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REVIEW

Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi

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Abstract Accurate species delimitations are crucial for ecological and conservation studies, assessments of biotic diversity, and identifying factors driving diversification. Estimates suggest that the vast majority of fungal species are currently unknown. Although many undescribed fungal taxa are expected to be indentified within understudied groups and from underexplored areas, mounting evidence suggests a substantial number of unrecognized fungal species are likely hidden within traditional phenotype-based species in lichen-forming fungi. Molecular genetics has revolutionized our ability to assess traditional species concepts and provides additional tools for robust species delimitation. In general, lichens display few taxonomically usefully characters; therefore molecular data have gained great importance in delimiting fungal species in lichen symbioses. As a result, the taxonomic value of phenotypical characters is now much better understood, and in many cases previously overlooked characters have been identified supporting molecular-based species circumscriptions. Although in some cases molecular research has verified traditional hypotheses, most studies repeatedly show that our current interpretation of morphological and chemical characters is inadequate to accurately characterize diversity. Here we report on the role of molecular data in understanding species-level diversity in lichenized fungi by reviewing current literature, focusing primarily on Ascomycota. While finding and applying the appropriate character sets and analytical tools remains one of the greatest challenges to empirical species delimitation in lichen-forming fungi, the available literature indicates that the inclusion of molecular data in species circumscription is crucial to establish robust hypotheses of species boundaries in this important group of fungi.

Keywords Cryptic species · DNA barcoding · Lichens · Monophyly of species · Phylogeny · Species circumscription · Taxonomy

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Introduction—Diversity in lichenized fungi

Accurate species delimitations are crucial for ecological and conservation studies, assessing biotic diversity, and identifying factors driving diversification. The number of fungi worldwide has been estimated as being about 1.5 million species (Hawksworth 1991, 2001), while more conservative estimates give a minimal number of 700,000 species (Schmit and Mueller 2007). However, results from recent environmental sampling projects indicate that the number of fungal species may even be much higher than 1.5 million (Blackwell 2011). Since approximately only 100,000 species are currently described (Kirk et al. 2008), it is reasonable to believe that the vast majority of fungal taxa are currently unknown. A large proportion of the undescribed fungal species is expected to be identified in poorly studied areas, such as tropical forests or in underexplored habitats, for example fungi growing in insects, plants or lichens (Hawksworth and Rossman 1997; Frohlich and Hyde 1999; Aptroot 2001; Sipman and Aptroot 2001; Lawrey and Diederich 2003; Arnold and Lutzoni 2007; Arnold et al. 2009). However, there is a growing body of evidence that in different groups of fungi, including lichens, species are hidden under names of supposedly widely distributed species. The phenotype-based approach to

species recognition vastly underestimates the number of species. For example, about 80 cryptic lineages are estimated being hidden under widely distributed or disjunct species in a sole family, Parmeliaceae (Crespo and Lumbsch 2010).

Lichenized fungi are an ecologically and evolutionary successful and diverse group of fungi forming mutualistic relationships with photoautotroph organisms (photobionts). More than 16,000 fungal species have been described that form lichens (Kirk et al. 2008). The photobionts are mostly green algae (Trebouxiophyceae and Ulvophyceae) and/or cyanobacteria, but rarely also species of xanthophytes and brown algae are used as symbiotic partners. Lichens occur in all terrestrial ecosystems on all continents and dominate the landscape in extreme environments, such as polar and alpine vegetations, but are especially diverse in tropical habitats (Sipman and Aptroot 2001). Since the fungi obtain carbon in the form of sugar or sugaralcohols from the photobiont, lichens can grow on any substrate, from rocks in alpine habitats, soil in deserts, and leaves in tropical rainforests. They play important ecological roles, including being pioneers of bare soil and rocks, helping to stabilize soil in arid and semi-arid regions, and contribute to the nitrogen influx in some ecosystems.

Lichen-forming fungi belong to the crown group of true fungi with the majority of them belonging to Ascomycota and only about 2% of species belonging to Basidiomycota. Within Ascomycota, lichen-forming fungi are found in five classes in the derived superclass Leotiomyceta, while the early-diverging subphyla Taphrinomycotina and Saccharomycotina, and the Pezizomycetes lack any lichenized taxa (Schoch et al. 2009; Lumbsch and Huhndorf 2010). This review focuses on the lichenized ascomycetes based on the experience of the authors, and also because the vast majority of studies aiming at addressing species delimitation in lichenized fungi has dealt with Ascomycota.

Lichen-forming fungi are important in understanding the evolution of fungi in general, especially Ascomycota. They represent the most diverse group of mutualistic ascomycetes and hence are important for an understanding of the transitions of antagonistic to mutualistic lifestyles (and *vice versa*) in this organismal kingdom (Gargas et al. 1995; Lutzoni et al. 2001, 2004; Schoch et al. 2009). They have also been shown to be crucial in understanding the ascoma-evolution (Schmitt et al. 2005, 2009) and ascoma-ontogeny in ascomycetes (Lumbsch and Huhndorf 2007) and the evolution of chemical diversity in fungi (Schmitt and Lumbsch 2009). As in other groups of fungi, molecular data have revolutionized our understanding of the evolution of lichenized fungi as discussed in several recent reviews (DePriest 2004; Lumbsch 2006; Printzen 2010).

Results and discussion

Morphological and chemical characters used in species delimitation

While ascomatal characters have traditionally played the major role in the higher-level classification of lichenized (and non-lichenized) fungi (Ott and Lumbsch 2001; Printzen 2010), species-level classification in lichen-forming fungi often include a wide array of vegetative characters as well. Morphological thalline characters used to distinguish species in lichen-forming fungi include various characters, such as thallus form and size, presence/form/color of attachment organs (e.g., rhizines, holdfast) and other supplementary organs (cilia, hairs, etc.), presence and form of pseudocyphellae and maculae, and the reproductive mode. In the latter, the type of reproduction (ascomata vs. vegetative diaspores), and the form and location of the reproductive structures are used to distinguish species. This includes different morphological types of vegetative diaspores, mainly corticated isidia and ecorticate soredia that are often formed in morphologically characteristic soralia, and the location and form of ascomata. Differences in ascoma morphology may include a number of characteristics, including location of ascomata (e.g., laminal or marginal on thallus), their position (e.g., sessile or immersed in areoles), presence or absence of thalline margins of ascomata, or the color of an apothecial disc and presence and color of a pruina. Anatomical thalline characters include the presence, size and type of a cortex of the thallus and/or thalline ascomatal margin, presence and form of crystals in the thallus and/or thalline ascomatal margin, and thickness of thalline layers. These vegetative characters are widely used by lichenologists to distinguish species in addition to ascomatal characters that are also widely used by mycologists studying non-lichenized ascomycetes. The latter include the form, color, size, and septation of ascospores, size and form and structure of asci, the hamathecium, type of epihymenium and hypothecium and the type of excipulum or peridium. Further, conidiomatal characters, especially form and size of conidia, are commonly used to distinguish species.

Ever since Nylander (Nylander 1866a,b) introduced simple spot tests for chemical examination of lichens, chemistry has played an important role in identification and classification of these organisms. Highlighting the role of chemistry in modern lichen taxonomy, Hawksworth (1976: 139) stated “any taxonomic revision not considering chemical data is likely to be regarded as incomplete”. Extrolites (secondary metabolites) found in lichens belong to various substance classes, most diverse are depsides, depsidones, and in some clades chlorinated xanthones or anthraquinones are common and diverse. Presence or

absence of specific substances or their replacements by another substance are widely used to distinguish species when correlated with geographic differences. If no morphological or geographical differences between populations containing different extrolites are found, the taxonomic significance has been disputed, with some authors distinguishing them as species and others preferring to regard them as chemical races within a species. The use of chemistry has been discussed in detail in numerous reviews (e.g., Culberson 1969, 1970; Hawksworth 1976; Brodo 1978; Leuckert 1985; Brodo 1986; Egan 1986; Rogers 1989; Lumbsch 1998a,b) and this is not reiterated here in detail. In addition to using the presence of substances, it has been proposed to arrange lichen substances into chemosyndromes of closely related substances (Culberson and Culberson 1976). The presence or absence of these chemosyndromes may potentially be used as characters to delimit species (Gowan 1986; Lumbsch 1994), while chemical differences involving the same chemosyndromes are often regarded as intraspecific variation.

It has long been known to evolutionary biologists that distinct species do not need to have diagnosable morphological differences, as summarized e.g. by Mayr (1942, 1963). This may be due to a selective advantage of maintaining a specific phenotype, parallel or convergent evolution. In a number of species, phenotypical differences are correlated with distinct lineages and this is the reason for a persistence of the pre-Darwinian morphological species concept (Ray 1686) in biological systematics. In contrast to other groups of fungi, the biological species concept, which includes members of populations that actually or potentially interbreed in nature within a species (Mayr 1942, 1963), has only rarely been used in lichenology. Notable exceptions are the studies by the Culberson's on gene flow and sibling speciation (Culberson et al. 1977; Culberson 1986; Culberson et al. 1993).

Cryptic and sibling species clearly demonstrate the inability of a morphological species concept to accommodate biological processes that are known to occur in speciation of all major clades of organisms. While the two terms were used interchangeably by numerous authors, including Mayr himself, others prefer to keep those terms separate (de Sa et al. 2005; Bickford et al. 2007). In the more restricted sense, the term sibling species is restricted to cryptic species that share a most recent common ancestor. In sibling species, the phenotypical similarity of distinct lineages is due to a morphological stasis. It was argued that this morphological stasis may be due to strong selective pressure on physiological characters for adaptation to a specific host (Schröngge et al. 2002) or to extreme environments (Nevo 2001). The discovery of many cryptic species in Polar Regions or other extreme habitats is consistent with this view (Vrijenhoek et al. 1994; Grundt et al. 2006; Lefebure et al. 2006; Vrijenhoek 2009). However, other studies suggest that cryptic species are homogeneously distributed among taxa and biogeographical regions (Pfenninger and Schwenk 2007). If cryptic species are not closely related, i.e. not sibling species, we expect the morphology to have a specific adaptive value to a habitat (Bickford et al. 2007).

Species delimitation and biogeography

In spite of the controversial issues associated with attempts to empirically circumscribe species, contemporary species concepts share the common view that species are segments of separately evolving metapopulation lineages, termed the general lineage concept, GLC; (de Queiroz 2007). This concept allows researchers to delimit species using different empirical properties associated with lineage formation and has facilitated the development of novel methods to test hypotheses of lineage separation (de Queiroz 2007). The continued interest in species delimitations has resulted in recently developed approaches used to investigate species boundaries using genetic data (Sites and Marshall 2004; Knowles and Carstens 2007; O'Brien et al. 2009; Vieites et al. 2009; Carstens and Dewey 2010; Yang and Rannala 2010). Under the GLC, more properties (e.g. monophyly, coalescence, morphology, geographic range, host preference, chemistry, etc.) supporting putative lineages are associated with a higher degree of corroboration (de Queiroz 2007). In lichen-forming fungi finding and applying the appropriate character sets and analytical tools remains one of the greatest challenges to empirical species delimitation (Wirtz et al. 2008; Crespo and Perez-Ortega 2009). Cross-validation using inferences from multiple empirical operational criteria and datasets have been shown to establish robust hypotheses of species boundaries (Dayrat 2005; Duminil et al. 2006; O'Brien et al. 2009; Ruiz-Sanchez and Sosa 2010).

Having knowledge about the circumscription of species is of major importance for evolutionary biology and diversity research, since species are the basic units of biodiversity and linked to the study of the process of speciation (de Queiroz 2005). This knowledge is also of crucial importance to other fields of biology, including ecology and conservation biology. This is especially true for a group of organisms, such as lichens, which are widely used in ecological studies. Lichenized fungi are ubiquitous elements in all terrestrial ecosystems and are important bioindicators for air pollution, forest age and health, and soil quality (Nash 2008). Hence, there is an increasing interest in documenting their diversity to understand the role of these organisms in ecosystems and their potential as sources of antibiotics or biocontrol agents.

Traditionally, biogeographical patterns were often neglected in both lichenized and non-lichenized fungi, and researchers accepted wide distribution ranges of species occurring on different continents. This was due to a common belief among mycologists and lichenologists that the "everything is everywhere" hypothesis applied to fungi. The "everything is everywhere" hypothesis, which claims that microorganisms have basically a worldwide distribution and that geographic patterns are only due to ecological requirements was revived by Finlay (2002). He suggested that eukaryotic microorganisms have global distributions. Advocates of the hypothesis that small eukaryotes lack endemism base this on studies of morphological species of ciliates (Fenchel and Finlay 2004). However, the validity of the morphology-based species recognition in these organisms has been challenged by recent molecular studies (Weisse 2006; Chantangsi et al. 2007), suggesting that the widespread occurrence of some microorganisms may be an artifact of inappropriate species circumscriptions. Further, several studies in different groups of microorganisms, including fungi, demonstrated geographically restricted distributions (e.g., Geiser et al. 1998; Kasuga et al. 1999; Koufopanou et al. 2001; Papke et al. 2003; Whitaker et al. 2003). The results of these recent studies indicate that a part of the undiscovered fungal diversity is to be found in widely distributed groups of species that are currently classified as single species based on morphological similarity. Specific cases in which molecular data to better understand the biogeography of lichenized fungi are discussed in the following chapter.

Molecular studies addressing species delimitations in lichenized ascomycetes

A number of studies using DNA sequence data have demonstrated incongruence between the phenotype-based species circumscriptions and the molecular phylogenetic reconstructions, often revealing the presence of distinct lineages within a single nominal taxon. Among those studies, several revealed subtle morphological or previously overlooked chemical differences between distinct lineages. These taxa are often referred to as "semi-cryptic" and have been identified in a wide array of species groups in different families of lichenized fungi. It may be expected to find cryptic species among the morphologically simple crustose lichens. These include for example, fungi in Acarosporaceae (Wedin et al. 2009), Graphidaceae (Lumbsch et al. 2008), and Lecanoraceae (Leavitt et al. 2011c), Lecideaceae (Ruprecht et al. 2010), and Teloschistaceae (Muggia et al. 2008; Vondrak et al. 2009); cryptic species with subtle morphological or chemical differences have also frequently found in foliose and fruticose lichens. These include, for example, species complexes in Lobariaceae (McDonald et al. 2003), several genera in Parmeliaceae (Kroken and Taylor 2001; Molina et al. 2004; Divakar et al. 2005b; Seymour et al. 2007; Wirtz et al. 2008; McCune and Schoch 2009), Peltigeraceae (Goffinet et al. 2003; O'Brien et al. 2009), some genera in Physciaceae (Cubero et al. 2004; Divakar et al. 2007; Lücking et al. 2008; Elix et al. 2009), and Sphaerophoraceae (Högnabba and Wedin 2003).

In some cases, the distinct clades were shown to have different distributions, such as *Melanelixia glabra* and *M. californica*, occurring in western Europe and western North America, respectively (Divakar et al. 2010). Other examples are disjunct species in *Xanthoparmelia* that represent distinct, cryptic species on different continents (Thell et al. 2009; Hodkinson and Lendemer 2011) or distinct species on different continents in the genus *Physcia* (Elix et al. 2009). In the *Leptogium furfuraceum-L. pseudofurfuraceum* complex sister-group relationships were found between populations from the same hemispheres, incongruent with previous classifications based on morphological differences (Otalora et al. 2010). The results of a dating estimate suggest that the species migrated via transoceanic dispersal with subsequent diversification on different continents. The most spectacular example, however, is the collective species *Parmelina quercina* sensu lato (s. l.), which was considered to be a single subcosmopolitan species, occurring in areas with Mediterranean climate in Europe and the Mediterranean, North America, and Australia. Molecular data clearly demonstrated that the populations on each continent represent distinct lineages, with two species present in western Europe and the Mediterranean (Arguello et al. 2007). The distinct clades were also supported by ultrastructural characters of the epicortex, and the size and form of the ascospores. Subsequent studies demonstrated that the Australian clade actually belongs to an unrelated clade of parmelioid lichens that was placed in the new genus *Austroparmelina*, which accommodates a number of species with a distribution center in Australasia and adjacent areas of the southern Hemisphere (Crespo et al. 2010a). This genus was shown to be unrelated to *Parmelina* but belonging to the *Parmotrema* clade as sister-group to a clade consisting of the genera *Flavoparmelia* and *Parmotrema*, while *Parmelina* forms the *Parmelina* clade with species of the genera *Bulbothrix, Myelochroa*, and *Remototrachyna* (Crespo et al. 2010b). This is a remarkable case highlighting the potential impact of convergent evolution of thallus morphology on species circumscriptions using morphological characters alone.

The frequency of the discovery of the so called "semi-cryptic" species indicates that our current interpretation of lichen morphology is inadequate, and numerous morphology-based species delimitations are based on oversimplifications. Currently, we lack a general understanding of the adaptive value of similar morphologies of distantly related taxa. At this point, limited studies explicitly

addressing growth forms and their adaptive value are available. For example, ecophysiological studies demonstrated that deeply divided fruticose lichens are able to use fog, snow or dew very efficiently (Lange and Redon 1983; Lange et al. 2006, 2007). Central cords are formed in unrelated groups of lichens and are either interpreted as ensuring stability of—an often large and hanging—fruticose thallus (*Usnea* spp.) or as water reservoirs in the genera *Seirophora* and *Simonyella* (Poelt 1983; Feige et al. 1992). However, for example, the adaptive value of a highly similar foliose thallus form in divergent lineages within the *Parmelina quercina* species-complex found in similar winter rain climates in Australia, western Europe, the Mediterranean, and western North America is completely unknown. An exciting new avenue of research in lichenology is assessing the adaptive value of morphologies, requiring closer collaborations between evolutionary biologists and ecophysiologists to adequately address these research questions.

Recently, an increasing number of studies have revealed the presence of cryptic species in lichen-forming fungi, without any recognizable morphological, chemical, or biogeographic support for distinct taxa within species complexes. Cryptic lineages have been reported in various, unrelated groups of fungi, including the *Cladia aggregata* group (Parnmen 2011), the genus *Letharia* (Kroken and Taylor 2001), the *Parmelina tiliacea* aggr. (Crespo and Lumbsch 2010), the *Parmotrema reticulatum* group (Divakar et al. 2005a), and the *Rhizoplaca melanophthalma* complex (Leavitt et al. 2011c). Further, there is evidence for the presence of cryptic lineages in tropical *Porina* spp. (Baloch and Grube 2009). The variability of mt SSU rDNA sequences within morphologically-based *Porina* spp. was found to be higher or about the same as the genetic divergence among distinct lineages in other genera of lichen-forming fungi (Högnabba and Wedin 2003; Divakar et al. 2005a). The studies on the *Cladia aggregata* and the *Rhizoplaca melanophthalma* complexes indicated the presence of a large number of cryptic species in these complexes. In the *Cladia aggregata* complex Parnmen (2011) found at least eleven putative species, all except one, being cryptic. In the *Rhizoplaca melanophthalma* complex, a total of ten candidate species were identified, four of which were previously recognized as distinct taxa, and six previously unrecognized lineages within what has thus far been considered a single nominal species (Fig. 1; Leavitt et al. 2011a,b,c). All cryptic *R. melanophthalma* s. l. species were chemically and morphologically polymorphic, although the greatest morphological and chemical variation was restricted to the most closely related lineages (*R. melanophthalma* 'cryptic species' 4–6 and vagrant forms in Fig. 1). These studies underscore the fact the cryptic species often remain undiscovered even within many well-known lichen taxa.

However, there are also other cases, in which molecular data indicate that putative morphologically/chemically distinct species likely belong to a single polymorphic lineage. This is especially true for species with different modes of reproduction. Traditionally, morphologically identical samples that form either ascomata or form vegetative diaspores (soredia), which propagate the fungal and photosynthetic partner simultaneously, were classified as distinct species (Poelt 1972). The taxonomic status of those so-called "species-pairs" has often been disputed (Tehler 1982; Mattsson and Lumbsch 1989), but molecular phylogenetic reconstructions often recover specimens with different reproductive modes together within a single monophyletic group (Lohtander et al. 1998a,b; Myllys et al. 1999, 2001; Articus et al. 2002; Molina et al. 2002; Cubero et al. 2004; Ott et al. 2004; Buschbom and Mueller 2006). These results suggest that species-pairs, i.e. morphologically and chemically identical populations that only differ in their reproductive mode (sorediate vs. fertile), usually represent a single species with variable reproductive mode. It was hypothesized that the changing reproductive mode in lichenized fungi can be explained by conflicting reproductive (favoring sexual reproduction) and nutritional (favoring vegetative reproduction that disperses both symbiotic partners) requirements imposed by the obligate symbiotic life-style of these fungi. These interacting constraints are interpreted as producing recurring selective sweeps within predominantly vegetatively reproducing lineages (Buschbom and Barker 2006; Buschbom and Mueller 2006). However, this phenomenon appears to be largely restricted to sorediate/fertile species pairs, while isidiate species have generally been confirmed as forming distinct lineages in phylogenetic studies (Crespo and Perez-Ortega 2009).

While most studies showing overestimation of diversity based on a morphological species delimitation focus on species pairs, some recent results indicate that the phenotype-based species delimitation in the hyper-diverse genus *Xanthoparmelia* with approximately 800 described species (Hale 1990; Elix 2003) led to an overestimation of the true diversity. In this genus, morphological and chemical characters, such as substrate, life-form (vagrant vs. attached to substrate), color of lower surface, form of rhizines, mode of reproduction, presence of conidiomata, and the presence of extrolites, were used in combination to delimit species. However, recent molecular studies do not support this classification (Leavitt et al. 2011a,b). In many cases, species-level genetic clusters inferred from six nuclear loci were shown to be morphologically and chemically polymorphic, containing up to eight traditionally circumscribed *Xanthoparmelia* species within individual

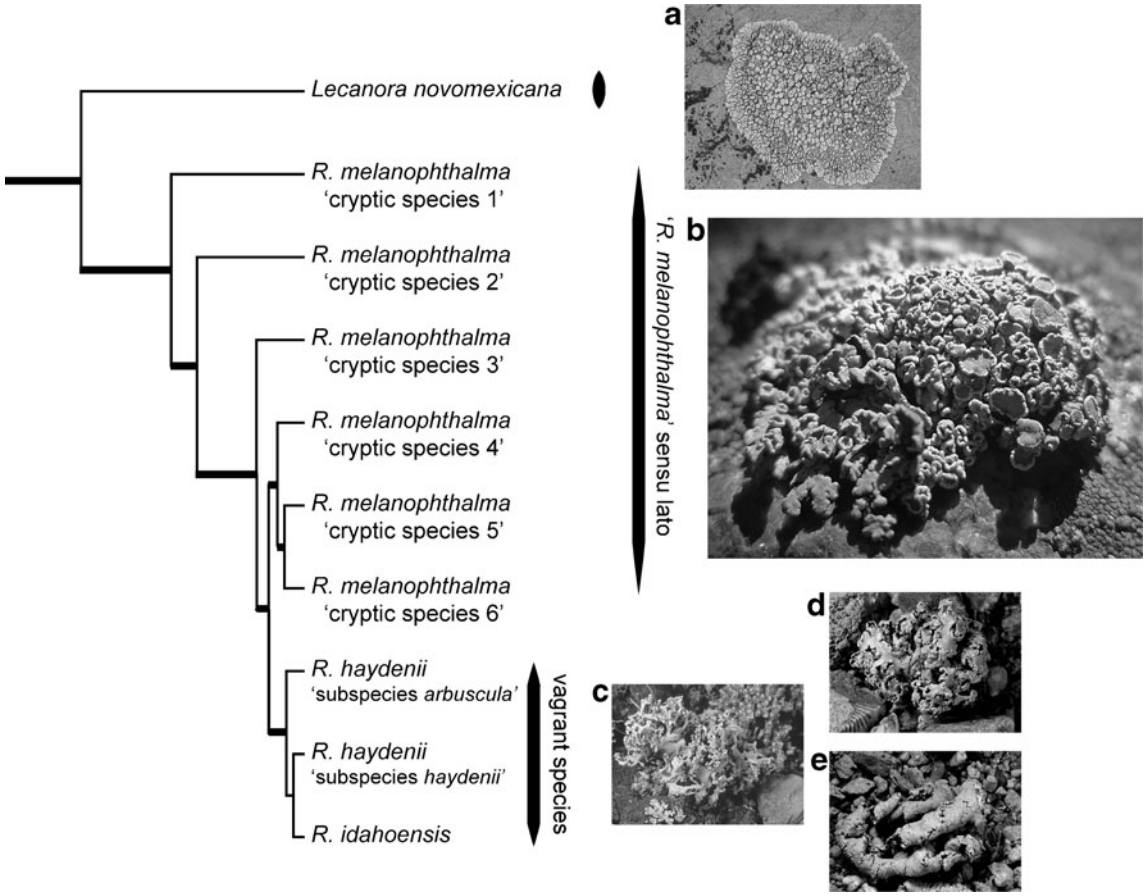

Fig. 1 Cryptic diversity within the *Rhizoplaca melanophthalma* species-complex shown in the multilocus species tree inferred from three ribosomal loci and two protein coding markers using a multispecies coalescent approach. The *R. melanophthalma* species-complex includes morphologically diverse taxa, including (**a**) *Lecanora novomexicana*, (**b**) *Rhizoplaca melanophthalma* sensu lato, and known vagrant *Rhizoplaca* species (**c**-**e**). A posteriori examinations of cryptic *R. melanophthalma* lineages did not reveal diagnostic morphological characters supporting distinct lineages within the nominal taxon *R. melanophthalma*. Thickened branches indicate posterior probability support >0.95, and speciation probabilities at all nodes (see Yang and Rannala [2010] were greater than 0.95. Images (**a**, **c**, **d**, **e**) are provided by S. Sharnoff. (see Leavitt et al. 2011a for complete details)

lineages. The results from this study indicate that frequent changes in morphological and chemical characters obscure the recognition of natural lineages with *Xanthoparmelia* (Fig. 2), and call for a major taxonomic revision within one of the best-studied genera of lichen-forming fungi.

Interestingly, chemical characters often outperform morphology in corroborating distinct lineages identified in molecular phylogenetic reconstructions, especially when different chemosyndromes are involved. For example, the distinction of the chemically distinct species *Parmeliopsis ambigua* and *P. hyperopta* (presence of usnic acid or atranorin in the upper cortex) was confirmed in a molecular study (Tehler and Kallersjo 2001). Other studies confirmed chemically distinct populations to represent different species (Lücking et al. 2008; Elix et al. 2009). However, in some groups no correlation of presence of extrolites and phylogenetic lineages could be found, indicating chemical polymorphism among interbreeding populations (LaGreca 1999; Wirtz et al. 2008; Nelsen and Gargas 2009; Parnmen 2011). Another study addressed putative species in the genus *Bryoria* that were distinguished based on the occurrence of the yellow pigment vulpinic acid (Velmala et al. 2009). While in *B. fremontii* the yellow pigment is restricted to the soralia and ascomata, it occurs throughout the thallus in *B. tortuosa* (Brodo and Hawksworth 1977). Molecular data did not support this distinction but rather demonstrated some genetic differentiation between European and North American populations of vulpinic acid-containing populations.

Using phylogenetic- and cohesion-based methods to address species delimitation

While it has become increasingly clear with recent studies that in many cases traditional phenotype-based species delimitations do not accurately reflect the true diversity of

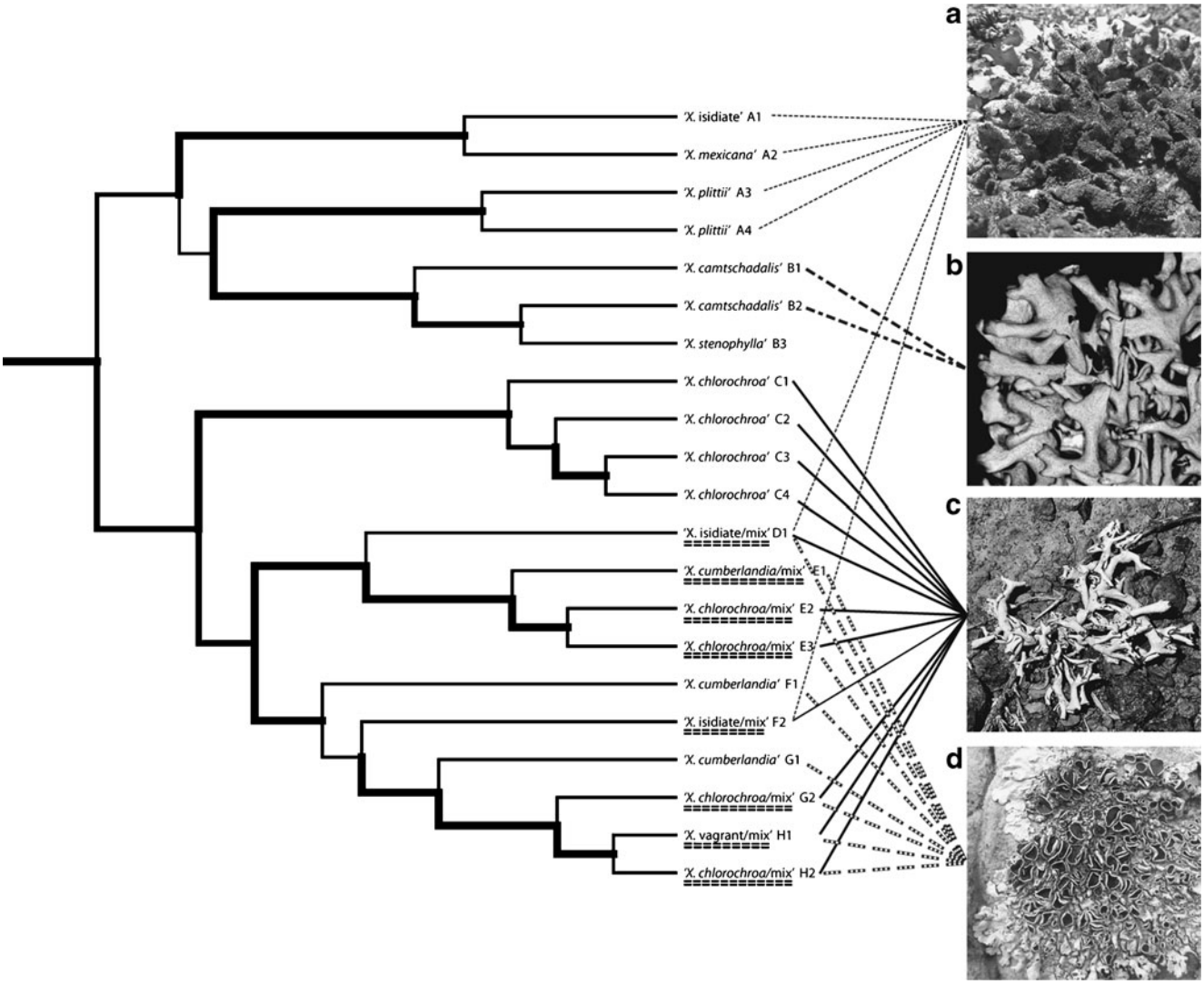

Fig. 2 The multilocus species tree, representing relationships between species-level genetic clusters inferred from four ribosomal loci and two protein coding markers generated from *Xanthoparmelia* specimens collected in western North America, estimated using a multispecies coalescent approach. Traditional combinations of morphological and chemical characters used for species recognition in *Xanthoparmelia* were not supported. Major diagnostic characters are distributed across divergent lineages, including (**a**) presence of isidia; (**b**) maculate upper cortex (in vagrant forms); (**c**) emaculate vagrant growth form; and (**d**) emaculate saxicolous forms. Underlined terminal labels indicate chemically polymorphic genetic clusters. Thickened branches are proportional to the level of posterior probability support, and speciation probabilities at all nodes (see [Yang and Rannala 2010] were greater than 0.95. (see Leavitt et al 2011b for complete details)

lichen-forming fungi, the delimitation of species based on molecular data is not without problems either. There may be problems with the molecular data themselves, particularly in recently diverged lineages (Knowles and Carstens 2007). In some cases the selected loci may simply not be variable enough to distinguish among closely related species or multiple copies of a target locus may be present. This could be due to a relaxation of concerted evolution in ribosomal DNA that has been demonstrated in different groups of fungi (O'Donnell and Cigelnik 1997; Rooney and Ward 2005; Simon et al. 2005; Chang et al. 2008; Simon and Weiss 2008) or presence of paralogous copies of duplicated, protein-coding genes (Landvik et al. 2001; Opanowicz et al. 2006; Feau et al. 2011).

As discussed elsewhere in detail, single-gene approaches cannot accommodate for incomplete lineage sorting between closely related species (Grube and Kroken 2000; Taylor et al. 2000). While single gene studies may be sufficient to support the distinction of disputed species, they fail to provide sufficient evidence to show that populations belong to a single species. Therefore approaches using multiple independent loci are necessary to test species delimitations. Further, application of a strict phylogenetic species recognition based solely on monophyly of taxa is problematic, since

it is unclear where the limit is between structured populations and cryptic lineages. Recently, the genealogical concordance phylogenetic species recognition has been employed to test species delimitations (Taylor et al. 2000). This recognition method uses concordance or discordance among loci as indication for the isolation of clades. If different gene trees have the same tree topologies it is assumed that this concordance is due to fixation of previously polymorphic loci following genetic isolation. Discordance, i.e. conflict among the gene trees of different loci is interpreted as being the result of recombination among individuals within a species. The transition from concordance to discordance determines the limits of species under this recognition method. Strong support for clades in nodes in multi-locus phylogenies is used as an indicator of species boundaries. The problem with this approach is, however, that homoplasy that leads to low support values can be caused by numerous factors, including parallel evolution, reversals or small number of differences.

Other methods attempt to circumvent these problems. Evaluation of gene flow among clades with population genetics statistics has been used in a study addressing species delimitation in the genus *Peltigera* (O'Brien et al. 2009). Another method, the general mixed Yule coalescent method, aims at identifying the transition of tokogenetic (intraspecific) and phylogenetic relationships on a chronogram (Pons et al. 2006; Monaghan et al. 2009). The most recent common ancestral node at the transition point is interpreted as distinguishing species. This method has been used to delimit species and detect cryptic species in various organisms, such as insects in Madagascar (Monaghan et al. 2009), skinks (Miralles et al. 2011), amphibians (Crawford et al. 2010), or rotifers (Fontaneto et al. 2011), and has recently also been applied to lichenized fungi (Parnmen 2011). However, this method also assumes monophyly of species and can only detect the minimal number of species present in the data set.

Among other scenarios, monophyly of species cannot be expected in the presence of peripatric speciation (Fig. 3; see Funk and Omland [2003] for detailed discussion). If speciation takes place in small, peripheral populations, these are expected to undergo rapid genetic change due to genetic drift, resulting in the founder-effect (Mayr 1963). There is evidence for the presence of peripatric speciation in ascomycetes (Wang et al. 2010) and hence non-tree based methods are an attractive alternative to accommodate for this speciation process. Network-based approaches, including the cohesion species recognition (Templeton 2001), have recently been used exclusively or in addition to phylogenetic methods to address species delimitation in lichen-forming fungi (Printzen and Ekman 2002; Printzen et al. 2003; Wirtz et al. 2008; Leavitt et al. 2011a,c).

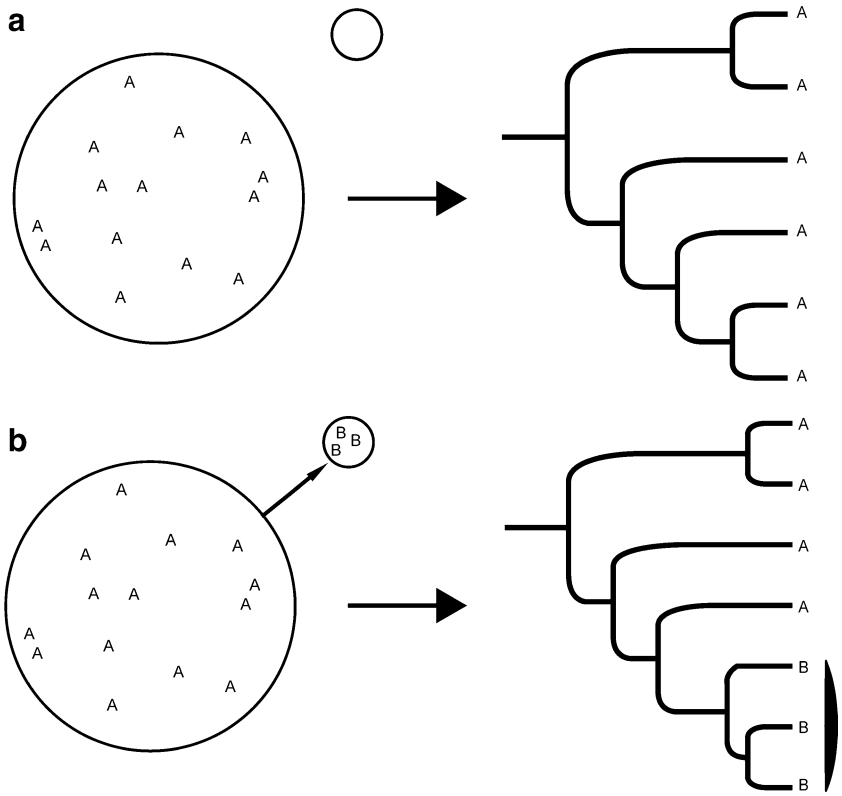

Fig. 3 A hypothetical example of peripatric speciation in which a small population along the periphery of a species range becomes isolated and undergoes speciation due to genetic drift and selective pressures. **a** The large circle represents the broad distribution of the 'parental' species, 'A', recovered as a monophyletic clade in phylogenetic reconstructions. In (**b**) the small circle represents a small and locally isolated population, species 'B', derived from the parental species. Phylogenetic reconstructions results in a monophyletic set of haplotypes, species 'B'—indicated by bar, nested within a widely distributed and paraphyletic parental species, 'A'

A further issue is the identification of problematic species complexes. If DNA sequence data are already available, a rapid and powerful method relying on comparisons of genetic distances can be used (Del-Prado et al. 2010). Intra- and interspecific distances derived from maximum-likelihood phylogenetic trees inferred from DNA sequences are compared. Based on these distance, the method uses thresholds of genetic distances to identify species. The method has been demonstrated to be powerful in identifying species complexes with unusually high genetic diversity in parmelioid lichens.

Since the distance-based method can be used with single gene data sets, it will be useful in evaluating DNA barcode data (Seifert et al. 2007; Seifert 2009) that will soon be developed with the expected announcement of a fungal barcoding gene. DNA barcoding is aiming at providing a universal tool for identification of organisms. However, DNA barcoding studies have also been instrumental in identifying cryptic species in a variety of organisms (e.g., Saunders 2008; Chantangsi et al. 2007; Le Gall and Saunders 2010; Johnson et al. 2008; Zemlak et al. 2009; Burns et al. 2008; Gomez et al. 2007; Moura et al. 2008).

Conclusion

Species delimitation in lichenized fungi remains a challenge in the molecular era. However, the appropriate use of DNA sequence data, in conjunction with other lines of evidence, allows researchers to identify species and to rigorously test species boundaries with precision that had been unimaginable a decade ago.

Although different datasets and operational criteria may give conflicting or ambiguous results due to multiple evolutionary processes associated with speciation, the use of independent suites of data and multiple empirical methods have been shown to establish robust species boundaries in many lichen-forming fungal lineages. Our current interpretation of morphological characters has been shown to vastly underestimate the diversity of lichenized fungi. However, the widespread occurrence of cryptic species, supported by previously unrecognized subtle morphological and/or chemical differences identified *a posteriori* with the help of a molecular phylogenetic hypothesis, demonstrate that finding and applying the appropriate morphological character sets may be a major reason for the discrepancies of traditional species concepts and molecular-based species delimitations. Also the schematic use of morphological characters in favor of an approach that uses a *gestalt* concept, has led to a misunderstanding of the diversity of lichen-forming fungi.

These are exciting times for lichenologists and new data will help us to better understand the evolution and diversity of lichenized fungi, appropriately interpret distribution patterns, more thoroughly assess ecological roles of lichen symbioses, and play an important role in effective conservation practices. We are hopeful that lichenologists, who traditionally have been eager to include new methods, such as chromatography, in their routine identifications, will be amenable to include molecular techniques to their routine examination of specimens for identification and classification. Although this may prove difficult to achieve by single individuals, especially civic scientists that traditionally play and important role in lichen taxonomy (Poelt 1992), the increasing number of collaborative projects in lichenology (e.g., Gueidan et al. 2009; Crespo et al. 2010b; Lumbsch et al. 2011) make us optimistic that broad-scale collaborative approaches will facilitate the inclusion of molecular data in lichen research at all levels. This approach is essential to successfully increase our knowledge of the diversity of lichenized fungi on this planet.

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