New lineages of *Asterochloris* photobionts in Bolivian lichens, and expand our knowledge on habitat preferences and distribution of *Asterochloris* algae

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Abstract

We studied biodiversity of Asterochloris photobionts found in lichen symbioses in Bolivian Andean vegetation and, to better understand global spatial distribution and adaptation strategies of this algae, in relation to worldwide phylogeny of the genus. Based on nuclear ITS rDNA, chloroplast rbcL gene and actin type I gene we constructed a phylogenetic tree that recovered 12 new Asterochloris lineages; and 29 Bolivian photobiont samples were assigned to 11 previously recognized Asterochloris lineages. We showed that some Asterochloris photobiont species and lineages known to date may occur in a broader spectrum of climatic conditions and mycobiont species and photobionts may show different preferences in the altitude gradient. To reveal general patterns of specificity of the mycobionts towards the photobiont in Asterochloris dependent symbiosis on global range, we tested the influence of climate, altitude, geographical distance and effects of symbiotic partner (mycobiont) at the species level of three genera of lichen forming fungi, i.e. Stereocaulon, Cladonia and Lepraria. Also, we compared specificity of mycobionts towards Asterochloris photobionts in cosmopolitan, Neotropical, and Pantropical lichen forming fungi. Interestingly, cosmopolitan species showed the lowest specificity to their photobionts', but also the lowest haplotype diversity. While, Neotropical and Paleotropical mycobionts were more specific.

1. Introduction

Lichenized fungi (lichens) are a marvelous example of ubiquitous mutualistic symbiotic organisms. Their thalli contain eukaryotic green algae and/or prokaryotic cyanobacteria, which represent the photosynthetic partners and are called photobionts, but also numerous bacteria and fungi occur in the lichen symbiosis [1,2,3] Many lichens are widely distributed, some of them being cosmopolitan, but it seems that photobionts in lichen symbioses may show their own habitat preferences independent of the lichenized fungus itself [4,5,6]. Since the organisms' environmental preferences may be closely related to their distribution, the geographical patterns of the photobionts may be different from their fungal partners – mycobionts [5]. Some phylogenetic lineages or even species and OTUs (operational taxonomic units) of photobionts are globally distributed, but many photobionts have only been recorded in a specific region or habitat [5,7,8,9]. In many cases, due to the uneven sampling of the lichen symbionts tested, it is too early to define exact biogeographic patterns for lichenized algae; this especially applies to photobionts from tropics [5,7].

*Asterochlori*s is one of the most common photobionts in lichen symbioses, but it is mostly restricted to certain phylogenetic lichen groups, of which undoubtedly the best sampled and studied are photobionts associated with members of *Cladoni*a, *Lepraria* and *Stereocaulon* [4,5,9,10,11,12,13,14,15,16,17,18,19,20,21]. So far eighteen species have been identified within *Asterochloris* [15,16,22,23,24,25]. Nevertheless, the latest research by e.g. Řídká et al. [5], Škaloud et al. [16], Vančurová et al. [9] and Kim et al. [25] showed that there are many phylogenetic *Asterochloris* lineages that can constitute still unrecognized species, which, apart from differences in the molecular markers, also differ in climate and substrate preferences. Moreover, Škaloud et al. [16] indicated that genetic diversity, ecology, biogeography and specificity to mycobiont partners should be taken into

account in species delimitation. Although, it was revealed that some lichens associate with *Asterochloris* spp. additional photobionts may occur in thalli, e.g., *Chloroidium* in *Stereocaulon* spp. [9] or *Vulcanochloris* in *Stereocaulon vesuvianum* [9,19].

To date, many analyses have been carried out to determine the distribution patterns of lichenized algae and many authors pointed out climate as the most important factor shaping their distribution [4,5,26,27,28,29]. In addition, phylogenetic photobiont lineages specific to a particular geographic region have been reported [26,27], but the exact factors driving photobiont selection remain still largely unexplored. Also, few researches proved that mycobiont can be highly specific to its photobiont [7,30,31,32,33,34], however, mycobiont generalists (*sensu* Yahr et al. [35], i.e. with low photobiont specificity) are also common, especially in lichens with wide geographical and ecological ranges [36,37,38]. In general, photobiont selection is driven by phylogenetic specialization, mycobiont reproductive strategy, availability of motile and airborne photobiont cells, as well as key ecological factors for the symbionts [35,36,39,40,41,42,43,44].

Despite extensive research efforts, there are many phylogenetic well supported lineages (OTUs) which are still unnamed. Few studies have been performed using the material from the Neotropics [45], and from this region most *Asterochloris* in lichen symbiosis have unverified taxonomic affiliation [9,14]. In this study we focused on the *Asterochloris* biodiversity in lichen symbioses in Bolivian Andean vegetation to better understand their global spatial distribution.

Bolivia is a country located in the center of South America. It represents the geographical and geological synthesis of South America - its geology is a cross-section of all geological eras [46]. The eastern Andean foothills were identified as the most biologically diverse region. Humidity, rainfall and temperature vary greatly in this region in space and time. These factors are important for many biological processes that led to a high speciation degree and endemism. In the Bolivian Andes, five ecoregions have been distinguished: Yungas and Tucuman-Bolivian forests, hilly Chaco, dry inter-Andean forest and Prepuna. Yungas forests are defined as a center of diversity for plants dependent on the humid seasonal climate [46] and potentially for lichens. This makes Yungas forests the most important center of endemism in Bolivia [46,47]. Furthermore, the territory of Bolivia is a puzzle of biogeographically different regions - Amazon, Andean, Chaco and Cerrado with ecotones forming unique transition points. Due to their location Yungas forest is a special point that is not heavily influenced by the seasonal regime and the interregional scale of taxa exchange is higher than in any other region [47].

The main aim of this work was to examine the genetic diversity of *Asterochloris* photobionts in lichens from Bolivia and to solve their phylogenetic relationships. Additionally, we studied the role of various factors that may determine the distribution of *Asterochloris* algae: mycobiont host, altitude, climatic conditions, substrate, and location. For some lineages we also assessed the level of selectivity of bionts. Furthermore, we compared the diversity of photobionts from different geographical distribution ranges and their adaptation strategies. Additionally, we present the first record of *Vulcanochloris* from the Neotropics.

2. Results

2.1. Phylogenetic analyses

In the present study we generated 54 new ITS rDNA sequences, 29 sequences of chloroplast *rbc*L gene fragment, and 14 sequences of *actin* type I of *Asterochloris* algae. In addition, we obtained one ITS rDNA sequence belonging to the genus *Vulcanochloris* (Table S1). The Bayesian phylogenetic tree inferred the genus *Asterochloris* to be divided into two major clades with high support values (Fig. 2), similarly to the results of Vančurová et al. [20]. The first major clade (PP=99, ML77/75) consists of three poorly supported subclades, although in Vančurová et al. only two subclades were found [20]. The second clade (PP=99, ML=77/75) consists of two subclades. We recovered 56 lineages (Fig. 2), 44 of them being previously identified [4,5,9,13,14,15,18,19,25] and for those we applied the same nomenclature as in original papers. 28 *Asterochloris* lineages obtained from Bolivian lichen samples grouped within 11 already recognized clades on the tree, of which only two were ascribed to the species, i.e. *A. mediterranea* [23] and *A. friedlii* [16] (Fig. 2). Remaining 26 photobiont sequences formed 12 new lineages of *Asterochloris*, probably representing undescribed species.

Our data have shown that some *Asterochloris* photobiont species and lineages known to date may occur in a broader spectrum of climatic conditions (data summarized in Table S8 and in Fig. 2 in reference to altitude). Thus, *A. mediterranea* known so far only from Mediterranean and temperate Europe can also occur in Neotropics. Annual precipitation determined for this species is between 18–974 mm, and precipitation of driest quarter: 2–95 mm (Fig. 3). *Asterochloris friedlii* was known from temperate Europe and USA and tropical areas of China and Korea, and it was also found in Bolivian sample of a widespread *Lepraria finkii*. Similar situation was observed for *A.* sp. clades StA1 and StA5, which till date were found to associate with *Stereocaulon* spp. in temperate regions or subtropical continental climate. *A.* sp. clade 9 found in South America, India and Southern North America associated only with cosmopolitan, Neotropical or Pantropical *Cladonia* spp. This lineage shows the highest annual mean temperature range (14.1–24.9 °C) and is characterized by drought resistance and rehydration (annual precipitation: 591–4771 mm, precipitation of driest quarter: 8–156 mm, precipitation of wettest quarter: 361–3968 mm). The widespread lineage *A.* sp. clade 12 associates with *Cladonia* spp., *Stereocaulon* spp. and *Diploschistes muscorum* and is characterized by widest temperature annual range: -5.5–18.1°C and high drought resistance (precipitation of driest quarter: 10–165 mm), and represents the lowest rehydration resistance of all *Asterochloris* photobionts. *Asterochloris* sp. clade P2 was found to associate with different lichen species and genera (*Cladia*, *Cladonia*, *Pilophorus* and *Stereocaulon*) similarly to *Asterochloris* sp. clade L14 (*Cladonia* and *Stereocaulo*n). Those lineages together with *A.* sp. clade L54 were found only in the Neotropics. So far, *A.* sp. clade L54 and clade A6 form a symbiosis exclusively with *Lepraria* spp. (Fig. 2).

Furthermore, in all 12 localities where lichen samples were collected in Bolivia, we found different *Asterochloris* species and lineages. In locality Chuquisaca 2 (N=7; for locality codes see Table S1), we found six different *Asterochloris* photobionts, of which four were detected for the first time. *Lepraria*

harrisiana from this locality is associated with three different *Asterochloris* lineages, while *Cladonia* aff. *ahtii* collected at different altitudes contains only one photobiont type. In locality Cochabamba 1 (N=7) we identified 7 photobionts lineages: *A. mediterranea*, *A. friedlii*, *A.* sp. StA1, clade 12, clade P2, and clades Bol 3 and Bol 9 associated with different lichen species. Furthermore, we identified the same haplotype of *A.* sp. clade Bol 2 in *Lepraria impossibilis* in a range of altitudes from 1943 to 2149 m a.s.l. (Table S13). The most abundant lineages identified in Bolivia, i.e. *A.* sp. clade Bol 1, Bol 2, P2 and StA1, were found to occur in most of the sampled localities of Bolivia. *Asterochloris* sp. clade Bol 1 were recorded in localities Chuquisaca 2, La Paz 1, Santa Cruz 1, Tarija 1 and 2, within thalli of Neotropical (*Cladonia* aff. *ahtii*, *C. ceratphyla*) or Pantropical (*Lepraria sipmaniana*) lichens in altitudes from 980 to 2950 m a.s.l., whereas, *Asterochloris* sp. clade Bol 2 associates with *Lepraria impossibilis* from localities Tarija 1 and 2 and with *L. harrisiana* from locality Chuquisaca 2. *Asterochloris* sp. clade P2, till date detected in *Pilophorus* cf. *cereolus* from Costa Rica, was also found in four Bolivian localities in altitudes between 2450 and 3860 m a.s.l. in different genera and species of lichens (Fig. 2).

Moreover, we observed dissimilarities between diversity of photobionts composition of four habitat types, i.e. lower montane cloud forest, upper montane cloud forest section 1, upper montane cloud forest section 2 and open high Andean vegetation (Table S1), e.g., in lower montane forest *Asterochloris* sp. clades Bol 1, Bol 2, Bol 3, Bol 6 and A14 were present, whereas in first section of upper montane cloud forest *A.* sp. clades 9, A14, L54, P2, S1 and Bol 1, 2, 5, 7, 12 and 13, *A. mediterranea* and *A. friedlii* occurred, whereas in second section of upper montane cloud forest *Asterochloris* sp. clades P2, S1, 12, A14, StA1, Bol 9. *A. medirerranea*, *A. friedlii* and *Vulcanochloris* sp. were also detected. In the open high Andean vegetation, *Asterochloris* sp. clades StA5, A6, Bol 8, 9, 10 and 11 were present. This shows that diverse photobiont types may occur within the gradient of altitude.

2.2. Statistical analyses

To identify the impact of selected factors on the distribution of *Asterochloris* photobionts, we performed variation partitioning analyses (Tables S5, S5, S7) that showed that selected variables explained 36-40% of the variation. 22-24% of the variation was explained by the species of the mycobiont (Table S6), which shows low correlation between photobiont distribution and mycobiont hosts. Remaining 14-16% was explained by environmental factors. 7% of the variability was shared between mycobiont host and climate in case of analyses when habitat was taken into account, and 6% of the variability was shared between mycobiont host, climate and geographical distance (when altitude was taken into account).

In the case of *Stereocaulon* 39% of the variation was explained by selected variables. The largest part of the variation in diversity of photobionts associated with *Stereocaulon* spp. was explained by mycobiont hosts (12%, Table S6). As independent factors, altitude explained 8% of the variation, while geographical distance - 4%. In *Cladonia* spp. 68% of the variability was explained by chosen variables (Table S6). 36% was explained by mycobiont species, whereas climate, altitude and geographical distance explained 1, 3 and 2%, respectively. However ecological factors shared 13% of the variability. In the case of *Lepraria* spp., altitude appeared to be insignificant and was excluded from analyses (Table S5). Results of PCoA

analyses for *Lepraria* spp. show moderate correlation between photobiont distribution and mycobiont host (35% of the variation explained by mycobiont). Remaining factors did not reveal significant influence.

In addition, we selected twelve cosmopolitan species, seventeen Neotropical and ten Pantropical from our dataset and data available in GenBank (Table S4). In the case of cosmopolitan species, variation partitioning analyses revealed that between 0 to 13% of the variability was explained by mycobiont hosts (Table S7). Furthermore, the climate explained 15% of the variability. In further analyses, altitude and substrate, appeared to be insignificant (dbRDA analyses Table S5). 59% of the variability of *Asterochloris* distribution in Neotropical and Pantropical lichens were explained by mycobiont host. We performed PCA analyses to identify which composition of photobionts was represented by selected groups of lichens, depending on climatic factors. PCA ordination of all analyzed species resulted in 72.7% cumulative variance explained on the first 2 axes (first – 47.8%, second – 24.9% of the variance). Lichen groups and their photobionts respectively showed indifference relative to climatic parameters, being scattered across the hyperdimensional climatic space (Fig. S2). However, Cosmopolitan, Neotropical and Pantropical species as groups represent different range of climatic conditions. In case of cosmopolitan species, the total explained variation in the biplot was 68.4% by component 1 and 2 (Fig. S3). PCA ordination of Neotropical species resulted in 85% (Fig. S4) cumulative variance explained on the first 2 axes and Pantropical in 79.3 % (Fig. S5).

However, in cosmopolitan, Neotropical and Pantropical groups of lichens and between them, different genera show indifferences, dependent from diverse climatic conditions (potentially climatic preferences). Tropical species prefer higher temperature in warm and wet periods of the year, whereas the cosmopolitan species are acclimated to extremes in temperatures (BIO4, 7).

Due to observed dissimilarities between diversity of photobionts composition of four habitat types in Bolivia, we performed PCA to visualize arrangement of photobionts of those habitat types depending on climatic factors (Fig. S6). This shows that on each habitat other conditions prevail, however, the ranges of photobionts from different habitats may overlap.

2.3. Haplotypes

Due to differences in the impact of the mycobiont species on distribution of photobionts in selected genera of lichens and differences in tolerance for different climatic conditions we performed haplotype analyses to study relations between mycobiont hosts and photobionts in selected groups of lichens. In case of *Asterochloris* photobionts from 200 samples of *Stereocaulon* spp. (Table S9) we found low haplotype diversity (0.28) and detected 55 photobiont haplotypes (Tables S9, S15), of which 24 were identified in samples from tropical areas, 36 in samples from temperate zone and 5 were identified in both temperate and tropical regions. However, haplotype diversity for samples from tropical regions appeared to be higher (0.52) than in case of samples from temperate area (0.23) (Table S16). *Asterochloris irregularis* appeared to be the most common photobiont associating with *Stereocaulon* spp.

in temperate climate (this haplotype found in 62 samples). In samples from Bolivia we found 5 distinct haplotypes:*A. mediterranea, Asterochloris* sp. clades P2, S1 and new lineages Bol 6 and 8.

In the case of *Asterochloris* associating with *Cladonia* spp., we found 144 ITS rDNA haplotypes (haplotype diversity = 0.36) based on 404 analyzed samples (Tables S10, S15). 65 of them originated from tropical region, 85 from temperate climate area and only 6 *Asterochloris* haplotypes were noted in both regions. Moreover, we observed differences in photobionts haplotype diversity in tropical and temperate samples associating with *Cladonia* spp. (tropical – 0.75, temperate – 0.27). The most common photobiont for *Cladonia* spp. from tropical areas seems to be *Asterochloris* clade 9 (found in 33 samples); furthermore this potentially new species is represented by fifteen haplotypes in our dataset. Also, *A.* sp. clade 9 was found in two lichen thalli of *Cladonia* spp. from temperate region. Samples from Bolivia are represented by fifteen haplotypes belonging to *A. mediterranea*, *A.* clades 9, 12, A14, S1, StA1, StA5, P2 and new lineages - Bol 1 and Bol 5.

Photobionts from *Lepraria* spp. (Tables S11, S15) represented by 44 haplotypes of *Asterochloris* showed high haplotype diversity (0.54). In tropical climate 14 haplotypes were found, while in temperate region we found 31 haplotypes. Moreover, haplotype diversity was higher in regions with tropical (0.70) than temperate (0.50) climate (Table S16.). Single haplotype of *A. friedlii* represented by fourteen sequences was found in both regions. In the case of groups representing cosmopolitan distribution patterns of lichen forming fungi, we observed 49 *Asterochloris* haplotypes (Table S12) with high haplotype diversity (0.65) (Table S15); together they represent 27 *Asterochloris* species or lineages. Samples from tropical climate were represented by 15 *Asterochloris* species or lineages, while specimens from temperate climate by 17. Some species of lichen forming fungi appeared to adopt different *Asterochloris* haplotypes in different climatic regions, e.g. *Cladonia furcata* associated with photobionts belonging to *A.* sp. clade 12 in temperate climate, while other haplotypes were found in tropical areas, i.e. *A.* sp. clades I1 and I2 in India and *A.* sp. clade P2 and clade Bol 5 in Bolivia. Moreover, *Stereocaulon alpinum* may associate with at least 10 different *Asterochloris* species or lineages. *Asterochloris* sp. clade Bol 8 was not noted in temperate climate, while *A.* sp. clade StA5 was found in Georgia, Austria (the same haplotype) and in Canada (different haplotype).

Seventeen Neotropical lichen species were analyzed; within them we identified nineteen haplotypes of *Asterochloris* belonging to 15 species or lineages (Table S13) with high haplotype diversity (0.76) (Table S15). In case of Pantropical group, ten lichen species associated with fourteen *Asterochlori*s haplotypes belonging to 9 lineages (Table S14) and showed the highest haplotype diversity (0.85) (Table S15). Within Neotropical and Pantropical lichens, we found *Asterochloris* haplotypes, either restricted to single species or with potentially wide selectivity (i.e., occurring in different species in similar or diverse localities).

3. Discussion

Little is still known about the nature of association between mycobionts and photobionts. In addition, only few works explored the diversity of *Asterochloris* photobionts in the tropical regions [5,9,13,14,21,34,35,45]. In this study we recovered 12 new *Asterochloris* lineages and 29 Bolivian photobiont samples were assigned to 11 previously recognized *Asterochloris* lineages, from which only two have been so far formally described (Fig. 2).

In this study, we showed that some *Asterochloris* photobiont species and lineages known to date may occur in a broader spectrum of climatic conditions, e.g. *A. mediterranea* so far not found in the Neotropics. On the other hand, alpine and psychrophilic *A.* clade StA5 lineage [9] may occur in open high Andean vegetation and probably may show higher drought resistance (Precipitation of Driest Quarter for sample UGDA-L 18963 = 30 mm) than previously thought (80–341 mm) (Fig. 3). In the case of *A.* sp. clade StA1, which was considered to be locally highly abundant, but globally rare [9], it was found that this lineage may show wider distribution in the Neotropical region (noted in 4 localities in Bolivia; for details see Tab. S1). *Asterochloris* clades P2, L14 and L54 are considered as Neotropical lineages, as well as *Asterochloris* clades Bol 1 and Bol 2 recovered in this study, which do not show close relationships with the species or lineages previously described.

Preferences of mycobionts for certain types of photobionts have been repeatedly recognized for *Trebouxia* [8,32,48], *Asterochloris* [9,16], or Trentepohliaceae [7]. Probably photobiont's ecological specialization determines the mycobiont selection for symbiont [4,49,50]. Furthermore, horizontal transmission probably favors the increase in the taxonomic range of compatible photobiont partners [18]. Vančurová et al. [9] showed that several algal species or lineages (e.g. *Asterochloris* clade StA1) have specificity towards a single mycobiont species. On the other hand, they recovered lineages that were not specific towards a single mycobiont. Here, we present that some of already described *Asterochloris* lineages were found to associate with broader ranges of mycobionts. *Asterochloris* clade StA1, as well as *A.* clade StA5 were previously found only within *Stereocaulon* spp., but we found these lineages associating with different *Cladonia* species, what suggests lower level of selectivity and specificity (*sensu* Beck et al. [10]). Furthermore, e.g., *Asterochloris* clades 9 and A6 were found to form thalli with only one genus of mycobiont each, i.e. *Cladonia* spp. and *Lepraria* spp., respectively, in diverse climatic conditions. Also, *A.* clade Bol 2 associated mostly with *L. impossibilis*, but also with *L. harrisiana*, however further analyses are necessary to detect the exact selectivity level. Interestingly, a newly recovered clade Bol 1 was found in two samples of *L. sipmaniana*, two of *Cladonia* aff. *ahtii* and two *C. ceratophyla* from different localities in altitude range from 980 to 2750 m a.s.l.

Ecological factors may modulate the availability of photobiont species/strains at various sites, thus reducing the extent of possible associations in the isolated populations. The influence of altitude gradient on photobiont population structure was previously described [7,29,51,52]. In our study we found changes in photobionts population in the altitude gradient, e.g., *Asterochloris* StA5, A6, Bol 8, 10 and 11 were noted in Bolivia only in open high Andean vegetation and *A.* clades Bol 1 and Bol 2 were present at lower montane cloud forest and upper montane cloud forest section 1, and *A.* clade Bol 9 in upper montane

cloud forest section 2 and open high Andean vegetation. However, we do not have data to define any of lineages listed above as specific for particular habitat.

Symbiotic interactions vary along environmental gradients [20,53] and can be affected by biotic and abiotic factors [4,9,54]. To reveal general patterns of specificity of the mycobionts towards the photobiont in *Asterochloris* dependent symbiosis on global range, we tested the influence of climate, altitude, geographical distance and effects of symbiotic partner (mycobiont) at the species level on three genera of lichen forming fungi, i.e. *Stereocaulon*, *Cladonia* and *Lepraria*. *Stereocaulon* species are widespread and with broad ecological requirements [9], and associate with many *Asterochloris* species [16], but also *Chloroidium* [9,10] and *Vulcanochloris* [19]. Diversity of photobionts in *Stereocaulon* and the association between this diversity and environmental conditions was previously conducted on global scale by Vančurová et al. [19]. The distribution of photobionts in *Stereocaulon* species in that study appeared to follow a pattern that was highly influenced by the type of substrate. However, this statement primarily concerned the genera *Chloroidium* and *Vulcanochloris*. Our analyses confirmed the hypothesis that photobiont variability may be dependent on geographical distance [35]. In the case of *Stereocaulon* [9], 4% of variability was explained by a net effect of geographical distance. Furthermore, distribution of photobiont diversity of *Stereocaulon* may be affected by altitude (8%). It has been previously shown that the majority of the *Stereocaulon* mycobiont species appeared to be specific towards phycobionts [9,20]. In our study *Stereocaulon* showed low level of selectivity toward photobiont lineages. Furthermore, we reported low haplotype diversity for *Asterochloris* photobionts of *Stereocaulon* that can be correlated with oversampled photobionts from temperate region.

Yahr et al. [35] demonstrated that geographic position and habitat are the best predictors of algal genotype distribution in *Cladonia*. Steinová et al. [18] showed that the photobionts are significantly structured by climate and geography, but the mode of reproduction was revealed to have the greatest impact on *Cladonia* photobionts diversity. In our analyses we found that 36% of the variability was explained by mycobiont host at species level. Furthermore, we reported that photobionts from *Cladonia* spp. represented higher haplotype diversity than *Stereocaulon* spp., where predominating photobiont species was *Asterochloris glomerata* (one haplotype represented by 52 sequences, or 12 haplotypes represented by 86 sequences). Nonetheless, *Cladonia* and *Lepraria* are known to associate with a wide range of *Asterochloris* species [4,16]. *Lepraria* species were found to associate with particular *Asterochloris* taxa from lineages B and C (*sensu* Vančurová, et al. [9]) and like *Cladonia* show moderate selectivity toward their photobionts, with moderate influence of mycobiont species and very low influence of climate and spatial structure and species of both genera may show different ranges of adaptation strategies. Those results confirm hypothesis of the ability to associate with numerous symbiotic partners in case of ubiquitous lichen species adapted to various ecological conditions [8,15,26]. Dominating species of *Asterochloris* found within *Lepraria* spp. is *A. friedlii*; this particular photobiont species was found in 22 samples, representing 27% of all tested samples. However, more research is needed to reveal the most common *Asterochloris* spp. in *Lepraria* and to explain factors affecting the distribution of photobionts.

Lichens exhibit various distribution patterns at the micro and macro levels [56]. In the case of lichen-forming fungi, 16 main biogeographic patterns were distinguished (including cosmopolitan, bipolar, paleotropical, Neotropical, Pantropical, Mediterranean), but still not much is known about the biogeography of lichen photobionts. Preferential association with locally adapted symbionts has been reported repeatedly [8,57,58]. It was hypothesized that low specificity of the host towards its symbiotic partner helps the host to take advantage of the locally adapted symbiotic partners and colonize broader geographic areas. In terms of higher specificity of the host, it is expected to have narrower ecological niche and restricted geographical distribution. In the case of lichen symbiotic associations, the generalist pattern is more common [8,26,52], however specialist pattern has been reported for *Nostoc*-associated lichen fungi [59]. We compared specificity of mycobionts towards *Asterochloris* photobionts in three groups of lichen forming fungi: cosmopolitan, Neotropical, and Pantropical. Interestingly, cosmopolitan species showed the lowest specificity, but also the lowest photobiont haplotype diversity. However, more haplotypes were identified in temperate region. In addition, the haplotype diversity of tropical species is higher. This may indicate a significant under sampling of tropical regions. In addition, the distribution of the diversity of photobionts within cosmopolitan lichen species was influenced by a moderate impact of climatic conditions (15%), while in the case of Neotropical and Pantropical lichens there was a correlation between the mycobiont species and climatic conditions. This indicates the selection of locally adapted photobionts in cosmopolitan lichens, while tropical species show primarily habitat preferences. However, in the light of the results obtained here and in previous research [8,60], we assume that the selection of a symbiotic partner also takes place based on selection of the best suited photobiont. To obtain more accurate results, more data on the species (preferably a model taxon) commonly found in the Neotropical and Pantropical regions are needed, with emphasis on the impact of habitat conditions.

Concluding, we suggest that a suitable species representative of each fungal genus associating with *Asterochloris* should be selected for the future studies before sampling. Furthermore, to avoid the formation of geographical gaps, sampling should be extended to the whole world. Moreover, on the basis of the results obtained here, it can be assumed that, due to its geological and ecological diversity, Bolivia may reflect the biodiversity of the entire Andean region in the Neotropics, where the issue of photobionts' biodiversity still remains unresolved. Additionally, the tropical area may be an underexplored source of hidden biodiversity of photobionts.

4. Materials And Methods

4.1. Material

54 lichens samples representing 4 genera: *Cladonia*, *Diploschistes*, *Lepraria* and *Stereocaulon* containing *Asterochloris* photobionts from various habitats in Bolivia were used. Lichens were collected from different substrata (rocks, soil, tree bark, wood, and bryophytes). Majority of samples were obtained from Yungas cloud forest, but also from Tucuman-Bolivian forest and dry inter-Andean forest (Fig. 1).

The ranges of distribution were defined for lichen species used in this study – Cosmopolitan, Neotropical or Pantropical. All voucher specimens are deposited in LPB and UGDA herbaria; detailed data on localities are presented in Supplementary Data Table S1.

4.2 Molecular methods

Well-preserved specimens lacking any visible symptoms of fungal infection were used for DNA isolation (total lichen DNA). DNA was extracted from thallus fragments following the CTAB protocol [61]. For the molecular identification of the photobionts (*Asterochloris*), the nuclear internal transcribed spacer (ITS, ITS1-5.8S-ITS2), chloroplast *rbc*L gene and in few cases of unique lineages of *Asterochloris*, *actin* type I gene were amplified. For the ITS rDNA region we used two combinations of primers, i.e. nr-SSU-1780-5' [34] with ITS4 [61] or a-nu-ssu-1752-5' [13] with ITS4. A fragment of the algal rbcL gene was amplified, using the following primers: PRASF1 [63] with a-ch-rbcL-991-30-MPN or achrbcL-203-50-MPN with a-ch-rbcL-991-30-MPN [64] or *rbc*La with *rbc*Lb [65]. A fragment of the algal *actin* type I gene was amplified, using the following primers: ActinF2 Astero-5', ActinR2 Astero-3' [10] The PCR condition were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C (or 50 °C for PRASF1 with a-ch-rbcL-991-30- MPN) for 1 min and elongation at 72°C for 1 min, with a final extension step at 72°C for 7 min. All amplification reactions were prepared in a total volume of 20µl with My Taq HS (Bioline) as described by Peksa & Škaloud [4] or 25µl with StartWarm HS-PCR Mix (A&A Biotechnology, Poland) consisted of 12.5 µl of StartWarm HS-PCR Mix (A&A Biotechnology, Poland), 1 µl of each of the 20 µM primers, 1 µl of genomic DNA and sterile, distilled water. The yield of the PCRs was verified by running the products on a 1% agarose gel using ethydium bromide or SimplySafe (EURx™, Poland). PCR products were purified using AMPure XP beads (Beckman Coulter) or Clean-Up Concentrator (A&A Biotechnology, Poland). Sequencing was performed in Macrogen® in Amsterdam, Netherlands using amplification primers. Sequences were compared to the sequences available in GenBank using Megablast searchers [66] to verify their identity and detect potential contaminations. The newly obtained sequences of the ITS rDNA, fragment of *rblc*L gene and *actin* type I region were deposited in GenBank (Table S1).

4.3. Phylogenetic analyses

Asterochloris datasets were analyzed as a concatenated dataset of ITS rDNA, chloroplast *rbc*L gene and *actin* type I loci consisted of 151 sequences with a total of 2591 sites. The ITS rDNA dataset consisted of 151 sequences, 54 newly obtained and 97 previously published representatives sequences of described species and lineages retrieved from GenBank. Reference sequences have been selected following the latest review work by Vančurová et al. [9] with additional new *Asterochlori*s spp. described by Kim et al. [25]. Nomenclature of some mycobiont species associating with *Asterochloris,* have not been updated according to the newest taxonomical works to avoid confusion; these are: *Lepraria borealis* and *L. caesiolaba* (both now subsumed under *L. neglecta*), *L. lobificans* auct. (now *L. finkii*), *L. nigrocincta* (now *L. yunnaniana*) and *Parmelinopis minarum* (now *Hypotrachyna minarum*). The chloroplast *rbc*L gene dataset consisted of 56 sequences: 29 newly obtained sequences, and 27 previously published

sequences. The *actin* type I dataset consisted of 104 sequences: 14 newly obtained sequences, and 90 previously published sequences. In cases for which *rbc*L or *actin* sequences were lacking, they were treated as missing data. The alignment was automatically performed using MAFFT - Multiple Alignment using Fast Fourier Transform [67] as implemented in UGENE [68]. Affiliation to individual species or *Asterochloris* phylogenetic lineages used in further analyses was estimated based on literature data. These data are available in Table S2. Nucleotide sequences of all samples received in this study were submitted to GenBank (accession numbers are given in Table S1). Phylogenetic relationships were inferred with Bayesian Inference (BI) carried out in MrBayes v.3.2.2 [69] using Metropolis-coupled Markov chain Monte Carlo (MCMCMC). Two parallel MCMC runs were performed, using four independent heated chains and 10 million generations, sampling every 1000th tree. The initial 2500 trees of each run (25%) were discarded as burn-in, and posterior probabilities were estimated by constructing a majority-rule consensus tree of all sampled post-burn-in trees. Maximum likelihood (ML) analyses were performed using RAxML-HPC v.8 with 1000 bootstrap replicates (ML-BS) and the GTRGAMMAI model [70] and the edgelinked partition model in *IQ-TREE* [71,72] on CIPRES Science Gateway [73]. The best-fit substitution models and block (Table S3.) were selected using Akaike Information Criterion (AIC) implemented in PartitionFinder 2 [74]. Tree topology obtained by maximum-likelihood method did not contradict the Bayesian tree, therefore only the Bayesian tree is shown. The consensus trees were visualized using FigTree v1.4.2 [75]. Branches with bootstrap support ≥70% and posterior probabilities ≥0.95 were considered as strongly supported. Analogically, the ITS rDNA dataset of *Vulcanochloris*, including a single new sequence of *Vulcanochloris* sp. detected in *Stereocaulon pityryzans* (UGDA-L 18522), was analyzed. It consisted of 16 sequences, of which 15 previously published sequences were retrieved from GenBank.

4.4. Statistical analyses

The relationship between species richness of Bolivian photobionts and lichen species as well as the relative effects of climate, altitude, geographical distances, substrate, and habitat type have been considered and analyzed. In case of data obtained from GenBank, only records with precise coordinates have been used. The comparative effects of selected variables were analyzed by variation partitioning in redundancy analyses, using the *varpart* function in the *vegan* package [76]. The phylogenetic distances of photobionts were used as a response variable, coded as the first 10 PCoA axes. Climatic data were obtained from the Global Climate Data – WorldClim Version 2 [77] at a resolution of 2.5 arc minutes. The 19 environmental variables as well as altitude, substrate and habitat type were transformed into principal component variables (PCs). Principal coordinates of neighbor matrices vectors (PCNM) representing the geographical distances at various spatial scales [78] were obtained due to transformation of geographical distance values (latitude and longitude). PCNM vectors were calculated based on the pairwise geographical distances obtained by the *distGPS* function in the BoSSA package [79]. Distance-based redundancy analyses (dbRDA) [80] were used to select statistically significant predictors for explaining variation for each data sets used in variation partitioning analyses.

Variation partitioning analyses were carried out separately for all Bolivian data obtained in this study (N=54) and for *Asterochloris* from selected lichen genera, i.e. *Cladonia* (N=156), *Stereocaulon* (N=169)

and *Lepraria* (N=34) due to better sampling. We found that the substrate influence appeared to be statistically insignificant and did not correlate with our datasets in case of all Bolivian data (Table S5). Furthermore, due to multiple missing data for this particular variable, we decided to omit this factor in the analyses of the datasets of selected genera of mycobiont. Variation partitioning analyses were also performed for selected group of lichen forming fungi representing different distribution patterns, i.e. cosmopolitan (N=76), Neotropical (N=23) and Pantropical (N=23) (pattern of distribution were specified based on literature data: Ahti [81]; Parnmen et al. [82]; Guzow-Krzemińska, et al. [83]; Sipman [84]; Saag et al. [85] (Supplementary materials Table S4)). Due to limited data for species showing different distribution patterns, different PCoA analyses schemes were used. A series of analyses were performed using mycobiont host as an explanatory variable everlastingly and one other variable, which was replaced in subsequent series (geographical distance, altitude, substrate and climate; Table S7). We took into account only morphospecies, not taking the possibility of the occurrence of different phylogenetic lineages, which can potentially be geographically or ecologically more restrictive as in Cladoniaceae [86].

To detect and visualize the differential ordination (tendency or strategies) of samples in the hyperspace, we performed Principal Component Analyses (PCA) according to climatic factors (BIO1-BIO19) and grouped results depending on distribution patterns of lichen species and genera of mycobiont host. Besides, we performed these analyses for Bolivian samples with grouping results by habitat type. All analyses were performed in R v 3.6.0 [87], using RStudio v.1.2.1335 [88].

4.5. Haplotypes

To visualize the exact interaction between mycobionts and photobionts we inferred haplotypes from pairwise character difference matrix of ITS rDNA sequences using *haplotype* function in *haplotype* package [89]. These analyses were carried out for 6 data sets (Table S11-14) consisting of sequences newly obtained for this paper and additional downloaded from GenBank of *Asterochloris* from selected lichen genera, i.e. *Cladonia*, *Stereocaulon* and *Lepraria* and for selected group of lichen forming fungi representing different distribution patterns, i.e. cosmopolitan, Neotropical, Pantropical. Furthermore, we measured nucleotide diversity, the number of haplotypes, and haplotype diversity for further estimations of differences between groups.

Declarations

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Data Availability

DNA Sequences will be deposited in GenBank upon approval of the above manuscript, accession numbers will be added in the final version.

Author contributions

M.K[1]. and B. G-K. designed the study. M.K conducted fieldwork, collected specimens and delimit the species of mycobionts. M.K, I.Č, and A.J. performed laboratory work with contributions from P.Š. M.K[1] analyzed the data with contributions from P.Š and B.G-K. M.K[1]., M.K., B.G-K. and P.Š. wrote the manuscript.

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Figures

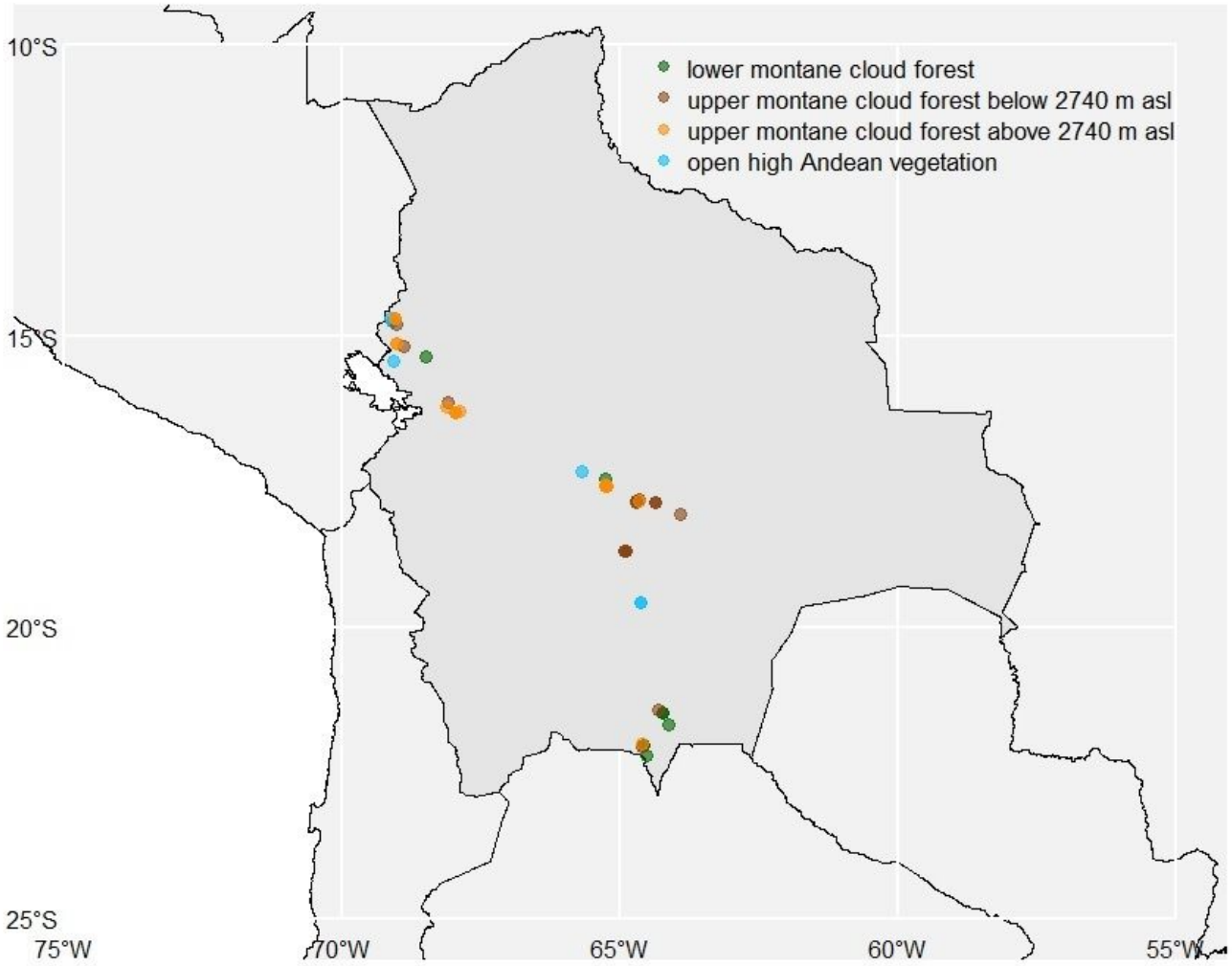
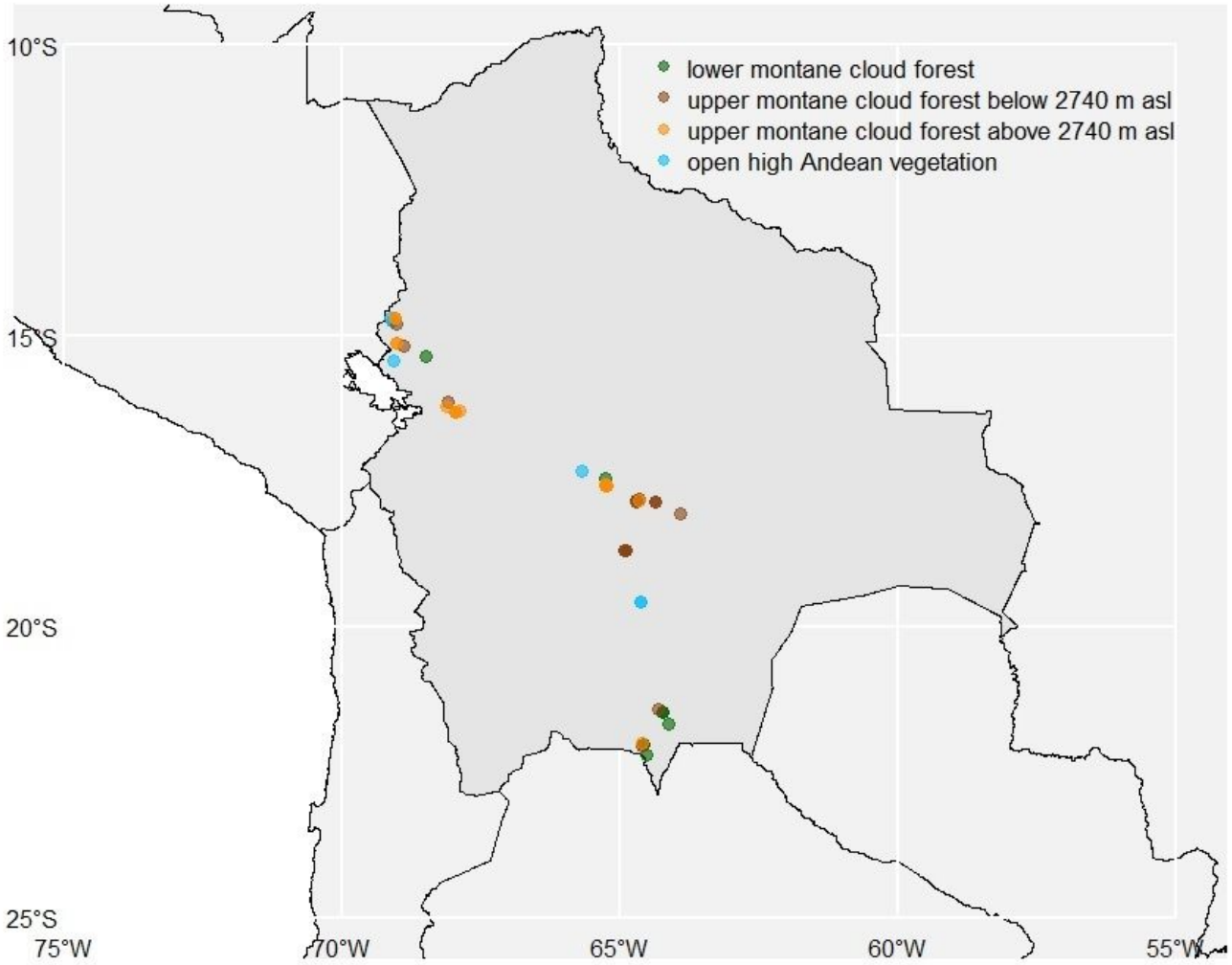


Figure 1

Map of sampling localities in Bolivia. Localities are marked in proper color depend from habitat.



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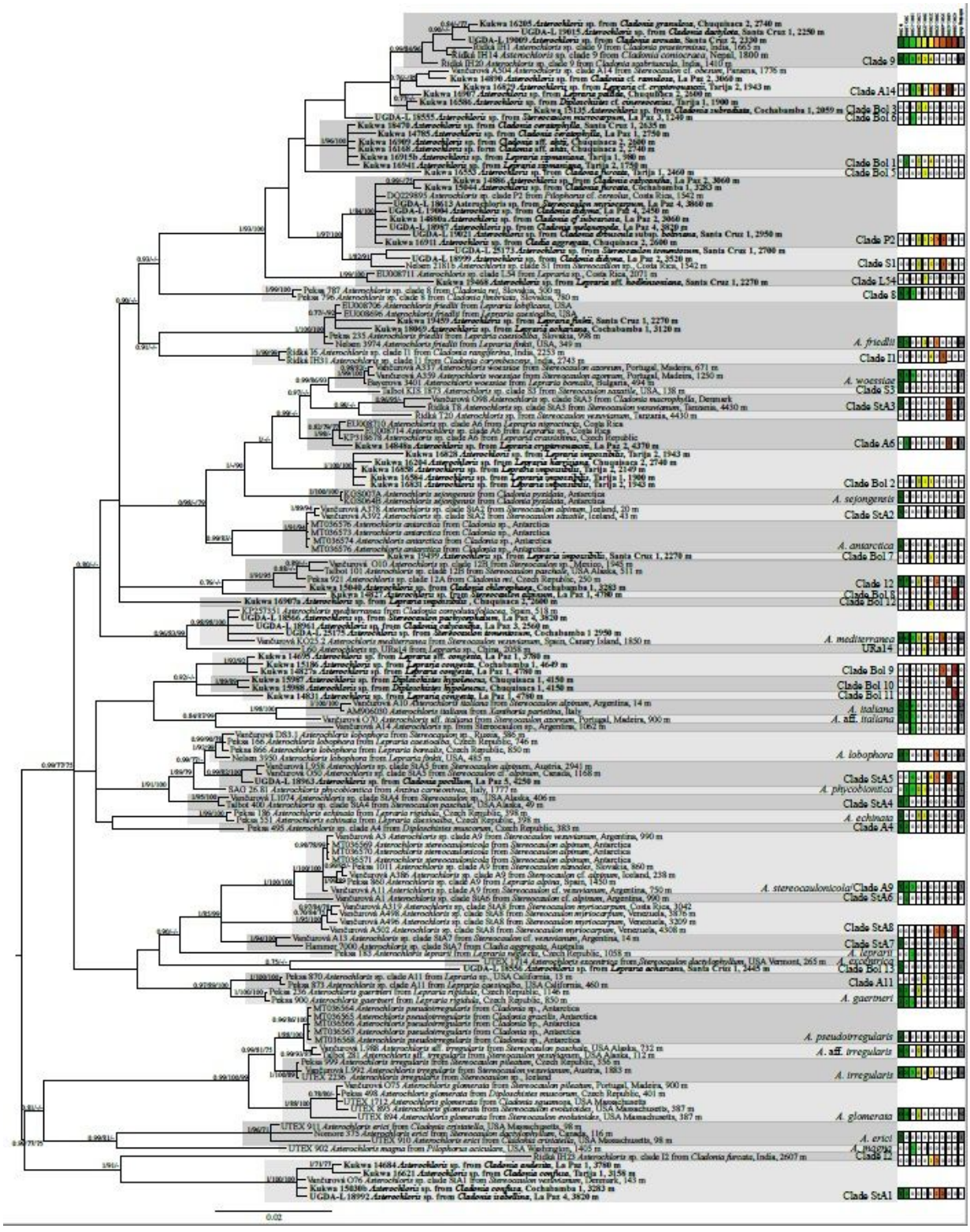
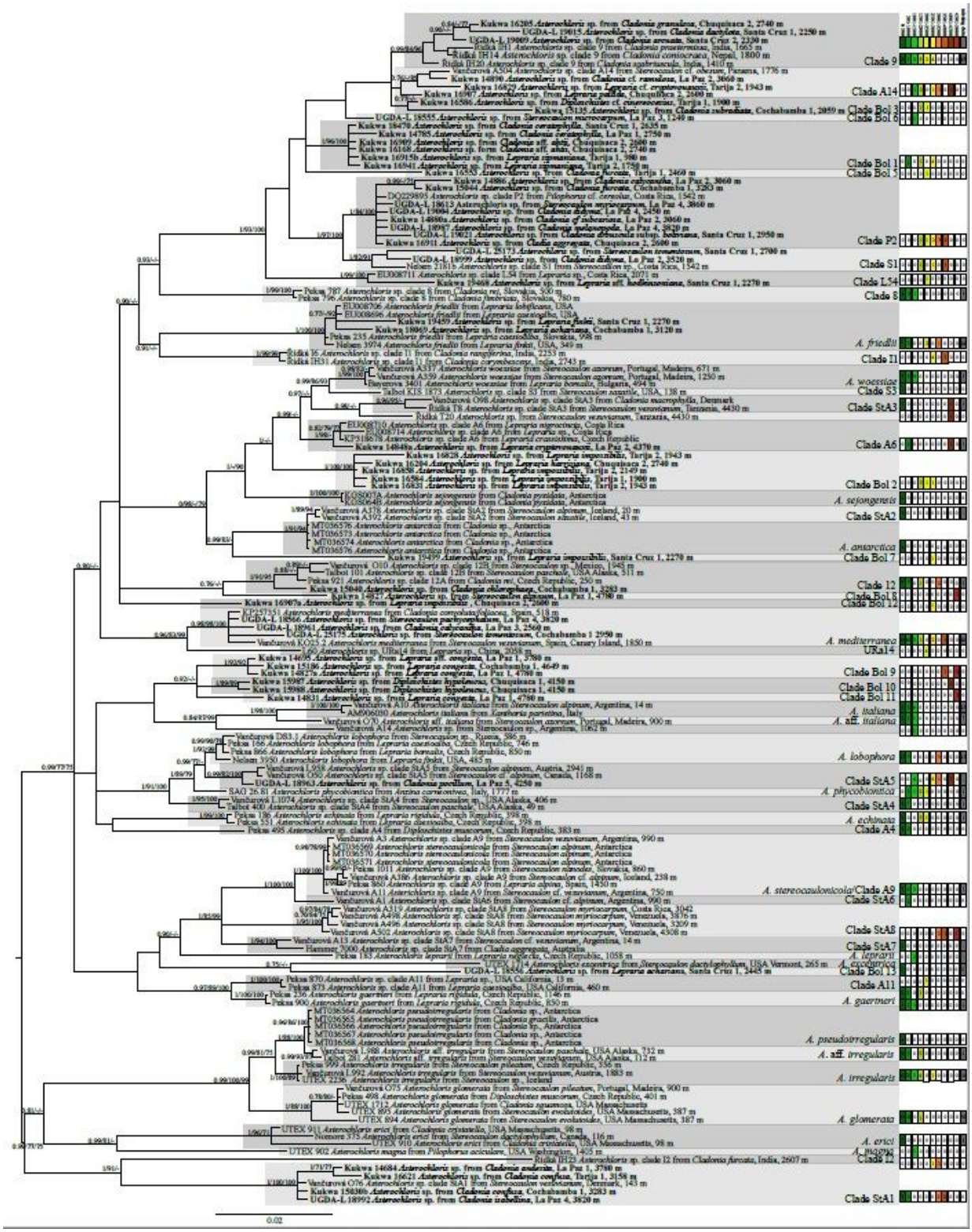


Figure 2

Majority- rule consensus tree from Bayesian analysis of Asterochloris based on ITS rDNA, rbcL and actin type I locus data set with posterior probabilities and bootstrap support values from RaxML and IQ-TREE analysis presented near the branches. For each record GenBank accession no. or voucher no. (for newly sequenced samples) are followed with photobiont name (if known), their mycobiont host name and the origin of specimen together with altitude (if known). Newly sequenced photobionts from Bolivia are

marked in bold. Species or phylogenetic lineages are marked in boxes with appropriate names. Based on altitude of the samples, which belongs to particular phylogenetic lineage, we added summary of abundance for each lineage. Samples from 0 to 500 m a.s.l. are in first box, from 501 to 1000 are in second box, from 1001 to 1500 are in third box, from 1501 to 2000 are in fourth box, from 2001 to 2500 are in fifth box, from 2501 to 3000 are in sixth box, from 3001 to 3500 are in seventh box, from 3501 to 4000 are in eighth box, from 4001 to 4500 are in ninth box, from 4501 to 5000 are in tenth box and samples with missing data of altitude are in eleventh box, marked in grey.



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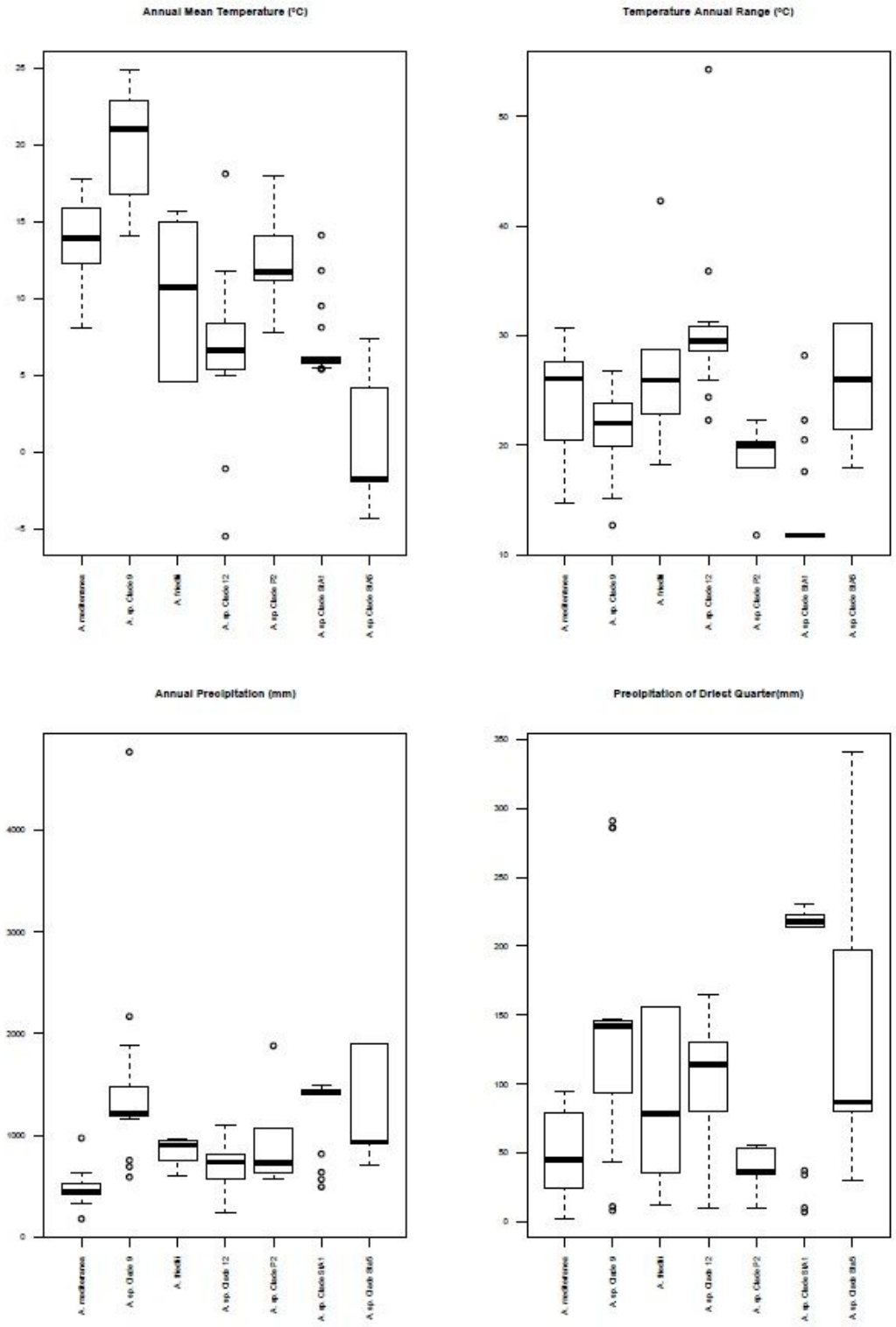
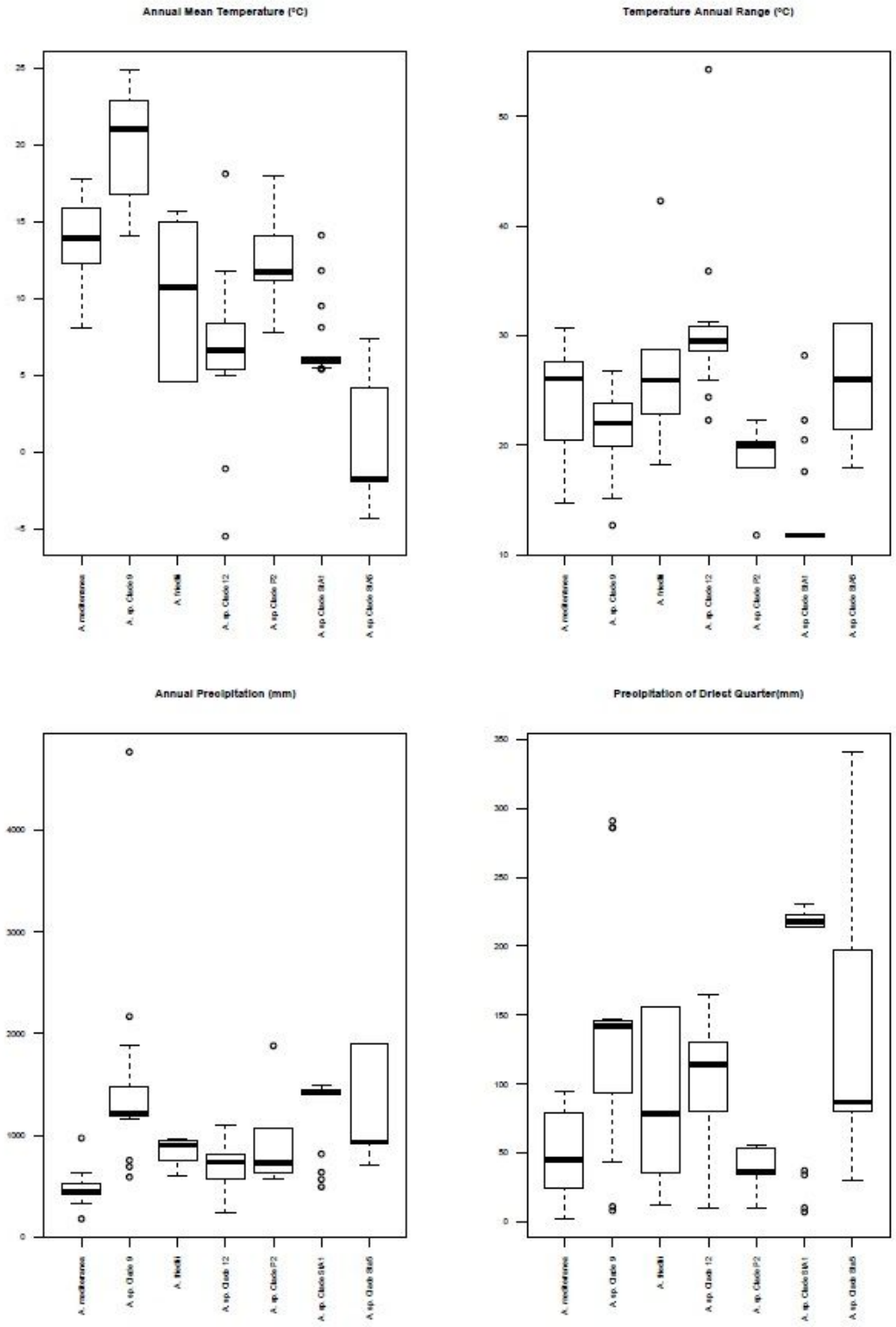


Figure 3

Box-plot diagram representing differences in climatic preferences for selected species and lineages of Asterochloris photobionts. Climatic data were obtained from the Global Climate Data – WorldClim. BIO1=Annual Mean Temperature (°C), BIO7= Temperature Annual Range (°C), BIO12= Annual Precipitation (mm), BIO17= Precipitation of Driest Quarter (mm).



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Supplementary Files

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