rikkinen et al., 2002; Rikkinen, 2003). These data strongly suggest that many pannarioid thalli share *Nostoc* strains between them and with other representatives of the Peltigerales that also have *Nostoc* in a well-defined thin layer. Furthermore collematoid thalli can share *Nostoc* with representatives of the Collemataceae that also have *Nostoc* chains throughout their thallus.

These results strongly suggest that the thallus type (collematoid versus pannarioid), and the organization of the *Nostoc* cells inside it, depend on the phylotype of the *Nostoc* with which the mycobiont associates. Therefore, it seems that in the family Pannariaceae, the *Nostoc* associated with the mycobiont would have more impact on the morphology of the thallus formed than the phylogenetic origin of the mycobiont. The corollary might be true as well, the *Nostoc* selection by the mycobiont is more affected by the morphological and ecophysiological characteristics of the association than by the phylogenetic position of the mycobiont. Extracellular polysaccharides substances (EPS) produced by many bacterial lineages, incl. cyanobacteria, are involved in the physiological and ecological characteristics of those organisms (Whitton and Potts, 2000); in *Nostoc*, the biochemistry and structure of the dense sheath of glycan strongly participate in the dessication tolerance of *Nostoc commune* (Hill et al., 1994). Although no clear evidence is available, we suspect that variations in the glycan sheath characteristics amongst the various strains of *Nostoc* involved in the lichenization events within the Pannariaceae drive the differences between the collematoid and the pannarioid thallus types.

2.4.2 Occurrence of Collematoid Thalli All across the Pannariaceae (Fig. 1)

We found collematoid thalli in the four main groups of the family. *Kroswia* and *Leciophysma* appear as part of the *Fuscopannaria* group, *Kroswia* being nested within *Fuscopannaria* s. str., excluding *F. sampaiana*; *Staurolemma* and *Ramalodium* are part of the *Pannaria* group and *Pannaria santessonii* was described as a collematoid thallus species; *Physma* is in the *Physma* group, along several taxa with pannarioid thalli; and finally *Leptogidium* is part of the *Parmeliella* s. str. group. These results suggest that thalli switched from pannarioid type to collematoid and possibly vice versa several times along the evolutionary history of the family.

These results also suggest that the thallus type organized by the association between a mycobiont and a photobiont is primarly driven by the identity of the latter, the *Nos-*

toc phylotype with which it associates rather than by the phylogenetic identity of the mycobiont. Indeed, unlike the original assumption that all collematoid thalli were part of the Collemataceae and all pannarioid thalli were part of the Pannariaceae, many collematoid thalli are actually members of the Pannariaceae, as already detected by Wedin et al. (2009); Otálora et al. (2010a). Moreover, they do not form a monophyletic group inside the Pannariaceae, but are present all across the family, suggesting the absence of phylogenetic pattern of the mycobiont related to the collematoid morphological and anatomical thallus type.

2.4.3 Evidence for Coincidence between Photobiont Switch and Change of Thallus Type

The most spectacular and straightforward example lies with the type species of *Kroswia* which is nested inside *Fuscopannaria* s. str.: it exhibits a drastic change of morphology (see figure 3d–e) of the thallus (all representatives of this genus so far have typical pannarioid thalli), and it associates with a *Nostoc* phylotype (phylotype F) that is totally different from the one associating with the closely related *Fuscopannaria leucosticta* (phylotype D). Moreover, phylotype F has also been found associated with the typically collematoid *Leptogium lichenoides*. The duo *Kroswia/Fuscopannaria* thus provides the best example of the influence of the *Nostoc* on the shape of the thallus. Actually, *K. crystallifera* is a species of *Fuscopannaria* with little genetic divergence with its related species such as *F. leucosticta* and *F. praetermissa*; this divergence however precludes any assumption that it could be considered as a photomorph of one of them. Its thallus is dramatically different because it switched to a different *Nostoc*, one that triggers the collematoid format for the thallus. Jørgensen (2007a), when studying the apothecia characters of the other species assigned to that genus (*K. gemmascens*), concluded that "the characters of the hymenium and the chemistry of the thallus certainly place it close to *Fuscopannaria* (...)". Quite interestingly another photobiont switch can be postulated in that group as the phylogenetic position of *Moelleropsis nebulosa* as sister to *F. leucosticta* has been retrieved by Ekman and Jørgensen (2002) and more recently announced as confirmed (Jørgensen et al., 2013). This species exhibits granulose thalli with clusters of *Nostoc* interwoven and covered by short-celled hyphae and very much different from the pannarioid thallus type, and thus most probably associated with a different *Nostoc* phylotype.

2.4.4 Occurrence of Tripartite Thalli All across the Pannariaceae (Fig. 1)

We could detect tripartite thalli in all main groups within the family, except in the *Fuscopannaria* group. This absence might be caused by incomplete sampling as the only tripartite species known in *Fuscopannaria* (*F. viridescens*, associated with a green algae and producing cephalodia; Nelson and Wheeler 2013) as well as both species

of *Degeliella* (forming tripartite thalli; Jørgensen 2004) could not be included in our dataset. *Psoroma*, *Psorophorus* and the tripartite representatives of *Pannaria* are resolved in the *Pannaria* group, *Xanthopsoroma* and the tripartite R969 belong to the *Physma* group, and the characteristic *Joergensenia* is included in the *Parmeliella* group. Until the seminal papers by Elvebakk and Galloway (2003); Passo et al. (2008), all tripartite Pannariaceae were assigned to a single genus (*Psoroma*) assumed to form a monophyletic group. Within the three main groups of the Pannariaceae where they are resolved, the species with tripartite thalli are mixed up with species with bipartite thalli, mainly of pannarioid type but also with collematoid type. These results suggest that several times through the history of the family, mycobionts switched from a tripartite to a bipartite thallus or vice versa.

2.4.5 Evidence for Cephalodia Emancipation

Switches from a tripartite to a bipartite thallus may involve the cephalodia and their emancipation from their green algae-containing thalli. Although cephalodia are usually associated with rather small, firmly attached, or even included, structures, there are many examples of tripartite *Pannaria* and *Psoroma* in which cephalodia are large and easily detached, or proliferating and developing large squamules that can be easily detached from their "host" thalli (examples in Elvebakk 2007; Elvebakk et al. 2008; Jørgensen and Wedin 1999; Passo et al. 2008). The cephalodia of the tripartite R969 start their development as modest blue gray squamules over the thallus, but eventually grow up to 0.7 cm across and develop a foliose habit with denticulate to deeply lobulate margin (see figure 3a).

More interestingly, the *Nostoc* photobiont in several accessions of *Physma byrsaeum* (annotated R1, R2, R2846 and R2847; phylotype E) is very closely related to the one found in the cephalodia of the tripartite R969. As the latter is basal to the clade containing all accessions of *Physma*, it can be postulated that several species belonging to this genus arose from cephalodia emancipation from their common ancestor. Indeed, the common ancestor of the whole *Physma* clade is recovered as producing tripartite thallus. Furthermore, the disposition of the *Nostoc* cells inside the cephalodia of R969 is similar to the one inside *Physma* thalli (see figure 3a–c): they are enclosed in ellipsoid chambers delimited by medulla hyphae, these structures being responsible for the maculate upper surface of thalli (*Physma*) or cephalodia (R969).

Besides the tripartite R969, the clade included both accessions of the recently described genus *Xanthopsoroma* (Elvebakk et al., 2010), which also develops tripartite thalli, with a green algae as the main photobiont and *Nostoc* included in cephalodia. The three species recognized within the *Parmeliella mariana* gr. may have arisen from cephalodia emancipation of their common tripartite ancestor or from a photobiont switch from a *Physma* ancestor. Quite interestingly, the pannarioid *Parmeliella borbonica*, nested within *Physma*, is associated with phylotype D of *Nostoc*, shared by most accessions of the *Pannaria* and *Parmeliella* s. str. groups (as well as other distantly

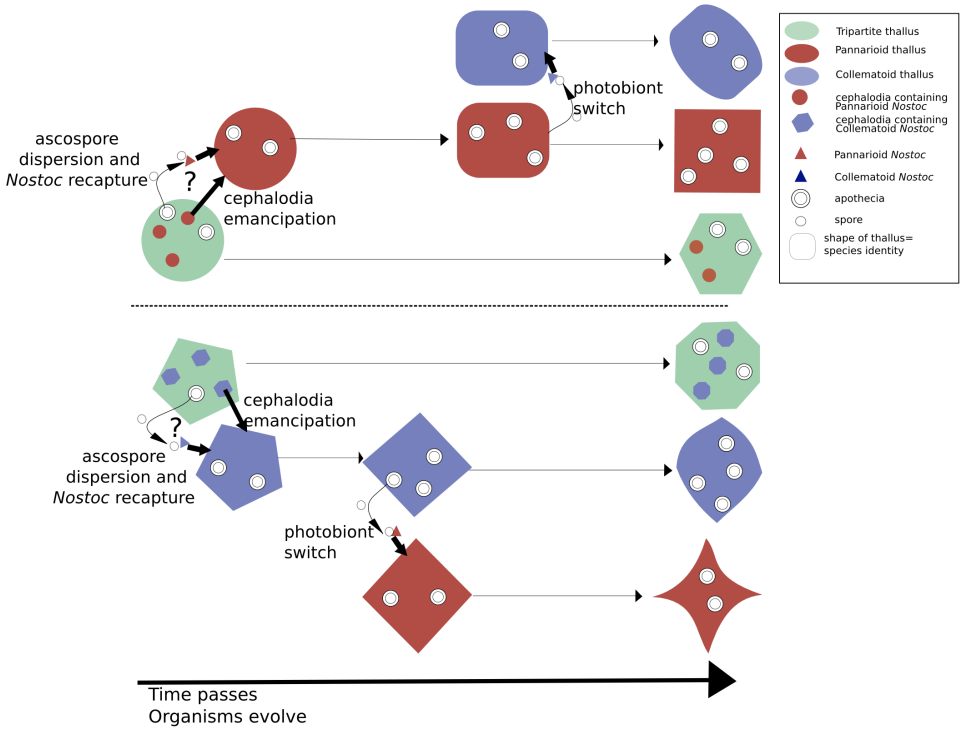


Figure 4: Scheme showing the different scenarios for switching from tripartite to bipartite thallus, and from collematoid to pannarioid thallus and vice versa. Changes in color represent the change of the thallus type. Changes in the shape of the thalli represent the phylogenetic divergence of the different thallus types.

related species of the Peltigerales), and not phylotypes C or G, chosen by all our accessions of its closely related species of the *Parmeliella mariana* gr. When excluding both accessions of *Xanthopsoroma*, the *Physma* gr. is a well-supported clade on a long branch and includes a tripartite species, species with pannarioid as well as collematoid thalli. The long branch may indicate that our sampling is too scarce and geographically too restricted. However, as both *Physma* and the *Parmeliella mariana* gr. have a pantropical distribution, we can confidently assume it would not collapse in future studies.

In figure 4, we illustrate the different possible scenarios to switch from tripartite to bipartite, and from collematoid to pannarioid thalli and vice versa, and emphasize on the possibility to obtain, with switches and time, the three types of thalli from the same tripartite ancestor.

As a matter of fact, earlier workers came close to the conclusion that cephalodia can emancipate and start their own evolutionary trajectory. Ekman and Jørgensen (2002) pointed to the "homology" between the cephalodia of the green algae-containing *Psoroma hypnorum* and the thallus of the cyanobacterial autonomous species *Santes-*

soniella polychidioides; Passo et al. (2008) retrieved the latter as sister to *Psoroma aphthosum*, a green algal species with coralloid-subfruticose cephalodia, very much akin the thallus of *Santessoniella polychidioides*. We strongly suspect this case represents a further case of cephalodia emancipation, and subsequent divergence. This scenario implies that emancipated cephalodia can reproduce sexually as most species of *Physma* and *Santessoniella polychidioides* produce apothecia and well-developped ascospores. There is indeed no reason to believe that thalli newly formed by cephalodia emancipation and containing only *Nostoc* as photobiont would not be able to produce apothecia, as only the mycobiont is involved in such formation. An interesting alternative would be that, when expelled out of the ascus, the ascospore produced by the mycobiont involved in the ancestral tripartite thallus, would collect or recapture the *Nostoc* of the cephalodia.

Several representatives of the Lobariaceae produce photomorphs, mainly within the genera *Lobaria* and *Sticta* (Magain et al., 2012; Moncada et al., 2013). These duos involving the same fungus lichenized either with a green algae or with a *Nostoc* comprize thalli morphologically rather similar or not (see Introduction), and living attached (thus forming tripartite thalli) or not. Although molecular studies on these duos have mainly sought to demonstrate the strict identity of the fungus involved in each part, the separation or "living apart" of one from the other has long been recognized in several taxa, such as *Lobaria amplissima* and its cyanomorph *Dendriscocaulon umhausense* and *Sticta canariensis* and its cyanomorph *S. dufourii* (James and Henssen, 1976). There is a priori no reason to exclude that the duos can separate on "a permanent basis" and thus emancipate; each morph would eventually run its own evolutionnary trajectory, as recently suggested for divergence patterns in *Sticta* photomorphs (Moncada et al., 2014). Such a scenario can be interpreted as a variant of cephalodia emancipation as advocated here for the evolution of thallus types within the Pannariaceae.

The alternative scenario for the complex phylogenies including bi- and tri-partite thalli implies that a cyanolichen would capture a green algae from the environment (or from another lichen), adopt it as its main photobiont and confine its Nostoc into cephalodia. This hypothesis has been suggested by Miadlikowska and Lutzoni (2000) for the sect. *Peltidea* in the genus *Peltigera* but so far has not been confirmed. Our data and reconstruction of ancestral state do not support it in the Pannariaceae, with a possible exception for *Joergensenia* cephalodina, but a better sampling is needed in that group to reconstruct the ancestral states.

2.5 Conclusions and Perspectives

Field observations of the lichen species belonging to the widespread and well-known order Peltigerales on the tiny and remote island of Reunion in the Indian Ocean instigated our studies on the relationships between photomorphs in the Lobariaceae (Magain et al., 2012) and the present study on the Pannariaceae. Indeed, we were intrigued by

the occurrence, several times at the same locality or even on the same tree, of representatives of that family with collematoid and pannarioid thalli, and more locally of tripartite thalli.

Collematoid and pannarioid thalli are represented throughout the Pannariaceae. Each thallus type mostly appears mingled within complex topologies. Switches between those thallus types are thus frequent throughout the family. We could demonstrate that both collematoid genera in the Pannariaceae we examined from Reunion material (*Kroswia* and *Physma*) are involved in photobiont switches. We suspect that such a scenario could be detected elsewhere in the Pannariaceae and may act as an important evolutionary driver within the whole family, and perhaps elsewhere within the fungi lineages containing lichenized taxa.

The tripartite thallus type is shown to be the ancestral state in the clade we could study (the *Physma* gr.). Although a larger sampling is needed before such an result could be confirmed, we can postulate that cephalodia emancipation and subsequent evolutionary divergence is the most likely scenario within that clade. The data available support the same scenario in other clades of the Pannariaceae, and it can be suspected in the Lobariaceae where it is represented by the separation and subsequent divergence of photomorphs.

The photomorph pattern in the Lobariaceae demonstrates that a single mycobiont can recognize and recruits phylogenetically unrelated photobiont partners and these associations result in morphologically differentiated thalli. We show here that the use of different lineages of *Nostoc* or the association with only one partner instead of two might lead to the same consequences. Recognition of compatible photobiont cells is carried out by specific lectins produced by the mycobiont, characterized by their ligand binding specificity (Galun and Kardish, 1995). *Peltigera* species have served as models in the studies of lectins and their involvment in the recognition of symbiotic partners (Lockhart et al., 1978; Petit et al., 1983; Díaz et al., 2011; Rikkinen et al., 2013). A lectin detects compatible *Nostoc* cells at the initiation of cephalodium formation in *P. aphthosa* and this process is highly specific (Lehr et al., 2000), as further demonstrated by experiment of inoculation of several *Nostoc* strains into the cephalodia of the same species (Paulsrud et al., 2001). The biochemical process sustaining the recognition of both partners in two lichen species associated with green algae has been elucidated by Legaz et al. (2004) and extended to cyanolichens with collematoid thalli by Vivas et al. (2010). The genes coding for two lectins assumed to be involved in photobiont recognition have recently been identified (Manoharan et al., 2012; Miao et al., 2012). Evaluation of the variation of those genes is of tremendous interest in the context of photobiont switching and cephalodia emancipation as lectins have been shown to be under selection pressure by the symbionts in corals (Hayes et al., 2010; Iguchi et al., 2011)and a coevolutionary process could thus be highlighted and demonstrated in lichenized fungi. A preliminary study with *Peltigera membranacea* material from Iceland could demonstrate a significant positive selection in LEC-2 but not due to variation in photobiont partner (Manoharan et al., 2012).

Further research should thus assemble larger dataset of tripartite taxa within the Pannariaceae and reconstruct their evolutionary history, especially as to the fate of their cephalodia. Numerous methods for detecting genes under positive selection are available (Aguileta et al., 2009) and could be applied to the Pannariaceae. Genomics studies of lectins associated with photobiont recognition on tripartite taxa as well as those involved in obvious photobiont switches (pannarioid to collematoid and vice versa) could therefore bring to light a nice model of coevolution (Thompson, 2005).

The taxonomical consequences of these results are published in a companion paper, dedicated to new taxa and new combinations.

2.6 Acknowledgments

Field studies in Reunion were made possible with the help and advice from the "Parc National de La Réunion", especially through the courtesy of Mr B. Lequette. Dr Cl. Ah-Peng and Prof. D. Strasberg of the University of La Réunion in Saint-Denis and Dr. J. Hivert of the Conservatoire Botanique National de Mascarin (St-Leu) were also very helpful. A first field trip to Reunion in 2008 was conducted with our colleagues and friends Maarten Brand and Pieter van den Boom. The field trip to Madagascar was organized with the logistical support of the "Parc Botanique et Zoologique de Tsimbazaza" in Antananarivo, and with collecting and export permits of scientific material issued by the "Ministère des Eaux & Forêts"; it was organized with our colleagues and friends Damien Ertz, Eberhard Fischer, Dorothee Killmann and Tahina Razafindrahaja. We thank them all very warmly. We further thank the curators of the following herbaria for the loan of type collections or relevant material: H, US. Field trip in Thailand was organized as an IAL post-symposium excursion by the Ramkhamhaeng University, and we warmly thank the Lichen Research Unit and the organizers of the field trip, Kawinnat Buaruang, curator of the herbarium, Wetchasart Polyiam and Pachara Mongkolsuk. We also thank Theerapat Luangsuphabool for his assistance during the field work. We further thank Mr I. Cremasco and L. Gohy for technical assistance in the molecular laboratory and herbarium at the University of Liège. Finally we thank Dr. Heath O'Brien and an anomymous referee for their critical and helpful notes and suggestions.

2.7 Data Accessibility

All newly produced sequences are deposited in GenBank.

All matrices used in the analyses are deposited in Treebase.

2.8 Supporting Information

Figure S1: Phylogenetic relationships in the family Pannariaceae, based on the best ML tree of the analysis on 3 loci (LSU, mtSSU, RPB1). Values above branches represent ML bootstrap.

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Chapter 3

The lichen genus *Kroswia* is a synonym of *Fuscopannaria* (Pannariaceae)

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Accepted for publication in The Lichenologist.

Abstract

Molecular inferences of three loci within a phylogenetic framework of a subset of the Pannariaceae confirm that the genus *Kroswia* is nested within the genus *Fuscopannaria*. The formal combination of the type species of *Kroswia* into *Fuscopannaria* is therefore made here, and *Kroswia* is reduced into synonymy with the latter genus.

Key Words: Peltigerales, ascomycota, taxonomy, morphology, *Nostoc*, cyanolichens

3.1 Introduction

A persistent question in the Pannariaceae, a well-known and cosmopolitan lichen family, lies with the assignment of taxa with collematoid thalli, that swell considerably and form gelatinous masses when wet and quickly return to a crispy and fragile form when dry, unlike typical members of the family that develop a "pannarioid" thallus that does not swell when wet (Wedin et al., 2009; Otálora et al., 2010). Species in the genus *Kroswia* P.M. Jørg. develop thalli of the former type, typically homoiomerous with indistinct cortex, the photobiont forming chains of cells with much swelling shealths and present thoughout the thallus ; species with typical " pannarioid " thallus such as in the genus *Fuscopannaria* P.M. Jørg. develop heteromerous thalli with a distinct upper cortex and a very distinct photobiont layer with photobiont cells compacted and assembled in clusters.

A further interesting matter within the same family is the occurrence of tripartite thalli, which are lichenized with green algae but further produce well differentiated structures, usually referred to as cephalodia, which are lichenized with cyanobacteria usually belonging to the genus Nostoc. Such cephalodia may develop thalloid forms, sometimes producing breaking off fragments that act as vegetative diaspores (Jørgensen and Wedin, 1999), or may resemble autonomous entities recognized as a different genus, namely *Santessoniella* Henssen.

A recent study conducted by the authors (Magain and Sérusiaux, 2014) could provide strong support for two interesting evolutionary patterns within that family. Indeed, a photobiont switch between two different strains of *Nostoc* is suspected to be the driver for the change in thallus type (pannarioid thallus switch to collematoid type) within a strongly supported clade comprizing the genera *Fuscopannaria*, *Kroswia*, *Leciophysma* Th. Fr. and *Protopannaria* (Gyeln.) P.M. Jørg. & S. Ekman. Photobiont switches have been shown or are suspected to play a crucial role in speciation processes of lichens (examples in Baloch and Grube 2006; Nelsen and Gargas 2008; Fernández-Mendoza et al. 2011; Printzen et al. 2013) and the molecular inferences in a phylogenetic context do support such a scenario for the genus *Fuscopannaria*.

Further, cephalodia emancipation from ancestral tripartite thalli followed by divergence is supported by the data and may represent an evolutionary pattern present throughout the family ; it may explain the morphological resemblance between the thalli of several genera with cephalodia of others as well as the complex phylogenetical relationships between species with tripartite thalli and others with collematoid or pannarioid thalli. A convincing example of this evolution pattern is provided by the free living *Santessoniella polychidioides* (Zahlbr.) Henssen, lichenized with *Nostoc*, which is nested with strong support within the tripartite genus *Psoroma* Ach. ex Michx. (Ekman et al., 2014) and can be interpreted as emancipated cephalodia of its tripartite ancestor that eventually diverged.

This study aims to confirm the findings by Magain and Sérusiaux (2014) that the collematoid genus *Kroswia* is nested in *Fuscopannaria* and resolve their relationships by producing a phylogenetic tree including all data available in *Fuscopannaria*. As three accessions of its type species (*K. crystallifera* P.M. Jørg.) are found nested within *Fuscopannaria* with strong support, the taxonomical and nomenclatural conclusions are drawn in this paper.

3.2 Material and Methods

All sequences used in the phylogenetic analyses were downloaded from GenBank (Table 1). Those produced by Ekman et al. (2014) in a revised classification of Pannariaceae and Magain and Sérusiaux (2014) for the taxa dealt with in this paper are thus included. We assembled a concatenated matrix of three loci: mtSSU, nuLSU *and* RPB1 using

MacClade v. 4.08 (Maddison and Maddison, 2002). Ambiguously aligned positions were delimited by eye and excluded from the phylogenetic analyses. The alignment was divided in six subsets: mtSSU, nuLSU, *RPB1* 1st, 2nd and 3rd codon positions, and the intron in *RPB1*. The best partition for the dataset was estimated using PartitionFinder (Lanfear et al., 2012) using AICc as a criterion and testing all models available with the greedy algorithm. The partition selected consisted of 5 subsets: LSU and the 1st codon of *RPB1* together, and every other subset by itself.

We produced a best ML tree using RaxML-HPC2 v. 8.0.24 (Stamatakis, 2006; Stamatakis et al., 2008) as implemented on the CIPRES portal (Miller et al., 2010) using the GTRGAMMA model and 1000 bootstrap iterations. A Bayesian analysis was performed using MrBayes v. 3.2.2 (Huelsenbeck et al., 2001) as implemented on the CIPRES portal, running for 20 million generations with 2 runs of 3 cold chains and 1 heated chain each, and sampling every 1000th generation. The first 25% of the trees sampled were discarded as burn-in, and a 50% consensus tree was produced using the remaining trees. Convergence of the analyses was assessed using Tracer v. 1.5. (Rambaut and Drummond, 2007) and AWTY (Nylander et al., 2008) as implemented on the website http://king2.scs.fsu.edu/CEBProjects/awty.

3.3 Results

The phylogenetic tree (Fig. 1) presented here is the Bayesian 50% consensus tree with evaluation of branch support from the Maximum Likelihood results and the Posterior Probabilities of the Bayesian search ; 2666 characters from four loci (5.8 S, mtSSU, nuLSU and *RPB1*) are included for 42 accessions representing 38 taxa.

As in earlier studies (Ekman et al., 2014; Magain and Sérusiaux, 2014), the genus *Fuscopannaria* is retrieved as a monophyletic group, divided into two strongly supported clades, pending that *F. sampaiana* (Tav.) P.M. Jørg. is assigned to a different genus (*Nevesia*: Ekman et al. 2014 and with the exception of *F. laceratula* (Hue) P.M. Jørg. which is resolved within a strongly supported and related lineage comprizing *Protopannaria pezizoides* P.M. Jørg. & S. Ekman. The first clade within *Fuscopannaria* includes i.a. the type species (*F. leucosticta* (Tuck.) P.M. Jørg.) and the three accessions of *Kroswia crystallifera*, whilst the second one includes i.a. the monotypic genus *Moelleropsis nebulosa* (Hoffm.) Gyeln..

Synonymy of *Kroswia* and new combination in the genus *Fuscopannaria*
The phylogenetic relationship of *Kroswia crystallifera*, the type species of *Kroswia*, is similar to *Moelleropsis nebulosa*: albeit the overall morphology strongly deviates from the typical pannarioid thallus type of all species assigned to that genus, the molecular data leave no doubt that both species must subsumed into *Fuscopannaria* (Ekman et al., 2014; Magain and Sérusiaux, 2014). Data on apothecial characters provided by

Figure 1: 50% consensus tree resulting from the Bayesian analysis of mtSSU, LSU and *RPB1* on representatives of the family Pannariaceae. The genus Fuscopannaria is highlighted. Rooting follows Magain and Sérusiaux (2014). Thick black branches have a pp ≥ 0.95 and a Maximum Likelihood Bootstrap ≥ 70. Thick grey branches have a pp ≥ 0.95 but MLBS < 70.

Jørgensen (2007a) on another species (*K. gemmascens* (Nyl.) P.M. Jørg.) referred to that genus are congruent : hymenium I+ blue-green rapidly turning red-brown, asci with amyloid ring structure. Two characters of the ascospores are deviating as they are globose and pale brown in *K. gemmascens*, while they are ellipsoid without any color in *Fuscopannaria leucosticta*, *F. praetermissa* (Nyl.) P.M. Jørg. and other related species (Jørgensen, 2007b). Further they lack a perispore, which makes a difference for many species of *Fuscopannaria*. The value of these characters have never been tested in a phylogenetic context, and they are thus difficult to interpret. They might be species-specific within the genus, or represent autapomorphies for a further generic entity within the *Fuscopannaria* clade. By all means, the genus has no close relationship with the *Pannaria lurida* (Mont.) Nyl. gr. as previously assumed (Jørgensen, 2002) as this group is resolved with strong support within *Pannaria* Delise ex Bory s. str.

A detailed description of *Kroswia* is available in Jørgensen (2002). Three species are currently recognized in the genus : *K. crystallifera*, known from Kenya, South Africa, Reunion, India/Tamil Nadu, Sri Lanka, Taiwan, Papua New Guinea and Australia (Jørgensen and Sipman, 2006) and Madagascar (Magain and Sérusiaux, 2014), *K. gemmascens*, reported from Japan and China/Sichuan and Xizang (Jørgensen, 2007a), and *K. polydactyla* P.M. Jørg., described and only known from New Caledonia (Jørgensen and Gjerde, 2012). The collematoid thallus of *K. crystallifera* (Fig. 2a) is homoiomerous, with a hardly distinct epicortex, or no cortex at all, with individual chains of *Nostoc* easily distinguished and spreading throughout the height of the thallus. Such a thallus is very different from the closely related species which have a distinct, multi-layered cortex and a well-delimited layer contains Nostoc cells, with hardly any chains distinguishable (Magain and Sérusiaux, 2014).

Interestingly, *Kroswia crystallifera* is closely related to the type species of *Fuscopannaria* (*F. leucosticta*, Fig. 2b), and to other species resolved all together as an unresolved polytomy (*F. ahlneri* (P.M. Jørg.) P.M. Jørg., *F. cheiroloba* (Müll. Arg.) P.M. Jørg., *F. confusa* (P.M. Jørg.) P.M. Jørg., *F. praetermissa*, *F. protensa* (Hue) P.M. Jørg., *F. sorediata* P.M. Jørg.). The hypothesis that *F. crystallifera* evolved from a duo of photomorphs, formed by the very same fungus and lichenized with two different strains of *Nostoc*, that eventually dissociated and diverged cannot be ruled out. Both photomorphs may even have formed a single thallus, such as in the case of cyano-chlorolichens (Henskens et al., 2012) or in cases of co-existence of two different photobionts within a single thallus (Casano et al. 2011; del Campo et al. 2013 for *Ramalina farinacea* (L.) Ach.). Further, another switch between different strains of *Nostoc* within the clade of *Fuscopannaria* is likely to explain the very different thallus of *Moelleropsis nebulosa*, formed by coarse, usually dispersed granules, as this monotypic genus is nested within the second group recognized within *Fuscopannaria*.

As no molecular data are available for *Kroswia gemmascens* and *K. polydactyla*, we refrain from formally proposing the combination of both epithets to *Fuscopannaria*. Indeed, the Pannariaceae have reserved so many surprises as to its evolutionary patterns that it must avoided to propose hardly confirmed nomenclatural changes.

Figure 2: Pictures of the thalli of the material studied; A, *Fuscopannaria* (*Kroswia*) *crystallifera*; B, *Fuscopannaria leucosticta*; Scale = 1cm.

Moelleropsis Gyeln. is a monotypic genus and is an older name than *Fuscopannaria*; a conservation proposal has been formally made (Jørgensen et al., 2013) and we therefore maintain the use of *Fuscopannaria* for this widespread and well-known species clade.

***Fuscopannaria* P. M. Jørg.** Journal of the Hattori Botanical Laboratory 76 : 198 (1994) ; type : *Fuscopannaria leucosticta* (Tuck.) P. M. Jørg. *Kroswia* P. M. Jørg., Lichenologist 34 : 297 (2002), syn. nov. ; type : *Kroswia crystallifera* P. M. Jørg.

***Fuscopannaria crystallifera* (P.M. Jørg.) Magain & Sérus. comb. nov.** MycoBank No 809865 *Kroswia crystallifera* P.M.Jørg., Lichenologist 34 : 299 (2002) ; type : India, Tamil Nadu, Palni Hills, 23 i 1975, M. E. Hale 43843 (US—lectotype !) (Fig. 2A) Selected material examined of *Fuscopannaria crystallifera* : Madagascar : Angavokely Forest Station, S 18'55 '37.9" E ''15.2", alt. 1770-1780m, degraded ericaceous shrub near the summit, 2008, E. Sérusiaux s.n. with E. Fischer, D. Ertz, D. Killmann & V. Razafindrahaja (LG M788). — Réunion : Cirque de Cilaos, Forêt du Grand Matarum S '07.416 'E 055 '.983 ', alt. 1400-1450m, disturbed montane forest, 2008, E. Sérusiaux with M. Brand & P. van den Boom (LG R1055) ; ibid., Col de Taïbit, S 21 ''42.5" E 55 '26 '34.0 ", alt. 1800m, disturbed montane forest, N. Magain & E. Sérusiaux (LG R1679).

3.4 Acknowledgements

Field studies in Reunion were made possible with the help and advice from the "Parc National de La Réunion", especially through the courtesy of Mr B. Lequette. Dr Cl. Ah-Peng and Prof. D. Strasberg of the University of La Réunion in Saint-Denis and Dr. J. Hivert of the Conservatoire Botanique National de Mascarin (St-Leu) were also very helpful. A first field trip to Reunion in 2008 was conducted with our colleagues and friends Maarten Brand and Pieter van den Boom. The field trip to Madagascar was organized with the logistical support of the "Parc Botanique et Zoologique de Tsimbazaza" in Antananarivo, and with collecting and export permits of scientific material issued by the "Ministère des Eaux & Forêts"; it was organized with our colleagues and friends Damien Ertz, Eberhard Fischer, Dorothee Killmann and Tahina Razafindrahaja. We thank them all very warmly. We further thank the curators of the following herbaria for the loan of type collections or relevant material: H, US. We further thank Mr I. Cremasco and L. Gohy for technical assistance in the molecular laboratory and herbarium at the University of Liège. Prof. A. Elvebakk recently corresponded with us regarding the generic status of several clades within the Pannariaceae and we thank him very warmly for providing us with interesting clues and hypotheses. Nicolas Magain is a Ph.D. Student at the University of Lièège and acknowledges the financial support by FRIA, an organ of the Belgian Scientific Research Foundation. Finally we warmly thank both referees (Arve Elvebakk and Mats Wedin) for their critical and helpful notes and suggestions.

Table 1: Voucher table of the specimens used in this study, with the species names and references to original publications; GenBank accessions of the sequences.

Taxon	Country	Paper	mtSSU	LSU	*RPB1*
Fuscoderma applanatum	New Zealand	Wedin et al. 2009	GQ259024	GQ258994	GQ259053
Fuscopannaria ahlneri	South Korea	Wedin et al. 2009	GQ259025	GQ258995	GQ259054
Fuscopannaria cheiroloba		Ekman et al. 2014	—	—	KC608113
Fuscopannaria confusa	Norway	Carlsen et al. 2012	GU570043	—	—
Fuscopannaria ignobilis		Miadlikowska et al. 2006	DQ917416	DQ917417	DQ986839
Fuscopannaria lacerulata		Ekman et al. 2014	KC608070	—	KC608115
Fuscopannaria leucosticta 1	Reunion Island	Magain and Sérusiaux 2014	JX494238	JX494264	JX494284
Fuscopannaria leucosticta 2	USA	Wedin et al. 2009	DQ900630	DQ900640	GQ259055
Fuscopannaria leucostictoides		Ekman et al. 2014	KC608071	—	KC608116
Fuscopannaria maritima		Ekman et al. 2014	KC608072	—	KC608117
Fuscopannaria mediterranea		Miadlikowska et al. 2006	DQ917418	DQ917419	—
Fuscopannaria olivacea		Ekman et al. 2014	KC608073	—	—
Fuscopannaria pacifica		Ekman et al. 2014	KC608074	—	KC608118
Fuscopannaria praetermissa 1	Reunion Island	Magain and Sérusiaux 2014	JX494239	—	JX494285
Fuscopannaria praetermissa 2	Sweden	Wedin et al. 2009	GQ259026	GQ258996	GQ259056
Fuscopannaria protensa		Ekman et al. 2014	—	—	KC608119
Fuscopannaria sorediata		Ekman et al. 2014	KC608067	—	—

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Table 1 – continued from previous page

Taxon	Country	Paper	mtSSU	LSU	*RPB1*
Kroswia crystallifera 1	Madagascar	Magain and Sérusiaux 2014	JX494235	JX494261	JX494281
Kroswia crystallifera 2	Reunion Island	Magain and Sérusiaux 2014	JX494236	JX494262	JX494282
Kroswia crystallifera 3	Reunion Island	Magain and Sérusiaux 2014	JX494237	JX494263	JX494283
Leciophysma furfurascens	Sweden	Wedin et al. 2009	GQ259028	GQ258998	GQ259058
Moelleropsis nebulosa		Ekman et al. 2014	KC608079	—	KC608122
Nevesia sampaiana	Norway	Carlsen et al. 2012 / Ekman et al. 2014	GU570030	—	KC608120
Pannaria calophylla	Argentina	Passo et al. 2008	EU885318	—	—
Pannaria implexa	Argentina	Passo et al. 2008	EU885333	—	—
Pannaria lurida	Reunion Island	Magain and Sérusiaux 2014	JX494248	JX494273	—
Pannaria microphyllizans	Argentina	Passo et al. 2008	EU885322	—	—
Pannaria multifida	Reunion Island	Magain and Sérusiaux 2014	JX494241	JX494266	KF704308
Pannaria pallida	Argentina	Passo et al. 2008 / Elvebakk et al. 2010	EU885323	GQ927270	—
Pannaria rubiginella		Wedin et al. 2009	GQ259037	GQ259007	GQ259074
Pannaria rubiginosa	Portugal	Wedin et al. 2009	AY340513	AY340558	GQ259073
Pannaria sp.	Thailand	Magain and Sérusiaux 2014	KF704289	KF704290	KF704306
Pannaria sphinctrina	Argentina	Passo et al. 2008 / Elvebakk et al. 2010	EU885324	GQ927271	—
Pannaria tavaresii	Argentina	Passo et al. 2008	EU885316	—	—

Continued on next page

Table 1 – continued from previous page

Taxon	Country	Paper	mtSSU	LSU	*RPB1*
Parmeliella parvula	Norway	Carlsen et al. 2012	GU570031	—	—
Protopannaria pezizoides	Sweden	Wedin et al. 2009	AY340519	AY340561	GQ259081
Psoroma hypnorum	Sweden	Wedin et al. 2009	AY340523	AY340565	GQ259085
Psoroma palaceum	Argentina	Passo et al. 2008	EU885327	GQ927305	—
Ramalodium succulentum	Australia	Wedin et al. 2009	GQ259043	GQ259013	GQ259086
Santessoniella sp.		Ekman et al. 2014	KC608105	—	KC608146
Staurolemma omphalarioides	Norway	Wedin et al. 2009	GQ259044	GQ259014	—
Staurolemma sp.	Reunion Island	Magain and Sérusiaux 2014	KF704288	KF704291	—

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Chapter 4

Phylogenetic placement, species delimitation, and cyanobiont identity of endangered aquatic *Peltigera* species (lichen-forming Ascomycota, Lecanoromycetes)

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Published in American Journal of Botany 101(7):1141-1156. 2014.

Abstract

• Premise of this study: Aquatic cyanolichens from the genus *Peltigera* section *Hydrothyriae* are subject to anthropogenic threats and, therefore, are considered endangered. In this study we addressed the phylogenetic placement of section *Hydrothyriae* within *Peltigera*. We delimited species within the section and identified their symbiotic cyanobacteria.

• Methods: Species delimitation and population structure were explored using monophyly as a grouping criterion (RAxML) and Structurama based on three protein-coding genes in combination with two nuclear ribosomal loci. The 16S and *rbcLX* sequences for the cyanobionts were analyzed in the broad phylogenetic context of free-living and symbiotic cyanobacteria.

• Key results: We confirm with high confidence the placement of section *Hydrothyriae* within the monophyletic genus *Peltigera*; however, its phylogenetic position within the genus remains unsettled. We recovered three distinct monophyletic groups corresponding to three species: *P. hydrothyria*, *P. gowardii* s.s., and *P. aquatica* Miadl. &

Lendemer, the latter being formally introduced here. Each species was associated with an exclusive set of *Nostoc* haplotypes.

• Conclusions: The ITS region alone provides sufficient genetic information to distinguish the three morphologically cryptic species within section *Hydrothyriae*. Section *Hydrothyriae* seems to be associated with a monophyletic lineage of *Nostoc*, that has not been found in symbiotic association with other members of *Peltigera*. *Capsosira lowei* should be transferred to the genus *Nostoc*. Potential threats to *P. aquatica* should be re-examined based on the recognition of two aquatic species in western North America.

Key Words: aquatic lichens, *Capsosira*, cyanobiont, elongation factor 2,fungal systematics, multilocus phylogenetics, mycobiont, *Nostoc*, section *Hydrothyriae*, species delimitation

4.1 Introduction

Lichens represent one of the most successful and widespread types of symbiosis (Nash, 2008). Although lichens are well known for their ability to survive in extreme environments, their biodiversity, distribution, and population structure can be seriously affected by anthropogenic environmental disturbances. The main threats to fauna and flora in general also affect lichens and include local and global scale changes (e.g., habitat degradation, loss, and fragmentation) resulting from urbanization, agriculture, pollution, and climate change (Nash, 2008; Scheidegger and Werth, 2009). The specific habitat requirements of lichens are hardly ever considered when establishing protected areas. Understanding species boundaries, especially in genera such as *Peltigera* Willd. that are suspected to include many cryptic species (O'Brien et al., 2009), is crucial for developing successful conservation strategies and manage- ment practices (Scheidegger and Werth, 2009).

The cosmopolitan genus *Peltigera* (Peltigerales, Lecanoromycetidae) includes predominantly muscicolous and terricolous foliose lichen-forming fungi. These lichens are associated with the cyanobacterium *Nostoc* Vaucher ex Bornet & Flahault and form bimembered thalli. A few species are also associated with the green alga *Coccomyxa* Léger & Hesse in addition to the cyanobiont *Nostoc* and thus form trimembered thalli. Although *Peltigera* is one of the earliest described lichen-forming fungi (Willdenow, 1787), this genus is still poorly known compared with other macrolichens. Variations in its morphological traits have been difficult to interpret by taxonomists using traditional taxonomic practices, which were unable to distinguish among phenotypic plasticity, genetically based phenotypic variation and cryptic speciation, all suspected to occur in *Peltigera*. Progress in understanding the biodiversity of this genus was thus greatly impeded even though molecular techniques have been available to lichenologists for more than 25 yr. Moreover, hybridization may also be a factor in this genus according to Goffinet and Hastings (1995), a process that has rarely been addressed by evolutionary

studies of fungi in general. Many unknown morphologically cryptic species are probably hidden under what are thought to be common *Peltigera* species (e.g., O'Brien et al. 2009; Lendemer and O'Brien 2011) such that some species are likely to be threatened by extinction, before they are recognized as distinct.

Molecular phylogenetic analyses coupled with traditional systematic studies, e.g., morphology, chemistry, biogeography, and ecology, have been useful in resolving some of these issues (e.g., Goffinet and Miadlikowska 1999; Miadlikowska et al. 2003; Sérusiaux et al. 2009). In addition to the ITS region (Goffinet and Miadlikowska, 1999; Goffinet et al., 2003), selected molecular markers, e.g., *RPB1* and *β -tubulin*, showed intra- and interspecies variation that allowed the recognition of biologically meaningful species within species complexes of *Peltigera* in North America (O'Brien et al., 2009). Using these three nuclear markers, these authors concluded that the genus *Peltigera* is more diverse in western North America than originally perceived and that morphological variability is due largely to the presence of undescribed species rather than hybridization or intraspecific variation. Currently, more than 90 species of *Peltigera* are recognized worldwide (Goffinet et al., 2003; MartÍnez et al., 2003; Vitikainen, 2006; Kirk et al., 2008; Sérusiaux et al., 2009; Han et al., 2013), and 37 of them occur in North America (Esslinger, 2010). To date, seven species (*P. castanea* Goward, Goffinet & Miadl., *P. cinnamomea* Goward, *P. chionophila* Goward & Goffinet, *P. gowardii* Lendemer & H. O'Brien, *P. hydrothyria* Miadlikowska & Lutzoni, *P. pacifica* Vitik., and *P. phyllidiosa* Goffinet & Miadlikowska) have been reported as endemic to North America (Goffinet and Miadlikowska, 1999; Goward and Goffinet, 2000; Miadlikowska and Lutzoni, 2000; Goffinet et al., 2003; MartÍnez et al., 2003; Lendemer and O'Brien, 2011). Prior to this study, only two Peltigera species were known to be aquatic (*P. gowardii* and *P. hydrothyria*).

In 2000, Miadlikowska and Lutzoni published the first phylogeny for the genus *Peltigera*. They proposed a new infrageneric classification consisting of eight monophyletic sections, one of which, section *Hydrothyriae* Miadlikowska & Lutzoni, was created for the aquatic monospecific genus *Hydrothyria* J. L. Russell. This genus was subsumed within *Peltigera* (*H. venosa* J. L. Russell = *Peltigera hydrothyria*) based on a single aquatic representative included in the phylogenetic analyses. Although the affiliation of *Hydrothyria* with *Peltigera* received significant support (100% bootstrap), its accurate placement within *Peltigera* was not settled by this phylogenetic study.

In the past, *Hydrothyria* was considered a member of the Collemataceae Zenker (Russell, 1856) because of its unique morphology (cyanolichen with an unstratified gelatinous thallus when wet and lacking rhizines) and its ecology (found in streams attached to rocks at or below water level). Due to its aquatic habitat and narrow ecological amplitude, *Peltigera hydrothyria* s.l. was the subject of various studies focusing mainly on potential anthropogenic threats to its populations and on developing potential conservation strategies (see summary by Poulsen and Carlberg 2007).

Despite the disjunct distribution (eastern and western areas of North America) and

chemical variation (populations with and without detectable secondary compounds) of *P. hydrothyria* s.l., its circumscription was never questioned until the recent systematic revision and molecular phylogenetic analyses (based on ITS) of material collected in the United States (Lendemer and O'Brien, 2011). The study revealed that *P. hydrothyria* represents a species complex consisting of three strongly supported monophyletic groups: the eastern *P. hydrothyria* and two allopatric western clades recognized within a single newly described species, *P . gowardii* s.s. and *P . gowardii* s.l. (Lendemer and O'Brien, 2011). The two western clades are partly sympatric, overlapping only in Washington State, with the range of *P. gowardii* s.s. extending northward and that of *P. gowardii* s.l. extending southward. Although morphologically cryptic, the western populations of *P. gowardii* are chemically distinct (lacking methylgyrophorate) from the eastern *P. hydrothyria*. The authors concluded that the recognition of *P. gowardii* s.l. as a third species within the section *Hydrothyriae* should be reexamined based on additional sampling and multiple unlinked molecular markers.

The cyanobiont of *P. hydrothyria* was identified as *Capsosira lowei* Casamatta, S. R. Gomez & J. R. Johansen, a new species of the family Capsosiraceae (A. Borzi) L. Geitler (Stigonematales), based on phenotypic characters of the cyanobacterium isolated from a lichen specimen collected in the southern Appalachian Mountains of North Carolina (Casamatta et al., 2006). Although morphologically similar to members of the Stigonematales Geitler (e.g., cell division in two planes), molecular evidence (16S phylogeny and the similarity in the ITS domains structure) supported close affiliation of *C. lowei* with *Nostoc* commune Vaucher ex Bornet & Flahault, a member of the Nostocales Cavalier-Smith (Casamatta et al., 2006). This finding raised questions about the validity of morphological synapomorphies used to circumscribe the genus *Nostoc* (Casamatta et al., 2006; Korelusová, 2008). Lendemer and O'Brien (2011) reported that *Nostoc* sp. was the cyanobiont associated with *P. gowardii*; however, no molecular data were included to sup- port their statement.

The main objectives of the current study were to (1) revisit the phylogenetic placement of section *Hydrothyriae* within the genus *Peltigera*; (2) reevaluate species delimitations within this section, especially the potential for the presence of two species within what is currently recognized as *P. gowardii*; (3) assess the identity of the cyanobionts associated with members of the section *Hydrothyriae* and their affiliation with free-living and other symbiotic cyanobacteria; and (4) provide an overview of the distribution, ecology, and threat factors to the conservation of aquatic *Peltigera* species.

To reach these goals, we sequenced three nuclear protein- coding genes and two nuclear ribosomal RNA loci for the my- cobiont representing all sections of *Peltigera*, as well as the *rbcLX* region and 16S ribosomal RNA of the cyanobiont found in members of section Hydrothyriae. We also incorporated new data on distribution, habitat requirements, and potential endangerment factors based on a recent inventory of aquatic *Peltigera* species in Canada.

4.2 Material and Methods

4.2.1 Taxon sampling and data acquisition

We selected 18 individuals of *P. gowardii* and 17 specimens of *P. hydrothyria* to address species delimitation and phylogenetic relationships among members of section *Hydrothyriae*, as well as to reveal the identity of their cyanobionts. Of these, 10 specimens of *P. gowardii* and nine of *P. hydrothyria* (collections from the USA only) were previously included in the systematic study by Lendemer and O'Brien (2011), where the ITS region was analyzed under maximum parsimony. We expanded the geographical range of the sampling by adding material collected mainly in western Canada (British Columbia) for *P. gowardii* (six individuals, plus two collections from the USA) and in three eastern provinces of Canada (Nova Scotia, New Brunswick, and Québec) for *P. hydrothyria* (eight individuals). All new collections of aquatic *Peltigera* from Canada were obtained as part of the Canadian inventory of both species performed by DR, FA, and RC. To revisit the phylogenetic position of section *Hydrothyriae* within the genus *Peltigera*, 45 individuals from 30 species were selected worldwide to represent the remaining seven sections (Appendix 1).

For the mycobiont, we targeted five molecular markers, including two nuclear ribosomal loci: ca. 0.6 kb of the internal transcribed spacer (ITS) region and ca. 1.2 kb of the large subunit (nrLSU), which are commonly used in molecular systematics of lichen-forming fungi including the genus *Peltigera* (e.g., Miadlikowska and Lutzoni 2000; Goffinet et al. 2003; Miadlikowska et al. 2003; Miadlikowska and Lutzoni 2004; Lendemer and O'Brien 2011). The three single-copy protein-coding genes we sequenced for the mycobiont are: ca. 0.7 kb of *β-tubulin* and ca. 0.8 kb of the RNA polymerase II largest subunit (*RPB1*), which were shown to be valuable markers for species delimitation within the genus *Peltigera* (O'Brien et al., 2009), and ca. 0.8 kb of the first region of the elongation factor 2 (*EFT2-1*), a new molecular marker developed as part of the Assembling the Fungal Tree of Life project (AFToL 2). For the cyanobiont, we sequenced ca. 1.0 kb of the *rbcLX* region (which includes the last 82 amino acids of the RUBISCO large subunit [*rbcL*], a puta- tive chaperone gene [*rbcX*] and two intergenic spacers; Li and Tabita 1997), and the ribosomal RNA small subunit (16S). We generated three new 16S and 33 new *rbcLX* sequences for cyanobionts associated with aquatic *Peltigera* species (Appendix 1). To these data sets we added 16S ribosomal RNA and *rbcLX* sequences from GenBank to represent the biodiversity of free-living and symbiotic cyanobacteria mainly from the Nostocales and Stigonematales.

We obtained a total of 296 new sequences: 260 for the mycobionts (16 ITS, 38 nrLSU, 70 *β-tubulin*, 63 *EFT2-1*, and 73 *RPB1*) and 36 for their cyanobionts (33 of *rbcLX* and three of 16S). Twenty four sequences, mostly ITS, were downloaded from GenBank (19 of which were generated by Lendemer and O'Brien 2011), and 46 sequences were missing (Appendix 1). All new sequences generated for this study were derived from DNA extracted directly from a single lichen thallus at a time.

Most of the new sequences were generated using the Sigma REDExtract-N- Amp Plant PCR Kit (St. Louis, Missouri, USA) for DNA isolation and R4775 Sigma REDExtract-N-Amp PCR ReadyMix for the PCR reaction (for detailed information, see Rivas Plata et al. 2013). Alternatively, a standard DNA isolation procedure employing 2% SDS lysis buffer (Zolan and Pukkila, 1986) was used. Sources for laboratory protocols and primers used for generating the new sequences of nrLSU can be found in the report of Lutzoni et al. (2004); Hofstetter et al. (2007); ITS, *RPB1* and *β-tubulin* in O'Brien et al. (2009); 16S and *rbcLX* in Elvebakk et al. (2008). PCR amplification of the *EFT2*-1 was performed using the following designed primers: EFT2-1F (5'-AAYATGWS- BGTBATYGC-3') and EFT2-4R (5'-GGVACCATYTTVGARAC-3'). Conditions for the touchdown PCR for *EFT2*-1 were as follows: 94°C for 30 s, 55°C for 30 s (-0.4°/cycle), 72°C for 1 min (+2 s/cycle) for 24 cycles; 94°C for 30 s, 45°C for 30 s, 72°C for 2 min (+3 s/cycle) for 12 cycles; 72°C for 10 min, followed by storage at 4°C . All PCR amplicons were cleaned with ExoSAP (Affymetrix, Santa Clara, California, USA) following the manufacturer's protocol.

Sequencing was carried out in 10 μL reactions using: 1 μL primer (10 μmol/L), 1 μL purified PCR product, 0.75 μL Big Dye (Big Dye Terminator Cycle sequencing kit, ABIPRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, California, USA), 3.25 μL Big Dye buffer, and 4 μL double-distilled water. Automated reaction clean-up and visualization was performed at the Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies (for details see Gaya et al. 2012).

All newly acquired sequences were subjected to BLAST searches to confirm the fungal or cyanobacterial origin of each sequence fragment. They were assembled and edited using the software package Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA) and aligned manually with the program MacClade 4.07 (Maddison and Maddison, 2003). The "Nucleotide with AA color" option was used for guiding (delimiting exons and introns) all alignments for protein-coding genes. Ambiguously aligned regions sensu Lutzoni et al. (2000) were delimited manually and excluded from subsequent analyses.

4.2.2 Data sets and analyses

We assembled and analyzed four data sets (Appendix 1), two for the mycobiont (ML1 and ML2) and two for the cyanobiont (ML3 and ML4). To address the first objective (i.e., the phylogenetic placement of the section *Hydrothyriae* within the genus *Peltigera*), we assembled a 4- locus (*β-tubulin* + *RPB1* + *EFT2*-1 + nrLSU) data set for 48 OTUs selected across the genus *Peltigera*, including three representatives of *P. hydrothyria* and two of *P. gowardii*, as well as two members of *Solorina* Ach. as the outgroup (ML1 in Appendix 1). To address the second objective (i.e., species delimitations within section *Hydrothyriae*), we assembled a 4-locus (*β-tubulin* + *RPB1* + *EFT2*-1 + ITS) data set for 52 OTUs, including 35 members of the section *Hydrothyriae* (18 individuals of *P. gowardii* and 17 individuals of *P. hydrothyria*) and 17 representatives from four other

sections [*Peltidea* (Ach.) Vain., *Chloropeltigera* Gyeln., *Phlebia* Wallr., and *Polydactylon* Miadlikowska and Lutzoni] serving as outgroups (ML2 in Appendix 1).

Compared with the sampling by Lendemer and O'Brien (2011), we increased the taxon sampling (from 21 to 35 individuals) by including mainly Canadian populations that were not previously sampled. Furthermore, we expanded molecular data by adding three single-copy protein-coding genes to the ITS region, the sole marker analyzed in that previous study (692 vs. 3231 characters) under the maximum parsimony optimization criterion. Due to the rapid sequence divergence of the ITS region resulting in a high proportion of ambiguously aligned regions (almost half of the ITS alignment; see also Lendemer and O'Brien 2011), ITS was not included for the ougroup taxa in ML2 analyses.

To address the third objective (i.e., to unveil the identity and phylogenetic affiliation of cyanobionts associated with *P. gowardii* and *P. hydrothyria*), we assembled two data sets: the 16S and the *rbcLX* data set for the ML3 and ML4 phylogenetic analyses, respectively. The ML3 data set incorporated 110 mostly published 16S sequences representing free-living and symbiotic cyanobacteria from the order Nostocales (families Nostocaceae Eichler, Rivulariaceae Frank, Microchaetaceae Lemmermann, and Scytonemataceae Frank) and selected taxa from the orders Stigonematales, Pleurocapsales Cavalier-Smith, and Gloeobacterales Cavalier-Smith. The latter was used to root this 16S-based tree. This data set contained 24 sequences used by Casamatta et al. (2006), including *Capsosira lowei*, a cyanobiont isolated from *P. hydrothyria*. We added two 16S sequences of the cyanobiont from *P. gowardii* collected in the state of Washington and one from *P. hydrothyria* collected in Queébec, Canada (Appendix 1).

ML4 consisted of 275 mostly published *rbcLX* sequences of free-living and symbiotic *Nostoc* spp. selected from the *Nostoc* clades I and II (Otálora et al., 2010; O'Brien et al., 2013) and 17 newly sequenced individuals of P. gowardii and 16 individuals of *P. hydrothyria* from several localities mainly in Canada (Appendix 1). Identical haplotypes were collapsed to one representative using the program Map (Aylor et al., 2006); however, we reincorporated some of the initial sequences that had been erroneously collapsed because the program excluded variable sites if they contained missing data.

Maximum likelihood analyses using the program RAxMLHPC-MPI-SSE3 (Stamatakis, 2006) were performed on all data sets (ML1–4) at the nucleotide level. Optimal tree and bootstrap searches were conducted with the rapid hill- climbing algorithm for 1000 replicates with GTR substitution model (Rodriguez et al., 1990) and gamma distribution parameter approximated with four categories in all analyses. Partitions for the ML1 and ML2 analyses were estimated with the program PartitionFinder v.1.1.0 (Lanfear et al., 2012) using greedy search and the Bayesian information criterion (BIC) model selection. For the ML1 data set, two partitions were defined. The first partition consisted of all introns, *β-tubulin* third codon position, and *RPB1* third codon position; the second partition incorporated the remaining sites of *β-tubulin*, *RPB1*, the entire *EFT2*-1 and nrLSU. For the ML2 data set, four partitions were defined. The first partition consisted

of ITS and all introns; the second partition consisted of *β-tubulin* first codon position, *RPB1* first codon position and *EFT2*-1 first codon position; the third partition consisted of *β-tubulin* third codon position, *RPB1* second codon position, and part of the *EFT2*-1 second codon position; and the fourth partition incorporated the remaining sites of *β-tubulin*, *RPB1*, and *EFT2*-1. For the ML3 analysis, a single partition corresponding to the 16S was used, whereas for the ML4 analysis, three partitions corresponding to the first, second, and third codon position of the *rbcLX* were defined. To detect topological incongruence among single-locus data sets, we implemented a reciprocal 70% ML bootstrap support criterion (Mason-Gamer and Kellogg, 1996; Reeb et al., 2004). A conflict was assumed to be significant if a group of taxa was supported as monophyletic at ≥70% with one locus but supported as nonmonophyletic, using the same bootstrap threshold, by another locus. No conflict was detected among the single-locus data sets part of the ML1 and ML2 concatenated data sets. Map and RAxML analyses were completed through the Mobyle SNAP Workbench version 1.0.5, a portal for evolutionary and population genetics analyses (North Carolina State University online facilities) developed as part of the Dimensions of Biodiversity project (DoB; Monacell and Carbone 2014). The ML2 concatenated data set and the resultant most likely RAxML tree were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S15894).

To infer population structure from genetic data for the aquatic *Peltigera* (ML2 data set restricted to 35 individuals from the section *Hydrothyriae*; Appendix 1), we used Structurama (Huelsenbeck et al., 2011). The program assumes that the sampled loci are in linkage equilibrium and implements a Markov chain Monte Carlo (MCMC) sampling strategy to approximate the posterior probability that individuals are assigned to specific populations. We ran the Markov chain for 1 million cycles sampling every 1000th cycle. We allowed the number of populations to be a random variable (following a Dirichlet process prior) with a gamma probability distribution (hyperprior).

4.2.3 Alignments

Ambiguously aligned sites, which were excluded from phylogenetic analyses (Table 1), were localized in introns of the protein-coding genes (especially *β-tubulin* with 40 excluded sites from ML2 analyses) and nrLSU (with 83 excluded sites from ML1 analyses). From the total of 3340 characters, 109 where ambiguously aligned and excluded, whereas the remaining 3231 characters were included in ML1 analyses. ITS and *RPB1* alignments did not contain any ambiguously aligned regions, and both loci were entirely included in the ML2 analyses (a total of 2578 characters, 50 of which were ambiguously aligned and excluded from ML2; Table 1). Among the five fungal loci analyzed in this study, nrLSU was the longest (1282 characters), while the other loci provided comparable numbers of unambiguously aligned characters (from 501 characters for *β-tubulin* to 783 characters for *EFT2*-1; Table 1). From a total of 979 cyanobacterial 16S sites, 32 sites were excluded (ambiguously aligned) and the remaining 947 characters were analyzed (ML3). Both spacers in *rbcLX* were too variable to be unambiguously aligned

Table 1: Contribution (number of sites) of each locus to the combined data sets (ML1 and ML2) and genetic variation within and among species of section *Hydrothyriae*. Numbers in parenthesis include indels. Ambiguously aligned sites excluded from phylogenetic analyses follow the plus sign.

Statistic	ITS/nrLSU	β-tubulin	*RPB1*	*EFT2*-1	Combined
Total no. of sites incl. in ML1	1282+83*	504+19	661+6	783+1	3231+109
Total no. of sites incl. in ML2	592+0	501+40	661+0	774+10	2528+50
No. of haplotypes in *Hydrothyriae*	4	3	3	4	N/A
No. of segregating sites in *Hydrothyriae*	16 (23)	20	11	18	65 (72)
No. of polymorphic sites with exclusively shared states for:					
P. gowardii s.str. + *P. gowardii* s.l.	7 (9)	5	3	5	20 (22)
P. gowardii s.str. + *P. hydrothyria*	3 (4)	2	3	3	11 (12)
P. hydrothyria + *P. gowardii* s.l.	5 (7)	12	5	10	32 (34)
No. of sites with uniquely different states for each aquatic species	1 (2)	1	0	0	2 (3)
No. of sites polymorphic within each aquatic species	-1	0	0	1	1 (2)

across the Nostocales, or even within the section *Hydrothyriae* alone, and were removed from subsequent phylogenetic analyses (ML4; 633 characters).

4.3 Results and Discussion

4.3.1 Phylogenetic placement of the section *Hydrothyriae*

Miadlikowska and Lutzoni (2000) reported a monophyletic delimitation of the genus *Peltigera* including *P. hydrothyria* (*Hydrothyria venosa*) and introduced section *Hydrothyriae* (one of the eight sections circumscribed within the genus), to accommodate this aquatic member of *Peltigera*. However, the phylogenetic placement of *P. hydrothyria* (only one specimen was included in that study), which represented the first diver- gence event within the genus, as well as the remaining deep relationships among sections, were poorly supported (bootstrap values below 50%) based on the combined nrLSU and morphological and chemical data analyzed under a maximum parsimony optimization criterion (fig. 8 of Miadlikowska and Lutzoni 2000).

In this study, we replaced phenotypic characters with data from three single-copy protein-coding genes (*β-tubulin*, *RPB1*, and *EFT2*-1) concatenated with the previously

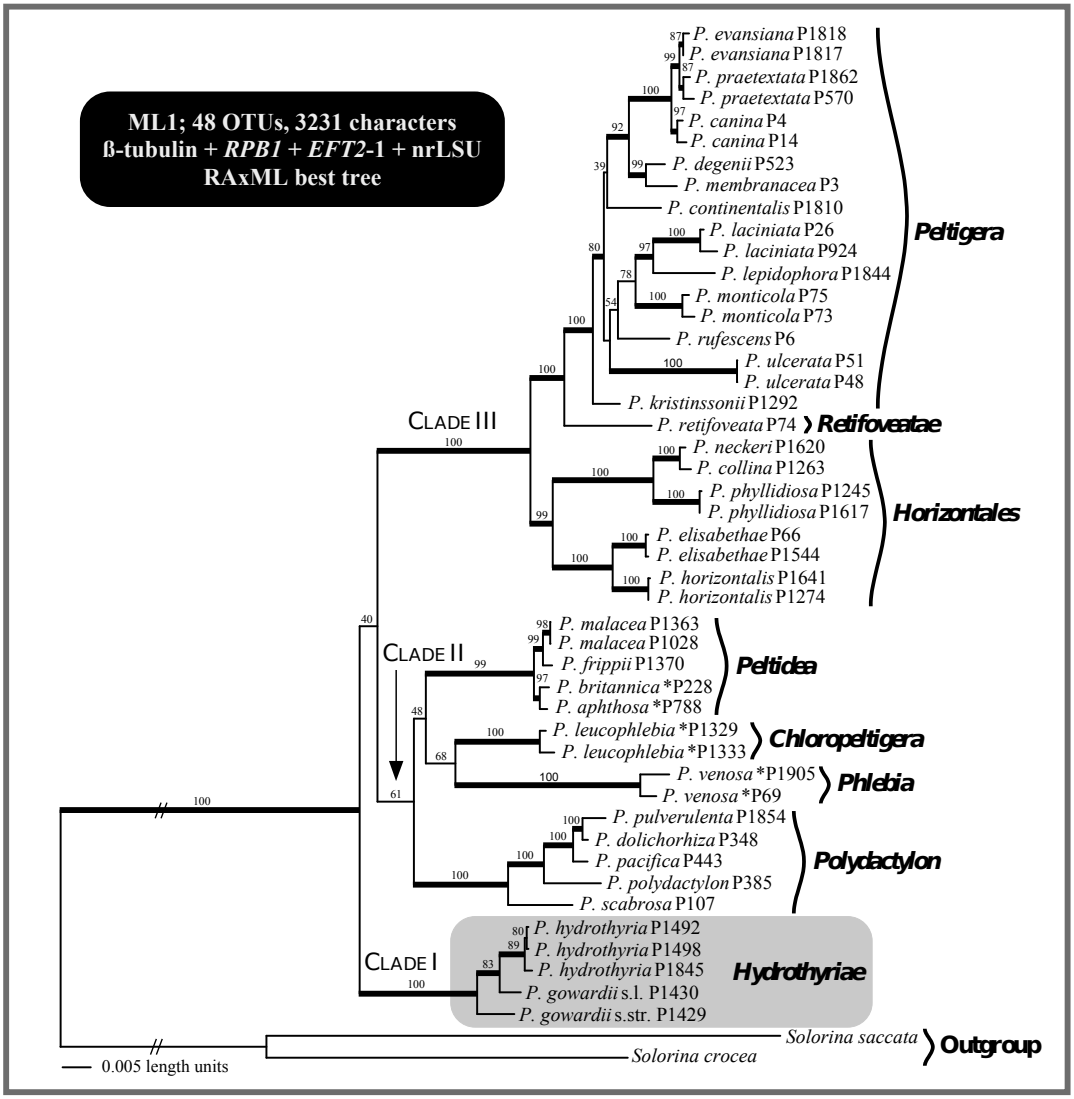

Figure 1: Placement of aquatic *Peltigera hydrothyria* and *P. gowardii* from section *Hydrothyriae* in the phylogenetic context of the genus *Peltigera* (represented by 46 members from all known sections) as revealed by maximum likelihood analysis based on combined *β-tubulin*, *RPB1*, *EFT2*-1, and nrLSU loci (ML1). Two individuals from the genus *Solorina*, the closest relative of *Peltigera* (e.g., Miadlikowska and Lutzoni 2000; Muggia et al. 2011), were used as outgroup to root the *Peltigera* tree. Values associated with internodes represent bootstrap support (BS). Thicker internodes indicate strongly supported (BS $\geq$ 80%) relationships. Stars indicate trimembered species of *Peltigera*.

used nuclear ribosomal LSU locus for a total of 3231 characters (898 variable characters) compared with 1209 characters in the previous study (Miadlikowska and Lutzoni, 2000). We extended the taxon sampling within section Hydrothyriae from one specimen to five individuals of *P. hydrothyria* (three collections) and *P. gowardii* (two collections) for a total of 46 operational taxonomic units (OTUs) comprising the ingroup (Fig. 1). They represented 31 of more than 90 currently recognized species (e.g., Vitikainen 2006; Kirk et al. 2008; Sérusiaux et al. 2009; Han et al. 2013) classified in all remaining sections of the genus *Peltigera* (Miadlikowska and Lutzoni, 2000). To minimize the number and size of ambiguously aligned regions, we restricted the outgroup to include only the genus *Solorina* (family Peltigeraceae), which was consistently shown to be the sister group to *Peltigera* in previous phylogenetic studies (e.g., Miadlikowska and Lutzoni 2000, 2004; Muggia et al. 2011; Spribille and Muggia 2013).

In the resulting most likely phylogeny (Fig. 1), all aquatic individuals of *P. hydrothyria* and *P. gowardii* are grouped together to form the monophyletic, highly supported, section *Hydrothyriae*. Its placement as the first evolutionary split within *Peltigera* (Miadlikowska and Lutzoni, 2000) was reconstructed but at a low level of confidence (BS = 40%). Our phylogeny confirms monophyletic delimitations of each section introduced by Miadlikowska and Lutzoni (2000) for which we have multiple species, as well as the relationships among sections that received high bootstrap support in 2000, e.g., sister group relationship between sections *Peltigera* and *Retifoveatae*, and their close affiliation with section *Horizontales* (clade III; Fig. 1). For the first time, sections *Peltidea*, *Chloropeltigera*, and *Phlebia*, which include all known trimembered *Peltigera* lichens, were grouped together within a single clade (BS = 48%), sister to section *Polydactylon* to form clade II (Fig. 1). However, these two sets of relationships were not strongly supported. The phylogenetic uncertainty associated with the deepest splits in the *Peltigera* phylogeny may have resulted from a rapid early radiation event, as illustrated by very short internodes holding longer branches representing most sections and clade III (Fig. 1). For determining the accurate placement of section *Hydrothyriae*, more loci and a more extensive taxon sampling are needed. The support of a single origin of the trimembered symbiotic state within *Peltigera* in future studies would also confirm a single acquisition of a green alga (*Coccomyxa* W. Schmidle) to form trimembered thalli within that genus. Consequently, the potential monophyly of the trimembered species would also support the hypothesis of a reversal to a bimembered symbiotic state during the evolution of the lineage leading to the speciation of *P. malacea* (Ach.) Funck and *P. frippii* Holt.-Hartw., which are two bimembered lichen species in the section *Peltidea* (Fig. 1) and might explain, in part, the high level of reciprocal (nearly one-to-one) specificity observed for symbionts of *P. malacea*, which is rarely encountered in lichen symbioses (Otálora et al., 2010; O'Brien et al., 2013).

4.3.2 Species delimitations within section Hydrothyriae

Lendemer and O'Brien (2011) introduced the possibility of recognizing three aquatic

Peltigera species: two cryptic taxa in western North America, *P. gowardii* s.s. and *P. gowardii* s.l. (highlighting that the later might be a new unnamed species), and *P. hydrothyria*, an eastern North American species. Our most likely tree for section *Hydrothyriae* (Fig. 2) fully agrees with the three main clades of the ITS phylogeny inferred with maximum parsimony by Lendemer and O'Brien (2011). Together the results of ML1 and ML2 revealed a strongly supported monophyletic section *Hydrothyriae*, with three distinct lineages corresponding to *P. hydrothyria*, its sister group *P. gowardii* s.l. (bootstrap value of 97%), and *P. gowardii* s.s.

Each locus (Tables 1, 2) provided a similar level of genetic information among populations within and among each of the three monophyletic groups (*P. gowardii* s.s., *P. gowardii* s.l., and *P. hydrothyria*; Fig. 2). No intraspecific variation in all analyzed loci was found among the sampled individuals of each potential species except for ITS and *EFT2*-1 of *P. hydrothyria* (Table 2). A single-site polymorphism within *P. hydrothyria* occurred in the ITS (two haplotypes differed by a single indel) and in *EFT2*-1 (two haplotypes differed by single nucleotide substitution present in one individual). However, each of the three monophyletic groups was uniquely distinct (Table 2). For the 72 polymorphic sites (including indels in the ITS) found across the concatenated loci (Table 1), the lowest level of genetic variation among all sampled individuals was found in *RPB1* (11), whereas the ITS, *β-tubulin*, and *EFT2*-1 provided a similar degree of polymorphism (23, 20, and 18 sites, respectively). The highest number of exclusively shared nucleotides at polymorphic sites was between *P. hydrothyria* and *P. gowardii* s.l. for all loci except for ITS where *P. gowardii* s.s. and *P. gowardii* s.l. shared more unique nucleotides at polymorphic sites. However, the difference was by a slight margin compared with *β-tubulin* and *EFT2*-1 where the number of sites segregating in favor of *P. hydrothyria* with *P. gowardii* s.l. (12/20 and 10/18, respectively) was at least twice as high as for *P. gowardii* s.l. with *P. gowardii* s.s. (5/20 and 5/18, respectively; Table 1).

Overall, *P. hydrothyria* exclusively shared the same nucleotide with *P. gowardii* s.l. at 34 polymorphic sites, whereas *P. hydrothyria* shared exclusively the same nucleotide with *P. gowardii* s.s. at only 12 polymorphic sites, and *P. gowardii* s.l. and *P. gowardii* s.s. shared exclusively the same nucleotide at 22 polymorphic sites (Fig. 2, Table 1). Fixed polymorphic sites unique to each of the three species were extremely rare across all loci (two in ITS and one in *β-tubulin*).

The Structurama analysis grouped all sequences into three distinct populations at the highest probability of 0.81. The presence of two *EFT2*-1 alleles in *P. hydrothyria* (a single point mutation shared with *P. gowardii* s.s.) enforced the alternative four-population scenario but with a low probability of 0.16. Notably, each of the 35 individuals was correctly allocated to the population ($p > 0.95$) corroborating the monophyletic groups revealed by phylogenetic analyses (Fig. 2).

As pointed out earlier by Lendemer and O'Brien (2011), *P. gowardii* s.l. is morphologically indistinguishable from both *P. hydrothyria* and *P. gowardii* s.s. and chemi-

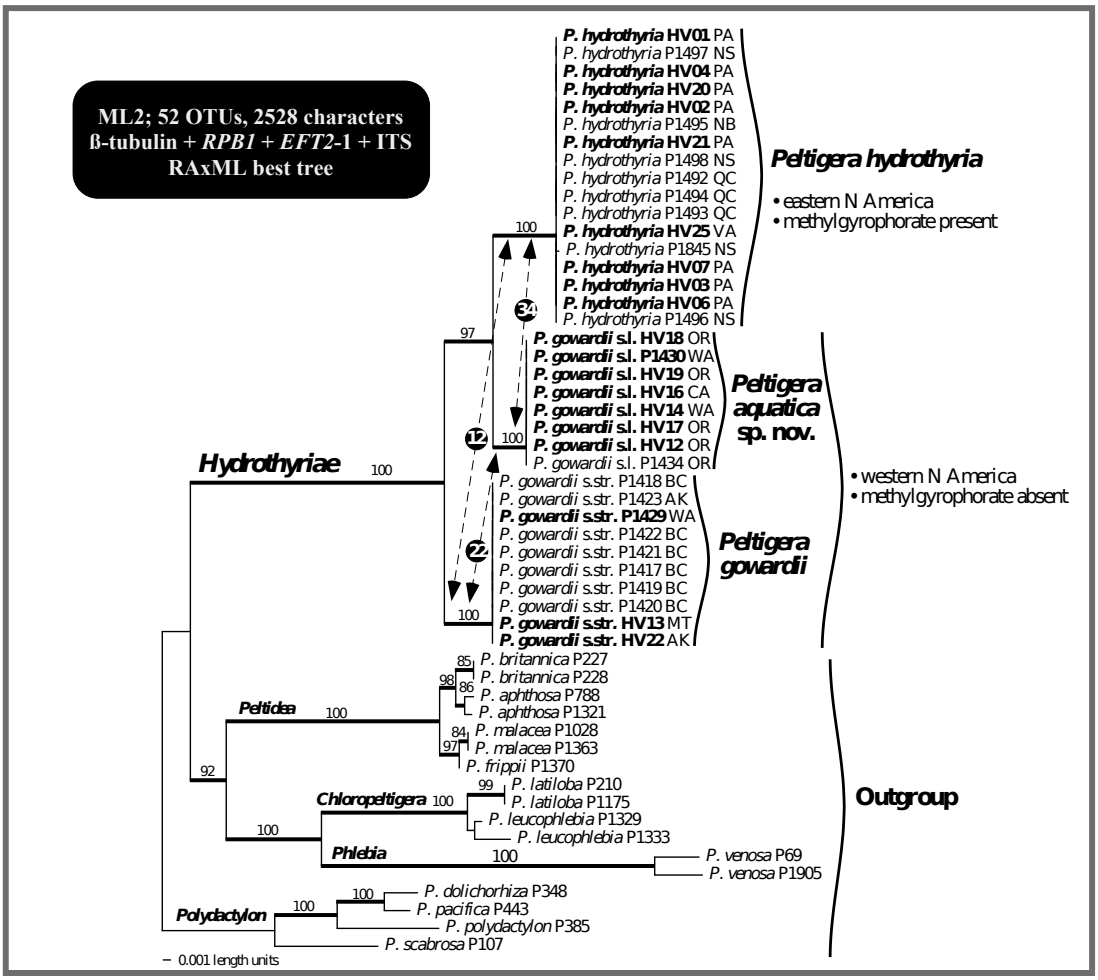

Figure 2: Phylogenetic delimitation of *P. hydrothyria*, *P. gowardii*, and the newly proposed *P. aquatica* (*P. gowardii* s.l.) as revealed by a maximum likelihood analysis of 35 individuals from section *Hydrothyriae* based on combined *β-tubulin*, *RPB1*, *EFT2*-1, and ITS loci (ML2). A total of 11 species from sections *Chloropeltigera*, *Peltidea*, *Phlebia*, and *Polydactylon* formed the outgroup. Values above internodes represent bootstrap support (BS). Thicker internodes indicate strongly supported (BS $\geq$ 80%) relationships. Abbreviations after taxon names within section *Hydrothyriae* indicate the geographical origin of sequenced individuals (states in the USA, Canadian provinces). Underlined names indicate specimens included in the study by Lendemer and O'Brien (2011). Numbers of polymorphic sites with shared nucleotides between pairs of species (dotted arrows) are shown in black circles (see Table 2).

Table 2: Haplotypes (H) sampled within each *Peltigera* species from the section *Hydrothyriae*, and their geographic origins. For the *rbcLX* locus, five spacer types are reported after the slash.Notes: AK, Alaska; BC, British Columbia; CA, California; MT, Montana; NB, New Brunswick; NS, Nova Scotia; OR, Oregon; PA, Pennsylvania; QC, Queébec; VA, Virginia; WA, Washington State.

Abbr.	Taxon	Voucher	ITS 4H	*β-tubulin* 4H	*RPB1* 3H	*EFT2*-1 4H	*rbcLX* 14H/5 spacers
P1430	*P. aquatica* (*P. gowardii* s.l.)	USA, WA	H4	H3	H2	H3	H14/S5
HV18	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	H3	H13/S5
HV19	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	H3	H11/S5
HV12	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	N/A	H12/S5
HV14	*P. aquatica* (*P. gowardii* s.l.)	USA, WA	H4	H3	H2	N/A	N/A
HV16	*P. aquatica* (*P. gowardii* s.l.)	USA, CA	H4	H3	H2	N/A	H11/S5
HV17	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	N/A	H14/S5
P1434	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	N/A	H2	N/A	H14/S5
All	*P. aquatica*(*P. gowardii* s.l.)	western	1H	1H	1H	1H	4H/1
P1417	*P. gowardii* s.str.	Canada, BC	H1	H1	H1	H1	H6/S2
P1418	*P. gowardii* s.str.	Canada, BC	H1	H1	N/A	H1	H5/S2
P1419	*P. gowardii* s.str.	Canada, BC	H1	H1	H1	H1	H5/S2
P1420	*P. gowardii* s.str.	Canada, BC	H1	H1	H1	H1	H7/S2
P1421	*P. gowardii* s.str.	Canada, BC	H1	H1	H1	H1	H5/S2
P1422	*P. gowardii* s.str.	Canada, BC	H1	H1	H1	H1	H5/S2
P1423	*P. gowardii* s.str.	USA, AK	H1	H1	N/A	H1	H10/S4
P1429	*P. gowardii* s.str.	USA, WA	H1	H1	N/A	H1	H10/S4
HV13	*P. gowardii* s.str.	USA, MT	H1	H1	H1	H1	H5/S2
HV22	*P. gowardii* s.str.	USA, AK	H1	H1	H1	N/A	H5/S2
All	*P. gowardii* s.str.	western	1H	1H	1H	1H	4H/2
P1492	*P. hydrothyria*	Canada, QC	H2	H2	H3	H2	H3/S1
P1493	*P. hydrothyria*	Canada, QC	H2	H2	H3	H2	H8/S3
P1494	*P. hydrothyria*	Canada, QC	H2	H2	H3	H2	H9/S3
P1495	*P. hydrothyria*	Canada, NB	H3	H2	H3	H2	H2/S1
P1496	*P. hydrothyria*	Canada, NS	H2	H2	H3	H2	H9/S3
P1497	*P. hydrothyria*	Canada, NS	H3	H2	H3	H2	H2/S1
P1498	*P. hydrothyria*	Canada, NS	H3	H2	H3	H2	H9/S3
P1845	*P. hydrothyria*	Canada, NS	H3	H2	H3	H4	H9/S3
HV25	*P. hydrothyria*	USA, VA	H3	N/A	N/A	N/A	H9/S3
HV07	*P. hydrothyria*	USA, PA	H3	H2	H3	N/A	H9/S3
HV06	*P. hydrothyria*	USA, PA	H3	H2	H3	N/A	H9/S3
HV04	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	H9/S3
HV03	*P. hydrothyria*	USA, PA	H3	H2	H3	N/A	H9/S3
HV21	*P. hydrothyria*	USA, PA	H3	H2	N/A	H2	H1/S1
HV20	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	H3/S1
HV02	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	H4/S1
HV01	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	N/A
All	*P. hydrothyria*	eastern	2H	1H	1H	2H	6H/2

cally and geographically more similar to *P. gowardii* s.s. Both *P. gowardii* s.s. and s.l. lack any detectable secondary compounds, and their ranges overlap in western North America (Washington state). However, all records, confirmed by molecular data (this study), for *P. gowardii* s.s. are from more northern localities (spreading up to Alaska) and a single occurrence in Montana, whereas *P. gowardii* s.l. has been reported from more southern states (e.g., Oregon and California) (Fig. 2, Appendix 1). The first diversification event within the section *Hydrothyriae* is represented by *P. gowardii* s.s. and therefore indicates that the aquatic *Peltigera* probably originated in the western area of North America, followed by a subsequent split into two lineages, *P. gowardii* s.l. (spreading south to California) and *P. hydrothyria* (isolated in the Appalachian mountain of eastern North America). Detailed morphological studies on fresh living specimens are needed to determine whether there are subtle differences that could distinguish the species and which might correlate with the genetic differentiation found in this section.

4.3.3 Cyanobiont identity within the section *Hydrothyriae*

Casamatta et al. (2006) circumscribed a new *Capsosira* species (*C. lowei*; Capsosiraceae, Stigonematales) to accommodate the cyanobiont isolated from the thallus of *P. hydrothyria* collected in North Carolina, which was reported as a filamentous cyanobacterium with true branching. However, the authors stated that phylogenetic analyses of the 16S, and structural similarity of the ribosomal ITS region supported this new species as being affiliated with *Nostoc* (Nostocales), i.e., sister to *N. commune* UTEX584 with high bootstrap support (Casamatta et al., 2006). As expected, based on previous phylogenetic studies on cyanobacteria (e.g., Turner et al. 1999; O'Brien et al. 2005; Korelusová 2008), most relationships in our 16S phylogeny were poorly supported (Fig. 3), but the overall topology and delimitation of major clades were in agreement with the existing 16S and *rbcLX* phylogenies (e.g., *Nostoc* clade I and II, see Otálora et al. 2010; O'Brien et al. 2009; H5 and H3, see Korelusová 2008).

The three cyanobacteria from the aquatic *Peltigera* thalli and *C. lowei* were nested within one of the few well-supported monophyletic groups (72% bootstrap support) within the *Nostoc* clade II, which contains the majority of symbiotic *Nostoc* associated with plants and lichens, as well as many free-living taxa of *Nostoc*, *including Nostoc commune* Vaucher ex Bornet & Flahault UTEX584 (Fig. 3). Phylogenetic relationships within the *Nostoc* clade II are mostly uncertain based on the 16S. The phylogenetic study of heterocystous cyanobacteria by Korelusová (2008) showed similar placement of *C. lowei* (in Nostocales; clade H5) among lichen cyanobionts of *Nostoc*. Our results suggest that cyanobacteria associated with the aquatic *Peltigera*, including *C. lowei*, represent *Nostoc* s.l., however, its closest relatives could not be established with high confidence. We could not evaluate the phylogenetic placement of the whole genus *Capsosira* (Capsosiraceae, Stigonematales) because the cultures for the remaining two species (*C. brebissonii* Kuützing ex Bornet & Flahault—type species and *C.*

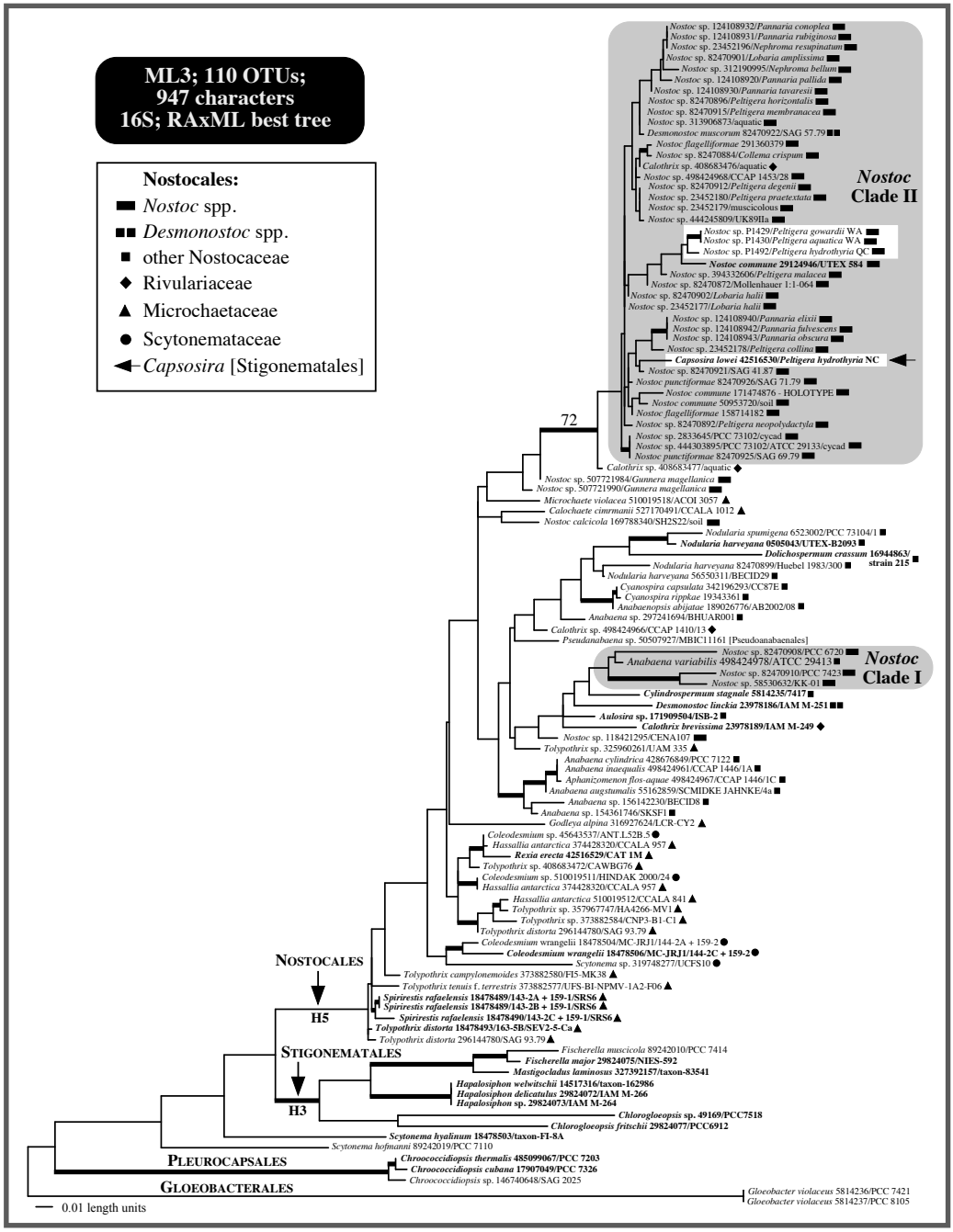
ML3; 110 OTUs;
947 characters
16S; RAxML best tree
Nostocales:
Nostoc spp.
Desmonostoc spp.
other Nostocaceae
Rivulariaceae
Microchaetaceae
Scytonemataceae
Capsosira [Stigonematales]
Nostoc Clade II
Nostoc Clade I
72
NOSTOCALES
H5
STIGONEMATALES
H3
PLEUROCAPSALES
GLOEOBACTERALES
0.01 length units

Figure 3: Placement of four cyanobionts (*Nostoc* sp. and *Capsosira lowei*) associated with section *Hydrothyriae* (white boxes) in the phylogenetic context of cyanobacteria (108 representatives) mostly from the Nostocales (families Nostocaceae, Microchaetaceae, Scytonemataceae, and Rivulariaceae), and related orders Stigonematales and Pleurocapsales, revealed by maximum likelihood analysis of the16S rRNA gene (ML3). Two representatives of Gloeobacterales (*Gloeobacter violaceus*) were used to root the tree. Thicker internodes indicate relationships with BS $\geq$ 70%. Taxon names in boldface indicate sequences included in the phylogeny presented in fig. 3 of Casamatta et al. (2006). *Nostoc* clade I and clade II correspond to clades delimited by O'Brien et al. (2005) in their fig. 2, whereas H3 (Stigonematales) and H5 (Nostocales) correspond to clades delimited by Korelusová (2008) in their fig. 1. GenBank identification numbers for all published sequences included are shown after each terminal name.

brasiliensis C. L. Sant'Anna & S. M. F. Silva) were not available in public depositories.

The genus *Nostoc* is shown to be a nonmonophyletic assemblage (Fig. 3), that is in need of a comprehensive molecular systematic treatment. Recently, an attempt toward disentangling this complex taxon was made by introducing a new genus, *Desmonostoc* Hrouzek & Ventura, to accommodate *Nostoc muscorum* Agardh ex Bornet & Flahault and related unnamed strains (Hrouzek et al., 2013). However, this change was based on a very restricted data set lacking completely lichen-associated strains and other commonly used reference cultures. Our phylogenetic results do not support a monophyletic genus *Desmonostoc* because *D. muscorum* (Agardh ex Bornet & Flahault) Hrouzek & Ventura (SAG 57.79) is nested in the *Nostoc* clade II (with strong support), whereas the phylogenetic placement of *D. linckia* (IAM M-251) falls outside clade II (Fig. 3).

Based on the *rbcLX* analysis, all *Hydrothyriae* cyanobionts were placed in subclade 3, extended cluster 1 (Fig. 4; Appendix S1, see Supplemental Data with the online version of this article), as a monophyletic group closely related to other symbiotic *Nostoc* strains associated with *Gunnera* L., *Blasia* L. and *Geosiphon* F. Wettst. As for previous *rbcLX* phylogenies of that scale, most relationships are not well supported (see Otálora et al. 2010; O'Brien et al. 2013). Because of its unusual habitat, it is possible that a unique lineage of *Nostoc* s.l. is associated with aquatic *Peltigera* and other co-occurring aquatic cyanolichens [i.e., *Leptogium rivulare* (Ach.) Mont. from Collemataceae] in western North America, but no data are available for the cyanobiont from aquatic members of the Collemataceae.

Four distinct *Nostoc* haplotypes were detected in each of the two western species (*P. gowardii* s.l. and *P. gowardii* s.s.), and six in the eastern species (*P. hydrothyria*). Phylogenetic relationships among these cyanobionts did not reflect monophyletic circumscription of the corresponding mycobiont species (Table 2; Fig. 2 vs. Fig. 4A). Two types of *rbcLX* spacers were present among the haplotypes associated with *P. gowardii* s.s. and *P. hydrothyria*, and a single spacer was found in the cyanobionts of *P. gowardii* s.l. (Table 2). Although the spacers were not alignable across all 14 haplotypes and therefore had to be excluded from the subsequent phylogenetic analysis, their sequences were unique for each mycobiont species. The presence of species-specific *rbcLX* sequences would suggest that each of the three aquatic species evolved in association with different strains of *Nostoc* (Table 2, Fig. 4A), which may be one of the factors

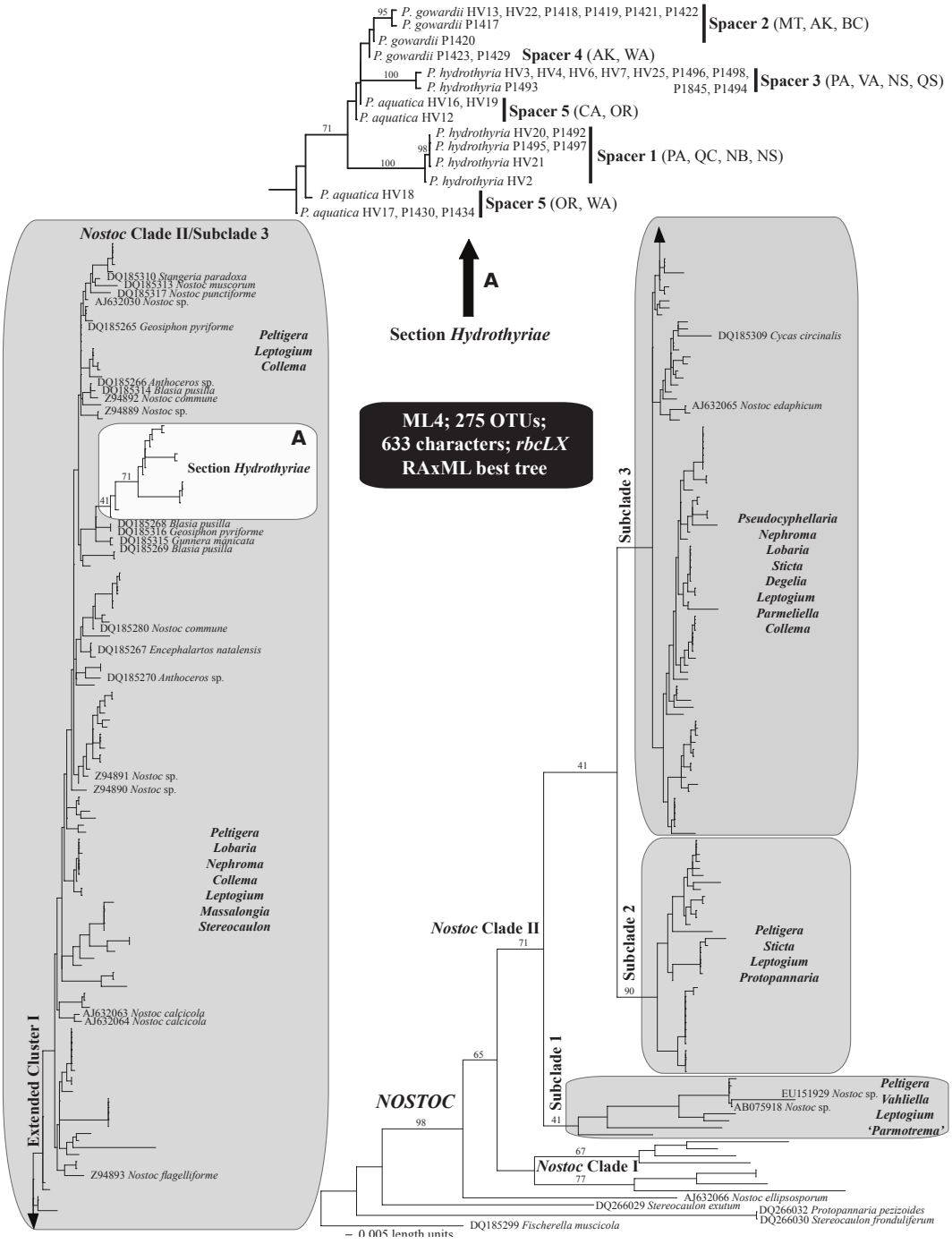
P. gowardii HV13, HV22, P1418, P1419, P1421, P1422
P. gowardii P1417
Spacer 2 (MT, AK, BC)
P. gowardii P1420
P. gowardii P1423, P1429
Spacer 4 (AK, WA)
P. hydrothyria HV3, HV4, HV6, HV7, HV25, P1496, P1498, P1845, P1494
P. hydrothyria P1493
Spacer 3 (PA, VA, NS, QS)
P. aquatica HV16, HV19
P. aquatica HV12
Spacer 5 (CA, OR)
P. hydrothyria HV20, P1492
P. hydrothyria P1495, P1497
P. hydrothyria HV21
P. hydrothyria HV2
Spacer 1 (PA, QC, NB, NS)
P. aquatica HV18
P. aquatica HV17, P1430, P1434
Spacer 5 (OR, WA)
A
Section Hydrothyriae
ML4; 275 OTUs;
633 characters; rbcLX
RAxML best tree
Nostoc Clade II/Subclade 3
DQ185310 Stangeria paradoxa
DQ185313 Nostoc muscorum
DQ185317 Nostoc punctiforme
AJ632030 Nostoc sp.
DQ185265 Geosiphon pyriforme
Peltigera
Leptogium
Collema
DQ185266 Anthoceros sp.
DQ185314 Blasia pusilla
Z94892 Nostoc commune
Z94889 Nostoc sp.
A
Section Hydrothyriae
DQ185268 Blasia pusilla
DQ185316 Geosiphon pyriforme
DQ185315 Gunnera magnicata
DQ185269 Blasia pusilla
DQ185280 Nostoc commune
DQ185267 Encephalartos natalensis
DQ185270 Anthoceros sp.
Z94891 Nostoc sp.
Z94890 Nostoc sp.
Peltigera
Lobaria
Nephroma
Collema
Leptogium
Massalongia
Stereocaulon
AJ632063 Nostoc calcicola
AJ632064 Nostoc calcicola
Extended Cluster I
Z94893 Nostoc flagelliforme
Nostoc Clade II
NOSTOC
Subclade 3
DQ185309 Cycas circinalis
AJ632065 Nostoc edaphicum
Pseudocyphellaria
Nephroma
Lobaria
Sticta
Degelia
Leptogium
Parmeliella
Collema
Subclade 2
Peltigera
Sticta
Leptogium
Protopannaria
Subclade 1
EU151929 Nostoc sp.
AB075918 Nostoc sp.
Peltigera
Vahliella
Leptogium
'Parmotrema'
Nostoc Clade I
AJ632066 Nostoc ellipsosporum
DQ266029 Stereocaulon exutum
DQ266032 Protopannaria pezizoides
DQ266030 Stereocaulon fronduliferum
DQ185299 Fischerella muscicola
0.005 length units

Figure 4: Monophyly of 33 newly sequenced cyanobionts (representing 14 haplotypes) associated with *Peltigera* section *Hydrothyriae* (white box) and their close affiliation with members of *Nostoc* clade II (subclade 3) revealed by maximum likelihood analysis of 271 individuals representing putative *Nostoc* spp. based on the *rbcLX* locus (ML4). *Fischerella muscicola* (Stigonematales) was used to root the tree. Names representing GenBank sequences of free-living and nonlichen-associated symbionts are shown. The remaining terminal branches correspond to GenBank sequences of cyanobionts (Appendix S1) from various lichen genera listed within each subclade. Delimitation of clades, subclades, and cluster I follows Otálora et al. (2010) and O'Brien et al. (2013). Bootstrap support values $\geq 70\%$, as well as support values for important internodes that are $< 70\%$, are shown above internodes. A: Phylogenetic relationships among 14 haplotypes representing cyanobionts found in the section *Hydrothyriae* (*P. gowardii* s.s., *P. aquatica* [*P. gowardii* s.l.] and *P. hydrothyria*) part of the ML4 phylogeny (white box). Cyanobionts with identical sequences are listed after species names of their fungal partner. Unique types of spacers within the *rbcLX* locus (part of the ambiguously aligned region that were excluded from the ML4 analysis) and geographical provenance of the lichen thalli are listed after the black vertical bars (for details see Table 2 and Appendix 1). Bootstrap support values $> 70\%$ are shown above internodes.

shaping speciation in the section *Hydrothyriae* as it has been proposed for other lichen-forming fungi (e.g., Kroken and Taylor 2000; Elvebakk et al. 2008; Fernández-Mendoza et al. 2011; O'Brien et al. 2013; Magain and Sérusiaux 2014).

4.3.4 Recognition of a new species within section *Hydrothyriae*

A clear segregation of all polymorphic sites for multiple loci, the lack of overlap between cyanobiont strains, and potentially distinct geographical patterns, strongly support the recognition of the three monophyletic groups within section *Hydrothyriae* as representing three distinct species (Fig. 2). Therefore, previously recognized as P*. gowardii* s.l. (Lendemer and O'Brien, 2011) is described here as a new species: *P. aquatica*.

Peltigera aquatica Miadl. & Lendemer sp. nov. (MycoBank # MB 809067)— Differs from two other recognized aquatic species (*P. gowardii*, GenBank JF837364; *P. hydrothyria*, JF837365) by having six molecular synapomorphies (including indels) within the nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) at the following positions: (1) 88–97: a chain of 10 adenines vs. 7 for *P. gowardii* and 9 for *P. hydrothyria*; (2) 107: cytosine vs. thymine for *P. gowardii* and adenine for *P. hydrothyria*; (3) 116: adenine vs. cytosine for *P. gowardii* and *P. hydrothyria*; (4) 120–128: a chain of 9 thymines vs. 7 for *P. gowardii* and *P. hydrothyria*; (5) 175: thymine vs. guanine for *P. gowardii* and *P. hydrothyria*; (6) 386: guanine vs. adenine for *P. gowardii* and *P. hydrothyria* (Appendix S2, see online Supplemental Data).

Type—USA, Oregon: Lane County, Ridge Creek, Cougar Reservoir, 44.058N 122.22W, D. Glavitch s.n. (with L. Geiser), 17 February 2007 (NY-01117843, holotype).

Morphology—Similar to *P . hydrothyria* and *P . gowardii* (Fig. 5C, D). Detailed description is provided in Lendemer and O'Brien (2011; Taxonomic section, *P. gowardii*). Chemistry—Similar to *P. gowardii* in that no substances were detected by thin layer chromatography (TLC) and standard spot tests (see Lendemer and O'Brien 2011). *Peltigera hydrothyria* contains methylgyrophorate and methyllecanorate and sometimes traces of gyrophoric or lecanoric acid (which give a C+ pink reaction to acetone extracts;

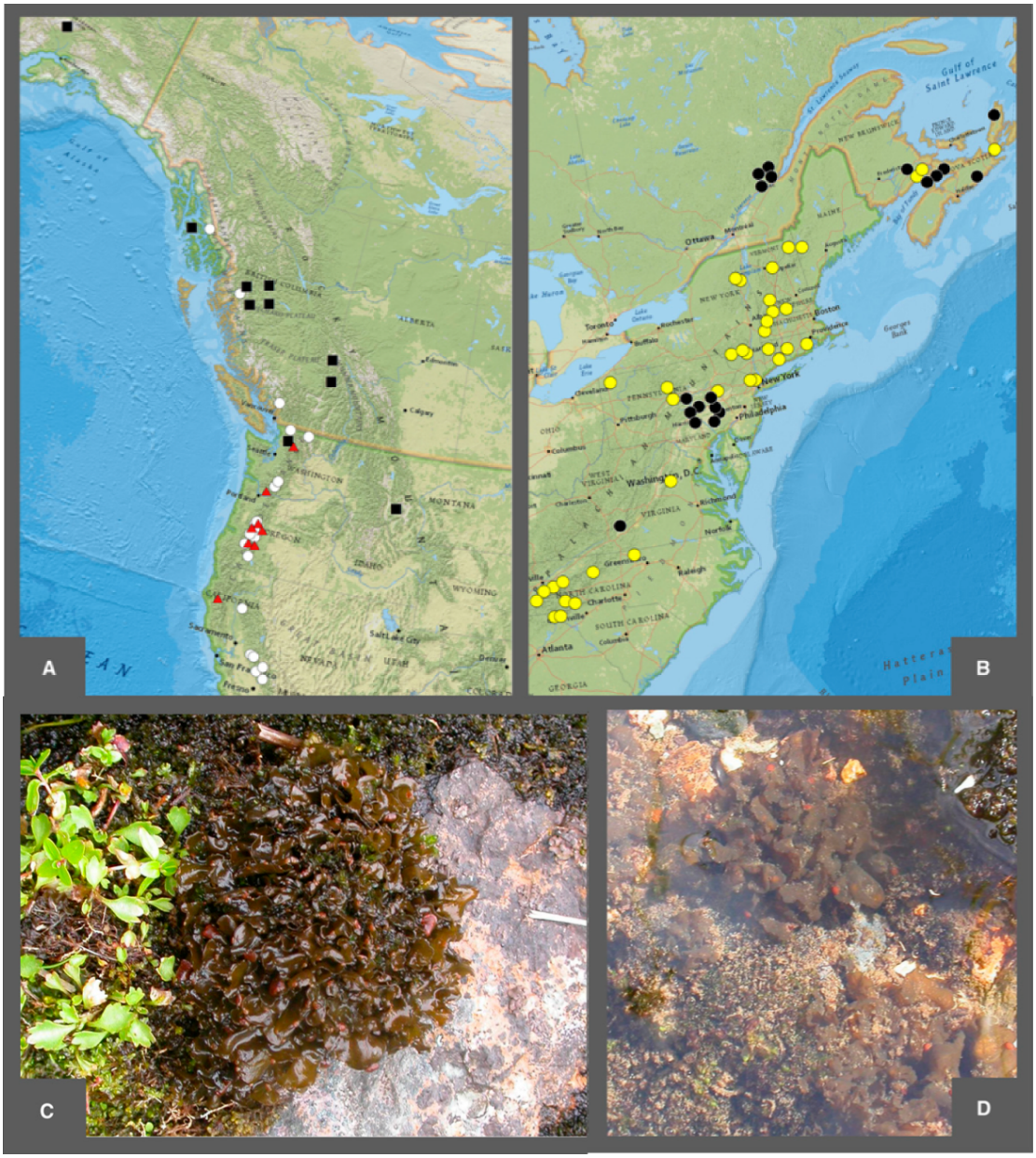

Figure 5: Geographic distribution and habit of aquatic *Peltigera*. The top left panel (A) shows the occurrences of *P. aquatica* (A; red triangles) and *P. gowardii* (A; black squares) confirmed by molecular data. The white circles represent other sites where aquatic *Peltigera* occur but from which specimens have not been examined using molecular techniques. The top right panel (B) shows the occurrences of *P. hydrothyria* (B; black circles) confirmed by molecular data along with the overall distribution of this species (yellow circles) in eastern North America. Note the four black circles shown in the province of Québec represent specimens from different parts of the same stream. The distribution maps were prepared using records from the NYBG herbarium, the Duke herbarium, the COSEWIC (2013a,b) reports, as well as the papers by Lendemer and O'Brien (2011); Lendemer and F (2012). The bottom panels show thalli of *P. gowardii*. One is colonizing a rock close to water level by a stream on Hudson Bay Mountain, Smithers, British Columbia, Canada (C) and a second is growing submerged in the stream (D). All three aquatic *Peltigera* species are morphologically very similar to one another and have not, to date, been differentiated on this basis. Photo credit: David Richardson.

Lendemer and O'Brien 2011).

Etymology— The name of this new species reflects its aquatic habit.

Ecology and distribution— As circumscribed here, the distribution of *P. aquatica* is restricted to the mountains of western United States, extending from central/northern California (Sierra ranges) northward to Oregon and Washington (Cascade ranges) where it co-occurs with *P. gowardii*. The latter species is found in the Northern Cordillera, but has a more maritime tendency and a more northern distribution. The geographical range of *P. gowardii* spans an area from Montana (Rocky Mountains) to British Columbia (Columbia Mountains) in Canada and Alaska.The actual geographical range of *P. aquatica* has to be verified by sequencing the ITS region of the existing collections of *P. gowardii* and populations that were never previously sampled in western North America (Fig. 5A). *Peltigera hydrothyria* is restricted to the eastern part of North America, extending from the southern Appalachian mountains of the USA (Georgia) to Nova Scotia (Canada) (Fig. 5B). The conservation and management status of the newly circumscribed species, *P. aquatica*, needs to be reevaluated in the western United States.

Currently based on the anticipated geographic distribution, its status varies from unranked to imperiled and vulnerable depending on the state (Peterson, 2010). More information about the geographical ranges, ecology, and potential threats to species within section *Hydrothyriae* is provided in online Appendix S3.

Specimens examined—See records of *P. aquatica* in Appendix 1 and Fig. 5A.

4.3.5 Conclusions

Although we expanded the taxon sampling and the number of molecular markers, this phylogenetic study demonstrated that four loci (three protein-coding genes and nrLSU) are not sufficient for reconstructing with high confidence the relationships among sections in the genus *Peltigera*, including the placement of the section *Hydrothyriae*. More characters (preferably single-copy protein-coding genes) are needed to capture deep evolutionary splits within the genus. However, the ITS region alone provided sufficient genetic information to distinguish three morphologically cryptic species within section *Hydrothyriae*. The addition of three protein-coding genes confirmed the ITS-based phylogenetic results from Lendemer and O'Brien (2011) and supported the recognition of a new species, *Peltigera aquatica*. Our phylogenies for nostocalean cyanobacteria indicate that molecular revisions of the genus *Nostoc* (known to be nonmonophyletic) and reliable identification of cyanobionts associated with lichens are needed. Future studies should be based on more complete sampling, with symbiotic strains and reference taxa well represented in the analyzed data and using more variable, but alignable, markers. Switches to different *Nostoc* strains might be associated with the cospeciation events within section *Hydrothyriae*. Future detailed morphological, anatomical, and chemical

revision of aquatic *Peltigera* based on freshly collected material may reveal phenotypic features correlated with the molecular data.

All collections of *P. gowardii* should be verified molecularly (based on the ITS region) to tease apart the actual geographical ranges of *P. aquatica* and *P. gowardii*, especially in the areas where both species co-occur. The molecular approach may not be possible for the old herbarium specimens especially if the material was collected in the last century. Potential threats to the populations of morphologically cryptic *P. aquatica* and *P. gowardii* should be then re-evaluated to assure survival of both taxa. Because of the aquatic habit, unique ecology (certain level of year-round humidity, stream-water flow, a generally low water temperature, pH close to neutral, and a lack of silt), and restricted geographical ranges, species from section *Hydrothyriae*, and very likely other co-occurring macrolichens (e.g., members of the Collemataceae and Verrucariaceae), are endangered as a result of anthropogenic activities negatively affecting lichen thalli and their habitat (e.g., through human recreation and communication infrastructure, forest management, global climate change, and pollution). Populations of aquatic *Peltigera* should continue to be monitored and protected.

4.4 Appendices

Appendices are available online and can be downloaded at
http://github.com/NicolasMagain/ThesisOnlineSupplementary

4.5 Bibliography

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Chapter 5

A further new species in the lichen genus *Arctomia*: *A. borbonica* from Reunion (Mascarene archipelago)

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Published in MycoKeys 4:9-21. 2012.

Abstract

Arctomia borbonica **sp. nov.** is described as new for science from montane natural and secondary habitats in Reunion in the Mascarene archipelago (Indian Ocean). It has a sterile, foliose, usually wrinkled, thallus whose margins produce goniocysts that disintegrate into a soredioid margin; it looks like a *Leptogium* species. Its phylogenetic position in the Arctomiaceae (Ostropomycetidae, Ascomycota) has been determined with 3 genes (nuLSU, mtSSU, *RPB1*) inferences.

Key Words :Ascomycota, Ostropomycetidae, Arctomiaceae, *Arctomia*, phylogenetic inferences, nuLSU, mtSSU, *RPB1*, Reunion, Mascarene archipelago

5.1 Introduction

Within the Lecanoromycetes, the subclass Ostropomycetidae Reeb, Lutzoni and Cl. Roux exhibits an impressive diversity of ascomata, thallus forms and ecological requirements. The phylogenetic relationships between genera and families are poorly resolved (Baloch et al., 2010), although impressive progress has been recently achieved for the Graphidaceae (incl. Thelotremataceae), the second largest family of lichenized fungi (Rivas Plata et al., 2012). Many taxa within the subclass still require detailed phy-

logenetic studies. Indeed, modern statistical methods within a phylogenetical context using several loci sequences yielded interesting and quite unexpected results, such as the polyphyly of two well-known genera. *Graphis* is now resolved into two strongly supported clades, nested within a large clade comprizing other well-known genera such as Diorygma, Glyphis and Phaeographis (tribe Graphideae; Rivas Plata et al. 2011). Further, *Pertusaria* is resolved into four strongly supported groups: *Pertusaria* s. str. (incl. the type species *P. pertusa*), *Pertusaria* s. l. 1 including *P. amara*, *P.* s. l. 2 including *P. lactea* and *P. velata*, and a fourth group, comprizing the species with gyalectoid ascomata and recently recognized as the new genus *Gyalectaria* (Schmitt et al., 2010).

Within such a large and very much unresolved variation, the case of the Arctomiaceae is rather simple. The family is strongly supported and includes three genera: *Gregorella* and *Wawea*, each with one species, and *Arctomia* with five species (Henssen, 1969; Henssen and Kantvilas, 1985; Jørgensen, 2003, 2007; Lumbsch et al., 2005; Øvstedal and Gremmen, 2001, 2006). They are lichenized with the cyanobacteria genus *Nostoc*, have a corticate thallus, gymnocarpous ascomata, asci with a non-amyloid thallus, and 1-10-septate, hyaline ascospores.

We here report the discovery of a further new species, which we assign to the genus *Arctomia*, found epiphytic in montane habitats in the island of Reunion (Mascarene archipelago, Indian Ocean). The material was first assigned to *Leptogium*, a genus belonging to the Collemataceae in the Lecanoromycetidae (Lumbsch and Huhndorf, 2011). It is an unusual species as it has a foliose, sometimes very much crumpled, thallus, producing corticate and easily detached "goniocysts", best developed at the lobes margins, disrupting when mature and then forming a soredioid margin. Three loci were amplified (nuLSU, mtSSU, *RPB1*) and inferences from the sequences produced from two collections left no doubt that the material belongs to the Arctomiaceae, and statistical support to include it in the genus *Arctomia* was found. A new species is thus described in this genus.

5.2 Methods

Well-preserved lichen specimens lacking any visible symptoms of fungal infection were used for DNA isolation. Extraction of DNA and PCR amplification were performed following the protocol of Cubero et al. (1999). The primers used were: for nuLSU, LR0R, LR3R, LR3, LR5R and LR6 (following the suggestions available on www.lutzonilab.net/primers), for mtSSU, mtSSU1 and mtSSU3R (Zoller et al., 1999), for *RPB1*, AFasc and 6R1asc (following the suggestions available on www.lutzonilab.net/primers). Amplicons were sequenced by Macrogen®. Sequence fragments were assembled with Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were subjected to megaBLAST searches (Wheeler et al., 2007) to detect potential contaminations.

We assembled matrices with most representatives of species included by Lumbsch et al. (2005) in their description of the new genus *Gregorella*, resolved within the strongly supported Arctomiaceae; we further added several other species belonging to the Ostropomycetidae included in the study of the gyalectoid representatives of *Pertusaria* s.l. by Schmitt et al. (2010), assigned to the new genus *Gyalectaria*. All accessions available on GenBank of representatives of the Arctomiaceae were included; they represent all species assigned to that family, except for both species of *Arctomia* described from subantarctic islands by Øvstedal and Gremmen (2001, 2006). The outgroup species (*Bacidia rosella*, *Lecanora intumescens* and *Toninia cinereovirens*) were chosen outside the Ostropomycetidae and within the Lecanorales (Miadlikowska et al., 2006) to avoid any putative homoplasy problem. Six new sequences were generated for this study, all belonging to the new species described in this paper (Table 1). The sequences were first aligned using MAFFT (online version available at http://mafft.cbrc.jp/alignment/server/) and eventually manually adjusted using MacClade v. 4.05 (Maddison and Maddison, 2002). Ambiguous characters have been detected by eye and excluded from the analyses.

Three matrices were assembled: 38 species with 927 included characters for nuLSU, 38 species with 668 included characters for mtSSU and 32 species with 675 included characters for *RPB1* (part 1). Incongruence between the matrices was tested with maximum likelihood analysis using GARLI (Zwickl, version 0.951 for OS X) with gaps treated as missing data, and a single most likely tree was produced. Support for the branches was estimated using bootstrap values from 1000 pseudoreplicates (all other parameters identical to the original ML search). A conflict was considered sig- nificant if a clade was supported with bootstrap support $\geq 75\%$ in a one-locus analysis and not in the other two. A further test for conflict was performed with LSU and *RPB1* concatenated in a single matrix versus mtSSU in another. No conflict was detected and therefore the available sequences for the three loci were concatenated. The assembled matrix is deposited in TreeBASE under the accession number 12710.

An unweighted maximum parsimony (MP) analysis was performed in PAUP* 4.0b10 (Swofford, 2003). All characters were equally weighted and gaps were treated as missing data. A first heuristic analysis was performed using NNI (Nearest Neigh- bor Interchange) branch-swapping, with 1000 replicates and saving 10 trees at each step, the functions Steepest descent and MulTrees being in effect. A second analysis was performed with the 10,000 saved trees using TBR (Tree Branch Swapping), with a maximum of 200 trees saved at each step, the function Steepest descent being inactivated. A 50% consensus tree is produced, and the strength of support for individual branches was estimated using bootstrap values (MPBS) obtained from 1000 heuristic bootstrap pseudoreplicates.

A partition of six subsets was implemented in the concatenated matrix: nuLSU, mtSSU, intron in *RPB1*, and three for each *RPB1* codon position. Models of evolution for the maximum likelihood and Bayesian analysis were selected based on the Akaike Information Criterion (Posada and Buckley, 2004) as implemented in Mr. Modeltest

v2.3 (Nylander, 2004). The selected model corresponds to the GTR model of nucleotide substitution (Rodriguez et al., 1990) including a proportion of invariable sites and a discrete gamma distribution of six rates categories. The maximum likelihood analysis was performed using RAxML-HPC2 (Stamatakis, 2006) on the Cipres Gateway (Miller et al., 2010), with 1000 bootstrap pseudoreplicates. Bayesian analyses were carried out using the Metropolis-coupled Markov chain Monte Carlo method (MC3) in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004). No priors values were assumed and gaps were treated as missing data. Four parallel runs were performed, each using four independent chains (three heated and one cold chain), with a single tree saved every 100th generation for a total of 6,000,000 generations. The incremental heating scheme was set by default. We used TRACER v1.4.1 (Rambaut and Drummond, 2007) to plot the log-likelihood values of the sample points against generation time, and determine when stationarity was achieved. Consequently the first 6,000 sampled trees were deleted as the burn-in of the chain. A majority rule consensus tree with average branch lengths was constructed for the remaining trees using the sumt option of MrBayes. Phylogenetic trees were visualized using FigTree v1.3.1 (Rambaut and Drummond, 2009). Branches support was considered as significant when Maximum Parsimony Bootstrap (MPBS) $\geq$ 70%, Maximum Likelihood Bootstrap (MLBS) $\geq$ 70% and Posterior Probabilities (PP) $\geq$ 0.95.

We tested the monophyly of the genus *Arctomia* by comparing the best unconstrained tree with the best tree obtained by constraining all *Arctomia* sequences to form a monophyletic group. Trees were generated in RaxML and then tested with two methods: the Shimodaira-Hasegawa (SH) test and the Expected Likelihood Weight (ELW) test as implemented in Tree-PUZZLE 5.2. (Shimodaira and Hasegawa, 2001; Strimmer and Rambaut, 2002; Schmidt et al., 2002)

5.3 Results

The concatenated matrix with aligned sequences for nuLSU, mtSSU and *RPB1* has 2781 characters, out of which 511 are excluded (330 for nuLSU out of which 250 represent introns in *Bacidia rosella*, 173 for mtSSU and 8 for *RPB1*), 983 are constant, 276 are parsimony-uninformative and 1011 are parsimony potentially informative. The most parsimonious tree has the following characteristics: length = 6295 steps, CI = 0.336 and RI = 0.428. The ML analysis yielded a tree with a likelihood value of Ln = -28660.4 and length of 6.175. Parameters of the partitions were as follows: LSU — p(A)= 0.2604, p(C)= 0.2216, p(G)= 0.2980, p(T)= 0.2199 a= 0.3134, r(A-C)= 0.7438, r(A-G)= 1.8229, r(A-T)= 0.7430, r(C-G)= 0.7409, r(C-T)= 4.5270, r(G-T)= 1.0000; mtSSU — p(A)= 0.3330, p(C)= 0.1606, p(G)= 0.2136, p(T)= 0.2926, a= 0.4207, r(A-C)= 0.9284, r(A- G)= 2.9298, r(A-T)= 1.6160, r(C-G)= 0.6649, r(C-T)= 3.4571, r(G-T)= 1.0000; RPB1 intron — p(A)= 0.2349, p(C)= 0.2056, p(G)= 0.2267, p(T)= 0,3287, a= 0.9412, r(A- C)= 6.9358, r(A-G)= 21.9085, r(A-T)= 11.1853, r(C-G)= 8.6280, r(C-

T)= 19.3378, r(G-T)= 1.0000; RPB1, 1st codon — p(A)= 0.2778, p(C)= 0.2440, p(G)= 0.3318, p(T)= 0.1463, a= 0.4211; r(A-C)= 4.0125, r(A-G)= 5.8268, r(A-T)= 3.1946, r(C-G)= 2.7176, r(C-T)= 2907386, r(G-T)= 1.0000; RPB1, 2nd codon — p(A)= 0.3521, p(C)= 0.2038, p(G)= 0.2319, p(T)= 0.2122, a= 0.3474, r(A-C)= 1.7253, r(A-G)= 3.1209, r(A- T)= 0.5159, r(C-G)= 1.9509, r(C-T)= 4.4498, r(G-T)= 1.0000; RPB1, 3rd codon — p(A)= 0.2683, p(C)= 0.2056, p(G)= 0.2545, p(T)= 0.2716, a= 0.5667, r(A-C)= 8.7546, r(A-G)= 24.9090, r(A-T)= 4.6296, r(C-G)= 5.8128, r(C-T)= 56.3087, r(G-T)= 1.0000.

All three analyses retrieve the family Arctomiaceae as a strongly supported clade (MPBS= 81%, MLBS = 97%, PP=1) (Fig. 1). All nodes within the Arctomiaceae clade are strongly supported: A. delicatula and A. teretiuscula form a clade supported with MLBS= 99% and PP=1.0; they further form a clade with both accessions of *A. borbonica* that is supported with MLBS = 94% and PP=1.0; *Gregorella humida* and *Wawea fruticulosa* form a clade supported with MLBS = 86% and PP= 1.0; and finally the latter is sister to the clade including all accessions of *Arctomia* (except for *A. interfixa*) in a node supported by MLBS= 95% and PP= 1.0.

SH test shows that the likelihood of the topology constraining all *Arctomia* sequences to form a monophyletic group is not significantly worse (at 0.05 significance level) than that with *Arctomia interfixa* being sister to all other accessions of the Arctomiaceae. Following that test, the monophyly of all species assigned to *Arctomia*, incl. *A. borbonica* sp. nov., cannot be rejected. The result of the ELW is the contrary: such a monophyly is rejected at 0.0473 significance level.

5.4 Discussion

The lichen family Arctomiaceae is fully recovered in our analysis (Fig. 1) and all other accessions are resolved in positions fully consistent with those published for the Ostropomycetidae (Lumbsch et al., 2005; Baloch et al., 2010; Schmitt et al., 2010), including the polyphyly of representatives of Pertusaria that are resolved in three distinct lineages, and the representative of the newly described genus *Gyalectaria* that is resolved as sister to the representative of *Coccotrema*. Our material is resolved without ambiguity within the Arctomiaceae. It is resolved with strong support as sister to a clade comprising the type species of *Arctomia* (*A. delicatula*). The monophyly of the three species of *Arctomia* for which DNA sequences are available, demonstrated with strong support in Lumbsch et al. (2005), is not recovered in our analysis but is not rejected by the topology tests. The assignment of our new species to the genus *Arctomia* can thus be considered legitimate. The apparent dismemberment of *Arctomia* in our analysis (with *A. interfixa* as sister to all other taxa of the Arctomiaceae) may be due to an incomplete dataset (sequences for the three loci are available for all accessions of Arctomiaceae, except for *A. interfixa* which lacks the most informative *RPB1* sequence): indeed, incomplete dataset may produce misleading results in likelihood-based analysis

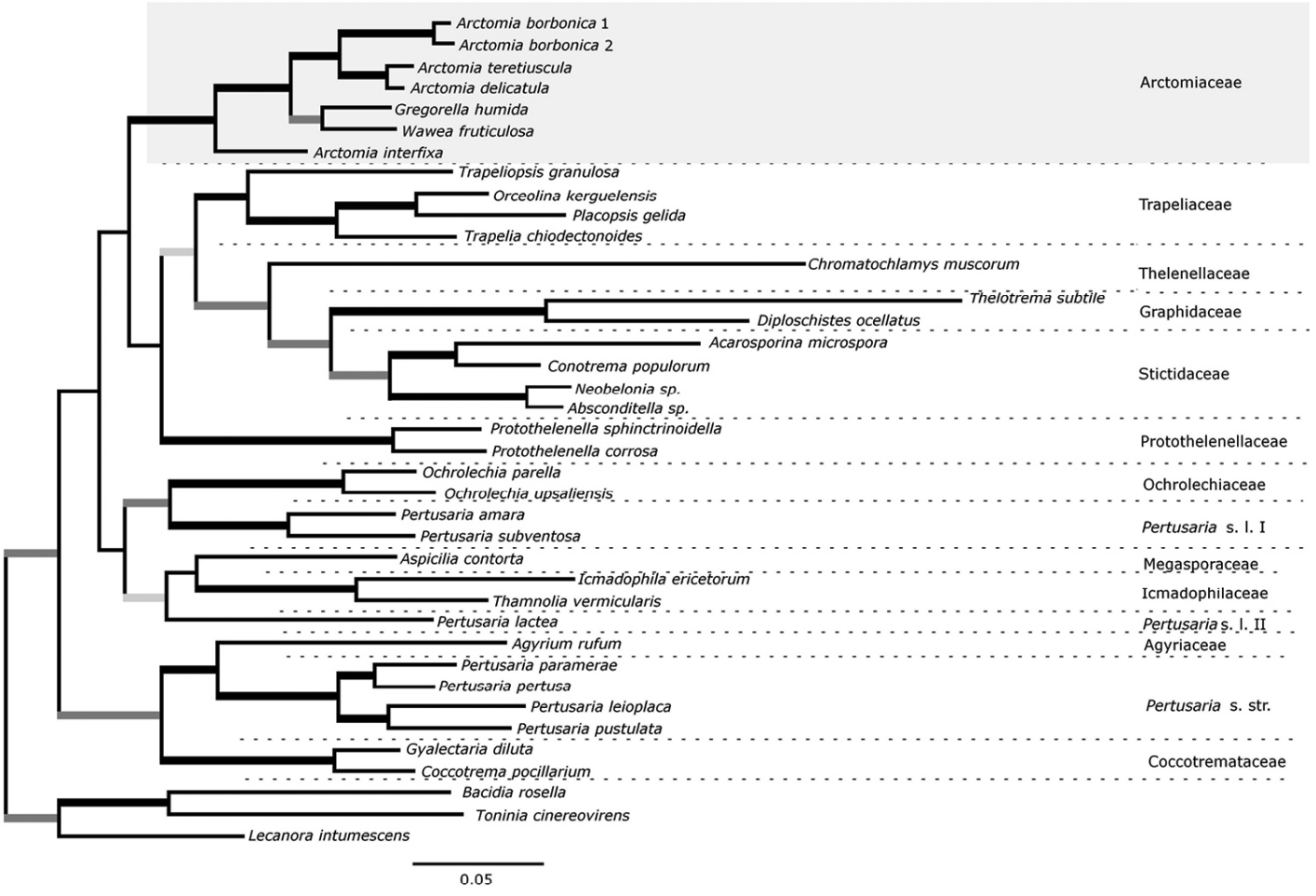

Figure 1: 50% consensus tree produced by the Bayesian analysis of a concatenated matrix with three loci (nuLSU, mtSSU and RPB1) with 2531 characters and highlighting the Arctomiaceae and the newly described Arctomia borbonica. Branches supported by MPBS and MLBS $\geq$ 70% and Bayesian posterior probabilities $\geq$ 0.95 are in black; those supported by MLBS $\geq$ 70% and Bayesian posterior probabilities $\geq$ 0.95 in dark grey and those only by Bayesian posterior probabilities $\geq$ 0.95 in light grey.

(Simmons, 2012). However, separate analyses of LSU and mtSSU sequences yielded the same topology, with *Arctomia* paraphyletic. The status of *Arctomia interfixa* should thus be studied in more details.

Diagnostic characters for the genera recognized within the Arctomiaceae are given by Lumbsch et al. (2005). In the absence of ascomata and conidiomata, they are: thallus crustose, composed of goniocysts for *Gregorella*, fruticose for *Wawea* and crustose to coralloid or squamulose for *Arctomia*. The other two species of *Arctomia*, described by Øvstedal and Gremmen (2001, 2006) and not included in Lumbsch et al. (2005) have a thallus "placodioid" or "foliose, [...] squamulose or elongate, forming rosettes". If assigned to *Arctomia*, our new species does not match the thallus description of that genus, as its thallus is foliose and produces typical goniocysts at its margin, disintegrating into a soredioid margin (Fig. 2). We suggest the thallus of *Arctomia borbonica* is much similar to that of *Wawea fruticulosa* which has a "fruticose, olive-grey to brown" thallus (Henssen and Kantvilas, 1985) but with lobes flattened or at least furrowed (see fig. 2 in Henssen and Kantvilas 1985; Kantvilas et al. 1999). Further, the structure of the cortex is quite similar in *Wawea* (cross section and surface view: see fig. 3A–B in Henssen and Kantvilas 1985) when compared with *A. borbonica* (Fig. 2C–E). Finally, it is interesting to note that the sister species of *Wawea* is *Gregorella humida* whose thallus is entirely made of goniocysts, very similar to those produced by *Arctomia borbonica* at its thallus margin. As long as ascomata and conidiomata are not found and could provide more information, the thallus characters of *Arctomia borbonica* confuse the generic delimitations within the family.

The hypothesis of describing a new genus for *Arctomia borbonica* has been carefully assessed. Indeed, the genus as circumscribed by Henssen (1969); Jørgensen (2007) is well-delimited and the inclusion of *A. borbonica* makes it morphologically heterogeneous. We refrained from describing a new genus because of the following points: (a) both subantarctic species recently described by Øvstedal and Gremmen (2001, 2006) in the genus, both assumed not to genuinely belonging to *Arctomia* s. str. and with generic affinities "under study", should be further studied; indeed, several characters put them aside of the genus such as a pluricellular cortex; the description of a new genus within such a small family as the Arctomiaceae is premature in that context; (b) ascomata and conidiomata are unknown, or not yet discovered, in *A. borbonica* and thus our dataset lacks important characters (Lumbsch et al. 2005, Table 2); (c) morphological and anatomical characters may be very much misleading for phylogenetic reconstruction and sound generic delimitations as demonstrated by many studies in lichenized or unlichenized ascomycetes (Gaya et al., 2008; Lantz et al., 2011; Prieto et al., 2012; Sérusiaux et al., 2010); and (d) two statistical topology tests applied to the likelihood tree gave opposite results to assess the monophyly of *Arctomia* when including all species studied, e.g. *A. borbonica*, *A. delicatula*, *A. interfixa* and *A. teretiuscula*.

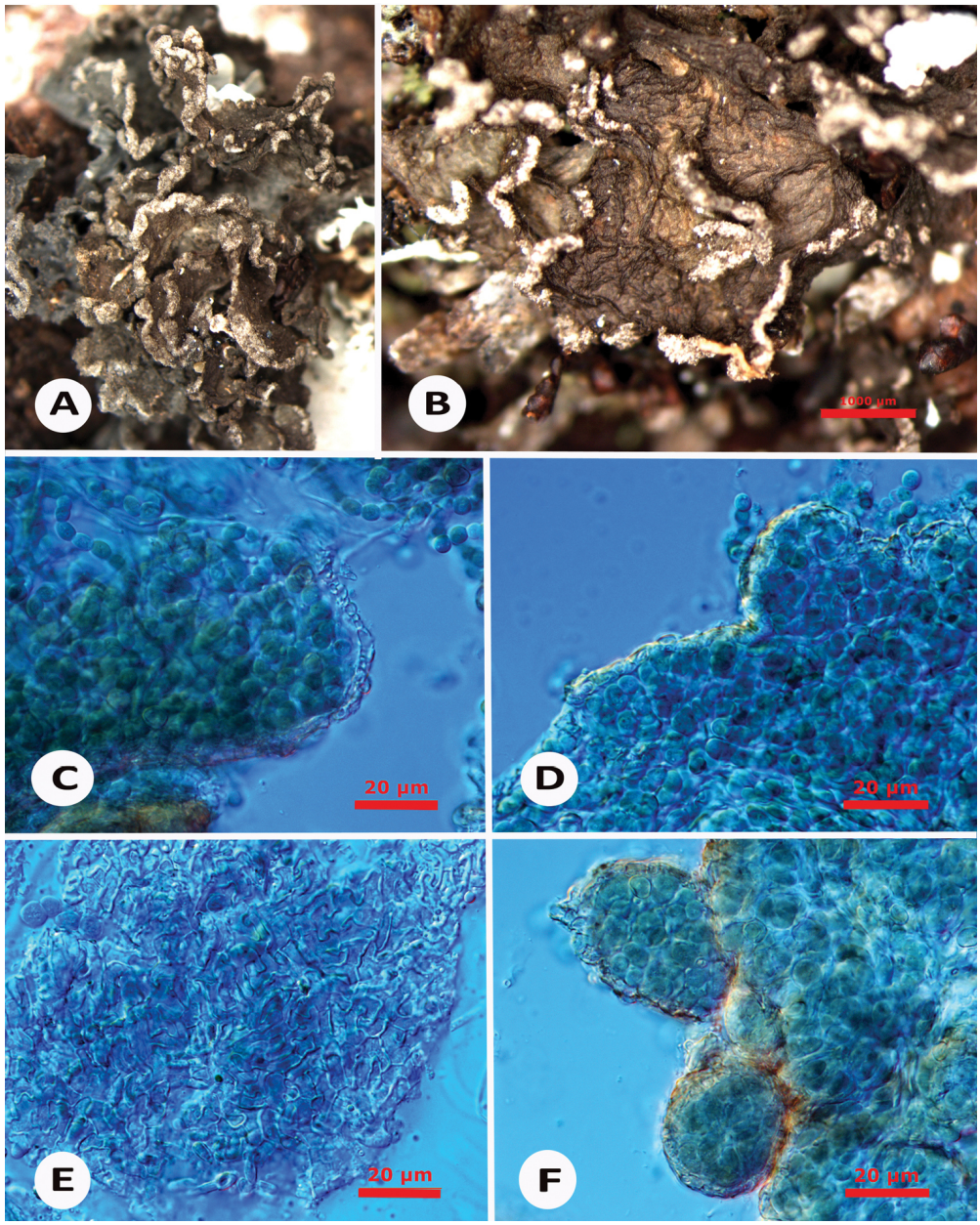

Figure 2: *Arctomia borbonica* (holotype). A–B macroscopic view of the thallus, with details of the wrinkled surface B and soredioid margin, made of disintegrating goniocysts C–D cross section through the thallus, showing the cortex with small, isodiametric cells, and the Nostoc chains E surface view of the cortex F young goniocysts formed at the lobes margins. Scale: A–B = 1 mm; C–E = 20 μm.

5.5 Taxonomy

Arctomia borbonica Magain & Sérus, sp. nov. Mycobank: MB 800279 Fig. 2

Diagnosis. Species recognized by its foliose, usually much crumpled, blue grey to brown thallus producing goniocysts at its margins, eventually forming a soredioid margin. Ascomata and conidiomata unknown.

Type. REUNION (Mascarene archipelago). Foreêt de Bébour, track starting at Gîte de Bélouve toward Piton des Neiges, 21'4'49" S, 55'31'24" E (DMS), 1850 m alt., 9 Nov 2009, wet montane ericoid tickets, N. Magain & E. Sérusiaux sn (holotype : LG).

Description. Thallus not exceeding 1 cm in diam., with distinct lobes when well-developed, lobes blue-grey to brown when dry, up to 0.2-0.3 mm wide and c. 200-400 μm thick, hardly distinguished in some specimens, with a surface typically wrinkled (even in young lobes), sometimes very much "crumpled", always developing small goniocysts, mainly at the margins but also on the upper surface; cortex (Fig. 2C–E) developed on upper and lower sides, formed by a single layer of small rounded (in cross section) and jigsaw-like (in surface view) cells, less than 5 μm thick; goniocysts (Fig. 2F) 20-80 μm across, always containing compact chains of *Nostoc* cells and covered by a layer of isodiametric to rounded cells, 2–5 μm, best developed at the lobes margins where they eventually form a typical pale brownish soredioid edge, due to cortical dis- integration. Photobiont belonging to the cyanobacteria genus *Nostoc* forming chains of small rounded cells 2–5 μm in diam. Ascomata and conidiomata unknown.

Chemistry. No secondary metabolites found by TLC.

Notes. The material looks like a species in *Leptogium*, a genus belonging to the Collemataceae in the Lecanoromycetidae (Lumbsch and Huhndorf, 2011). Soredia or soredioid propagules are however unknown in that genus as well as in the closely related *Collema*. *Arctomia borbonica* is easily recognized by its foliose, sometimes very much crumpled, blue grey to brown thallus, producing corticate and easily detached "goniocysts", best developed at the lobes margins, disrupting when mature and then forming a soredioid margin.

Distribution and ecology. *Arctomia borbonica* has been collected at three different sites on the island of Reunion in the Mascarene archipepago, incl. in highly disturbed secondary tickets with Eucalyptus plantations; it grows on trunks (*Eucalyptus*, *Acacia*

heterophylla) or on main stems of *Erica* tickets. It is probably widespread on the island. The two localities with natural vegetation belong to two different and typical habitats. The first one is the margin of the "Foreêt de tamarins des hauts" with large boles of the endemic tree *Acacia heterophylla* (locality at the nature reserve "Roche Ecrite", at 1500 m) and corresponds to the "Acacia mountain forest" in Strasberg et al. (2005). The other one is the wet upper montane ericoid tickets (type locality; locality in the Beébour forest at 1800–1850 m) and corresponds to the "Philippia mountain ticket" in Strasberg et al. (2005). Here the vegetation does not exceed 4–5 m in height and is formed by *Erica arborescens*, *E. montana*, *Eugenia buxifolia*, *Agauria buxifolia*, *Cordyline mauritiana* (locally very abundant), *Cyathea* sp., *Phylica nitida*, *Astelia hemichrysa*, *Blechnum attenuatum*; ground is covered by very thick (up to 80 cm) layer of *Sphagnum* and other bryophytes. It is one of the most rewarding habitat for lichens on Reunion, with many interesting species, including representatives of the austral element (Sérusiaux et al., 2011), such as *Gomphillus morchelloides*, *G. pedersenii* and *Sporopodiopsis mortimeriana*.

Other specimens examined. REUNION (Masarenes archipelago). Nature reserve at Roche Ecrite, track to the summit, 20'58 '6" S, 55'26'26" E (DMS), c. 1500 m alt., 4 nov 2009, montane forest dominated by *Acacia heterophylla*, N. Magain & E. Sérusiaux sn (LG). S part of the island, N of St-Philippe, near "giîte Bernard Brice", 21'20'23" S, 55'41'55" E (DMS), 650 m alt., 10 Nov 2009, Eucalyptus plantations and secondary tickets, N. Magain & E. Sérusiaux sn (LG).

5.6 Acknowledgements

Field studies in Reunion were made possible with the help and advice from the Parc National de La Reéunion, especially through the courtesy of Mr B. Lequette and Mr J. M. Pausé. Dr Cl. Ah-Peng and Prof. D. Strasberg of the University of La Réunion in Saint-Denis and Dr. J. Hivert of the Conservatoire Botanique National de Mascarin (St-Leu) were also very helpful. We thank them all most sincerely. We further thank Mr I. Cremasco and L. Gohy for technical assistance in the molecular laboratory and herbarium at the University of Liège. Nicolas Magain is a Ph.D. Student at the University of Liège and acknowledges the financial support by FRIA, an organ of the Belgian Research Foundation. Finally we thank both referees for their critical and helpful notes and suggestions.

5.7 Bibliography

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Table 1: Species and specimens used for this study, with GenBank accessions numbers for the three loci examined. Newly produced sequences for *Arctomia borbonica* are in bold.

Species name	**LSU**	**mtSSU**	***RPB1***
Absconditella sp.	AY300825	AY300873	—
Acarosporina microspora	AY584643	AY584612	DQ782818
Agyrium rufum	EF581826	EF581823	EF581822
Arctomia borbonica 1 (holotype)	**JX030030**	**JX030032**	**JX030034**
Arctomia borbonica 2	**JX030031**	**JX030033**	**JX030035**
Arctomia delicatula	AY853355	AY853307	DQ870929
Arctomia interfixa	DQ007345	DQ007348	—
Arctomia teretiuscula	DQ007346	DQ007349	DQ870930
Aspicilia contorta	DQ986782	DQ986876	DQ986852
Bacidia rosella	AY300829	AY300877	AY756412
Chromatochlamys muscorum	AY607731	AY607743	FJ941910
Coccotrema pocillarium	AF274093	AF329166	DQ870940
Conotrema populorum	AY300833	AY300882	—
Diploschistes ocellatus	HQ659183	HQ659172	DQ366252
Gregorella humida	AY853378	AY853329	—
Gyalectaria diluta	GU980982	GU980974	—
Icmadophila ericetorum	DQ883694	DQ986897	DQ883723
Lecanora intumescens	AY300841	AY300892	AY756386
Neobelonia sp.	AY300830	AY300879	—
Ochrolechia parella	AF274097	AF329173	DQ870959
Ochrolechia upsaliensis	GU980986	GU980979	GU981009
Orceolina kerguelensis	AY212830	AY212853	DQ870963
Pertusaria amara	AF274101	AY300900	DQ973048
Pertusaria lactea	AF381557	AF381564	DQ870971
Pertusaria leioplaca	AY300852	AY300903	DQ870973
Pertusaria paramerae	DQ780326	DQ780293	GU981012
Pertusaria pertusa	AF279300	AF381565	DQ870978
Pertusaria pustulata	DQ780332	DQ780297	GU981013
Pertusaria subventosa	AY300854	DQ780302	DQ870981
Placopsis gelida	AY212836	AY212859	DQ870984
Protothelenella corrosa	AY607734	AY607746	DQ870988
Protothelenella sphinctrinoidella	AY607735	AY607747	DQ870989
Thamnolia vermicularis	AY853395	AY853345	DQ915599
Thelotrema subtile	DQ871013	DQ871020	DQ870998
Toninia cinereovirens	AY756365	AY567724	AY756429
Trapelia chiodectonoides	AY212847	AY212873	DQ870999
Trapeliopsis granulosa	AF274119	AF381567	DQ871001
Wawea fruticulosa	DQ007347	DQ871023	DQ871005

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Chapter 6

Further photomorphs in the lichen family Lobariaceae from Reunion (Mascarene archipelago) with notes on the phylogeny of *Dendriscocaulon* cyanomorphs

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Published in The Bryologist, 115(2):243-254. 2012.

Abstract

Two new photomorphs in the Lobariaceae have been found on the remote island of Reunion in the Mascarene archipelago: the free-living *Dendriscocaulon*-like cyanomorph of the pantropical *Lobaria discolor*, and the cyanomorph of *Sticta dichotoma*, a species apparently endemic to the western parts of the Indian Ocean, known only from its chloromorph. Inferences from three loci demonstrate that the fungus involved in each morph of either pair belongs to the same species. Phylogenetic analyses resolve all genera of the Lobariaceae as polyphyletic, and all *Dendriscocaulon*-like cyanomorphs within *Lobaria*, except for *D. dendroides*, which belongs to *Sticta*.

Key Words: Peltigerales, *Lobaria discolor*, *Sticta dichotoma*, phylogeny.

6.1 Introduction

A lichen can be described as the intersection of two destinies, that of its mycobiont and photobiont, leading to a composite thallus with distinct, emergent properties (Sanders,

2010). Although typically described as the association between two partners, it has long been known that some fungi can recruit both a green alga and a cyanobacterium and form tripartite associations such as in *Nephroma articum* or *Peltigera aphthosa* (Tønsberg and Holtan–Hartwig, 1983).

Furthermore, genotypification of photobionts reveals that two or more species of green algae may coexist within a single thallus (Bačkor et al., 2010; Casano et al., 2011; Guzow-Krzeminska, 2006; Piercey-Normore, 2006) and that fungi may be associated with distinct algal partners along environmental or geographical gradients (Fernández-Mendoza et al., 2011). Such ability extends even to obligatory sterile taxa, and may occur repeatedly over evolutionary timescales (Nelsen and Gargas, 2008). Such latitude in specificity for algal partners may allow the fungus to colonize a wider spectrum of ecological niches (Fernández-Mendoza et al., 2011; Yahr et al., 2006), to the extend that the ecological distribution of the fungus may be shaped primarily by that of the photobiont (Peksa and Škaloud, 2011; Rikkinen et al., 2002).

The words "photosymbiodemes", "photopairs" or "photomorphs" apply to the most extreme photobiont switching, from a cyanobacterium to a chlorococcoid alga (Chlorophyta) or vice versa, to form thalli that differ in their physiology and habitat preferences, and in some cases also in their morphological and chemical traits. Photomorph pairs are best known from the Peltigerales but have recently also been discovered in the Lecanorales (Lücking, 2008) and Peltulales (Aptroot and Schumm, 2010). Comparison of discrete genetic loci of the mycobiont involved in the alternative photomorphs in the Peltigerales suggests that they indeed belong to the same species (Armaleo and Clerc, 1991; Goffinet and Bayer, 1997; Högnabba et al., 2009; Stenroos et al., 2003).

The most spectacular dimorphism is known within the Lobariaceae, where some species of *Lobaria* and *Sticta* form foliose chloromorphs and densely branched, coralloid cyanomorphs. These alternative thalli are either free-living or attached, with typically the cyanomorph emerging from the chloromorph. These cyanomorphs have historically been accommodated in a distinct genus, *Dendriscocaulon* established by Nylander (1886), a concept that should be abandoned considering the polyphyly of the group and more importantly, the conspecificity of the fungal symbionts involved in these thalli and known chloromorphs of *Lobaria* and *Sticta*.

We here report the discovery of two cyanomorphs on the island of Reunion, and test their affinities to sympatric Lobariaceae associated with a green alga. Inferences from ITS data suggest that these cyanomorphs are formed by *Lobaria discolor* and *Sticta dichotoma*, both so far known only as lichenized with a green alga. For the former, the cyanomorph was *Dendriscocaulon*-like and was found free-living, and for the latter, lobes with a green photobiont developed on lobes formed by the cyanomorph. Furthermore, we assess the phylogenetic affinities of *Dendriscocaulon* cyanomorphs throughout the Lobariaceae in continuation of the seminal papers by Stenroos et al. (2003); Takahashi (2006) and Högnabba et al. (2009).

6.2 Material and Methods

The material was collected during two field trips to Reunion in 2008 and 2009, the latter being dedicated to a detailed study of Peltigerales. Material from the Albertine Rift in Democratic Republic of Congo (RDC) and accessions from GenBank have been added to expand our sampling of related taxa. Identification of voucher material follows Galloway (1994, 2001, 2007); Galloway et al. (2001); Swinscow and Krog (1988) and Yoshimura (1971).

Well-preserved lichen specimens lacking any visible symptoms of fungal infection were sampled for DNA isolation. Great care was taken to extract DNA from the target material only, e.g. the *Dendriscaulon*-like free living cyanomorph (Fig. 1D) and the cyano- and chloromorph of *Sticta* growing as an autonomous assemblage (Figs. 1A

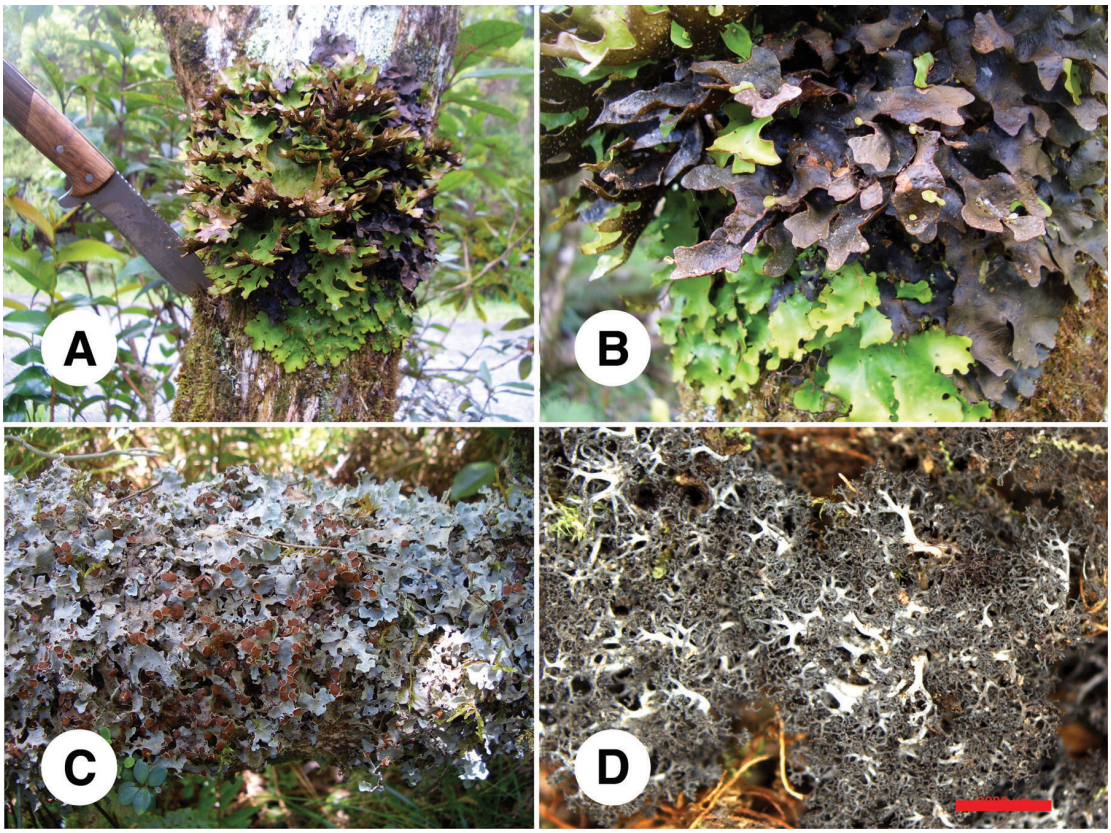

Figure 1: Photomorphs of Lobariaceae found on Reunion. A. Assemblage of both morphs of the common species *Sticta dichotoma* (Forêt de Bébour, Nov. 2009). B. Close-up showing details of chloromorphs lobes overgrowing the cyanomorph. C. Most common chloromorph of *Lobaria discolor* (Forêt du Grand Matarum, Nov. 2009). D. Free-living *Dendriscocaulon*-like cyanomorph of *Lobaria discolor* (Above St-Philippe, Nov. 2009, N. Magain & E. Sérusiaux s. n., LG). A-C: photographs taken in the field. Scale= 2 cm.

& B). We broadened our sampling to additional species including those originally described based on material collected on Reunion (Table 1). Extraction of DNA and PCR amplification were performed following the protocol of Cubero et al. (1999). The following primers were used: (1) for ITS: ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990); (2) for mtSSU: mrSSU1 and mrSSU3R (Zoller et al., 1999); for nLSU: LIC2044, LR0R, LR3R, LR3, and LR6 (Kauff and Lutzoni, 2002; Rehner and Samuels, 1994; Vilgalys and Hester, 1990). Amplicons were sequenced by Macrogen®. Sequence fragments were assembled with Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were subjected to BLAST searches to detect potential contamination. The sequences were aligned manually using MacClade 4.05 (Maddison and Maddison, 2002). Ambiguous regions were delimited using the online version of GBLOCKS v 0.91b (Castresana, 2000) at http://molevol.cmima.csic.es/castresana/Gblocks.html, allowing for gap positions within the final blocks then carefully checked manually.

We assembled two matrices. Matrix 1 was assembled to detect the phylogenetic affinities of both photomorphs studied here within the Lobariaceae, a family whose generic delimitations are debated (Högnabba et al., 2009). It includes sequences of three loci, nuLSU, ITS and mtSSU for representative species of the three genera of the Lobariaceae (*Lobaria*, *Pseudocyphellaria* and *Sticta*), with a focus on species assumed to be closely related to the photomorphs studied here. *Massalongia carnosa* and *Nephroma antarcticum* were chosen as outgroups as they belong respectively to the Massalongiaceae and Nephromataceae, two strongly supported sister families (Wedin et al., 2007).

Matrix 2 includes ITS sequences only for selected exemplars of Lobariaceae but including all available sequences of *Dendriscocaulon*-like cyanomorph as either free-living or attached photomorphs, to test their affinities within the genera *Lobaria* and *Sticta*. *Massalongia carnosa* was selected as outgroup as it belongs to a family (Massalongiaceae) forming an unresolved clade with the Lobariaceae (Wedin et al., 2007). No representative of *Pseudocyphellaria* was included as no *Dendriscocaulon*-like cyanomorph has ever been detected in that genus. Congruence between the three loci partitions in matrix 1 was assessed, with datasets considered congruent if relationships characterized by bootstrap proportions for MP and ML or posterior probabilities above 70% or 0.95, respectively, were identical or at least not in direct conflict among the inferences from individual loci. Since all partitions were shown to be congruent they were concatenated. The two matrices are deposited in TreeBASE under the accession numbers S12431. For each matrix phylogenetic relationships were reconstructed based on Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inferences. The MP analysis was performed in PAUP* 4.0b10 (Swofford, 2003) with characters equally weighted and gaps treated as missing data. An initial run was performed by using the NNI (Nearest Neighbor Interchange) branch swapping, with 10 trees saved for each of 1000 replicates, and was followed by a second analysis using the Tree Bisection Reconnection (TBR) branch swapping algorithm whereby all saved NNI trees

Table 1: Voucher information and associated GenBank accession numbers for nLSU, mtSSU and ITS loci. New accessions are in bold. Collections made in Reunion were gathered by N. Magain and E. Sérusiaux and are deposited at LG. The number following the herbarium acronym LG refers to the lichen DNA database at the University of Liège.

Name	Voucher	ITS	nLSU	mtSSU
Dendriscocaulon dendroides (Nyl.) R. Sant.	New Zealand: Thomas et al. 2002	AF350303	—	—
Dendriscocaulon sp. 1	Reunion (LG 1020)	JQ735971	JQ735988	JQ736005
Dendriscocaulon sp. 2	China: Takahashi 2006	AB239338	—	—
Dendriscocaulon sp. 3	China: Hur et al. 2005	DQ001281	—	—
Dendriscocaulon sp. 4	China: Takahashi 2006	AB239337	—	—
Dendriscocaulon sp. 5	China: Takahashi 2006	AB239340	—	—
Dendriscocaulon umhausense (Auersw.) Degel.	United Kingdom: Kelly et al. 2011	FR799178	—	—
Lobaria subexornata Yoshim.	Mexico: Stenroos et al. 2003; Högnabba et al. 2009	AF524902	EU558770	EU558804
Lobaria amplissima (Scop.) Forss.	Norway: Stenroos et al. 2003; Högnabba et al. 2009	AF524924	EU558749	EU558805
Lobaria discolor (Bory) Hue	Reunion (LG 1035)	JQ735972	JQ735989	JQ736006
Lobaria linita (Ach.) Rabenh.	Japan: Stenroos et al. 2003; Högnabba et al. 2009	AF524914	EU558800	EU558809
Lobaria orientalis (Asahina) Yoshim.	Japan: Stenroos et al. 2003; Högnabba et al. 2009	AF524907	EU558796	EU558810
Lobaria patinifera (Taylor) Hue	Reunion (LG 947)	JQ735973	JQ735990	—
Lobaria pulmonaria (L.) Hoffm.	Russia: Stenroos et al. 2003; Högnabba et al. 2009	AF524910	EU558797	EU558811
Lobaria quercizans Michaux	Canada: Stenroos et al. 2003; Högnabba et al. 2009	AF524921	EU558747	EU558814
Lobaria retigera (Bory) Trevis.	China: Lohtander et al. 2002 (ITS and mtSSU), and Canada: Wiklund and Wedin 2003 (LSU)	AY124094	AY340550	AY124159
Lobaria sachalinensis Asahina	Japan: Stenroos et al. 2003; Högnabba et al. 2009	AF524906	EU558795	EU558815
Lobaria scrobiculata (Scop.) DC.	Finland: Stenroos et al. 2003; Högnabba et al. 2009	AF524913	EU558787	EU558816
Massalongia carnosa (Dickson) Körber	Finland: Högnabba et al. 2009	EU558742	EU558697	EU558817
Nephroma antarcticum (Wulfen) Nyl.	Argentina: Stenroos et al. 2003; Högnabba et al. 2009	AF524897	EU558743	EU558818
Pseudocyphellaria argyracea (Delise) Vain.	Reunion (LG 1024)	JQ735974	JQ735991	JQ736007
Pseudocyphellaria aurata (Ach.) Vain.	Reunion (LG 1246)	JQ735975	JQ735992	JQ736008
Pseudocyphellaria clathrata (De Not.) Malme	Brazil: Högnabba et al. 2009	EU558729	EU558784	EU558828

Continued on next page

Table 1 – continued from previous page

Name	Voucher	ITS	nLSU	mtSSU
Pseudocyphellaria crocata (L.) Vain.	Reunion (LG 688)	JQ735976	JQ735993	JQ736009
Pseudocyphellaria freycinetii (Delise) Malme	Argentina : Högnabba et al. 2009	EU558717	EU558771	EU558843
Pseudocyphellaria intricta (Delise) Vain.	D.R. Congo (LG 883)	JQ735977	JQ735994	JQ736010
Sticta ambavillaria (Bory) Ach.	Reunion (LG 992)	JQ735978	JQ735995	JQ736011
Sticta caperata (Nyl.) Bory	Reunion (LG 962)	JQ735979	JQ735996	JQ736012
Sticta caulescens De Not.	Argentina: Högnabba et al. 2009	EU558737	EU558793	EU558858
Sticta cyphellulata (Müll.Arg.) Hue	Reunion (LG 1023)	JQ735980	JQ735997	JQ736013
Sticta dichotoma Bory 1 (free living chloromorph)	Reunion (LG 945)	JQ735981	JQ735998	JQ736014
Sticta dichotoma Bory 2 (chloromorph attached to cyanomorph)	Reunion (LG 984)	JQ735982	JQ735999	JQ736015
Sticta dichotoma Bory 3 (cyanomorph attached to chloromorph)	Reunion (LG 985)	JQ735983	JQ736000	JQ736016
Sticta duplolimbata (Hue) Vain.	Reunion (LG 1040)	JQ735984	JQ736001	JQ736017
Sticta filix (Sw.) Nyl.	New Zealand: Thomas et al. 2002	AF350304	—	—
Sticta gaudichaudia Delise	Argentina: Högnabba et al. 2009	EU558736	EU558792	EU558860
Sticta hypochroa Vain.	Argentina: Högnabba et al. 2009	EU558714	EU558767	EU558861
Sticta lacera (Hook. f. & Taylor) Müll. Arg.	New Zealand: Thomas et al. 2002	AF350305	—	—
Sticta latifrons A.Rich.	New Zealand: Thomas et al. 2002	AF350307	—	—
Sticta macrophylla Bory	Reunion (LG 946)	JQ735985	JQ736002	JQ736018
Sticta oroborealis Goward & Tønsberg	Canada: Tønsberg and Goward 2001	AF208015	—	—
Sticta sublimbata (Steiner) Swinscow and Krog	D. R. Congo (LG 885)	JQ735986	JQ736003	JQ736019
Sticta variabilis Ach.	Reunion (LG 1037)	JQ735987	JQ736004	JQ736020
Sticta weigelii (Ach.) Vain.	Guyana : Stenroos et al. 2003; Högnabba et al. 2009	AF524905	EU558794	EU558865
Sticta wrightii Tuck	China: Takahashi 2006	AB239343	—	—

were swapped to completion with no limit to the number of trees saved. Throughout the analysis, MulTrees option was activated. Support for the branches was estimated using the bootstrap approach (Felsenstein, 1985) with a heuristic search algorithm on 500 pseudoreplicates, all other parameter settings being identical. Bootstrap frequencies (MPBS) were obtained by constructing a majority-rule consensus tree of all trees saved during the analysis.

Models of evolution for the ML and Bayesian analyses were selected based on the Akaike Information Criterion (Akaike, 1974; Posada and Buckley, 2004) as implemented in MrModeltest v2.3 (Nylander, 2004). We used RAxML 7.0.4 (Stamatakis, 2006; Stamatakis et al., 2008) for the ML analysis on the CIPRES gateway (Miller et al., 2010). Support for each branch was evaluated using the "fast bootstrap" with 1000 pseudoreplicates. Bayesian analyses were carried out using the Metropolis-coupled Markov chain Monte Carlo method (MC3) in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). No prior values were assumed. Four parallel runs were performed each using four independent chains (three heated and one cold chain), with a single tree saved every 100th generation for a total of 4,000,000 generations. The incremental heating scheme was set by default. We used TRACER v1.4.1 (Rambaut and Drummond, 2007) to plot the log-likelihood values of the sample points against generation time, and determine when stationarity was achieved. Consequently the first 400,000 generations were deleted as the burn-in of the chain. A majority-rule consensus tree with average branch lengths was constructed for the remaining trees using the sumt option of MrBayes. Phylogenetic trees were visualized using FigTree v1.2.3 (Rambaut, 2009). Branches support values were considered significant when MP bootstrap (MPBS) $\geq 75\%$, ML bootstrap (MLBS) $\geq 75\%$ and Bayesian posterior probabilities (PP) $\geq 95\%$.

6.3 Results

For both matrices, the General Time Reversible substitution model accommodating a proportion of invariant sites and a rate heterogeneity distributed with a parameter gamma among six categories "GTR + I + G" (Rodriguez et al., 1990) was selected. Matrix 1 was composed of nuLSU, ITS and mtSSU sequences for 34 exemplars of the Lobariaceae. Of the 2825 characters, 781 characters were excluded, 1415 constant and 402 potentially parsimony-informative. Under MP analysis, 35 equally parsimonious trees were retained, with a tree length of 1774 steps, CI = 0.526 and RI = 0.661. The single most likely tree had a likelihood score of 211510.547757. Matrix 2 was composed of ITS sequences for 32 exemplars of Lobariaceae comprising all available *Dendriscocaulon*-like cyanomorphs sequences, and included 664 characters, of which 289 were excluded, 229 were constant and 99 were potentially parsimony-informative. Under MP analysis, 34 equally parsimonious trees were retained, with a tree length of 337 steps, CI = 0.562 and RI = 0.766. The single most likely tree had a likelihood score of -22329.674464.

The phylogenetic inference within the Lobariaceae (Fig. 2) suggests a) that *Lobaria*

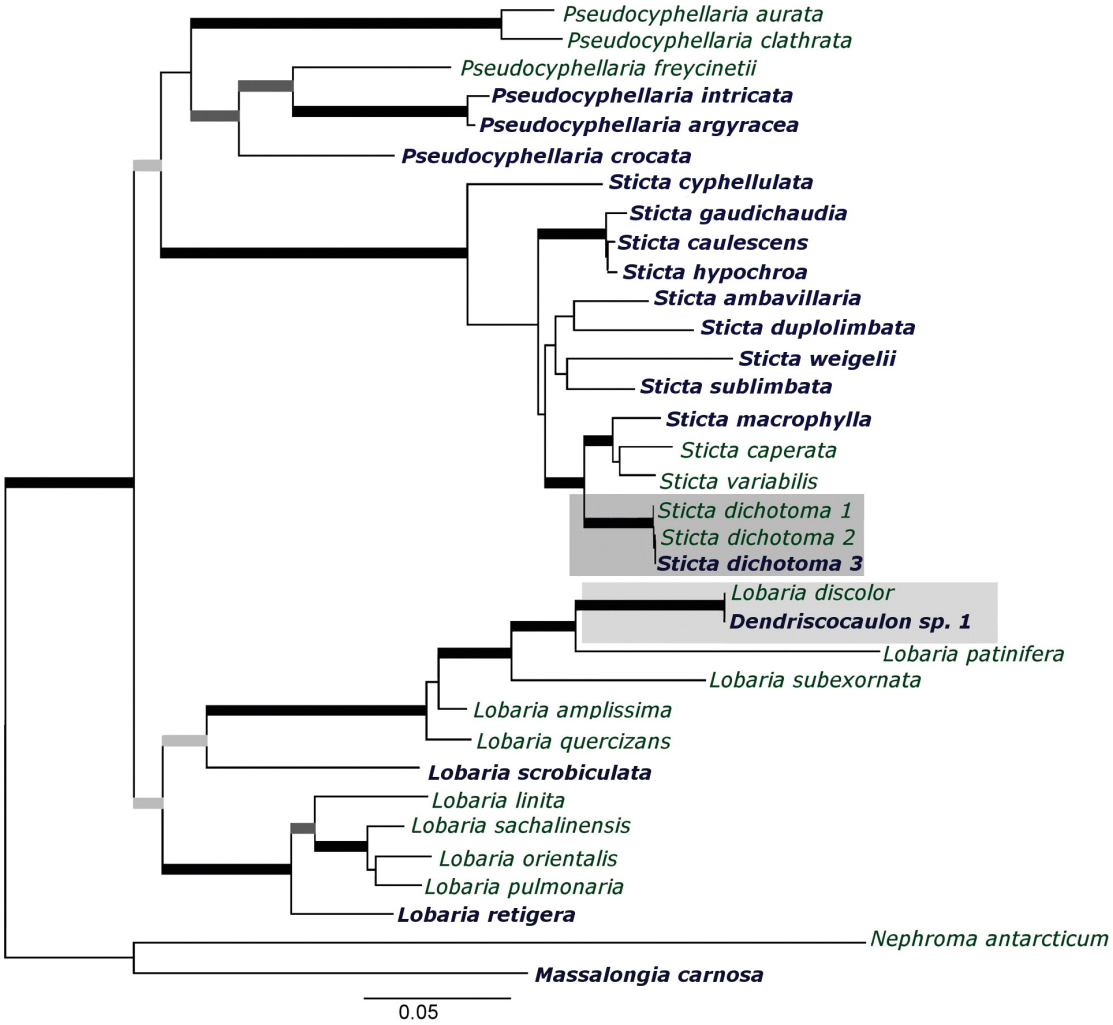

Figure 2: Most likely phylogenetic tree of the Lobariaceae, including the two photomorphs found in Reunion (*Lobaria discolor* and its free-living *Dendriscocaulon*-like cyanomorph; *Sticta dichotoma*, 1 free-living chloromorph, 2 chloromorph attached to cyanomorph, 3 cyanomorph attached to chloromorph). Concatenated matrix with nLSU, ITS and mtSSU sequences. Black branches are characterized by bootstrap percentages (MPBS and MLBS) above 75% and posterior probabilities (PP) above 0.95; dark grey branches have MLBS $\geq$ 75% and PP $\geq$ 0.95 but MPBS $<$ 75%; and light grey branches have PP $\geq$ 0.95 but MPBS and MLBS $<$ 75%. Taxa in bold blue are associated with *Nostoc* as the main photobiont while taxa not bold but in green are associated with a green alga.

is composed of two clades (i.e., the strongly supported *pulmonaria* group and a clade comprising *L. scrobiculata* sister to the strongly supported *L. amplissima group*), b) that the monophyly of *Pseudocyphellaria* comprising the robust *aurata* and *argyracea* groups and *P. crocata*, is unsupported, and c) that *Sticta* is a strongly supported but poorly resolved genus. Within this phylogenetic scenario, the sample of *Dendriscocaulon* from Reunion is resolved as a member of *Lobaria*. Furthermore, the sequences of three loci obtained for this sample are identical to their homologous sequences in chlorolichen *L. discolor*. Similarly the genotypes of the mycobiont in the attached foliose cyano- and chloromorphs are identical. They differ only by a single substitution in ITS2 from the mycobiont of an independent chloromorph of *S. dichotoma*, collected in a different locality on the island.

Inferences from ITS loci (Fig. 3) retrieved two strongly supported groups: a) one comprising all accessions of *Sticta* (except *S. oroborealis* and *S. wrightii*) and *Dendriscocaulon dendroides* which is resolved within a strongly supported group with *S. filix* and *S. lacera*; and b) one composed of all accessions of *Lobaria* and all other accessions of *Dendriscocaulon*. Within the latter *D. umhausense* shares a common ancestor with *L. amplissima*, the exemplar from Reunion is sister to *L. discolor*, and all samples compose a strongly supported group with *Sticta oroborealis* and *S. wrightii*. *Dendriscocaulon* 4 & 5 share a common ancestor with *S. wrightii* but their relationships remain ambiguous, whereas samples 2 & 3 are sister and potentially closely related to *S. oroborealis*.

6.4 Discussion

The species producing photomorphs in Reunion. *Lobaria discolor* (Fig. 1C) is a pantropical species (Joshi and Awasthi, 1982; Yoshimura, 1971; Yoshimura et al., 1998), described from Reunion (Delise, 1822) where it is common in montane forests ("Bois de couleurs des Hauts"; Doumenge and Renard 1989). Throughout its range, it is quite variable as reflected by the numerous varieties and forms recognized by Yoshimura (1971); the taxonomic status of these taxa has never been assessed within a phylogenetic framework. The species was only known to associate with a green alga as primary photobiont. Its cyanomorph, reported for the first time, was found free-living in a heavily disturbed forest at c. 650 m elev. on the SE part of the island, where the green photomorph has not been seen. It is a typical *Dendriscocaulon* morph with a bushy habit, numerous arbusculoid and coralloid bluish-grey secondary branches, and attached to the substrate by a single conspicuous pale orange, terete or more typically slightly flattened rooting stalk (Fig. 1D).

Sticta dichotoma Delise (Figs. 1A & B) has also been described from Reunion (Delise, 1822) and its distribution range seems to be restricted to the western parts of the Indian Ocean: Reunion (where it is locally very abundant), Madagascar (no data published are available but a picture of a collection from Madagascar is available at http://www. tropicallichens.net/3127.html) and a single locality in NE Tanzania

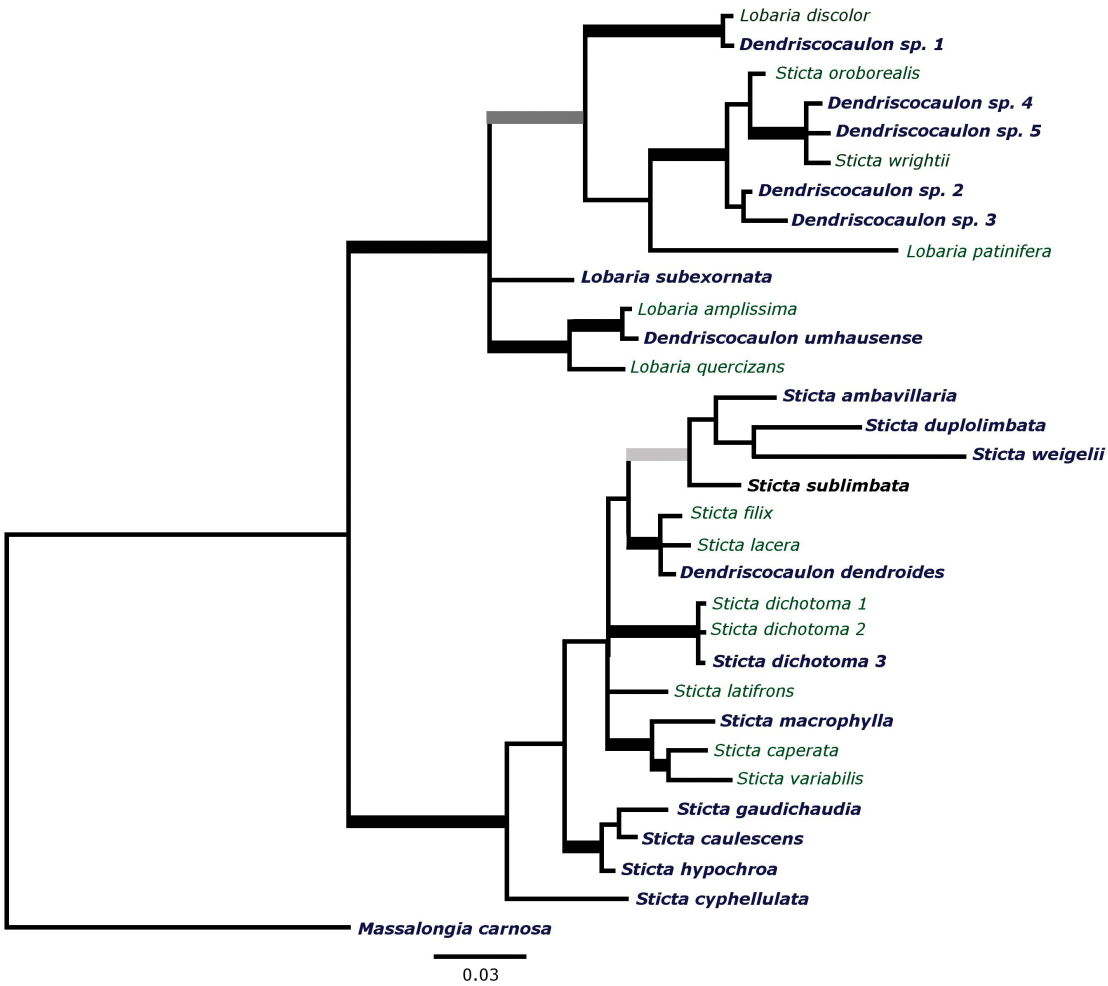

Figure 3: Most likely phylogenetic tree of the Lobariaceae, including the two photomorphs found in Reunion (*Lobaria discolor* and its free-living *Dendriscocaulon*-like cyanomorph; *Sticta dichotoma*, 1 free-living chloromorph, 2 chloromorph attached to cyanomorph, 3 cyanomorph attached to chloromorph). Concatenated matrix with nLSU, ITS and mtSSU sequences. Black branches are characterized by bootstrap percentages (MPBS and MLBS) above 75% and posterior probabilities (PP) above 0.95; dark grey branches have MLBS $\geq$ 75% and PP $\geq$ 0.95 but MPBS $<$ 75%; and light grey branches have PP $\geq$ 0.95 but MPBS and MLBS $<$ 75%. Taxa in bold blue are associated with *Nostoc* as the main photobiont while taxa not bold but in green are associated with a green alga.

(Krog, 2000). The chloromorph is almost exclusively present on Reunion and the assemblage of its chloromorph developing over its cyanomorph has been detected only once. The thallus developed by either photobiont is similar, although the cyanomorph has more irregularly dichotomous lobes with a maculate upper surface versus regularly dichotomous lobes and a smooth shiny upper surface in the chloromorph.

Phylogenetic distribution of *Dendriscocaulon*. The Lobariaceae are one of nine families composing the Peltigerales (Wedin et al., 2007, 2011), and include three genera of large foliose lichen forming fungi: *Lobaria*, *Pseudocyphellaria* and *Sticta*. These genera are defined by anatomical features of their lower surface, namely the presence of cyphellae (*Sticta*), pseudocyphellae (*Pseudocyphellaria*) or neither (*Lobaria*). Such straightforward morphological diagnosis has been widely adopted but recent phylogenetic inferences from sequence data reveal that neither cyphellae nor pseudocyphellae are derived characters that arose once and were retained by all descendant species (synapomorphies). Indeed, all three genera as currently defined, are polyphyletic: *Pseudocyphellaria anomala* Brodo & Ahti, *P. anthraspis* (Ach.) H. Magn. and *P. rainierensis* Imsh. are included in *Lobaria* (Högnabba et al., 2009), and *Sticta oroborealis* Goward & Tønsberg and *S. wrightii* Tuck., two *Sticta* species known to develop *Dendriscocaulon* cyanomorphs (Takahashi, 2006; Tønsberg and Goward, 2001), are also resolved within *Lobaria*. Furthermore, the monophyly of *Lobaria* and *Pseudocyphellaria* as currently circumscribed is supported only by Bayesian analysis (Fig. 2).

The genus *Lobaria* has been split into several genera (Yoshimura et al., 1998; Yoshimu ra, 2002): *Lobarina* (Vain.) Cromb. for *L. scrobiculata*, *Lobariella* Yoshim. (= *Lobaria* subgenus *Durietzia* C. W. Dodge) for the *L. crenulata* group, all remaining species belonging to *Lobaria* (Schreb.) Hoffm. (type species: *L. pulmonaria*). *Lobarina* was segregated on the basis of denuded portions of the thallus lower surface made of periclinal hyphae (versus sclero- or paraplectenchymatous lower cortex for all other species of *Lobaria* s. l.), whereas *Lobariella* was diagnosed by the production of pseudocyphellae on the upper surface of the thallus (versus absent in all other species). Phylogenetic inference (Högnabba et al., 2009) suggests, however, that species of *Lobaria* compose two well-supported clades: the *L. amplissima* group with the two species of *Lobariella* (*L. crenulata* and *L. subexornata*) forming a robust group nested in it, and the *L. pulmonaria* group including *L. scrobiculata*. Our results reveal a similar pattern except for *L. scrobiculata*, resolved as sister to the *amplissima* group. All phylogenetic reconstructions converge on all species producing *Dendriscocaulon*-like cyanomorphs, including *Sticta oroborealis* Goward & Tonsberg and *S. wrightii* Tuck., being resolved in a single clade (the *Lobaria amplissima* group), except for *D. dendroides*, which is unambiguously resolved in *Sticta* (Fig. 2).

The phylogenetic affinities of *Sticta oroborealis* and *S. wrightii* within the *Lobaria* clade is inconsistent with the current morphological definition of these genera, since *Lobaria* lacks cyphellae, a diagnostic feature of *Sticta*, on its lower surface. The phylogenetic significance of cyphellae is further questioned by the resolution of three species of

Pseudocyphellaria (i.e., *P. anomala*, *P. anthraspis* and *P. rainierensis*), a genus defined by pseudocyphellae on its lower surface, within *Lobaria*, a genus lacking pseudocyphellae on its lower surface. Furthermore, pseudocyphellae on the upper surface are used as a diagnostic character for the segregation of *Lobariella* whereas they occur in several species of *Pseudocyphellaria*, e.g. the *P. argyracea* group (Swinscow and Krog, 1988). Whether cyphellae and pseudocyphellae on either thallus surface have been acquired independently in multiple lineages or repeatedly lost during the diversification of the Lobariaceae remains to be critically tested.

Morphological classification of the photomorphs. James and Henssen (1976) recognize four photomorphs morphotypes in their seminal paper on "cephalodia". A. *Dendriscocaulon*-like cyanomorph attached by a conspicuous rooting stalk, with chloromorph developing dorsi-ventral lobes; B. dorsivental thallus as the chloromorph and attached or free-living *Dendriscocaulon*-like cyanomorph; C. as the former but further with chloromorph developing dorsiventral lobes on the *Dendriscocaulon*; D. dorsiventral thallus as cyanomorph and chloromorph developing over it and also forming a dorsiventral thallus. All four types occur in the Lobariaceae. Type (A) occurs in *Sticta* s. str. with *Dendriscocaulon dendroides*, an Australasian, free-living cyanomorph, sometimes found attached to species of *Sticta* belonging to the *latifrons-filix* group (Galloway, 2007). It is also found in the *Lobaria amplissima* group, with species such as *Sticta oroborealis* and *S. wrightii*. Type (B) belongs to the *L. amplissima* group, and the identity of fungi in both morphs has been demonstrated on molecular basis for *L. amplissima* (Stenroos et al., 2003) and here for *L. discolor*. Other cases mentioned by James and Henssen (1976), such as *L. ornata* or *S. glomulifera* have not been further documented. Type (C) could be considered as a more morphologically complex variant of the former, with the chloromorph growing over the cyanomorph, which is growing on the chloromorph. James and Henssen (1976) mentioned two examples, one with *L. amplissima* and the other with *L. cf. erosa*. Foliose cyanomorphs (Type D) are best known within *Nephroma* and *Peltigera* (Goffinet and Bayer, 1997; Tønsberg and Holtan–Hartwig, 1983; White and James, 1988) [see illustration of *P. britannica* (Gyeln.) Holtan-Hartwig & Tonsberg in Brodo et al. (2001)]. In such cases, both morphs develop rather similar thalli, except that the upper surface is most usually rather dull and typically maculate (white-marbled) in the cyanomorph and mostly shiny and smooth in the chloromorph.

Type (D) is indeed rare in the Lobariaceae, but is hereby shown to characterize *S. dichotoma* on Reunion, and is otherwise well documented for *Sticta canariensis* (Florke) Delise for which the cyanomorph was originally distinguished and named *S. dufourii* Delise. The latter develops a margin minutely dissected in small, terete to coralloid isidia compared to the entire margin of the chloromorph. In another case within *Sticta* (unidentified species in Brazil: Sanders 2001), both photomorphs develop similar thalli, except for a dull and maculate surface in the cyanomorph and a smooth and shiny one for the chloromorph. However, the composite thalli could not be identified to species

level and no molecular analysis could be conducted to support that a single fungus was involved in the assemblage. A further case is hereby reported with *S. dichotoma*, for which the fungus is identical in both photomorphs as demonstrated by their strictly identical sequences for the three loci studied here. Phylogenetic inferences demonstrate that none of these morphotypes represent an autapomorphic character within the Lobariaceae. However, as long as the relationships within the whole family are not fully resolved, the evolution of such characters as those offered by the photomorphs cannot be assessed.

6.5 Acknowledgements

Field studies in Reunion were made possible with the help and advice from the Parc National de La Réunion, especially through the courtesy of Mr B. Lequette and Mr J. M. Pausé. Dr Cl. Ah-Peng and Prof. D. Strasberg of the University of La Réunion in Saint-Denis and Dr. J. Hivert of the Conservatoire Botanique National de Mascarin (St-Leu) were also very helpful. We thank them all most sincerely. Our warm thanks are also due to our colleague and friend, Prof. Dr. Eberhard Fischer who organized the field trip to RDC, South Kivu (Kahuzi-Biega National Park) where many interesting species of the Peltigerales were collected. Nicolas Magain is a Ph.D. Student at the University of Liège and acknowledges the financial support by FRIA, an organ of the Belgian Research Foundation. Finally we thank both referees for their critical and helpful notes and suggestions.

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Chapter 7

Species delimitation in the cosmopolitan *Peltigera* section *Polydactylon* group (Peltigerales, Lecanoromycetes): comparison of methods based on molecular data and information about geography, morphology and association with the photobiont

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Abstract

We reconstructed the phylogeny of the mycobiont of *Peltigera* section *Polydactylon* based on molecular data from eight loci, including three newly designed intergenic *Peltigera*-specific markers (IGS1, IGS3 and IGS16) and applied five species delimitation methods. We focused on two major clades of the section: the Scabrosoid clade, where the lineages representing putative species are well delimited and most phylogenetic relationships among them are highly supported by bootstrap values; and the Dolichorhizoid clade, where substantially lower levels or resolution and bootstrap support was obtained and where species delimitation was more challenging. All methods resulted in mostly congruent species delimitations within the Scabrosoid clade. A total number of 12 species including 9 previously unrecognized species was detected. In the Dolichorhizoid clade, methods relying on different models and assumptions provided different species delimitations. The species delimitations we propose were based on a consensus among these various methods. We concluded that the Dolichorhizoid clade comprises 29 species, for which only 7 have already been described and named. The consensus approach revealed that most "evolutionary significant" species have relatively

well-defined distribution ranges (usually panboreal or restricted to a single biogeographic region), and cosmopolitan species names usually referred to assemblages of distinct evolutionary lineages. The majority of newly-delimited species showed a high specificity towards their cyanobionts. Information about geographic origin and patterns of photobiont association can be useful for species delimitation and identification.

Key Words: Structurama, bGMYC, bPTP, spedestem, bPP, coalescence, mycobiont, lichen, Nostoc, biogeography, ascomycota, IGS

7.1 Introduction

Species are key units to understand relationships among organisms, ecosystem dynamics, as well as to understand the dynamics of evolution. Defining biologically significant species units is thus very important for many aspects of the study of life.

For long, the delimitation and identification of lichen-forming fungi was based solely on morphology. However, molecular data highlighted the difficulty to correctly define boundaries among species (defined as "separately evolving metapopulation lineages"; De Queiroz 1998) in order to bridge the morphological and other commonly used species concepts (e.g., the biological species concept).

Indeed, the morphological species concept can be very difficult to apply in fungi, including lichens, because the absence of diagnostic traits can lead to the recognition of fewer species than phylogenetically defined (e.g., Crespo and Pérez-Ortega 2009; Miadlikowska et al. 2014). Intraspecific plasticity can be often higher than interspecific differences, leading to the circumscription of species representing different phenotypes within a single evolutionary lineage (e.g., Pino-Bodas et al. 2011). It can also be very difficult to detect morphological convergence when characters lack distinct developmental signature, and as a consequence unrelated lineages were sometimes embedded within the same species (e.g., Lumbsch et al. 2005; Otalora and Wedin 2013; Passo et al. 2008). Moreover, cryptic species that cannot be recognized based solely on the morphology have been frequently detected in lichen-forming fungi, including well-studied taxa from well-sampled areas (e.g., Lumbsch and Leavitt 2011). Biogeographical factors shaping the systematics of lichen-forming fungi was often neglected. For example, the same species name was often applied to morphologicaly similar individuals from different continents, when they might be drastically different genetically (e.g., Leavitt et al. 2011). As a result, species based on morphological concepts (morphospecies), might not always represent biologically or phylogenetically meaningful units. Moreover, recognition and circumscription of morphological traits are sometimes arbitrary and authors may diverge on boundaries among morphologically defined species. Chemotypic variation (differences in the set of secondary compounds) as an alternative tool for species delimitation was proved to be unreliable because the chemical traits often vary depending on the stage of lichen development, part of the thallus or ecological conditions (Lumbsch,

1998).

Recognizing biological species sensu Mayr (1940; "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups"), by testing the mating compatibility, is problematic in lichens because most lichenized fungi do not grow in artificial conditions. Moreover, lichens grow very slowly and their spores are tiny, difficult to observe and can be carried on very long distances, resulting in a great difficulty to monitor and test lichen reproduction (but see Zoller et al. 1999). Asexuality or homothallism in some species are also factors that complicate mating tests on lichens (Taylor et al. 2000; but see Scherrer et al. 2005).

However, the availability of molecular data has made possible to apply in lichen-forming fungi the phylogenetic species concept ("a diagnosable cluster of individuals within which there is a parental pattern of ancestry and descent, beyond which there is not, and which exhibits a pattern of phylogenetic ancestry and descent among units of like kind" ; Eldredge and Cracraft 1980) and the genealogical species concept ("basal group of organisms whose members are all more closely related to each other than they are to any organisms outside the group"; Hudson and Coyne 2002) concepts. In particular, Taylor et al. (2000) recommended the use of the Genealogical Concordance species concept or "exclusive concordance of alleles, where different gene topologies have to be congruent for interspecific relationships" (Avise and Ball, 1990) for species delimitation of fungi. Once phylogenetic or genealogical species are defined based on molecular data, it is possible to select a posteriori a set of phenotypic and chemotypic traits that are species-specific (Lumbsch and Leavitt, 2011).

7.1.1 *Peltigera* section *Polydactylon*

Peltigera section *Polydactylon* is one of the eight sections defined by Miadlikowska and Lutzoni (2000). It only comprises mycobiont species associating solely with the cyanobacterial genus *Nostoc*. It has been shown that *Peltigera* section *Polydactylon* is a well-supported monophyletic group, and is composed of three main clades, named the Dolichorhizoid (containing *P. dolichorhiza*), Polydactyloid (containing *P. polydactylo*n) and Scabrosoid (containing *P. scabrosa*) clades (see chapter 1). Most species from this section reproduce sexually, but a few members produce also vegetative propagules (mostly phyllidia). This section as a whole, has a broad, almost cosmopolitan, distribution. However, the distribution range of individual species varies greatly within the group ranging from endemism in small geographical areas such as the Azores to broad distributions covering North America, Europe and Asia (Martínez et al., 2003). Nineteen species have been described in this group but it has been suspected that section *Polydactylon* contains several species complexes encompassing cryptic as well as morphologically distinct but unrecognized species. Because most species in this group have relatively uniform morphology, the implementation of a morphological species concept is likely to lead to an underestimation of the number of species. However, the recognition of geographically structured morphotypes and chemotypes within broadly distributed

taxa such as for *P. neopolydactyla*, *P. scabrosa* (Holtan-Hartwig, 1993; Vitikainen, 1994), and *P. dolichorhiza* (Sérusiaux et al., 2009) strongly suggest the presence of multiple undescribed species.

Distinct morphological, chemical and geographical patterns detected within the section *Polydactylon* makes it a good model system for testing if cosmopolitan lichen species represent single "evolving metapopulation lineage" (De Queiroz, 1998) or assemblages of morphologically similar but evolutionary distinct lineages.

7.1.2 Objectives

I selected eight loci, including three protein-coding two ribosomal genes, and three newly developed *Peltigera*-specific molecular markers (intergenic spacers; IGS), to reconstruct the phylogeny of the mycobiont based on a worldwide sampling of most of the known species from the section *Polydactylon*. The multi-locus data were used to test and compare several species delimitation methods on the Scabrosoid clade, where lineages representing putative species are well defined; and on the Dolichorhizoid clade, where boundaries among species are not clear because of their putative recent origin. The tree topologies for these two groups, are very different (see Chapter 1); the Scabrosoid clade is well-resolved, with many long, well-supported branches representing different lineages, whereas the Dolichorhizoid clade includes several polytomies, many short branches, and unsupported relationships. I also reconstructed a species tree, once species have been defined, and compared its topology with phylogenies obtained from concatenated datasets.

I tested if cosmopolitan species represent single evolutionary lineages or the assemblages of several distinct entities and if unrecognized, cryptic or morphologically meaningful species are present in this group. I assessed, the taxonomic status of phylogenetic lineages derived most likely from recent radiation events within the South American clade. I also tested if geographical data on species distributions and patterns of association with *Nostoc* phylogroups can be used, in addition to the morphology to support newly delimited species.

Because molecular data were more limited in the Polydactyloid clade (higher proportion of missing sequences, fewer representatives per species, lack of data for species from remote regions of the world with no recent material available), this clade was not included in the comparison of the species delimitation methods.

7.1.3 Species delimitation methods and approaches tested

Structurama (Huelsenbeck et al., 2011) is a software using multilocus genotype data to infer population structure and assign individuals to populations (Pritchard et al., 2000). Each cluster (population) is modeled by a characteristic set of allele frequencies.

Its main modeling assumptions are Hardy-Weinberg equilibrium within populations and complete linkage equilibrium between loci within populations. Under these assumptions each allele at each locus in each genotype is an independent draw from the appropriate frequency distribution. It attempts to find population groupings that are not in desequilibrium.

The difference between the popular software Structure and Structurama is that, while Structure can only assign specimens to a number of populations fixed by the user, Structurama can estimate the number of populations, based on the data and on priors determined by the users (Huelsenbeck et al., 2011).

Structurama has been widely used for species delimitation, assuming that the reconstructed populations in equilibrium are distinct, isolated species (see for instance Pinzon and LaJeunesse 2011; Salicini et al. 2011).

The **GMYC** (General Mixed Yule Coalsecent) model considers branching between species as a Yule model (Yule, 1924), a stochastic birth-only model, which allows to calculate the likelihoods of the times before a new species appears, in a phylogeny with assumed constant average speciation rates. The GMYC model assumes neutral coalescence within each species; and a coalescent branching rate parameter for each species. It attempts to fit the location of the switches from speciation to coalescent nodes; which correspond to the most recent common ancestral node defining each species. In particular, it assumes that there is a threshold time before which all nodes reflect interspecific relationships and after which all nodes reflect intraspecific variation (Pons et al., 2006). This model can be tested on ultrametric phylogenetic trees. **bGMYC** (Reid and Carstens, 2012) is a bayesian implementation of the GMYC approach that account for tree topologies uncertainty.

bPTP (bayesian Poisson Tree Process, Zhang et al. 2013) implements a similar approach, but models speciations using the number of substitutions (based on branch lengths) instead of the time. It considers that each substitution has a small probability of generating a speciation, and that it follows a Poisson distribution in continuous time. It assumes that a tree has been generated by two distinct Poisson process classes, one describing speciation, and the other describing within-species branching events, and searches for the transition points where the branching pattern changes from an among-species to a within-species branching pattern. This model can be tested on non-ultrametric phylogenetic trees.

spedeSTEM (Ence and Carstens, 2011) incorporates the program STEM (Kubatko et al., 2009) which calculates the maximum likelihood species tree from a set of gene trees, under the assumption that the incongruences between gene trees are due to coalescence only. It takes an a-priori assignment of species and single-locus gene trees as input, and compares the likelihoods of the species tree when the units tested are considered as distinct species, or merged following certain hierarchical permutations, and proposes an optimal species delimitation to maximize the likelihood of the species tree.

bPP (Yang and Rannala, 2010) is a bayesian approach that generates the posterior probabilities of species assignments based on multi-locus datasets, taking the uncertainties due to unknown gene trees and the ancestral coalescent process into account . It requires the user to provide a species guide tree, and tests if the lineages defined in the species tree should be considered as distinct lineages or merged into more inclusive species.

These two methods thus try to define the best species delimitation to maximize a species tree in a coalescent framework. One of the main differences of approach is that spedeSTEM takes fixed gene topologies into account, and don't take gene trees uncertainties into account, but computes different species trees, while on the other hand bPP takes the alignments as input, allowing to take gene topologies uncertainties into account, but requires a fixed species tree, so it doesn't consider species tree uncertainties. These two methods try to rearrange predefined lineages among more inclusive species, but don't test the splitting of predefined lineages.

The concept of **barcoding gap** (Hebert et al., 2003) suggests that there should be a big difference between interspecific genetic variation and intraspecific variation so that there would be a gap, and therefore sequences with variation below a certain threshold could be assigned to a certain species.

7.2 Materials and methods

7.2.1 Development of three new markers: IGS1, IGS3, IGS16

Existing genetic markers do not provide a sufficient phylogenetic resolution and support for relationships among closely related individuals at the population and species levels in the genus Peltigera, as well as in other lichen genera in Lecanoromycetes.. With the aim of potentially discovering novel standing variation within genera, we used available genomic, metagenomic and metatranscriptomic data to develop and test three novel molecular markers (Gajdeczka et al., in prep). We sampled genomic sequence data for *Cladonia grayi* and *Xanthoria parietina* and three *Peltigera* species, largely from non-coding regions in order to choose potential regions that could be amplified. We targeted highly polymorphic, approximately neutrally-evolving regions of the genomes.

We scanned the 30 largest scaffolds of the *Cladonia grayi* genome assembly (Clagr2; http://genome.jgi.doe.gov/Clagr2/Clagr2.home.html), as aligned to corresponding scaffolds of the *Xanthoria parietina* assembly (Xanpa1; http://genome.jgi-psf.org/Xanpa1/Xanpa1.home.html) in the DoE JGI Vista Browser Synteny tool (Frazer et al., 2004; Grigoriev et al., 2011). We ranked nearly two hundred conserved syntenic blocks according to the following criteria: 1) greatest sequence variability in non-coding regions; 2) greatest sequence conservation in potential primer sites; 3) highest proportion of non-protein coding sequence; 4) lack of obvious linkage to other markers; and 5) po-

Table 1: Names and sequences of the primers used for the amplification of the IGS markers in the *Peltigera* section *Polydactylon* group

Primer name	Primer sequence (5'-3")
IGS-1F	GCTGTCGGCGAAGAGCTGAA
IGS-1R-B	CCATTTCTCCGCCGTTCTGGTA
IGS-3F-A	GGAGACGTTGCTAATGCATT
IGS-3R-B	CCGAAGTCCGCTCTGAAGACA
IGS-16Fout	GCGGAKGCGCAGATGATTTG
IGS-16Rmid1	TGTGGCACGGTGAACACTAG

tential for development of internal primers. We used a custom implementation of the BLAST-P 2.3X tool (Deng et al., 2007) to rank the 40 most promissing regions according to shared homology (in conserved regions) with existing assemblies of meta-genomic and meta-transcriptomic data. These data were derived from low-coverage sequencing of three *Peltigera-associated* lichen species: *P. dolichorhiza* (Magain et al., 2010),P*P. membranacea* (Xavier et al., 2012) and *P. praetextata* (Hodkinson et al., 2014). For developing PCR-based markers, we selected 20 regions that shared the most homology in potential primer annealing sites (typically in flanking exons). Based on *Peltigera* data, if possible, we designed one to ten primers (including degeneracies) per Watson-Crick strand, resulting in a total of two to twelve primer combinations per region.

We designed three markers, IGS1, IGS3 and IGS16 using our conserved synteny comparative genomic approach.. Each of these markers comprises an intergenic region and two flanking gene parts were the primers were placed. IGS1 is located within a two-gene microsyntenic region on the first scaffold of the Clagr2 genome assembly (between base pairs 283,015 and 285,166) and on scaffold 19 of the Xanpa1 assembly (between base pairs 632,451 and 634,199). IGS3, is located within a microsyntenic region on the fourth scaffold of the Clagr2 genome assembly (between base pairs 508,539 and 513,282), and in the seventh scaffold of the Xanpa1 assembly (between base pairs 1,104,171 and 1,108,382). IGS16 is located within a microsyntenic region on scaffold 27 of the Clagr2 assembly (between base pairs 335,996 and 340,907) and on scaffold 1 of Xanpa1 (between base pairs 2,640,063-2,644,705). The sequences of the newly generated primers used for the amplification of the three new markers are shown in Table 1. These primers were tested on *Peltigera* samples from most sections of the genus and showed great success in amplifying the targeted loci.

7.2.2 Taxon Sampling

Over 2000 specimens of *Peltigera* section *Polydactylon* (identified based on morphology) obtained as loans from several herbaria world-wide (AMNH, B, BG, CGMS, CONN, DUKE, H, LG, MAF, MEXU, NSPM, NY, O, PTZ, QFA, UBC, UDBC, UGDA, UMEX, UPS) and various private collections, as well as collected during numerous field trips

part of this study (Reunion Island in 2009; Norway, Canada: Québec, USA: North Carolina and Alaska in 2011; Russia, Peru and Brazil in 2012) were examined to select a set of representative specimens for DNA extraction and sequencing of the mycobiont and cyanobiont.

7.2.3 DNA extraction and Sequencing

We extracted DNA from approximately 950 well-preserved lichen specimens lacking any visible symptoms of fungal infection following two extraction protocols: Cubero et al. (1999) or modified Zolan and Pukkila (1986) using a 2% sodium dodecyl sulphate (SDS) as the extraction buffer. We amplified the internal transcribed spacer (ITS) of the nuclear ribosomal tandem repeat of the mycobiont from about 950 lichen thalli representing a broad geographic and morphological diversity of the group, using the ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primers. The PCR conditions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 45 sec, 52°C for 45 sec, and 72°C for 90sec, with a final extension of 72°C for 10 min. Based on these preliminary results, we further amplified 7 other loci on 164 specimens (94 for the Dolichorhizoid clade, 35 for the Scabrosoid clade, and 34 for the Polydactyloid clade) representing the diversity in ITS sequences. We also selected specimens with identical ITS haplotypes if they came from very distinct geographic regions (e.g. different continents) or exhibited very different morphology.

We amplified the nuclear ribosomal large subunit (LSU) using primers LR0R and LR7 (Vilgalys and Hester, 1990), three protein-coding genes: RNA II polymerase largest subunit (*RPB1*) using primers RPB1-AF (Stiller and Hall, 1997) and RPB1-CR (Matheny et al., 2002), elongation factor 2 region 1 (EFT2.1) using primers EFT2.1-1F (Miadlikowska et al., 2014) and EFT2.1-3R (Magain et al. in prep, see Chapter 1), and β-tubulin using the reverse primer BT2B (Glass and Donaldson, 1995) and the forward primer T1 (O'Donnell and Cigelnik, 1997) or alternatively bt-34F (O'Brien et al., 2009), and the three newly developed intergenic spacers, IGS1, IGS3 and IGS16 (primers are provided in Table 1. Amplification of RPB1 and LSU follows O'Brien et al. (2009) whereas the amplification of ß-tubulin, EFT2.1, IGS1, IGS3 and IGS16 were as follows: 94°C for 30 s, 55°C for 30 s (-0.4°/cycle), 72°C for 1 min (+2 s/cycle) for 24 cycles; 94°C for 30 s, 45°C for 30 s, 72°C for 2 min (+3 s/cycle) for 12 cycles; 72°C for 10 min, followed by storage at 4°C. All PCR amplicons were cleaned with ExoSAP (Affymetrix, Santa Clara, California, USA) following the manufacturer's protocol.

Sequencing was carried out in 10 μL reactions using: 1 μL primer (10 μmol/L), 1 μL purified PCR product, 0.75 μL Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, California, USA), 3.25 μL Big Dye buffer, and 4 μL double-distilled water. Automated reaction clean-up and visualization was performed at the Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies (for details see Gaya et al. 2012)

The list of specimens used for this study can be found in online supplementary Table S1.

7.2.4 Alignment, model selection and partitioning

Sequences were edited using Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan) and aligned using MacClade v. 4.08 (Maddison and Maddison, 2005). In order to reduce the number of ambiguously aligned characters that must be excluded from phylogenetic analyses, single-locus and multi-locus separate datasets were assembled for the whole section, as well as for each of the three clades.

For each single-locus dataset the best model for nucleotide substitution was determined by MrModelTest v. 2.3 (Nylander, 2004) using the greedy algorithm and the BIC criterion on all models available. For all the concatenated datasets, we used PartitionFinder (Lanfear et al., 2012) to determine the best partition to use in subsequent phylogenetic analyses. The following 18 data subsets were pre-delimited: LSU, ITS1+ITS2, 5.8S, IGS1 (not included in the dataset for the Scabrosoid clade), IGS3, IGS16, and four subsets for each protein-coding locus, (each of the three codon positions,and the non-coding parts.

7.2.5 Phylogenetic analyses

We generated single-locus phylogenetic trees for the section and for each of the three clades using RAxML v. 7.4.2 (Stamatakis, 2006) or alternatively RAxML v. 8.0.9 (Stamatakis, 2006; Stamatakis et al., 2008) as implemented on the CIPRES portal (Miller et al., 2010). Optimal tree and bootstrap searches were conducted with the rapid hill-climbing algorithm for 1000 replicates with GTR substitution model (Rodriguez et al., 1990) and gamma distribution parameter approximated with four categories in all analyses. Phylogenetic relationships that received bootstrap values of 70% and above were considered highly supported. We also generated phylogenetic trees on the concatenated datasets, using the same settings as above, and the best schemes determined by PartitionFinder to partition the data. We generated 50% consensus phylograms with MrBayes v. 3.2.2 (Huelsenbeck et al., 2001) on the CIPRES portal using the best BIC scheme determined by PartitionFinder to partition the data and determine the substitution models; completing 15 million generations for each clade, and 25 million generations for the whole Section; with 2 runs of 4 chains (3 cold chains and a heated one) each; sampling every 1000th generation; and discarding the 25% first trees as burn-in.

We generated chronograms for the Dolichorhizoid and Scabrosoid clades with BEAST v. 1.8 (Drummond and Rambaut, 2007) as implemented on the CIPRES portal by completing 50 million generations and discarding 20% of the trees as burn-in. For the concatenated datasets, we used the best BIC scheme determined by PartitionFinder to partition the data and determine the substitution models. For single-locus analyses on

the Dolichorhizoid and the Scabrosoid clade, we applied the substitution models determined by MrModelTest. The concatenated and single-locus BEAST analyses on the Scabrosoid clade were performed using a strict molecular clock. For the Dolichorhizoid clade, the concatenated analysis, as well as the single-locus analysis on ß-tubulin were performed with a lognormal relaxed clock, while the remaining analyses were performed with a strict clock. Convergence of Bayesian results was explored using Tracer v. 1.5 (Rambaut and Drummond, 2007) and AWTY (Nylander et al., 2008) as implemented on the portal http://king2.scs.fsu.edu/CEBProjects/awty/.

7.2.6 Pairwise distances

For the Scabrosoid and the Dolichorhizoid clades, pairwise-distances between ITS sequences were computed using PAUP v. 4.0a134 (Swofford, 2003).These distances were used to generate a heatmap using R (R Development Core Team, 2008) package ggplot2 (Wickham, 2009).

7.2.7 Species delimitation methods

Structurama

Using Sequencher, for each individual we coded alleles represented in each locus sequenced (eight loci for the Dolichorhizoid clade and seven loci for the Scabrosoid clade) using 100% similarity as the criterion to collapse samples in a single allele. We ran Structurama (Huelsenbeck et al., 2011) for one million generations, sampling every 1000th generation and tested several gamma hyperpriors on the expected number of populations (a constant gamma scale of 1 and gamma shape values of 3, 5, 8, 10, 12, 15, 18, 22, 24, 27 and 30, successively). We also tested the impact of each locus on species delimitation, by assembling datasets with one locus removed, resulting on eight 7-locus datasets for the Dolichorhizoid clade, and seven 6-locus datasets for the Scabrosoid clade. We ran four analyses on each of these datasets, completing one million generations, sampling every 1000th generation, with gamma shapes of 3, 8, 15 and 30 respectively, and gamma scale of 1 for the hyperprior on the expected numbers of populations.

bGMYC

For the Scabrosoid clade, we ran a bGMYC (Reid and Carstens, 2012) analysis on chronograms derived from the seven loci (ITS, β-tubulin, LSU, *RPB1*, *EFT2*.1, IGS3, IGS16). For the Dolichorhizoid clade, we ran a bGMYC analysis on the five most variable loci only: ITS, β-tubulin, IGS1, IGS3, and IGS16. For each analysis, we selected 200 chronograms from the tree distribution resulting from the single-locus BEAST analyses. Each of the files contained 50.000 trees, of which we discarded the first 5000 trees

as burn-in. We then selected one tree out of each 225th sample using R and the package APE (Paradis et al., 2004) to obtain a a 200-trees file. We ran bGMYC on each set of 200 trees for 50,000 generations on each tree, discarded 40,000 generations as burn-in with a thinning value of 100 and threshold values (corresponding to the interval of possible number of species) from 1 to 15 for the Scabrosoid clade and from 2 to 40 for the Dolichorhizoid clade.

bPTP

For the the Scabrosoid and Dolichorhizoid clades, we ran bPTP (Zhang et al., 2013) as implemented on the website http://species.h-its.org/ on the best ML tree resulted from the RAxML analyses on the concatenated datasets. We completed 500,000 generations with a thinning value of 1000 and discarding the first 25% generations as burn-in.

spedeSTEM

We ran spedeSTEM (Ence and Carstens, 2011) as implemented on the website http://spedestem.osu.edu, using the discovery method. Because spedeSTEM tests the merging but does not split predefined species, we tested species corresponding to all the individual lineages delimited in Fig. 2.

The program requires single-locus ultrametric trees as input. For the Scabrosoid clade, we used the seven single-locus chronograms generated with BEAST (see above). We also ran the analysis without the *RPB1* chronogram, because this locus is not variable enough in the Scabrosoid clade. We estimated the relative rates of each locus based on the subsitution rates derived from phylogenetic analyses. We tested a wide range (0.0001, 0.001, 0.005, 0.01, 0.05, and 0.1) of theta values (parameter depending on the population size and the substitution rate) because good estimates for our group are not available. We also ran an analysis with specimens attributed to *P. neopolydactyla* 4 randomly split in two species in the traits file, to check if the method merges them into one unit, as a test for the adequacy of the input parameters.

Because single-locus chronograms were poorly resolved in the Dolichorhizoid clade, we generated ultrametric trees by transforming the best ML single-locus trees resulting from the RAxML analyses using non-parametric rate smoothing with TreeEdit (Rambaut and Charleston, 2002). We used the same approach as for the Scabrosoid clade for assigning a species trait to our samples. We also assigned samples of *P. hymenina* and *P. dissecta* (which have been shown conspecific in phylogenetic reconstruction and other species delimitation methods) to distinct species as a test for the adequacy of the parameters. We tested the following theta values: 0.00001, 0.0001, 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5.

bPP

We ran bPP v. 2.2 (Yang and Rannala, 2010) on the Scabrosoid and Dolichorhizoid clades using seven and eight single-locus alignments, respectively. We used the same species assignment as for spedeSTEM and a guide-tree reflecting the topology found in the MrBayes analyses on the concatenated datasets. We used the species delimitation algorithm, keeping all sites containing missing data. We estimated the relative rates of single loci based on substitution rates from the ML analyses.

For the Scabrosoid clade, for the tau prior, we set the gamma shape to 4 and the gamma scale to 100. For the theta prior, we set the gamma shape to 2, and tested scale values of 200,000; 20,000; 2000 and 200; so that the mean of the theta prior is 0.00001; 0.0001; 0.001; 0.001 and 0.01 respectively.

We also tested the adequacy of the parameters by randomly splitting *P. neopolydactyla* 4 and *P. scabrosa* 2 in two species each, under two theta priors: 0.0001 and 0.01.

For the Dolichorhizoid clade, for the tau prior we set the gamma shape to 3 and the gamma scale to 100. For the theta prior, we set the gamma shape to 2, and tested the scale values of 2000; 200, 100 and 40 so that the mean of the theta prior is 0.001, 0.01, 0.02 and 0.05 respectively.

As for the spedeSTEM analysis, we tested the adequacy of the parameters by splitting *P. hymenina* in two (*hymenina* and *dissecta* morphotypes).

7.2.8 Final species delimitation and species tree

We used the consensus species delimitation resulted from all species delimitation methods as the species assignment for our specimens and generated species tree from eight (for the Dolichorhizoid clade) and seven (for the Scabrosoid clade) loci using *BEAST (Heled and Drummond, 2010). We ran the program for 50 million generations, sampling every 1000th generation and used lognormal relaxed clocks. For each locus, we attributed the nucleotide substitution model according to MrModelTest results (see online supplementary Table S2).

7.3 Results and discussion

7.3.1 Sequencing, alignment and concatenation

Within the Polydactyloid clade, for a total of 35 taxa, all of them are represented by at least three loci (100%), 30 taxa by four loci (85.7%), 24 taxa by five loci (68.6%), 15 taxa by six loci (42.9%), 9 taxa by seven loci (25.7%), and 3 taxa by eight loci (8.6%).

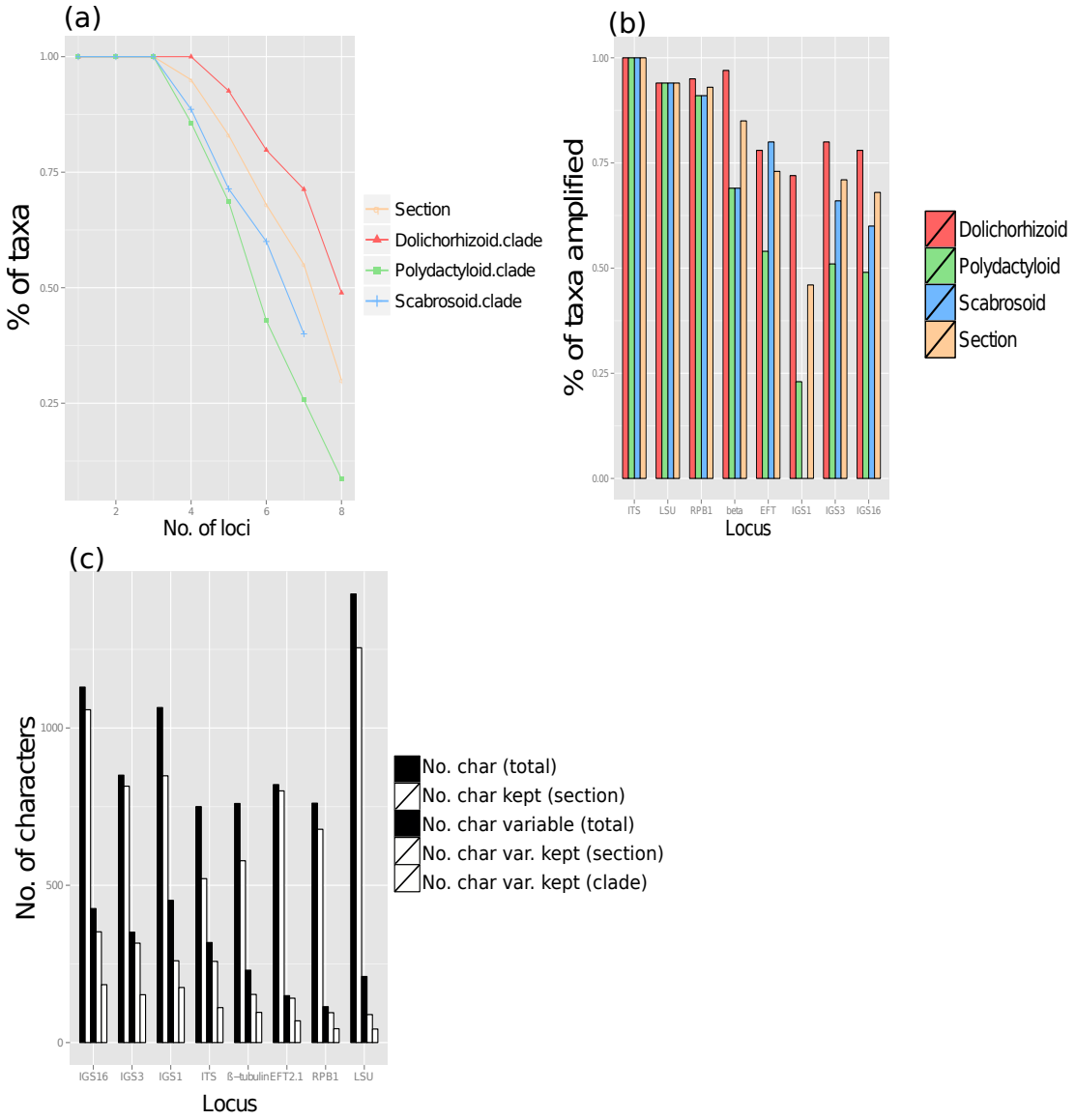


Figure 1: (a) Proportion of taxa in each clade and the whole section in respect to the number of sequenced loci; (b) Proportion of taxa in each clade and the whole section in respect to the number of sequences from each targeted locus; (c) For each locus, respectively from left to right: total number of characters, total number of unambiguous characters; total number of variable characters including ambiguous regions; total number of variable unambiguous characters for the whole section and for the Dolichorhizoid clade only.

Within the Scabrosoid clade, for a total of 35 taxa, all of them are represented by at least three loci (100%), 31 taxa by four loci (88.6%), 25 taxa by five loci (71.4%), 21 taxa by six loci (60%), and 14 taxa by seven loci (40%). We could not obtain IGS1 sequences for members of this clade.

Within the Dolichorhizoid clade, for a total of 94 taxa, all of them are represented by at least four loci (100%), 87 taxa by five loci (92.6%), 75 taxa by six loci (79.8%), 67 taxa by seven loci (71.3%), and 46 taxa by eight loci (48.9%). The highest proportion of missing sequences occurs in the Polydactyloid clade whereas the Dolichorhizoid clade is represented by the most complete sequence data (Fig. 1).

Overall each specimen is represented by the ITS sequence and 90% of individuals have LSU and *RPB1* loci in all datasets. Polydactyloid clade contains the highest proportion of missing sequences for *EFT2*.1, IGS3 and IGS16. Dolichorhizoid clade has the best coverage of IGS1 locus, which is the least represented marker across the clades (in less than 50% of taxa) while *EFT2*.1, IGS3 and IGS16 were successfully sequenced for 65-75% of targeted taxa (Fig. 1).

7.3.2 Comparison of length and variability of loci, including the IGS markers

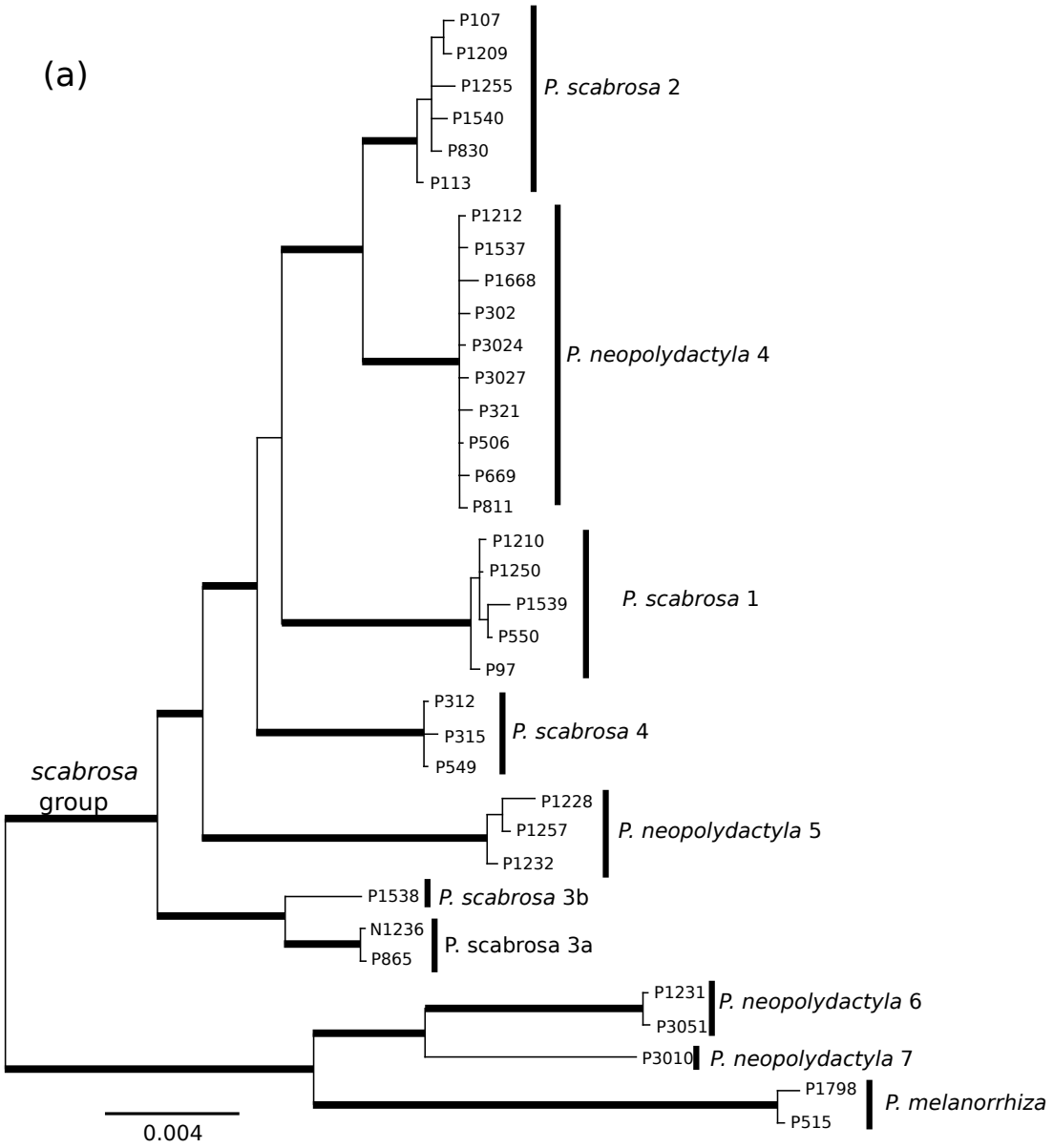
For each targeted locus, we compared: (a) the total number of characters (the length of the longest sequence, excluding introns in the IGS markers); (b) the total number of characters included in the phylogenetic analyses (after the exclusion of ambiguous regions); (c) the total number of variable characters before the exclusion of ambiguous regions; (d) the total number of variable characters after the exclusion of ambiguous regions for the whole section and (e) for the Dolichorhizoid clade only. This information can be found in Table 2 and Fig. 1.

The largest amplicons among our targeted loci and after the removal of ambiguously aligned regions of the alignments were the LSU (1255bp), however this locus contained the least number of variable characters included in phylogenetic analyses (89 characters). Besides LSU, the three IGS markers were the longest (from 1058 bp to 815 bp), and overall the most variable ones , ranging from 452 variable characters in IGS1, 426 in IGS16 to 351 in IGS3, and therefore outperforming the level of genetic variation provided by the ITS (351 characters) even after the exclusion of ambiguously aligned regions (260 character in IGS1 versus 258 in the ITS). β-tubulin and LSU were much less variable (230 and 210 variable characters, respectively), while *EFT2*.1 and *RPB1* contained the lowest numbers of variable characters (149 and 114, respectively).The three protein-coding genes and the ITS were of similar total length (750-820bp) but the number of unambiguously aligned characters for the *EFT2*.1 and *RPB1* (800 and 678bp respectively) was greater than for ITS and β-tubulin (521 and 578 characters at the section level, respectively), which contain large regions that could not be unambiguously aligned, including introns Following LSU, the locus with the least number of variable

Table 2: Variation of each locus at the level of the section and each of the tree clades.Number of taxa and proportion of taxa with the locus amplified, total number of characters, number of unambiguously aligned characters, total number of variable characters, and numbers of variable characters kept at the section and the Dolichorhizoid clade levels

	Dolichorhizoid clade				Scabrosoid clade		
	No. taxa	% taxa	No. characters kept	No. variable char	No. taxa	% taxa	No. characters kept
ß-tubulin	91	0.97	575	96	24	0.69	575
EFT2.1	73	0.78	817	60	28	0.80	817
RPB1	89	0.95	678	44	32	0.91	678
LSU	88	0.94	1309	43	33	0.94	1313
ITS	94	1.00	620	111	35	1.00	653
IGS1	68	0.72	793	175	0	0	0
IGS3	75	0.80	771	152	23	0.66	800
IGS16	73	0.78	946	184	21	0.6	995
Concatenated	94		6509	865	35		5831

	Section Polydactylon						Polydactyloid clade		
	No. taxa	% taxa	characters tot.	No. characters kept	variable tot.	No. variable kept	No. taxa	% taxa	No. characters kept
ß-tubulin	139	0.85	760	578	230	153	24	0.69	605
EFT2.1	120	0.73	820	800	149	141	19	0.54	818
RPB1	153	0.93	761	678	114	95	32	0.91	678
LSU	154	0.94	1426	1255	210	89	33	0.94	1307
ITS	164	1	750	521	318	258	35	1.00	640
IGS1	76	0.46	1065	848	452	260	8	0.23	788
IGS3	116	0.71	850	815	351	316	18	0.51	714
IGS16	111	0.68	1130	1058	426	352	17	0.49	885
Concatenated	164			6553		1575	35		6435


(a)
P107
P1209
P1255
P1540
P830
P113
P. scabrosa 2
P1212
P1537
P1668
P302
P3024
P3027
P321
P506
P669
P811
P. neopolydactyla 4
P1210
P1250
P1539
P550
P97
P. scabrosa 1
P312
P315
P549
P. scabrosa 4
scabrosa group
P1228
P1257
P1232
P. neopolydactyla 5
P1538
P. scabrosa 3b
N1236
P865
P. scabrosa 3a
P1231
P3051
P. neopolydactyla 6
P3010
P. neopolydactyla 7
P1798
P515
P. melanorrhiza
0.004

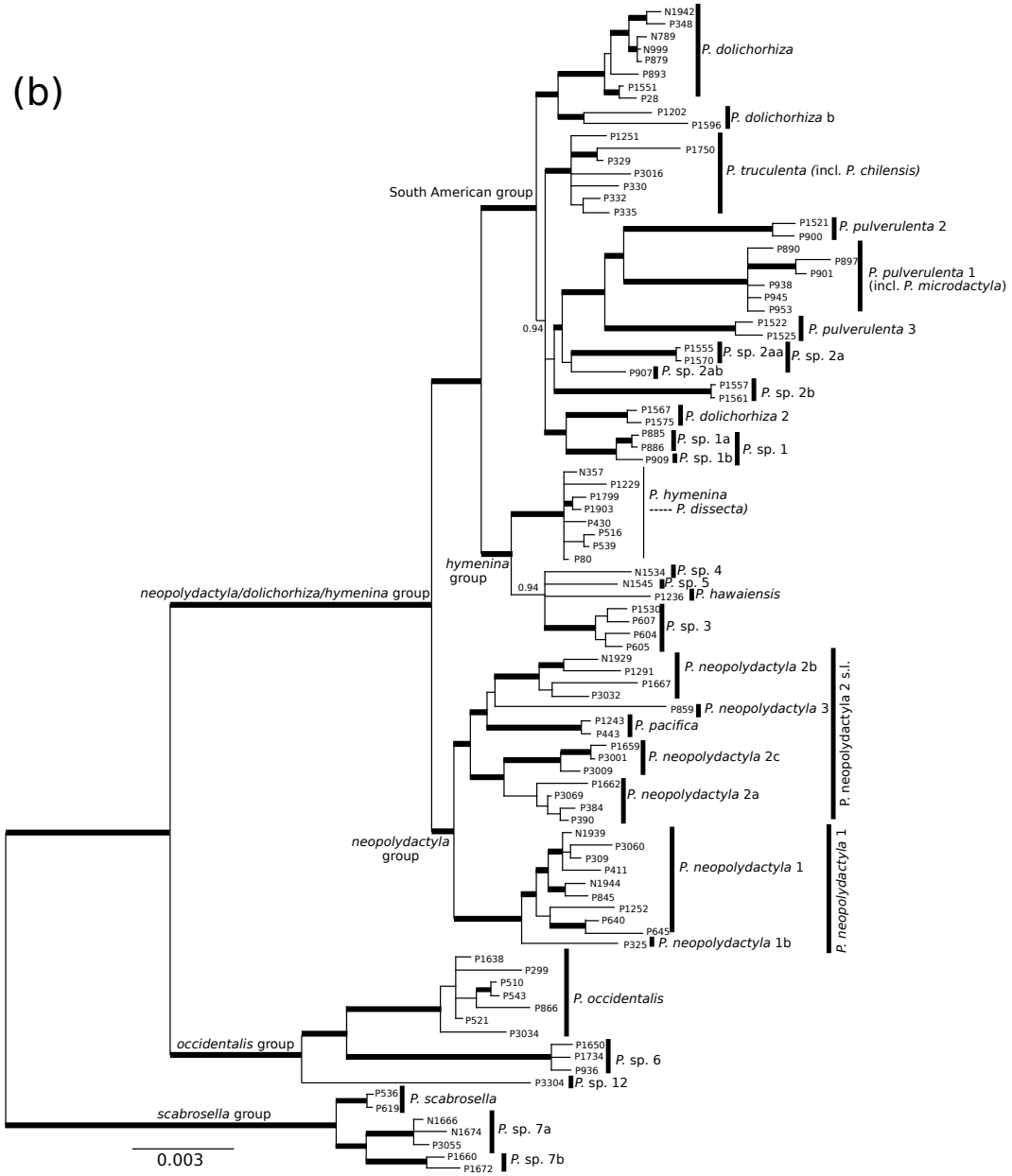


Figure 2: 50% bayesian consensus trees from the analysis on the concatenated datasets of (a) the Scabrosoid clade and (b) the Dolichorhizoid clade; the Scabrosoid clade. Thick internodes have bayesian pp ≥ 0.95. Rooting follows the topology found on the tree of the whole section (see online supplementary material). Vertical bars represent the putative species defined based on monophyly, morphology and geography, and correspond to the a priori species assignment for the spedeSTEM and bPP analyses

characters kept in phylogenetic analyses was *RPB1* (95 characters) whereas *EFT2*.1 and β-tubulin contributed equally (141 and 153 characters, respectively).

Among the three new markers, IGS16 has the highest number of variable characters included in the analyses at the section level (352 characters) followed by IGS1 (316 characters) and IGS3 (260 characters).

All three IGS markers are extremely useful at this taxonomic level, compared to the other loci. They are among the longest fragments amplified, with LSU, but have much more variable characters that can be kept for phylogenetic analyses. They are the three markers with the highest number of variable characters, at the section level, and more strikingly at the clade level, where ITS loses much of its variation. These markers are thus of great interest when working at this very low taxonomic level, at the specific or intraspecific level. ITS, β-tubulin and to some extent *EFT2*.1 still have enough variable characters to be useful at this level, whereas *RPB1*, and especially LSU, have for long proven their use for studies at higher taxonomic levels, but are less helpful when working at the specific or intraspecific level, as in the present study.

7.3.3 Phylogenetic reconstructions

The list of all the performed phylogenetic analyses along with the nucleotide substitution models determined by MrModelTest, as well as the partition and nucleotide substitution models determined by PartitionFinder for the concatenated datasets can be found in online supplementary Table S2. Single-locus topologies are available in the online supplementary material.

1. Single-locus phylogenies

Within the *Polydactylon* section, the Scabrosoid clade was revealed as a strongly supported monophyletic group in all single locus ML trees. The monophyly of the Polydactyloid clade received high support based on the analyses of the three IGS markers, ß-tubulin and *RPB1* and was weakly supported in *EFT2*.1 tree. In ITS and LSU trees, this clade is paraphyletic, however, without strong support. The Dolichorhizoid clade is monophyletic and includes the scabrosella group strongly supported in the *RPB1*, LSU, IGS1 and IGS3 trees but not in ITS phylogeny (without strong support). However, based on the ß-tubulin, *EFT2*.1 and IGS16 topologies, the placement of the *scabrosella* group is unresolved. No significant conflict was detected among the main clades within the section based on the single locus phylogenies.

Most putative species within the Scabrosoid clade represent well-defined and highly supported lineages. Two conflicting relationships include close relationship of *P. scabrosa* 1 with *P. neopolydactyla* 4 and *P. scabrosa 2* in the *RPB1* phylogeny (highly supported), whereas LSU and IGS3 strongly support the affiliation of *P. scabrosa* 1 with *P. scabrosa* 4. Similarly, *P. scabrosa* 3 represents the first split in a clade containing *P. neopolydactyl*a 5 and other species from the *scabrosa* group based on the ITS and IGS3

phylogenies, while *EFT2*.1 placed *P. neopolydactyla* 5 as the early diverged lineage in this clade.

A few cases of conflicting relationships among single locus topologies occurred in the Dolichorhizoid clade. For example, *P.* sp. 7a and *P.* sp. 7b in the *scabrosella* group are sister based on *RPB1* and IGS3 phylogenies whereas close relationship between *P.* sp. 7a and *P. scabrosella* was revealed by ß-tubulin.

The IGS1 and IGS3 grouped the south-american group together with the *hymenina* group, and each of them are strongly supported clades in the ß-tubulin, IGS3 and IGS16 phylogenies. Overall phylogenetic relationships among the putative species in the Dolichorhizoid clade received a low support is the single locus phylogenies.

The most robust phylogenies for this clade were obtained based on the IGS3, IGS16 and ß-tubulin.

The large amount of missing data in the Polydactyloid clade makes it difficult to test the discrepancies between the single loci.

The majority of conflicts detected among the single locus phylogenies may occur due to complex gene histories, but more likely because most of the loci are not very variable at this taxonomic level, and therefore include a small number of phylogenetically informative characters that are crucial for inferring relationships among individuals that diverged recently.

The topologies resulted from the new IGS markers were highly congruent with the remaining loci that have been commonly used in molecular systematic studies in lichen-forming fungi at the intrageneric and species levels. These three markers provided higher level of the resolution compared to the traditional loci, especially within the clades representing the most recent radiations, e.g., the *neopolydactyla/dolichorhiza/hymenina* group.

2. Multi-locus phylogenies

Fig. 2 shows the 50% consensus trees derived from the MrBayes analyses for the Dolichorizoid and the Scabrosoid clades. The 50% consensus tree of the Section Polydactylon can be found in Online Supplementary Material

In the Scabrosoid clade, most putative species, resolved as monophyletic groups on long, well-supported branches, seem to perfectly fit the concept of "distinct evolutionary lineages" (sensu De Queiroz 1998).

The first split in the clade divides a group of 3 distinct lineages corresponding to *P. neopolydactyla* 6 and *P. neopolydactyla* 7 supported together, and *P. melanorrhiza*; from the rest of the group, referred to as the the *scabrosa* group.

In the *scabrosa* group, *P. scabrosa* 3 is the most basal lineage, and possibly composed of 2 distinct lineages (*P. scabrosa* 3a :P865-N1236 and *P. scabrosa* 3b: P1538). *P.*

neopolydactyla 5 is basal to remaining species: *P. scabrosa* 4, *P. scabrosa* 1, *P. neopolydactyla* 4 and *P. scabrosa* 2, the two latter being grouped together with support. The only branch without support is the one holding *P. scabrosa* 1 with *P. neopolydactyla* 4 + *P. scabrosa* 2. As we saw in single gene phylogenies several loci place *P. scabrosa* 1 as sister to *P. scabrosa* 4, whereas other place it in the same position as in the concatenated analysis.

In the Polydactyloid clade (see online supplementary material), *P. polydactylon* from North America and Europe are in two well-supported distinct groups, possibly representing distinct evolutionary lineages. They appear as sister to *P.* sp. 10. *P. nana* 1 and *P. nana* 2, grouped together, are sister to the *P.* sp. 8 complex. *P.* sp. 11 and *P.* sp. 9 are basal. In the tree of the section, *P.* sp. 9 is the most basal species of the Polydactyloid clade, but without support (pp=0.86)

In the Dolichorhizoid clade, the *scabrosella* group is basal (pp=1) to the rest of the clade (see online supplementary material for the rooting and the tree of the whole section). Then the next split divides the *occidentalis* group (pp=1) from the *neopolydactyla/dolichorhiza/hymenina* group. In the *scabrosella* group, *P.* sp 7a and *P.* sp7b are more closely related (pp=1) than they are to *P. scabrosella* (there were conflicts about this relationships in the single gene topologies). In the *occidentalis* group, *P. occidentalis* and *P.* sp. 6 are more closely related than they are to *P.* sp. 12 .

In the *neopolydactyla/dolichorhiza/hymenina* group, the *hymenina* and South-American groups are resolved together (pp=1), the *neopolydactyla* group is basal. In the *neopolydactyla* group, the first split resolves *P. neopolydactyla* 1 s.l as basal to the rest of the group (pp=1), *P neopolydactyla* 1b is sister to the rest of *P. neopolydactyla* 1 (pp=1). *P. neopolydactyla* 2b, *P. neopolydactyla* 3 and *P. pacifica* group together (pp=0.997), *P. neopolydactyla* 2a and *P. neopolydactyla* 2c also group together (pp=0.994).In the *hymenina* group, *P.* sp. 3, *P.* sp.4 *P.* sp. 5 and *P. hawaiensis* group together with pp=0.94, this group is sister to *P. hymenina*. *P. dissecta* is nested inside *P. hymenina* with no structure to suggest that it might represent a distinct species, as already shown in Chapter 1.

In the South American group, *P. dolichorhiza* is basal, sister to P1202 and P1596 ("*P. dolichorhiza* b"). The rest of the group is supported as monophyletic at pp=0.94, and forms a three-branches polytomy: *P. truculenta* (including *P. chilensis*, confirming what was found in chapter 1) on one branch, *P. dolichorhiza* 2 and *P.* sp. 1 supported together on the second branch, and the third branch composed of *P. pulverulenta* 1, *P. pulverulenta* 2, *P. pulverulenta* 3 (the three resolved together at pp=1); *P.* sp. 2a (grouped with them at pp=0.95); and *P.* sp. 2b resolved with them at pp=0.62.

For the Scabrosoid clade, the topology of the BEAST chronogram (see Fig. 9a) is in agreement with the topology of the MrBayes phylogram. In the chronogram of the Dolichorhizoid clade alone (see Fig. 9b), the *occidentalis* group and the *scabrosella* group appear grouped together (without support, pp=0.52) as opposed to the *neopolydactyla/dolichorhiza/hymenina* group. This is due to a rooting issue, and is even the

case with a lognormal relaxed clock. In the BEAST chronogram of the whole section, the positions of the *scabrosella* group appears as basal to the *occidentalis* group and the *neopolydactya/dolichorhiza/hymenina* group (see online supplementary material). The rest of the topologies are congruent.

7.3.4 Species delimitation: comparison of the methods

Structurama

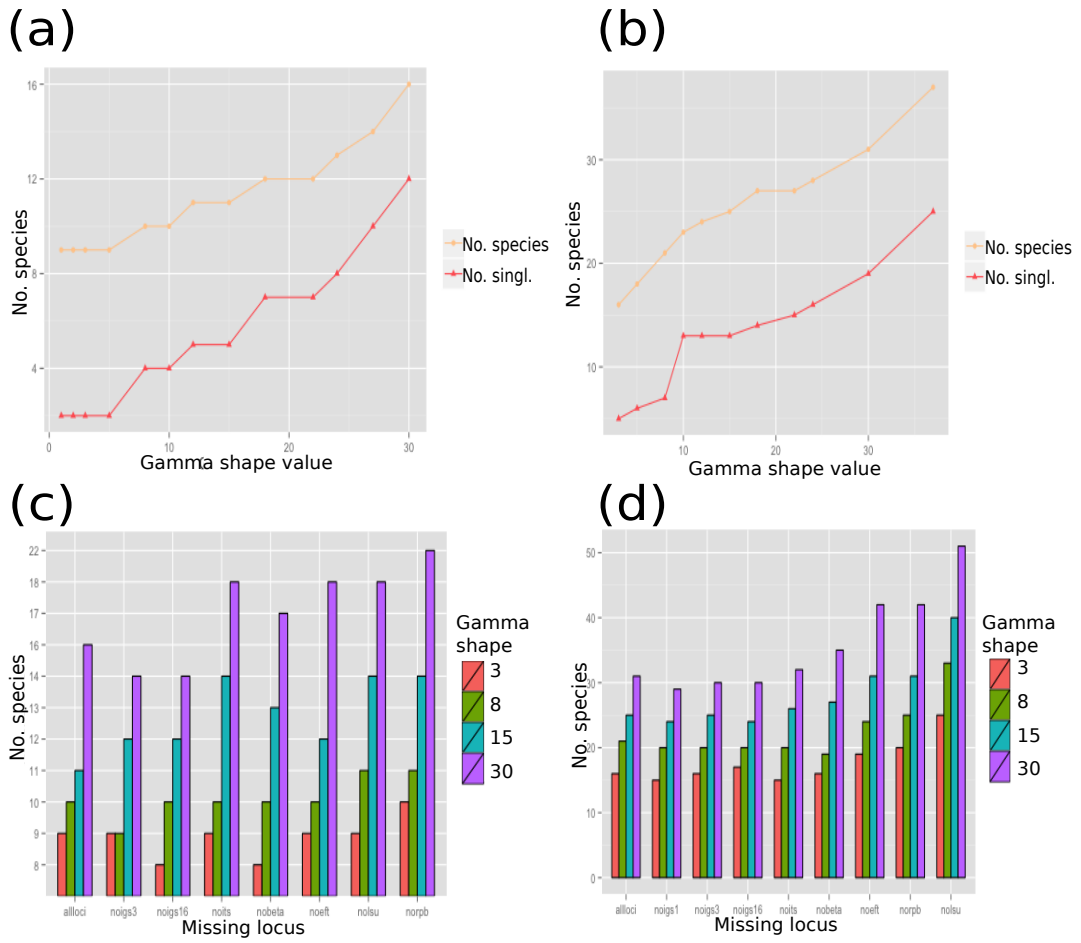
Impact of the gamma shape hyperprior and individual loci on the number of delimited species. In the Scabrosoid clade, the number of species remains stable until the gamma shape value reaches 5. Higher values (e.g., 8) lead to splitting lineages and increase the number of delimited species, especially singletons (species represented by only one individual, see Fig. 3a). We chose the gamma shape value of 3 for the final analysis. In the Dolichorhizoid clade, the number of species increases almost linearly with the increase of gamma shape hyperprior, but the number of singleton species goes up drastically when gamma shape reaches 8 and fluctuates above this value. (see Fig. 3b)

Analysis with a low gamma shape hyperprior (= 3) groups some potentially unrelated (well accepted and circumscribed morphologically and geographically) lineages, e.g., *P.* sp. 5 (N1534) with *P. pacifica*, and *P. neopolydactyla* 3 (P859) with a subset of *P. neopolydactyla* 1 and *P. hymenina* despite that these species do not share any allele for any of the loci. We selected the intermediary gamma shape value of 15 for the final analysis (see Figure 4).

In the Scabrosoid clade, in general a single locus does not affect the number of delimited species in a meaningful way when gamma shape prior is low (= 3). Removal of each of the following loci: IGS3, ITS, *EFT2*.1 or LSU from the combined dataset resulted in the same nine species delimitation; the exclusion of IGS16 or ß-tubulin decreased the number of species by one (*P. scabrosa* 4 and *P. neopolydactyla* 5 collapsed); without *RPB1* the number of species increased to ten (Fig. 3c).

A similar pattern of fluctuation in the number of recognized species (from one less to two extra species) was obtained with the gamma shape value of 8. For higher gamma values, the removal of one locus increased the number of species regardless of the locus removed (for the gamma shape of 30, the number of species can increase by six). In general, removing a single locus from the combined dataset leads to greater number of species in the Scabrosoid clade. It is very likely that having fewer loci decreases the chance that specimens share an allele at any locus and lowers the probability that they will cluster together.

In the Dolichorhizoid clade, the exclusion of any of the three IGS markers (or ITS/ ß-tubulin for low gamma shape values) usually slightly decreases the number of species; whereas removing *EFT2*.1, *RPB1* or especially LSU strongly increases the number of



species retrieved (see Fig. 3d and e). Overall, removing a variable locus results in less species, meaning that these loci tend to increase the number of species, whereas removing a less variable locus results in more species, meaning that these loci tend to decrease the number of species. It makes sense as in a less variable locus, the chance that samples share alleles is higher, so the chance that they will cluster together is higher too.

For the IGS regions, almost every individual is represented by a unique allele while for the LSU, *RPB1* and *EFT2*.1 many specimens share the same alleles (Fig. 3f). It is clear that the loci with the lower number of alleles within the sampled individuals (the left part of Fig. 3f) are the ones who tend to reduce the number of species (Fig. 3e) whereas the ones with the highest number of alleles (the right bar) tend to increase the number of species (Fig. 3e).

Removing *EFT2*.1, *RPB1* or LSU increases the number of species, but especially the number of singletons (Fig. 3g). For example, a broadly delimited paraphyletic species (corresponding to the grey zone in Fig. 4), which members share one or several alleles

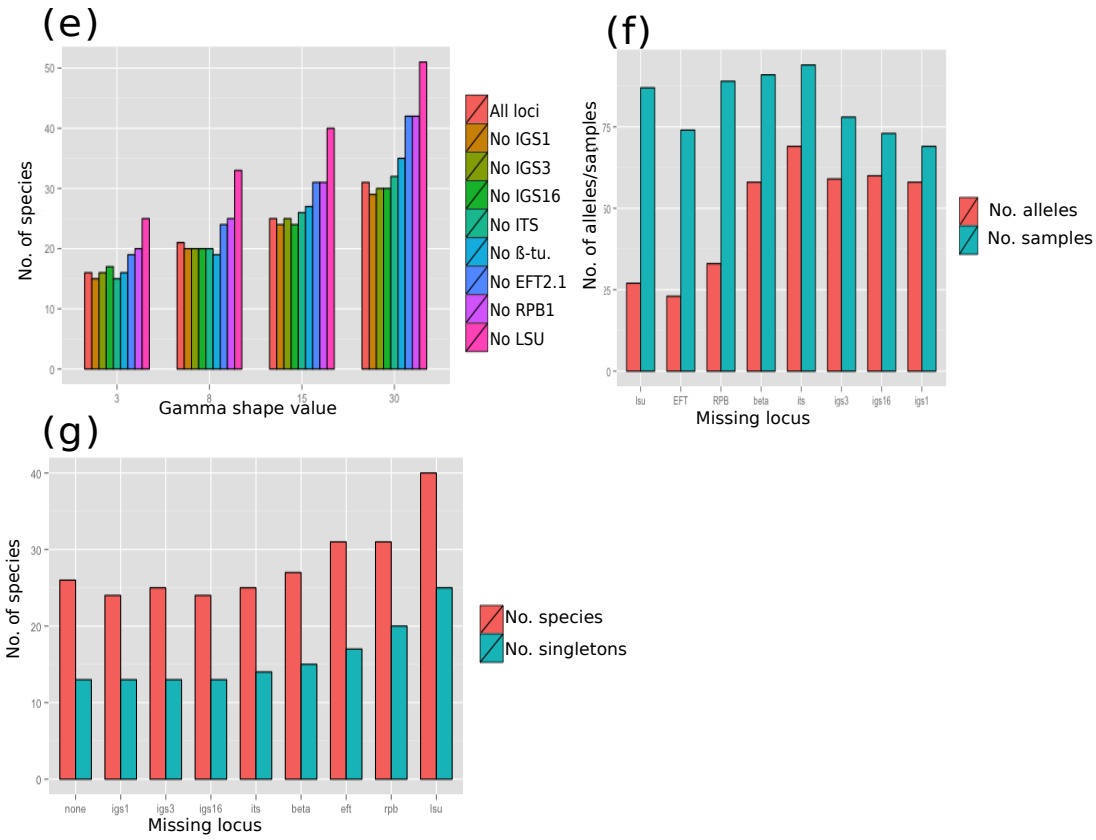


Figure 3: (a) Impact of the gamma shape hyperprior on the number of species represented by a single (in red) and multiple specimens (in orange) as delimited by Structurama in the Scabrosoid clade; (b) Impact of the gamma shape hyperprior on the number of species represented by a single (in red) and multiple specimens (in orange) as delimited by Structurama in the Dolichorhizoid clade; (c) Number of species retrieved in the Scabrosoid clade when removing each locus, for gamma shape values of 3, 8, 15 and 30 respectively; Number of species retrieved in the Dolichorhizoid clade after removal of each locus from the combined dataset for gamma shape values of 3, 8, 15 and 30.; (e) Number of species retrieved in the Dolichorhizoid clade for gamma shape values of 3, 8, 15 and 30 depending on the locus removed; (f) Number of alleles and the total number of individuals represented in each locus; (g) Number of species and singleton species in the Dolichorhizoid clade when removing a locus, for a gamma shape value of 15

for these three loci was reconstructed, regardless of the gamma shape value On the other hand IGS markers are too variable (almost every individual has a different allele), and therefore contributes to the overestimation of the number of species. However, their high variability makes them suitable loci for the phylogenetic reconstructions at the species level.

To improve Structurama performance on the *Polydactylon* section, more markers with an intermediary genetic variation (like ß-tubulin and ITS) are needed. In addition including more loci and more representatives from each putative species should increase the chance of specimens from an actual species to share alleles and cluster together.

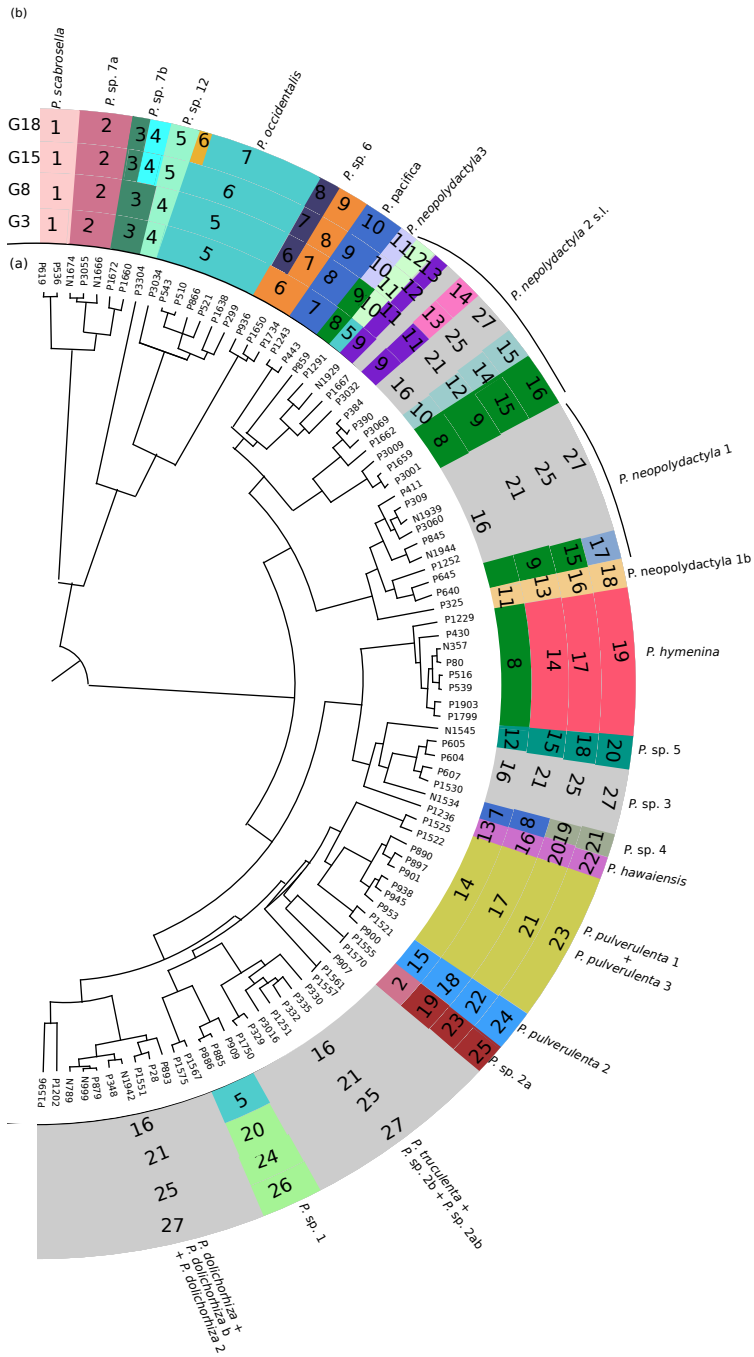


Figure 4: (a) chronogram resulted from the BEAST analysis on the concatenated dataset of the Dolichorhizoid clade and (b) assignment of each sample by Structurama when analyzed with four different gamma shapes of the hyperprior; inner circle: species assignment with a gamma shape of 3; second circle: species assignment with a gamma shape of 8; third circle: species assignment with a gamma shape of 15; outer circle: species assignment with a gamma shape of 18. Each color and number inside circles refers to a different species assignment. Species names outside of the circles correspond to the lineages defined in Fig. 2.

Assignment of samples to species. In the Scabrosoid clade, when including all loci, the only difference when rising the gamma shape from 3 to 8 is the split of *P. melanorrhiza* in two. Then from gamma a gamme shape of 8 to 15, it splits *P. neopolydactyla* 5 in two.

At gamma shape= 3, all lineages retrieved but one coincide with monophyletic groups and correspond to *P. scabrosa* 2, *P. scabrosa* 3 a and *P. scabrosa* 3b (as two distinct species), *P. scabrosa* 4, *P. neopolydactyla* 5, *P. neopolydactyla* 6, *P. neopolydactyla* 7 and *P. melanorrhiza*. The only non-monophyletic group retrieved is the clustering of *P. neopolydactyla* 4 and *P. scabrosa* 1 together. These two speices share the same allele for the locus *RPB1*, which exhibits a low variation in this group. When analyzing the dataset without *RPB1*, *P. scabrosa* 1 and *P. neopolydactyla* 4 appears as distinct species, the rest of the lineages delimited are the same. We therefore decided to implement the Structurama analysis without *RPB1* for our final consensus.

In our group, with few loci and haploid data, it seems that in most cases, when distinct lineages share an allele, they are clustered together.

Fig. 4 shows the species delimitation by Structurama in the Dolichorhizoid clade, in function of four different gamma shapes.

Some of our putative species are always well-defined regardless on the gamma shape of the hyperprior, this is the case of *P. scabrosella*, *P.* sp. 7a, *P. occidentalis* (at gamma shape of 18, P3034 is however considered as a singleton), *P. pulverulenta* 2, *P. neopolydactyla* 1b, *P.* sp. 5 and *P. hawaiensis*.

On the one hand, some putative species are well defined with low gamma values, but are split in several species with higher gamma values, like *P.* sp. 6 (split in two with a gamma shape of 8), or *P.* sp. 7b (split in two with a gamma shape of 15).

On the other hand, some putative species are well-defined at high gamma values, but are clumped with other unrelated taxa at low gamma values (*P. hymenina*, *P.* sp. 1, *P.* sp. 2a, are well defined with a gamma shape of 8 and above, *P. neopolydactyla* 3, *P. pacifica* and *P.* sp. 5 at a gamma shape of 15 and abobe, *P. neopolydactyl*a 2c at a gamma shape of 18 or above, but these species are part of non-monophyletic assemblages at lower gamma values).

If Structurama seems to perform well in some parts of our tree, the fact that it is not tree-based, and that specimens which share an allele will often be grouped together, result in a large paraphyletic species (in grey in Fig. 4) comprizing *P.* sp. 3, *P. neopolydactyla* 2a, a subset of *P. neopolydactyla* 1, *P. truculenta*, *P. dolichorhiza*, *P. dolichorhiza* b, *P. dolichorhiza* 2 and *P.* sp. 2b. This paraphyletic species has no geographical or morphological significance. Therefore, Structurama alone can't infer species boundaries in our whole group.

Some singleton species retrieved by Structurama are credible based on phylogeny, morphology and geography, as P325 (*P. neopolydactyla* 1b), P859 (*P. neopolydactyla*

3), P1236 (*P. hawaiensis*), N1534 (*P.* sp4), N1545 (*P.* sp5), P3304 (*P.* sp. 12). On the contrary, we consider that other singletons such as P1291, N1929, P3032, P1662 (parts of *P. neopolydactyl*a 2 s.l.), or the pair P640-645 (parts of *P. neopolydactyla* 1 s.l.) are likely to represent samples from species with high intraspecific allelic variation, that Structurama fails to cluster together.

At the low gamma shape of 3, in the Dolichorhizoid clade, some samples even cluster together while they don't even share an allele (e.g., the very distantly related *P.* sp. 7a and *P.* sp. 2a cluster together). The value of the gamma shape of the hyperprior is thus very important, a value too low will result in paraphyletic assemblages of unrelated taxa, whereas a value too high will result in splitting some species in several singletons.

Removing IGS3, IGS16 don't affect the species delimitation, suggesting that their high variability is not informative in this analysis.

bGMYC and bPTP

Posterior probabilities to support a species in bGMYC are usually relatively low, because it takes the tree uncertainties into account. Indeed, in the present case, a posterior probability of 0.5 means that half of the 200 trees tested support the delimitation of a certain species, which is considerable, especially considering all the other possible combinations.

We will consider that a species is delimited by bGMYC when the probability of grouping these samples together exclusively is higher than any of the probabilities of other groupings including at least one of these samples.

Missing samples differ from one locus to the other but if the bGMYC analysis on a locus assigns A, B and C to a species, and the analysis on another locus where C is missing assigns A and B to a species, we will consider that the bGMYC results are congruent on these two loci.

Online supplementary Table S4 contains the posterior probabilities for each species delimited by bGMYC.

Scabrosoid clade. bGMYC analyses on each locus agree with the final species delimitation from the Structurama analysis (performed without *RPB1*), at the exception of the analysis on ITS, where *P. scabrosa* 3a and *P. scabrosa* 3b are assigned together as a single species. Fig. 5 shows the species delimitation by bGMYC in the Scabrosoid clade.

The delimitation from bPTP on the best ML tree from the RAxML analysis on the concatenated dataset gives similar result, at the exception of *P. scabrosa* 3a, which is split in 2 with probability = 0.5 and P. *neopolydactyla* 5 is also split in two. This is a very surprizing result, as the topology of the tree don't support such a delimitation

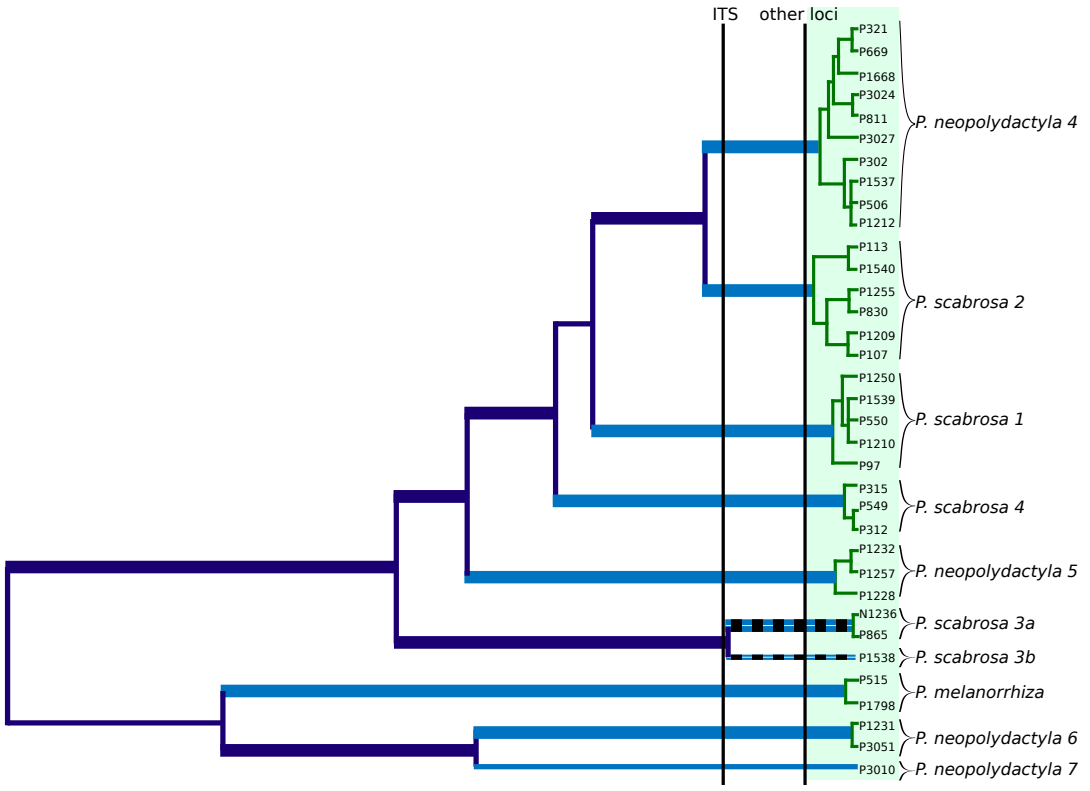


Figure 5: Consensus of the bGMYC results in the Scabrosoid clade on the chronogram resulted from the BEAST analysis on the concatenated dataset. Thick branches have pp value ≥ 0.95. Navy blue branches are branches representing interspecific relationships, blue branches are the branches supporting species, and green branches represent intraspecific variation, according to the bGMYC results. Stripes indicate the alternative results from ITS versus the other loci. The two vertical branches show putative thresholds on the tree where shifts from interspecific to intraspecific branching took place. The green background color highlights the zone where intraspecific variation occur.

(Fig. 2). By comparison, when applying bPTP to the 50% bayesian consensus tree, the species delimitation is the same as in bGMYC and Structurama.

Dolichorhizoid clade. In the Dolichorhizoid clade, we tested bGMYC on the 5 most variable loci: ITS, ß-tubulin, IGS1, IGS3 and IGS16.

Fig. 6 shows the results of the species delimitation by bGMYC on each locus, as well as the species delimitation by bPTP.

Some species are retrieved by the analyses on every locus: it is the case of the two singleton species *P. neopolydactyla* 3 (pp varying from 0.19 to 0.86 in bGMYC, pp=1 in bPTP) and *P.* sp. 12 (pp from 0.53 to 0.84 in bGMYC, pp=1 in bPTP). *P.* sp. 6 is also retrieved as a species according to all loci (pp ranging from 0.56 to 0.81, bPTP pp=0.86), as well as *P. pulverulenta* 2 is supported as a species by all loci (pp ranging

from 0.25 to 0.55, bPTP pp=0.64).

P. occidentalis is also retrieved as a species by all bGMYC analyses (pp from 0.3 to 0.51) but not by bPTP.

P. sp. 2a (P1555 and P1570) is supported as a species by all loci (pp 0.28-0.59), but in ITS, P907 is included in the species (pp=0.38).

Several species are supported by all loci but 1: it is the case of *P. pulverulenta* 1 (pp from 0.28 to 0.56, bPTP pp=0.75 but not retrieved in IGS1); *P. pulverulenta* 3 (pp from 0.35 to 0.54 and bPTP pp=0.65, but retrieved in IGS16); *P. pacifica* (pp from 0.27-0.47, bPTP pp=0.88 but not retrieved in IGS3); *P. dolichorhiza* (pp from 0.18-0.46, bPTP pp=0.62, not retrieved in IGS16); *P. neopolydactyla* 3 (pp varying from 0.19 to 0.86, not retrieved in IGS3).

Among the disagreements between loci, P1202 and P1596 (*P. dolichorhiza* b) are part of a same species in ITS and ß-tubulin, but separate species in IGS3 and IGS16.

Similarly, *P. scabrosella*, *P.* sp. 7a and *P.* sp. 7b are merged in a single species by four loci (with pp from 0.52-0.8) but ß-tubulin and bPTP consider them as three distinct species, as did Structurama .

There are a lot of uncertainties in the species delimitation in the group of *P. neopolydactyla* 1 s.l. and *P. neopolydactyla* 2 s.l. (see Fig. 6).

For instance, *P. neopolydactyla* 2a and *P. neopolydactyla* 2c are grouped together in ITS, but not in the other loci. *P. neopolydactyla* 1 is delimited without *P. neopolydactyla* 1b in ITS and IGS1, but with *P. neopolydactyla* 1b in IGS3 and ß-tubulin.

The cases where bGMYC performs poorly are in most cases due to uncertainties in the trees analyzed. For instance in ITS, most of the characters segregating *P. hymenina*, *P.* sp. 1 and *P. dolichorhiza* 2 are excluded, resulting in a lack of resolution in the tree, and bGMYC group them together, whereas these species are resolved as distinct in the bGMYC analyses on the other loci.

The delimitation of *P. truculenta* is also problematic, it appears as a distinct species in ITS and IGS1 but is grouped with *P.* sp. 1 in ß-tubulin, part of a non-resolved group in IGS3, and is divided in many singletons in IGS16 as well as in the bPTP analysis. It could be due to the lack of resolution in single locus trees, especially because there is not much variation in the South American group. It could also reveal that speciation is under process in this little group that has a high degree of haplotype and morphological diversity.

P. sp. 1 is defined as a species in ITS, IGS16 and bPTP, but is grouped with *P. truculenta* in ß-tubulin and with *P. dolichorhiza* 2 in IGS1, and part of a non-resolved group in IGS3. This is probably linked with the lack of resolution in single loci in the South American group due to the very recent radiation.

bGMYC gives mostly congruent results in the majority of cases, but shows differences depending on the loci, so delimiting species based on GMYC or bGMYC on a single locus can be inaccurate, as we haven't found two loci giving the exact same results.

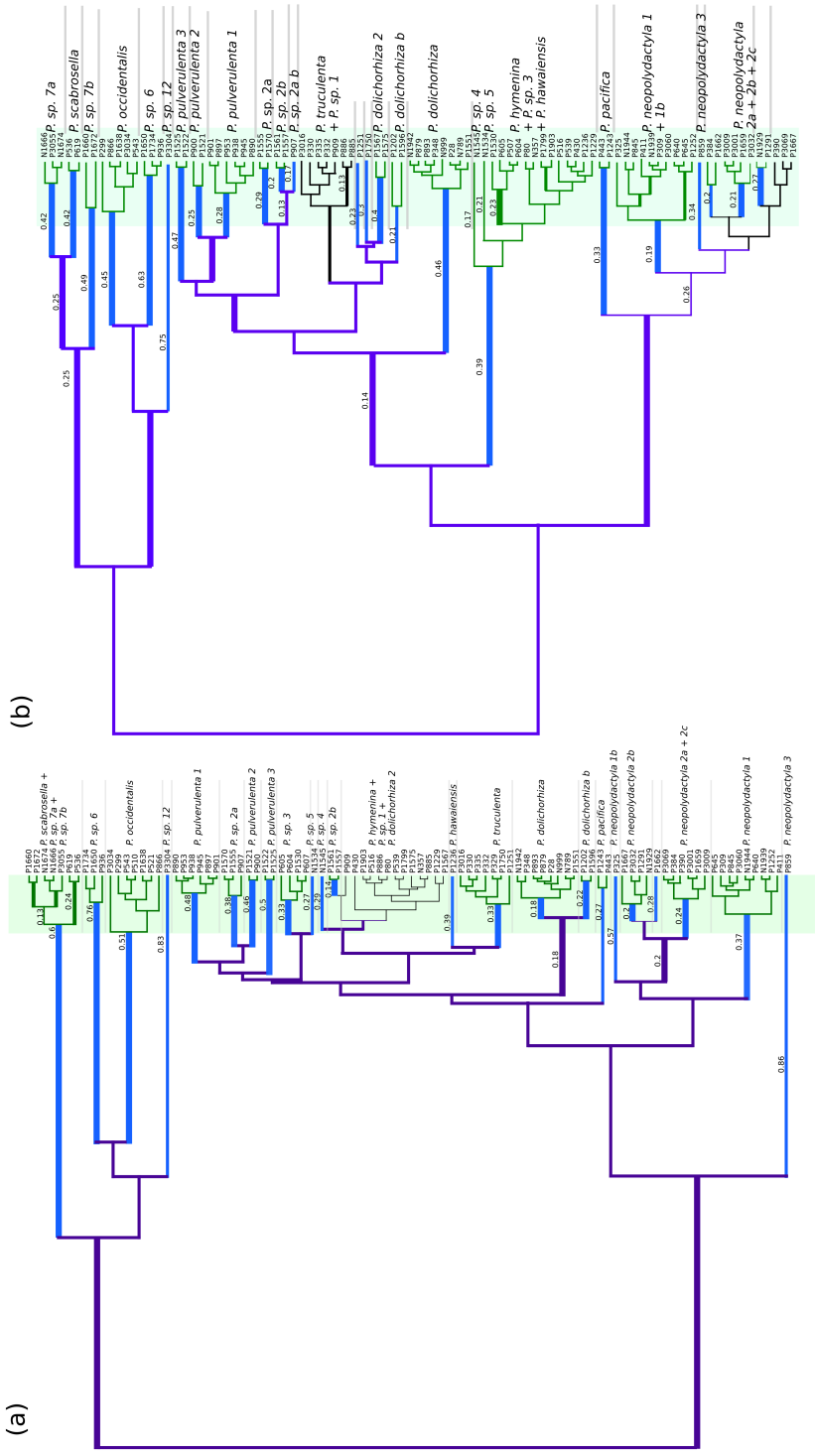
Moreover, single gene topologies don't always match the topology of the "real" species tree, so GMYC or bGMYC must be used with caution, even if in most cases it gave us good and congruent results. Applying bGMYC to several loci and establishing a consensus might be a more effective approach.

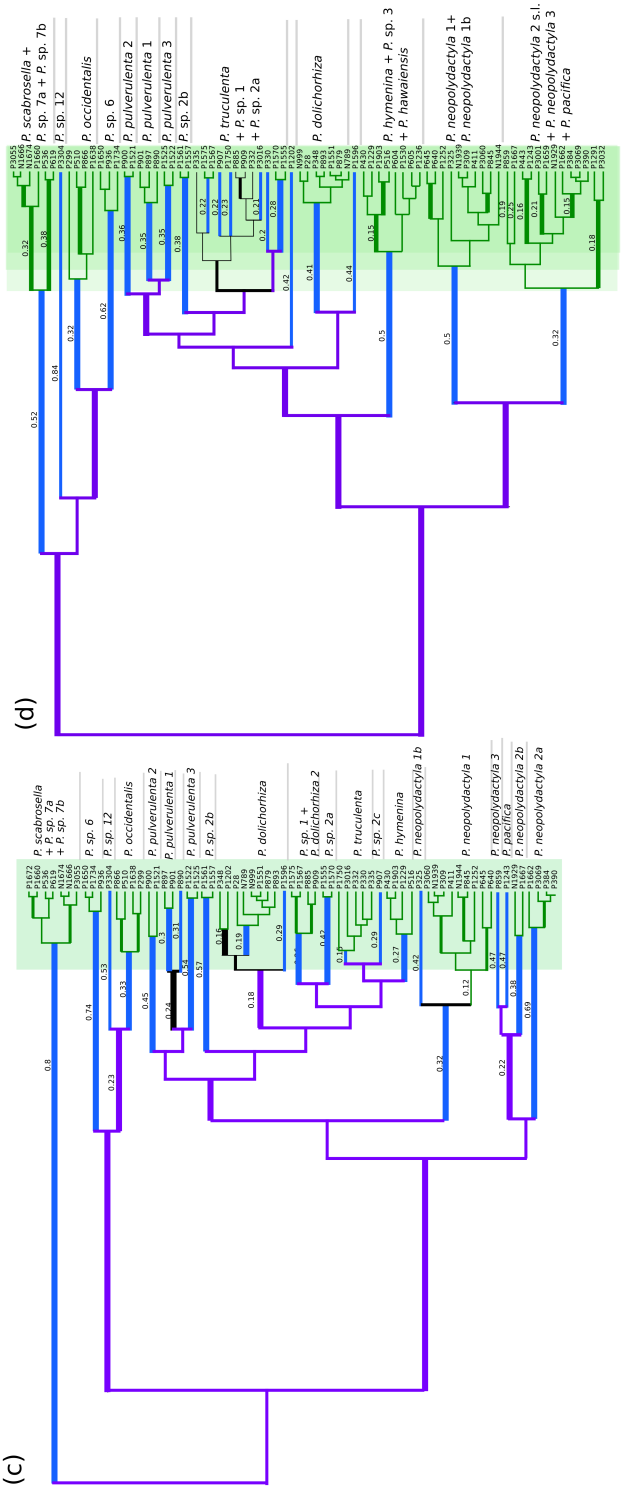
Among the advantages of bGMYC, it can be used on a single locus, and therefore is more cost-effective than multi-locus methods. It is tree-based so it will always return monophyletic species.

However, it assumes that all the transitions from interspecific to intraspecific events took place at the same time (single threshold), whereas it is not always the case (the example of a rapid radiation in one part of the tree). A multi-threshold implementation of the GMYC model exists, where several thresholds can be implemented, but this model did not improve the species delimitation results in our group.

Different results from a locus to another might be explained by different gene histories, but more likely in our case by the lack of resolution in the single-locus topologies, due to their lack of variation. The more variable the locus is, the best it is for this method (as long as there is no saturation and that it can be used to accurately reconstruct the phylogeny).

bPTP seems to perform well in most cases and is usually congruent with bGMYC. Its advantage is that it can be used on multi-locus phylograms, taking advantage of the best resolution available from the tree provided, and it does not require ultrametric trees, which can be problematic to generate, because it is based directly on substitutions, and not on time. However, in several cases in our study, it split species that seemed to represent homogeneous, well-supported lineages in many singletons. It is the case for *P. occidentalis* (split in 6 units), *P. neopolydactyla* 1 (split in 4 units), *P. neopolydactyla* 2 s.l. and *P. truculenta* (split in 6 units). It seems that even in what appears to be a species, based on phylogeny and other methods, if one tip is a little longer, it has a high probability to be considered as a distinct species. This must be due to the specificity of this model, which links the probability of belonging to a distinct species to a Yule process, depending on the number of substitutions (the branch lengths).


(b)
P. sp. 7a
P. scabrosella
P. sp. 7b
P. occidentalis
P. sp. 6
P. sp. 12
P. pulverulenta 3
P. pulverulenta 2
P. pulverulenta 1
P. sp. 2a
P. sp. 2b
P. sp. 2a b
P. truculenta + P. sp. 1
P. dolichorhiza 2
P. dolichorhiza b
P. dolichorhiza
P. sp. 4
P. sp. 5
P. hymenina + P. sp. 3
P. hawaiensis
P. pacifica
P. neopolydactyla 1 + 1b
P. neopolydactyla 3
P. neopolydactyla 2a + 2b + 2c
(a)
P. scabrosella + P. sp. 7a + P. sp. 7b
P. sp. 6
P. occidentalis
P. sp. 12
P. pulverulenta 1
P. sp. 2a
P. pulverulenta 2
P. pulverulenta 3
P. sp. 3
P. sp. 5
P. sp. 4
P. sp. 2b
P. hymenina + P. sp. 1
P. dolichorhiza 2
P. hawaiensis
P. truculenta
P. dolichorhiza
P. dolichorhiza b
P. pacifica
P. neopolydactyla 1b
P. neopolydactyla 2b
P. neopolydactyla 2a + 2c
P. neopolydactyla 1
P. neopolydactyla 3


(d)
P. scabrosella + P. sp. 7a + P. sp. 7b
P. sp. 12
P. occidentalis
P. sp. 6
P. pulverulenta 2
P. pulverulenta 1
P. pulverulenta 3
P. sp. 2b
P. truculenta + P. sp. 1 + P. sp. 2a
P. dolichorhiza
P. hymenina + P. sp. 3 + P. hawaiensis
P. neopolydactyla 1+ P. neopolydactyla 1b
P. neopolydactyla 2 s.l. + P. neopolydactyla 3 + P. pacifica
(c)
P. scabrosella + P. sp. 7a + P. sp. 7b
P. sp. 6
P. sp. 12
P. occidentalis
P. pulverulenta 2
P. pulverulenta 1
P. pulverulenta 3
P. sp. 2b
P. dolichorhiza
P. sp. 1 + P. dolichorhiza 2 + P. sp. 2a
P. truculenta
P. sp. 2c
P. hymenina
P. neopolydactyla 1b
P. neopolydactyla 1
P. neopolydactyla 3
P. pacifica
P. neopolydactyla 2b
P. neopolydactyla 2a

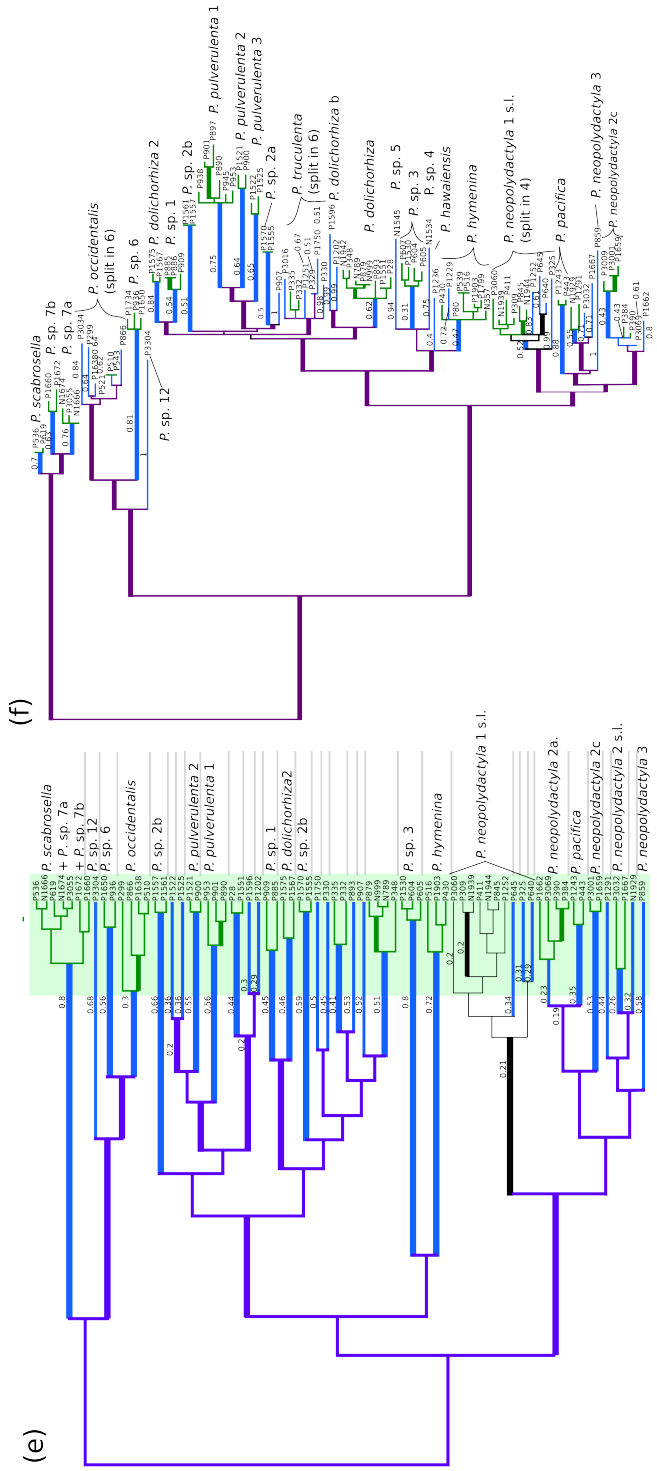

(f)
P. scabrosella
P. sp. 7b
P. sp. 7a
P. occidentalis (split in 6)
P. sp. 6
P. dolichorhiza 2
P. sp. 1
P. sp. 2b
P. pulverulenta 1
P. pulverulenta 2
P. pulverulenta 3
P. sp. 2a
P. truculenta (split in 6)
P. dolichorhiza b
P. dolichorhiza
P. sp. 5
P. sp. 3
P. sp. 4
P. hawaiensis
P. hymenina
P. neopolydactyla 1 s.l. (split in 4)
P. pacifica
P. neopolydactyla 3
P. neopolydactyla 2c
P. sp. 12
(e)
P. scabrosella
P. sp. 7a
P. sp. 7b
P. sp. 12
P. sp. 6
P. occidentalis
P. sp. 2b
P. pulverulenta 2
P. pulverulenta 1
P. sp. 1
P. dolichorhiza2
P. sp. 2b
P. sp. 3
P. hymenina
P. neopolydactyla 1 s.l.
P. neopolydactyla 2a.
P. pacifica
P. neopolydactyla 2c
P. neopolydactyla 2 s.l.
P. neopolydactyla 3

Figure 6: Comparison of the bGMYC results on each of the five loci tested, and bPTP results on the concatenated dataset in the Dolichorhizoid clade. Thick branches have pp value ≥ 0.95. Dark purple branches are branches representing interspecific relationships, blue branches are the branches supporting species, and green branches represent intraspecific variation, according to the bGMYC results. Black branches represent parts of the tree were bGMYC did not resolve species according to our criterion. The green background color highlights the zone where intraspecific variation occur. Values above branches represent the posterior probability for the delimitation of the species. Results of bGMYC on (a) the ITS trees; (b) the ß-tubulin trees; (c) the IGS1 trees; (d) the IGS3 trees and (e) the IGS16 trees; and (f) results of the bPTP analysis on the best ML tree resulted from the RAxML analysis on the concatenated dataset

spedeSTEM

Scabrosoid clade, without RPB1. When testing the species assignment with *P. neopolydactyla* 4 split in two random sets of 5 samples each, the two parts of *P. neopolydactyla* 4 appears as a single species from a theta value of 0.02 and values above. For these theta values, *P. scabrosa* 3a and *P. scabrosa* 3b are merged as a single species. Other lineages are considered as distinct species, even for high values of theta. The species delimitation is the same with the species assignment following the lineages represented in Fig. 2.

Scabrosoid clade, with *RPB1*. Testing the species assignment with *P. neopolydactyla* 4 split in two random sets of 5 samples each, *P. neopolydactyla* 4 appears as a single species for values of theta of 0.035 and above. At these theta values, all the species are supported as distinct lineages. With these values of theta and the species assignment following Fig. 2, all species tested are supported as distinct lineages.

There is thus a discrepancy when testing spedeSTEM with or without *RPB1*, on whether *P. scabrosa* 3a and *P. scabrosa* 3b represent distinct species. For the final consensus, we selected the results from the analysis without *RPB1* and a theta value of 0.02

Dolichorhizoid clade

The number of species supported by spedeSTEM varies quite much depending on the theta value, from 12 species with theta=0.5 or 4 species with theta = 0.00001 or 0.000001, to 17 to 26 species for theta values between 0.0001 and 0.1. Actually, theta values of 0.001, 0.005, 0.01 and 0.02 return the same species delimitation, that we will keep for the final consensus. We will select a theta value of 0.02, the same as the one selected for the Scabrosoid clade.

We selected a theta value of 0.02 (the same value as the one we selected for the Scabrosoid clade). This species delimitation, merges *P. hymenina* and *P. dissecta*, which was our test for the adequacy of the parameters as these two OTUs are conspecific based

on phylogenetic reconstructions. It also merges *P. scabrosella*, *P.* sp. 7a and *P.* sp. 7b; and *P.* sp. 1 and *P.* sp. 1b (as most methods). *P. dolichorhiza* is merged with *P. dolichorhiza* b and also with *P. truculenta*; and *P.* sp. 3, *P.* sp. 4 and *P.* sp. 5 are merged together. These two last mergings haven't been suggested by any other method (see Fig. 9 for the delimitation of species and online supplementary material for the species tree supported by spedeSTEM).

bPP

In the Scabrosoid clade, when running bPP with the species assignment where *P. neopolydactyla* 4 and *P. scabrosa* 2 are randomly split in two groups, the two half of each species are merged, suggesting that the method performs well. In this analysis, and in all other analyses, regardless of the mean of the theta prior, every lineage is supported as a distinct species, including *P. scabrosa* 3a and *P. scabrosa* 3b, which are supported as distinct species (e.g., with pp=0.97 for the analysis with a theta mean of 0.02), all the other species are supported as distinct with pp$\geq$0.99.

In the Dolichorhizoid clade, when the mean of the theta prior equals 0.001, *P. dissecta* and *P. hymenina* appear as distinct species, suggesting that the priors are not adequate.

When the mean of the theta prior equals 0.01 and 0.02, *P. hymenina* and *P. dissecta* are merged in a single species, whereas all the other species are supported as distinct lineages.

When the mean of the theta prior equals 0.05, *P. scabrosella*, *P.* sp. 7a and *P.* sp. 7b are merged, but *P. hymenina* and *P. dissecta* are considered as distinct species.

We decided to select the analysis with the mean of the theta prior equal to 0.02, as for the other analyses. The species delimitation supported by bPP is shown in Fig. 9 and the species tree with the support values in online supplementary material.

7.3.5 ITS pairwise distances: existence of a barcoding gap?

Fig. 7 shows the heatmaps summarizing the pairwise differences between ITS sequences within each clade. In the Scabrosoid clade, no real difference can be seen between *P. scabrosa* 3a and *P. scabrosa* 3b (otherwise supported by most methods as two distinct species). For the other species, the intraspecific variation is way smaller (light colors inside the squares) than the interspecific variation (darker colors outside the squares). In the Scabrosoid clade, it therefore seems that a barcoding gap approach could be implemented.

However, it seems difficult to apply the concept of barcoding gap in the Dolichorhizoid clade. If some species appear to have lower intraspecific variation than interspecific

variations with other species (e.g., *P. occidentalis*, and to some extent *P. hymenina*), in other groups such as in the South-American group or in *P. neopolydactyla* 2. s.l. we can't see a difference between intraspecific variation inside putative species and interspecific variation with the closely related lineages. In the *neopolydactyla/dolichorhiza/hymenina* group, there is clearly no such thing as a barcoding gap between ITS intraspecific and interspecific variation, probably due to the fact that the different species diverged too recently.

7.3.6 Comparison of the species delimitation methods

The delimitation by each method and the final consensus are shown in Fig. 9.

In our study, most methods performed well on the Scabrosoid clade, which shows that they perform well when the species are separated for large amounts of time, and are well-separated (based on the topology of the phylogenetic tree and the ITS distances between species).

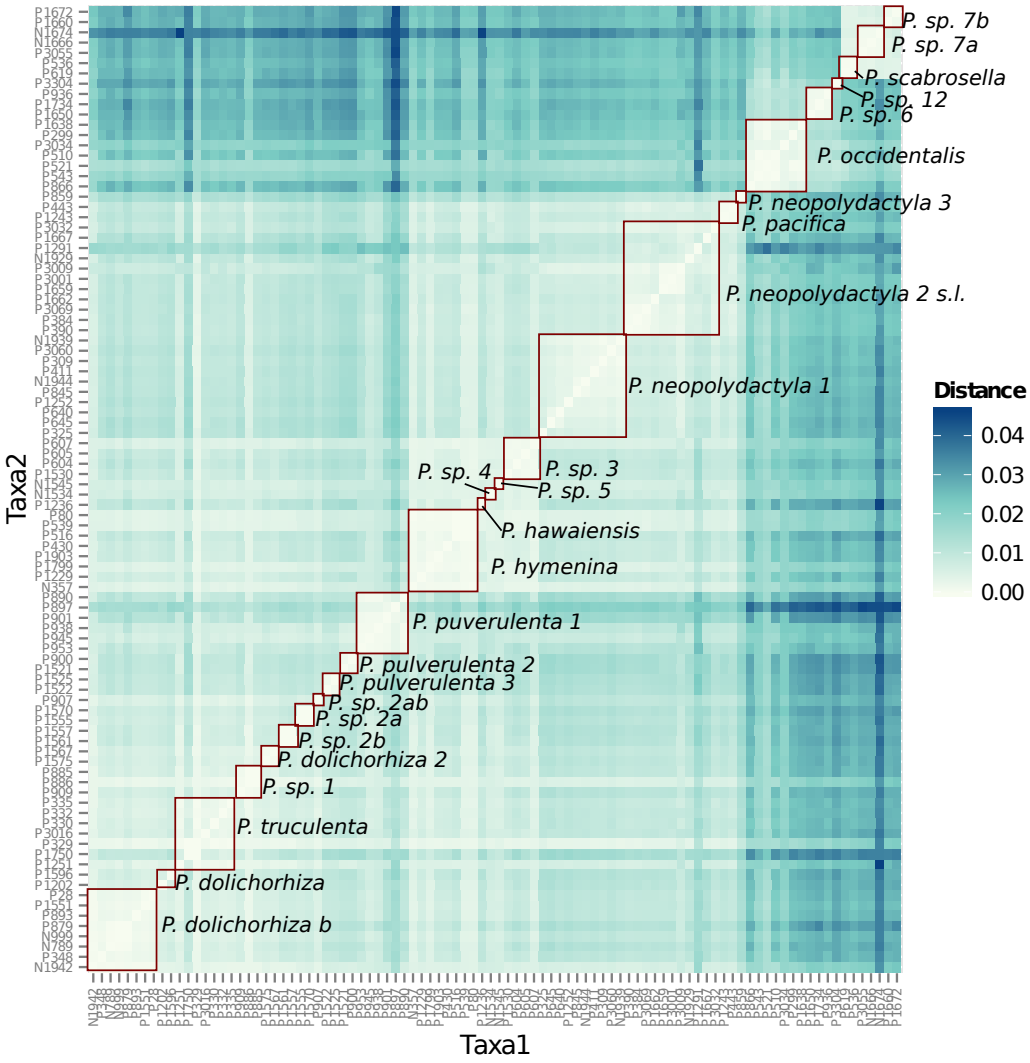
In the Dolichorhizoid clade, Structurama does not seem to perform well. It is probably because we did not include enough taxa per putative species. We would also probably need more loci, especially loci with an intermediary level of variation, to obtain better results. The fact that this approach is not based on phylogenetic results in the delimitation of non-monophyletic species, unlike the other methods tested. However, in the case of the Scabrosoid clade, and in parts of the Dolichorhizoid clade, species are accurately delimited (based on the phylogenetic reconstructions and the other species delimitation methods). When Structurama and tree-based methods are congruent, it is a strong indication that our putative species are genuine species, because different assumptions and models led to the same delimitation.

bGMYC performed well in most cases, even if it is sometimes negatively impacted by the lack of resolution of single-locus trees, when there is not enough variation in the locus. However, bGMYC can be a very powerful tool if implemented on very variable markers that are congruent with the real species tree.

bPTP also performed well and has the advantage that it can be ran on multi-locus phylograms, allowing to use a tree with all the the resolution provided by the different loci. However, it apparently has a tendancy to over-split some species, when branches inside species are not very short.

These two methods do not require big computational power, and only take phylogenetic trees as input, which make them very unexpensive and easy to use.

spedeSTEM takes gene trees as input, therefore uncertainty in the single-locus topologies cannot be taken into account; but the species tree is inferred by the program, so relationships between species can vary. It is the opposite approach in bPP: the alignments are provided, and therefore single-locus topologies are not fixed; but



a guide-tree is provided, and the relationships between species are thus fixed. In our case, it seems that spedeSTEM has a higher tendency to merge species, whereas bPP keeps them separate. The advantages of these two methods is that they take conflicts between the different topologies and histories of the loci tested in consideration, whereas bGMYC and bPTP only take a fixed topology into account.

spedeSTEM and bPP require estimates of theta, which is problematic in our group, because neither the estimation of the population sizes nor the accurate estimates of the substitution rates exist for lichen-forming fungi. However, testing a variety of theta values allowed us to test these methods.

Overall, bPP gave estimates that better match our species concepts based on mor-

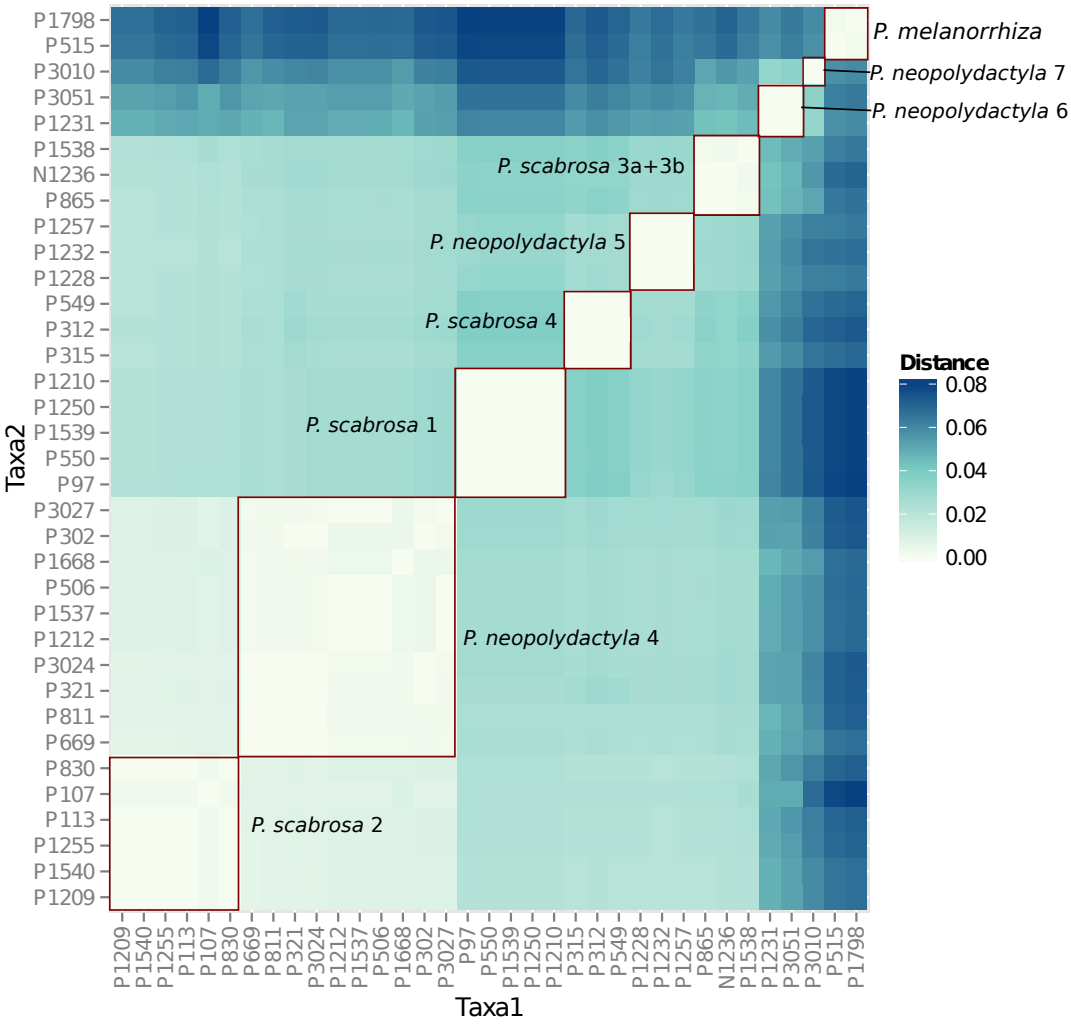
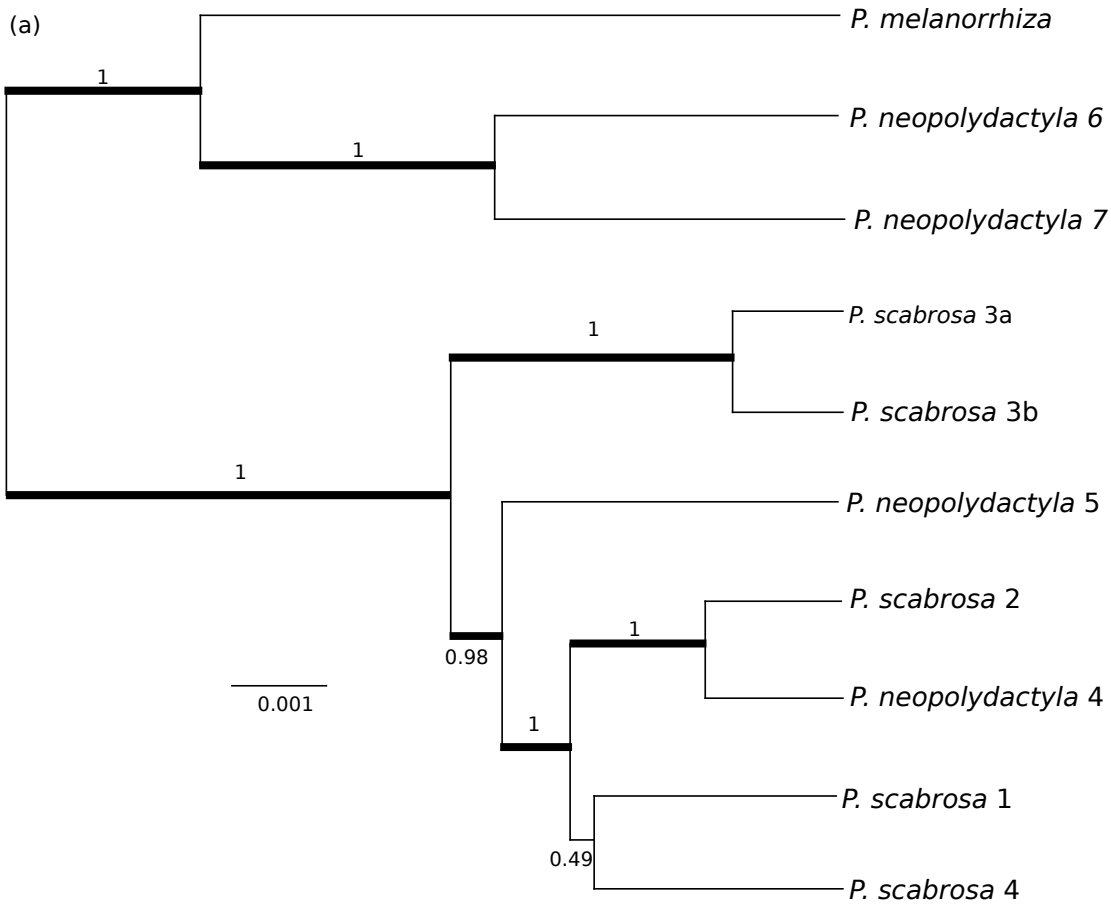


Figure 7: Heatmaps showing the pairwise ITS genetic distances between samples of (a) the Dolichorhizoid clade and (b) the Scabrosoid clade. Light colors represent low genetic distance, darker colors represent higher distances (see the scale on the right). Red squares represent the delimitation of the putative species, and the names to which they correspond are indicated on the side.

phology and geographic distributions, and seem to be less sensitive to the parameters that it takes as input. However, more loci would probably give better results and use more information about different gene histories, and would reduce the impact of a single locus on the final results. Unfortunately, the more loci used, the more expensive and computationally-intensive these methods would be.



7.3.7 Comparison of the species trees and the concatenated 8-locus and 7-locus trees

Fig. 8 shows the species trees generated by *BEAST.

In the Scabrosoid clade, the species tree has the same topology as the concatenated tree, except that *P. scabrosa* 1 is grouped with *P. scabrosa* 4 in the species tree (pp=0.49), whereas it is grouped with *P. scabrosa* 2 and *P. neopolydactyla* 4 in the concatenated analyses (for instance, pp=0.7 in the MrBayes analysis). However, none of these relationships are supported.

In the Dolichorhizoid clade, the topology is globally the same. There is one difference in the *P. neopolydactyla* 2. s.l. group, where *P. neopolydactyla* 2b is grouped with *P. neopolydactyla* 2a and *P. neopolydactyla* 2c in the species tree but without support (pp=0.36). These three lineages are then grouped with *P. pacifica* (pp=0.54) and with *P. neopolydactyla* 3 (pp=0.91).

In the concatenated analysis, *P. neopolydactyla* 2b is grouped with *P. neopolydactyla*

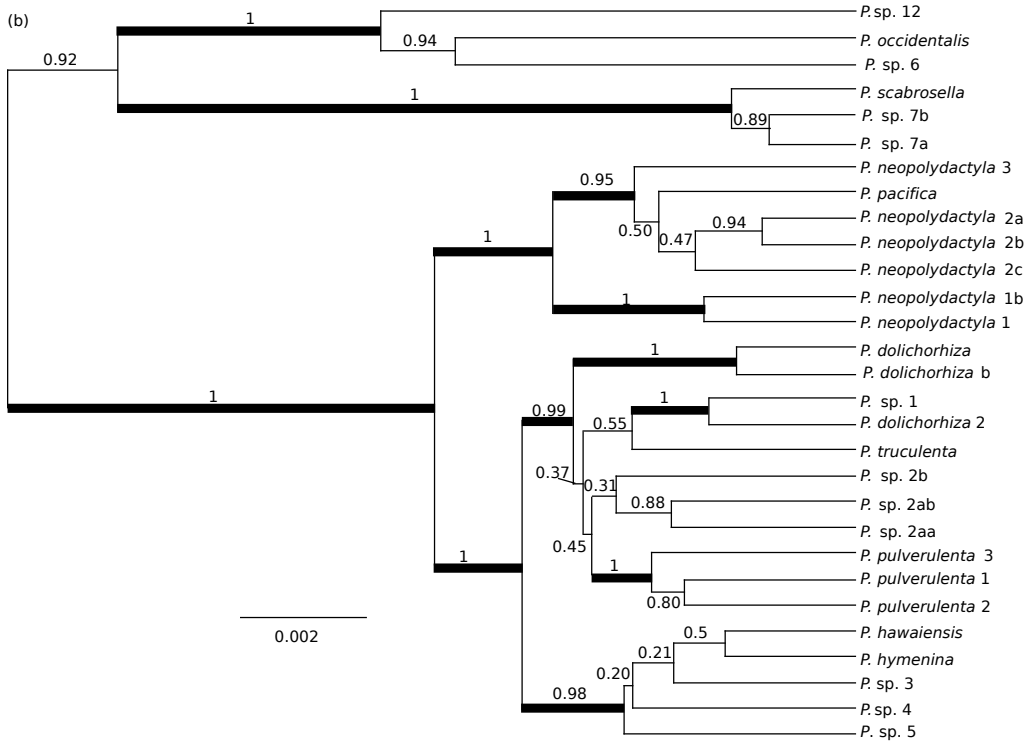


Figure 8: Species trees resulted from the *BEAST analyses, for (a) the Scabrosoid and (b) the Dolichorhizoid clade. Thick internodes have a pp $\geq$ 0.95 and values above the branches represent the posterior probabilities.

3 (pp=0.84) then with *P. pacifica* (pp=0.99), whereas *P. neopolydactyla* 2a and *P. neopolydactyla* 2c are grouped together (pp=0.99). This conflict actually mostly concerns the position of *P. neopolydactyla* 2b, whereas both methods agree to group these 5 species together.

The *hymenina* group is well supported in both analyses, but *P. hawaiensis* is grouped with *P. hymenina* in the species tree whereas it is grouped with *P.* sp. 3, *P.* sp. 4 and *P.* sp. 5 in the concatenated analysis. However none of these relationships are supported (pp=0.38 in the species tree, pp=0.94 in the Bayesian analysis).

Finally in the South-American group, the topologies are congruent, which is remarkable given the recent radiation and the lack of resolution and amount of short branches in the single locus trees. The only difference is the position of *P.* sp. 2a and *P.* sp2b, grouping with *P. pulverulenta* 1, 2 and 3 in the concatenated analysis, and with *P.* sp. 1 and *P. dolichorhiza* 2 in the species tree, but none of these relationships are supported (pp=0.33 in the species tree, pp=0.62 in the Bayesian concatenated tree).

In general, posterior probabilities are lower in the species tree, which makes sense because it takes the uncertainty of the conflicting single-locus topologies into account,

whereas in concatenated analyses, the tendency is that many characters supporting one relationship will mask the impact of the conflicting characters.

Examples include the grouping of *P.* sp. 7a and *P.* sp. 7b (pp=0.75 in the species tree, pp=1 in the MrBayes tree), *P. occidentalis* with *P.* sp. 6 (pp=0.9 in the species tree, pp=1 in the concatenated dataset), *P. pulverulenta* 1 with *P. pulverulenta* 2 (pp=0.78 in the species tree vs pp=1 in the concatenated analysis). For the two latter cases, conflicts came from only one locus (see the results about the single locus topologies), but still significantly affect the pp.

7.3.8 Influence of the *Nostoc* on the phenotype and on species delimitation

We noticed that all specimens associating with the *Nostoc* phylogroup VIIa (see chapter one for the nomenclature of *Nostoc* phylogroups) had a typical emerald green color when wet, especially in the field. Because some species (*P. neopolydactyla* 1, *P. occidentalis*, *P. scabrosa* 1, *P. scabrosa* 4) always associate with this phylogroup in the panboreal zone, and other morphologically similar species (*P. neopolydactyla* 2, *P. neopolydactyla* 4, P. *scabrosa* 2) never associate with it, the identity of the *Nostoc* phylogroup can be very useful to identify the species, especially in the field or when no molecular data is available.

More detailed study of the influence of other *Nostoc* phylogroups on the phenotype of the thallus should be conducted, to determine if this character could be used for the identification of a wide variety of species.

As many species have shown a strong specificity towards the *Nostoc* phylogroup they associate with, this information might also be helpful as a criterion for species delimitation. For instance, methods performed poorly to delimit species in the group of *P. neopolydactyla* 2 s.l., however *P. neopolydactyla* 2a and *P. neopolydactyla* 2c were always found associating with *Nostoc* phylogroup XIII, whereas *P. neopolydactyla* 2b was always found with *Nostoc* phylogroup X. This different pattern of association might be a good indication that they actually belong to different species, even if it could also be specialization to different ecological conditions. The fact that the sympatric and morphologically similar pair *P. neopolydactyla* 1 and *P. neopolydactyla* 2a, or *P. scabrosa* 1 and *P. scabrosa* 2 actually belong to different species is consolidated by the fact that they never share the same *Nostoc* phylogroup even in the same localities.

We suggest that when a new haplotype of the mycobiont is found, if it cannot be clearly assigned to a species based on the phylogeny, and there is no time to repeat all the species delimitation methods, the identity of the *Nostoc* haplotype it associates with can help to assign the specimen to a species, if a clear pattern of specificity exists within this group.

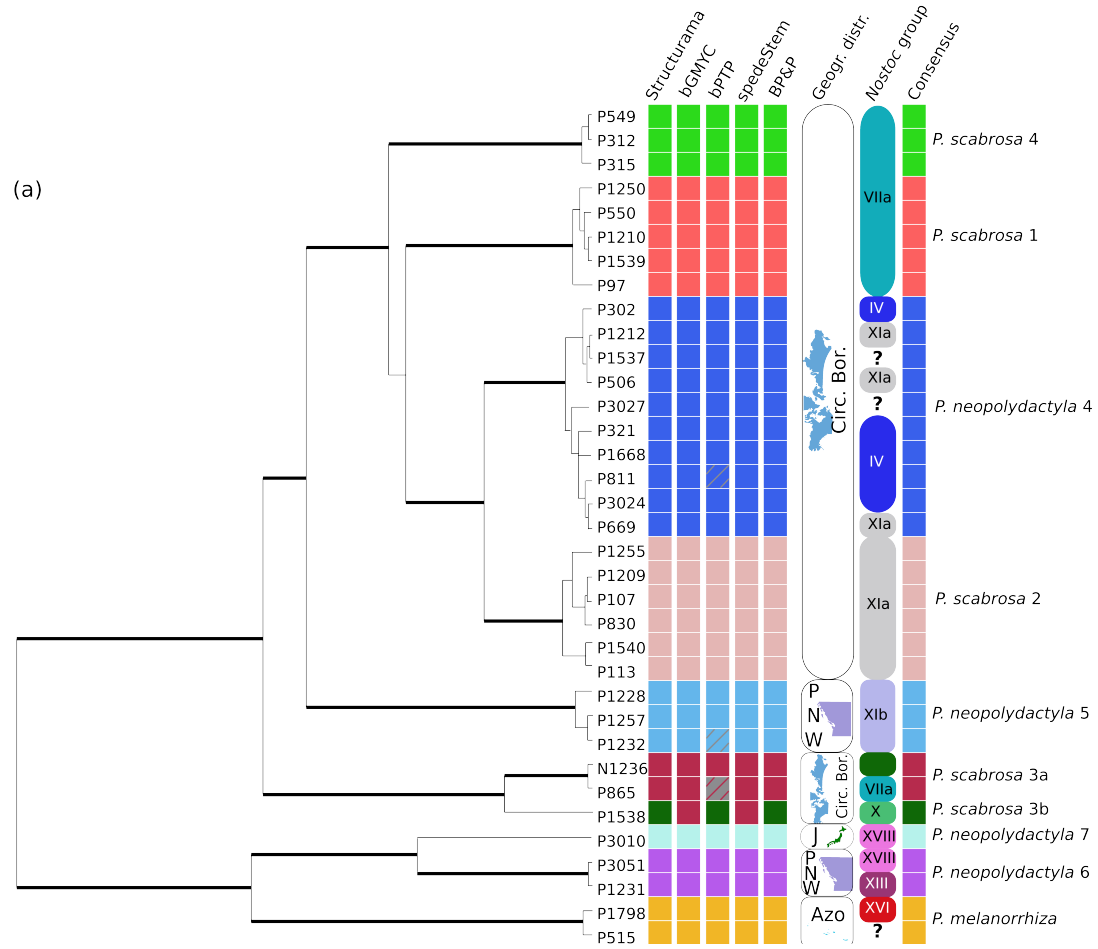
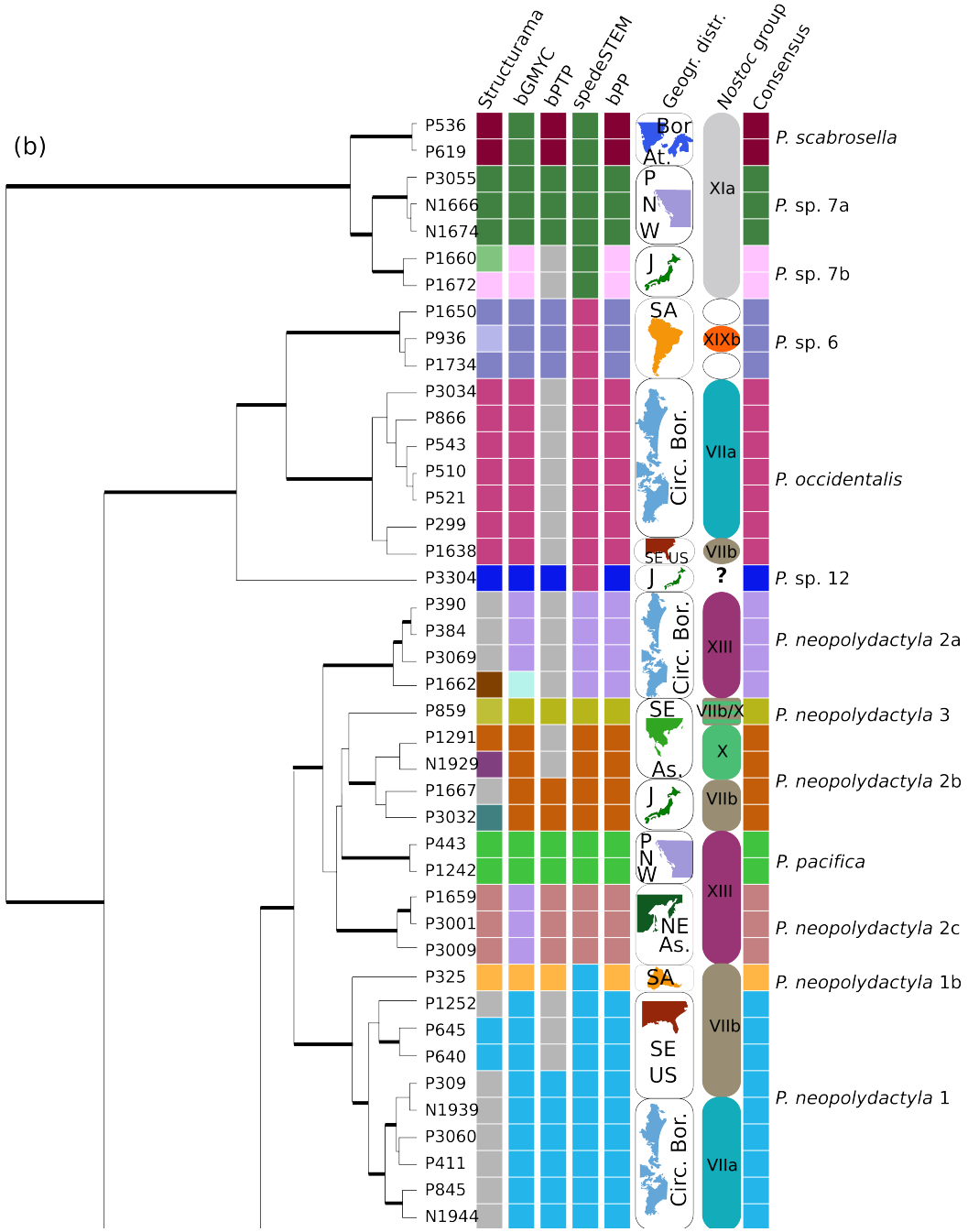
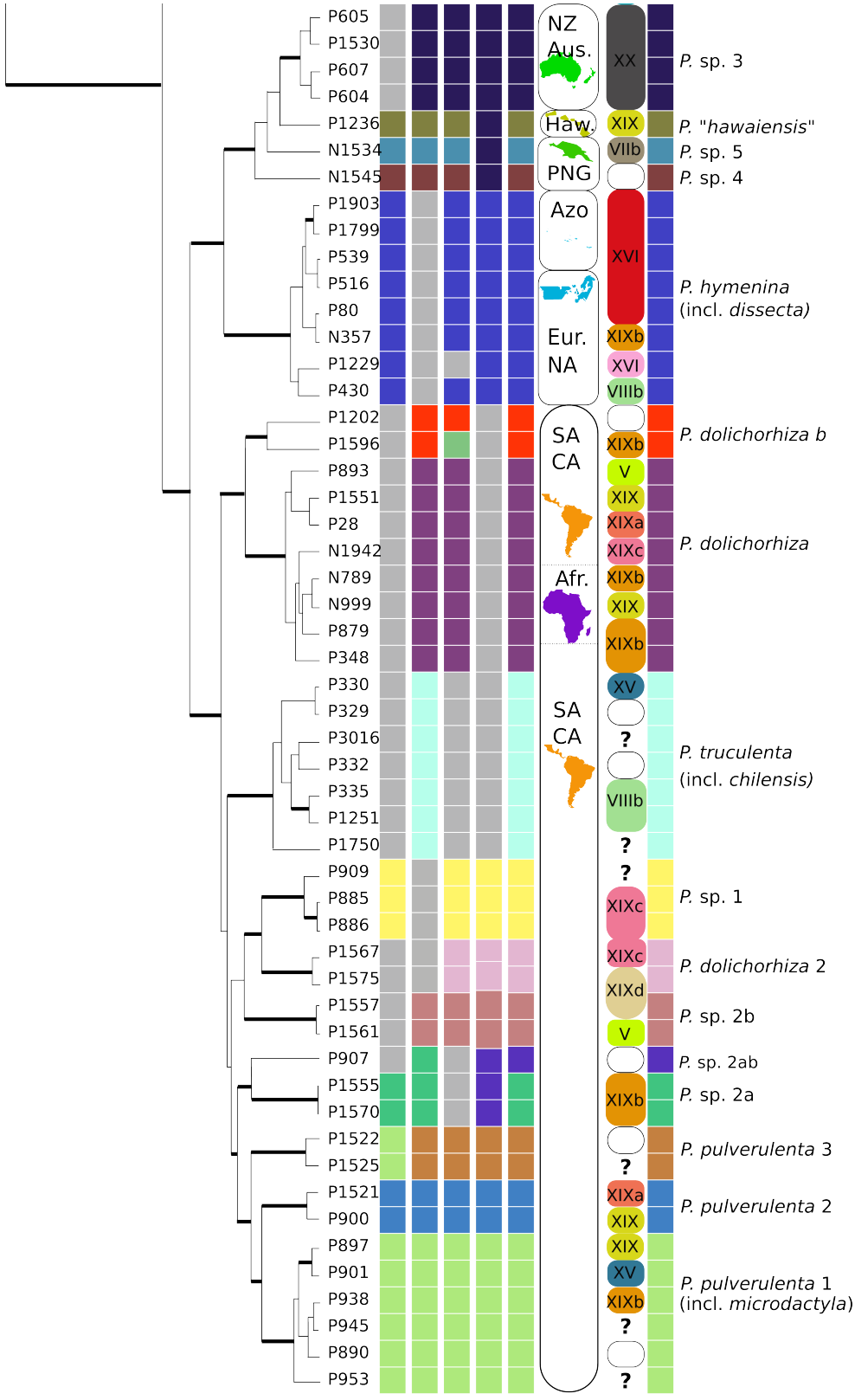


Figure 9: (a) consensus on the species delimitation for the Scabrosoid clade and (b) the Dolichorhizoid clade. On the left part, chronogram resulted from the BEAST analysis on the concatenated datasets of Section *Polydactylon*. Thick branches in the chronograms have pp ≥ 0.95. Chronograms for both clades are subsets of a chronogram generated by the same BEAST analysis (164 taxa, 8 loci, 6553 characters). On the right part, from left to right: species assignment by Structurama (with a gamma shape of 3 for the Scabrosoid clade, and with a gamma shape of 15 for the Dolichorhizoid clade); species assignment from the bGMYC analysis on the ITS locus; species assignment from the bPTP analysis on the concatenated dataset; species assignment from the spedeSTEM analysis; species assigment from the bPP analysis; geographic distribution of the specimens; *Nostoc* phylogroup that each specimen associates with (colors and numbering refer to the definition of phylogroups in chapter one; missing data are represented by question marks, white circles represent unique haplotypes, not included in any color-coded *Nostoc* phylogroup) and, in the last column, final species delimitation from our consensus. In species delimitation boxes, grey means that the method poorly performed; stripes mean that the probability for the alternative delimitations was similar. Abbreviations for the geographic zones: Afr.: Africa, As.: Asia, At.: Atlantic, Aus: Australia, Azo: Azores, CA: Central America, Circ. Bor.: Circumboreal, Eur: Europe, J: Japan, NA: North America, NE: North-East, NZ: New Zealand, PNG: Papua New Guinea, PNW: Pacific Northwest, SA: South America, SE: South-East


(b)
Structurama
bGMYC
bPTP
spedeSTEM
bPP
Geogr. distr.
Nostoc group
Consensus
P536
P619
P3055
N1666
N1674
P1660
P1672
P1650
P936
P1734
P3034
P866
P543
P510
P521
P299
P1638
P3304
P390
P384
P3069
P1662
P859
P1291
N1929
P1667
P3032
P443
P1242
P1659
P3001
P3009
P325
P1252
P645
P640
P309
N1939
P3060
P411
P845
N1944
Bor. At.
P N W
J
SA
Circ. Bor.
SE US
J
Circ. Bor.
SE As.
J
P N W
NE As.
SA
SE US
Circ. Bor.
XIa
XIXb
VIIa
VIIb
?
XIII
VIIb/X
X
VIIb
XIII
VIIb
VIIa
P. scabrosella
P. sp. 7a
P. sp. 7b
P. sp. 6
P. occidentalis
P. sp. 12
P. neopolydactyla 2a
P. neopolydactyla 3
P. neopolydactyla 2b
P. pacifica
P. neopolydactyla 2c
P. neopolydactyla 1b
P. neopolydactyla 1


P605
P1530
P607
P604
P1236
N1534
N1545
P1903
P1799
P539
P516
P80
N357
P1229
P430
P1202
P1596
P893
P1551
P28
N1942
N789
N999
P879
P348
P330
P329
P3016
P332
P335
P1251
P1750
P909
P885
P886
P1567
P1575
P1557
P1561
P907
P1555
P1570
P1522
P1525
P1521
P900
P897
P901
P938
P945
P890
P953
NZ Aus.
Haw.
PNG
Azo
Eur. NA
SA CA
Afr.
SA CA
XX
XIX
VIIb
XVI
XIXb
XVI
VIIIb
XIXb
V
XIX
XIXa
XIXc
XIXb
XIX
XIXb
XV
?
VIIIb
?
?
XIXc
XIXc
XIXd
V
XIXb
?
XIXa
XIX
XIX
XV
XIXb
?
?
P. sp. 3
P. "hawaiensis"
P. sp. 5
P. sp. 4
P. hymenina (incl. dissecta)
P. dolichorhiza b
P. dolichorhiza
P. truculenta (incl. chilensis)
P. sp. 1
P. dolichorhiza 2
P. sp. 2b
P. sp. 2ab
P. sp. 2a
P. pulverulenta 3
P. pulverulenta 2
P. pulverulenta 1 (incl. microdactyla)

7.3.9 Consensus on species delimitation

Species delimitation, geographic ranges, *Nostoc* specificity and morphological variation in the Scabrosoid clade

The delimitation of species in the Scabrosoid clade was congruent in most methods (see Fig. 9), and samples are assigned to ten species as follow:

P1798 and P515 as *P. melanorrhiza*, a rare species endemics to the Azores and easy to recognize morphologically.

P1231 and P3051 as *P. neopolydactyla* 6, a species endemics to the Pacific Northwest region of North America. It is not a strict specialist, but shows affinity to two *Nostoc* phylogroups (XIII and XVII, for the numbering of phylogroups, see chapter 1). It corresponds to very specific morphotypes of *P. neopolydactyla* s.l. and is easy to recognize morphologically.

P3010 as *P. neopolydactyla* 7, a rare species only known from this specimen from Japan.

P1228, P1232 and P1257 as *P. neopolydactyla* 5, a species endemics to the Pacific Northwest region of North America. It is highly specialized, always found with *Nostoc* phylogroup XIb. It corresponds to very specific morphotypes of *P. neoplydactyla* s.l. and is easy to recognize morphologically.

P312, P315 and P549, as *P. scabrosa* 4, a rare species known from Québec and Norway, restricted to boreal zones. It is highly specialized, always associating with *Nostoc* phylogroup VIIa.

P97, P550, P1210, P1250 and P1539 as *P. scabrosa* 1, a widespread species from the panboreal zone. Specimens included in this study come from Québec, Alberta, Norway, British Columbia and Siberia, Russia. It is highly specialized, always associating with *Nostoc* phylogroup VIIa.

P107, P113, P830, P1209, P1255 as *P. scabrosa* 2, a widespread species from the panboreal zone. Specimens included in this study also come from Québec, Norway, British Columbia and Siberia, Russia. It is not a strict specialist, but shows high selectivity towards *Nostoc* phylogroup XIa, even if it has also been found with phylogroup VIId.

P302, P321, P506, P669, P811, P1212, P1537, P1668, P3024, P3027 as *P. neopolydactyla* 4, a widespread panboreal species. Specimens included in this study come from Québec, Norway, Michigan, Siberia, Eastern Russia and Japan. It is not a strict specialist, but shows very high specificity towards *Nostoc* phylogroup IV and XIa. It morphologically resembles *P. neopolydactyla* 1 s.l. and *P. neopolydactyla* 2 s.l.

The only open question concerns *P. scabrosa* 3a and *P. scabrosa* 3b: several methods considered these two as one species (bGMYC on ITS, spedeSTEM on 6 loci), others as two species (Structurama, bGMYC on other loci, bPTP, spedeSTEM on 7 loci, bPP). Most methods placed them in two distinct lineages, and it makes sense when looking at the concatenated phylogenetic analyses, and the number of differences in the ITS sequences (see haplotype network in Chapter 1, figure 5). However, these species are very rare and we only had 3 samples (2 of *P. scabrosa* 3a and one of *P. scabrosa* 3b, respectively) in the present study. We suggest that it might represent two distinct species and we included two lineages in the species tree but more material should be examined before any final conclusion is made.

Out of these ten species, seven represent previously unrecognized species. We hypothesize that *P. neopolydactyla* 4 represents *P. neopolydactyla* s. str. and *P. scabrosa* 2 represents *P. scabrosa* s. str., based on morphological, chemical and geographical data about the type speciemens. Except these two species and *P. melanorrhiza*, all the other species need a formal description.

Species delimitation in the Dolichorhizoid clade

In the Dolichorhizoid clade, the species delimitation was straightforward in several groups (see Fig. 9 for the comparison of results):

In the *occidentalis* group, *P. occidentalis*, *P.* sp. 6 and *P.* sp. 12 are three distinct species. Even if some methods split *P. occidentalis* (bPTP) or *P.* sp. 6 (Structurama) in several singletons, we believe that this is a problem with the datasets and the methods, as these species are well-defined, based on the phylogeny and the morphology and their geographic distributions, and do not appear to include cryptic variation, based on their sequences.

P. occidentalis has a typical morphology and is easy to identify. It always associates with the *Nostoc* phylogroup VIIa in the boreal zone, and has a panboreal distribution. It can also very rarely be found in the Appalachians, where it associates with the *Nostoc* phylogroup VIIb. In our study, specimens from Norway, Québec, Alaska, Japan and North Carolina were included.

P936, P1650 and P1734 belong to *P.* sp. 6, which is only found in tropical Central and South America, and does not seem to have specificity in its association with *Nostoc*. Our study included specimens from Peru, Colombia and Honduras

P3304 belongs to *P.* sp. 12, which is a very rare species, only known from one specimen from Japan.

In the *hymenina* group, *P. hymenina*, *P. hawaiensis*, *P.* sp. 3, *P.* sp. 4 and *P.* sp. 5 are supported as distinct species by most methods. *P. dissecta* appears as conspecific to *P. hymenina*, despite his unique ITS haplotype and its very different morphology, as

shown in Chapter one.

P604, P605, P607 and P1530 represent *P.* sp. 3, only known from, but widespread in Australia and New Zealand, where it was usually identified as *P. dolichorhiza*, *P. polydactylon* or *P. nana*. It has an important ITS haplotype diversity and morphological variation.

P1236, *P. "hawaiensis"* is only known from Hawaii. *P.* sp. 4 (N1534 in this study) and *P.* sp. 5 (N1545) are only known from Papua New Guinea, where they were described as variants of *P. dolichorhiza* in Sérusiaux et al. (2009).

P80, P430, P516, P539, P1229, P1799, P1903 belong to *P. hymenina*, which has a wide distribution, but is only present in Northern regions, in North America, Europe and Atlantic Islands; and was never found in Pacific Islands, where the other species of this group can be found. It is easy to recognize morphologically, but is not specialized to a single *Nostoc* phylogroup, even if it is often found with phylogroup XVI. Specimens from our study came from the Pacific Northwest of North America, Newfoundland (Canada), Iceland, Norway, Tenerife and the Azores.

The geographic distributions of species is a good factor to distinguish species in the *hymenina* group, as only *P.* sp. 4 and *P.* sp. 5 occur in the same region.

In the *neopolydactyla* group, *P. pacifica*, composed of P443 and P1243, might be the only non-problematic species. It was delimited as a species by almost every method. It is easy to recognize morphologically, endemic to the Pacific Northwest of North America, and is specialized toward *Nostoc* phylogroup XIII.

P. neopolydactyla 1b (P325) seems to be a distinct species from *P. neopolydactyla* 1 (N1939, P309, P411, P640, P645, P845, P1252, P3060), as supported by most methods.

P. neopolydactyla 1b is only known from one specimen from Peru whereas *P. neopolydactyla* 1 is only found in Northern regions, including the Appalachian mountains, Arizona, and boreal zones in Norway, Québec and Russia. In the boreal zone, it always associates with *Nostoc* phylogroup VIIa, whereas it was found with phylotype VIIb in the Appalachians and VIIc in Arizona.

P859, *P. neopolydactyla* 3 is also considered as a distinct species by most methods. It is only known from two specimens from Yunnan (China) and Vietnam.

The delimitation of lineages inside *P. neopolydactyla* 2 s.l. was difficult for most methods. It is mainly composed of three lineages: *P. neopolydactyla* 2a, 2b and 2c, which are considered as distinct species in several methods (and supported as distinct by bPP and spedeSTEM), unresolved in others, rarely grouped together (for instance, *P. neopolydactyla* 2a and 2c are grouped together in the bGMYC analysis of ITS). Specimens such as P1662 were also considered as distinct singleton species in several methods.

It seems that *P. neopolydactyla* 2b (N1929, P1291, P1667, P3032), known mainly

from Yunnan (China) and Japan and associating with *Nostoc* phylogroup X would be a distinct lineage according to several methods, or would even represent several species according to other methods.

P. neopolydactyla 2c (P1659, P3001, P3009), known mainly from Northern Japan and Russia, and *P. neopolydactyla* 2a (P384, P390, P3069, P1662), widespread in the boreal zone, including Norway, Canada, Russia, Japan, but also moutainous regions from France, for instance, both show specificity towards phylotype XIII. If they are distinct species, as suggested by most methods, they would be sympatric in Hokkaido, Japan.

The delimitation in the *scabrosella* group (composed of *P. scabrosella*: P536 and P619, *P.* sp. 7a: N1666, N1674 and 3055; *P.* sp. 7b: P1660 and P1672) is problematic. Some methods assigned them to a single species, whereas other assigned them to three distinct species. They all associate with *Nostoc* phylogroup XIa. However, *P. scabrosella* has a very different morphology (small scabrid lobes without veins; *P.* sp. 7a and *P.* sp. 7b have wide glabrous lobes with veins) and thalli from *P.* sp. 7a and *P.* sp. 7b also differ considerably in terms of morphology. Moreover, they have distinct geographical distributions. *P. scabrosella* occurs in boreal zones of Scandinavia and the Atlantic coast of North America, *P.* sp. 7a is endemic to the Pacific Northwest of America, whereas *P.* sp. 7b has only been found in Hokkaido (Japan). Moreover, if their haplotypes are always genetically very similar, they are never identical from one species to the other. Therefore, allelic exclusivity, morphological differences and distinct geographic ranges led us to consider them as three distinct species.

In the South American group, most methods agreed to distinguish *P. pulverulenta* 1 (P890, P897, P901, P938, P945, P953), *P. pulverulenta* 2 (P900 and P1521) and *P. pulverulenta* 3 (P1522 and P1525) as three distinct lineages, while they are sympatric. (*P. pulverulenta* 1 is widespread, found in Mexico, Guatemala, Colombia, Bolivia, Brazil...) whereas the two other lineages are rare, and only found in Colombia. They cannot be recognized morphologically and they might represent sibling species (Steyskal, 1972), closely related species where divergence and genetic isolation was not correlated with significant change in morphology

Most methods also agreed to recognize the widespread *P. dolichorhiza* (N789, N999, N1942, P348, P28, P879, P893, P1551), found in Central and South America, from Mexico to Brazil, including Colombia, Bolivia and Galapagos Islands, as well as in Africa, including Rwanda, South Africa, Madagascar and Reunion Island, and the rare *P.* sp. 1 (P885, P886, P909, from Colombia and Bolivia), *P.* sp. 2a (P1555, P1570 from Colombia), *P.* sp. 2b (P1557, P1561 from Brazil) , and *P. dolichorhiza* 2 (P1567, P1575 from Brazil) as distinct species. Most of these species can be distinguished morphologically, but they were poorly resolved in the phylogenetic trees, as they originated from a very recent radiation, and further testing should be investigated, to determine if they are completely reproductively isolated or if some gene flow might still occur.

The status of *P. truculenta* (including *P. chilensis*) known from the Neantarctic

parts of Chile and Argentina as well as several remote islands (e.g., Kerguelen islands, Crozet islands and Gough Island), and exhibit significant morphological plasticity, should be further assessed, as some methods suggested that it might comprise several distinct lineages.

The status of the very rare P1202 and P1596 ("*P. dolichorhiza* b"), known from only one specimen from Mexico and one specimen from Brazil, on whether they represent one species, two species, or if they represent genetically distinct variants of *P. dolichorhiza*; and the status of P907 ("*P.* sp. 2ab" from Colombia) as whether it belongs with *P.* sp. 2a (P1555 and P1570 from Brazil) or represent a distinct species should be further assessed, but more material would be needed to answer these questions.

This would result in a total of 27-29 species in the Dolichorhizoid clade, whereas only seven are currently described (*P. dolichorhiza*, *P. pulverulenta*, *P. truculenta*, *P. pacifica*, *P. hymenina*, *P. occidentalis* and *P. scabrosella*). The name "*P. hawaiensis*" was used by several naturalists but never formally described. There would thus be nineteen to twenty-one species that need formal description in the group.

7.3.10 Occurence of cosmopolitan species in the section *Polydactylon*?

P. scabrosa, *P. neopolydactyla*, *P. dolichorhiza* and *P. polydactylon* were the taxa which were considered to have a cosmopolitan, or almost cosmopolitan distribution.

P. scabrosa s.l. is actually composed of 4 or 5 lineages, which all have a panboreal distribution.

When defined based on phylogeny and species delimitation methods, *P. dolichorhiza* is only present in the Neotropics and Afrotropics.

P. neopolydactyla s. l. is composed of at least 10 species according to the species delimitation methods.

P. neopolydactyla 1, 2a and 4 are panboreal, sometimes expanding to moutain regions in temperate zones.

P. neopolydactyla 2b and *P. neopolydactyla* 3 seem restricted to temperate Asia, and *P. neopolydactyla* 2c to Hokkaido and Eastern Russia.

P. neopolydactyla 5 and *P. neopolydactyla* 6, as well as *P.* sp. 7a are endemics to the Pacific Northwest of North America, *P.* sp. 12 and *P.* sp. 7b to Japan.

Based on phylogenetic inferences, *P. polydactylon* is actually composed of three different lineages in Europe (*P. polydactylon* 1), East Coast of North America (*P.* sp. 10), and West Coast of America (*P. polydactylon* 2). , Morphotypes identified as *P. polydactylon* in Asia or South America belong to distinct species.

It seems that cosmopolitan lineages don't exist in *Peltigera* section *Polydactylon*.

Several widespread species have a panboreal distribution, or are widespread in one continent (e.g., South America, Asia...) , but no species has been found with an actual or an almost cosmopolitan distribution. The names used to identify similar morphotypes across continents and climatic zones were assigned to distinct evolutionary species, which need formal description.

It is now clear that most species, once correctly delimited, only occur within well-delimited geographic range. This information can be used to facilitate species delimitation. Once that morphologically similar species that are known not to occur in a specific zone have been dismissed, it will be easier to identify *Peltigera* specimens based on the remaining possibilities.

7.4 Supplementary Material

Supplementary Material from this chapter can be downloaded online at https://github.com/NicolasMagain/ThesisOnlineSupplementary/.

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Appendices

Many supplementary files can be found at
http://github.com/NicolasMagain/ThesisOnlineSupplementary.

These appendices include:

- A PDF version of the thesis.

- All figures presented in the thesis, in larger size and high resolution.

- Online Supplementary figures and tables from several chapters of the thesis.

- PDF versions of the published articles.

- Posters and slideshow presentations realized in the context of this thesis.

- Sequence matrices used to generate the results presented.

- Phylogenetic trees from the last chapter.
- More.