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Photobiont genetic variation in *Flavocetraria nivalis* from Poland (*Parmeliaceae*, lichenized Ascomycota)

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Abstract: Molecular sequence data of the nuclear ITS region was used to investigate the diversity of photobionts in Polish samples of *Flavocetraria nivalis*. The samples came both from alpine habitats, as well as from lowland localities near the coast. All green algal symbionts were identified as members of the *Trebouxia simplex* aggregate. These were compared with those of additional samples from *Flavocetraria nivalis* collected in different parts of Europe and also with photobionts assigned to *T. simplex* from other lichens. Within the *T. simplex* aggregate, the *Trebouxia* ITS sequences from *F. nivalis* formed four clades. In the Polish lowland populations only a single clade of *T. simplex* was detected which also occurs in Polish mountains, south Sweden and Austria. A further clade of *T. simplex* is present in *F. nivalis* from Polish mountains and is also known from *F. nivalis* further north in Scandinavia and Greenland, as well as from other lichens in Sweden, the Austrian Alps, and Antarctica.

Key words: *Flavocetraria nivalis*, ITS, photobiont, selectivity, *Trebouxia simplex*.

Introduction

The cetrarioid lichen *Flavocetraria nivalis* (L.) Kärnefelt & Thell is geographically centred in the Northern Hemisphere being found in Europe, Asia, and North America, but it is also reported from South America (Rassadina 1950; Oxner 1974; Tobolewski & Kupczyk 1976; Calvelo & Liberatore 2000). In the Northern Hemisphere it is a typical arctic-alpine species, and is especially abundant in tundra heathlands, where it can attain a high percentage cover and provide an important winter food source for reindeer and caribou. *Flavocetraria nivalis* grows on soil in open habitats and forms clusters of yellow erect flat-lobed foliose thalli, which are loosely attached to the substratum. Although apothecia can occasionally be found within the predominantly sterile populations, the principal mode of reproduction is by thallus fragmentation.

In Poland, *F. nivalis* occurs in two lowland localities (Tuchola Forest and Mierzeja Wiślana) and in the highest Polish mountains (Tatra Mts, Karkonosze Mts and Babia Góra Mt). The lowland populations are rather small in size (*c.* 80 thalli each) and the thalli (Fig. 1) differ slightly by having bigger and flatter lobes of more greenish colour. Moreover, although some morphological polymorphisms have been observed (Motyka 1960; Fałtynowicz & Tobolewski 1980; Fałtynowicz & Budzbon 1983, Opanowicz 2002), morphological characters are generally insufficient to warrant separation at the subspecies level in *F. nivalis*. However, there are no thorough genetic studies of these or other *F. nivalis* populations. Internal transcribed spacers (ITS) of nuclear mycobiont rDNA have been used to reconstruct the phylogeny of cetrarioid lichens (e.g. Thell & Miao 1999; Thell 1999; Thell *et al.* 2000). Only a small number of

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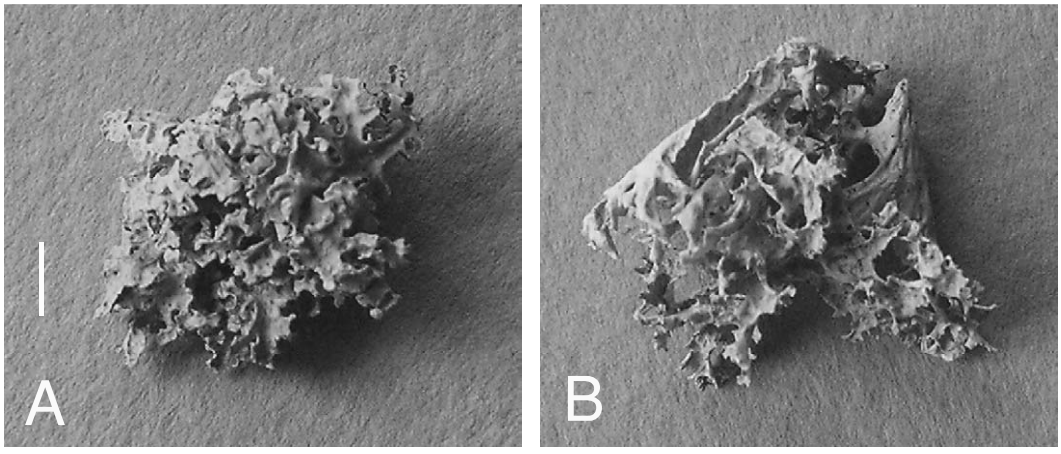

FIG. 1. *Flavocetraria nivalis*. A, Tatra Mts, Swinica; B, specimen from Polish lowlands, Tuchola Forest. Scale = 5 mm.

specimens was included in these analyses and intraspecific diversity in *F. nivalis* is unknown. Hence characterization of the mycobiont genetic variability in this species requires further studies.

In this study we were interested in whether the lowland and highland populations of *F. nivalis* differ in characteristics other than those of the mycobiont. Previously, Friedl (1989) cultivated the photobionts of several *Parmeliaceae*, including cetrarioid species, and determined *Trebouxia jamesii* as a symbiont of *Flavocetraria cucullata*, a species that occurs at similar sites to *F. nivalis*. Friedl, however, did not examine the photobiont in *F. nivalis*. Now molecular data can be used to identify photobionts of lichens without the need for isolation and culture, because an infrageneric, phylogenetic framework for *Trebouxia* is available. While Friedl & Rokitta (1997) investigated photobiont species relationships with large subunit ribosomal DNA sequences, a number of recent publications have focused on the ITS region of trebouxioid photobionts (e.g. Beck 1999; Helms *et al.* 2001; Beck *et al.* 2002; Romeike *et al.* 2002). Beck (2002) indicated that all photobionts which were assigned to *Trebouxia jamesii* using molecular methods actually belong to the *T. simplex* aggregate. In this study we have examined the genetic diversity of the photobionts of *F. nivalis*, in particular those present in lowland and highland populations in Poland.

Material and Methods

The thalli used for DNA extraction were first checked for externally visible contamination. Clean fragments of thalli were then selected for DNA isolation following the protocol of Cubero *et al.* (2000). The primers used in this study were ITS1T and ITS4T, which are specific for the trebouxioid photobionts, as described by Kroken & Taylor (2000). PCR was performed in 40-µl reaction volume (17·8 µl of H_2O, 4 µl of dNTPs [1 mM], 2 µl of each primer [10 pM], 0·2 µl *Taq* polymerase, 10 µl DNA extraction) in a thermal cycler (Gene Amp®, PCR System 2400) using the following protocol: denaturation at 94°C for 3 min; 35 cycles of 94°C for 0·45 min; annealing temperature 55°C for 0·45 min; extension at 72°C for 1·30 min; final extension of 7 min. The PCR products were cleaned with QIAquick PCR purification kit from QIAGEN and diluted into 30 µl of H_2O.

Both complementary strands were sequenced using the Big Dye Cycle Sequencing Kit (ABI) according to the manufacturer's protocol. Sequences were run on an ABI310 automated sequencer (ABI). The alignment was produced using BioEdit 5.0.6 (Hall 1999), using the accessory application ClustalW with default parameters. The specimens sequenced for molecular analysis are presented with their GenBank numbers in Table 1. Because an appropriate outgroup was not available for *Trebouxia* (see also Dahlkild *et al.* 2001), the tree was rooted with *Trebouxia impressa*, which is not present as a photobiont in *Flavocetraria*.

The dataset generated in this study was combined with the following sequences retrieved from GenBank:

TABLE 1. *Localities of* Flavocetraria nivalis *specimens sequenced in this study and GenBank accession numbers*

Locality	Code in Fig. 2.	Date	Collector, number & herbarium*	Genbank accession number
Poland, Mierzeja Wiślana, between Krynica Morska and Piaski	Poland 9	11 vii 2001	M. Opanowicz, 25	AY444759
Poland, Tuchola Forest, between villages Lubnia and Wiele	Poland 1, Poland 2	02 vii 2001	M. Opanowicz, 30	AY444757
Poland, Babia Góra Mt, 1680 m	Poland 11	22 viii 2001	M. Opanowicz, 40	AY444762
Poland, Karkonosze Mts, Czarny Grzbiet, 1400 m	Poland 12	08 vii 2002	M. Opanowicz, 67	AY444760
Poland, West Tatra Mts, Chuda Turnia, Czerwone Wierchy, 1750 m	Poland 17	18 vii 2002	M. Opanowicz, 80	AY444752
Poland, High Tatra Mts, Świnica, 2200 m.	Poland 19	23 vii 2002	M. Opanowicz, 90	AY444753
Norway, Ulvik Kommune, Finse, 1205 m	Norway 14	11 viii 2002	M. Grube, GZU	AY444751
Sweden, Öland Island, The Great Alvar, Gösslunda Hutterstad	Sweden 1	14 v 2002	T. Johansson, 74	AY444761
Sweden, Öland Island, Stenåsa, Frösslunda, 1300 m	Sweden 2	06 iv 2002	H. Lundkvist, 77	AY444763
Iceland, Eyjafjardarsýsla, Krossanesborgir, N of Akureyri	Iceland 1	25 ix 2002	E. Stocker-Wörgötter	AY444765
Iceland, Eyjafjardarsýsla, Öxnadalur Valley	Iceland 2	25 ix 2002	E. Stocker-Wörgötter	AY444764
Denmark, Greenland, Qeqertannguit	Greenland 2	23 vii 1998	E. S. Hansen, GZU	AY444755
Finland, Lappland, Nuorgam	Finland	04 viii 1995	R. Skytén 6373, MBU, Helsinki	AY444757
Ukraine, East Carpathians, Swidowiec, 1878 m	Ukraine	31 vii 2001	M. Kukwa 856, Ex UGDA	AY444754
Austria, Styria, Alp Mts, Gleinalpe S of St Michael, 1780 m	Austria 7	13 viii 2002	J. Hafellner 56468, GZU	AY444766
Austria, Styria, Alp Mts, Handalpe, 1800 m	Austria 13	28 ix 2002	M. Opanowicz	AY444768
Austria, Styria, Alp Mts, Handalpe, 1800 m	Austria 14	28 ix 2002	M. Opanowicz	AY444767

*Specimens collected by MO are deposited in her private herbarium.

AF249576 (*T. impressa* from *Melanelia glabra*), AF128271 (*T. angustilobata* from *Lecidea lapicida*), AF453260 (*T. simplex* from *Chaenotheca subroscida*), AF431582 (*T. simplex* from *Umbilicaria kappenii*), AF315855 (*T. simplex* from *Umbilicaria antarctica*), AF431586 (*T. simplex* from *Umbilicaria umbilicarioides*), AJ249571 (*T. simplex* from *Imshaugia placorodia*), Z68702 (strain of cultured *T. usneae*), AJ249573 (*T. usneae* from *Usnea filipendula*), AJ249566 (strain of cultured *T. corticola*), AJ249574 (*T. higginsiae* from *Buellia straminea*), AJ249567 (*T. galapagensis* from *Ramalina* sp.), AJ249564 (*T. arboricola* from *Punctelia subrudecta*), AF242470 (*T. showmanii* from *Lecanora hagenii*) and AJ249565 (*T. asymmetrica* from *Diploschistes diacapsis*). In addition, we included the photobiont sequence from *Lecanora swartzii*, collected in Styria, Stuhleck, *c.* 1760 m, by M. G. (GZU, AY444769).

The phylogenetic hypothesis was constructed using a Bayesian approach as implemented in the program MrBayes (Huelsenbeck & Ronquist 2001). The general time reversible substitution model with among-site variation (GTR+Γ, rates for variable site were drawn from a gamma distribution with 4 discrete categories) was used for likelihood calculations. The Markov Chain Monte Carlo (MCMC) analysis was run for 1 000 000 generations, with 4 chains starting from a random tree, and using the default temperature of 0·2. Every hundredth tree was sampled, while the first 30 000 generations were discarded as burn-in. A consensus phylogram showing mean branch lengths was calculated with the sumt command in MrBayes.

Results

The primer pair (ITS1T and ITS4T) is specific to trebouxioid lichens and was used to amplify an ITS fragment of 700 nts uniform size from DNA-isolations from *Flavocetraria nivalis*. The phylogenetic analysis shows that all the sequences obtained represent *Trebouxia simplex*.

The tree with posterior probabilities of topologies higher than 50% is presented in Fig. 2. The likelihood parameters in the tree sample (arithmetic mean of marginal likelihood = −3381·57) had the following average values (variance): rate matrix r(GT) = 1·000 (± 0), r(CT) = 8·86 (7·79), r(CG) = 1·36 (0·30), r(AT) = 4·95 (2·93), r(AG) = 7·61 (6·03), r(AC) = 2·69 (1·05), base frequencies π(A) = 0·21 (0), π(C) = 0·24 (0), π(G) = 0·27 (0), π(T) = 0·26 (0), gamma shape parameter α = 0·34 (0).

The *T. simplex* clade (node A in the tree) is strongly supported and consists of several lineages (Fig. 2). At the base of this clade symbionts from *Letharia* are found (Kroken & Taylor 2001). Another clade includes symbionts from *Pseudevernia* and *Imshaugia*, whereas *T. angustilobata* (Beck) Beck ined. is here branching as the sister clade to all symbionts found in *Flavocetraria* (node B). This group in the tree also includes symbionts of other lichens.

Within the *T. simplex* aggregate, the *Trebouxia* ITS sequences from *F. nivalis* show distinct variability and form four clades. The first clade contains only one specimen from Iceland, which was collected in a valley at low altitude (E. Stocker-Wörgötter pers. comm.). The second group is formed by specimens from the Polish High Tatra Mts (alt. 1750 m and 2200 m), as well as from Norwegian highlands (alt. 1205 m), Ukrainian Swidowiec Mts (Eastern Carpathians; alt. 1878 m), Greenland, and Finland. Photobionts of this group were also present in other unrelated lichens, for example in *Chaenotheca subroscida* (Tibell & Beck 2002) from Sweden, in *Umbilicaria* spp. from Antarctica, and in *Lecanora swartzii* from the Alps. The third group contains only three specimens, two from the Austrian Alps (alt. 1800 m), and one from Iceland. The fourth group is represented by samples from the Polish lowlands (i.e. altitudes <600 m), the Polish Karkonosze Mts (alt. 1400 m), Babia Góra Mt (alt. 1680 m), as well as from Öland (on Alvars and Stenåsa), and the Austrian Alps (at alt. 1780 m). A photobiont of *Pseudevernia furfuracea* (Kroken & Taylor 2000), collected in Austria (Kroken pers. comm.) also belongs to this group.

Discussion

Trebouxia simplex is a wide-spread photobiont in many unrelated groups of lichens and is reported here for the first time in *Flavoceteria nivalis*. The species is also known in other foliose cetrarioid species (Friedl 1989), for example in foliose *Parmeliaceae*, *Anzia colpodes*, *Hypogymnia physodes*, *Imshaugia pseudoracodia*, and in species

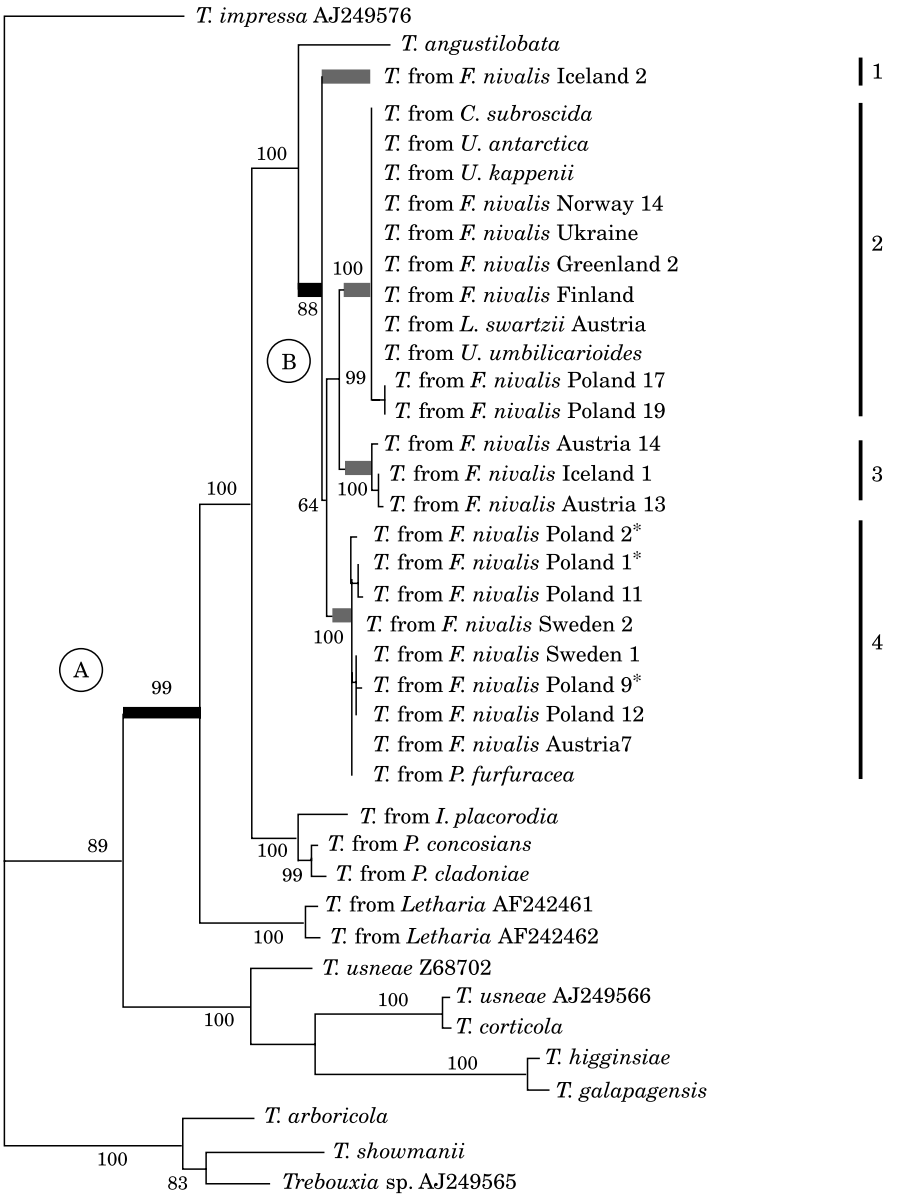

FIG. 2. Phylogenetic tree of ITS sequences from photobionts in *Flavocetraria nivalis*. Node A represents the *Trebouxia simplex* aggregate, node B the photobiont genotypes found in *Flavocetraria*. The vertical bars with numbers correspond with the clades as discussed in the text. *C.* = *Chaenotheca*, *F.* = *Flavocetraria*, *I.* = *Imshaugia*, *L.* = *Lecanora*, *P.* = *Pseudevernia*, *T.* = *Trebouxia*, *U.* = *Umbilicaria*. Bayesian posterior probabilities are given with the nodes. Samples from lowland localities in Poland are marked with an asterisk (*).

of *Parmelia*, *Pseudevernia*, and *Letharia* species. Those occurring in the last genus were studied in detail by Kroken and Taylor (2000). They showed that ITS sequences of *T. simplex* (as *T. jamesii*) are diverse in *Letharia*, and with additional sequences from the actin gene, it was concluded that *T. simplex* consists of several phylogenetic

species. Our study shows a similar diversity in ITS sequences of photobionts in *Flavocetraria nivalis*. We have not attempted to test whether the sequence diversity represents different species, as this would require more data.

The geographic distribution patterns and host specificity of photobionts are not well understood so far, but recent data show that some patterns seem to exist. While the presence of only two photobiont species in foliose *Physciaceae* was found in Scandinavia (Dahlkild *et al.* 2001), at least one different photobiont was found in this family (in *Anaptychia runcinata*) in the Mediterranean (Helms *et al.* 2001), suggesting that a broad geographic sampling is required to assess the range of photobionts selected by a mycobiont species. Recently, Romeike *et al.* (2002) found that *Umbilicaria* spp. along a transect in Antarctica may associate with different photobiont species. Interestingly, *U. antarctica* with dispersal units that include only the mycobiont, is associated with several photobiont species, while *U. kappenii*, a sorediate species, has only a single photobiont. Re-association with photobionts is apparently possible with one of several algal species in lichens that propagate via ascospores or conidia, while lichens with joint propagation of myco- and photobionts may generally have less variation of photobionts. *Flavocetraria nivalis* develops fruitbodies only occasionally and vegetative dispersal is the main propagation method. This may account for the restriction of photobionts to those in the *T. simplex* aggregate, and a prevalence of certain photobiont strains at particular localities. The sandbars on which the Polish lowland populations are found emerged only seven thousand years ago, and it is likely that these sites have been colonized by a founder population of *F. nivalis* with a single photobiont strain.

Because the photobiont strains from lowland areas in Poland are also found at two sites in Öland and the Alps, it might be suggested that the lowland population originated from one of these localities. The relationship with Öland is noteworthy because it has sometimes been argued that migrating birds could contribute to the dispersal of lichens. In fact, there are birds that move between Sweden and Poland, but these birds, which are typically found at Alvar, do not migrate to the sandbars of the Polish lowlands. The origin of the Polish lowland population remains a matter of speculation without further data, for example the lowland localities of *F. nivalis* in Baltic countries also need to be studied.

An investigation of the origin of the Polish lowland populations of *F. nivalis* using molecular data from the mycobiont has not been possible so far. Sequence data from numerous specimens (*c.* 60) have as yet not shown any variation in the genes investigated (ITS, parts of IGS, mtLSU rDNA, and an unidentified locus) (M. Opanowicz unpublished), thus study of more variable loci is required. However, the subtle morphological characteristics of the Polish lowland populations are shared by the samples from Öland. Future studies will investigate the photobiont variation throughout the whole geographic range of *F. nivalis* and will estimate genotype frequencies within smaller geographic areas.

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