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Molecular studies of photobionts of selected lichens from the coastal vegetation of Brazil

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Abstract

A light microscopic and molecular analysis of photobionts in *Ramalina* and *Cladonia* from coastal habitats of Brazil is presented. A Bayesian phylogenetic analysis of ITS rDNA sequences suggests a *Trebouxia* lineage which is preferentially tropical in geographic distribution. This highly diverse clade also includes the morphological similar species *Trebouxia higginsiae* and *galapagensis*. Within the predominantly tropical clade of *Trebouxia* we distinguish several subclades, three of which are represented in our samples of *Ramalina* species. Since sexuality has not been recognized in coccal lichenised photobionts until recently, we cannot apply a biological species concept, but when compared with the sequence diversity between known species we conclude that several new species need to be described in this clade. The mutually exclusive presence of other *Trebouxia* lineages in temperate samples of *Ramalina* suggests an evolution towards higher selectivity in this genus. A strictly tropical lineage is not conspicuous in the photobionts of the genus *Asterochloris* sampled from *Cladonia* so far.

Keywords: Photobionts; Lichen; *Trebouxia*; *Asterochloris*; *Ramalina*; *Cladonia*; ITS rDNA

1. Introduction

Compared with the mycobiont partner of lichens, still little is known about the geographic distribution and ecology of the associated photobionts. Historically, this can be explained by the general difficulty to recognize the species in the lichen thallus. Previous identifications therefore included the isolation and axenic culture of algal symbionts. With the use of molecular tools, the interest in this symbiotic partner has increased. Friedl and Rokitta [1] showed a clear heterogeneity of trebouxioid photobionts. Species with a chloroplast closely appressed to the cell wall at certain stages and an indistinct pyrenoid, containing regular thylakoids, were found to be distantly related to the core *Trebouxia* cluster. These data agree with the findings of Tschermak-Woess [2] who splitted *Trebouxia* in two subgenera *Trebouxia* and *Eleutherococcus*. Piercey-Normore and DePriest [3] compared the sequences of several photobionts of Cladoniineae, including the subgenus *Eleutherococcus* sensu Tschermak-Woess and the isolated photobionts from *Anzina carneonivea*, i.e. *Asterochloris phycobiontica* [4]. They found 93% of similarity on their ITS sequences, suggesting that all these photobionts belong

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to the same genus *Asterochloris*. Phylogenetic studies of ITS rDNA from *Trebouxia* allowed the assignment of photobionts to certain clades [5,6] or focused on selectivity/specificity of fungi for photobionts [7]. These studies show that there are clear differences in photobiont selectivity in lichens. Only two species of *Trebouxia* were observed in some foliose Physciaceae by Dahlkild et al. [8], while in *Parmelia* and in the lichen genus *Umbilicaria* from Antarctica, several photobionts were found in different thalli of a single species [6,9]. There are few studies dealing with the diversity of photobionts of particular habitats. Beck et al. [5,10] indicated that a green algal species can generally be selected from a more diverse ''pool of locally available algae''. A similar situation has been found in lichens that are associated with cyanobacteria [11]. The ''pool'' or guild of photobionts comprise a number of species that are adapted to a certain habitat. It is not clear whether the composition of the ''pool'' is changing in different geographic regions and still little is known about the biogeography of photobionts. While the systematics of tropical lichens are now increasingly understood taxonomically and floristically, much less is still known about their photobionts. Practically nothing is known about cyanobacterial symbionts of tropical lichens, and the state of knowledge of the very common representatives of Trentepohliaes in lichens is quite limited [12,13]. While morphological data of growth form could assist in the recognition of taxa in the latter group, this is hardly possible in the second large group of eukaryotic tropical lichen photobionts, the coccale green algae of the Chlorophyceae. Phenotypic characters of this group are best studied using cultivated isolates, while molecular sequence data can readily be used to assess their phylogenetic relationships. Photobionts of lichen from tropical regions are included in a clade (Clade IV) in Friedl et al. [14], which includes lichens from Galapagos Islands and Florida – USA [9]. Some tropical members of Physciaceae are also included in this clade [7].

The Brazilian coast is composed of diverse environments. Ecologically outstanding are the ''restinga'' (sand bank vegetation) and mangrove. Restinga is an assemblage of coastal sandy ecosystems with floristically and physiognomically distinct communities. These plant communities colonize sediments of diverse origin, forming a edaphic vegetational complex that occupies a narrow belt along the coast, including such distinct regions as beaches, dunes and associated depressions, sand ridges, terraces and plains [15]. Although, this environment in Brazil is rich in lichens, detailed information is available only for some few species such as *Ramalina* [16] and *Cladonia* [17–21]. Differently from the restinga, the mangrove represents tropical vegetation developed in areas which are periodically inundated from the tides and are particularly present in areas where riverine habitats border to the sea [22]. However, the lichen flora in the mangrove does not seem to be much different from that in other tropical rain forest close to the coast [23].

In the present paper, we describe the phylogenetic position (ITS rDNA sequence comparison) and light microscopic characters of photobionts from some tropical lichens belonging to the families Ramalinaceae and Cladoniaceae, from different coastal environments of Brazil.

2. Material and methods

2.1. Lichen material

Samples utilized in this study were collected in different environments of Brazil: *Restinga*: *Ramalina sprengelii* 68; *Ramalina gracilis* 63; *Ramalina peruviana* 67 – 25°32′05″S/48°20′30″W; sea level (0 m); Ilha do Mel, Pontal do Paraná – PR, Brazil (February/2004). *Ramalina sorediosa* 59; *Ramalina anceps* 70 – 25°19′52″S/48°25′10″W; 8 m; Ilha Rasa, Guaraquecaba – PR, Brazil (February/2004). *R. gracilis*, *Ramalina complanata* – 27°35′48″S/48°32′57″W, 3 m, Campeche Beach, Santa Catarina Island – SC, Brazil (August/2001). *Cladina confusa* – 25°32′05″S/48°20′30″W; on sandy soil, sea level, Ilha do Mel, Pontal do Paraná – PR, Brazil (August/2001).

Mixed rain forest: R. peruviana 58 – 25°26′15″S/49°03′45″W; 940 m; Piraquara – PR, Brazil (February/2004).

Mangrove: Ramalina sorediosa 60 – 25°19′51″S/48°27′40″W; sea level; Guaraquecaba – PR, Brazil (February/2004). *R. anceps* 78 (6) 25°19′31″S/48°25′49″W; sea level; Guaraquecaba – PR, Brazil (February/2004). *Ramalina dendroides* 79 (8) 25°19′04″S/48°26′41″W; sea level; Guaraquecaba – PR, Brazil (February/2004).

Recovery area of Atlantic rain forest: R. peruviana 81 (5) 25°14′36″S/48°29′38″W; 40 m; Guaraquecaba – PR, Brazil (February/2004).

Caatinga: Cladonia verticillaris – 7°26′S/34°56′W; Alhandra – PB, Brazil.

Atlantic rain forest – Savanna (contact zone): Cladonia crinita; *Cladonia fissidens* – 20°05′S/43°29′W; Sandy Soil, Monastério do Caraça – MG, Brazil.

Some samples from non-tropical environments were included: *Cladonia perforata*, Eric Mendes Biological Station, Florida, USA (subtropical; gift from Rebecca Yahr). *Ramalina fraxinea* FB30 and *Ramalina farinaceae* FB22: Slovenia, 1997.

All the voucher specimens are placed in the UPCB Herbarium (UFPR-Curitiba, Brazil).

2.2. DNA extraction, PCR and sequencing

Total DNA was extracted according to a modified CTAB method [24]. DNA-extracts were used for PCR-amplification of the ITS regions including the 5.8S gene

of the nuclear rDNA. The algal-specific ITS primers ITS1T and ITS4T [25] were used for amplification of the rDNA from lichens with *Trebouxia* photobionts and the cultures of the photobionts of *R. complanata* and *R. gracilis* while the primers ITS1R and ITS 4 [26] were used to amplify the rDNA from the lichens with "*Asterochloris*" photobionts and the culture of the photobiont of *Cladina confusa*.

Amplifications were performed in 30 μL reactions (7.05 μl H_2O, 3 μl dNTPs [1 mM], 1.5 μl of each primer [10 pM], 2 units *Taq* DNA-polymerase, 12 μl DNA extract and 1.8 μl $MgCl_2$ [1.5 mM]). Amplification conditions were: 35 cycles of a denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and elongation at 72 °C for 1 min 30 s for the primer pair ITS1T/ITS4T. The annealing temperature was decreased to 52 °C for the primer pair ITS1R/ITS4. PCR products were cleaned using QIAGEN quick spin columns (Qiagen) and quantified on 1% agarose gel stained with ethidium bromide. Both complementary strands were sequenced using the primers ITS1T, ITS4T [25], ITS 2 and ITS 3 [26]. The sequencing was performed using Dye Terminator Cycle Sequencing Ready Reaction Kit (APPLERA, Vienna) according to the manufacturer's instructions, with detection on an ABI 310 automated sequencer (APPLERA, Vienna). Sequences were assembled into full-length sequences using Autoassembler.

2.3. Data analysis

The ITS rDNA sequences obtained in this study were aligned with available *Trebouxia* and *Asterochloris*-ITS sequences from GenBank (Table 1) using Clustal W included in the BioEdit Sequence Alignment Editor [27]. The phylogenetic hypothesis for *Trebouxia* and *Asterochloris* species was constructed using a Bayesian approach as implemented in the program MrBayes [28]. The nucleotide substitution models (GTR + G: *Trebouxia* Tree; K80 + G: "*Asterochloris*" tree) were selected using a likelihood ratio test [29] with a program MrModelTest [30], a simplified version of ModelTest v3.06. For other parameters default settings were applied. The Markov Chain Monte Carlo (MCMC) analysis was run for 2,000,000 generations, with chains starting from a random tree, and using the default temperature of 0.2. The run was repeated 5 times to assess whether the chains converged to a stable phylogeny. For each run every hundredth tree was sampled, while the first 50,000 generations were discarded as burn-in. The trees from each run were joined in one file and a consensus phylogram was calculated with showing mean branch lengths using the *sumt* command in MrBayes. In addition, we assessed branch support by parsimony bootsrapping with 1000 replicate heuristic searches using PAUP*4.0b10 [31]. Phylogenetic trees were drawn using the program Treeview [32].

2.4. Photobiont isolation and culture conditions

Photobiont cultures were obtained from thallus fragments of the lichens *Ramalina gracilis*, *R. complanata* and *Cladina confusa*, following the method of Yamamoto [33]. Preferably the green fragments containing the photobiont cells and remaining on the 150 μm filter, were picked up with a sterile needle under a dissecting microscope and transferred to slanted Sabouraud-2% sucrose-agar medium [34,35].

After three weeks, the tubes containing contaminant microorganisms, such as fungi, yeast or bacteria, were discarded. Cultured lichen photobiont appeared from the small segments after about 6–8 weeks and the growing algae free of contamination were transferred to slants or Petri dishes with inorganic Bolds Basal Medium (BBM) – 2% agar medium [36]. For morphological evaluation, the photobiont was cultivated for 4 weeks and kept in the culture chamber at 24 °C with an alternating daily cycle of 12/12 h. Its morphological characters and life cycle were observed using light microscopy.

3. Results

3.1. Molecular data

Photobiont ITS rDNA sequences from Ramalinaceae analysed together with available ITS sequences from GenBank showed four major ITS clades (Fig. 1) according to Friedl et al. [14]. Clade I is 100% supported and is represented by *T. gigantea* (UTEX2231), *T. showmanii* (UTEX2234), *T. incrustata* (UTEX784), *T. arboricola* (SAG 219-1a) and *T. arboricola* (92011C5). The two non-tropical *Ramalina* specimens used in this work (*R. farinaceae* FB22, *R. fraxinea* FB30) are placed in this clade. Clade II (85% supported) containing *T. potteri* (UTEX900), *T. flava* (UTEX181) and *T. impressa* was used as outgroup. Clade III (supported 100%) is represented by *Trebouxia* specimens, that according Beck [37] and Helms [7] are closely related to *T. simplex* TW-1A2. All the tropical samples from *Ramalina* and their isolated photobionts were placed in Clade IV, together with *T. usneae*, *T. higginsiae*, *T. corticola* and *T. galapagensis.* This main clade was strongly supported in the phylogenetic tree, and, with exception of *T. corticola* and *T. usneae*, all the samples originated from tropical environments. These data agree with Helms [7], who found that some of the tropical samples of Physciaceae were placed together with *T. galapagensis*, in a clade that was denominated Clade G by him.

The Clade IV is divided in four well-supported monophyletic subclades (all with 100% support). The subclade a presents two monophyletic lineages, the first (a1) contains the photobiont from *R. peruviana 67* (from rest-

Table 1
Trebouxia and *Asterochloris* species used in this study

Lichen species	Photobiont[b]	GenBank[a]	References
Anzina carneonivea	*A. phycobiontica*	AF345374	[3]
Cladia aggregata	*Asterochloris* sp.	AF345437	[3]
Cladina confusa	*Asterochloris* sp. (culture)	AY842279	This Study
Cladonia bellidiflora	*Asterochloris* sp.	AF345413	[3]
Cladonia chlorophaea	*Asterochloris* sp.	AF345431	[3]
Cladonia crinita	*Asterochloris* sp.	AY842277	This Study
Cladonia crispata	*Asterochloris* sp.	AF345378	[3]
Cladonia cristatella	*Asterochloris* sp.	AF345425	[3]
Cladonia didyma	*Asterochloris* sp.	AF345415	[3]
Cladonia farinacea	*Asterochloris* sp.	AF345432	[3]
Cladonia fimbriata	*Asterochloris* sp.	AF345434	[3]
Cladonia fissidens	*Asterochloris* sp.	AY842278	This Study
Cladonia furcata	*Asterochloris* sp.	AF345429	[3]
Cladonia grayii	*Asterochloris* sp.	AF345376	[3]
Cladonia grayii	*Asterochloris* sp.	AF345385	[3]
Cladonia ochrochlora	*Asterochloris* sp.	AF345438	[3]
Cladonia parasitica	*Asterochloris* sp.	AF345426	[3]
Cladonia perforata	*Asterochloris* sp.	AY842280	This Study
Cladonia pyxidata	*Asterochloris* sp.	AF345436	[3]
Cladonia scabriuscula	*Asterochloris* sp.	AF345424	[3]
Cladonia strepsilis	*Asterochloris* sp.	AF345401	[3]
Cladonia subulata	*Asterochloris* sp.	AF345427	[3]
Cladonia symphycarpa	*Asterochloris* sp.	AF345430	[3]
Cladonia turgida	*Asterochloris* sp.	AF345428	[3]
Cladonia verticillaris	*Asterochloris* sp.	AY842276	This Study
Cladonia. rappii	*Asterochloris* sp.	AF345417	[3]
Cladonia cristatella	*T. erici* (UTEX912)	AF345441	[3]
Cladonia sp.	*T. magna* (UTEX67)	AF345423	[3]
Stereocaulon dactylophyllum	*Asterochloris* sp.	AF345442	[3]
Stereocaulon dactylophyllum	*T. excentrica* (UTEX1714)	AF345433	[3]
Stereocaulon pileatum	*T. glomerata* (UTEX897)	AF345405	[3]
Stereocaulon pileatum	*T. pyriformis* (UTEX1713)	AF345407	[3]
Stereocaulon sp.	*T. irregularis* (UTEX2236)	AF345411	[3]
Anaptychia runcinata	*Trebouxia* sp.	AJ293781	[38]
Buellia georgei	*Trebouxia* sp.	AJ293783	[38]
Buellia straminea	*T. higginsiae* (UTEX 2232)	AJ249574	[14]
Caloplaca cerina	*T. gigantea* (UTEX2231)	AJ249577	[14]
Diploschistes diacapsis	*T. asymmetrica*	AJ249565	[14]
Free-living	*T. corticola* (UTEX 909)	AJ249566	[14]
Hypogymnia physodes	*T. simplex* Hp-MT1	AJ511357	[46]
Lecanora dispersa	*T. incrustata* (UTEX784)	AJ293795	[38]
Lecanora hageni	*T. showmannii* (UTEX2234)	AF242470	[25]
Lecanora rubina	*T. potteri* (UTEX900)	AF242469	[25]
Letharia vulpina	*T.simplex*	AF242457	[25]
Melanelia glabra	*T. impressa*	AJ249576	[14]
Parmotrema tinctorium	*Trebouxia* sp. (UBT-87.019A1)	Z68702	[45]
Physcia pulverulenta	*T. flava* (UTEX181)	AF242467	[25]
Pseudevernia furfuracea SK	*T.simplex*	AF242459	[25]
Punctelia subrudecta	*T. arboricola*	AJ249564	[14]
Ramalina anceps (70)	*Trebouxia* sp.	AY842269	This study
Ramalina anceps (78)	*Trebouxia* sp.	AY842268	This study
Ramalina complanata	*Trebouxia* sp. (culture)	AY842262	This study
Ramalina dendroides (79)	*Trebouxia* sp.	AY842267	This study
Ramalina farinacea (FB22)	*Trebouxia* sp.	AY842264	This study
Ramalina fraxineae (FB30)	*Trebouxia* sp.	AY842265	This study
Ramalina gracilis (63)	*Trebouxia* sp.	AY842273	This study
Ramalina gracilis	*Trebouxia* sp. (culture)	AY842263	This study
Ramalina peruviana (58)	*Trebouxia* sp.	AY842270	This study
Ramalina peruviana (67)	*Trebouxia* sp.	AY842266	This study
Ramalina peruviana (81)	*Trebouxia* sp.	AY842275	This study
Ramalina sorediosa (59)	*Trebouxia* sp.	AY842272	This study
Ramalina sorediosa (60)	*Trebouxia* sp.	AY842271	This study
Ramalina sp.	*T. galapagensis* (UTEX2230)	AJ249567	[14]

Table 1 (*continued*)

Lichen species	Photobiont[b]	GenBank[a]	References
Ramalina sprengelii (68)	*Trebouxia* sp.	AY842274	This study
Umbilicaria antarctica	*Trebouxia* sp.	AJ431580	[6]
Umbilicaria decussata	*Trebouxia* sp.	AJ431583	[6]
Umbilicaria. antarctica	*Trebouxia simplex*	AJ431575	[6]
Unknown	*T. arboricola* (SAG219-1a)	Z68705	[45]
Usnea arizonica	*Trebouxia* sp.	AF242471	[25]
Usnea filipendula	*T. usneae* (UTEX2235)	AJ249573	[14]

[a] Acession number for ITS rDNA sequence.
[b] Culture strain between brackets.

inga) together with the isolated strains of *T. higginsiae* and *T. galapagensis*. According to Friedl et al. [14] the latter sequences are identical, although their GenBank sequences differ by a gap of 45 nucleotides in *T. higginsiae*. The close distance with these sequences strongly suggest that the photobiont of *R. peruviana 67* belongs to the same species. The second (a2) is characterized exclusively by samples from restinga. One of the branches of clade a2 is represented by the isolated strain of *Ramalina gracilis*. Its life cycle and morphological characteristics are described below.

The subclade (b) is well supported (100%), and includes the photobiont isolated from *Parmotrema tinctorum* (*strain 87019A1*) a typical sub-tropical lichen (collected in Florida-USA, Friedl [9]). This photobiont was first classified as *T. usneae* [9], however, recent publications refer to it as *Trebouxia* sp. [7,38]. Moreover this subclade contains the isolated photobiont of *R. complanata*, which is also described in detail below. More diversity of environments was found in the subclade (b), with samples from mangrove, restinga, Atlantic rain forests and from Florida.

The subclade (c), present in clade IV comprises samples from mangrove and restinga, however, the restinga area where one of the samples (*R. anceps* 70) was collected is an island, ''Ilha Rasa, Baia de Guaraqueçaba – PR (Brazil)'', which is surrounded by mangrove trees where the lichen *Ramalina anceps* is very common, and develops rather large thalli, sometimes exceeding 50 cm in length.

Subclade (d) contains the UTEX isolated strains *T. usneae* and *T. corticola* (*Usnea filipendula* and free-living, respectively). This is the only subclade in clade IV without tropical samples. Both samples originated from the USA according to Ettl and Gärtner [39].

Besides the high supporting values found for all the subclades, their interrelationships are not well resolved.

Because the ITS sequences from *Asterochloris* are hardly alignable to those of *Trebouxia* sensu strictu, they were analysed separately, but together with other *Asterochloris* sequences from Genbank. We found a tree with the same structure that was also obtained by Piercey-Normore and DePriest [3] with three main clades (Fig. 2), and clade III, which included the isolated strain UTEX 912 from *Trebouxia erici*, was used as an outgroup [3,40].

Similar to Piercey-Normore and DePriest [3], clade I is very well supported (100%) but not well resolved. It comprises the isolated strains of ''*Trebouxia*'' *glomerata* (UTEX 897), ''*Trebouxia*'' *pyriformis* (UTEX 1713), ''*Trebouxia*'' *irregularis* (UTEX2236) and the isolated photobiont from *Anzina carneonivea*, *Asterochloris phycobiontica* [4].

Although the clade II itself is not well supported (69%), we may distinguish three more or less distinct subclades. The subclades a and c are higly supported (97% and 100%, respectively), but do not include any of the isolated UTEX strain. The subclade (b) is not well supported (74%), and contains the isolated strain of ''*Trebouxia*'' *magna* (UTEX 67), and also the isolated photobionts from the subtropical lichen *C. perforata*.

All the sequences sampled from tropical lichens are placed in the subclade (c). Our samples from the South (*C. confusa*) and Northeast (*C. verticillaris*, *C. crinita* and *C. fissidens*) of Brazil are placed together with the Guyana samples related by Piercey-Normore and DePriest [3] (data not shown). This subclade is not strictly tropical, as also *Cladonia rappii* and *Cladonia didyma* from the USA are included [3]. However, these widespread species are present from the south of Brazil up to the southeast of USA [18,41].

3.2. Morphological data

Photobionts of *R. gracilis*, *R. complanata* and *C. confusa*, isolated from thallus fragments, produced axenic dark-green, rough colonies on the surface of Sabouraud-2% sucrose-agar (Fig. 3(a)). When transferred to inorganic Bolds Basal Medium (BBM) and cultivated for 4 weeks, mature vegetative cells of *Trebouxia* sp. from *Ramalina* species are spherical (Figs. 3(b) and 4(a)), 8–12 μm in diameter, uninucleate, with the nucleus located in the invagination of the chloroplast, nucleolus sometimes visible. The chloroplast is axial, with short lobes, and contained one pyrenoid that is distinct by a continuous starch sheath (discontinuous in *Ramalina complanata* isolated) in the central portion. Asexual reproduction by autospores, zoo- and aplanospores

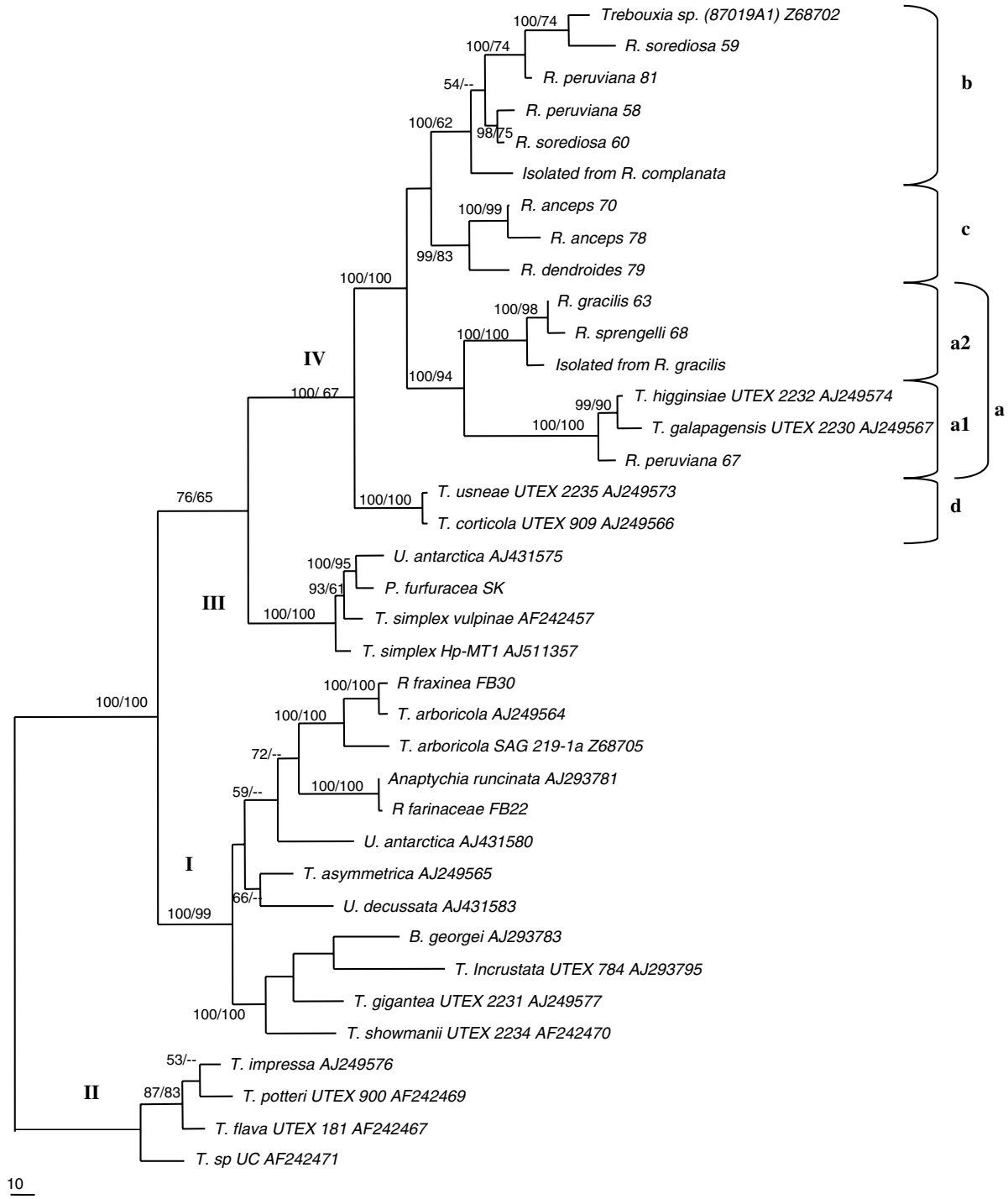

Fig. 1. *Trebouxia* phylogeny based on the ITS rDNA sequences. Branch support is shown as posterior probability (Bayesian analysis) followed by parsimony bootstrap values. Missing support and dashes indicate that the branch was not resolved.

was observed (*sensu* Tschermak-Woess [2]). The mother cells gave rise to 4, 8 or 16 autospores (Figs. 3(c) and 4(b)). Within these autosporangia the sporangial wall is visible and a tiny, triangular interspace between two neighbouring cells and the sporangial wall persists (Fig. 3(c), arrow). Different stages of protoplast division were seen, in which the original protoplast had divided into a few to numerous new protoplasts (Fig. 3(d) and (e)). The transformation of trophic cells into zoo-/aplanosporangia started with the formation of a local thickening of the cell wall that marks the prospective opening (Figs. 3(f) and 4(c)). The zoosporangia (Fig. 3(g)) contained 64 or 128 zoospores, which were released by rupture of the mother cell wall. When their release was arrested, the zoosporangia developed into aplanosporangia (Fig. 3(h) and 4(d)), the zoospores absorbed

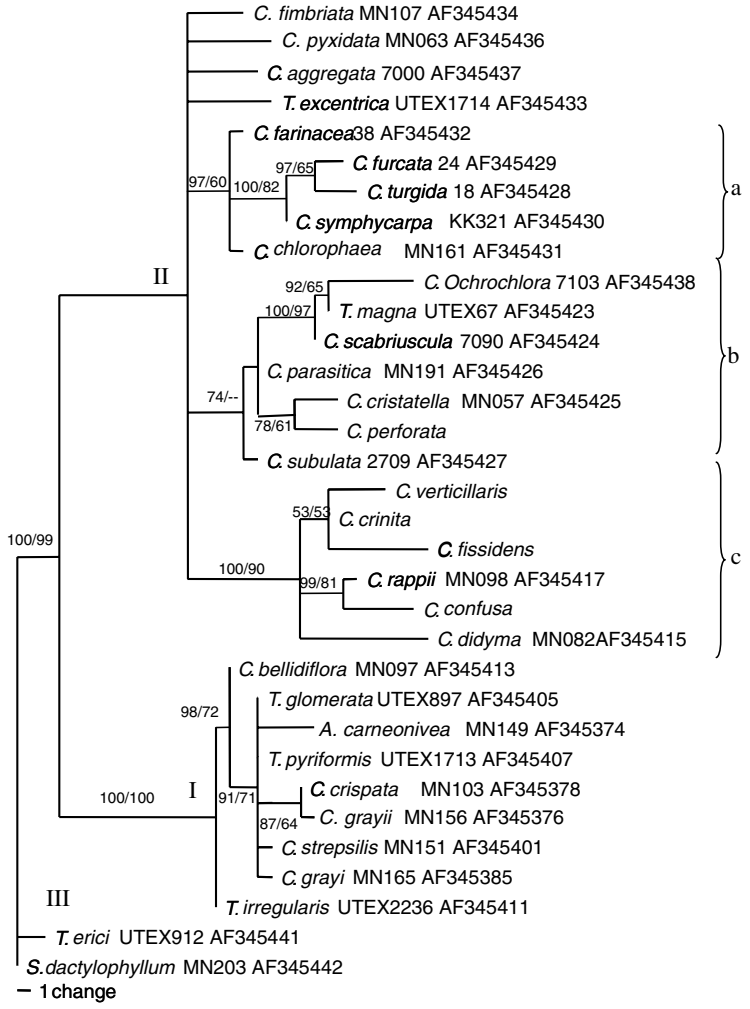

Fig. 2. *Asterochloris* phylogeny based on the ITS rDNA sequences. Branch support is shown as posterior probability (Bayesian analysis) followed by parsimony bootstrap values. Missing support and dashes indicate that the branch was not resolved.

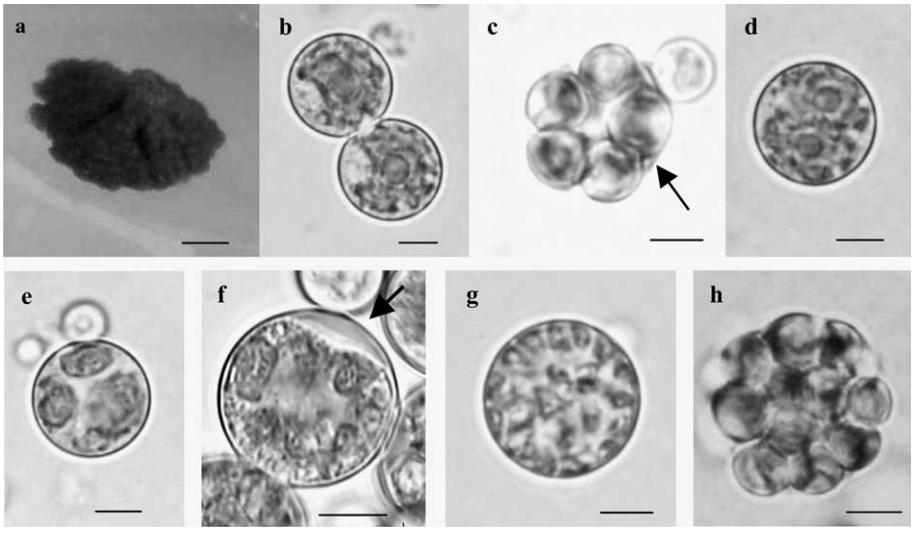

Fig. 3. Photobiont *Trebouxia* sp. isolated from *Ramalina gracilis*. (a) Axenic dark-green colony grown on solid Sabouraud-2% sucrose-agar after 4 months of incubation. Bar, 1 mm. (b) mature vegetative cells showing pyrenoid and structure of chloroplast; (c) autosporangium with 8 daughter cells; (d, e) successive divisions of the protoplast; (f) developing zoosporangium with bulging thickening of cell wall (arrow); (g) fully developed zoosporangium; (h) aplanosporangium. Bars, 5 μm.

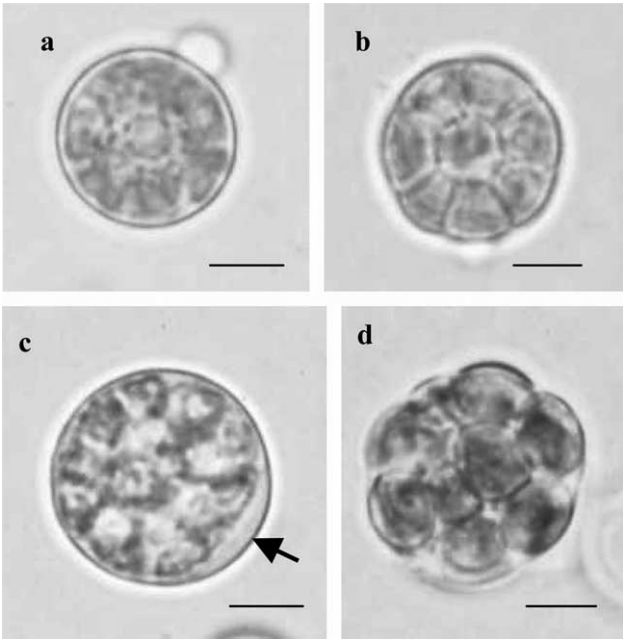

Fig. 4. Photobiont *Trebouxia* sp. isolated from *Ramalina complanata*. (a) mature vegetative cell showing central pyrenoid and lobation of chloroplast; (b) autosporangium with 16 daughter cells; (c) mature zoosporangium with thickening of cell wall (arrow); (d) aplanosporangium. Bars, 5 μm.

their flagella, rounded up and produced their own cell wall.

On the other hand, in the log growth phase, mature vegetative cells of the photobiont *Asterochloris* sp. of *Cladina confusa* are slightly ellipsoidal or spherical (Fig. 5(a) and (b)) and asexual reproduction only occurs by zoo- (Fig. 5(d)) and aplanospores (Fig. 5(g)). Autospore production is absent. The thickening of the mother cell wall was also observed (Fig. 5(c)), before the zoosporangia finally opened and released the zoospores. These were naked, 3–5 μm in width, 5–7 μm in length, had two whip-like flagella and a chloroplast in posterior position (Fig. 5(e)). After the zoospores were released, also a few cells remained within the sporangium and developed into young vegetative cells within the old sporangial wall (Fig. 5(f)). Moreover, many empty cell walls were adhering together, forming large packages.

4. Discussion

The molecular data from two genera of lichens from coastal vegetation of Brazil indicate that poorly investigated lineages of lichen photobionts are predominant in tropical environments. Helms [7] analysed a larger number of Physciaceae, and all tropical samples are distributed in one clade (G), related to *Trebouxia galapagensis* or *T. higginsiae*. This clade is here subdivided in several distinct lineages, which might represent new species. These merit a valid description at a later stage, when ultrastructural data become available. Further lineages could be present also in other regions of the tropical belt (Reis, unpublished) and we assume that photobiont diversity of tropical habitats is still underestimated.

None of the tropical isolations contained photobionts of clade I, which was detected in *Ramalina farinacea* and *R. fraxinea* of temperate regions. The photobiont of *R. fraxinea* represents *Trebouxia arboricola*, while the

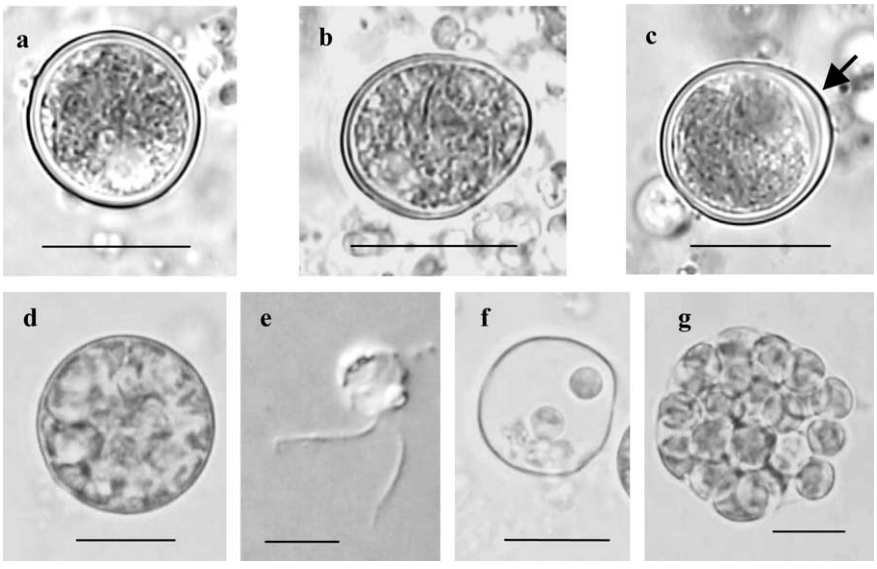

Fig. 5. Photobiont *Asterochloris* sp. isolated from *Cladina confusa*. (a, b) ellipsoidal vegetative cells showing structure of chloroplast. Nucleus and nucleolus visible. (c) developing zoosporangium with four chloroplasts and thickening of cell wall (arrow); (d) fully developed zoosporangium; (e) zoospore, showing two whip-like flagela and chloroplast in position posterior. Bar, 5 μm; (f) young zoospores within the old sporangial wall; (g) aplanosporangium. Bars, 10 μm. In (e) was used Normarski interference contrast.

photobiont of *R. farinaceae* belongs to a still unnamed clade with a photobiont of an Antarctic lichen (*Umbilicaria antarctica*) seems to be related to this lineage. In contrast, none of the tropical photobionts is found in a larger sample of temperate *Ramalina* species (Grube, unpublished data).

The classification of *Trebouxia* placed in the clade IV is somehow controversial. The species *T. higginsiae* and *T. galapagensis* were first described by Hildreth and Ahmadjian [42] (as *Pseudotrebouxia*). The descriptions are very similar: one of the important distinguishing characters was the presence of a discontinuous starch sheath in *T. higginsiae*. In 1989, Friedl [9] based on the pyrenoid structure, suggested that both are the same species together with *T. corticola*. Recently, Ettl and Gärtner [39] kept the original interpretation of Hildreth and Ahmadjian [42], being the color of the colonies in the *Trebouxia*-agar the main difference in their key, i.e., light green in *T. galapagensis* and dark green in *T. higginsiae*.

Our molecular and morphological data suggest that the subclade IVa, that include the UTEX strains *T. galapagensis* and *T. higginsiae* and our isolated photobiont from *R. gracilis*, represents only one specie. However, the cultivated photobiont from *R. complanata*, placed in subclade IVb may represent another specie, beside its morphological similarities (chloroplast and pyrenoid) with *Trebouxia* from *R. gracilis* [39,42].

The presence of a predominantly tropical clade of photobionts is not conspicuous in the case of *Asterochloris* detected in *Cladonia*. While most of the tropical members have a photobiont belonging to the lineage c in our *Asterochloris* tree, this lineage is also found in non-tropical samples of *Cladonia* from the Southern United States. Clade c is well supported in our tree, however, the remainder of the *Asterochloris* phylogeny is not completely resolved. Therefore, to assess the infrageneric relationships equally well as in *Trebouxia* is not possible at present, since data from another locus for further analyses are not available.

However, our data result in an interesting question about the evolution of selectivity patterns in lichen symbionts. Usually, with certain exceptions, a rather strong pattern of selectivity is found in some foliose and fruticose species so far studied; e.g., *Flavoparmelia nivalis* and *Letharia vulpina*, respectively, accept only a subset of photobionts lineages in *Trebouxia simplex*, while *Xanthoria parietina* only associates with *Trebouxia arboricola*. While we notice a low selectivity in certain crustose species (e.g. *Lecanora rupicola*; Blaha et al., submitted), Schaper and Ott [43] have shown by in vitro experiments that symbiotic stages of the crustose *Fulgensia bracteata* are rapidly formed with their genuine photobionts, while formations of lichens association are clearly delayed in resynthesis experiments when other photobiont are used. However, the finding of different photobiont groups in tropical and temperate species of the same mycobiont genus poses the question whether the phylogenetic diversification of *Ramalina* is accompanied by an evolution of increasing selectivity for photobionts, or if selectivity was equally strong also in the ancestors of *Ramalina* and the diversification of photobionts co-evolved with the host. Since both, temperate and tropical photobiont lineages are also present in a wide range of other mycobionts, a pattern of co-evolution is unlikely. Rather, the generally low photobiont selectivity so far found in crustose and squamulose lichen groups let us assume that photobiont selectivity was relaxed in the ancestors of *Ramalina*, which likely evolved from crustose groups of lichens [44]. The less conspicuous pattern with *Cladonia* photobionts could either be due to the low resolution of ITS sequences or to the fact that *Cladonia* contains both strictly fruticose species and groups with primary thalli of squamulose growth. It need to be further studied, by more thorough sampling, whether photobiont selectivity are correlated with different growth forms in *Cladonia*.

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