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Integrating coalescent and phylogenetic approaches to delimit species in the lichen photobiont *Trebouxia*

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ABSTRACT

The accurate assessment of species boundaries in symbiotic systems is a prerequisite for the study of speciation, co-evolution and selectivity. Many studies have shown the high genetic diversity of green algae from the genus *Trebouxia*, the most common photobiont of lichen-forming fungi. However, the phylogenetic relationships, and the amount of cryptic diversity of these algae are still poorly understood, and an adequate species concept for trebouxiophycean algae is still missing. In this study we used a multifaceted approach based on coalescence (GMYC, STEM) and phylogenetic relationships to assess species boundaries in the trebouxioid photobionts of the lichen-forming fungus *Lasallia pustulata*. We further investigated whether putative species of *Trebouxia* found in *L. pustulata* are shared with other lichen-forming fungi. We found that *L. pustulata* is associated with at least five species of *Trebouxia* and most of them are shared with other lichen-forming fungi, showing different patterns of species-to-species and species-to-community interactions. We also show that one of the putative *Trebouxia* species is found exclusively in association with *L. pustulata* and is restricted to thalli from localities with Mediterranean microclimate. We suggest that the species delimitation method presented in this study is a promising tool to address species boundaries within the heterogeneous genus *Trebouxia*.

1. Introduction

Cryptic diversity is a common phenomenon in many groups of organisms (e.g. Bálint et al., 2011; Bickford et al., 2007; Hebert et al., 2004; Lumbsch and Leavitt, 2011). Different lineages of species can display different adaptive responses to environmental changes, thus overlooking cryptic diversity may lead to inefficient conservation practices (Pauls et al., 2013). Furthermore, in symbiotic associations, it is important to assess diversity of symbiotic partners at the species level to understand patterns of co-evolution and co-distribution. A correct assessment of species boundaries may provide information about species-to-species and species-to-community interactions, as well as co-distribution and co-speciation. For example, it has been proposed that lichen-forming fungi form ecological guilds by sharing the same algae. These horizontally linked fungal networks may become evident once the system is studied at the level of species and communities (Rikkinen, 2002).

Lichens are symbiotic systems composed of at least one fungal partner (mycobiont) and green algae and/or cyanobacteria (photobiont). Generally, more than one lichen-forming fungus forms associations with a single algal lineage (Friedl and Büdel, 2008). Recent studies using a combination of molecular, chemical and morphological data have shown that several groups of lichen-forming fungi may include cryptic species (Crespo and Lumbsch, 2010; Crespo and Perez-Ortega, 2009). Some studies have also highlighted the presence of cryptic diversity in algae associated with lichen-forming fungi, e.g. in the genera *Asterochloris* and *Trebouxia* (Casano et al., 2011; Škaloud and Peksa, 2010). The coccoid green algae of the genus *Trebouxia* are the most common

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and widespread photobionts involved in symbiotic associations with fungi (Friedl and Büdel, 2008; Tschermak-Woess, 1988). Studies exploring the genetic diversity of *Trebouxia* did usually not go beyond designating phylogenetic clades with letters and numerals (Helms, 2003; Ruprecht et al., 2012; Yahr et al., 2006). Despite the fact that molecular phylogenies are often not entirely congruent with current taxonomy of *Trebouxia* (Blaha et al., 2006; Romeike et al., 2002), attempts to assign species in a systematic way based on molecular and morphological data are rare. A self-organizing classification tool based on algal sequence information and single strand conformation polymorphism (SSCP) analysis has been proposed (Grube and Muggia, 2010), however, it is not widely employed. At present we lack a clear understanding of species boundaries and species richness within this genus (Škaloud and Peksa, 2010).

The traditional way to describe species is based on phenotypical characters (e.g. Gärtner, 1985). When working with groups that show limited morphological characters, this approach may lead to underestimation of the real number of species. To improve the assessment of species boundaries there is need to test morphological characters together with molecular data (e.g. Knowles and Carstens, 2007; Welton et al., 2013). Some species delimitation methods assign samples to groups without a priori information, e.g. the general mixed Yule coalescent model (GMYC, Pons et al., 2006), Gaussian Clustering (Hausdorf and Hennig, 2010), Structurama (Huelsenbeck et al., 2011), or O'Meara's heuristic method (O'Meara, 2010), while others require prior assignment of samples to putative lineages such as species tree estimation using maximum likelihood for gene trees under coalescence (e.g. STEM, Kubatko et al., 2009). One of the first widely used methods based on molecular data was the genealogical concordance phylogenetic species approach comparing the presence of putative species in single-locus analyses (e.g. Avise and Ball, 1990; Kroken and Taylor, 2001; Taylor et al., 2000). This method accommodates for incomplete lineage sorting of several genes and evaluates concordance among single-gene trees. Although this method is widely used, it has been shown to be flawed when attempting to delimitate closely related species (Carstens et al., 2013). Clades that are present in the majority of single-locus genealogies are likely to represent isolated lineages (Dettman et al., 2003). In the GMYC method the species delimitation is based on branch length differences (Monaghan et al., 2009; Pons et al., 2006). This approach has been widely applied to delimit species in many different groups such as bats (Esselstyn et al., 2012), insects (Hamilton et al., 2011), snails (Puillandre et al., 2012), lizards (Wiens and Penkrot, 2002) and lichen-forming fungi (Leavitt et al., 2013, 2012; Parnmen et al., 2012). The assumption of GMYC models is that the independent evolution leads to appearance of genetically distinct clusters, which are separated by long internal branches (Barraclough et al., 2003; Fujisawa and Barraclough, 2013; Queiroz, 2007). Another widely used coalescence model is STEM (Kubatko et al., 2009), which was developed to assess species boundaries in systems with existing subspecies taxonomy (Carstens and Dewey, 2010). This test computes the gene tree probability for all hierarchical permutations of lineage groupings. In this method the species delimitation is not affected by the phylogenetic uncertainty in the species tree, however the correctness of the method depends on the accuracy of the gene tree (Carstens and Dewey, 2010).

In the present study we used a multifaceted approach combining phylogenetic and coalescent methods to delimit species boundaries in the trebouxioid photobiont of the lichen *Lasallia pustulata* (L.) Mérat. *Lasallia pustulata* is an umbilicate macrolichen reproducing mainly by asexual propagules (isidia). From this mode of reproduction we would expect little variability of the photobiont as a result of predominantly clonal dispersal. However, genetic variability of the photobiont in *L. pustulata* populations is in fact high (Sadowska-Deś et al., 2013), an observation that has also been made in other asexually reproducing lichen species (Dal Grande et al., 2012; Nelsen and Gargas, 2009; Ohmura et al., 2006; Opanowicz and Grube, 2004; Piercey-Normore, 2006, 2009; Werth and Sork, 2010; Wornik and Grube, 2010). These studies suggest that multiple photobiont species associate with a single fungal species, and that various photobionts may be commonly available in a given environment. In this study we aimed to answer the following questions: (i) is *Lasallia pustulata* associated with a single or multiple *Trebouxia* species?, and (ii) does the range of compatible photobiont partners found in *L. pustulata* overlap with that of other lichen-forming fungi?

2. Materials and methods

2.1. Taxon sampling

We collected specimens of *L. pustulata* across the species distribution range. Out of a total of 469 thalli we selected 22 samples that had a unique haplotype at either of four photobiont loci. Fresh samples were dried and stored at −20 °C until preparation. Information about sampling locality, haplotypes and GenBank accession numbers are given in Table S1. Specimens are deposited in the herbaria Berlin (B), Frankfurt (FR), and Oslo (O).

2.2. DNA isolation, polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was extracted from a small part of the thallus using the CTAB method (Cubero and Crespo, 2002). We sequenced the algal symbiont at the following loci: internal transcribed spacer region (nrITS rDNA), chloroplast intergenic spacer (psbJ-L), cytochrome C oxidase II (*COX2*) and ribulose-bisphosphate carboxylase (*rbcL*). Primers for PCR amplification were: nrITS: nrITS1T, nrITS4T (Kroken and Taylor, 1990) and nrITSaJOFOR2, nrITSaJOREV2 (Sadowska-Deś et al., 2013); psbJ-L: psbF and psbR (Werth and Sork, 2008); *COX2*: Cox2-P2fw-50, Cox2-P2rv-30 (Fernandez-Mendoza et al., 2011); rbcL: a-ch-rbcL-203, a-ch-rbcL-991 (Nelsen et al., 2011).

Standard PCR amplification (25 μl) contained 0.65 U Ex Taq polymerase (TaKaRa BIO INC.), 1× buffer, 0.2 mM dNTP mixture, 0.5–1.0 μM of each primer, 2–50 ng DNA template, and H_2O. Thermal cycling parameters for all loci were: initial denaturation 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 40 s, 72 °C for 1 min, and final elongation 72 °C for 5 min. PCR products were separated on 1% agarose gels stained with ethidium bromide. When multiple bands were present, fragments of the expected length were extracted using the peq-GOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH). The amplicons were sequenced using Big Dye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The following cycle sequencing program was used: initial denaturation for 1 min at 95 °C, followed by 30 cycles of 96 °C for 10 s, 50 °C for 10 s, 60 °C for 2 min. Products were run on an ABI PRISMTM 3730 DNA Analyzer (Applied Biosystems).

2.3. Sequence alignment

Sequences of each locus were aligned using the MUSCLE alignment algorithm (Edgar, 2004) as implemented in Geneious v5.4.2 (Drummond et al., 2011). Before the analysis, ambiguous regions from all loci were manually removed. We confirmed sequence identity by using BLAST searches in GenBank.