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Identifying algal symbionts in lichen symbioses

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Abstract — Lichens are a ubiquitous terrestrial symbiosis of fungi with photoautotrophic microorganisms. The identification of the hosted photoautotrophs is notoriously difficult. Molecular data to clarify evolutionary relationships on the involved algal and cyanobacterial lineages are accumulating, but the assignment to species is challenging for various reasons. One of the challenges is the limited knowledge on the alpha diversity of photoautotrophs. New lineages are being discovered with increasing amounts of sequencing. Identification tools could incorporate these aspects, by routinely updating the assignment process. We propose the establishment of a classification tool using algal sequence data from public databases.

Index Terms — lichens, symbionts, photobionts, ITS, actin.

1 Introduction

Lichens are symbioses of fungi and photoautotrophic partners (algae and/or cyanobacteria). Lichens are widespread in all climatic zones and cover more than 8% of the land surface [1]. Lichens are generally named after the morphology-determining fungal partner which represents more than 18.800 known species of Ascomycetes [2]. Contrarily, the knowledge about photobiont species diversity is still limited. The determination of lichen photobionts is complicated due to the lack of diagnostic characters for routine analyses. Algae in lichenized stage do not express useful characters at all, and cultivation of algae is time-consuming and not yet possible for some lineages [3].

Recent DNA sequence analyses have studied phylogenetic diversity of algal partners in lichen symbioses. About 50% of the lichen fungal species associate with single-celled green algae, and most of these belong to the genus *Trebouxia* (Trebouxiophyceae, Chlorophyta). Although morphologically similar, different genetic lineages of these photobionts are detected in wide geographic ranges of the same lichen fungal species.

Algal symbiont sequence information is usually obtained by using algal specific primers for amplification from total lichen (=holobiont) extracts, which avoids the contamination by sequences of the fungal partner, and multiple co-occurring bacteria. The phylogenetic analyses of the internal transcribed spacer

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(ITS) nuclear region uncovered relationships among trebouxioid photobionts and selectivity of the fungal partners for their algae. Sequence data of this group of algae have accumulated significantly in the past years and meanwhile a search in GenBank using "*Trebouxia* ribosomal ITS" returns 1356 hits (as of 07.07.2010).

Phylogenetic analyses are used to assign sequenced strains to named species. This is not at all a trivial procedure, because sequence divergence among recognized species is far from equal: some taxa are separated by few nucleotide changes while more pronounced sequence divergence can be detected among other species. Thus there is uncertainty about the assignment of sequences within the range of divergence among two species.

Previous study found that sequence divergence of *Trebouxia* is recognized in several main phylogenetic clades, which have been designated by letters A, I, G, S [4]. Within these clades, subclades are distinguished by numerals. This has lead to a fairly resolved phylogenetic classification of lineages in *Trebouxia*. This phylogeny does not agree perfectly with phenotypical classification and species taxonomy. It has also been revealed that diversity of these algae was previously underestimated and includes many yet to be described species. This includes entirely new lineages as well as the better characterization of yet cryptic lineages within broadly understood species. Nevertheless, new names for species are rarely introduced, e.g. when morphological characters correlate with distinct phylogenetic positions. Fig. 1 (modified from [5]) displays the challenges. In a recent publication [3] we could show that the sister clade (*Trebouxia* sp. 1) of *Trebouxia arboricola* cannot be distinguished by ultrastructural data of cultured algae from that species, whereas the distinct clade *Trebouxia* sp. 2 could not be cultured with standard methods. Thus the phylogenetic suggest a distinct clade but phenotypic support is still missing. Whether the basal lineages of that clade could represent a further species still needs to be awaited and supported by further sequence data.

Variation within an algal species will more precisely be estimated with more sequence data. Because sexual stages are cryptic in lichenizing *Trebouxia*, sequence evolution in clonal lineages could blur species delimitation. We expect that species are increasingly recognizable as 'clusters' in the sequence space with appropriate gene loci. Sequence divergence of ITS is suitable for DNA barcoding of green algal lichen symbionts. We therefore suggest establishing an automated assignment tool that tests query sequences against a regularly updated database of lichen algal ITS sequences. Automated classifiers have been incorporated in the RDP database of bacterial rDNA [6], but do not yet exist for eukaryotes. Moreover, assignments have to consider the growing amount of environmental sequences without assignment to taxonomic names. We are therefore exploring methods to assess the coherence of related sequence as clusters in the sequence space, and the confidence of their assignment to species names. This work is still in progress and more details will be presented at the Bioidentify meeting in Paris.

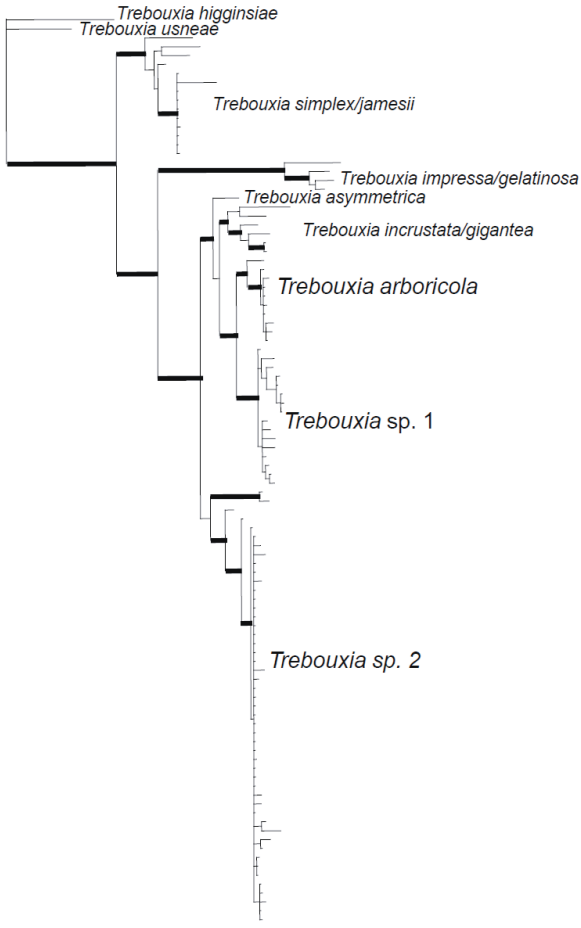

Fig. 1 – Phylogenetic tree of *Trebouxia* species as algal symbionts in lichens (modified from [3]). The tree is constructed using ITS rDNA sequence data. Symbionts in Mediterranean samples of the lichen *Tephromela atra* are named informally as *Trebouxia* sp. 1 and sp. 2. Further information is required for taxonomic recognition of these cryptic species.

2 Multiple phototrophs in lichens

Lichen fungi may internalize more than one algal symbiont. This is clearly observed when a green algal lichen thallus contains nitrogen-fixing cyanobacteria in specialized organs. Less clear, however, are cases where several algae of the same green algal lineages are involved. Culture based studies have previously shown that a single individual of lichen can host several lineages of *Trebouxia*. This variation is often poorly detected with conventional PCR approaches using whole thallus DNA extracts, whereby usually only one distinct sequence

is detected. Any additionally occurring algal sequences are obscured by the exponential amplification of the most common sequence during PCR. Because diagnostic characters of algae in the lichenized stage are hardly available it is still unclear how multiple strains of algae are distributed in lichens. Are additional algae merely epibionts, are they evenly distributed in low abundance throughout the thallus, or are they localized in certain parts of a lichen thallus?

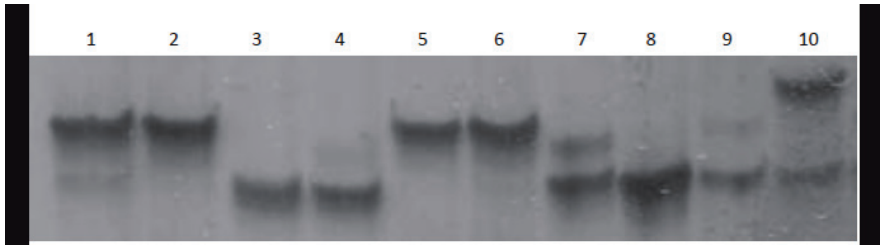

Fig. 2 – Identification of multiple algal symbionts in the lichen *Lecanora muralis* by single strand conformation polymorphism (SSCP) detection. External (odd lanes) and internal (even lanes) areoles from 5 lichen individuals were analysed. Bands of equal position represent distinct algal genotypes. *Lecanora muralis* associates with several algal genotypes, and areoles 1, 7, 9, and 10 display heterogeneity for the algal partner.

Several methods are available to analyse the composition of microbial communities. One of these is single strand conformation polymorphism (SSCP) analysis. With this method single-stranded DNA fragments can be separated on a gel according to their nucleotide sequence variation. The separated bands can then be excised from the gels for sequencing. Each band with different run-length represents a different sequence/strain of algae. Best results are obtained with sequence fragments of 200-300 nucleotides length, i.e. within the size range of the ITS subregions flanking the internal 5.8S rDNA. We are now using this method to explore the algal composition in lichens in greater detail. Fig. 2 shows a detail of a SSCP analysis of individual areoles of 5 specimens of the euryoecious lichen *Lecanora muralis*. Odd lanes are from external areoles, even lanes are from internal areoles. Symbiont heterogeneity is observed among thalli and within thalli (especially in the two samples representing lanes 7-10).

We assume that different strains are not evenly distributed at dissimilar abundances in the entire thallus. We rather think that thallus outgrowths can newly associated with algae, and that different algae could be detected in different areoles or lobes of a lichen individual. We expect that this flexibility could contribute to the ecological adaptivity of euryoecious lichens.

3 Conclusion

The ITS rDNA sequences of lichen photobionts are useful DNA barcodes to study partner selectivity in symbioses. Here we focused on algal partners in lichens. The major challenge of this work is the still unsettled taxonomy of algae, and that several algal symbionts may be present in lichen individuals. A self-organising classification tool that uses regularly updated sequence information

on algal lichen symbionts is under development.

Acknowledgement

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