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High photobiont diversity associated with the euryoecious lichen-forming ascomycete *Lecanora rupicola* (Lecanoraceae, Ascomycota)

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The diversity and phylogenetic position of photobionts in the widespread saxicolous, crustose lichen-forming ascomycete *Lecanora rupicola s.l.* is presented. The algal partners of this lichen species complex belong to diverse and unrelated lineages in the genus *Trebouxia*. Specimens were sampled from different habitats and geographical origins. Either whole thallus DNA extractions or minute fragments of the algal layer of the lichen thallus were subjected to polymerase chain reaction, using primers that specifically amplify internal transcribed spacer rDNA of the photobionts. No correlations between different chemical races of *L. rupicola* with particular lineages of *Trebouxia* spp. were found. Irrespective of the different algal partners, all lichen thalli abundantly developed ascomata. *L. rupicola* apparently maintains full fecundity with a low degree of selectivity for photobionts, which promotes the occurrence of this lichen-forming species in ample ecological situations. *Biological Journal of the Linnean Society*, 2006, **88**, 283–293.

ADDITIONAL KEYWORDS: *Lecanora rupicola* – lichens – phylogeny – selectivity – symbiosis – Trebouxiophyceae – *Trebouxia*.

INTRODUCTION

The lichen thallus offers a special habitat for algal symbionts by forming a natural 'growth chamber'. In the thallus, fungal structures form protective layers around the algae and direct the flux of water and air, while fungal compounds act as light filters in the upper cortex (Rikkinen, 1995). Thus, by living in symbiosis with fungi, algae are able to survive and propagate under environmental conditions that appear to be generally less suitable for their free-living relatives. However, there is still limited knowledge about the range of accepted photobionts in lichen species.

Members of Trebouxiophyceae are the photobionts of more than 60% of lichen-forming fungi (Ahmadjian, 1982). Traditional classification within this class of green algae improved considerably in the past two decades using light and electron microscopy techniques (Gärtner, 1985; Friedl, 1989; Tschermak-Woess, 1989; Ettl & Gärtner, 1995). Such investigations also revealed that different photobiont strains or species can exist in different thalli of the same species (Friedl, 1989), but extensive studies with many samples of a single species were not carried out because these would have required the time-consuming establishment of axenic cultures. Meanwhile, molecular approaches have been used to resolve the relationships among photobionts (Friedl & Rokitta, 1997; Beck, 1999; Helms *et al*., 2001) and to address issues of symbiont selectivity using a molecular phylogenetic framework. These works showed, for example, that apparently only two species of *Trebouxia* are present in foliose Physciaceae in Northern Europe (Dahlkild *et al*., 2001), or that few genotypes of *Asterochloris* are shared among related and unrelated *Cladonia* spp. (Piercey-Normore & DePriest, 2001). In a comprehensive study of the *Trebouxia* spp. associated with Physciaceae, Helms *et al*. (2001) and Helms (2003) found diverse species of photobionts in crustose lineages, for the most part comprising the paraphyletic genus *Rinodina*. However, the diversity of accepted photobionts in a single fungal species is insufficiently known from

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these studies because, often, only one sample of each crustose species was analysed. In an extended sampling of the *Letharia vulpina* complex (Kroken & Taylor, 2000), and in *Flavocetraria nivalis* (Opanowicz & Grube, 2004), it was shown that these species only associate with strains of a single photobiont species, whereas representatives of the foliose lichen genus *Umbilicaria* in Antarctica can form symbioses with several *Trebouxia* spp. (Romeike *et al*., 2002).

To date, few investigations are available about phenotypic effects in re-synthesized lichen symbioses with non-native photobionts. In pioneering studies, Ahmadjian, Russell & Hildreth (1980) and Ahmadjian & Jacobs (1981) observed that isolates of *Cladonia cristatella* and *Rhizoplaca chrysoleuca* associate with several lichen photobionts *in vitro* and may form well differentiated thalli, but only undifferentiated, sorediate prethallus stages of development were achieved with distantly related photobiont species or with free-living algae. Similar results were obtained by Schaper (2003) for *Fulgensia* species, which developed a proper lichen thallus with the genuine algal partner, whereas other lichen photobionts caused delays or failures to develop lichenized stages. These studies were unable to reveal which phenotypic effects may occur in natural associations when diverse photobionts are involved (e.g. whether photobionts may have any influence on the fecundity of lichen-forming fungi, on morphological features, or on the production of secondary metabolites). If there are no effects, especially with regard to fecundity, it could be expected that these lichens are ecologically 'successful' by easily establishing on newly available substrates in different habitats.

In the present study, we investigated the geographically widespread lichen-forming ascomycete *Lecanora rupicola* (Fig. 1), which has a rather broad ecological amplitude and is found both in cold alpine habitats as well as in hot Mediterranean sites. *Lecanora rupicola* and closely related species are well circumscribed by their saxicolous growth on siliceous and intermediary rocks and by the presence of the unique chromone sordidone (Leuckert & Poelt, 1989). Recently, Grube, Baloch & Arup (2004) showed the monophyly of the sordidone containing *Lecanora* spp. [i.e. *Lecanora* subgen. *Glaucomaria* (including *Lecanora bicincta*, *Lecanora carpinea*, *Lecanora leptyrodes*, *Lecanora lojkaeana*, *Lecanora rouxii*, *Lecanora rupicola*, *Lecanora subcarpinea*, and *Lecanora swartzii*)] using molecular data. In the present study, we accept these species according to their traditional morphological concept, and despite *L. rupicola* and *L. bicincta* not being supported as separate species by internal transcribed spacer (ITS) data. *Lecanora rupicola* is variable in chemistry and usually fertile, producing fruitbodies and ascospores. Thus, ascospore germlings relichenize when suitable photobiont cells are available. By contrast, two sterile species of the group, *L. lojkaeana* and *L. rouxii*, propagate both symbionts jointly via soredia. To ensure a broader sampling we included samples of different chemotypes of *L. rupicola*, but also samples of other fertile species in the *L. rupicola* group, and the sterile *L. lojkaeana*.

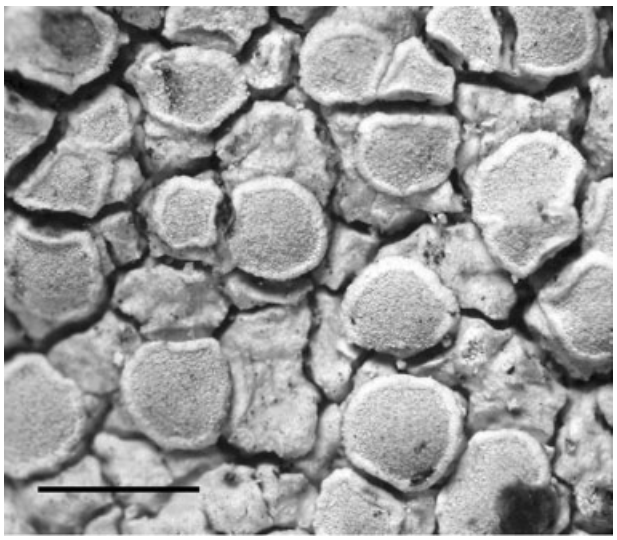

Figure 1. Habitus of richly fruiting thallus of *Lecanora rupicola* (Spain, Sierra de Guadarrama, Blaha B425). Scale bar = 1 mm.

MATERIAL AND METHODS

Lichen specimens listed in Table 1 were used for the analysis of photobiont ITS rDNA sequences. All voucher specimens are stored in the herbarium of the Institute of Plant Sciences, Graz (GZU), except for samples collected by U. Arup, U. Kirschbaum, H. Komposch, and M. Opanowicz, which were from the private herbaria of the collectors. To avoid sequencing miscellaneous other algae that might be present on the surface of the lichen thalli, samples were carefully checked for any externally visible contaminations using a stereomicroscope. To confirm that the thalli usually contained only a single strain of photobionts, we analysed various parts of the thalli separately in several selected specimens.

Total DNA was extracted from thallus parts, according to a modified CTAB method (Cubero *et al*., 1999) or using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions, except that the DNA was eluted with sterile water. In a few cases (e.g. when some areoles of larger thalli showed symptoms of infections by lichenicolous fungi or when we suspected the presence of epilichenic algae), we amplified the DNA directly from thallus fragments. For this purpose, we concentrated on clean parts of the thalli and

Table 1. Lichen samples analysed for their photobionts and their GenBank accession number

ID number	Mycobiont	Geographic origin (collector)	GenBank accession number
A13	*Lecanora bicincta*	Greece, Crete (M. Grube)	DQ166576
A34	*Lecanora bicincta*	USA, Arizona, Coconino Co., Nr. 36810 (J. Hafellner)	DQ166577
B383	*Lecanora bicincta*	Spain, Catalonia, N. von Lleida, Pirineos centrales, Cabdella (J. Blaha)	DQ166578
E146	*Lecanora bicincta*	Turkey, Prov. Aksaray (V. John)	DQ166579
M15	*Lecanora bicincta*	Greece, Crete (M. Grube)	DQ166580
MG100	*Lecanora bicincta*	Norway, Knutshoe (M. Grube)	DQ166581
A9	*Lecanora carpinea*	Spain, Canary Islands, Gran Canaria, Nr. 48014 (J. Hafellner)	DQ166582
M39	*Lecanora carpinea*	Slovenia, Trnovski gozd, Nr. 61232 (J. Prügger)	DQ166583
J144	*Lecanora lojkaeana*	Austria, Styria, Frauenalpe (J. Blaha)	DQ166584
MG101	*Lecanora lojkaeana*	Norway, Soebo (M. Grube)	DQ166585
O9076	*Lecanora lojkaeana*	Austria, Styria, Reiteralm (W. Obermayer)	DQ166586
A10	*Lecanora rupicola*	Greece, Crete, Nomos Rethimnio (M. Grube)	DQ166587
A31	*Lecanora rupicola*	Spain, Canary Islands, Gran Canaria, Pico de las Nieves, Nr. 47898 (J. Hafellner)	DQ166588
A36	*Lecanora rupicola*	Denmark, Bornholm, Sandkaas, Nr.150 (E. St. Hansen)	DQ166589
A41	*Lecanora rupicola*	Spain, Canary Islands, La Palma, Nr. 29612 (J. Hafellner)	DQ166590
B309	*Lecanora rupicola*	France, Corsica, Cole de vergio (E. Baloch)	DQ166591
B313	*Lecanora rupicola*	France, Corsica, Cole de vergio (E. Baloch)	DQ166592
B317	*Lecanora rupicola*	France, Corsica, Cole de vergio (E. Baloch)	DQ166593
B331	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, El Ventorrillo (J. Blaha)	DQ166594
B332	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, El Ventorrillo (J. Blaha)	DQ166595
B339	*Lecanora rupicola*	Spain, Catalonia, N von Lleida, Pirineos centrales, Cabdella (J. Blaha)	DQ166596
B348	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, Cuerda de las Cabrillas (J. Blaha)	DQ166597
B349	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, Cuerda de las Cabrillas (J. Blaha)	DQ166598
B395	*Lecanora rupicola*	Austria, Osttirol, Innergschlöß (J. Blaha)	DQ166599
B419	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, NE von Madrid, Cercedilla (J. Blaha)	DQ166600
B420	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, NE von Madrid, Cercedilla (J. Blaha)	DQ166601
B428	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, NE von Madrid, Cercedilla (J. Blaha)	DQ166602
B431	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, NE von Madrid, Cercedilla (J. Blaha)	DQ166603
B434	*Lecanora rupicola*	Spain, Madrid, Sierra de Guadarrama, Callado Mediano (J. Blaha)	DQ166604
B452a	*Lecanora rupicola*	Portugal, Alentejo, Serra de Sao Mamede (J. Blaha)	DQ166605
B452b	*Lecanora rupicola*	Portugal, Alentejo, Serra de Sao Mamede (J. Blaha)	DQ166606
J152	*Lecanora rupicola*	Austria, Styria, Hartberg (H. Komposch)	DQ166607
J181	*Lecanora rupicola*	Portugal, Madeira Nr. 5136 (U.Kirschbaum)	DQ166608
M14	*Lecanora rupicola*	Greece, Crete (M. Grube)	DQ166609
M71	*Lecanora rupicola*	Greece, Crete (M. Grube)	DQ166610
M9	*Lecanora rupicola*	Australia (T. Lumbsch)	DQ166611
MG103	*Lecanora rupicola*	Norway, Soebo (M. Grube)	DQ166612
MG104	*Lecanora rupicola*	Norway, Kongsvold (M. Grube)	DQ166613
MG105	*Lecanora rupicola*	Norway, Urnes (M. Grube)	DQ166614
MG106	*Lecanora rupicola*	Norway, Grimsdalen (M. Grube)	DQ166615
MG108	*Lecanora rupicola*	Sweden, Åhus par. Åhus, Ö. Täppet (U. Arup)	DQ166616
MG17	*Lecanora rupicola*	France, Haute Languedoc (M. Grube)	DQ166617
MOPA79	*Lecanora rupicola*	Poland, Suche Czuby, West Tarta Mts. (M. Opanowicz)	DQ166618
B346	*Lecanora swartzii*	Spain, Madrid, Sierra de Guadarrama, Cuerda de las Cabrillas (J. Blaha)	DQ166619
M62	*Lecanora swartzii*	Austria, Styria, Stuhleck (M. Grube)	DQ166620

removed the upper cortex of a well-developed and healthy areole with a razor-blade. Thin sections of the algal layer were then cut out and immersed directly into the amplification cocktail prior to the cycling reaction. The same approach was also followed with small specimens or to confirm the uniformity of the algal partner in the thalli.

Primers for amplification were ITS1T and ITS4T, which are specific for trebouxioid photobionts (Kroken & Taylor, 2000). Fifty microlitres of polymerase chain reaction (PCR) mix (10 mM Tris pH 8.3/50 mM KCl/ 1.5 mM $MgCl_2$/50 μg gelatine) contained 1.25 units of Taq DNA Polymerase (Amersham Pharmacia Biotech Inc.), 0.2 mM of each of the four dNTPs, 0.5 μM of each primer and either a section of the algal layer or *c*. 10–50 ng DNA of the metagenomic isolate. Products were cleaned using QIAquick PCR Purification Kit (Qiagen). Both complementary strands were sequenced using the BigDyeTerminator Cycle Sequencing Ready Reaction Kit (Applera) according to the manufacturer's instructions. Sequences were run on an ABI 310 automated sequencer (Applera) and raw data were assembled using AutoAssembler (Applera). The sequences were submitted to NCBI (http://www.ncbi.nlm.nih.gov/) and their accession numbers are listed in Table 1.

To assess the phylogenetic relationships of *Trebouxia* spp. in the *L. rupicola* group, we first constructed an alignment that also included sequences of *Trebouxia* spp. from other lichens retrieved from GenBank (Table 2). For an initial alignment, we used the CLUSTAL algorithm as implemented in BioEdit (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). After optimization by eye, the alignment revealed distinct sequence insertions at four positions. The corresponding sites were excluded from the analysis as was also the case for otherwise ambiguously aligned positions. The phylogenetic hypothesis was constructed using a Bayesian approach as implemented in the program MrBayes version 3.0B4 (Huelsenbeck & Ronquist, 2001). Because the results of a Bayesian analysis are sensitive to the model chosen, the appro-

Table 2. ITS sequences downloaded from GenBank

Species	Source	GenBank accession number
Trebouxia asymmetrica	*Diploschistes diacapsis*	AJ249565
Trebouxia corticola	UTEX 909	AJ249566
Trebouxia decolorans	*Xanthoria parietina*	AJ007387
Trebouxia decolorans	*Punctelia subrudecta*	AJ249564
Trebouxia flava	UTEX 181	AF242467
Trebouxia gigantea	UTEX 2231	AJ249577
Trebouxia higginsiae	UTEX 2232	AJ249574
Trebouxia impressa	*Melanelia glabra*	AJ249576
Trebouxia impressa	*Physcia adscendens*	AJ007383
Trebouxia incrustata	UTEX 784	AJ293795
Trebouxia potteri	UTEX 900	AF242469
Trebouxia showmanii	UTEX 2234	AF242470
Trebouxia simplex		AF128271
Trebouxia simplex		AJ249571
Trebouxia simplex	*Pseudevernia furfuracea*	AF242459
Trebouxia simplex	*Chaenotheca subroscida*	AF453263
Trebouxia sp.	*Dimelaena oreina*	AJ293785
Trebouxia sp.	*Phaeophyscia orbicularis*	AJ293786
Trebouxia sp.	*Rinodina milvina*	AJ293794
Trebouxia sp.	*Rinodinella controversa*	AJ293790
Trebouxia sp.	*Rinodina artrocinerea*	AJ293791
Trebouxia sp.	*Anaptychia ciliaris*	AJ293770
Trebouxia sp.	*Amandinea punctata*	AJ293780
Trebouxia sp.	*Rimularia insularis*	AF534386
Trebouxia sp.	*Rimularia insularis*	AF534387
Trebouxia sp.	*Anaptychia runcinata*	AJ293781
Trebouxia sp.	*Physcia stellaris*	AJ293778
Trebouxia usneae	UTEX 2235	AJ249573

priate model of nucleotide substitution was obtained by the program MrModeltest (written by J. A. A. Nylander and available at http://morphbank.ebc.uu.se/mrbayes/). According to the Akaike Information Criterion, the general time reversible substitution model (Rodriguez *et al*., 1990) with estimation of invariant sites and assuming a discrete gamma distribution (GTR + I + G) was suggested as the optimal model. The Markov Chain Monte Carlo analysis was run for 2 000 000 generations, with 12 chains starting from a random tree, and using the default temperature for the heated chain of 0.2. Every hundredth tree was sampled, while the first 30 000 generations were discarded as burn-in. The consensus phylogram showing mean branch lengths was calculated with the sumt command in MrBayes (Fig. 2). Posterior probabilities higher than 90% and 95% are indicated by the increased thickness of the branches. Because an appropriate outgroup was not available for *Trebouxia* (Dahlkild *et al*., 2001), the tree was arbitrarily rooted with *Trebouxia higginsiae*, which was not present as photobiont in *Lecanora*. A number of sequences were retrieved from GenBank for comparison (Table 2).

To assess the fecundity of *L. rupicola*, we considered the central part of the thalli (*c*. 1–1.5 cm in diameter) because the growing periphery is generally devoid of ascomata. A simple ordinal scale was applied and three categories were considered: (1) no to very few ascomata present; (2) ascomata sparse to moderately developed (i.e. area covered by ascomata distinctly smaller than area without ascomata); and (3) ascomata frequent to abundant (i.e. area with hymenia approximately equal or larger than the area without ascomata). The arrangement of photobiont cells was studied with a stereomicroscope (Wild M3B) in horizontally dissected thalli upon removal of the cortex with a razor blade. Because the abundance of algal cells generally diminishes towards basal parts of the algal layer, only the uppermost zone was considered. The interaction of fungi with the photobionts was investigated using thin, hand-made sections (*c*. 15 μm thick) of the algal layer. The sections were mounted in tap water and observed using a compound microscope (Zeiss Axioskop).

Lichen substances were analysed using high-performance thin-layer chromatography according to Arup *et al*. (1993).

RESULTS

Forty-five new algal nITS rDNA sequences (Table 1) were obtained and a total of 74 taxa were included in the analyses. After exclusion of insertions and ambiguously aligned regions (234 sites), the total dataset contained 675 characters; 266 were parsimony informative. The likelihood parameters in the sample had the following estimated values (mean ± variance): rate matrix r(CT) = 6.496 ± 1.239, r(CG) = 0.856 ± 0.042, r(AT) = 2.898 ± 0.316, r(AG) = 4.614 ± 0.635, and r(AC) = 1.833 ± 0.154; base frequencies π(A) = 0.226 ± 0, π(C) = 0.238 ± 0, π(G) = 0.268 ± 0, and π(T) = 0.267 ± 0; gamma shape parameter α = 0.788 ± 0.036; and the proportion of invariable site p(invar) = 0.147 ± 0.004.

The phylogenetic analysis of algal ITS sequences shows that several *Trebouxia* spp. are photobionts of *L. rupicola* (Fig. 2). The nomenclature proposed by Helms (2003) for main clades in the *Trebouxia* phylogeny was adopted. Clade S3 comprises the phenotypic species *Trebouxia simplex* (syn. *Trebouxia jamesii*), which is reported as photobiont of *L. rupicola* for the first time. All specimens from the Alps and samples from Mediterranean areas, France (Corsica), and Spain (Sierra de Guadarrama), at altitudes higher than 1000 m have *T. simplex* as photobiont. Furthermore, symbionts of both samples of *L. swartzii* and of one sample each of *L. bicincta* and *L. carpinea* belong to this clade. Clade I1, representing the phenotypic species *Trebouxia impressa* and *Trebouxia potteri*/*Trebouxia flava*, includes photobionts of *L. rupicola* and *L. bicincta* from the Pyrenees, France (Corsica), Spain (Sierra de Guadarrama), and Turkey. Clade A6, with one Australian representative, may belong to another *Trebouxia* spp. (*T*. spec. 1). Clade A10, comprising specimens from Spain, is assigned to the phenotypic species *Trebouxia incrustata*. The ITS sequences of this lineage contained two longer insertions between alignment positions 501–544 and 557–605. Clade A4 includes samples from a wide geographical range, both Greece (Crete), France (Corsica), Portugal, Sweden, and Denmark (Bornholm). This clade is also present in thalli of *Lecidella* and *Protoparmelia* (data not shown), which grew adjacent to *L. rupicola* on the same piece of rock. *Lecanora lojkaeana*, a sorediate member of the *L. rupicola* group, and the corticolous *L. carpinea* contain *Trebouxia decolorans* (syn. *Trebouxia arboricola*, Beck, 2002; Helms, 2003) as photobiont (clade A1). Another presumably undescribed species is represented by clade A2, found in samples from the Mediterranean, in *L. rupicola* MG106 from Norway, and in *L. lojkaeana* J144 from Austria. This clade is characterized by two distinct insertions (one in position 172–200, exclusively in clade A2 and another one in position 698–707, in clade A1 and A2).

Slight differences in the arrangement of algal cells within the lichen thalli were found in specimens of the *L. rupicola* complex. The algal cells of clade A1, A2, A4, A6, A9, A10, and I1 often form clusters, which may be scattered throughout the algal layer of the lichen and are well separated by hyphal strands (Fig. 3A). By contrast, the algal cells of the *T. simplex* complex (clade S3) usually are more regularly distributed in

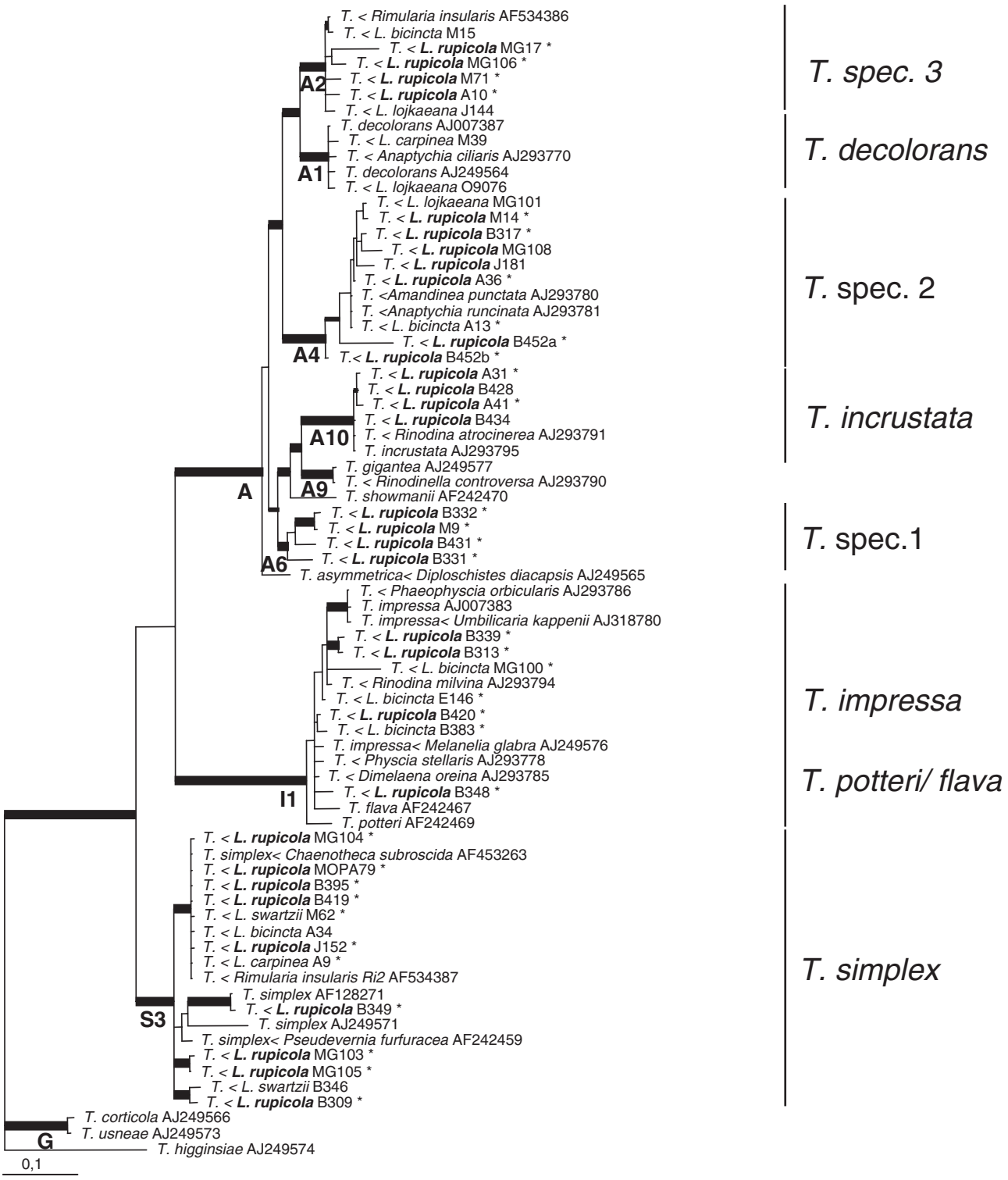

Figure 2. Phylogenetic hypothesis of trebouxioid photobionts in *Lecanora rupicola* and related species. Bayesian analysis of DNA sequence data. Posterior probabilities are indicated by the increased thickness of the branches: < 90%, 90–94%, and ≥ 95%. Asterisks indicate the occurrence of xanthones in the upper cortex of the corresponding lichen thalli.

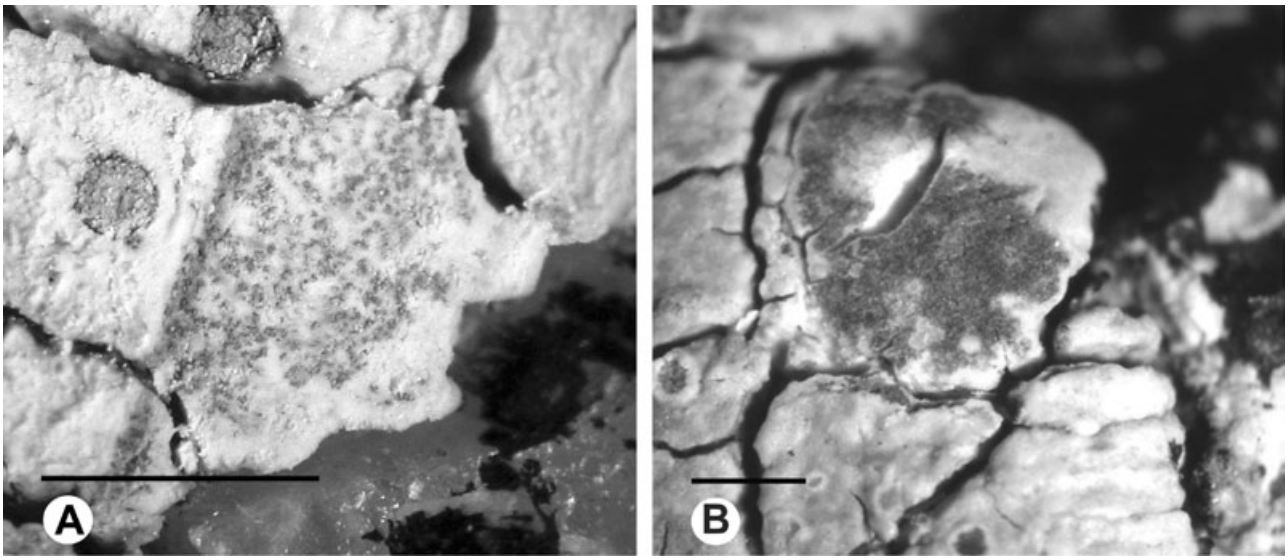

Figure 3. Different arrangements of algae in horizontally dissected thalli of *Lecanora rupicola* found in geographically distant species: A, Clusters of algal cells scattered in the algal layer of the lichen typical for photobiont species of clades A1 to I1, from Spain (Blaha B357). B, Homogeneous distribution of the photobiont in thalli with algae of the *Trebouxia simplex* complex (clade S3), from Norway (Grube MG103). Scale bar = 1 mm.

the thalli (Fig. 3B). This was also seen, albeit to a lesser extent, in thalli with *T. simplex* from sites where other thalli contained different algae. No differences were observed in the morphology of the intraparietal haustoria, by which the fungi establish the physiological contact with their photobionts. All investigated thalli could be assigned to the third category of fecundity, representing frequent to abundant ascomata (area with hymenia equal or larger than area without ascomata). In some thalli, the abundance of ascomata varied within the thallus, which is apparently caused by other effects than the photobiont.

The chemotypes of all specimens were investigated and the presence of xanthones (arthothelin, thiophanic acid, and different dichloronorlichexanthones) was mapped on the photobiont phylogeny and is summarized in Table 3. Nearly all lineages are associated with mycobionts that either produce or lack xanthones. Only clade A1 was exclusively found in *L. carpinea* and *L. lojkaeana* without xanthones, and A6 and I1 solely in specimens producing these compounds.

Table 3. Different clades of *Trebouxia* found and occurrence of xanthones in the upper cortex of corresponding thalli

Clade	Thalli with xanthones	Thalli without xanthones
S3	11	2
I1	7	–
A1	–	2
A2	4	2
A4	6	3
A6	4	–
A10	2	2

DISCUSSION

Until the introduction of molecular methods, phenotypic species concepts have been applied in *Trebouxia*, leading to the recognition of approximately 20 species. Ultrastructural features observed by transmission electron microscopy of cultured samples, especially the fine structure of chloroplasts and pyrenoids, were regarded as highly significant for classification. Recently, phylogenetic hypotheses showed that these characters do not necessarily reflect monophyletic lineages in *Trebouxia* (Helms *et al*., 2001), but only in a few cases have molecular data contributed to a re-evaluation of the species concept so far. Kroken & Taylor (2000) implemented a phylogenetic species concept, using genealogical concordance of ITS and actin sequence data, to demonstrate several cryptic species in the *T. simplex* complex. Although sexual stages have never been observed in *Trebouxia* spp., neither in the thallus nor in culture (*Trebouxia* photobionts are hardly ever found in the free-living state), a recombinant population structure was detected by Kroken & Taylor (2000) in one of these species. It thus remains

an open question to what extent a single locus such as ITS rDNA reflects different species, but with an increasing number of nucleotide differences, the analysis of ITS data readily reflects the relationships among different species complexes. The species delimitation will clearly require additional studies, but our data provide further evidence for three distinct, yet unnamed, lineages in *Trebouxia*. According to our concept, these lineages likely represent different species complexes, which appear to occur preferentially in Mediterranean regions. A formal description of species within these complexes is pending until ultrastructural data are available.

The geographical range of different *Trebouxia* spp. is still incompletely known, but it generally appears that several lichen photobionts of this genus are widely distributed. This is certainly true for lineages in the *T. simplex* complex (Kroken & Taylor, 2000; Opanowicz & Grube, 2004). In *Letharia* spp., Kroken & Taylor (2000) found three phylogenetic species of the *T. simplex* complex exclusively in California, whereas other lineages in this complex have much wider geographical ranges and can be found in different continents. One of them detected in our samples, which is found at higher altitudes in the Mediterranean, might represent an independent species in the *T. simplex* complex (samples B309 and B346). In the present study, we provide further evidence for the wide distribution of other lineages. For example, photobionts in clade A4 are present in *Umbilicaria* spp. from Antarctica (Romeike *et al*., 2002) and were found in *L. rupicola* from the Mediterranean and Denmark (Bornholm).

The present analysis of photobiont diversity in the *L. rupicola* complex (from the Eastern Alps, Arizona, Australia, France, Greece, Norway, Portugal, Spain, and Sweden) shows that photobiont selection is not limited to a particular clade within the genus *Trebouxia*; rather, members of diverse species complexes of *Trebouxia* readily serve as a symbiotic partner. Similar results were presented for a few foliose species (Friedl, 1989; Romeike *et al*., 2002), but we are also able to show an associated pattern in the present study. Alpine samples of the *L. rupicola* group commonly contain photobiont lineages that can be assigned to the *T. simplex* complex, whereas this species was not detected in the lowland samples from Mediterranean habitats. These latter samples comprised other photobionts that can be assigned to different unrelated lineages (clades A2, A4, A6, A10, and I1). *Trebouxia* spec. 3 (clade A2) was found also by De los Rios, Ascaso & Grube (2002) in a Mediterranean sample of *L. rupicola* and its lichenized parasite *Rimularia insularis*. Another undescribed *Trebouxia* spp., *T.* spec. 2 (clade A4) was also found by Helms *et al*. (2001) in Physciaceae (in *Amandinea punctata* and *Anaptychia runcinata* from Elba). *Trebouxia* spec. 1 (clade A6) is basal to the lineages of *T. incrustata* (clade A10, also detected in *L. rupicola*), *Trebouxia gigantea*, and *Trebouxia showmanii* and, finally, clade I1, which corresponds to the phenotypic complex of *T. impressa*/*T. potteri*/*T. flava*. Thus, together with the *T. simplex* complex, six distinct *Trebouxia* clades contribute to photobionts of *L. rupicola*, whereas other known *Trebouxia* species, such as *Trebouxia corticola*, *Trebouxia usneae*, and *Trebouxia galapagensis*/*Trebouxia higginsiae* (clade G according to Helms, 2003) are not known to associate with members of the *L. rupicola* group. It appears that the concept of 'lichen guilds', previously introduced for species assemblages of cyanobacterial lichens with ecologically similar requirements and closely related photobionts (Rikkinen, Oksanen & Lohtander, 2002), is of limited use in communities with *L. rupicola* because this species contributes to several 'guilds' and can associate with unrelated *Trebouxia* spp. in different habitats.

Recent studies elucidated some ecological preferences of trebouxioid photobionts. It is known that *T. simplex* is common in lichens growing on acid and heavy metal rich substrates (Beck, 2002), whereas lichen species of Physciaceae from calcareous rocks appear to be restricted to clade A (Helms, 2003: particularly subclades A5–A11). In the present study, we found representatives of this clade in many samples. *Lecanora rupicola* is generally not known from calcareous rocks, but may readily occur on acid to subneutral to base-rich rocks, which may host lichens containing diverse lineages of clade A. The absence of *T. simplex* from lowland Mediterranean regions (i.e. in samples collected at altitudes lower than *c*. 1000 m) could be due to unfavourable climatic conditions on the sun-exposed rock surfaces in European-Mediterranean habitats. It is possible that temperatures are too high at these sites for the species. Studies of cultured *T. simplex* demonstrated that the optimal conditions for reproduction by zoo- and aplanospores are at comparatively low temperatures, *c*. –2 to 10 °C (Tschermak-Woess, 1988, 1989). In more temperate climates, this photobiont species appears less selective for the substrate and can also occur in corticolous parmelioid lichens from Switzerland or Germany (on *Abies*, *Acer*, *Betula*, *Juglans*, and *Quercus*; Friedl, 1989).

Irrespective of the different photobionts in *L. rupicola*, the interaction between the symbionts appears to be determined entirely by the mycobiont. All samples showed intraparietal haustoria of similar shapes. Differences were only found in the arrangement and abundance of the algae in the algal layer (Fig. 3). However, careful interpretation is warranted because such characters might well be influenced by habitat conditions or by seasonal variations (Fiechter & Honegger,

1988). The latter do not explain the differences alone because almost all samples were collected during summer, and slight differences were also found in samples with different algae but from the same habitat (Cercedilla, Spain).

Presently, it is not clear how *L. rupicola* recognizes its preferred symbionts because the functional details of symbiont selectivity in lichens are not explored and recognition mechanisms of matching partners remain unknown. Only in one mycobiont species was a protein detected that specifically binds to the cells of the proper photobiont (Bubrick & Galun, 1980; Bubrick, Frensdorff & Galun, 1985). In this case, the selectivity of the fungal partner for the algal symbiont is very high: only *Trebouxia decolorans* (syn. *T. arboricola*) is known as a partner of *Xanthoria parietina*. It still remains to be clarified whether analogous recognition proteins are present in *L. rupicola* and whether these have a broader binding capacity for different photobionts, or whether several such proteins are expressed, each potentially recognizing another photobiont.

Photobionts can generally be selected from a 'pool of locally available algae' (Beck, 1999; Beck, Friedl & Rambold, 1998; Beck, Kasalicky & Rambold, 2002). In the present study, the local photobiont 'pools' seem to contribute a variable number of algal species. For example, in one locality in Spain (Sierra de Guadarrama/Cercedilla), four different algal lineages (*T. simplex, T. impressa, T. incrustata*, and *T.* spec. 1, see Table 4) were detected, whereas fewer photobionts were present in *L. rupicola* populations elsewhere. However, more samples from other lichens are needed to show to what extent the species composition of the local photobiont 'pool' varies in different geographical regions with similar habitat conditions (e.g. Mediterranean habitats in different continents). A large local photobiont diversity can be detected also in other crustose *Lecanora* spp. with a rather broad ecology (e.g. in *Lecanora muralis*; B. Guzow-Krzeminska, pers. comm.).

Table 4. Selected localities with more than one species of *Trebouxia* found in different individual thalli of *Lecanora rupicola*. The single collecting sites showed homogenous environmental conditions

Locality	Spain (Cercedilla)	France (Corsica)	Spain (Cabrillas)
Species			
Trebouxia simplex	x	x	x
Trebouxia impressa	x	x	x
Trebouxia incrustata	x		
Trebouxia spec. 1	x		
Trebouxia spec. 2		x	

Several possibilities exist regarding how newly establishing thalli may generally recruit their photobionts from the available pool. A germinating ascospore can associate with a free-living photobiont, which can be air-borne, disseminated by invertebrates (Meier, Scherrer & Honegger, 2002), or persisting in microscopic rock crevices. Lichens with juvenile parasitic stages may also steal photobionts by entering other lichens. Juvenile parasitism is often associated with particular host lichens, but these have not been clearly observed in *L. rupicola*. However, it appears that at least some strains of this species are rather competitive and may overgrow neighbouring lichen thalli of other species (e.g. if young colonies develop convex outlines and slightly raised margins when bordering to other lichens). As we have commonly found both the same and different photobionts in other adjacent crustose lichens, but generally only a single photobiont in a *L. rupicola* thallus, we assume that once *L. rupicola* has gained its photobiont after ascospore germination, this photobiont is maintained throughout the ontogeny. In species that propagate exclusively via joint dispersal of both symbiotic partners (e.g. by soredia), we would expect a strictly vertical inheritance of photobionts, but we have no evidence for this from our data. Three studied samples of *L. lojkaeana*, two from the Alps (O9076 and J144) and one from Norway (MG101), associate with three different algal lineages (Fig. 2), suggesting that occasional photobiont switches occur in this sterile lichen species.

Mapping of the secondary compounds of the mycobionts on the *Trebouxia* phylogeny of the according photobionts demonstrated no correlation between the chemistry of the mycobiont and the associated photobiont. Due to the fact that different chemotypes of *L. rupicola* can associate with the same *Trebouxia* lineage, and that the same chemical compounds are produced irrespective of the associated *Trebouxia*, we conclude that the photobiont has no impact on the production of secondary compounds in *L. rupicola*. This is in agreement with previous findings in other green algal lichens (Culberson, Culberson & Johnson, 1985).

The finding that *L. rupicola* coll. may select various photobionts without reduction in fertility may shed new light on the ecology of some lichen species. A mycobiont species that can select its photobiont from a set of possible photobionts with differing ecological preferences may have a selective success due to the adaptation to a broader ecological amplitude. A more distinct case of such an ecologically successful strategy is possibly provided by phycosymbiodemes, with respect to the facultative symbiosis with either cyanobacteria or green algae. Also in these cases, the cyano-

bacterial and green algal morphs of the same lichen can be found in ecologically different habitats (Renner & Galloway, 1982; Poelt, 1986).

It might be argued that a high diversity of trebouxioid photobionts could be a secondary phenomenon when the mycobiont itself is euryoecious. However, Ahmadjian *et al.* (1980), Ahmadjian & Jacobs (1981) and, more recently, Schaper (2003) provided evidence from resynthesis experiments demonstrating that the photobiont preference of the mycobiont is genetically determined. It is therefore clear that even an euryoecious mycobiont can only establish lichen symbioses in ample ecological situations when it has evolved acceptance of the locally adapted photobiont species.

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