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Cophylogenetic patterns in algal symbionts correlate with repeated symbiont switches during diversification and geographic expansion of lichen-forming fungi in the genus *Sticta* (Ascomycota, Peltigeraceae)

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ABSTRACT

Species in the fungal genus *Sticta* form symbiotic associations primarily with either green algae or cyanobacteria, but tripartite associations or photosymbiodemes involving both types of photobionts occur in some species. *Sticta* is known to associate with green algae in the genus *Symbiochloris*. However, previous studies have shown that algae from other genera, such as *Heveochlorella*, may also be suitable partners for *Sticta*. We examined the diversity of green algal partners in the genus *Sticta* and assessed the patterns of association between the host fungus and its algal symbiont. We used multi-locus sequence data from multiple individuals collected in Australia, Cuba, Madagascar, Mauritius, New Zealand, Reunion and South America to infer phylogenies for fungal and algal partners and performed tests of congruence to assess coevolution between the partners. In addition, event-based methods were implemented to examine which cophylogenetic processes have led to the observed association patterns in *Sticta* and its green algal symbionts. Our results show that in addition to *Symbiochloris*, *Sticta* associates with green algae from the genera *Chloroidium*, *Coccomyxa*, *Elliptochloris* and *Heveochlorella*, the latter being the most common algal symbiont associated with *Sticta* in this study. Geography plays a strong role in shaping fungal-algal association patterns in *Sticta* as mycobionts associate with different algal lineages in different geographic locations. While fungal and algal phylogenies were mostly congruent, event-based methods did not find any evidence for cospeciation between the partners. Instead, the association patterns observed in *Sticta* and associated algae, were largely explained by other cophylogenetic events such as host-switches, losses of symbiont and failure of the symbiont to diverge with its host. Our results also show that tripartite associations with green algae evolved multiple times in *Sticta*.

1. Introduction

Mutualisms are ubiquitous in nature and include a variety of associations many of which are crucial to the functioning of ecosystems. Mutualistic systems involve at least two partners living in close association and interacting in a way that is beneficial to both partners, usually by providing goods or services to one another (Schwartz and Hoeksema, 1998; Herre et al. 1999). The degree of association between mutualistic partners can vary from tight associations where mutualistic partners live on or inside their hosts, to more loose associations, where the partners may interact with each other only a short period of time during their life cycle.

The selection of the proper partner is important in mutualistic systems, as it influences the survival of the mutualistic association and its partners in a given environment. Feedback mechanisms in partner selection will also increase the fitness of the mutualistic association and

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its partners over evolutionary time (Leigh, 2010). In tight mutualistic interactions, where one partner lives in close association with or even inside the other, partners may be vertically transmitted from one generation to the next, which can lead to the cospeciation of the partners and can also translate into concordant patterns in the phylogenies of the hosts and symbionts (Herre et al., 1999; Funk et al., 2000). In horizontal transmission, new generations need to acquire their partners anew, which bears the risk of not obtaining a partner at all, but also offers the possibility to associate with a new partner with a different genetic make-up or physiological performance (e.g. Piercey-Normore and DePriest, 2001; Ertz et al., 2018). However, even in systems where the transmission of mutualistic partners is predominantly vertical, horizontal transmission of new partners may occur (Nelsen and Gargas, 2008; Dal Grande et al., 2012). Therefore, vertical transmission of symbionts does not necessarily lead to congruent host and symbiont phylogenies (Herre et al., 1999).

Lichens are traditionally viewed as mutualistic systems consisting of fungi that form tight symbiotic associations with green algae and/or cyanobacteria. The fungal partner, the mycobiont, encloses the algal or cyanobacterial partner, the photobiont, and protects it from excessive light, providing optimal conditions for the photobiont to perform photosynthesis (Kranner et al., 2005). The photobiont, on the other hand, provides carbohydrates for the mycobiont (Smith, 1973; Green and Smith, 1974). The majority of lichens have a green algal partner as a primary photobiont, *Trebouxia* and *Trentepohlia* being the most common symbiotic green algae recruited by lichen-forming fungi (Friedl and Büdel, 2008). Approximately 10% of lichenized fungi associate primarily with a cyanobacterial partner, *Nostoc* and *Rhizonema* being the most common cyanobionts in lichens (Friedl and Büdel, 2008; Lücking et al., 2009).

The successful establishment of the lichen symbiosis requires that the mycobiont encounters and recognizes a suitable photobiont with which it can form a new lichen thallus. Lichens can obtain their symbionts in several different ways. In vegetative reproduction, fungal and algal partners are vertically transmitted to the next generation in propagules that contain cells from both partners. In sexual reproduction where the partners are horizontally transmitted, meiotic spores of the mycobiont must find a proper photobiont in order to form the symbiotic association de novo. Suitable photobionts may be obtained from a pool of free-living algae or even from thalli of other lichens or vegetative propagules (Bubrick et al., 1984; Mukhtar et al., 1994; Beck et al., 1998; Beck, 1999; Friedl and Büdel, 2008; Nelsen et al., 2011).

Lichen mycobionts are selective towards photobionts and usually associate with a particular species or genus of photobionts, with the exception of certain mycobionts that can simultaneously associate with green algae and/or cyanobacteria (Galun and Bubrick, 1984; Rambold et al., 1998). Symbiotic associations in lichens are considered specific when the interacting partners come from a relatively narrow taxonomic range (Smith and Douglas, 1987). Lichen mycobionts can be classified as generalists or specialists based on how many different partners they are able to associate with. Generalists show low levels of specificity for their symbionts, i.e. they accept a wider range of partners, whereas specialists only associate with a one or a few types (Yahr et al., 2004). Most mycobionts are selective towards their photobionts, at least at higher taxonomic levels, with many lichenized families exclusively associating with photobionts from particular genera such as *Trebouxia*, *Asterochloris* and *Nostoc* (Rambold et al., 1998; Miadlikowska et al., 2006; Miadlikowska et al., 2014). Lichens that reproduce sexually and need to encounter a suitable partner upon reproduction may have lower selectivity towards their partners, as the ability to associate with algae from multiple lineages increases the chances of finding a proper partner and to re-establish the symbiotic association (O'Brien et al., 2013; Steinová et al., 2019). Low selectivity is frequently linked to a wider ecological amplitude and distributional range, then often in association with locally adapted photobionts (Blaha et al., 2006; Muggia et al., 2014). Low selectivity has also been shown to occur in lichens occurring in ecologically extreme habitats (Wirtz et al., 2003). Chiefly or exclusively vegetatively reproducing lichens with vertical transmission of symbionts appear to be more selective towards their photobionts through the retention of the same symbiont across generations, and are also considered to have narrow ecological niches or occur under specific environmental conditions (Wornik and Grube, 2010; Otálora et al., 2010; Otálora et al., 2013; Pardo-De la Hoz et al., 2018). However, even in species where vertical transmission of symbionts is the predominant reproductive mode, rare events of symbiont switches can uncouple the symbiotic association and produce new combinations of partners helping the lichen to adapt to new environmental conditions and expand its distribution range (Nelsen and Gargas, 2008; Fernández-Mendoza et al., 2011; Dal Grande et al., 2012).

The lichenized genus *Sticta* (Schreber) Ach. contains foliose macrolichens in the family Peltigeraceae (subfamily Lobarioideae) with subcosmopolitan distribution, reaching the greatest species diversity in tropical montane and Andean regions (Brodo et al., 2001; Galloway, 2001; Moncada et al., 2013a; Kraichak et al., 2018; Lücking, 2019). Species in this genus associate with green algae or cyanobacteria, but green algal species usually form tripartite associations, either containing cyanobacteria in internal cephalodia or forming morphologically distinct lobes or even thalli, so-called photosymbiodemes (Moncada et al., 2013b; Tønsberg et al., 2016). The ability to form symbiotic associations with either green algae or cyanobacteria has made it possible for *Sticta* to colonize a variety of habitats, as the two types of photobionts differ in physiological performance under different environmental conditions (Galloway, 1998). In contrast to green algal lichens, cyanobacterial lichens are capable of fixing atmospheric nitrogen and can thrive in nutrient-poor habitats, also being important contributors to the nitrogen cycle of ecosystems (Green et al., 1980; Nash, 2008). Cyanobacteria have also shown higher photosynthetic activity in wet environmental conditions than green algae, whereas green algae outperform cyanobacteria in drier environments (Green et al., 1993).

Our understanding of the diversity and diversification of the mycobionts of the genus *Sticta* has developed significantly in recent years (Simon et al., 2018; Widhelm et al., 2018; Widhelm et al., 2019), but complementary progress regarding the photobionts is hampered by the scarcity of studies. In this study, we examined symbiont association patterns in the green algal species of the genus *Sticta*. Our aim was to assess the diversity and identity of green algal partners of selected species of *Sticta* using a molecular phylogenetic approach. We also examined the levels of specificity and selectivity and performed tests of congruence on multi-locus phylogenies obtained from both partners to explore the coevolutionary history of *Sticta* and its green algal symbionts. In addition, we utilized event-based methods to gain insight into what cophylogenetic events are likely to have been responsible for the observed pattern of association between *Sticta* and associated algae.

2. Material and methods

2.1. Taxon sampling and molecular data

A total of 101 specimens representing 30 species of *Sticta* that associate primarily with green algae were included as core group in this study (Supplementary Table 1). In addition, we assembled a broader, ITS-based phylogeny of 150 species to assess the phylogenetic placement of green algal *Sticta* species (Supplementary Table 2). *Sticta* species with green algal photobionts are mostly distributed in the Southern Hemisphere and therefore collections made in areas with high diversity of *Sticta*, i.e. South America, Madagascar, Mauritius, Reunion, Australia, and New Zealand were selected for this study.

For newly generated sequences, total genomic DNA was extracted from *Sticta* thalli using the *Quick*-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, California, USA), following the manufacturers' protocol, with the following exception: lichen thalli were ground with

mortar and pestle in 750 µl Lysis solution and after that placed in an incubator at 56 °C for one hour. DNA was eluted with 50 µl DNA elution buffer.

For the algal partner, we amplified part of the nuclear small subunit ribosomal RNA gene (18S) and part of the chloroplast ribulose-bisphosphate carboxylase-RuBisCO gene (*rbc*L). For the fungal partner, the nuclear internal transcribed spacer region (ITS), part of the nuclear large subunit ribosomal RNA gene (nuLSU), and the DNA replication licensing factor (MCM7) were amplified. PCR amplification was performed in 12.5 µl reaction volume consisting of 6.25 µl of MyTaq™ Red Mix DNA polymerase (Bioline, London, UK), 5.25 µl of water, 0.25 µl of primer and 0.5 µl of DNA.

Primers and PCR conditions are listed in Supplementary Tables 3 and 4, respectively. PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio, USA) and labeled with Big Dye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA). Cycle sequencing conditions were as follows: 25 cycles of 10 s 96 °C, 5 s 50 °C and 4 min 60 °C. Purified and labeled PCR products were detected on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence contigs were assembled and manually edited using Geneious v.11 (Biomatters Limited, Auckland, New Zealand). Sequences of each locus were aligned with MAFFT v.7.309 (Katoh et al., 2002; Katoh and Standley, 2013) as implemented in Geneious v.11 using default parameters.

2.2. Phylogenetic placement of algae associated with Sticta

To determine the identity of algae associated with *Sticta* and to examine the phylogenetic placement of these algae within Trebouxiophyceae, we incorporated 99 *rbc*L sequences obtained from the algal partners of *Sticta* in the *rbc*L dataset of Trebouxiophyceae from Dal Grande et al. (2014) for a broad phylogenetic analysis. A number of sequences belonging to algae in the genus *Ulva* were removed from the dataset, because they were not relevant for our study. We also inferred an 18S rDNA phylogeny for the algae associated with *Sticta*. For this purpose, we included 79 sequences obtained from algal symbionts of *Sticta* in the 18S dataset of Trebouxiophyceae from Dal Grande et al. (2014). We chose to analyze *rbc*L and 18S datasets separately because a large portion of the 18S sequences did not originate from the same specimens as the *rbc*L sequences.

Maximum likelihood (ML) analysis was performed as implemented in RAxML-HPC BlackBox v.8.2.10 (Stamatakis, 2014) on the CIPRES Science Gateway v.3.3. (Miller et al., 2010). The universal substitution model GTRGAMMA + I was selected for both datasets and node support was estimated with 1000 bootstrap replicates. We also performed a Bayesian analysis using MrBayes v.3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on the CIPRES Science Gateway v.3.3. We used jModelTest2 2.1.1 (Guindon and Gascuel, 2003; Darriba et al., 2012) to select nucleotide substitution model for the Bayesian analysis. The model GTR + I + G was selected for both *rbc*L and 18S datasets. Bayesian analyses were performed using fours chains and 10 million generations. Every 500th generation was sampled and the first 25% of the samples was discarded as burnin-in. A 50% majority rule consensus tree was calculated from the post-burnin trees.

We performed an additional ML and Bayesian analysis for algal partners of *Sticta* identified as belonging to the genus *Symbiochloris* for species delimitation purposes as the *rbc*L and 18S phylogenies suggested that *Sticta* might associate with several different species of that genus. *Symbiochloris rbc*L and 18S sequences obtained from our *Sticta* samples were incorporated in the *Symbiochloris rbc*L and 18S dataset from Škaloud et al. (2016). We also added two *Symbiochloris* sequences obtained from GenBank. One of these was a *rbc*L sequence of the algal partner of *Sticta subcaperata* collected in New Zealand (GenBank accession: KC333593), and the other was an 18S sequence belonging to the photobiont of *S. latifrons*, also collected in New Zealand (GenBank accession: KC333498). The parameters of the RAxML and Bayesian analysis for the concatenated *Symbiochloris rbc*L and 18S dataset were the same as stated above. For the Bayesian analysis, the substitution model GTR + I + G was selected for both *rbc*L and 18S datasets.

2.3. Mycobiont phylogeny

The placement of green algal *Sticta* species was first assessed with a broad, ITS-based phylogeny of 150 species, including *Lobaria pulmonaria* as outgroup (Supplementary Table 2). Since ITS-based phylogenies in *Sticta* are congruent with multilocus phylogenies (e.g. Moncada et al. 2013a; Magain & Sérusiaux 2015; Widhelm et al. 2018), we relied on ITS for this purpose, in order to have a broad sampling of species. The phylogeny was established using ML as outlined above.

To construct a multi-locus phylogeny for the *Sticta* mycobionts of the green algal target group, ITS, nuLSU and MCM7 alignments were concatenated into a single matrix using Geneious v.11 (Biomatters Limited, Auckland, New Zealand). One specimen of *Sticta cinereoglauca* from New Zealand and three *S. laciniosa* specimens collected in Cuba were excluded from the analysis due to missing data. Maximum likelihood and Bayesian analyses were performed using the same configurations as in the analyses mentioned above. For the Bayesian analyses, the following substitution models were selected: ITS1: K80 + G; 5.8S: K80; ITS2: GTR + G; nuLSU: GTR + I + G, and MCM7: GTR + I.

2.4. Ancestral character state analysis of phylogenetic photobiont distribution

The broad ITS dataset was subjected to molecular clock analysis in BEAST v.1.7.5 (Drummond et al., 2012). Based on earlier studies (Kraichak et al. 2018; Widhelm et al. 2018), the root node was calibrated at 45 mya and the *Sticta* crown node at 26 mya, both under a normal distribution with a SD of 5 my. The analysis was run with 10 million generations, sampling every 1000th tree. We first ran a strict molecular clock under default settings to obtain an initial rate estimate under a uniform prior. The posterior rate was then used under a log-normal distribution as prior for a log-normal relaxed clock, with all other parameters set to default. The results from this run were inspected using Tracer v.1.5 (Drummond et al., 2012) and the posteriors for all priors were used as priors in a subsequent run. This was repeated five times until no visible change of posteriors occurred. From the final run, the maximum-clade-credibility tree was generated.

We then reconstructed the phylogenetic distribution of photobionts along the internal nodes of the phylogeny using a binary state file assigning either green algae or cyanobacteria to each terminal, with documented photosymbiodemes coded as occurring with green algae. Analyses were performed in BayesTraits v.3.0.1 (Pagel et al., 2004), as implemented in RASP v.4.1 (Yu et al., 2015), and were run for 1.01 million generations using 10,000 generations as burn-in.

2.5. Cophylogenetic analyses of symbionts

One sample from 27 species of *Sticta* and five species of algae associated with *Sticta* were included in the cophylogenetic analysis. We were unable to perform the cophylogenetic analysis on species trees due to the large amount of missing sequence data from several mycobionts and photobionts. The inference of a species tree requires the inclusion of several samples per species in the analysis and our data did not meet this requirement. Therefore, a ML tree was inferred from the concatenated *rbc*L and 18S datasets for the algal partner and from ITS, nuLSU and MCM7 datasets for the fungal partner using RAxML-HPC BlackBox v.8.2.10 (Stamatakis, 2014) on the CIPRES Science Gateway v.3.3. The model GTRGAMMA was selected for both partners and bootstrapping was performed with 1000 replicates.

Two global-fit methods were used to assess the congruence of fungal and algal phylogenies: ParaFit (Legendre et al., 2002) and Procrustean Approach to Cophylogeny (PACo; Balbuena et al., 2013). Both methods test for global congruence of host and symbiont phylogenies and also

consider the contribution of individual host-symbiont links to the global congruence. ParaFit tests for random association of host and symbiont phylogenies, whereas PACo evaluates the dependence of symbiont phylogeny upon the host phylogeny. Both methods require that host and symbiont phylogenies are first transformed into patristic distances. We performed a global ParaFit test in R using the package APE v.4.1 (Paradis et al., 2004), with 999 permutations and implementing individual host-symbiont links, to test the null hypothesis of random association of *Sticta* and their green algal symbionts. PACo analysis was performed in R with 100,000 permutations using packages APE v.4.1 (Paradis et al., 2004) and VEGAN v.2.4.6 (Oksanen et al., 2018).

We also utilized the event-based tree reconciliation method implemented in JANE v.4 (Conow et al., 2010), to see which of the five possible cophylogenetic events (cospeciation, duplication, duplication and host switch, loss, failure to diverge) were the most likely to have caused the observed pattern of association in *Sticta* and their green algal partners. Each of these events was assigned an associated cost and seven different cost scenarios were tested in search for a cost scenario with the lowest total cost (Supplementary Table 5). JANE analysis was performed with a population size of 23 and the number of generations was 45, as recommended in Conow et al. (2010). The association patterns of *Sticta* and their algal partners were plotted in R using the function "cophyloplot" included in the package APE v.4.1 (Paradis et al., 2004).

3. Results

3.1. Phylogenetic placement of algae associated with Sticta

We obtained 99 *rbc*L and 79 18S rDNA sequences from green algae associated with *Sticta* (Supplementary Table 1). The large *rbc*L alignment of trebouxiophycean algae consisted of 295 taxa and 1018 sites, whereas the 18S alignment contained 363 taxa and 1054 sites. The *rbc*L and 18S ML and Bayesian phylogenies showed no conflicts between the trees and therefore only the *rbc*L ML likelihood phylogeny is shown (Fig. 1). 18S ML phylogeny is shown in Supplementary Fig. 1.

The green algal partners of *Sticta* grouped in five strongly supported clades. All 17 specimens of *Sticta* collected in South America associated with algae of the genus *Symbiochloris*. Three specimens of *Sticta laciniosa* collected in Cuba associated with algae that formed a strongly supported sister clade to *Coccomyxa*. A total of 34 specimens representing four species of *Sticta* collected in Australia and New Zealand grouped in a clade with algae of the genus *Elliptochloris*. Four specimens belonging to *Sticta latifrons* and *S. subcaperata* collected in New Zealand associated with algae in the genus *Chloroidium*. A total of 46 specimens representing 18 species of *Sticta* collected in Madagascar, Mauritius, Reunion and New Zealand associated with algae in the genus *Heveochlorella*.

The phylogenetic analysis of the *Symbiochloris rbc*L and 18S sequence data revealed that *Sticta* associates with potentially up to three different species of *Symbiochloris* (Fig. 2). The majority of *Sticta* with this photobiont, all from Central and South America, associate with a yet undescribed species of *Symbiochloris* provisionally named as *Symbiochloris* sp. 3 in the study of Škaloud et al. (2016). *Sticta cinereoglauca*, the only species in our study collected in New Zealand with *Symbiochloris* as photobiont, associated with *S. pauciautosporica*. Two photobiont sequences obtained from Genbank one belonging to *S. latifrons* (KC333498) and the other to *S. subcaperata* (KC333593), and both originating from collections made in New Zealand, were also identified as *S. pauciautosporica*. The algal partner of *Sticta ainoae* collected in Chile grouped with the photobiont of *Chaenotheca brunneola* which represents yet another undescribed species of *Symbiochloris* (*Symbiochloris* sp.6 in Škaloud et al., 2016).

3.2. Mycobiont phylogeny

We generated 35 new ITS, 63 nuLSU, and 68 MCM7 sequences from mycobionts of green algal *Sticta* species. The concatenated ITS, nuLSU and MCM7 alignment consisted of 98 taxa and 1724 sites. In the ML phylogeny, most of the specimens grouped in three large clades correlating with geography (Fig. 3). Clade I contained samples from Australia and New Zealand, clade II comprised specimens from Madagascar, Mauritius and Reunion, and clade III included specimens from South America; the only exception was *S. ainoae* from Chile, which formed a sister group to clade II from Madagascar, Mauritius and Reunion clade (Fig. 3). In addition, *Sticta squamata* from New Zealand formed an unsupported sister group to all other *Sticta*. The backbone of the phylogeny was poorly supported, likely because of the exclusive focus on green algal species, but is largely congruent with studies based on more inclusive taxon sampling (e.g. Moncada et al. 2014; Widhelm et al., 2018). The Bayesian phylogeny (not shown) was otherwise identical to the ML phylogeny with the exception of South American *Sticta* that formed a sister group to all other *Sticta*.

3.3. Phylogenetic placement of green algal Sticta species

Green algal species of the genus *Sticta* are not uniformly dispersed over the phylogeny but are more concentrated among two early diverging clades and in several smaller, late diverging clades, with most of the middle portion of the phylogeny featuring almost exclusively cyanobacterial species (Fig. 4). The early diverging green algal species in the first clade are exclusively from the Southern Hemisphere and in the second clade almost exclusively from Madagascar and the Mascarenes, whereas the late diverging species are neotropical and in one case from eastern Europe and Macaronesia. Ancestral character state reconstruction is ambiguous as to whether the two large, early diverging clades together constitute a single origin of green algal associations, but suggests with high likelihood that the late diverging green algal species were secondarily derived several times from cyanobacterial ancestors. This finding strongly correlates with photobiont affinities (see above). Whereas the first early diverging clade from the Southern Hemisphere associated mostly with the green algal genus *Elliptochloris* and in part also with *Chloroidium* and *Heveochlorella*, the second clade from Madagascar and the Mascarenes associated exclusively with *Heveochlorella*, thus showing some evolutionary connection in photobiont choice through the shared genus *Heveochlorella* in both clades. In contrast, the tested green algal species from the Neotropics associated mostly with algae of the genus *Symbiochloris* and in a few instances with a clade related to *Coccomyxa*. The interpretation of a secondary acquisition of green algal photobionts in neotropical species from cyanobacterial ancestors is therefore supported by the different algal phylogroups involved.

3.4. Cophylogeny and association patterns of hosts and symbionts

Parafit supported congruence between host and symbiont phylogenies, rejecting the null hypothesis of random association of the partners (ParaFit Global = 0.01086802, $P = 0.001$). Twenty-one out of the 31 symbiont links (67.7%) contributed significantly to the Parafit Global based on ParaFit1 values and 26 links (83.8%) based on ParaFit2 values ($P \leq 0.05$). Similarly, PACo provided evidence for congruence between the phylogeny of *Sticta* mycobionts and that of its green algal symbionts (PACo $m^2 = 0.07795$, $P = 0$ ($P \leq 0.0001$).

Out of the seven cost scenarios tested in JANE, scenario G had the lowest total cost. This scenario assigned highest cost to cospeciation, but did not penalize loss and failure to diverge. Cost scenario G suggested two duplications, two duplications with host-switches, 12 losses and 26 failure to diverge events (Supplementary Fig. 2).

The association patterns of *Sticta* and their algal partners showed substantial variation between and within mycobiont species (Fig. 5).

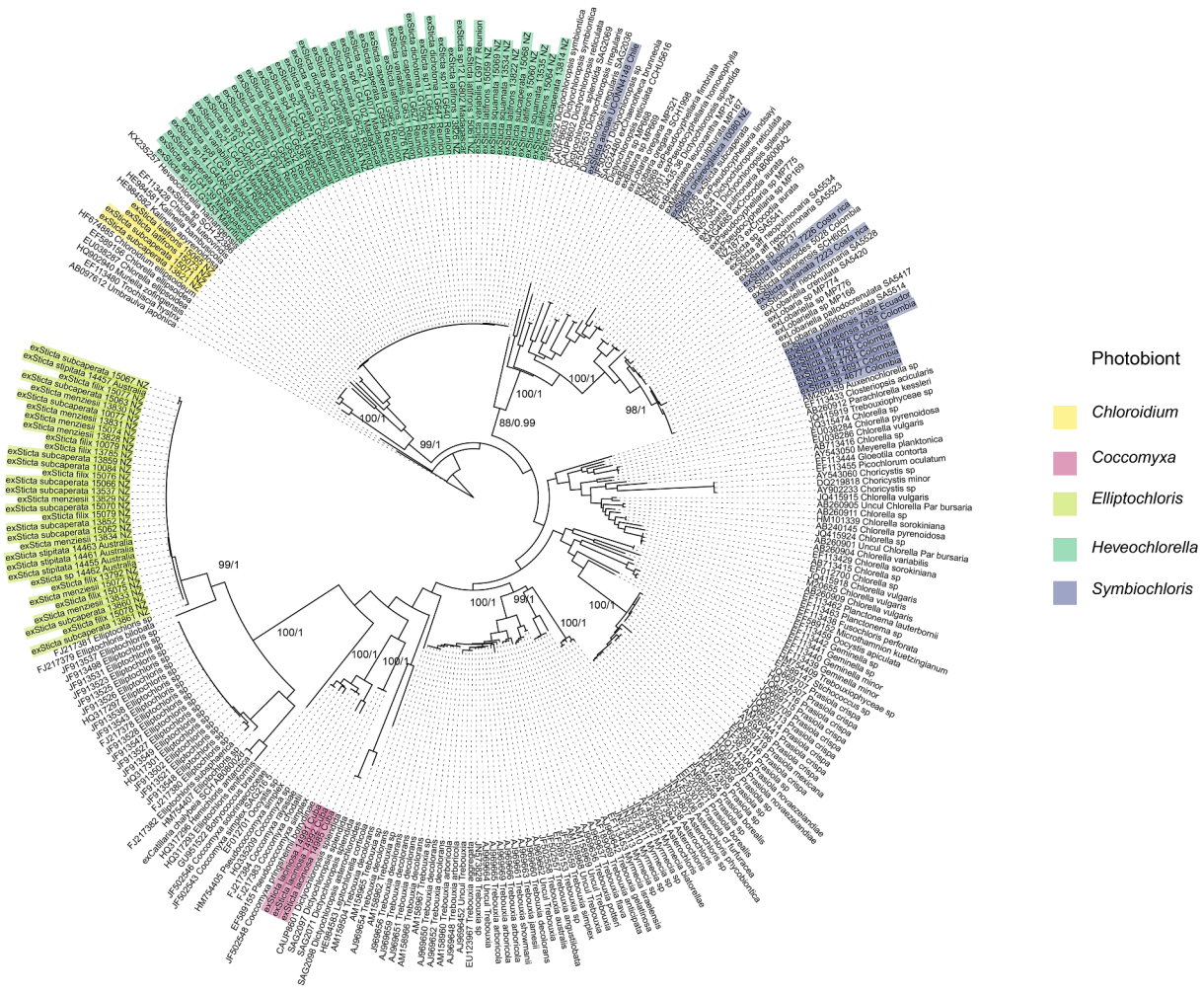

Fig. 1. *Rbc*L ML phylogeny of algae in Trebouxiophyceae. Highlighted clades represent different genera of algae associated with *Sticta*. Bootstrap support values ≥ 75 from ML analysis and Bayesian posterior probabilities ≥ 95 are shown at nodes.

Sticta latifrons and *S. squamata* both associated with algae from two genera (*S. latifrons*: *Chloroidium* and *Heveochlorella*; *S. squamata*: *Elliptochloris* and *Heveochlorella*), whereas *S. subcaperata* was found to associate with algae from three genera (*Chloroidium*, *Elliptochloris* and *Heveochlorella*). All other species of *Sticta* were found to associate with an algal partner from a single genus only.

Of all the algal partners of *Sticta* examined here, *Heveochlorella* had the highest diversity of mycobiont associations. It associated with 18 species of *Sticta* and was the most commonly encountered photobiont in our study. *Elliptochloris* and *Symbiochloris* sp. 3 both associated with five species of *Sticta,* whereas *Chloroidium* and *Symbiochloris* sp. were found in association with two and one mycobionts, respectively.

4. Discussion

4.1. Identity and diversity of algae associated with Sticta

We examined the identity and diversity of green algal partners associated with lichenized fungi in the genus *Sticta*, and assessed association patterns of both partners. We found species from the fungal genus *Sticta* associated with a diverse pool of green algal partners from five genera in the class Trebouxiophyceae: *Chloroidium*, *Coccomyxa*, *Elliptochloris*, *Heveochlorella* and *Symbiochloris*. *Heveochlorella* was found to exclusively associate with *Sticta* in Madagascar, Mauritius, Reunion, and was also found in some New Zealand taxa, making it the most commonly encountered photobiont in this study. Simon et al. (2018) found that *Sticta* in Madagascar and the Mascarenes originated from a single colonization event, followed by adaptive radiation and diversification that resulted in a high number of endemic species, some of which represent microendemics known only from particular mountains. This suggests that the green algal associations in this clade evolved secondarily from cyanobacterial ancestors, explaining the different green algal photobiont present in this clade, although *Heveochlorella* was also found in some Southern Hemisphere species from New Zealand (see below). Three mycobiont species of the Madagascar-Mascarenes clade have been reported outside that area: *Sticta caperata*, *S. dichotoma* and *S. variabilis*. *Sticta caperata* is known to occur in Australia, the Comoro Islands and several archipelagos in the western Pacific (Galloway, 2001). *Sticta variabilis* has also been recorded from Australia and the Comoro Islands, and it is also known from East Africa and Papua New Guinea (Galloway, 2001). *Sticta dichotoma* on the other hand has been reported from Panama (Büdel et al., 2000) and Tanzania (Krog, 2000), but these reports have not been confirmed by DNA studies (Simon et al., 2018) and may be based on erroneous identifications (e.g. Moncada et al. 2018). Whether these species associate with *Heveochlorella* remains to be studied.

Three species of *Sticta* collected in New Zealand were also found to associate with *Heveochlorella*: *S. latifrons*, *S. subcaperata*, and *S. squamata*. However, all three species also associated with other algae, namely *Chloroidium* and *Elliptochloris*, although our results indicate that *S. latifrons* seems to prefer *Heveochlorella* over other algal partners, as the majority of specimens from this species had *Heveochlorella* as a

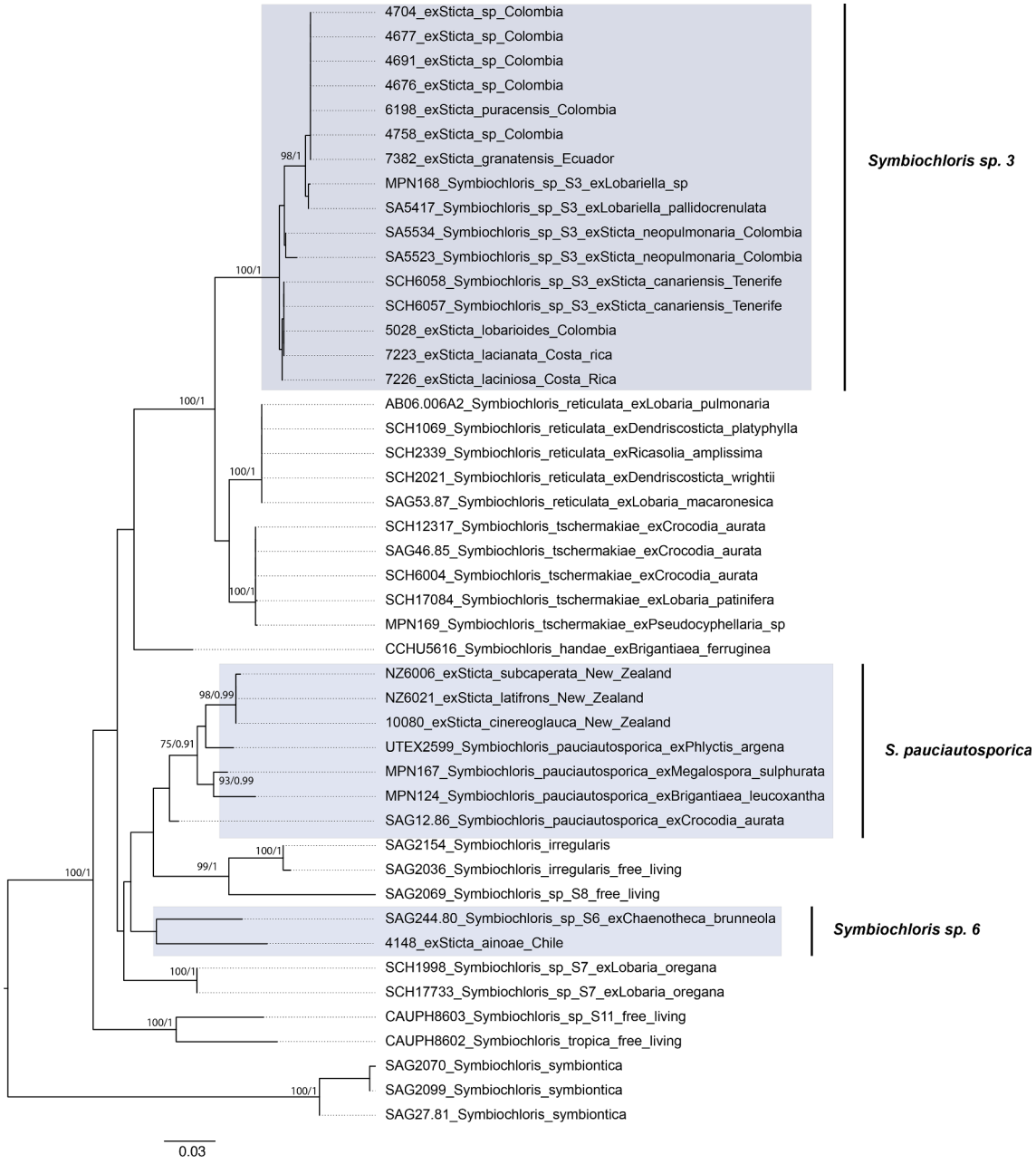

Fig. 2. ML phylogeny of concatenated *Rbc*L and 18S data sets of *Symbiochloris* algae. Highlighted clades indicate the placement of *Symbiochloris* associated with *Sticta*. Bootstrap support values ≥ 75 from ML analysis and Bayesian posterior probabilities ≥ 95 are shown at nodes. The nomenclature of *Symbiochloris* follows Škaloud et al. (2016).

photobiont. *Sticta latifrons* was recently analyzed as a potential indicator of old-growth forest in New Zealand (Ranft et al., 2018), and photobiont choice may depend on microhabitat in this species. Also, *S. latifrons* frequently alternates with a dendriscocauloid cyanomorphs, requiring reassociation with green algae during its life cycle, which may favor a lower level of green algal photobiont selectivity.

Heveochlorella was first reported from two unidentified specimens of *Sticta* from Taiwan by Dal Grande et al. (2014). In our study, the photobiont of those specimens did not group among *Heveochlorella* obtained from our *Sticta* specimens, suggesting it might belong to a different lineage of *Heveochlorella*. Dal Grande et al. (2014) also found a heveochlorellan photobiont in two *Pseudocyphellaria* specimens, also from Taiwan. We sequenced the *rbc*L region from two *Pseudocyphellaria* specimens collected in New Zealand and both were found to have *Heveochlorella* as photobiont. Algae in the genus *Heveochlorella* were recently reported to be rather common photobionts also in foliicolous (leaf-dwelling) lichens in families Gomphillaceae and Pilocarpaceae in tropical and subtropical regions (Sanders et al., 2016). Our results add to the increasing evidence of *Heveochlorella* being an overlooked genus of photobionts in lichens and their diversity is likely to increase as more lichens are studied especially those formed by Peltigeraceae subfamily Lobarioideae.

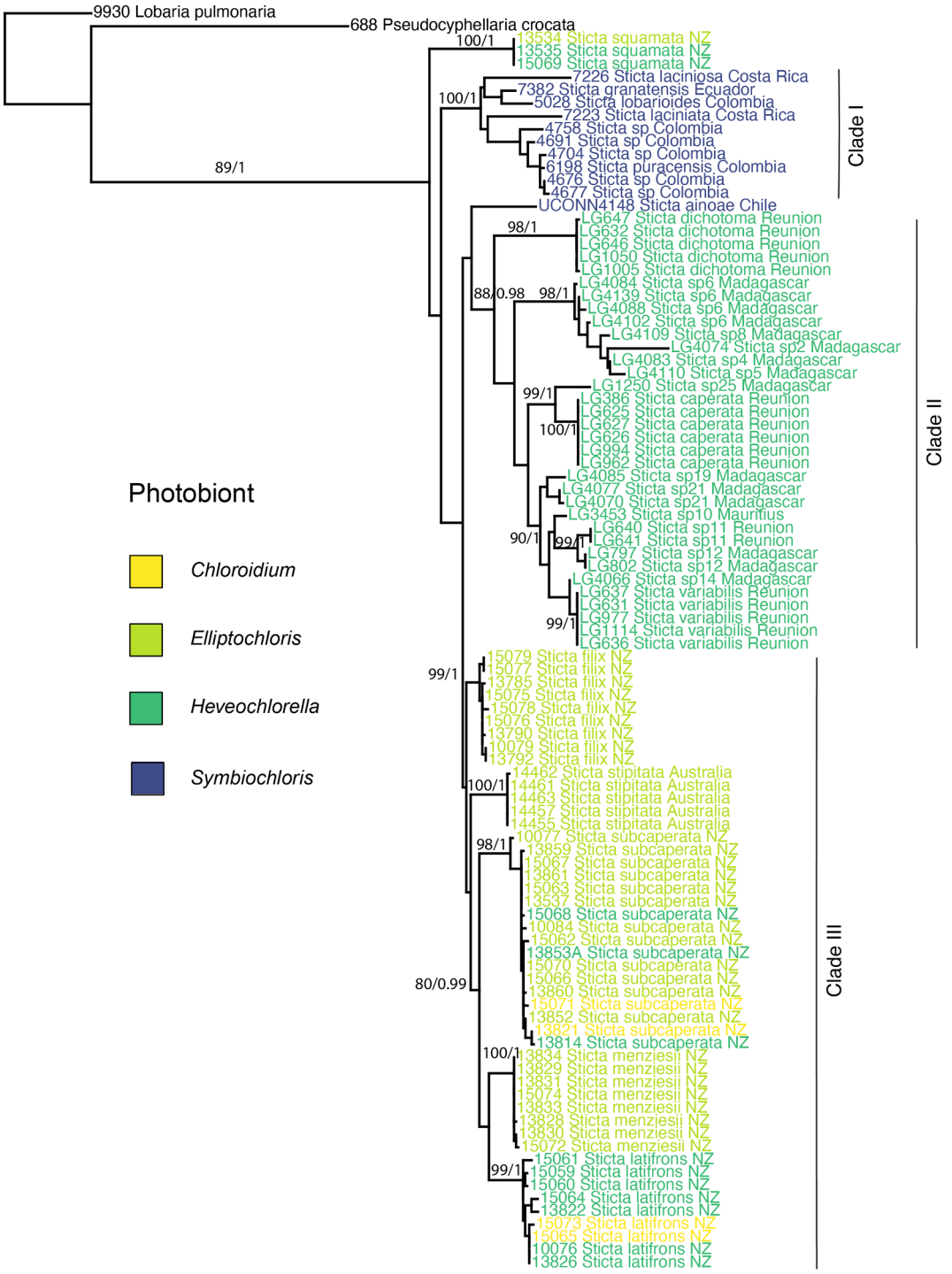

Fig. 3. ML phylogeny of concatenated ITS, nuLSU, and MCM7 datasets of *Sticta* associated with green algae. Taxa are highlighted according to the identity of the algal partner. Bootstrap support values ≥ 75 from ML analysis and Bayesian posterior probabilities ≥ 95 are shown at nodes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Elliptochloris was the second most common algal partner of *Sticta* in our study, and it was found to associate with *Sticta* collected in Australia and New Zealand. *Sticta filix*, *S. menziesii*, and *S. stipitata* associated exclusively with *Elliptochloris*, whereas for *S. subcaperata* and *S. squamata* it was one of several acceptable partners. Notably, both *S. filix* and *S. menziesii* frequently form dendriscocauloid cyanomorphs (Ranft et al., 2018) but still appear to associate only with a single genus of green algae, contradicting the aforementioned notion that such photosymbiodemes may drive lower photobiont selectivity. The *Elliptochloris* sequences generated here grouped with a sequence from *E.*

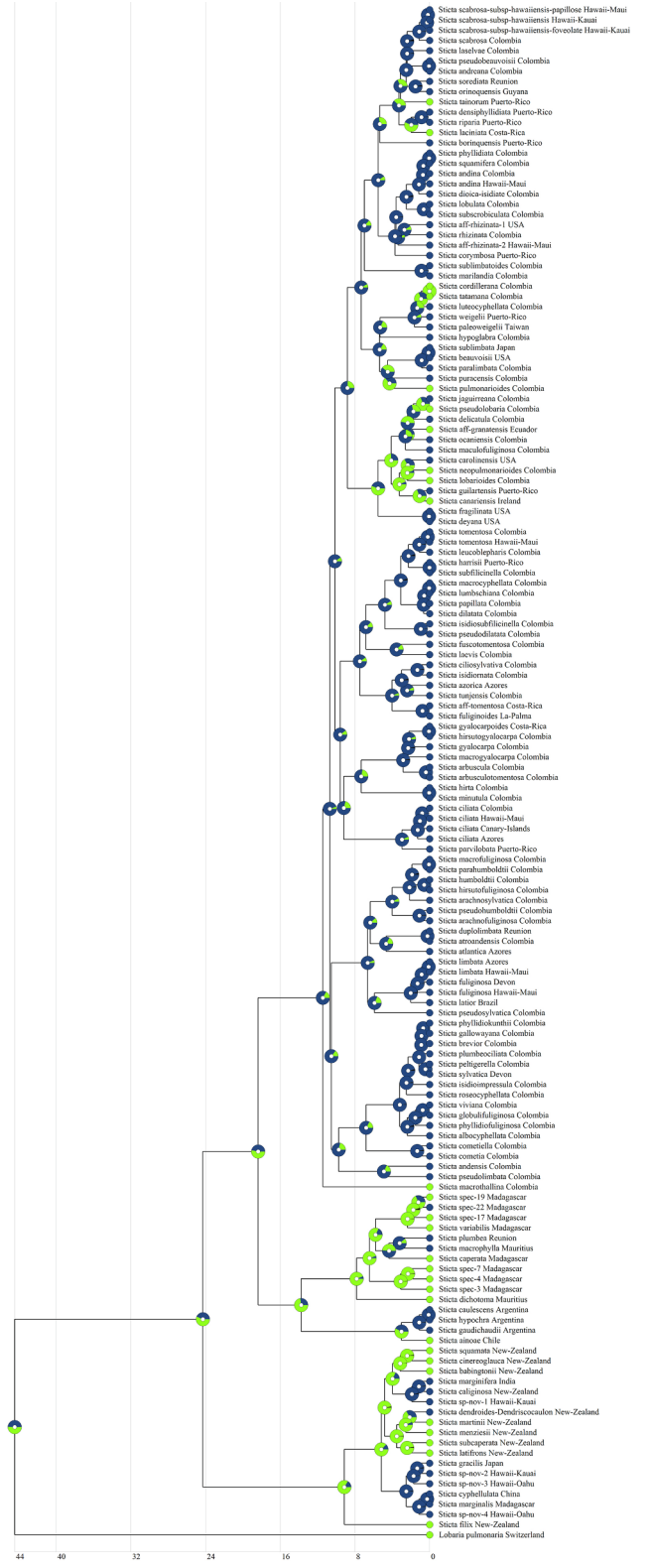

Fig. 4. ITS phylogeny indicating phylogenetic placement of *Sticta* associated with green algae. Nodes are shaded in proportion to the probability of the type of association occurring in each state (green = green alga; blue = cyanobacterium). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

perforata (Fig. 1), a species known as a photobiont in *Micaria prasina* (Yahr et al., 2015). Other species of *Elliptochloris* associate with lichenized fungi in genera such as *Catillaria*, *Micarea* and *Verrucaria*, which all form crustose lichens (Thüs et al., 2011; Voytsekhovich et al., 2011; Dal Grande et al., 2014), and some have even been found as symbionts in sea anemones (Letsch et al., 2009; Gustavs et al. 2017). Species of *Elliptochloris* are also known to occur free-living (Darienko et al., 2016).

Green algal *Sticta* had previously been reported to associate with algae of the genus *Dictyochloropsis* (Dal Grande et al., 2014). In their taxonomic revision of *Dictyochloropsis*, Škaloud et al. (2016) split the genus into two genera, *Dictyochloropsis* and *Symbiochloris*, based on 18S sequence data, morphological characteristics such as chloroplast structure, and the type of asexual spores, and ecological characteristics (whether free-living or in symbiotic association). Škaloud et al. (2016) assigned all lichenized algae, including those associated with species belonging in the former lichen family Lobariaceae, to the genus *Symbiochloris*, and described several new species within the latter. In our study, all *Sticta* species collected in South America and one collected in New Zealand (*S. cinereoglauca*) associated with *Symbiochloris* algae. *Sticta ainoae* from Chile and *S. cinereoglauca* from New Zealand both associated with a different *Symbiochloris* (*Symbiochloris* sp.6 and *S. pauciautosporica*, respectively), while the truly neotropical, late diverging species of *Sticta* collected in Costa Rica, Colombia and Ecuador all associated with *Symbiochloris* sp. 3. Our ancestral character state analysis, lends strong support to the conclusion that these green algal lichens are secondarily derived from cyanobacterial ancestors (see also: Miadlikowska and Lutzoni, 2004; Högnabba et al., 2009; Magain and Sérusiaux, 2014). Cyanobacterial photomorphs of photosymbiodemes in these Neotropical taxa are exclusively macrofoliose (Moncada et al. 2013b), as are those of the Madagascar-Mascarenes clade (Simon et al. 2018), whereas photosymbiodemes in the early diverging clades feature dendriscocauloid cyanomorphs (Ranft et al. 2018). This not only supports a secondary acquisition of green algal photobionts in the neotropical and the Madagascar-Mascarene species largely associated with *Symbiochloris* or *Heveochlorella*, but also a secondary acquisition of photosymbiodemism, with a much lower degree of morphological divergence between the photomorphs. We therefore hypothesize that in the earliest diverging, Southern Hemisphere clade, green algal photomorphs are primary and dendriscocauloid cyanobacterial photomorphs are secondarily derived from internal cephalodia, whereas at least in the late diverging, neotropical clades, the cyanomorphs represent the primary stage and the green algal photomorphs are derived from these, hence with a morphology more similar between the photomorphs.

Symbiochloris was previously found to associate with *Sticta latifrons* and *S. subcaperata* in New Zealand (Dal Grande et al., 2014). We included algal sequences from these two specimens in our *Symbiochloris* dataset and found that they belong to the same photobiont that associates with *S. cinereoglauca*: *Symbiochloris pauciautosporica*. However, our study implies that *Symbiochloris* algae are not likely to be common photobionts of *Sticta* in New Zealand, as none of our nine specimens from *S. latifrons* or our 17 specimens from *S. subcaperata* included in this study associated with *Symbiochloris*. We examined photobionts from six species of *Sticta* in New Zealand and in our data, only one specimen of *Sticta cinereoglauca* associated with *Symbiochloris*. *Symbiochloris* has also been found in *Sticta canariensis* in the Canary Islands (Dal Grande et al., 2014). Our data did not contain any specimens from Africa, Asia, Europe or North America and therefore we cannot make any conclusions about how common *Symbiochloris* photobionts are in *Sticta* in these regions.

Algae belonging in the genera *Chloroidium* and *Coccomyxa* were found as photobionts in three species of *Sticta*. *S. latifrons* and *S. subcaperata* both associated with *Chloroidium*, whereas three specimens of *S. laciniosa* from Cuba associated with *Coccomyxa*. *Chloroidium* is a

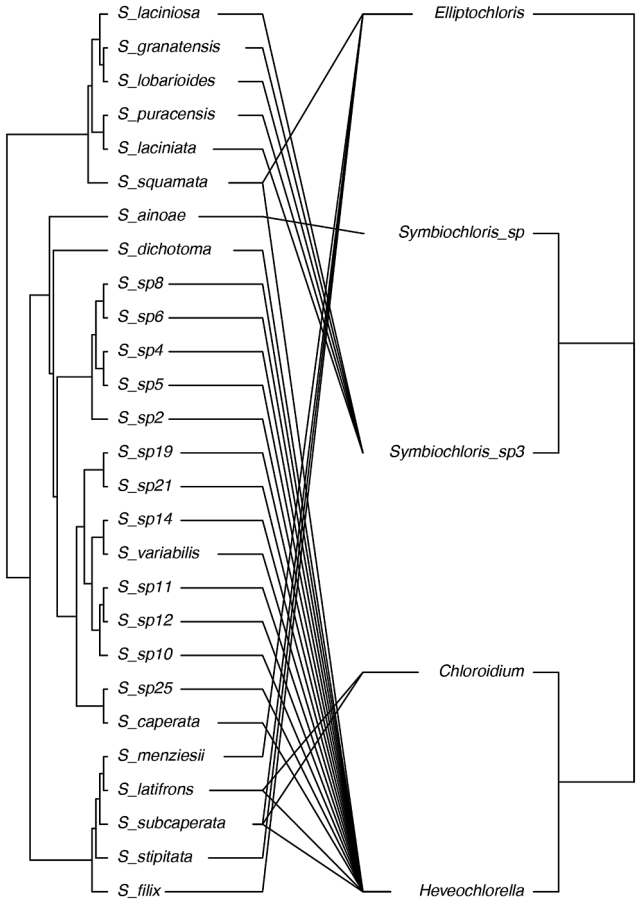

Fig. 5. ML Cophylogeny of *Sticta* and their green algal partners.

common alga in terrestrial and aquatic ecosystems and it can also occur as a symbiont in protozoa (Darienko et al., 2010). It has been previously reported from the lichen genera *Bacidia*, *Gyalidea*, *Gomphillus*, *Lecidea*, *Psilolechia*, *Stereocaulon*, *Trapelia*, and *Verrucaria*, but it is not considered a common photobiont in lichens (Tschermak-Woess, 1988; Thüs et al., 2011; Sanders et al., 2016; Vančurová et al., 2017). Like *Chloroidium*, *Coccomyxa* is also widely distributed and can thrive in both terrestrial and aquatic habitats (Gustavs et al., 2017). It is known to associate with many lichen-forming asco- and basidiomycetes, such as *Baeomyces*, *Icmadophila*, *Multiclavula*, and *Lichenomphalia* (Tschermak-Woess, 1988) and is also known to form symbiotic associations with lichenized fungi in the family Peltigeraceae, such as *Nephroma*, *Peltigera*, and *Solorina* (Tschermak-Woess, 1988), as well as associate with optionally lichenizing fungi in the family Stictidaceae such as *Schizoxylon albescens* (Muggia et al. 2011). Therefore, it is not surprising that this photobiont was also found from three specimens of *Sticta laciniosa* collected in Cuba.

4.2. Specificity and selectivity of Sticta towards its algal partners

The specificity and selectivity of *Sticta* towards its algal partners varied among species. In general, *Sticta* species exhibit high levels of specificity for algal partners, as the great majority of *Sticta* included in this study associated with only one photobiont genus. This was particularly the case with *Sticta* collected in Australia, Cuba, Madagascar, Mauritius, Reunion and South America. In New Zealand, *Sticta filix* and *S. menziesii* both associated with only one algal partner. However, three species of *Sticta*, all sampled in New Zealand, showed lower levels of specificity, as they associated with at least two distinct genera: *S. latifrons* associated with *Heveochlorella* and *Chloroidium* and *S. squamata* associated with *Heveochlorella* and *Elliptochloris*. *Sticta subcaperata* associated with three different algae in our study: *Chloroidium*, *Elliptochloris* and *Heveochlorella*. The cause for this level of variation is unclear but could be related to ecological conditions or photobiont switches between photomorphs. *Sticta latifrons* and *S. subcaperata* have previously been found to associate with an alga in the genus *Symbiochloris* in New Zealand, increasing the number of potential partners for these species to three and four, respectively (Dal Grande et al., 2014). Even though *Sticta latifrons* and *S. subcaperata* are able to establish a lichen thallus with one of multiple partners, they seem to be largely selective towards their symbiotic partners, as they both prefer one genus over the others; *Heveochlorella* was the most common partner of *S. latifrons*, whereas *S. subcaperata* was most commonly associated with *Elliptochloris*.

Due to the high prevalence of vertical transmission of the partners and the metabolically integrated nature of the lichen symbiosis, lichenized fungi and their symbiotic partners are often expected to have undergone coevolution or even cospeciation and, as a testament to this, are expected to have congruent phylogenies (Hawksworth, 1982; Ahmadjian, 1987). The phylogenies of *Sticta* and its algal symbionts were congruent, but according to the event-based methods used here, this congruence was not the result of cospeciation, but was more likely caused by failure of the algal symbiont to diverge with its mycobiont host, as many of the algal symbionts were found to associate with multiple species of *Sticta*. It has been shown that phylogenetic congruence between hosts and symbionts or hosts and parasites is rarely caused by cospeciation (de Vienne et al., 2013). Instead, switches to closely related partners or hosts can also lead to observed patterns of phylogenetic congruence in many symbiotic and parasitic associations (de Vienne et al., 2007, 2013; Millanes et al., 2014; Singh et al., 2017), as here also shown for the genus *Sticta*.

In *Sticta*, as in many other lichens, both sexual ascospores and asexual propagules can easily be carried by wind even to remote locations. In their study on diversification in the genus *Sticta*, Widhelm et al. (2018) for instance discovered multiple colonization events of *Sticta* in Hawaii. Being able to associate with locally adapted partners has been suggested to be an important strategy for lichens to expand their distributional range and colonize new areas (Muggia et al., 2014; Werth & Sork, 2014). In our study, the preference of *Sticta* for different green algal partners is strongly correlated with geographic regions. In South America, *Sticta* associated exclusively with *Symbiochloris* whereas in the Madagascar and the Mascarenes *Heveochlorella* was the only accepted algal partner. In Australia and New Zealand *Sticta* preferentially associated with *Elliptochloris* even though other suitable algae were available. However, this does not appear to be a consequence of dispersal and de novo lichenization, but rather reflects the apparent evolutionary history of the genus, which originated in the Southern Hemisphere, with a dominance of primarily green algal species, and then expanded northwards through various routes in the Paleo- and Neotropics. At least for the neotropical route, this expansion appears to be associated with the subsequent loss of green algal symbionts and secondary gain of green algal associations in the late diverging clades, then with a different green alga (Fig. 4). The geographic patterns are therefore a mere reflection of the apparent expansion routes of this genus in connection with paleoecological variations. On the other hand,

in the case of the insular territory of Madagascar and the Mascarenes, it is possible that the observed geographic pattern is caused by long distance dispersal followed by association with a locally adapted photobiont and subsequent diversification of the mycobionts. Our cophylogenetic analyses observed a rather high number of events where the algal symbiont had failed to diverge with its host. This would at least in part help to explain why in this insular region a high number of *Sticta* associate with one species of green alga.

5. Conclusions

We used multi-locus sequence data to infer phylogenies for fungal and green algal partners in the lichen genus *Sticta* in order to examine the diversity and identity of the algal symbiont and to study association patterns between the symbiotic partners. Our study highlights the remarkable diversity of green algal partners associated with fungi in the genus *Sticta* as species in this genus are able to form symbiotic associations with algae from five different genera in the Trebouxiophyceae, *Heveochlorella* being the most common algal partner in *Sticta*. Algae from genera *Chloroidium*, *Coccomyxa* and *Elliptochloris* were reported from *Sticta* for the first time pointing out to the important role proper sampling has in discovering hidden diversity. *Sticta* exhibits high levels of selectivity for algal symbionts as many mycobiont species only associated with a single species of alga. Our results also add to the increasing evidence of evolutionary events other than cospeciation having a significant role in shaping association patterns of hosts and symbionts. According to the event-based methods used in this study, losses of algal symbiont, host-switches and failure of the algal symbiont to diverge with its host have most likely led to the observed association patterns of the symbionts and also congruent phylogenies. Fungal-algal association patterns in *Sticta* are also structured according to geography as mycobionts associated with different algae in different geographic locations. Our study supports the view that photosymbiodemes with green algae evolved multiple times in *Sticta*.

CRediT authorship contribution statement

Hanna Lindgren: Conceptualization, Data curation, Methodology, Writing - original draft. **Bibiana Moncada:** Data curation. **Robert Lücking:** Conceptualization, Data curation, Methodology, Writing - review & editing. **Nicolas Magain:** Data curation, Writing - review & editing. **Antoine Simon:** Data curation, Writing - review & editing. **Bernard Goffinet:** Data curation, Writing - review & editing. **Emmanuël Sérusiaux:** Data curation, Writing - review & editing. **Matthew P. Nelsen:** Conceptualization, Writing - review & editing. **Joel A. Mercado-Díaz:** Data curation, Writing - review & editing. **Todd J. Widhelm:** Data curation, Writing - review & editing. **H. Thorsten Lumbsch:** Conceptualization, Writing - review & editing.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106860.

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