2 The Dynamic Discipline of Species Delimitation: Progress Toward Effectively Recognizing Species Boundaries in Natural Populations

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Abstract

Species represent a fundamental unit in evolutionary biology and provide a valuable context for organizing, evaluating, and communicating important biological concepts and principles. Empirical species delimitation is a dynamic discipline, with ongoing methodological and bioinformatical developments. Novel analytical methods, increasing availability of genetic/genomic data, increasing computational power, reassessments of morphological and chemical characters, and improved availability of distributional and ecological records offer exciting avenues for empirically exploring species delimitation and evolutionary relationships among species-level lineages. In this chapter, we aim to contribute a contemporary perspective on delimiting species, including a brief discussion on species concepts and practical direction for empirical species delimitation studies. Using lichen-forming fungi as an example, we illustrate the importance and difficulties in documenting and describing species-level biodiversity.

Keywords

Barcoding · Coalescence · DNA taxonomy · Fungi · Gene tree · Genomics · Lichens · Species circumscription · Species concept · Species tree

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2.1 Introduction: What's in a Name? The Importance of Accurate Species Delimitations

Although there are over 1.5 million species formally named by scientists, current estimates of the number of species alive on the planet today range from approximately two million to over one hundred million (Caley et al. 2014). Documenting, describing, and naming this diversity is paramount for conservation, human health, food security, and recreation (Tewksbury et al. 2014). In a broad sense, species delimitation is the process of identifying how individuals and populations fit into natural, species-level clusters, and not simply constructs of classification (Carstens et al. 2013). Empirical species delimitation is a dynamic discipline, with ongoing methodological and bioinformatical developments due to a growing interest in empirical species delimitations. Novel analytical methods, increasing availability of genetic/genomic data, increasing computation power, reassessments of morphological and chemical characters, and improved availability of distributional and ecological records offer exciting avenues for empirically exploring species delimitation and evolutionary relationships in species all over the world. In this chapter, we aim to contribute a contemporary perspective on delimiting species and offer practical direction for empirical species delimitation studies.

To illustrate the importance and difficulties in documenting and describing biodiversity, we will use the lichens as an example. Lichens describe a mutualism between a fungus (mycobiont) and a photosynthetic partner (photobiont), which can be either a green algae and/or cyanobacterium. Lichens are ubiquitous components of most terrestrial ecosystems, playing important ecological roles and contributing to global biogeochemical cycles (Porada et al. 2014; Bonan and Shugart 1989; Longton 1997). Due to the fact that many lichens live and grow continuously for decades, or even hundreds of years, showing cumulative responses to changes in atmospheric pollution levels, land management practices, and fluctuation climate, these are commonly used as bioindicators to monitor the impacts of air pollution, forest age, soil quality, and climate change (McCune 2000). As iconic examples of symbiosis, lichens also provide crucial insight into general patterns and processes in symbiotic systems. Central to understanding the dynamic roles of lichens is our ability to accurately delimit and recognize species boundaries. Increased accuracy in recognizing species boundaries in lichenized fungi has major implications for enhancing our perspective on biological diversity, evolution, ecology, symbiotic interactions, biomonitoring research, and conservation policy.

2.1.1 Species: Concepts and Criteria

Species serve as a central unit for categorizing biological diversity. Humans, including birdwatchers, doctors, fisherman, gardeners, politicians, scientists, and others, rely to varying degrees on recognizing species for distinguishing different kinds of organisms and effective communication. In the biological sciences, species represent one of the most fundamental units and provide a valuable context for organizing, evaluating, and communicating important biological concepts and principles (Coyne and Orr 2004; Mayr 1963; Darwin 1859). Due to the fact that biological information is commonly provided with reference to a species unit, accurate species circumscriptions are integral to interpreting biological patterns and processes across a wide range of subdisciplines in biology (e.g., anatomy, behavior, ecology, evolution, physiology, etc.).

In spite of the underlying importance of species in biology, the conceptualization of the term "species" remains somewhat contentious (de Queiroz 2007; Hausdorf 2011; Coyne and Orr 2004; Mayden 1997; Simpson 1951; Mayr 1963). Most biologists agree that biological nature is aggregated into discrete, evolutionarily independent entities, i.e., "species" (Coyne and Orr 2004), although theorists and empiricists alike continue to debate over an all-encompassing species concept and appropriate operational criteria for delimiting species (Hausdorf 2011;

de Queiroz 2007; Hey 2006; Donoghue and Gauthier 2004; Cracraft 1983; Mishler and Brandon 1987; Mayr 1970). Over two-dozen different and at least partially incompatible species concepts have been proposed, each based on distinct biological properties, e.g., differences in genetic or morphological features, adaptive zones or ecological niches, mate recognition systems, reproductive compatibility, monophyly, etc. (de Queiroz 2007; Mayden 1997). Hausdorf (2011) argues that most species concepts are useable, but acceptance of a specific concept requires an appropriate adaptation of the term "species" and of species delimitation. In contrast, de Queiroz (1998) and Mayden (1997) argue that distinct species concepts emphasize different properties of species rather than fundamental conceptual differences, and all modern species concepts share an important commonality—equating species with segments of metapopulation lineages. This "general lineage concept" (GLC; de Queiroz 1999) highlights that no single property should be regarded as defining for the recognition of species, apart from forming lineages (Simpson 1951), and segments of metapopulation lineages (i.e., "species") may exist regardless of our ability to empirically delimit them (Camargo and Sites 2013).

We concur that the GLC provides a practical solution to the species concept impasse, and our discussion of species delimitation is based on the GLC. Arguably, the major implication of the GLC is that most of the earlier species concepts should be regarded as secondary species "criteria," rather than "concepts," that can provide evidence of lineages separation (Sites and Marshall 2003; Camargo and Sites 2013; Mayden 1997; de Queiroz 2007). This pivotal distinction disentangles the conceptual issues of defining "species" from methodological issues of delimiting species boundaries (Camargo and Sites 2013). The GLC allows researchers to delimit species using different empirical properties and facilitates the development of new methods to test hypotheses of lineage separation (de Queiroz 2007). Although different datasets and operational criteria may give conflicting or ambiguous results due to multiple evolutionary processes occurring within and between populations (e.g., Miralles and Vences 2013; Satler et al. 2013), the use of several independent suites of characters, such as genetic data, morphology, geographic range, host preference, chemistry, and cross-validation using inferences from multiple empirical operational criteria, can provide robust hypotheses of species boundaries (Carstens et al. 2013; Fujita et al. 2012).

2.1.2 Species in Lichenized Fungi: Cases of Cryptic Diversity, Polymorphic Lineages, and Striking Biogeographic Patterns

Similar to most major biological groups, including birds (McKay et al. 2013), fish (Wagner et al. 2013), plants (Griffin and Hoffmann 2014), arthropods (Schlick-Steiner et al. 2010; Moreau 2009), and many others, finding and applying the appropriate character sets and analytical tools is one of the greatest challenges with empirical species delimitation in lichen-forming fungi (Lumbsch and Leavitt 2011). Understanding the differences between morphological variation within a species and among closely related groups is central to identifying diagnostic characters required for establishing accurate phenotype-based taxonomic boundaries. However, in practice, a clear demarcation between intraspecific and interspecific variation is commonly subject to observational bias and individual interpretation.

Traditionally, differences in morphological, chemical, and ecological features have been the predominant source of diagnostic taxonomic characters for circumscribing lichen-forming fungal species (Printzen 2009). However, lichens generally display few taxonomically useful characters, relative to other groups (e.g., vascular plants, vertebrates, and arthropods) (Printzen 2009), and varying levels of intraspecific variation among different species groups may confound accurate taxonomic circumscriptions. While some species may have little variation, high levels of intraspecific phenotypic variation are well documented in some lichen-forming fungi (e.g., *Xanthoparmelia*; Hale 1990). Therefore, molecular

genetic data now play a prominent role in delimiting fungal species and understanding evolutionary relationships in lichens (Lumbsch and Leavitt 2011; Printzen 2009).

Arguably, the use of molecular data has led to an improved perspective on the taxonomic value of many phenotypic characters in lichenized fungi and species delimitation in general. Cryptic species-level lineages are commonly identified using molecular data, and in some cases, these previously unrecognized lineages are corroborated by formerly overlooked phenotypic characters (Pino-Bodas et al. 2012a; Spribille et al. 2011; Divakar et al. 2010; Argüello et al. 2007). Other studies have revealed the fact that some species-level lineages are likely comprised of chemically and morphologically polymorphic individuals, which are conventionally considered as separate species (e.g., Leavitt et al. 2011a; Pino-Bodas et al. 2011; Velmala et al. 2009). While these studies highlight the limitations of using traditional taxonomic characters for distinguishing natural groups in lichen-forming fungi, they also provide a valuable perspective on the importance of ongoing research in even the best-studied lichen groups. Furthermore, an improved perspective of species boundaries has led to a striking improvement in understanding diversification and distribution in many groups of lichenized fungi.

Traditional phenotype-based approaches to species recognition appear to vastly underestimate diversity in lichen-forming fungi. While molecular research has corroborated traditional, phenotype-based hypotheses of species boundaries in a number of cases, studies repeatedly demonstrate that our current interpretation of morphological and chemical characters often fails to accurately characterize species-level diversity (reviewed in Lumbsch and Leavitt 2011). A growing body of evidence reveals that a significant proportion of unknown diversity estimated for fungi, including lichen-forming fungi, is hidden under names of supposedly common and widespread species. For example, approximately 80 unrecognized species-level lineages are estimated to occur in Parmeliaceae (Crespo and Lumbsch 2010). Even higher levels of unrecognized species diversity are estimated to occur in other families, such as Graphidaceae (Rivas Plata and Lücking 2013; Rivas Plata and Lumbsch 2011; Lücking 2012).

The topic of cryptic species, cases where two or more distinct species-level lineages are erroneously classified (and hidden) under one nominal taxon (Bickford et al. 2007), has been frequently reviewed for lichen-forming fungi (Lumbsch and Leavitt 2011; Crespo and Lumbsch 2010; Crespo and Pérez-Ortega 2009; Printzen 2009). Although novel diagnostic phenotypic characters may potentially be identified corroborating "cryptic" species, these previously unrecognized lineages generally remain difficult to classify within a traditional phenotype-based taxonomy (Leavitt et al. 2013c, d). In some cases, it appears that similar phenotypic characters may arise in parallel at local or regional scales, but may not be correlated with natural groups or genetic isolation (Muggia et al. 2014; Rivas Plata and Lumbsch 2011; Grube and Hawksworth 2007). For example, in the cosmopolitan species *Tephromela atra* (Fig. 2.1), up to 15 independent lineages were identified using phylogenetic analyses of molecular sequence data. However, the continuum of morphological and chemical variability in the *T. atra* complex currently prevents the description of new species using traditional phenotype-based characters (Muggia et al. 2014).

Recent research on the genus *Cladonia* (Cladoniaceae) highlights a fitting example of the complexities associated within using phenotypic characters for delimiting species in lichen-forming fungi (Fig. 2.1; Pino-Bodas et al. 2011, 2012a, b, 2013a, b). In the *Cladonia gracilis* group, most currently accepted species were not recovered as monophyletic clades and traditional diagnostic morphological characters were shown to be highly homoplasious (Pino-Bodas et al. 2011). Similarly, *C. iberica* and *C. subturgida* have been shown to constitute a single morphologically and chemically polymorphic species (Pino-Bodas et al. 2012b). Similar patterns of high degrees of morphological and chemical polymorphisms have also been observed in the

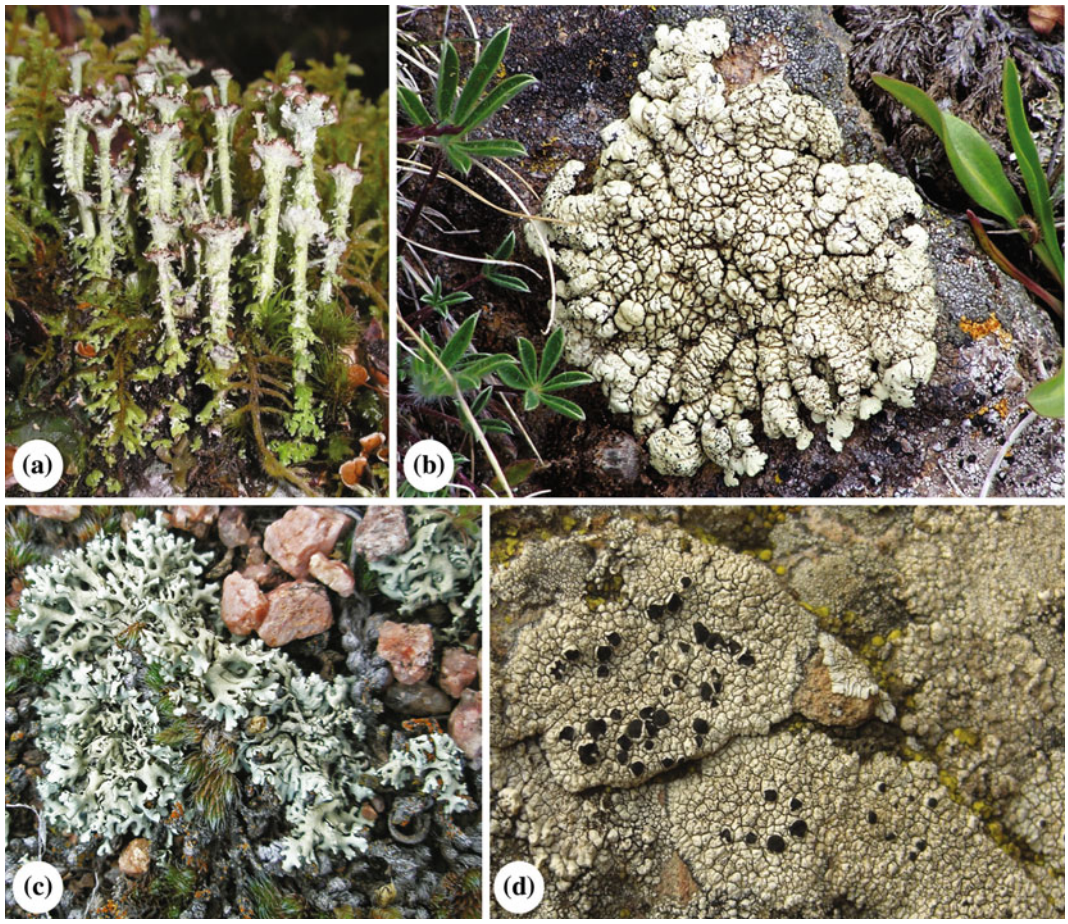

Fig. 2.1 Examples of common lichens in which traditional morphology-based species circumscriptions fail to reflect natural species-level fungal lineages. **a** *Cladonia gracilis* photographed in the Clearwater Valley, British Columbia, Canada (see Pino-Bodas et al. 2011) (*photograph credit* Curtis Björk). **b** *R. shushanii*, a member of the *Rhizoplaca melanophthalma* species group, from the Aquarius Plateau, Utah, USA (see Leavitt et al. 2011a) (*photograph credit* S. Leavitt). **c** *Xanthoparmelia* aff. *wyomingica* occurring in Colorado's Front Range, USA (see Leavitt et al. 2011b, c; Leavitt et al. 2013e) (*photograph credit* S. Leavitt). **d** *Tephromela atra* sensu lato found in the Santa Monica Mountains, California, USA (see Muggia et al. 2014) (*photograph credit* J. Hollinger)

C. cariosa group (Pino-Bodas et al. 2012a) and *C. humilis* species complex (Pino-Bodas et al. 2013a). High levels of intraspecific morphological and chemical polymorphisms are not restricted to *Cladonia*. A clear demarcation between intraspecific and interspecific morphological and/or chemical variation does not exist for a large proportion of *Xanthoparmelia* species in western North America, and high intraspecific morphological and chemical variation are common for a number of species-level genetic groups (Fig. 2.1; Leavitt et al. 2011b, c, 2013e). In some cases, as many as eight traditionally circumscribed *Xanthoparmelia* species were recovered in a single species-level genetic group (Leavitt et al. 2011c). High levels of intraspecific phenotypic variation have been observed in a number of other genera in Parmeliaceae, including *Bryoria* (Velmala et al. 2009), *Cetraria* (Pérez-Ortega et al. 2012), *Vulpicida* (Mark et al. 2012), and others.

The importance of biogeography in lichen-forming fungal evolution has remained somewhat

ambiguous due to the occurrence of phenotypically similar lichens occurring across broad, intercontinental distributions and uncertainty of appropriate species circumscriptions. While a number of lichen-forming fungal species have been found to be truly widespread (e.g., Lindblom and Søchting 2008; Fernández-Mendoza et al. 2011; Ahti and Hawksworth 2005; Del-Prado et al. 2013), improved recognition of species boundaries has provided insight into important biogeographical patterns in lichens previously assumed to have cosmopolitan distribution patterns (Leavitt et al. 2013d; Del-Prado et al. 2013; Amo de Paz et al. 2012; Sérusiaux et al. 2011; Otálora et al. 2010; Elix et al. 2009). Cryptic diversity and complex biogeographic patterns are highlighted in the *Rhizoplaca melanophthalma* species group (Fig. 2.1; Leavitt et al. 2013d). Analyses of *R. melanophthalma* sensu lato collected from five continents supported the presence of cryptic species within this complex. Two of these lineages were found to have broad intercontinental distributions, while the other four were limited to western North America (Leavitt et al. 2013d). Most strikingly, of the six lineages, five were found on a single mountain in the western USA and the sixth occurred no more than 200 km away from this mountain. A recent study of *Pseudocyphellaria* (Lobariaceae) sensu lato in Hawaii revealed a surprising number of previously unrecognized species hidden within nominal taxa with putative broad geographic distributions, suggesting a high degree of endemism in Hawaii (Moncada et al. 2014). These studies provide crucial impetus to reevaluate species boundaries in order to improve our understanding of diversity, distributions, and evolution in lichenized fungi.

2.2 A Practical Guide to Contemporary Species Delimitation

As it has become clear that conventional phenotype-based criteria for species circumscriptions are often problematic (Bickford et al. 2007), molecular data are thereby particularly valuable for assessing traditional species boundaries and for species delimitation in general (Lumbsch and Leavitt 2011; Fujita et al. 2012). Below, we provide an overview of relevant operational species delimitation methods, the majority of which are reliant on molecular data coupled with molecular phylogenetic and population genetic analyses. Assuming that species do in fact represent "segments of metapopulation lineages" (de Queiroz 1998), direct genetic evidence of lineage status is particularly relevant to species delimitation studies when analyzed within a rigorous statistical framework, regardless of whether lineages differ in phenotypic characters that are apparent to human observers (Fujita et al. 2012). This perspective should not be taken as support for disregarding phenotypic characters in species delimitation studies (Edwards and Knowles 2014; Fujita et al. 2012; Yeates et al. 2011). Rather, hypotheses of species boundaries should be considered more robust with increasing corroboration from independent data sources (i.e., molecular, chemistry, morphology, ecology, etc.), and the integration of independent data for empirical species delimitation studies should be a major focus of species delimitation research (Fujita et al. 2012).

The majority of molecular phylogenetic studies of lichenized fungi focus on generating sequence data from a number of specimens representing the focal group, inferring a gene tree from the genetic data matrix, and assessing the monophyly of the sampled taxa. This approach has provided valuable acumen into evolutionary relationships, the value of morphological characters for taxonomy, and insight into diversity of lichenized fungi (Thell et al. 2009; Reese Næsborg et al. 2007; Westberg et al. 2007; Martín et al. 2003; Arup and Grube 2000; Lohtander et al. 2000; Stenroos and DePriest 1998). However, simply assessing the monophyly of traditional phenotype-based species often offers an incomplete perspective on species boundaries. Studies explicitly designed for empirically delimiting species are pivotal to advancing our understanding of speciation and species diversity in lichens. For example, although a nominal species may be recovered as monophyletic in a gene tree, intraspecific phylogenetic substructure may correspond to evolutionarily

independent lineages (e.g., Leavitt et al. 2011a). As a construct of taxonomy, recognizing a monophyletic clade comprised of multiple morphological indistinguishable species-level lineages as a single species may hold some appeal; however, failing to formally recognize this diversity can have far-reaching implications in our biological (e.g., ecology, evolution, reproduction) interpretation of the group. Alternatively, well-supported intraspecific phylogenetic substructure may be the result of stochastic evolutionary processes, uniparental inheritance, etc., rather than evolutionary independence. Interpreting this type of phylogenetic substructure as species-level diversity can introduce detrimental bias.

Empirical species delimitation methods have broadly focused on four main areas: detecting putative species, individual specimen assignment to a species group or operational taxonomic unit (OTU), validation of candidate species or OTUs as evolutionarily distinct lineages, and inferring species relationships (i.e., species tree inference). Ideally, operational species delimitation criteria should be based on explicit statistical protocols in order to objectively assess species boundaries and minimize the need of subjective interpretations or taxonomic expertise. In this section, we provide a brief overview on a number of empirical species delimitation methods that to varying degrees fit these criteria. For convenience, we divide these methods into three general categories: (i) species discovery methods for assigning samples to populations without a priori information; (ii) species validation approaches and coestimating individual assignments and species trees; and (iii) species delimitation using genomic data (Table 2.1).

Empirical species delimitation has received increasing attention, including ongoing development of bioinformatical approaches, and the methods and programs provided in this chapter are by no means intended to be a comprehensive list of all available analytical approaches. Rather, the methods provided here have been shown to be useful in a number of previous studies or show particular promise for future species delimitation studies. Our aim is that these can serve as a starting point when designing studies to assess species limits. Not surprisingly, various approaches to species delimitation may yield different estimates of species boundaries, and the researcher may be required to make some degree of subjective interpretation of the most biologically appropriate species boundaries. Complementarily, recently developed metrics for quantifying the congruence between two taxonomies (C_{tax}) and the potential for an approach to capture a high number of species boundaries (R_{tax}) provide a means to objectively assess discrepancies among species delimitation methods (Miralles and Vences 2013). More sophisticated approaches, including selection of species delimitation models using approximate Bayesian computing (Camargo et al. 2012; Fan and Kubatko 2011; Beaumont et al. 2010) and designing and conducting a simulation study that matches the characteristics of the empirical study (Carstens et al. 2013; Camargo et al. 2012), can be used to more objectively evaluate competing hypotheses of species boundaries. In most contexts, it is likely better to fail to delimit species than it is to falsely circumscribe entities that do not represent actual species, and therefore, the inferences drawn from species delimitation studies should generally be conservative (Carstens et al. 2013; Miralles and Vences 2013).

2.2.1 Corroborating Traditional Taxonomy and Discovering Cryptic Species Using Single-Locus Data

Objectively defining a threshold separating intraspecific population substructure from interspecific divergence is the general aim of species delimitation studies using single-marker datasets. Most species delimitation methods based on single-locus sequence data fall under two general categories: either genetic distance or tree-based approaches (Sites and Marshall 2004). Distance-based approaches attempt to detect a difference between intra- and interspecific distances (i.e., "barcode gap") where the pairwise genetic

Table 2.1 Some methods used for species delimitation, including species discovery methods for assigning samples to populations without a *priori* information (BAPS, Gaussian clustering, Guillot's Unified Model, STRUCTURE, STRUCTURAMA), genetic distance-based method for sorting sequences into hypothetical species (ABGD), tree-based species discovery methods (bGMYC, GMYC, bPTP, PTP, "Species Delimitation" plug-in for Geneious), and joint discovery and validation methods (BP&P, Brownie, DISSECT, SpeDeStem)

Method	Description	Input data
BAPS—population assignment using Bayesian clustering	A program for Bayesian inference of the genetic structure in a population clustering molecular data and performing admixture analyses. Genetic mixture analyses can be performed at both group and individual levels using either a non-spatial or spatial model. BAPS treats both the allele frequencies of the molecular markers (or nucleotide frequencies for DNA sequence data) and the number of genetically diverged groups in population as random variables. In the "clustering with linked loci" model, a genetic mixture analysis can be done using haploid sequence data or other linked genetic markers. Analyses and model comparisons can also be performed using a fixed number of genetically diverged groups or prespecified population structures *Limitations* Temporal divergence and relationships among putative groups are not explicitly estimated. Equivalence to genetic clusters to species-level groups is uncertain, and validation approaches can be used to assess evolutionary independence of clusters *Source* Available from http://www.helsinki.fi/bsg/software/BAPS/; described in Corander et al. (2004, 2006, 2008); Corander and Marttinen 2006)	Genotypic data, haploid sequence data, or linked markers (AFLP or SNPs)
Gaussian clustering—population assignment using Gaussian clustering	A program for Bayesian inference of the genetic structure in a population. Model groups sample into populations using genotypic data by searching for clusters that can be attributed to being mixtures of normal allele frequency distributions. Gaussian clustering requires a dataset where the cases are defined by variable of metric scale and has been used with genetic, environmental, and morphological datasets individually, in addition to integrated datasets *Limitations* Temporal divergence and relationships among putative groups are not explicitly estimated. Equivalence to genetic clusters to species-level groups is uncertain, and validation approaches can be used to assess evolutionary independence of clusters *Source* Implemented in R using the prabclus (Hausdorf and Hennig 2010) and mclust packages (Fraley and Raftery 2007)	Genotypic data (flexible)

(continued)

Table 2.1 (continued)

Method	Description	Input data
Guillot's Unified Model—population assignment using Bayesian clustering	This approach provides a statistical model that allows one to analyze genetic and phenotyptic data within a unified model and inference framework and optionally incorporate information about the spatial distribution of samples. A Bayesian clustering algorithm assumes that each cluster in a geographical domain can be approximated by polygons that are centered around points generated by a Poisson process. Guillot's Unified Model is flexible in terms of the genetic data that it can utilize and capable of accurately delimiting species *Limitations* Genetic and phenotypic data can trace different evolutionary histories, for instance, phylogenetic divergence for neutral genetic markers and adaptation for a morphological structure *Source* Available as an extension of the R GENELAND package (Guillot et al. 2005, 2012) (http://www2.imm.dtu.dk/~gigu/Geneland/)	Genotypic and non-genetic (e.g., phenotypical, ecological, geographical, behavioral) data
STRUCTURE—population assignment using Bayesian clustering	A model-based clustering method using multilocus genotype data to infer population structure and assign individuals to populations. Individuals in the sample are assigned probabilistically to populations, or jointly to two or more populations if their genotypes indicate that they are admixed. The model does not assume a particular mutation process, and it can be applied to most of the commonly used genetic markers, provided that they are not closely linked. The method can produce highly accurate assignments using modest numbers of loci (Pritchard et al. 2000). The most appropriate level of population structure can be inferred by assessing likelihood scores or the ad hoc ΔK statistic (Evanno et al. 2005) *Limitations* Identifying the most appropriate number of genetic clusters is challenging; clusters produced by STRUCTURE can be strongly influenced by variation in sample size; clusters created by STRUCTURE may not be consistent with the evolutionary history of the populations when there are relatively long divergence times within evolutionary lineages. Temporal divergence and relationships among putative groups is not explicitly estimated. Equivalence to genetic clusters to species-level groups is uncertain, and validation approaches can be used to assess evolutionary independence of clusters *Source* http://pritchardlab.stanford.edu/structure.html; described in Falush et al. (2003) and Pritchard et al. (2000)	Genotypic data

(continued)

Table 2.1 (continued)

Method	Description	Input data
Structurama—population assignment using Bayesian clustering	Implements the clustering algorithm used in STRUCTURE (see above) that clusters samples into populations by minimizing Hardy–Weinberg disequilibrium for a given partitioning level. However, Structurama also includes the addition of reversible-jump MCMC to identify the optimal partitioning level. Nearly any type of genetic data can be input into Structurama, and the program can assign individuals to population with or without the admixture *Limitations* Temporal divergence and relationships among putative groups is not explicitly estimated. Equivalence to genetic clusters to species-level groups is uncertain, and validation approaches can be used to assess evolutionary independence of clusters *Source* http://cteg.berkeley.edu/~structurama/index.html; described in Huelsenbeck et al. (2011)	Genotypic data
ABGD—barcode gap using genetic distances	"Automatic Barcode Gap Discovery" sorts sequences into hypothetical species based on the barcode gap. The method uses a recursive approach to partition the data and test for significant gaps. ABGD is fast, simple method to split a sequence alignment dataset into candidate species that should be complemented with other evidence in an integrative taxonomic approach *Limitations* Single-locus data alone should only be used to provide a preliminary perspective of species boundaries and not as the sole evidence in species circumscriptions. *Source* http://wwwabi.snv.jussieu.fr/public/abgd/; described in Puillandre et al. (2012)	Single-locus sequence alignment
GMYC&bGMYC—gene tree	The GMYC approach combines a coalescent model of intraspecific branching with a Yule model for interspecific branching, which is then fit to an inferred single-gene topology to estimate species boundaries and a statistical measure of confidence for the inferred boundaries. As an input, the GMYC approach requires an ultrametric gene tree, and recent refinements can account for uncertainty in phylogenetic relationships and parameters of the GMYC model. The GMYC is generally stable across a wide range of circumstances, including various methods of phylogenetic reconstruction, the presence of a high number singletons, high numbers of sampled species, and gaps in intraspecific sampling; the accuracy of the GMYC is most significantly affected by the mean population size relative to divergence times between them *Limitations* The GMYC may delimit well-supported clades of haplotypes as independent lineages and as such may be prone to over-delimitation. Single-locus data alone should only be used to provide a preliminary perspective of species boundaries and not as the sole evidence in species circumscriptions *Source* (http://r-forge.r-project.org/projects/splits, https://sites.google.com/site/noahmreid/home/software); described in Monaghan et al. (2009), Fujisawa and Barraclough (2013) and Pons et al. (2006)	Single-locus ultrametric gene tree

(continued)

Table 2.1 (continued)

Method	Description	Input data
PTP&bPTP—gene tree	The Poisson tree processes (PTP) model can be used to infer putative species boundaries on a given phylogenetic input tree. PTP can infer putative species boundaries that are consistent with the PSC. An important advantage of this method is that it models speciation in terms of the number of substitutions, thereby circumventing the potentially error-prone and compute-intensive process of generating ultrametric trees, which are required as an input for GMYC model (see above). Furthermore, it appears that the PTP model may outperform the GMYC and other OTU-picking methods when evolutionary distances are small *Limitations* Single-locus data alone should only be used to provide a preliminary perspective of species boundaries and not as the sole evidence in species circumscriptions *Source* http://sco.h-its.org/exelixis/web/software/PTP/index.html; described in Zhang et al. (2013)	Single-locus gene tree
Geneious Species Delimitation plug-in—gene tree	A plug-in to the Geneious software provides an exploratory tool allowing the user to assess phylogenetic support and diagnosability of species defined as user-selected collections of taxa on user-supplied trees. The plug-in computes statistics relating to the probability of the observed monophyly or exclusivity having occurred by chance in a coalescent process and assesses the within- and between-species genetic distances to infer the probability with which members of a putative species might be identified successfully with tree-based methods *Limitations* The plug-in summarizes measures of phylogenetic support and diagnosability of species defined as user-selected collections of taxa, but it does not provide definitive support for species groups. It assumes species are monophyletic *Source* Implemented as a plug-in to Geneious (geneious.com); described in Masters et al. (2011)	Single-locus gene tree
BP&P—multispecies coalescent model for species validation	This approach to species delimitation uses a Bayesian modeling approach to generate the posterior probabilities of species assignments taking account of uncertainties due to unknown gene trees and the ancestral coalescent process. The method relies on a user-specified guide tree, implementing a reversible-jump Markov chain Monte Carlo search of parameter space that includes θ, population divergence, and estimated distributions of gene trees from multiple loci *Limitations* Misspecifications of priors and the guide tree can result in inflated speciation probabilities, it assumes no recombination, and computational limitations restrict its utility with next-generation datasets with 100s of loci *Source* http://abacus.gene.ucl.ac.uk/software.html; described in Yang and Rannala (2010) and Rannala and Yang (2003)	Multilocus sequence alignments and group membership

(continued)

Table 2.1 (continued)

Method	Description	Input data
DISSECT—multispecies coalescent model for species delimitation	DISSECT explores the full space of possible clusterings of individuals and species tree topologies in a Bayesian framework. To avoid the need for reversible-jump MCMC, it uses an approximation in the form of a prior that is a modification of the birth–death prior for the species tree. It is implemented as part of BEAST and requires only a few changes from a standard *BEAST analysis. Analyses of simulated and empirical data suggest that the method is shown to be insensitive to the degree of approximation, but quite sensitive to other parameters *Limitations* Recently described method lacking a thorough theoretical and empirical evaluation. It appears that a large number of sequences are required to draw firm conclusions *Source* http://code.google.com/p/beast-mcmc/&http://www.indriid.com/dissectinbeast.html; described in Jones and Oxelman (2014)	Multilocus sequence data
SpeDeSTEM—multispecies coalescent model for discovery, validation, and joint estimation	This maximum likelihoodand/or information theory-based method was developed to test species boundaries in a system with existing subspecies taxonomy (Carstens and Dewey 2010), and computes the probability of the gene trees given the species tree for all hierarchical permutations of lineage grouping. Species boundaries are compared using Akaike information criteria, and phylogenetic uncertainty in the species tree topologies does not affect species delimitations *Limitations* Accuracy is dependent on quality of the gene tree estimates *Source* (http://carstenslab.org.ohio-state.edu/software.html).criteria; described in Ence and Carstens (2011)	Multilocus sequence alignments and group membership
Brownie—multispecies coalescent model for species delimitation	The nonparametric heuristic species delimitation approach implemented in the program Brownie (O'Meara 2010) jointly sorts anonymous samples into species and infers a species tree from input gene trees from different loci, assuming that for a speciation even the corresponding nodes on gene trees will be more consistent with each than the divergences within species *Limitations* Finding both the optimum species tree and species boundaries remains computationally challenging, and Brownie has been shown to frequently yield incorrect results. The accuracy of the method is likely correlated with nodal support values in the individual gene topologies *Source* http://www.brianomeara.info/brownie; described in O'Meara (2010)	Individual gene trees

(continued)

Table 2.1 (continued)

Method	Description	Input data
BFD*—multispecies coalescent model for species delimitation	The recently developed method, Bayes factor delimitation (*with genomic data; BFD*), combines a dynamic programming algorithm for estimating species trees that bypasses the computationally intensive MCMC integration over gene trees to provide a rigorous technique for species delimitation studies using genome-wide SNP data. Competing species delimitation models are compared using Bayes factors, and it appears that this approach is robust to sample sizes (i.e., few loci and limited samples per species) and misspecification of the prior for population size (θ) *Limitations* Recently described method lacking a thorough theoretical and empirical evaluation *Source* http://www.beast2.org/wiki/index.php/BFD*; described in Leaché et al. (2014)	Genome-wide SNP data

distances among organisms belonging to the same species are smaller than distances among organisms from different species (Puillandre et al. 2012; Hebert et al. 2003). Genetic distance approaches hold particular promise as an identification tool shortcutting the difficulties of morphology-based identification (Hebert et al. 2004), although in practice a barcode gap may not exist for many groups (Wiemers and Fiedler 2007). Furthermore, the role of distance-based approaches using a single genetic marker for species discovery remains more controversial (Rubinoff 2006), and without other corroborating evidence, OTUs inferred from single-locus dataset should only be considered "candidate" species. Tree-based methods aim to detect monophyletic clades corresponding to species-level diversity by detecting discontinuities associated within inter- and intraspecific branching patterns (Fujisawa and Barraclough 2013; Zhang et al. 2013; Monaghan et al. 2009; Pons et al. 2006). Tree-based species delimitation methods can also be used on the basis of other properties related to phylogenetic tree topologies (monophyly, concordance with geography, etc. (Sites and Marshall 2003, 2004). Both distance- and tree-based methods have been applied for assessing species boundaries in lichen-forming fungi (Leavitt et al. 2012c, 2014; Parnmen et al. 2012; Del-Prado et al. 2010, 2011; Molina et al. 2011).

A number of tree-based methods partially automate the species delimitation process with specific bioinformatical analyses (Table 2.1), including the general mixed Yule coalescent (GMYC) approach (Fujisawa and Barraclough 2013; Monaghan et al. 2009; Pons et al. 2006) and the Poisson tree processes (PTP) model (Zhang et al. 2013). These methods provide relatively straightforward and objective pipelines for delimiting putative species-level lineages from inferred gene trees by fitting within- and between-species branching models to an inferred single-locus topology. A number of other tree-based methods for species delimitation are effectively summarized in Sites and Marshall (2004).

When only single-locus data are available, the GMYC has been shown to be a relatively robust tool for species delimitation. The GMYC approach combines a coalescent model of intraspecific branching with a Yule model for interspecific branching, which is then fit to an inferred single-gene topology to estimate species boundaries and a statistical measure of confidence for the inferred boundaries. As an input, the GMYC approach requires an ultrametric gene tree, and recent refinements can account for uncertainty in phylogenetic relationships and parameters of the GMYC model (Fujisawa and Barraclough 2013; Reid and Carstens 2012). Regardless of these improvements, it may be difficult to accurately infer an adequate ultrametric tree for large datasets. Although it appears that the GMYC is generally stable across a wide range of circumstances, including various methods of phylogenetic reconstruction, the presence of a high number singletons, high numbers of sampled species, and gaps in intraspecific sampling, the accuracy of the GMYC is most significantly affected by the mean population size relative to divergence times between them (Fujisawa and Barraclough 2013; Talavera et al. 2013). Furthermore, research suggests that the so-called single-threshold version of the GMYC method likely outperforms the multiple-threshold approach (Fujisawa and Barraclough 2013; Monaghan et al. 2009). However, other studies suggest that the GMYC method may often provide higher estimates for the total number of OTUs than other molecular species delimitation methods (Hamilton et al. 2014; Miralles and Vences 2013; Talavera et al. 2013), warranting a cautious interpretation of results from GMYC analyses.

Compared to the GMYC approach, the recently introduced PTP method for species delimitation has been suggested to be more accurate for preliminary species delimitation (Zhang et al. 2013). Relative to the GMYC, PTP offers a more straightforward implementation, requiring a simple phylogenetic tree, rather than an ultrametric chronogram. However, more research is required to assess the general performance of PTP across wide range of empirical and simulated species delimitation studies. At this point, data support the use of the GMYC and PTP methods as objective and reasonable starting

points for species delimitation using single-gene topologies.

The "Species Delimitation" plug-in to the Geneious software provides statistical approaches for exploring species boundaries in single-gene topologies (Masters et al. 2011). Using a priori specimen assignments to putative species, the "Species Delimitation" plug-in computes statistics relating to the probability of the observed monophyly or exclusivity having occurred by chance in a coalescent process and assesses the within- and between-species genetic distances in order to infer the probability with which members of a putative species might be identified successfully with tree-based methods. An important contribution of the "Species Delimitation" plug-in is that it provides an objective means for users to assess putative species within an empirical, statistic-based framework, rather than qualitative evaluations of what level of hierarchy constitutes a species in a phylogeny.

In contrast to simple sequence similarity thresholds (OTU-picking) for delimiting putative evolutionarily significant units, the Automatic Barcode Gap Discovery (ABGD) method is an automated procedure that sorts sequences into hypothetical species based on the existence of a barcode gap, observed whenever intraspecific genetic distances are less than those among organisms from different species (Puillandre et al. 2012). Ultimately, ABGD is a fast, simple method that can be used to group individuals represented in a single-locus sequence alignment into candidate species that should be complemented with other lines of evidence in an integrative taxonomic approach (Kekkonen and Hebert 2014; Puillandre et al. 2012).

In general, the ABGD, GMYC, and PTP analytical protocols using single-locus data are repeatable and computationally relatively fast, providing a valuable starting point for a preliminary perspective into species boundaries in understudied groups that can be validated with subsequent studies (Kekkonen and Hebert 2014). Similarly, analyses of single-locus data can be used to corroborate traditional phenotype-based species boundaries and identify candidate species that have previously been hidden within nominal species. However, single-locus species delimitation methods may be particularly prone to failure in recognizing significant proportions of species-level biodiversity due to the strict criterion for reciprocal monophyly or, alternatively, provide spurious inflations of estimated species diversity based on genetic differences that do not correspond to species-level lineages. For example, recent estimates suggest that the incidence of species-level gene tree paraphyly is approximately 20 % (Ross 2014), suggesting that analyses of single-locus datasets would likely fail to accurately delimit species in one in every five cases. In contrast, recent empirical studies suggest that in some cases the GMYC provides a striking overestimate of species diversity (Miralles and Vences 2013).

The internal transcribed spacer region (ITS) has played a central role in molecular phylogenetic studies of lichenized fungi and has recently been adopted as the official barcoding marker for fungi (Schoch et al. 2012). In many cases, the ITS is sufficiently variable to resolve species boundaries for lichenized fungi (Schoch et al. 2012; Kelly et al. 2011), although accurate specimen identification using sequence-based identification approaches requires a thoroughly curated reference database (Leavitt et al. 2014; Orock et al. 2012; Kelly et al. 2011). In spite of the general utility of the ITS marker, a number of issues may potentially limit its effective use in species delimitation studies, including the potential for intragenomic variation of the nuclear ribosomal tandem repeat and difficulties in aligning ITS sequences from divergent taxa (Kiss 2012). Because the ITS has been formally adopted as the official barcoding marker for fungi, we recommend that species delimitation studies attempt to include this region for consistency across studies. However, we recognize that in some taxonomic groups the inclusion of the ITS may be problematic and therefore suggest a careful screening of other markers to identify appropriate loci for resolving species-level relationships.

Ultimately, the success of any single-locus species delimitation method largely depends on the evolutionary history of the species group under study and the variability of the selected marker (Fig. 2.2). Species delimitation will be

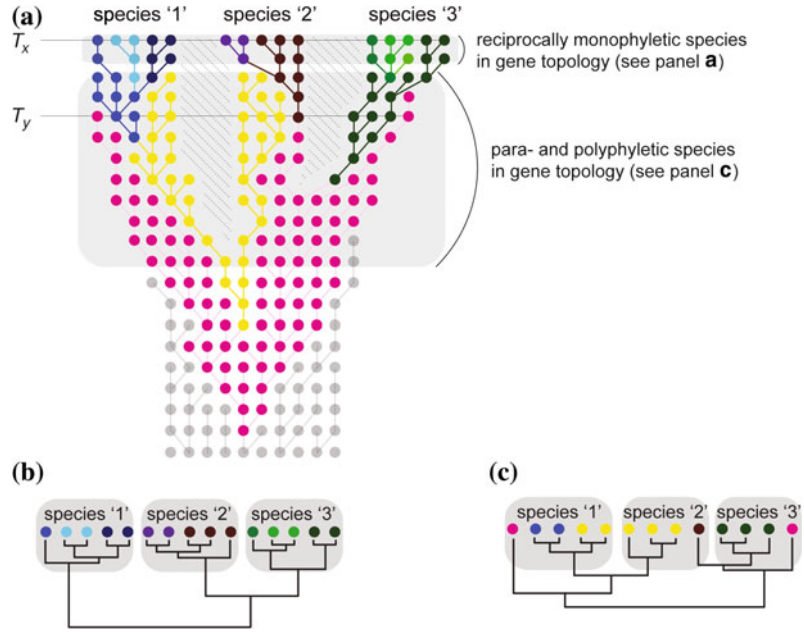

Fig. 2.2 A simplified diagram illustrating the process of speciation through time in a single gene history and resulting gene topologies sampled at two points in the speciation history (modified from Leliaert et al. 2014). **a** Each *dot* represents a distinct gene copy and each row one non-overlapping generation, with lines connecting gene copies to their ancestors in the previous generation (*one row below*); *dashed diagonal lines* represent reproductive barriers; two hypothetical sampling intervals at different points in the speciation history are shown, T_x and T_y; species delimitation methods using single-locus data are effective only when species are reciprocally monophyletic in the sampled gene tree; genetic clustering and coalescent-based species delimitation methods can circumscribe species when species may not be monophyletic in sampled genetic loci. **b** Gene topology representing sampled haplotypes at time T_x (shown in panel **a**); hypothetical species are reciprocally monophyletic. **c** Gene topology representing sampled haplotypes at time T_y (shown in panel **a**); hypothetical species are para- and polyphyletic

more difficult in more recently diverged lineages and in cases with some level of interspecific gene flow, relative to older, well-diverged lineages (e.g., Leavitt et al. 2012c, 2013e). In the end, we reemphasize that single-locus data alone should only be used to provide a preliminary perspective of species boundaries and not as the sole evidence in species circumscriptions.

2.2.2 Sampling Across the Genome: Multilocus Sequence Data and Genome-Wide Markers

Although single-locus methods can provide an efficient approach for preliminary large-scale assessments of species diversity, there are significant limitations, particularly in recently diverged species groups where retention of ancestral polymorphisms and incomplete lineage sorting will likely result in different neutral loci having unique gene topologies that do not mirror the speciation process (Knowles and Carstens 2007; Heled and Drummond 2010; Taylor et al. 2000). In contrast to single-locus and strictly phenotype-based approaches for species delimitation, analyses of genetic data collected from independent genomic regions can provide robust hypotheses of species boundaries with increasing confidence (Satler et al. 2013; Zhang et al. 2011; Yang and Rannala 2010). Sequences from multiple independent loci provide an important source of data for species delimitation studies, including recently developed models that

combine individual gene genealogies and species phylogenies via modeling the coalescent history of markers (Yang and Rannala 2010; Edwards 2009; Knowles and Carstens 2007). As a response to advances in sequencing technologies, bioinformatical approaches for multimarker species delimitation analyses continue to be developed (Table 2.1; Camargo and Sites 2013).

Long-term reproductive isolation of candidate species can be assessed with multilocus sequence data by evaluating genealogical concordance of unlinked markers (Baum and Shaw 1995; Avise and Ball 1990). Within species, the mixing effects of recombination cause unlinked loci to have distinct genealogical histories, but genetic drift and long-term divergence leads to concordant genealogical histories at loci across the genome. Relationships of individuals belonging to distinct candidate species can be evaluated using gene genealogies (e.g., haplotype networks or single-gene topologies) to identify lineages that exhibit genealogical exclusivity across unlinked neutral loci (Hudson and Coyne 2002; Dettman et al. 2003b; Avise and Ball 1990). The presence of the same clades in the majority of single-locus genealogies is taken as evidence that the clades represent reproductively isolated lineages (Dettman et al. 2003a; Pringle et al. 2005). In practice, the criteria of reciprocal monophyly and genealogical concordance of unlinked loci provide a conservative approach for assessing species boundaries due to the fact that a substantial amount of time is required after the initial divergence of species until ancestral polymorphisms have fully sorted (Knowles and Carstens 2007; Hudson and Coyne 2002). Consequently, groups with recent divergence histories will likely go undiscovered under a genealogical concordance criterion due to the fact that species boundaries likely are not reflected in gene genealogies. For example, it would take more than 1 million years after speciation before species would be delimited if 15 loci were sampled in species with an effective population size (N_e) of 100,000, assuming one generation a year under a strict reciprocal monophyly criterion (Knowles and Carstens 2007). The amount of time required for a species to be recognized using a criterion of reciprocal monophyly increases proportionally with increasing population sizes (Hudson and Coyne 2002). In a number of studies of lichen-forming fungi, a genealogical concordance criterion has been used to both delimit previously unrecognized species-level lineages and validate some conventional phenotype-based species (Leavitt et al. 2012a, c, 2013b; Molina et al. 2011; Kroken and Taylor 2001).

Although analyses of molecular sequence data relying on reciprocal monophyly or fixed character differences for species delimitation can serve as important criteria for identifying species, these criteria are not commonly met across multiple loci, particularly in recent speciation histories (Fig. 2.2). To accommodate the observed conflict among genealogies from multiple loci with the underlying speciation history, the recent merge of coalescent theory with phylogenetics has driven a major paradigm shift in species delimitation and molecular systematics in general (Fujita et al. 2012; Edwards 2009). The multispecies coalescent model can be applied to genealogical histories from multiple independent loci to assemble separate coalescent processes occurring in populations into a species tree (Degnan and Rosenberg 2009; Rannala and Yang 2003). Within this coalescent-based framework, multiple individuals can be assigned to a single species/population and the speciation history of ancestral and descendant species-level lineages can be inferred as a "species tree," in contrast to estimating gene genealogies from individual samples (Degnan and Rosenberg 2006, 2009; Rannala and Yang 2003).

A number of multispecies coalescent-based species delimitation methods have recently been introduced, offering an exciting framework for empirically assessing species boundaries by selecting the best species tree model from a set of alternative models representing different hypotheses of species boundaries (Table 2.1). For example, SpeDeSTEM (Ence and Carstens 2011) finds the maximum likelihood values for a species tree assuming all putative species are separate lineages and for alternative species trees where two or more species are collapsed into a

single lineage. The best fitting model is then selected using the Akaike information criterion, under the assumption of constant population sizes and fixed gene topologies across the species tree. The species delimitation program Bayesian Phylogenetic and Phylogeography (BP&P;Yang and Rannala 2010) accommodates gene tree uncertainty and variable population sizes and samples from the Bayesian posterior distribution of species delimitation models using reversible-jump Markov chain Monte Carlo (rjMCMC) sampling. BP&P requires a user-provided guide tree with resolution finer than the species level and evaluates alternative modes derived from all possible subtrees that are generated by collapsing or splitting nodes on the guide tree (Yang and Rannala 2010; Rannala and Yang 2003). The proportion of time spent on each model is proportional to the posterior probability of the model, i.e., "speciation probabilities." While BP&P ranks among the most popular coalescent-based species delimitation programs (Fujita et al. 2012), misspecifications of the guide tree and/or the prior distribution for population size (θ) can result in strong support for models containing an inflated number of species (Leaché and Fujita 2010; Zhang et al. 2011). The nonparametric heuristic species delimitation approach implemented in the program Brownie (O'Meara 2010) jointly sorts anonymous samples into species and infers a species tree from input gene trees from different loci, assuming that for a speciation event, the corresponding nodes on gene trees will be more consistent with each than the divergences within species. However, finding both the optimum species tree and species boundaries remains computationally challenging, and Brownie has been shown to frequently yield incorrect results (O'Meara 2010).

Most recently, available coalescent-based species delimitation methods require individual assignments to a species or population a priori. In a number of scenarios, correct assignments of samples to species may be difficult, including presence of cryptic species, incongruence between conventional species and molecular data, or simply the fact that accurate specimen identification in complex groups with subtle or difficult to discern diagnostic characters is a nontrivial task (Leavitt et al. 2013e). Statistical methods for assessing individual assignment and species detection prior to coalescent-based species delimitation and species tree reconstruction and species provide a more objective approach for understanding species boundaries.

Population assignment tests using a variety of clustering algorithms using genetic data offer a useful approach for grouping individuals into putative reproductively isolated groups (Table 2.1; Hausdorf and Hennig 2010; Corander et al. 2004, 2008; Falush et al. 2003; Pritchard et al. 2000), particularly for species delimitation problems that exist at the interface of traditional population genetic and phylogenetic analyses (Carstens et al. 2013; Weisrock et al. 2010; Knowles and Carstens 2007).

The programs STRUCTURE (Falush et al. 2003; Pritchard et al. 2000) and STRUCTURAMA (Huelsenbeck et al. 2011) cluster samples into populations by minimizing Hardy–Weinberg disequilibrium for a given number of population clusters using unlinked genetic markers. In general, unlinked markers are not available for most groups of lichen-forming fungi and a number of studies have used information from multilocus sequence data for STRUCTURE analyses (Leavitt et al. 2011a, b, c, 2013e; Fernández-Mendoza and Printzen 2013; Fernández-Mendoza et al. 2011). Varying approaches have been implemented to convert DNA sequence data to allelic data for Bayesian clustering (see O'Neill et al. 2013). STRUCTURE is expected to perform well when there is sufficient independence across regions such that linkage disequilibrium within regions does not dominate the data (STRUCTURE manual), but can also be effective using multilocus sequence data and treating all SNPs as independent loci regardless of physical linkage within each locus (O'Neill et al. 2013; Falush et al. 2003). A recent study of the lichen-forming genus *Letharia* showed that Bayesian clustering implemented in the program STRUCTURE was generally able to recover the same putative *Letharia* lineages circumscribed employing a gene genealogical approach in Kroken and Taylor's iconic species delimitation

study (Altermann et al. 2014; Kroken and Taylor 2001). Altermann et al. (2014) show that population assignments were largely consistent across a range of scenarios, including extensive amounts of missing data, the exclusion of SNPs from variable markers, and inferences based on SNPs from as few as three gene regions. In contrast to STRUCTURE and STRUCTURAMA, the program BAPS (Corander et al. 2008) can explicitly infer genetic structure using haploid sequence data or other linked genetic markers in the "clustering with linked loci" model. Another advantage of BAPS is that the program is much more computationally efficient than STRUCTURE or STRUCTURAMA. Simulation studies indicate that both BAPS and STRUCTURE perform well at low levels of population differentiation and when clusters are not well differentiated (Latch et al. 2006).

In practice, inferring the most appropriate level of population structure using Bayesian clustering algorithms remains challenging (Latch et al. 2006; Evanno et al. 2005). BAPS and STRUCTURAMA can infer both individual assignments and the most likely number of genetic clusters (Corander et al. 2008; Huelsenbeck et al. 2011), and the ad hoc ΔK statistic proposed by Evanno et al. (2005) provides an objective approach for identifying the uppermost hierarchical level of structure. However, careful consideration of other levels of population structure may ultimately reveal more biologically meaning results and researchers should examine individual assignments across a range of genetic groupings. Some markers generated from independent genomic regions, such as SNPs and fast-evolving microsatellites, can be used to distinguish fine-scale population structuring on the basis of allele frequencies, and species delimitation analyses based on these markers may imply the risk of severe taxonomic oversplitting. In these cases, validation approaches, such as BP&P and SpeDeSTEM, and corroboration among different species delimitation approaches can provide perspective between intraspecific population structure and species-level clusters.

Another limitation of clustering approaches is that they do not assess or take into account evolutionarily divergences and relationships among population clusters. Coalescent-based species tree methods (discussed below) provide a means to assess the diversification histories of populations inferred from clustering analyses. Therefore, a potential working protocol for an informed species delimitation study that takes into account population structure could consist of first applying a genetic clustering analysis under a population genetics criterion (e.g., BAPS, STRUCTURE, STRUCTURAMA) to identify genetically distinct population clusters that can be considered "candidate species." From these candidate species, a species tree can be inferred for focal group using coalescent-based species tree reconstruction methods (e.g., *BEAST). Subsequently, a coalescent-based validation method can be applied to assess whether the distinct population clusters represent independent evolutionary lineages (e.g., BP&P). This multistep approach would provide a consistent and standard criterion for distinguishing between population- and species-level lineages (Camargo and Sites 2013), and has been applied to a number of cases, including lichen-forming fungi (Leavitt et al. 2011a, b, c, 2013e; Leaché and Fujita 2010).

High-throughput sequencing methods provide the means to effectively sample hundreds to thousands of loci from across a species genome for a large number of species and continue to revolutionize studies that can be performed even in non-model organisms. Targeted high-throughput sequencing approaches, such as anchored phylogenomics, transcriptome sequencing, reduced-representation genomic library sequencing (restriction-site-associated DNA sequencing: RAD-Seq and genotype-by-sequencing: GBS), and high-throughput amplicon sequencing, provide important insight into species boundaries for a number groups, although none of these approaches has yet been applied to studies of lichenized fungi.

For example, restriction-site-associated (RAD-Seq) DNA can simultaneously detect and genotype thousands of genome-wide SNPs by focusing the sequencing effort on a reduced representation of the entire genome (Baird et al. 2008) and has been successfully applied to

intraspecies (Lewis et al. 2007; Miller et al. 2007; Emerson et al. 2010) and interspecies studies (Rubin et al. 2012; Eaton and Ree 2013; Wagner et al. 2013). This approach has provided striking insight into the recent adaption radiation of Lake Victoria cichlids (Wagner et al. 2013). An alternative approach targeting the sequencing of specific loci using next-generation sequencing platforms provides an efficient means of generating data for loci of known genomic location, orthology, size, and expected level of variation (O'Neill et al. 2013). Markers can be targeted using well-established PCR techniques or newer hybridization techniques and subsequently pooled for high-throughput sequencing using parallel-tagged sequencing of multiple individual samples within a single sequencing run (O'Neill et al. 2013). A recent study of North American tiger salamanders (*Ambystoma tigrinum*) demonstrated the potential for amplicon-based parallel-tagged sequencing to rapidly generate large-scale genomic data for species delimitation and species tree research (O'Neill et al. 2013).

Although these methods provide an enormous amount of data and insight into diversification at an unprecedented scale, computational limitations restrict the applicable analytical methods for large datasets, although computational and analytical advances are happening rapidly. Most recent coalescent-based species delimitation and species tree models using gene trees have been limited to handle tens of loci for multiple individuals, and combining hundreds or thousands of gene trees into a single species delimitation framework presents considerable computational challenges (Camargo and Sites 2013). The recently developed method, Bayes factor delimitation (*with genomic data; BFD*), combines a dynamic programming algorithm for estimating species trees that bypasses the computationally intensive MCMC integration over gene trees to provide a rigorous technique for species delimitation studies using genome-wide SNP data (Leaché et al. 2014). Competing species delimitation models are compared using Bayes factors, and it appears that this approach is robust to sample sizes (i.e., few loci and limited samples per species) and misspecification of the prior for population size (θ) (Leaché et al. 2014).

Fungi are an ideal model for assessing divergence in eukaryotes due to their simple morphologies, small genomes, broad ecological roles, and diverse lifestyles (Gladieux et al. 2014). However, the use of genomic data from high-throughput sequencing methods have not yet been included in species delimitation studies of lichen-forming fungi. This is due, in part, to challenges in dealing with metagenomic data containing genomic information from a plethora of symbionts associated with a lichen thallus, scant genomic resources, and discipline-specific inertia. A number of lichen-forming fungal genomes are currently available, including *Endocarponpusillum* (Wang et al. 2014), *Cladonia* spp. (Park et al. 2013a, 2014), *Caloplaca flavorubescens* (Park et al. 2013b), and *Cladonia greyi* and *Xanthoria parientina* through the Fungal Genomics Program at Joint Genome Institute of the United States Department of Energy (http://www.jgi.doe.gov). The foreseeable ongoing expansion of genomic resources for lichen-forming fungi will be central to developing approaches for delimiting species using high-throughput sequencing. Specifically, genomic resources will be crucial in identifying novel markers (variable genes regions, SNPs, microsatellites, etc.), identifying conserved fungal markers for targeted high-throughput sequencing approaches, and references for entire genome comparisons (Devkota et al. 2014; Werth et al. 2013).

2.3 Can We Make Species Delimitation in Lichen-Forming Fungi Truly Integrative?

The widespread availability of genetic data has created a biased viewpoint that only genetic data should be used for statistical species delimitation. However, an ongoing appeal to researchers to assess species boundaries from multiple and complementary perspectives (phylogenetics, population genetics, comparative morphology, development, ecology, etc.) has resulted in an

integrative taxonomic framework bringing these conceptual and methodological perspectives together (Hamilton et al. 2014; Fujita et al. 2012; Salicini et al. 2011; Yeates et al. 2011; Padial et al. 2010; Dayrat 2005; Will et al. 2005; Wiens and Penkrot 2002). In practice, any study linking different kinds of data to support hypotheses of species boundaries, including mapping morphological characters onto a molecular phylogeny, can be considered integrative. Integrative methods for species delimitation fall across a broad spectrum, ranging from verbal and qualitative assessments of data classes to quantitative methods that allow different data types to contribute to statistical species delimitation (Yeates et al. 2011). In most taxonomic studies utilizing both molecular and morphological data, expert opinion is eventually used to some degree to evaluate the final status of a candidate species (e.g., Bond and Stockman 2008). In this sense, many studies using evidence for independent data sources for delimiting species boundaries use an iterative approach (Yeates et al. 2011) where species boundaries can be tested within a *hypothetico*-deductive framework with diverse datasets.

From a practical perspective, we advocate a process of iterative taxonomy (sensu Yeates et al. 2011) to circumscribe and refine species limits using multiple lines of evidence. This iterative process involves comparisons of morphological data with a phylogenetic hypothesis (i.e., single-locus gene tree or concatenated multilocus phylogeny) to identify the least inclusive monophyletic clade in the topology characterized by at least one unambiguously diagnostic morphological character (Miralles and Vences 2013). This phylogenetic tree-informed approach to assessing species boundaries represents a common practice in studies of lichen-forming fungi. Previously unrecognized species-level clades with corresponding subtle, or overlooked, phenotypic characters have been commonly observed in both crustose lichens [e.g., Acarospraceae (Wedin et al. 2009), Graphidaceae (Rivas Plata and Lumbsch 2011; Papong et al. 2009), Lecanoraceae (Leavitt et al. 2011a), Lecideaceae (Ruprecht et al. 2010), Mycoblastaceae (Spribille et al. 2011), and Teloschistaceae (Vondrák et al. 2009; Muggia et al. 2008)] and foliose and fruticose lichens [e.g., Lobariaceae (Moncada et al. 2014; McDonald et al. 2003), Parmeliaceae (Leavitt et al. 2013a; Wirtz et al. 2012; Divakar et al. 2005; Molina et al. 2004; Kroken and Taylor 2001), Peltigeraceae (O'Brien et al. 2009; Goffinet et al. 2003), Physciaceae (Elix et al. 2009; Divakar et al. 2007), and Sphaerophoraceae (Högnabba and Wedin 2003)]. As a specific example, Lücking et al. (2008) used a combination of medullary chemistry and underside pigmentation in specimens from the *Heterodermia obscurata* group in Costa Rica to corroborate monophyletic clades in an ITS gene tree as species-level lineages.

While this iterative approach to species delimitation and taxonomy has proven valuable for understanding species boundaries and describing new taxa in some groups of lichenized fungi, a posteriori examination of morphological and chemical features has failed to reveal diagnostic phenotypic characters in a number of studies (Muggia et al. 2014; Leavitt et al. 2011a; Otálora et al. 2010; O'Brien et al. 2009). Furthermore, a study of widespread species in the genus *Melanelixia* (Parmeliaceae) indicated that phenotypically cryptic lichen-forming fungal species-level lineages may be relatively ancient and diagnosable phenotypic differences may be absent even millions of years after the initial divergence (Leavitt et al. 2012b). The latter study highlights the fact that species-level lineages may commonly exist without any observable diagnostic phenotypic characters, calling into question the impetus for a universal application of integrative taxonomy.

In keeping with the principle that as many lines of evidence as available should be combined to delimit species (Dayrat 2005), the formalized integrative taxonomic approach (ITAX; Miralles and Vences 2013) uses a mtDNA guide tree and observations from different types of data that might provide conclusive evidence for the independence of lineages and thus their identity as different species. Miralles and Vences (2013) provide a non-exhaustive list of species delimitation criteria to be integrated in the ITAX approach, including (a) sympatric occurrence without admixture as revealed by consistent

differences in morphological or molecular characters at the same geographic location; (b) strong differences in a behavioral, morphological, or genetic character known to mediate premating isolation; (c) unviability or infertility of hybrids; (d) lack of gene flow across a geographical hybrid zone; (e) congruent diagnostic differences between sister lineages in various unlinked morphological character; (f) absence of haplotype sharing in several unlinked nuclear loci; and (g) a combination of criteria e–f. Species boundaries are based on seeking the least inclusive monophyletic group in the mtDNA tree which fulfills at least one of the criteria listed above. Clearly, the ITAX approach is sensitive to sample size, and in order to reliably support the distinctiveness of a given species, it has been recommended that the sampling strategy includes at least five individuals per species (Miralles and Vences 2013). A similar approach could be adopted for lichenized fungi using an ITS gene topology as guide tree, rather than mtDNA.

A total evidence approach, including concatenation, has been a common approach for integrating information from different sources, including independent genetic markers in phylogenetic reconstructions (Kluge 1989; Wiens 1998; de Queiroz et al. 1995). Proponents for concatenation of independent data in phylogenetic analyses argue that when combining all data, the underlying signal of speciation may emerge even if weakly contradictory signal is contained in the individual data partitions (Gatesy et al. 1999). For example, establishing a preliminary perspective of species boundaries from multilocus sequence data using concatenation may provide a reasonable starting point for screening for cryptic species and species tree inference (Šlapeta et al. 2006; Leavitt et al. 2011a; Le Gac et al. 2007; Leaché 2009). However, concatenation and consensus methods imply a risk of obtaining inflated support for incorrect relationships and information about variance in gene coalescence is lost (Degnan and Rosenberg 2006, 2009; Kubatko and Degnan 2007; Edwards 2009). In spite of the impetus for integrating evidence from independent data sources into empirical species delimitation studies and taxonomy (Fujita et al. 2012; Padial et al. 2009), most currently available species delimitation methods are unable to accommodate non-genetic data sources in a statistical framework.

2.3.1 Selecting the Appropriate Data

In the face of increasing availability of genetic data and associated bioinformatical approaches for delimiting species, researchers should carefully consider what information is being sacrificed by the failure to consider non-genetic data in species delimitation studies and whether accuracy could be improved by the addition of multiple data types. Morphological data have historically served as a proxy to identify reproductively isolated groups (i.e., "species") (Ray 1686; Fujita et al. 2012). Current methods for delimiting species using non-genetic data (e.g., chemistry, morphology, and ecology) remain woefully understudied. For example, morphology-based species circumscriptions are generally based on one or more qualitative (or quantitative) morphological characters that do not appear to overlap with other species. However, ascertaining that a given trait is truly fixed within a population with statistical confidence requires unrealistic sample sizes, even when allowing for some level of polymorphism in the diagnostic character (Wiens and Servedio 2000). Now various combinations of data—from morphology, genetics, geography, and ecology—are accepted as standard information for species delimitation studies (Ruiz-Sanchez and Sosa 2010; Ross et al. 2010; Edwards and Knowles 2014).

Below, we briefly discuss appropriate data sources for species delimitation studies of lichen-forming fungi. However, homoplastic characters (similar traits that are not derived from a common ancestor) are common among many traits commonly used to circumscribe fungal taxa, the biological significance of secondary metabolite variation remains largely unknown (Lawrey 1986), and ecological niches may be difficult to adequately characterize and model due to

microhabitat requirements and data resolution. Therefore, we advocate a cautious approach to selecting appropriate and relevant data for assessing species boundaries.

In spite of the potential challenges and limitations of using phenotypic data, these traits have provided a plethora of valuable information for understanding species boundaries. For example, in the *Melanelixia fuliginosa* group (Parmeliaceae), a morphometric analysis, using color, isidia, and marginal zone free of isidia as characters, revealed a general pattern of differentiation between material formerly recognized as subspecies *fuliginosa* and *glabratula,* in spite of the fact that considerable overlap between groups occurs in some characters individually (Arup and Berlin 2011). Furthermore, the distinction of the two groups was supported by a phylogenetic analysis of the ITS marker and ecological differences, with *M. fuliginosa* occurring predominantly on rock and *M. glabratula* on bark (Arup and Berlin 2011). This study of the *M. fuliginosa* group provides a fitting example of using multiple independent suites of data, including ecology, morphology, and genetic information, to establish a robust hypothesis of species boundaries.

In lichen systematics, phenotypic data, including thallus organization, secondary metabolites, mode of reproduction, ascoma-type and ontogeny, ascus and ascospore characters, have historically played a prominent role (Printzen 2009). Ascomatal characters have traditionally held a major role in higher-level classification (Printzen 2009), in contrast to species-level classification which also tends to include a wide array of vegetative and chemical characters. Commonly assessed morphological characters include thallus form and size, cortical features (e.g., maculae and pseudocyphellae), presence/form/color of attachment structure (e.g., rhizines), and reproductive mode (ascomata vs. vegetative diaspores) (Printzen 2009). Different morphological types of vegetative diaspores—corticated isidia and ecorticated soredia—and their location are commonly used to distinguish species. Ascomatal characters, including morphology, location (laminal vs. marginal), position (sessile vs. immersed), presence of thalline margins, color of an apothecial disc, and presence or color of pruina, are also commonly used in species delimitations of lichen-forming fungi (Printzen 2009). Other important characters may include thalline characters, 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, conidiomatal characters, etc. (Printzen 2009).

Assessments of secondary metabolites has played an important role in lichen taxonomy, beginning with the introduction of simple spot tests by Nylander (1866a, b). The use of chemistry in lichen taxonomy has been discussed in detail in numerous reviews (Lumbsch 1998a, b; Rogers 1989; Brodo 1986; Egan 1986; Leuckert 1985; Brodo 1978; Hawksworth 1976; Culberson 1969, 1970), and we refer readers to these valuable sources for a more comprehensive perspective on lichen chemistry. In short, extrolites (secondary metabolites) belong to various classes; the most common and diverse include depsides, depsidones, chlorinated xanthones, and anthraquinones (Lumbsch 2002; Culberson 1969). The presence or absence of specific extrolites, or their replacements by another substance, is widely used to distinguish species, particularly when correlated with differences in geographic distributions. However, if morphological or geographical differences between populations containing different extrolites are not apparent, 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. In addition to simply using the presence or absence of extrolites, Culberson and Culberson (1976) proposed to arrange lichen substances into chemosyndromes of closely related substances. The presence or absence of these chemosyndromes may potentially be used as characters to delimit species, regarding differences involving the same chemosyndromes as intraspecific variation and distinct chemosyndromes as evidence for interspecific populations (Lumbsch 1994; Gowan 1986).

Due to the fact that species delimitation studies often incorporate a substantial biogeographical or ecological component, ecological niche modeling plays an increasing role in phylogenetic and taxonomic research. Niche modeling can provide evidence for ecological isolation between populations based on either conserved or divergent ecological niches and therefore can provide additional evidence supporting lineage independence between putative species. The application of ecological niche modeling has been applied to species delimitation studies in a number of cases (Ruiz-Sanchez and Sosa 2010; Leaché et al. 2009; Raxworthy et al. 2007; Rissler and Apodaca 2007), although it has not been explicitly applied to assess species boundaries in lichen-forming fungi. By mapping the spatial distribution of environmental suitability of climatic variables for candidate species, the application of ecological niche modeling can be particularly important in cases where species have allopatric distributions (Raxworthy et al. 2007).

Ecological niche modeling utilizes known associations between a species' occurrence, localities, and environmental variables to define abiotic conditions within which populations can be maintained (Guisan and Thuiller 2005). The methodological approach for modeling is based on four general properties: (i) The current known species' localities is the dependent variable, (ii) the distribution is modeled as a map composed of grid cells at a specified resolution, (iii) a range of environmental variables (e.g., temperature, precipitation, and solar exposure) are collected to describe the characteristics of each cell, and (iv) classifying the degree to which each cell is either suitable or unsuitable for each species under a range of models (Guisan and Thuiller 2005).

Ecological data also have the potential to play an important role in understanding species boundaries in lichen-forming fungi. For example, Nash and Zavada (1977) demonstrated that *Xanthoparmelia* populations with distinct chemistries occurring in the northern portion of the Sonoran Desert exhibit habitat selection among different rock substrates within a region with relatively uniform climate and topography. In another case, the parmelioid species, *Parmelia mayi*, is morphologically indistinguishable from *P. saxatilis*, but can be separated by bioclimatic features and genetic and chemical characters (Molina et al. 2011). McCune and Printzen(2011) assess distributions and climatic niches of species in the *Lecanora varia* group in western USA and provide a model that uses continental influence and annual temperature as the major factors predicting species distributions. The distribution of *Usnea hirta*, a lichen commonly used in air quality biomonitoring research, was modeled for a section of the White River National Forest in central Colorado, based on the presence of *U. hirta* at 72 biomonitoring reference sites distributed in the intermountain western United States (Shrestha et al. 2012). The best model for predicting *U. hirta* distribution included four variables—solar radiation, average monthly precipitation, and average monthly minimum and maximum temperatures (Shrestha et al. 2012). These studies support the potential use of ecological niche modeling methods in species delimitation studies of lichen-forming fungi.

2.4 Conclusions: What About Taxonomy?

In most cases, species circumscription and taxonomy requires some degree of qualitative judgment and individual interpretation. Integrating multiple types of data into an empirical framework for delimiting species boundaries where species boundaries can be tested within a *hypothetico*-deductive framework with diverse datasets can provide robust hypotheses of species boundaries and taxonomic stability (Yeates et al. 2011). However, it has long been known to evolutionary biologists that distinct species do not need to have diagnosable morphological differences (Mayr 1963), and increasing availability of genetic data has allowed researchers to identify species and to rigorously test species boundaries with a level of precision that was unimaginable a decade ago. While analytical advances in statistical species delimitation have been largely based on genetic data, the utility of

these approaches to formal taxonomy remains elusive. Due to these challenges, an eclectic approach to delimiting species and caution against the reliance on any single dataset or method is required when delimiting species.

While results from many recent studies may contradict traditional species boundaries across many groups of lichen-forming fungi, we are optimistic that this research represents substantial progress toward a more accurate perspective on species boundaries and diversity in fungi. As a result of ongoing research of species boundaries in lichen-forming fungi, the taxonomic value of many phenotypes is now better understood; our understanding of ecological, evolutionary, and biogeographic patterns has improved, and we can begin to better understand patterns of symbiont interactions in lichens. Integrating new data (including novel morphological characters and genetic data) will be essential to accurately represent species-level diversity across all groups of lichenized fungi. Hopefully, an improved perspective on lichen diversity also increases our appreciation of these incredible symbiotic systems. While we are strong advocates for the application of independent data types in developing an integrative taxonomy, there is an increasing need to formally recognize the existence of phenotypically cryptic species-level lineages in lichen-forming fungi (see Hibbett et al. 2011). In some cases, a molecular taxonomy may provide the most practical approach to consistent treatment of mycobiont species within lichen groups where diagnostic morphological characters are unidentifiable or practically not feasible (Leavitt et al. 2013c, d).

These are exciting times for taxonomists and phylogeneticists. A closer look at lichen taxonomy, with the inclusion of new data, will help us to better understand the diversity of these fascinating organisms, accurately interpret distribution patterns, and play a more important role in meaningful conservation practices. However, some level of uncertainty will accompany progress. In many taxonomic groups, our traditional approach for species identification will likely need to be substantially modified. The search for corroborating morphological support for cryptic species identified using molecular data will require meticulous and creative approaches to assess phenotypic variation in potentially unorthodox ways. 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 citizen scientists that traditionally play an important role in lichen taxonomy, the increasing number of collaborative projects in the discipline (e.g., Lumbsch et al. 2011; Crespo et al. 2010; Gueidan et al. 2009) make us optimistic that broad-scale collaborative approaches will facilitate the inclusion of molecular data in lichen research at all levels.

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