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Green-algal photobiont diversity (*Trebouxia* spp.) in representatives of *Teloschistaceae* (Lecanoromycetes, lichen-forming ascomycetes)

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Abstract: The green algal photobionts of 12 *Xanthoria*, seven *Xanthomendoza*, two *Teloschistes* species and *Josefpoeltia parva* (all *Teloschistaceae*) were analyzed. *Xanthoria parietina* was sampled on four continents. More than 300 photobiont isolates were brought into sterile culture. The nuclear ribosomal internal transcribed spacer region (nrITS; 101 sequences) and the large subunit of the RuBiSco gene (*rbc*L; 54 sequences) of either whole lichen DNA or photobiont isolates were phylogenetically analyzed. ITS and *rbc*L phylogenies were congruent, although some subclades had low bootstrap support. *Trebouxia arboricola, T. decolorans* and closely related, unnamed *Trebouxia* species, all belonging to clade A, were found as photobionts of *Xanthoria* species. *Xanthomendoza* species associated with either *T. decolorans* (clade A), *T. impressa, T. gelatinosa* (clade I) or with an unnamed *Trebouxia* species. *Trebouxia gelatinosa* genotypes (clade I) were the photobionts of *Teloschistes chrysophthalmus, T. hosseusianus* and *Josefpoeltia parva.* Only weak correlations between distribution patterns of algal genotypes and environmental conditions or geographical location were observed.

Key words: *Asterochloris*, *Josefpoeltia parva*, nrITS, *rbc*L, *Teloschistes*, *Xanthomendoza*, *Xanthoria*

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Introduction

Lichens, as found in nature, are the symbiotic phenotype of lichen-forming fungi in association with their photoautotrophic partner. Species names of lichens refer to the fungal partner. Lichen photobionts, mostly green algae or cyanobacteria, very rarely *Xanthophyceae* or *Phaeophyceae* (Tschermak-Woess 1988; Peršoh *et al.* 2004), have their own names and phylogenies. Traditionally, species of lichen-forming fungi were described on the basis of morphological and chemical characters (morpho- and chemospecies). Morphological criteria also formed the basis of species descriptions in lichen photobionts. In less than 2% of the *c.* 13 500 species of lichen-forming fungi known to science has the photobiont ever been identified at species level (Honegger 2008); this estimate is based on Tschermak-Woess (1988) and on the recent literature.

As lichen-forming fungi do not easily re-lichenize under sterile culturing conditions, the range of compatible photobiont taxa per lichen-forming fungal species cannot be experimentally approached with re-lichenization experiments in the Petri dish. Instead, the photobiont of lichen specimens, as collected in the wild, is investigated. Traditionally, isolation and culturing under defined sterile conditions, followed by light or electron microscopic analysis and comparison with reference strains, were used (Ahmadjian 1958, 1967; Tschermak-Woess 1988). Accordingly, only a few experts worldwide were able to identify the photobionts of lichen-forming fungi at species level. Since the

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advent of molecular techniques, the time-consuming isolation and culturing has been largely avoided; instead, photobiont-specific molecular markers applied to whole lichen DNA has facilitated photobiont identification at the species level (Kroken & Taylor 2000; Dahlkild *et al.* 2001; Helms *et al.* 2001; Piercey-Normore & DePriest 2001; Tibell 2001; Romeike *et al.* 2002; Tibell & Beck 2002; Helms 2003; Piercey-Normore 2004, 2006; Yahr *et al.* 2004, 2006; Blaha *et al.* 2006; Guzow-Krzeminska 2006; Muggia *et al.* 2008, 2010; Francisco De Oliveira *et al.* 2012). Based on increasing numbers of entries in databases, the studies above have gained novel insights into photobiont diversity and phylogenies. Isolation and culturing are, however, still crucial as reference material, for genetic analyses at the subspecific level and for diverse experimental approaches.

The range of compatible photoautotrophic partners per fungal species and their inter- and intraspecific diversity are ideally studied in a large set of samples from a wide geographical range. However, even the analysis of one or a few samples gives valuable first insights into the taxonomic affiliation of compatible photobionts. The majority of morphologically advanced species of lichen-forming fungi are moderately specific to specific with regard to their photobiont selection, that is a fungal species associates with one or few species of green algae or cyanobacteria (Honegger 1993). A lower specificity towards their photobiont was observed in a few of the lichen-forming ascomycetes forming morphologically less advanced crustose thalli (Friedl 1987; Tschermak-Woess 1988; Beck 2002; Helms 2003; Blaha *et al.* 2006; Pérez-Ortega *et al.* 2012; but see Vargas Castillo & Beck 2012). Moreover, lichen mycobionts growing in extreme habitats such as Antarctic or alpine ecosystems tend to associate with a wide range of photobiont strains (Romeike *et al.* 2002; Wirtz *et al.* 2003; Muggia *et al.* 2008; Domaschke *et al.* 2012; Pérez-Ortega *et al.* 2012). Interestingly, the most common and widespread aerophilic unicellular green algae, often forming conspicuous green layers on bark or rock surfaces, are very rarely acceptable partners of lichen-forming fungi (Tschermak-Woess 1988; Peršoh *et al.* 2004).

More than 80% of the lichen-forming fungi studied associate with green algal photobionts, representatives of the genera *Trebouxia* de Puymaly and *Asterochloris* (Tscherm.-Woess) T. Friedl *ined.* (*Trebouxiophyceae sensu* Friedl 1995) being the most common and widespread partners in all climates (Ahmadjian 1988; Tschermak-Woess 1988; Rambold *et al.* 1998; Peršoh *et al.* 2004; Beck & Peršoh 2009). Probably due to their ability to survive desiccation unharmed, *Trebouxia* spp. are the photobionts of most lichen-forming fungi in climatically extreme habitats such as Antarctic, Arctic, alpine or desert ecosystems, where the whole thallus is continuously subjected to drought and temperature extremes.

Sexually reproducing lichen-forming fungi are assumed to re-lichenize at each reproductive cycle, that is germinating asco- or basidiospores have to find a compatible photobiont. Contradictory views are found in the literature concerning the abundance of free-living *Trebouxia* cells and their availability for asco- or basidiospore-derived germlings of lichen-forming fungi. According to Ahmadjian (1988, 2002*a*, *b*), *Trebouxia* species do not normally exist outside lichen thalli. Tschermak-Woess (1978) found free-living *Trebouxia* cells, but pointed out that they are rare in aerophilic algal communities. Bubrick *et al.* (1984) found free-living *Trebouxia* cells near thalli of *Xanthoria parietina*, and according to Mukhtar *et al.* (1994) *Trebouxia arboricola* de Puymaly is one of the most common colonizers of bare rock surfaces after fires in Israel. In a series of elegant *in situ* re-lichenization studies, Sanders (2005) observed large numbers of free *Trebouxia* cells on plastic slides which had been exposed in oak trees (*Quercus ilex*) with lichen cover in Spain, and germ tubes of *Xanthoria parietina* ascospores in contact with them. In the phycological literature, the aerophilic *T. arboricola*, type species of the genus, is referred to as abundant and widespread on saxicolous and corticolous substrata in Europe (Ettl & Gärtner 1995; John *et al.* 2002; Rindi & Guiry 2003).

The present study aims to explore the identity, diversity and phylogeny of the photobionts in *Teloschistaceae* (Teloschistineae, Lecanoromycetes), the focus being on the genera *Xanthoria* and *Xanthomendoza*. *Teloschistaceae* are lichen-forming ascomycetes with a worldwide distribution. They comprise species with a very wide geographical range such as the ubiquitous *Xanthoria elegans* and the very widespread *X. parietina*, alongside species with a small area of distribution such as the South African endemics *X. capensis, X. flammea* and *X. karrooensis*. *Teloschistaceae* are associated with trebouxioid green algal photobionts. Best investigated is the widely distributed type species of the genus, *Xanthoria parietina*, and the closely related European *X. calcicola* and *X. ectaneoides*, here referred to as the *X. parietina* complex (fungal phylograms in Scherrer & Honegger 2003; Eichenberger 2007). According to the literature, the *X. parietina* complex is associated with *Trebouxia arboricola*, *T. crenulata* Archibald, *T. decolorans* (Ahmadjian) Archibald, and *T. italiana* Archibald [syn. *Asterochloris italiana* (Archibald) T. Friedl ined.] (Ahmadjian 1960, 2002*b*; Gärtner 1985*a*, *b*, *c*; Honegger & Peter 1994; Beck *et al.* 1998). *Trebouxia decolorans* has been determined to be the photobiont of *Xanthomendoza hasseana* and *Xanthoria tenax* in southern California (Werth 2012), whereas *Trebouxia asymmetrica* has been reported as the photobiont of *Fulgensia fulgida* (Beck *et al.* 2002). In contrast, *Teloschistes chrysophthalmus* associates with *T. gelatinosa* (Werth 2012; Nyati *et al.* 2013*a*). The *Trebouxia* photobiont of *Teloschistes flavicans* was found to be related to *Trebouxia galapagensis* and *T. higginsiae* (Reis *et al.* 2005). Antarctic photobionts of *Caloplaca* belong to the genus *Trebouxia* (Pérez-Ortega *et al.* 2012). *Trebouxia arboricola, T. decolorans*, and *T. gigantea* were found to be the photobionts of *Caloplaca* spp. from northern Chile (Vargas Castillo & Beck 2012). No data are available on the taxonomic affiliation of the photobionts of other *Teloschistaceae*.

The goals of the present study are: 1) to evaluate photobiont diversity and phylogenies in a range of *Xanthoria* and *Xanthomendoza* spp.; 2) to explore the range of compatible photobionts in a large sample of the *X. parietina* complex from worldwide locations. As *X. parietina* was most likely introduced to Australia and New Zealand (Galloway 1985; Rogers 1992), we were interested to see whether it associates with different photobionts in these areas than in Europe or North America; 3) to isolate *Trebouxia* photobionts of *Teloschistaceae* into sterile culture as reference strains and for diverse future investigations. Most of the corresponding fungal partners were brought into sterile culture (Honegger 2003), their taxonomic affiliation and phylogenies being analyzed in parallel experiments (Eichenberger 2007; Itten & Honegger 2010).

Materials and Methods

Lichen collection and storage

Freshly collected lichens were either immediately processed or stored, in a desiccated state, at −20°C, where they stay viable for prolonged periods of time (Honegger 2003). Voucher specimens were deposited in the herbarium of ETH Zürich (Z+ZT). Collectors and collecting sites are listed in Table 1. A few experiments were carried out with specimens from the lichen herbarium of the University of Graz, Austria. From a set of samples originating from the campus of the University of Zürich (Zürich-Irchel, numbers 319–320), the thalli were left *in situ* and only small fragments were removed after photographic documentation.

Photobiont isolation and culture

With a sterile platinum needle, photobiont cells were scraped from the thalline margin of apothecia, or alternatively from the algal layer of lobes in samples with no or few fruiting bodies. Photobiont cells were spread on the surface of agarized non-nutrient, mineral medium [Bold's basal medium (BBM) according to Deason & Bold 1960] contained in Petri dishes, with double amount of nitrogen and with 0·005% (w/v) doxycycline (Sigma-Aldrich, MA, USA) as an antibiotic. These plates were maintained at 15 ± 1°C at a 16:8 h light-dark cycle at *c.* 5 μE m^{-2} s^{-1} for 2–3 weeks until cells started to divide. All cultures were screened regularly; fungal contaminants were immediately cut out. Groups of dividing algal cells were either transferred to *Trebouxia* medium II according to Ahmadjian (1967), with only ¼ amount of glucose and casamino acids (Honegger 2004), and cultured for 8–12 weeks, or left on BBM 2N. Most cultures are multi-cell isolates, cells originating from a

TABLE 1. *Photobionts isolated from members of the* Teloschistaceae *used in the present study, their country of origin, collectors and collection numbers and ITS and rbcL GenBank Accession numbers*

Lichen Species	Collecting site	Country	Collector	Photobiont sp.*	Isolate†*	Genbank Acc. No.	
						ITS	rbcL
Josefpoeltia parva (Räsänen) Fröden & L. Lindblom (*syn. J. boliviana* S.Y. Kondr. & Kärnefelt)	Tucuman	Argentina	*B. Marrazzi, R. Vanni, G. Lopez*	*Trebouxia gelatinosa*	L-447-I-P2	AM159212	
Teloschistes chrysophthalmus (L.) Th. Fr.	Canary Islands	E	*R. Stalder*	*T. gelatinosa*	P-270-I-a	AJ969579	AJ969640
Telo. hosseusianus Gyeln.	Tucuman	Argentina	*B. Marrazzi, R. Vanni, G. Lopez*	*T. gelatinosa*	L-447-t1	AM159211	
Xanthomendoza borealis (R. Sant. & Poelt) Søchting *et al.*[1]		Greenland		*Trebouxia sp.*	G9306	AJ969505	AJ969662
Xm. borealis		Greenland		*T. decolorans*	G9307	AJ969506	
Xm. borealis		Greenland		*T. decolorans*	G9308	AJ969507	
Xm. fallax (Hepp) Søchting *et al.*[2]	California	USA	*R. Robertson*	*T. impressa*	L-46	AJ969525	AM158968
Xm. fallax	Chur	CH	*R. Honegger & U. Jauch*	*T. impressa*	L-68	AJ969533	AM158969
Xm. fallax	Minnesota	USA	*S. Scherrer*	*T. impressa*	L-329-t1	AM159203	
Xm. fulva (Hoffm.) Søchting *et al.*[3]	Sevan	Armenia	*M. Käppeli*	*T. decolorans*	L-247-t1	AM159215	
Xm. hasseana (Räsänen) Søchting *et al.*[4]	California	USA	*R. Robertson*	*T. decolorans*	P-69-I-a-Sc	AJ969534	AJ969652
Xm. hasseana	California	USA	*S. Werth*	*T. decolorans*	P-400-I-a-Sc	AM159210	
Xm. novozelandica (Hillmann) Søchting *et al.*[5]	Roxburgh	NZ	*D. J. Galloway*	*T. gelatinosa*	L-66	AM159502	
Xanthomedoza sp.	Colorado	USA	*C. Eichenberger*	*T. impressa*	L-475-t2	AM159209	
Xanthomedoza sp.	Colorado	USA	*C. Eichenberger*	*T. decolorans*	L-477-t1	AM159207	
Xanthomedoza sp.	Colorado	USA	*C. Eichenberger*	*Trebouxia sp.*	L-478-t1	AM159208	
Xm. ulophyllodes (Räsänen) Søchting *et al.*[6]	Minnesota	USA	*S. Scherrer*	*T. impressa*	P-330-I-b	AJ969605	AJ969636
Xm. weberi (S.Y. Kondratyuk & Kärnefelt) L. Lindblom[7]	Delaware	USA	*O. Crichton*	*T. gelatinosa*	P-57-I-a	AJ969532	AJ969642
Xm. weberi	Roussillon	F	*R. Honegger*	*T. gelatinosa*	L-114-t1	AM159214	
Xm. weberi	Massachusetts	USA	*V. Ahmadjian*	*T. gelatinosa*	P-350a-III	AM159213	
Xanthoria calcicola Oxner	Burgdorf	CH	*R. Honegger*	*T. arboricola*	P-44-I-a1	AJ969524	
X. calcicola	Lausanne	CH	*S. Scherrer*	*T. arboricola*	P-105-I-a	AJ969542	
X. calcicola	Avenches	CH	*R. Honegger*	*T. arboricola*	P-141-II	AJ969552	
X. calcicola	Hampshire	GB	*P. W. James*	*T. arboricola*	L-80	AJ969536	
X. candelaria (L.) Th. Fr.[8]	Myvatn	IS	*J. Achermann & G. Schuwey*	*T. decolorans*	P-205-II-a	AJ969569	
X. candelaria	Nove Mesto	CZ	*J. Lentjes*	*T. decolorans*	L-264	AJ969576	AJ969655
X. capensis Kärnefelt *et al.*[9]	Cape Town	ZA	*A. Möhl*	*T. arboricola*	P-306-I-a	AJ969591	
X. aureola (Ack.) Erichsen	Cornwall	GB	*J. M. Gray*	*T. arboricola*	P-83-I-a	AJ969611	
X. aureola	Mt. Eros, Hydra	GR	*O.W. Purvis*	*T. arboricola*	P-85-II-a	AJ969537	
X. aureola	Hydra	GR	*O.W. Purvis*	*T. arboricola*	P-86-I-b	AJ969538	AJ969666
X. aureola	Bretagne	F	*R. Honegger*	*T. arboricola*	P-158-IV-mc	AJ969560	
X. aureola	Sicily	I	*R. Honegger*	*T. arboricola*	L-43	AJ969523	
X. aureola	Karthago	TN	*U. Zippler*	*T. arboricola*	P-174-II-adA	AJ969565	
X. elegans (Link) Th. Fr.[10]	Manasulu	Nepal	*F. Rutschmann*	*T. decolorans*	L-269	AJ969578	
X. elegans	Gemmi Pass	CH	*H. P. Schöb*	*Trebouxia sp.*	L-398-t1	AM159204	

TABLE 1. *Continued*

Lichen Species	Collecting site	Country	Collector	Photobiont sp.*	Isolate†*	Genbank Acc. No. ITS	Genbank Acc. No. rbcL
X. elegans	Bishkek	KS	*L. E. Tapernoux*	*Trebouxia sp.*	L-459-t1	AM159206	
X. flammea (L. f.) Hillmann[11]	West coast	ZA	*H.P. Ruffner & E. Ruiz*	*T. arboricola*	L-101	AJ969540	AJ969664
X. karrooensis S.Y. Kondratyuk & Kärnefelt[12]	Western Cape	ZA	*H. Gansner*	*T. arboricola*	P-360-I	AM159216	
X. ligulata (Körb.) P. James	South Island	NZ	*W. Malcom*	*T. arboricola*	P-17-II-a	AJ969519	
X. ligulata	South Island	NZ	*J. Bannister & A. Knight*	*T. arboricola*	P-53-I-a	AJ969528	AJ969670
X. ligulata	South Island	NZ	*J. Bannister & A. Knight*	*T. arboricola*	P-54-II-a	AJ969530	
X. parietina (L.) Th. Fr.	Tasmania	AUS	*G. Kantvilas*	*T. decolorans*	P-10-I-a	AJ969515	AJ969659
X. parietina	Tasmania	AUS	*G. Kantvilas*	*T. arboricola*	L-11-II-a	AJ969516	
X. parietina	Port Fairy	AUS	*U. & R. Stidwill*	*T. decolorans*	P-133-I-a	AJ969551	AM158961
X. parietina	Barossa Valley	AUS	*J. Pokorny*	*T. decolorans*	L-275-II	AJ969580	
X. parietina	Canberra	AUS	*J. Pokorny*	*T. arboricola*	P-276-I-a	AJ969581	
X. parietina	Grampians	AUS	*J. Pokorny*	*T. arboricola*	L-277-I	AJ969582	
X. parietina	Roxburgh	NZ	*D.J. Galloway*	*T. decolorans*	L-1-II-A	AJ969508	
X. parietina	South Island	NZ	*J. Bannister & A. Knight*	*T. decolorans*	L-51-I	AJ969527	
X. parietina	Oregon	USA	*B. Mc Cune*	*T. decolorans*	P-6-I-a	AJ969511	
X. parietina	California	USA	*R. Robertson*	*T. decolorans*	L-8	AJ969513	
X. parietina	California	USA	*R. Robertson*	*T. decolorans*	L-9	AJ969514	
X. parietina	Maine	USA	*J. Hinds*	*T. arboricola*	L-26	AJ969521	
X. parietina	Maine	USA	*J. Hinds*	*T. decolorans*	P-28-I-a	AJ969522	
X. parietina	Massachusetts	USA	*V. Ahmadjian*	*T. arboricola*	L-348	AJ969607	
X. parietina	Oslo	N	*T. Tønsberg*	*T. decolorans*	L-16-I-A	AJ969517	
X. parietina	Northamptonshire	GB	*J. J. Pittet*	*T. arboricola*	P-18-I-a	AJ969520	AJ969647
X. parietina	Gotland	S	*S. Scherrer*	*T. decolorans*	P-97-I-a	AJ969539	AM158965
X. parietina	Canary Islands	E	*M. Trembley*	*T. decolorans*	P-104-II-a	AJ969541	AJ969651
X. parietina	Madrid	E	*R. Schönthal*	*T. decolorans*	L-265-II	AJ969577	
X. parietina	Mallorca	E	*M. Trembley*	*T. decolorans*	P-280-II-a-Sc	AJ969583	AJ969660
X. parietina	Mallorca	E	*M. Trembley*	*T. decolorans*	P-281-I-a-Sc	AJ969584	
X. parietina	Mallorca	E	*M. Trembley*	*T. decolorans*	P-282-I-a-Sc	AJ969585	
X. parietina	Paphos	CY	*A. Birchmeier*	*T. arboricola*	P-5-I-a-A	AJ969510	
X. parietina	Keflavik	IS	*J. Achermann & G. Schuwey*	*T. arboricola*	P-198-II-a	AJ969568	
X. parietina	Thingvellir	IS	*J. Achermann & G. Schuwey*	*T. arboricola*	P-210-I-a	AJ969570	AJ969648
X. parietina	Sevan	Armenia	*M. Käppeli*	*T. decolorans*	P-246-I-a-Sc	AJ969574	
X. parietina	Sevan	Armenia	*M. Käppeli*	*T. decolorans*	P-249-I-a	AJ969575	
X. parietina	Bretagne	F	*R. Honegger*	*T. arboricola*	P-7-I-a	AJ969512	AJ969646
X. parietina	Cerdagne	F	*R. Honegger*	*T. decolorans*	P-116-II-b-A	AJ969543	
X. parietina	Roussillon	F	*R. Honegger*	*T. decolorans*	P-120-I-bd	AJ969544	
X. parietina	Roussillon	F	*R. Honegger*	*T. decolorans*	P-121-a1Dark	AJ969547	
X. parietina	Roussillon	F	*R. Honegger*	*T. decolorans*	P-121-a1Light	AJ969548	
X. parietina	Roussillon	F	*R. Honegger*	*T. decolorans*	P-121-II-cd	AJ969550	AM158967
X. parietina	Roussillon	F	*R. Honegger*	*T. decolorans*	P-121-II-gd		AJ969656
X. parietina	Bourgogne	F	*R. Honegger*	*T. decolorans*	P-144-III-bd	AJ969554	

TABLE 1. *Continued*

Lichen Species	Collecting site	Country	Collector	Photobiont sp.*	Isolate†*	Genbank Acc. No. ITS	Genbank Acc. No. rbcL
X. parietina	Bourgogne	F	*R. Honegger*	*T. decolorans*	P-145-I-dj	AJ969559	
X. parietina	Bretagne	F	*R. Honegger*	*T. decolorans*	P-164-I-a	AJ969561	
X. parietina	Bretagne	F	*R. Honegger*	*T. decolorans*	P-164-IX-a-2	AJ969563	
X. parietina	Corsica	F	*L. Walthert & K. Boschi*	*T. arboricola*	P-218-I-a	AJ969573	
X. parietina	Zürich	CH	*S. Nyati & R. Honegger*	*T. decolorans*	P-319-I-g	AJ970889	AM159504
X. parietina	Zürich	CH	*S. Nyati & R. Honegger*	*T. decolorans*	P-319-II-c1	AJ969596	
X. parietina	Zürich	CH	*S. Nyati & R. Honegger*	*T. decolorans*	P-319-IV-c2	AJ969598	
X. parietina	Zürich	CH	*S. Nyati & R. Honegger*	*T. decolorans*	P-320-II-c	AJ969601	
X. parietina	Zürich	CH	*S. Nyati & R. Honegger*	*T. decolorans*	P-320-II-f	AJ969603	AM158963
X. parietina	Zürich	CH	*S. Nyati & R. Honegger*	*T. arboricola*	P-320-III-a	AJ969604	AJ969668
X. parietina	Nekrasova	RUS	*T. Horath*	*T. decolorans*	P-191-I-a	AJ969567	AJ969654
X. parietina	Stavropol	RUS	*K. Bouke*	*T. decolorans*	P-213-I-a	AJ969571	
X. parietina	Cape Point	ZA	*H. Gansner*	*T. decolorans*	L-356	AJ969608	
X. polycarpa (Hoffm.) Th. Fr. ex Rieber[13]	Otago	NZ	*J. Bannister & A. Knight*	*T. arboricola*	P-48-III-a	AJ969526	
X. polycarpa	Oregon	USA	*B. Mc Cune*	*T. decolorans*	P-56-II-a	AJ969531	
X. polycarpa	California	USA	*R. Robertson*	*T. decolorans*	P-71-II-b	AJ969535	
X. polycarpa	Zürich	CH	*R. Honegger*	*T. decolorans*	P-215-I-a	AJ969572	AM158962
Xanthoria sp.	Adelaide	AUS	*M. Federer*	*T. decolorans*	L-184	AJ969566	
Xanthoria sp.	Sevan	Armenia	*M. Käppeli*	*T. decolorans*	L-243-t1	AM159202	
Xanthoria sp.	Tilos	GR	*U. & R. Stidwill*	*T. decolorans*	P-287-VI-b	AJ969586	AJ969649
Xanthoria sp.	Tilos	GR	*U. & R. Stidwill*	*T. decolorans*	P-288-I-a	AJ969587	AJ969650
Xanthoria sp.	Tilos	GR	*U. & R. Stidwill*	*T. decolorans*	P-303-III-a	AJ969589	AJ969667
Xanthoria sp.	Tilos	GR	*U. & R. Stidwill*	*T. decolorans*	P-304-I-a	AJ969590	
Xanthoria sp.	Canary Islands	E	*C. Eichenberger*	*T. arboricola*	L-337	AJ969606	AJ969645
X. sorediata (Vain.) Poelt[14]	Langwies	CH	*S. Scherrer & C. Eichenberger*	*Trebouxia sp.*	L-454-t1	AM159205	
X. turbinata Vain.[15]	Port Nolloth	ZA	*R. Dudler*	*T. arboricola*	P-3-I	AJ969509	AJ969669

* Photobiont species were determined based on ITS and *rbc*L sequence data where available, authorities are mentioned in Table 2; †*P: photobiont isolated, L: whole lichen DNA used for PCR amplification and sequencing where axenic cultures could not be established, followed by voucher number, thallus number and apothecia or lobe number; Sc: single cell isolate, G: lichen specimens obtained from the herbarium of the University of Graz (GZU). [1]syn. *Gallowayella borealis* (R. Sant. & Poelt) S.Y. Kondr. *et al.*; [2]syn. *Oxneria fallax* (Hepp.) S.Y. Kondr. & Kärnefelt; [3]syn. *Gallowayella fulva* (Hoffm.) S.Y. Kondr. *et al.*; [4]syn. *Gallowayella hasseana* (Räsänen) S.Y. Kondr. *et al.*; [5]syn. *Jesmurraya novozelandica* (Hillmann) S.Y. Kondr. *et al.*; [6]syn. *Oxneria ulophyllodes* (Räsänen) S.Y. Kondr. & Kärnefelt; [7]syn. *Honeggeria rosmarieae* (S.Y. Kondr. & Kärnefelt) S.Y. Kondr. *et al.*; [8]syn. *Massjukiella candelaria* (L.) S.Y. Kondr. *et al.*; [9]syn. *Xanthodactylon capense* (Kärnefelt, Arup & L. Lindblom) S.Y. Kondr. *et al.*, 2009; [10]syn. *Rusavskia elegans* (Link) S.Y. Kondr. & Kärnefelt; [11]syn. *Xanthodactylon flammeum* (L. f.) C.W. Dodge; [12]*karrooensis*; [13]syn. *Massjukiella polycarpa* (Hoffm.) S.Y. Kondr. *et al.*; [14]syn. *Rusavskia sorediata* (Vain) S.Y. Kondr. & Kärnefelt; [15]syn. *Xanthodactylon turbinatum* (Vain.) C.W. Dodge.

very small area, but a few are single cell isolates. Approximately 300 sterile photobiont cultures from 12 identified and a few unidentified *Xanthoria* species, seven *Xanthomendoza* and two *Teloschistes* spp. were established. All isolates are stored in liquid nitrogen in our laboratory (Honegger 2003). Reference strains obtained from culture collections are listed in Table 2.

DNA extraction

Genomic DNA was isolated and purified using GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, NJ, USA), following the protocol of the manufacturer with slight modifications. Briefly, algal isolates or lichen samples, respectively, were frozen in liquid nitrogen prior to grinding. After addition of 100 µl of capture buffer to the ground material, the samples were incubated at 60°C for 10 min and subsequently centrifuged. The supernatant was transferred to a GFX column, which had been preloaded with 100 µl of capture buffer, incubated for 3 min at room temperature, centrifuged and washed with 500 µl of washing buffer. The DNA was eluted in 50 µl of elution buffer (10 mM Tris-HCL, pH 8·0) and stored at 4°C.

ITS amplification

The complete internal transcribed spacer region of nuclear ribosomal ITS region (ITS1, 5.8S rDNA and ITS2) of algal isolates was amplified in both directions using primer pair ITS5 and ITS4, as described by White *et al.* (1990). For whole lichen DNA, 1·5 µM of forward primer AL1500bf (Helms *et al.* 2001) and reverse primer LR3 (Friedl & Rokitta 1997) were used in 50 µl reactions containing 1U Taq polymerase (Sigma-Aldrich), 200 µM of each dNTP, 1× PCR buffer containing 1·5 mM $MgCl_2$, 10 mM Tris-HCl pH 8·3, 50 mM KCl, and 0·001% gelatin (final concentrations). Amplifications were run on a PTC 200 DNA engine (MJ Research, Watertown, MA, USA) with the following PCR conditions: initial denaturation at 95°C for 3 min, followed by 32 cycles (94°C for 40 s, 50°C for 40 s, and 72°C for 80 s), with a final extension step at 72°C for 10 min. Internal primers at 5.8S rDNA were newly designed (Table 3). PCR products were purified with GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences), following the standard protocol provided by the manufacturers and sequenced directly.

When direct sequencing did not give satisfactory results, the samples were cloned using pGEM®-T Easy Vector System (Promega Corp., WI, USA) and competent XL10-Gold® *Escherichia coli* cells (Stratagene, CA, USA). Plasmid DNA was isolated using GFX™ Micro Plasmid Prep Kit according to the manufacturer's protocol (Amersham Biosciences).

*rbc*L amplification

Six different primers were newly designed (Table 3) for amplification and sequencing of the large subunit (*rbc*L) of the plastid gene ribulose-1, 5-biphosphate carboxylase/oxygenase. Concentrations of PCR ingredients were the same as in the ITS amplifications. PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles (95°C for 45 s, 52°C for 60 s, and 72°C for 80 s), with a final extension at 72°C for 10 min.

Agarose gel electrophoresis

PCR fragments were run on 1·2% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer at 80 V, stained with ethidium bromide and visualized by a UV transilluminator at 302 nm wavelength. Cut gel fragments were purified with the GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences), following standard protocol provided by the manufacturer.

Sequencing

Purified PCR fragment (10–20 ng DNA) or plasmid (150–300 ng DNA) was used for sequencing in 10 µl reaction mix containing 120 nM primer, 0·8 µl BigDye Terminator Mix V3.1 (Life Technologies, Rotkreuz, Switzerland), and 1× reaction buffer following the protocol of the manufacturer. Amplification conditions were as follows: initial denaturation at 94°C for 2 min, followed by 60 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 3 min (0·9°C/s ramp). The products were analyzed on a HITACHI ABI 3730 DNA Analyzer (Life Technologies).

Phylogenetic analysis

Sequences were analyzed with Sequencher™ 4.2.2 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned automatically with Clustal X 1.81 (Thompson *et al.* 1997), with a gap opening penalty of 10·0 and gap extension penalty of 0·20. Aligned sequences were imported in MacClade 4.06 (Maddison & Maddison 2002) and aligned manually. Phylogenetic analysis was carried out using PAUP 4.0b10 (Swofford 1998) by Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbour-joining (NJ) methods on each locus separately and on a combined dataset containing 39 samples. Ambiguous characters were removed from the analysis. A separate analysis was carried out where missing and ambiguous sites were included, which resulted in a similar phylogram (data not shown). Jackknife values for 500 replicates were calculated separately by MP and NJ analyses. ITS analyses were carried out with complete ITS1, ITS2 and 5.8S rDNA sequences. Intron sequences were cut out from the nrITS alignment since these were present in only 25% of newly generated ITS sequences. In ITS analyses, *T. simplex* sequences were used as outgroup while in *rbc*L analyses, *Asterochloris* sequences were used as outgroup. For the combined analysis, neither *Asterochloris* sp. sequences nor *T. simplex* were available and, hence, a midpoint rooted neighbour-joining tree is shown. The displayed tree is a neighbour-joining tree, constructed with MEGA version 5.1 (Tamura *et al.* 2007) and annotated with support values from PAUP.

TABLE 2. *List of reference* Trebouxia *strains and their ITS and rbcL accession numbers*

Photobiont species*	Strain†*	Lichen species	Collecting site	Genbank Acc. No. rbcL	Genbank Acc. No. ITS	Reference**
Trebouxia de Puymaly						
T. aggregata (Archibald) Gärtner	UTEX 180/IB 325	*Xanthoria* sp. (Fr.) Th. Fr.	Delft, Netherlands	AJ969643	unpublished[1]	This study
T. anticipata Ahmadjian ex Archibald	UTEX 903/IB 340	*Parmelia rudecta* (Ach.) Krog	USA	AJ969638		This study
*T. arboricola** de Puymaly	SAG 219-Ia ‡	Free living?	MA, USA	AM158960	Z68705	Bhattacharya *et al.* (1996)
T. arboricola de Puymaly	M-92.025C1	*Xanthoria parietina* (L.) Th. Fr.	Munich, Germany		AJ007387	Beck *et al.* (1998)
T. asymmetrica T. Friedl & Gärtner	B207	*Toninia sedifolia* (Scop.) Timdal	France		AF344177	Beck *et al.* (2002)
*T. corticola** (Archibald) Gärtner	UTEX 909	Free living?	MA, USA		AJ249566	Friedl *et al.* (2000)
*T. crenulata** Archibald	CCAP 219-2/IB 359	*X. calcicola* Oxner	England	AJ969639	unpublished[1]	This study
*T. decolorans** Ahmadjian	UTEX 901/IB 327	*X. parietina* (L.) Th. Fr.	USA	AJ969657	unpublished[1]	This study
*T. flava** Archibald	UTEX 181/IB 346	*Physconia distorta* (with.) J. R. Laundon	Delft, Netherlands	AJ969637	AF242467	Kroken & Taylor (2000)
*T. galapagensis** (Hildreth & Ahmadjian) Gärtner	UTEX 2230	*Ramalina sp.*	Galapagos Islands		AJ249567	Friedl *et al.* (2000)
*T. gelatinosa** Ahmadjian ex Archibald	UTEX 905/IB 347	*Flavoparmelia caperata* (L.) Hale	USA	AJ969641		This study
T. gelatinosa Ahmadjian ex Archibald	87.072B1	*Punctelia subrudecta* (Nyl.) Krog			AJ249575	Friedl *et al.* (2000)
*T. gigantea** (Hildreth & Ahmadjian) Gärtner	UTEX 2231	*Caloplaca cerina* (Hedw.) Th. Fr.	Ohio, USA		AF242468	Kroken & Taylor (2000)
*T. higginsiae** (Hildreth & Ahmadjian) Gärtner	UTEX 2232/IB 335	*Buellia straminea* Tuck.	Galapagos Islands		AJ249574	This study; Friedl *et al.* (2000)
T. impressa Ahmadjian	87.017E1	*Parmelina carporrhizans* (Taylor) Poelt & Vězda			AJ249570	Friedl *et al.* (2000)
*T. incrustata** Ahmadjian ex Gärtner	UTEX 784	*Lecanora dispersa* (Pers.) Röhl.	USA		AJ293795	Helms *et al.* (2001)
*T. jamesii** (Hildreth & Ahmadjian) Gärtner	UTEX 2233/IB 336	*Schaereria fuscocinerea* (Nyl.) Clauzade & Cl. Roux	England	AJ969663	unpublished[1]	This study
*T. potteri** Ahmadjian ex Gärtner	UTEX 900/IB 332	*Lecanora rubina* (Vill.) Ach.	MA, USA	AJ969635	AF242469	Kroken & Taylor (2000)
*T. showmanii** (Hildreth & Ahmadjian) Gärtner	UTEX 2234/IB 337	*Lecanora hageni* (Ach.) Ach.	USA	AJ969661	AF242470	Kroken & Taylor (2000)
T. simplex Tscherm.-Woess	TW-1A2	*Chaenotheca chrysocephala* (Turner ex Ach.) Th. Fr.	Austria		unpublished[1]	

TABLE 2. *Continued*

Photobiont species*	Strain†*	Lichen species	Collecting site	Genbank Acc. No. rbcL	Genbank Acc. No. ITS	Reference**
Asterochloris (Tscherm.-Woess) T. Friedl (*ined.*) (isolates are kept under the genus name *Trebouxia* de Puymaly in culture collections)						
*A. erici** (Ahmadjian) T. Friedl (ined.)	UTEX 910/IB 342	*Cladonia cristatella* Tuck.	USA	AJ969631		This study
A. erici	UTEX 912	*Cladonia cristatella* Tuck.	MA, USA		AF345441	Piercey-Normore & DePriest (2001)
*A. excentrica** Archibald	UTEX 1714/IB 345	*Stereocaulon dactylophyllum* Flörke	USA	AJ969629		This study
A. glomerata (Ahmadjian) T. Friedl (ined.)	UTEX 894/IB 349	*Stereocaulon evolutoides* (H. Magn.) Frey	MA, USA	AJ969633		This study
A. glomerata	UTEX 897	*Stereocaulon pileatum* Ach.	Princeton, USA		AF345405	Piercey-Normore & DePriest (2001)
*A. italiana** (Archibald) T. Friedl (ined.)	CCAP 219-5b/IB 358	*X. parietina* (L.) Th. Fr.	Italy	AJ969632		This study
*A. magna** (Archibald) T. Friedl (ined.)	UTEX 67	*Cladonia* sp.	Delft, Netherlands		AF345423	Piercey-Normore & DePriest (2001)
A. magna	UTEX 902/IB 354	*Pilophorus acicularis* (Ach.) Th. Fr.	USA	AJ969630		This study
*A. pyriformis** (Archibald) T. Friedl (ined.)	UTEX 1713/IB 356; UTEX 1712/IB 355	*Stereocaulon pileatum* Ach.; *Cladonia squamosa* (Scop.) Hoffm.	USA	AJ969634	AF345407	Piercey-Normore & DePriest (2001)

* type strains are indicated with an asterisk; †*UTEX: Algal Culture Collection at University of Texas; IB: Algal Culture Collection at University of Innsbruck; SAG: Algal Culture Collection at University of Göttingen; other isolates are in private culture collections; ‡Type species of the genus *Trebouxia* de Puymaly; **references applicable only for nrITS sequences already published, all *rbc*L sequences were generated in the present study; [1]ITS sequences generated by Thomas Friedl or Gert Helms, who kindly provided access to their unpublished sequence data for comparison.

TABLE 3. *List of primers used in the present study.*

Primer (orientation)	Sequence (5′–>3′)	Target locus	Position*	Reference
ITS4 (rev)	TCCTCCGCTTATTGATATGC	LSU	1-18 (Z95381)	White *et al*. (1990)
ITS5 (fwd)	GGAAGTAAAAGTCGTAACAAGG	SSU	2072-2093 (Z68705)	White *et al*. (1990)
AL1500bf (fwd)	GATGCATTCAACGAGCCTA	SSU	1800-1818 (Z68705)	Helms *et al*. (2001)
LR3 (rev)	CCGTGTTTCAAGACGGG	LSU	591-607 (Z95381)	Friedl & Rokitta (1997)
TreSeq1 (fwd)	CAACTCTCAACAACGGATATC	5.8 s nrDNA	2859-2879 (Z68705)	This study
TreSeq2 (rev)	GACGCTGAGGCAGACATGCTC	5.8 s nrDNA	2992-3012 (Z68705)	This study
TreSeq3 (rev)	CCGAAGCCTCGAGCGCAATTT	5.8 s nrDNA	2967-2987 (Z68705)	This study
rbcLfwd (fwd)	GAMACTGATATTCTTCTTGCAGC	*rbc*L	59-74 (AF189069)	This study
rbcLrev (rev)	GCAGCTAATTCAGGACTCCA	*rbcL*	1314-1331 (AF189069)	This study
rbcL1 (fwd)	CGTGGTGGTTTAGATTTTAC	*rbc*L	543-562 (AF189069)	This study
rbcL2 (rev)	ATTTGCGTTGACGACCATGA	*rbc*L		This study
rbcL3 (rev)	ATTTACGTTGTCGTCCATGT	*rbc*L		This study
rbcL4 (fwd)	GCAGCDTTYCGTATGACTCCTCAA	*rbc*L	86-109 (AF189069)	This study

* The position of the primers given with respect to a reference sequence (accession number given in brackets)

Results

Isolation and culturing

In the course of ongoing projects, *c.* 300 photobiont isolates were obtained from most of the freshly collected lichen specimens, with or without prior storage at −20°C. On non-nutrient mineral medium (BBM) all isolates grew well, albeit more or less slowly, and kept their green colouration. There was no evidence of bleaching under the light conditions provided, nor of any dependence on external nutrient supply, as suggested by Ahmadjian (1960, 2002*a*, *b*). In our laboratory, the type strain of *T. decolorans* retained its colour with the same intensity after four months culturing on either BBM 2N or *Trebouxia* ¼ media. Different growth rates were observed among different isolates, partly even among isolates from samples collected next to each other (e.g. among isolates 319 and 320). Only one algal isolate was normally taken per lichen sample. All except one isolate were phenotypically homogenous, and RAPD-PCR analyses of diverse subsamples per isolate turned out to be homogenous (five subsamples each of five isolates tested with three primers, data not shown). However, two phenotypically different isolates (P-121-I-a, either light green, or dark green to brownish) were obtained from the same apothecium of a *X. parietina* sample. As concluded from ITS phylogenetic analyses, both isolates represented different genotypes of the same algal species (*T. decolorans*) (see Fig. 1).

ITS phylogeny

A total of 101 photobiont nrITS sequences were obtained in this study, originating from 12 *Xanthoria* species, seven *Xanthomendoza* species, two *Teloschistes* species, *Josefpoeltia parva* and 10 unnamed *Xanthoria* and *Xanthomendoza* samples. A total of 781 characters were included in nrITS (ITS1, 5.8S rDNA and ITS2) phylogenetic analyses, 358 of which were constant, 118 were variable but uninformative, and 305 were parsimony informative. Primer binding sites and adjacent flanking regions were omitted from the analyses. Tree topologies for main clades were identical in ML, MP and NJ analyses. Only one most likely tree resulted in ML analyses (Fig. 1). In 30 out of 101 ITS sequences, a longer ITS fragment was found due to a group I intron at position 1512 as described by Bhattacharya *et al.* (1996, 2002) and Helms *et al.* (2001). Intron sequences were removed from ITS alignments prior to phylogenetic analysis and investigated in a separate study (Nyati *et al.* 2013*b*).

The major clades in both phylogenies were in accordance with the *Trebouxia* clade system as proposed by Beck (2002) and Helms (2003). In their system, clade A includes *T. arboricola* (including *T. aggregata* and *T. crenulata*), *T. decolorans*, *T. asymmetrica*, *T. showmanii*, *T. incrustata* and *T. jamesii*. In the present study, clade A was subdivided into an *arboricola* cluster (subclades Aa and Ab) and a *decolorans* cluster (subclades Ac and Ad); unnamed *Trebouxia* species formed subclade Ae. Clade I comprised the *impressa* (subclade Ia) and *gelatinosa* (subclade Ib) clusters. Photobionts of all identified and unidentified *Xanthoria* spp. analyzed in this study belonged either to *T. decolorans*, *T. arboricola* or closely related, unnamed *Trebouxia* sp. within clade A (Fig. 1). The best represented photobionts in our sample set were from associations with *X. parietina*, with 49 ITS sequences from specimens collected on 4 continents. Photobionts of *Xanthomendoza* species belonged to either the *arboricola* (A) or *impressa* (I) clades, but none of the *Xanthomendoza* species associated with both.

Photobionts of eight identified and four unnamed *Xanthoria* species were represented in the *arboricola* cluster (subclades Aa and Ab), which is characterized by a 28 nucleotides long insert within ITS1 (Helms *et al.* 2001). Subclade Aa has jackknife support of 87% (MP) and 92% (NJ). It includes the type species of the genus, *Trebouxia arboricola* (strain SAG 219-I-a, arrowhead), *T. aggregata* (UTEX 180) and photobiont isolates of *X. calcicola*, *X. aureola*, *X. ligulata*, *X. parietina* and an unnamed *Xanthoria* species, phenotypically resembling *X. parietina* (L-337, Canary Islands). Subclade Ab of the

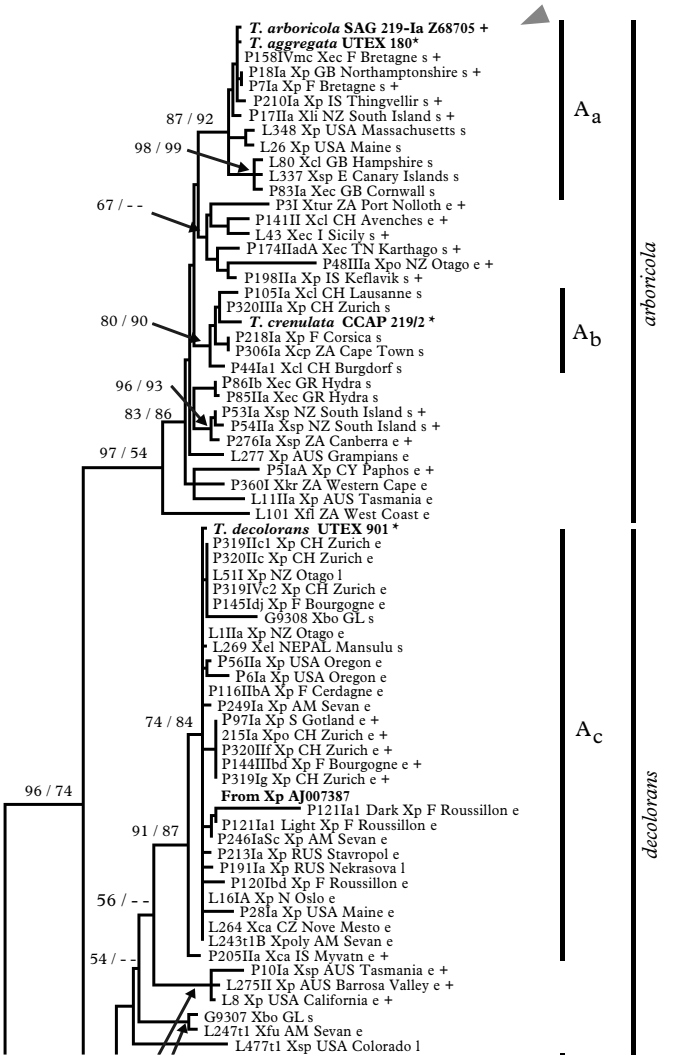

FIG. 1. ML phylogram of nrITS region (combined ITS1, ITS2 and 5.8S rDNA). Jackknife values calculated separately for 500 replicates by MP (first number) and NJ (second number) analyses and indicated at the nodes. *Trebouxia simplex* sequences were used as outgroup. Arrowhead points to the type species of the genus *Trebouxia* de Puymaly. Samples were labelled as follows: example P 270 I a Telochry E Canary Islands e : P, sterile cultured photobiont of *Teloschistes chrysophthalmus*, voucher number 270, thallus I, apothecium a, collected from Spain (E), Canary Islands, epiphytic (e). Abbreviations used: ***Xanthoria***: *Xca: X. candelaria, Xcl: X. calcicola, Xcp: X. capensis*, *Xec: X. aureola*, *Xel: X. elegans*, *Xfl: X. flammea*, *Xkr: Xanthoria karrooensis; Xli: X. ligulata*, *Xp: X. parietina*, *Xpo: X. polycarpa*, *Xsp:* unidentified *Xanthoria* or *Xanthomendoza* sp., *Xtu: X. turbinata*. ***Xanthomendoza***: *Xbo: Xanthomendoza borealis*, *Xfa: Xm. fallax*, *Xfu: Xm. fulva*, *Xha: Xm. hasseana*, *Xnovo: Xm. novozelandica*, *Xul: Xm. ulophyllodes*, *Xweb: Xm. weberi;* synonyms see Table 1. ***Teloschistes***: *Telochry: Teloschistes chrysophthalmus*, *Telohos: Telo. hosseusianus.* ***Josefpoeltia***: *Jb: Josefpoeltia parva* (syn. *J. boliviensis)*. Letters A, C, I & S indicate *Trebouxia* clades (A: *arboricola*; C: *corticola*; I: *impressa*; S: *simplex*) as proposed by Helms (2003). P: photobiont isolated; L: whole lichen DNA used for amplification; e: epiphytic; s: saxicolous; l: lignicolous/ corticolous; +: sequence contained a 1512 intron. Sequences obtained from databases are in bold and indicated with strain number and accession number; *: unpublished sequence provided by G. Helms. Arrowhead points to type species of the genus.

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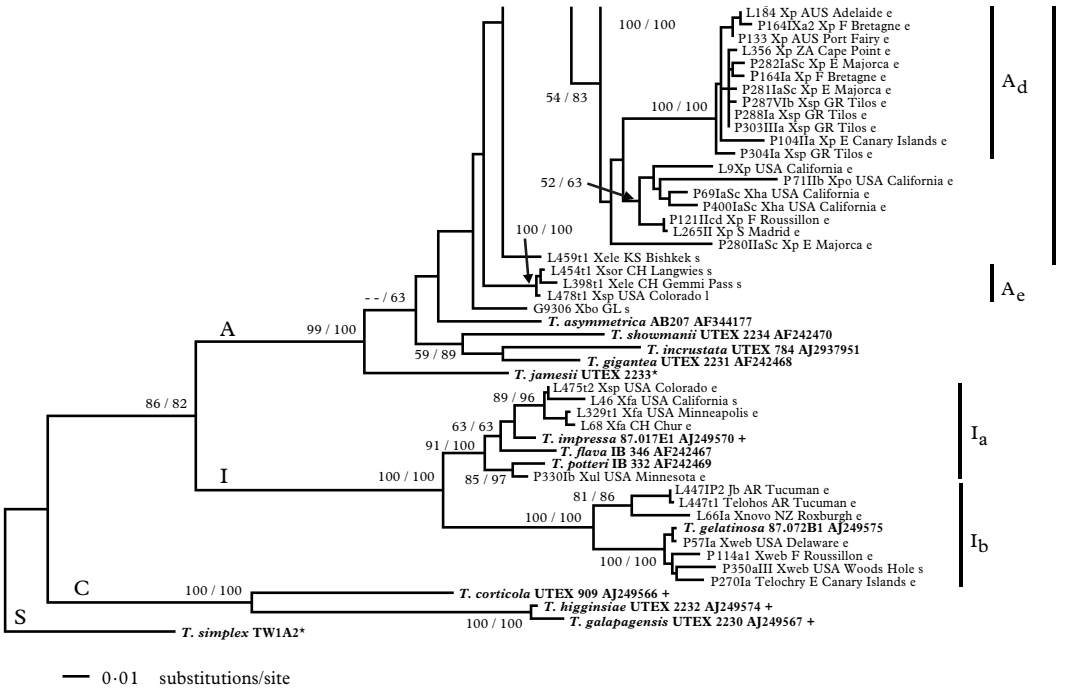

FIG. 1. *Continued*

arboricola cluster includes *T. crenulata* (strain CCAP 219/2), photobionts of *X. calcicola, X. parietina*, and of the South African endemic species *X. capensis*. Photobiont isolates of *X. turbinata*, *X. calcicola*, *X. aureola*, *X. polycarpa* and *X. parietina* formed a cluster which was only weakly supported in MP analysis and not supported by NJ analysis. Photobionts of unnamed *Xanthoria* species from New Zealand and Australia formed a small cluster with high support (96% in MP, 93% in NJ), although photobionts from other *Xanthoria* species in this region fall in separate lineages. Photobionts of *X. flammea* (ZA), *X. aureola*, *X. karrooensis* and *X. parietina* formed unresolved basal lineages. Subclade Ac, being part of the *decolorans* cluster, has high jackknife support in MP and NJ analyses (91% and 87% respectively). It includes *T. decolorans* (UTEX 901) and photobiont sequences of *X. parietina* (CH, NZ, F, RUS, S, USA), *X. candelaria* (CZ, IS), *X. elegans* (Nepal), *X. polycarpa* (Armenia, CH, USA) and *Xm. borealis* (Greenland). The ITS sequence of the *X. parietina* photobiont identified as *T. arboricola* (AJ007387) by Beck *et al.* (1998) also falls in this subclade. Subclade Ad, also being part of the *decolorans* cluster, is very well supported (100%). It comprised photobiont sequences of four unidentified *Xanthoria* species clustering within the *X. parietina* complex (GR), along with photobiont sequences of *X. parietina* s. str. (AUS, F, E, USA & ZA). The phylogenetic position of several photobionts, which cluster outside subclades Ac and Ad, could not be properly resolved. This part of the tree includes the photobionts of *Xanthomendoza fulva* (Armenia), *Xm. hasseana* (USA), *Xm. borealis* (Greenland), *X. candelaria* (IS), *X. parietina* (AUS, F, E and USA), *X. polycarpa* (USA), and unidentified *Xanthoria* species (AUS, USA). Subclade Ae has a high jackknife support in both ML and NJ analyses (100%) and includes photobiont sequences of *X. elegans* (CH), *X. sorediata* (CH), and an unidentified

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Xanthomendoza sp. (USA). This ITS subclade Ae most likely represents a cryptic *Trebouxia* sp. The exact phylogenetic positions of photobionts of *Xanthomendoza borealis* (G 9306; Greenland) and *Xanthoria elegans* (KS) could not be resolved. Two morphologically different thalli of *Xm. borealis*, a narrow and a broad-lobed specimen growing side by side, which were collected at the same locality in Greenland, had different *T. decolorans* genotypes from different subclades (Fig. 1); their fungal partners turned out not to be conspecific (Eichenberger 2007).

Subclade Ia has a very high jackknife support (91% in MP, 100% in NJ; Fig. 1). This might be partly due to the small sample size. It includes *T. impressa*, *T. potteri*, *T. flava* and photobiont sequences of *Xanthomendoza fallax* (CH, USA), *Xm. ulophyllodes* (USA) and an unidentified *Xanthomendoza* sp. (USA). Helms (2003) found the authentic strain of *T. impressa* (UTEX 893) to be very similar to *T. potteri* (UTEX 900) and most probably conspecific with *T. flava* (UTEX 181), as inferred from ITS p-distances. Therefore, all our isolates in this cluster are referred to as *T. impressa*. The well-supported (100%) *gelatinosa* cluster (subclade Ib) comprises two separate groups, one of them harbouring the type strain of *T. gelatinosa* with photobiont sequences from *Teloschistes chrysophthalmus* (E), and *Xanthomendoza weberi* (F, USA). A sister clade, also with high support, comprised photobiont isolates of *Xm. novozelandica* (NZ), *Teloschistes hosseusianus* (Argentina) and *Josefpoeltia parva* (Argentina). *Teloschistes hosseusianus* and *Josefpoeltia parva* grew side by side and were locally overgrowing each other; it is interesting to see that they associate with largely the same photobiont (one nucleotide difference).

*rbc*L phylogeny

A total of 1155 characters were included in phylogenetic analyses of the *rbc*L gene, 925 of which were constant, 34 variable but uninformative, and 196 were parsimony informative. ML (Fig. 2), MP and NJ analyses resulted in similar tree topologies. *Asterochloris* sequences formed an outgroup. The *rbc*L phylogeny was largely congruent with ITS phylogeny. *Trebouxia arboricola* (SAG 219-Ia) and *T. aggregata* (UTEX 903) were part of subclade Aa (bootstrap support 99%) while *T. crenulata* (CCAP-219-2) was part of subclade Ab, as was also the case in the ITS phylogram. The *rbc*L clade Ab had very low (52% in NJ) or no jackknife support (MP). Photobiont isolates of *X. flammea* (ZA), *X. aureola* (GR) and of an unidentified *Xanthoria* sp. (NZ) fell outside subclade Ab. Deduced amino acid sequences within subclade Aa were identical and differed from subclade Ab sequences only marginally (data not shown). Subclade Ac with low support (53% MP, 64% NJ) comprised *T. decolorans* (UTEX 901) along with isolates which were also part of subclade Ac in the ITS phylogeny. Subclade Ad, which is highly supported in the ITS phylogram had low support ($<50\%$) in *rbc*L phylogeny (dotted line). The photobiont isolates of *Xanthomendoza fallax* (CH, USA) and *Xm. ulophyllodes* (USA) clustered with *T. impressa, T. potteri* and *T. flava* in subclade Ia, which was very well supported (100%). All *rbc*L sequences in subclade Ib, including type strains *T. gelatinosa* and *T. anticipata*, were nearly identical. Six representatives of the genus *Asterochloris* formed the outgroup.

Combined phylogeny

A total of 1921 characters were included in the combined phylogenetic analysis of ITS and *rbc*L. ML and NJ analyses resulted in similar topologies, containing the clades Aa and Ab belonging to *T. arboricola* and clades Ac and Ad belonging to *T. decolorans*, as well as clades Ia and Ib (Fig. 3). As in the separate analyses for each locus, *Trebouxia arboricola* (SAG 219-Ia) was part of subclade Aa whereas *T. potteri* (IB 332) and *T. flava* (IB 346) belonged to subclade Ia. The position of *T. showmanii* (UTEX 2234) in the tree was associated with a high degree of uncertainty, as indicated by lack of support. Clade I, subclade Ad and subclade Ib were well supported (100% NJ, 100% ML), as was subclade Ia (100% NJ, 95% ML). In contrast, subclade Ab received no statistical support in the combined analysis, and clade A

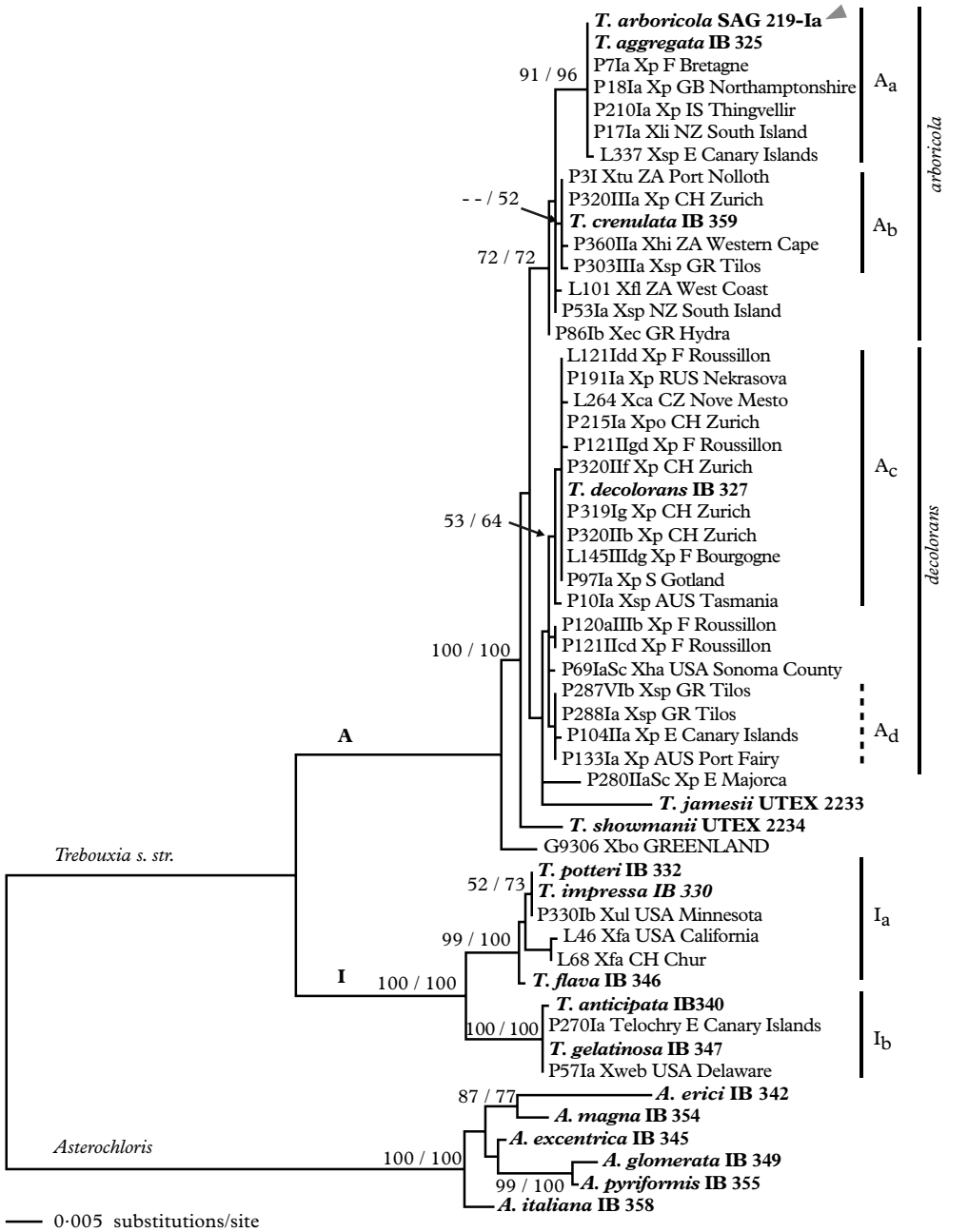

FIG. 2. ML phylogram of *rbc*L locus. Jackknife values calculated separately for 500 replicates by MP (first number) and NJ (second number) analyses are given at the nodes. *Asterochloris* sequences form the outgroup. Abbreviations used: ***Xanthoria***: *Xca: X. candelaria, Xec: X. aureola, Xfl: X. flammea, Xli: X. ligulata, Xp: X. parietina, Xpo: X. polycarpa, Xtu: X. turbinata, Xsp:* unidentified *Xanthoria* or *Xanthomendoza sp.* ***Xanthomendoza****: Xbo: Xanthomendoza borealis, Xfa: Xm. fallax, Xha: Xm. hasseana, Xul: Xm. ulophyllodes, Xweb: Xm. weberi;* ***Teloschistes****: Telochry: Teloschistes chrysophthalmus.* A, C and I indicate *Trebouxia* clades as proposed by Helms (2003). P: photobiont isolated; L: whole lichen DNA used for amplification.

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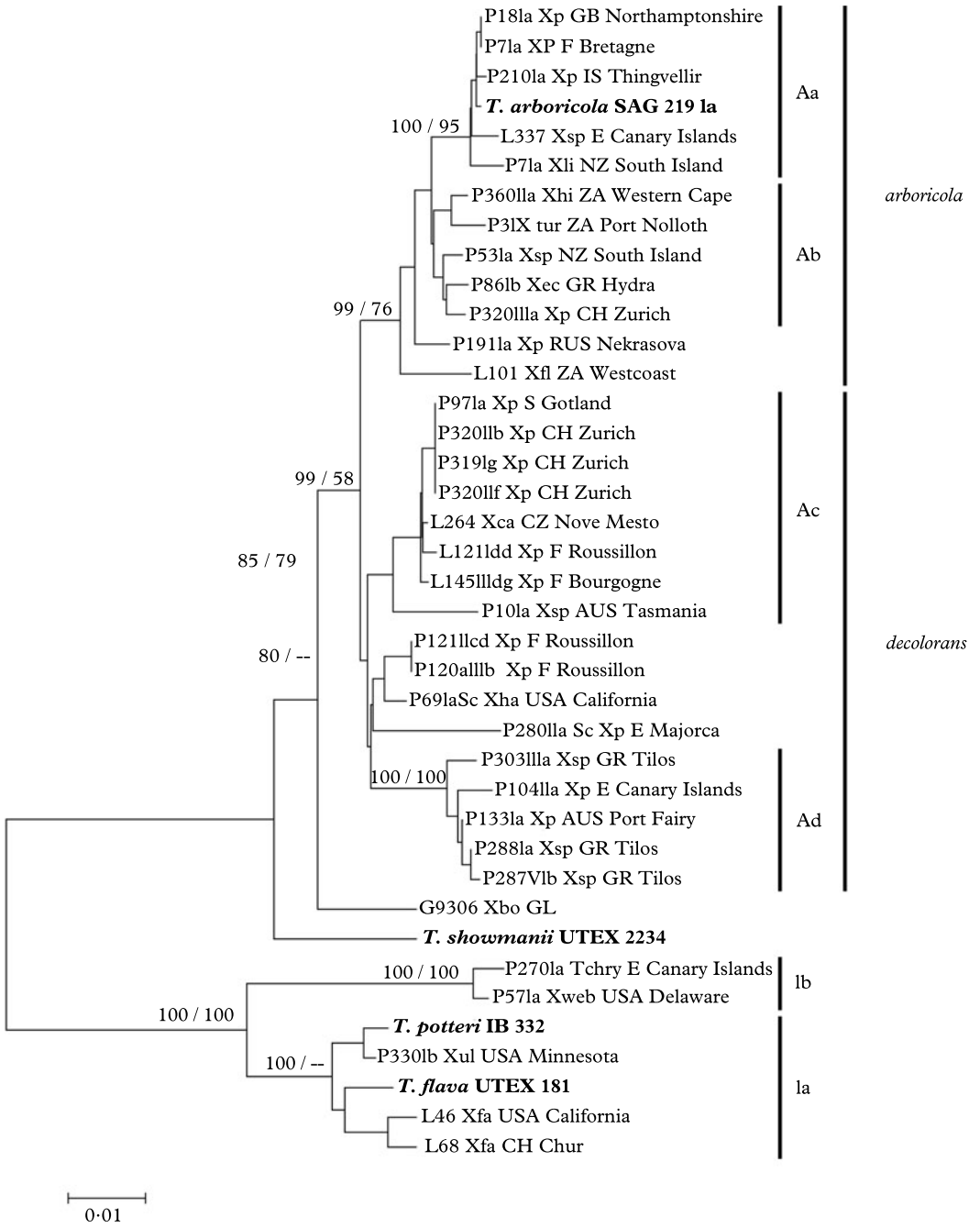

FIG. 3. NJ tree of the combined phylogenetic analysis of ITS and *rbc*L loci. A total of 1921 characters were included in the analysis. For the combined analysis, neither *Asterochloris* sp. sequences nor *T. simplex* was available and hence, a midpoint rooted neighbour-join tree is shown. ML and NJ analyses resulted in similar topologies.

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had high support in NJ (99%) but low support in ML (58%). The *arboricola* clade had relatively high support (99% NJ, 76% ML), but the *decolorans* clade received support only in NJ jacknifing (80% NJ, 0% ML).

Discussion

Photobionts of the *Teloschistaceae*

All foliose (*Xanthoria*, *Xanthomendoza*) and fruticose (*Teloschistes*) *Teloschistaceae* investigated in the present study associated with *Trebouxia* species belonging either to ITS clade A or I *sensu* Helms (2003), or *rbc*L clades A or I, respectively (Figs 1 & 2). None of the *Xanthoria*, *Xanthomendoza* and *Teloschistes* species associated with photobionts from two clades, and none of the samples had photobionts of ITS clades S or C or of the genus *Asterochloris*. The present findings refer to a moderate specificity at genus level within foliose and fruticose *Teloschistaceae*, species of few subclades of the same *Trebouxia* clade being acceptable partners. It has to be admitted that the sample size was very small in many of the taxa examined. The range of compatible photobionts in representatives of the genera *Teloschistes* and *Josefpoeltia* remains unclear, *Trebouxia gelatinosa* (clade Ib) being the only green-algal partner so far found associated with these taxa. Other studies have found the same algal species in association with *Teloschistes* (Reis *et al.* 2005; Werth 2012). Only one freshly collected specimen per species was available for South African endemic species, hence the range of compatible photobionts remains unclear for these taxa. It would be interesting to investigate photobiont diversity among crustose *Teloschistaceae* (genera *Caloplaca*, *Ioplaca* etc.). Two studies have investigated the photobionts of *Caloplaca* spp. In northern Chile, *Caloplaca* associated with three species of *Trebouxia* (*T. arboricola, T. decolorans*, and *T. gigantea*) (Vargas Castillo & Beck 2012), and in Antarctica, a *Caloplaca* sp. was found in association with three ITS haplotypes, but the species was not determined (Pérez-Ortega *et al.* 2012). Crustose taxa of lichen-forming ascomycetes turned out to be less specific than foliose and fruticose ones at either the generic (Tibell 2001; Tibell & Beck 2002) or species level (Beck 2002; Helms 2003; Blaha *et al.* 2006). Also, the habitat may influence photobiont selectivity: in extreme climates, lichen-forming fungi tend to associate with a wide range of photobionts (Romeike *et al.* 2002; Wirtz *et al.* 2003; Muggia *et al.* 2008). No co-speciation is evident in the present data set. This is not surprising as the photobionts of *Teloschistaceae* are also partners of numerous other lichen-forming ascomycetes. A similar situation was found in *Cladoniaceae* (Piercey-Normore & DePriest 2001) and *Physciaceae* (Helms 2003), but Dahlkild *et al.* (2001) reported co-speciation for *Physciaceae*.

In studies on green-algal phylogenies, the *rbc*L locus, which encodes for the large subunit of Rubisco (ribulose-1,5-biphosphate-decarboxylase), was shown to be a highly suitable molecular marker (McCourt *et al.* 1995; Nozaki *et al.* 1997, 2002; Sherwood *et al.* 2000). The same applies for green-algal photobionts of lichen-forming ascomycetes, as shown in the present study. Rubisco was located in the pyrenoid of *Trebouxia* species with immunocytochemical techniques (Ascaso *et al.* 1995). In *Trebouxia decolorans* photobionts associated with *Ramalina menziesii*, *rbcL* turned out to be variable at the population level (Werth & Sork 2010).

Comparison of morphological and molecular data sets

Comparisons of morphological data, as compiled by Friedl (1989), and molecular data within the genera *Trebouxia* and *Asterochloris* (present study) are summarized in Table 4. ITS clade A, comprising most of the photobionts of *Teloschistaceae* investigated in this study, includes *Trebouxia* species from several morphological groupings. The morphology of the samples genetically identified in this study will have to be analyzed in future investigations. The ITS sequence data obtained by Helms *et al.* (2001), Helms (2003) and in the present study indicate that *T. crenulata* and *T. aggregata* are

TABLE 4. *Comparison of molecular markers and morphological characters in the genera* Trebouxia *and* Asterochloris *(Trebouxiophyceae, Chlorophyta)*

Phylogeny (present study)				Fine structure & morphology *sensu* Friedl (1989), modified				
ITS clade Helms (2003)	ITS clade present study	*rbc*L clade present study	Photobiont species	Arrangement of thylakoids	Pyrenoid type	Chloroplast shape	Cell shape	Cell cycle
Trebouxia s. str.†								
A7	A	n.d	*T. asymmetrica*	I	gi	6	ovoid	A
A9	A	n.d	*T. gigantea*	I	gi	6	ovoid	A
A8	A	n.d	*T. showmanii*	I	gi	6	ovoid	A
A10	A	n.d	*T. incrustata*	I	gi	6	ovoid	A
A2	Aa	Aa	*T. aggregata*	I	ar	3	globose	A
A2	Aa	Aa	*T. arboricola*	I	ar	3	globose	A
A2	Ab	Ab	*T. crenulata*	I	ar	4*	ovoid	A
A1	Ac	Ac	*T. decolorans*	I	ar	4*	globose	A
I1	Ia	Ia	*T. flava*	I	im	1	globose	B
I1	Ia	Ia	*T. impressa*	I	im	1	globose	A
A4	A	A	*T. jamesii*	I	im	2	globose	A
S3	S	n.d	*T. simplex*	I	im	2	globose	A
I1	Ia	Ia	*T. potteri*	I	im	5	globose	A
n.d	n.d	Ib	*T. anticipata*	I	ge	7	globose	B
I2	Ib	Ib	*T. gelatinosa*	I	ge	7	globose	B
C1**	C	n.d	*T. corticola*	I	co	9	globose	A
C2**	C	n.d	*T. galapagensis*	I	co	9	globose	A
C2**	C	n.d	*T. higginsiae*	I	co	9	globose	A
C1**	n.d	n.d	*T. usneae*	I	co	8	globose	B
Asterochloris (Tscherm.-Woess) T. Friedl (ined.) (Rambold *et al.* 1998)								
n.d		outgroup	*A. magna*	I	ma	12	ovoid	B
n.d		outgroup	*A. excentrica*	II	ir	11	ovoid	B
n.d		outgroup	*A. glomerata*	II	ir	10	ovoid	B
n.d		n.d.	*A. irregularis*	II	ir	10	ovoid	B
n.d		outgroup	*A. italiana*	II	ir	10	ovoid	B
n.d		outgroup	*A. pyriformis*	II	ir	10	ovoid	B
n.d		outgroup	*A. erici*	II	er	10	ovoid	B

† authorities given in Table 2, *chloroplast shape distinctly different in *T. crenulata* and *T. decolorans* (Gärtner 1985*b*). **termed G in Helms (2003), now changed into C ("*corticola*"; G. Helms, pers. comm.)

conspecific with *T. arboricola*. Peršoh *et al.* (2004) consider *T. arboricola* synonymous with *T. decolorans*. As both are morphologically distinguishable by the shape of their chloroplast (Gärtner 1985*b*; Friedl 1987) and cluster within different ITS and *rbc*L subclades, both species names were retained in the present investigation. Some authors refer automatically to *T. arboricola* when ITS sequences fall into clade A *sensu* Helms (2003). Morphospecies names given to taxa among the genera *Trebouxia* and *Asterochloris* need to be revised in future studies, based on additional genetic and morphological data.

A wide range of algal genotypes was found in each ITS and *rbc*L subclade, comparable to the situation among photobionts of the genera *Letharia* (Kroken & Taylor 2000; Altermann 2009), *Cladonia* (Piercey-Normore 2004), *Evernia* (Piercey-Normore 2006), *Ramalina* (Werth & Sork 2010; Francisco De Oliveira *et al.* 2012) or *Parmotrema* (Ohmura *et al.* 2006). Studies based on microsatellite markers yielded similar results for *Lobaria* (Dal Grande *et al.* 2012; Werth & Scheidegger 2012; Widmer *et al.* 2013). It is interesting to see that identical algal ITS genotypes occurred in the same or even in

different *Xanthoria* spp. from geographically different locations; examples are the photobionts of *X. parietina* from Corsica and of *X. capensis* from South Africa (subclade Ab; Fig. 1), of *X. parietina* from Otago (NZ), Zürich (CH) and Burgundy (F) (subclade Ac; Fig. 1), or of *X. polycarpa* from Zürich, *X. parietina* from Zürich (CH), Götland (S) and Burgundy (F) (subclade Ac; Fig. 1). On the other hand, *X. parietina* thalli collected side by side (populations 144 & 145 from Roussillon, SW France, 120 & 121 from Burgundy, France, and 319 & 320 from Zürich, Switzerland) had partly the same, partly different ITS genotypes of the same subclade (Fig. 1). RAPD-PCR analyses of the sterile cultured fungal partners revealed considerable genetic variation within the populations (populations 120 & 121, 144 & 145, 319 & 320 plus 164 from Brittany analyzed; Itten & Honegger 2010).

Photobionts of the genus *Xanthoria*

All *Xanthoria* species investigated in the present study associated with photobionts of ITS clade A *sensu* Helms (2003) (Fig. 1). No clear geographical pattern can be seen in the present data set, but *T. decolorans* (subclades Ac and Ad) was almost exclusively found in epiphytic samples (marked with e in Fig. 1), whereas *T. arboricola* occurred in saxicolous specimens (forming subclade Aa) in the Northern and Southern Hemispheres and in many of the corticolous samples in the Southern Hemisphere (subclade Ab). The *T. arboricola* photobiont of saxicolous *X. parietina*, growing under a willow tree in Zürich, was more closely related to the photobiont of a saxicolous *X. parietina* from Corsica than to the *T. decolorans* genotypes isolated from corticolous samples on the respective willow tree. *Xanthoria candelaria* (CZ, IS) associated with *T. decolorans*, whereas Aoki *et al.* (1998), using microscopy techniques, identified *T. incrustata*, another representative from clade A *sensu* Helms (2003), from a sample collected in Antarctica. *Fulgensia fulgida* was shown to associate with *T. asymmetrica* (Beck *et al.* 2002), another representative of ITS clade A.

Photobionts of *Xanthoria parietina* s. lat.

Early investigators had already discovered a range of phenotypically different strains among *Trebouxia* isolates derived from thalli of *X. parietina*, which they interpreted as ecotypes (Thomas 1939; Werner 1954; Tomaselli 1956). Our present findings are in agreement with earlier reports, based on light and electron microscopic as well as molecular investigations, on *T. arboricola*, *T. decolorans* and *T. crenulata,* all members of ITS clade A *sensu* Helms (2003) and partly conspecific, being photobionts of *X. parietina* s. lat. (Ahmadjian 1960; Gärtner 1985*b*; Honegger & Peter 1994; Beck *et al.* 1998), *X. calcicola* and *X. aureola* included (Scherrer & Honegger 2003).

Asterochloris photobionts are found in *Cladoniaceae* (Rambold *et al.* 1998; Peršoh *et al.* 2004; Yahr *et al.* 2004, 2006) and in *Stereocaulaceae* (Peksa & Skaloud 2011), whereas most foliose *Lecanorineae* and *Teloschistineae* select *Trebouxia* spp. as photobiont. Nevertheless, there are some reports, based on microscopic investigations, of *Asterochloris* photobionts among *Parmeliaceae* (summarized by Rambold *et al.* 1998); these deserve re-investigation with molecular tools. *Asterochloris* species were reported twice as photobionts of *X. parietina*, which was postulated to reveal low specificity (Ahmadjian 2002*b*). *Asterochloris italiana* was originally isolated from an Italian sample (Tomaselli 1956) as *Cystococcus Xanthoriae parietinae*. No details are given on isolation techniques, nor is a voucher deposited. One out of Tomaselli's three different photobiont isolates, originating from three different *X. parietina* specimens, was kept in the Cambridge Culture Centre (CCC) as *T. decolorans*. It became the type strain of *A. italiana* (sub *Trebouxia italiana*), the cells of which are mentioned to be multinucleate (Archibald 1975). Peršoh *et al.* (2004) speculate in this particular case on confusion of strains. The fate of this type species cannot be reconstructed. Ahmadjian (2002*b*) mentioned *A. irregularis* (sub *Trebouxia irregularis*) as photobiont of *X. parietina*,

without giving any further details. The few *Xanthoria parietina* specimens investigated, and the phenotypically very similar but phylogenetically different *Xanthoria* samples from Australia, Tasmania and New Zealand, all corticolous, had photobiont genotypes either from subclade Ab (*T. arboricola*), Ad (*T. decolorans*) or from the assembly of *T. decolorans* genotypes which fall between subclades Ac and Ad. The genetic diversity of some of the corresponding fungal partners was studied with fingerprinting techniques (RAPD-PCR applied to sterile cultured single- or multispore-isolates, Honegger *et al.* 2004). These data suggest a relatively high similarity of Australian *X. parietina* with samples from the Western Mediterranean, including the Balearic and Canary Islands. The photobiont of *X. parietina* from Port Fairy, Australia (voucher no. 133) falls in subclade Ad, which comprises an interesting assembly of *Trebouxia decolorans* genotypes isolated from corticolous samples growing in coastal areas from Brittany to Majorca, Canary Islands, Greek Islands, South Africa and South-Eastern Australia. The mycobiont of an unnamed epiphytic *Xanthoria* species from Canberra (AUS; voucher no. 276), which is morphologically similar to *X. parietina*, was strongly dissimilar and formed an outgroup in the fingerprinting experiments (Honegger *et al.* 2004); its *Trebouxia* photobiont was found in the unresolved part of the "*arboricola* cluster" (Fig. 1).

Algal theft by *Xanthoria* spp. from *Physcia* species?

Based on the assumption of scarcity of free-living *Trebouxia* photobionts outside lichen thalli, Ott (1987*a*, *b*; Ott *et al.* 2000) addressed the question of how germinating ascospores of the always richly fertile *X. parietina* and *X. polycarpa*, both with no vegetative symbiotic propagules, acquire a compatible photobiont. She postulated temporary association of *Xanthoria* germlings with ultimately incompatible green-algal cells and/or invasion by ascospore-derived germ tubes into the thalli of adjacent *Physcia* spp. (Lecanorineae, Lecanoromycetes), theft of their *Trebouxia* photobiont and subsequent development of a brightly yellow-coloured thallus on or within the grey *Physcia* thalli. However, upon careful dissection, presumed chimaerae of *X. parietina* and *Physcia tenella* and/or *P. adscendens* were invariably found to be juvenile thalli of *Xanthoria polycarpa* which, at a young age, may be as grey as adjacent, small-lobed *Physcia adscendens* due to very small amounts of anthraquinones in their vegetative thallus, only pycnidial ostioles and apothecial discs being coloured by yellow anthraquinones (Honegger *et al.* 1996). In their inventory of photobiont diversity within crustose and foliose species of the *Physcietum adscendentis*, *X. parietina* being part of this community, Beck *et al.* (1998) showed with molecular markers that the photobionts of *Physcia* spp. are not associated with *X. parietina*. Similar results were found for lichen communities of southern California: *Physciaceae* associated with different photobiont clades than *Teloschistaceae*, and no algal sharing was detected among representatives of the two families when thalli growing side by side were examined (Werth 2012). Extensive studies on photobiont diversity within the *Physciaceae* (Dahlkild *et al.* 2001; Helms *et al.* 2001; Helms 2003) support this view.

The present findings on photobiont diversity in *X. parietina* and *X. polycarpa* indicate that both species associate with photobionts of clade 'A', that is with genotypes of *T. decolorans* (corticolous samples in the Northern Hemisphere) or *T. arboricola* (saxicolous *X. parietina* in the Northern Hemisphere, corticolous *X. polycarpa* in NZ). Thus photobionts of *Physcia tenella* and *P. adscendens* (subclade I1 *sensu* Helms 2003) are unlikely acceptable algal partners of either mycobiont. Sorediate structures, as described by Ott *et al.* (2000) as evidence for colonization of sorediate *Physcia* thalli by *X. polycarpa*, are within the range of phenotypic plasticity of *X. polycarpa* (Eichenberger 2007). However, as already described by Ahmadjian (1960) with microscopy techniques and confirmed with molecular methods (Beck *et al.* 1998), *Buellia punctata*, an inconspicuous crustose

species of the *Physcietum adscendentis*, has the same *T. decolorans* photobiont as *X. parietina* and, according to the present findings, as *X. polycarpa*.

Are *Trebouxia* spp. free-living?

Ahmadjian (2002*a*, *b*) wrote about "lingering lichen myths" such as the belief that *Trebouxia* spp. occur free-living outside lichen thalli and that they are photoautotrophic. Instead, he suggests *Trebouxia* spp. are not independent organisms, but heterotrophic ones, "both in the lichen thallus and also growing independently in culture". Our long-term culturing experiments on agarized non-nutrient mineral media leave no doubt about the ability of *Trebouxia* species to live as independent, photoautotrophic organisms. Based on diverse microscopic observations on free-living *Trebouxia* cells in nature (Tschermak-Woess 1978; Bubrick *et al.* 1984; Gärtner 1985*a*; Mukhtar *et al.* 1994; Ettl & Gärtner 1995; Schroeter & Sancho 1996; John *et al.* 2002; Rindi & Guiry 2003; Sanders 2005; Handa *et al.* 2007; Hedenås *et al.* 2007), it seems reasonable to assume *Trebouxia* species are very widespread and distinctly more common than previously hypothesized. The fact that closely related *Trebouxia* genotypes occur in thalli of different sexually-reproducing *Xanthoria* spp. with no vegetative propagules on different continents, as shown in the present investigation (Fig. 1), indirectly indicates that these photobionts must be available in nature for re-lichenization events. Molecular probes might be used in future experiments to detect the availability of free-living *Trebouxia* photobionts of lichen-forming fungi in environmental samples.

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