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DNA-based species delimitation in algae

REVIEW

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Given the problems of species delimitation in algae using morphology or sexual compatibility, molecular data are becoming the standard for delimiting species and testing their traditional boundaries. The idea that species are separately evolving metapopulation lineages, along with theoretical progress in phylogenetic and population genetic analyses, has led to the development of new methods of species delimitation. We review these recent developments in DNA-based species delimitation methods, and discuss how they have changed and continue to change our understanding of algal species boundaries. Although single-locus approaches have proven effective for a first rapid and large-scale assessment of species diversity, species delimitation based on single gene trees falls short due to gene tree–species tree incongruence, caused by confounding processes like incomplete lineage sorting, trans-species polymorphism, hybridization and introgression. Data from unlinked loci and multi-species coalescent methods, which combine principles from phylogenetics and population genetics, may now be able to account for these complicating factors. Several of these methods also provide statistical support regarding species boundaries, which is important because speciation is a process and therefore uncertainty about precise species boundaries is inevitable in recently diverged lineages.

Key words: coalescence, DNA barcoding, DNA taxonomy, evolution, gene genealogy, lineage sorting, molecular systematics, speciation, species concepts, taxonomy

Introduction

The idea that biological nature is aggregated in discrete entities named species is widespread but not unanimously accepted (Mayr, 1996; De Queiroz, 2007; Barraclough, 2010). Most biologists agree that the species represents a fundamental category of biological organization. Yet, they have debated endlessly over an all-encompassing definition and the criteria used to delimit species (Hey, 2006; Wilkins, 2009) and phycologists are no exception to this (Guiry, 1992; John & Maggs, 1997; Mann 1999, 2010).

Disagreement over the nature of species and their defining properties has not dissuaded phycologists from documenting algal diversity and describing new species. A screening of papers published in *European Journal of Phycology* and *Phycologia* in 2012 and 2013 revealed that half (68 out of 137 research papers; Supplementary Table S1) describe new taxa or make explicit statements on species boundaries. In virtually all of these papers, species delimitation is based on molecular data combined with at least some information about the morphology, ecology or (eco-)physiology of the organisms. It seems that, at least in the peer-reviewed literature, it has become rare for extant species to be defined solely by morphology. In some algal groups (e.g. diatoms) the majority of new taxa, however, are still being described based on morphology in book series, such as *Bibliotheca Diatomologica* and *Iconographia Diatomologica* (Kusber & Jahn, 2003).

Our survey of papers also revealed that whereas molecular and phylogenetic techniques are usually explained in detail, the criteria or methods used to delimit species based on sequence data are rarely specified. Typically, a phylogeny containing multiple specimens per species is constructed, and species are delimited based on topological criteria: monophyly and distinctness from sister species expressed as branch lengths, and some measure of support (bootstrap most commonly). Alternatively, but following a similar

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Glossary

Ancestral polymorphism: genetic variation that originated prior to a speciation event. The presence of ancestral polymorphism in closely related species (shared ancestral polymorphism) may result in gene tree – species tree incongruence (Maddison & Knowles, 2006) (see also Trans-species polymorphism).
Barcoding gap: a gap observable in the frequency distribution of intraspecific and interspecific genetic distances; a corresponding distance threshold may be used to delimit species.
Biological species concept (BSC): a species concept that defines a species as 'a group of interbreeding natural populations that are reproductively isolated from other such groups' (Mayr & Ashlock, 1991).
Coalescence: looking back in time, the point at which two alleles converged on a single ancestral copy, known as the most recent common ancestor (MRCA) (Figs 2–5).
Coalescent theory: 'the mathematical and probabilistic theory underlying the evolutionary history of alleles' within a population lineage (Fujita *et al.*, 2012).
Deep coalescence: coalescence of alleles occurring significantly earlier than the divergence of the species containing those alleles (Fig. 3).
DNA barcoding: a method of species identification, which identifies specimens based on DNA sequence similarity against a sequence database of *a priori* defined species (Hebert *et al.*, 2003).
DNA taxonomy: DNA-based species delimitation, which defines species boundaries based on sequence data (Tautz *et al.*, 2003).
Evolutionary species concept: a species concept that defines a species as 'a lineage of ancestral descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate' (Wiley, 1978).
General Mixed Yule Coalescent (GMYC) model: a model of a phylogenetic tree that separately considers branching within species (neutral coalescent model) and branching between species (Yule model).
Incomplete lineage sorting (ILS): the maintenance of genetic variation within a metapopulation lineage from one speciation event to the next, resulting in deep coalescence and gene tree–species tree incongruence (Baum & Smith, 2012).
Lineage sorting: the process by which alleles are inherited and lost over time.
Metapopulation lineages: 'Metapopulations' refers to a set of populations connected by gene flow over a relatively short time interval. 'Lineage' refers to an ancestral-descendent sequence. Thus, a 'metapopulation lineage' is a set of populations connected by gene flow on an evolutionary timescale (de Queiroz, 2005).
Multispecies coalescent: coalescent model extended to the interspecific level, i.?e. applied to gene trees in a species tree (Degnan & Rosenberg, 2009).
Phylogenetic species concept: a species concept that defines species as 'the smallest biological entities that are diagnosable and/or monophyletic' (Mayden, 1997).
Reciprocal monophyly: monophyletic with respect to each other. Two sister taxa are reciprocally monophyletic when all alleles within each taxon are more closely related to one another than to any alleles in the other taxon.
Trans-species polymorphism: presence of similar alleles in related species generated by the passage of alleles from ancestral to descendant species (Klein *et al.*, 1998) (Fig. 5).
Yule model: a birth-only model of a phylogenetic tree in which each branch is associated with a birth rate determining the rate at which the branch bifurcates into two branches.

rationale, species are delimited based on a ratio of intra- versus interspecific divergence (the so-called barcoding gap) (Hebert *et al.*, 2004). Sometimes, other thresholds, such as the presence of compensatory base pair changes (CBCs) in the secondary structure of the ribosomal RNA internal transcribed spacer (ITS), are used as a proxy for species divergence (Coleman, 2009). In several of the surveyed papers, intraspecific divergence could not be estimated because only a single representative per species was included, and comparable interspecific branch lengths or p-distances are taken as evidence for species boundaries. Another often-used criterion is concordance between data from independent sources, when individuals belong to the same clades in phylogenies based on different molecular markers, or when there is morphological divergence or sexual incompatibility between lineages in a phylogeny. In the two journals surveyed, we did not find systematic differences in how species delimitation was approached in either macro- or microalgae.

It is relevant to ask the question whether and how current practices of species delimitation reflect our perceived knowledge of algal species diversity. For example, if two individuals share identical 18S rDNA sequences, does this make them conspecific? Is it possible for two individuals lacking CBCs in their ITS2 sequences to represent different biological species? By extension, is reproductive isolation a prerequisite for species delimitation and do species need to be monophyletic with all markers chosen? These and other questions highlight an important gap in our understanding of what algal species are and how to delimit them.

In this review we first aim to address species delimitation from a conceptual perspective, followed by an overview of the common practices in the phycological literature, and finally we look ahead to see how new developments in sequencing technologies, and advances in phylogenetic and population genetic approaches, are taking centre stage in species delimitation.

Species concepts and species delimitation

In the years following the publication of Darwin's 'On the Origin of Species', the emerging evolutionary

biological community struggled to reconcile the gradual process of evolution with the discrete species that it appeared to have produced (Coyne & Orr, 2004). The Modern Evolutionary Synthesis brought a better understanding of how species evolve, and the view that species are not just arbitrary divisions of an evolutionary continuum but discrete entities gained broader acceptance (Shaw, 1998). Ernst Mayr, amongst others, regarded a species as a group of interconnected populations that form an extended gene pool (metapopulation), or incorporating a time dimension, an ancestral-descendant sequence of metapopulations (metapopulation lineage) evolving separately from others, and thus having its own evolutionary tendencies and fate (Coyne & Orr, 2004). The application of this so-called 'evolutionary species concept' (Simpson, 1951), however, was problematic because it was purely theoretical and at that time of little practical use for species delimitation. The search for operational criteria to delimit species has led to a proliferation of species concepts, each based on different biological properties of a species (Mayden, 1997; Hausdorf, 2011).

The morphological species concept uses discontinuities in morphological variation to distinguish species. This concept has, for practical reasons, dominated algal systematics for many decades (John & Maggs, 1997; Mann, 1999). Although discontinuities in morphological variation will in many instances correspond to species boundaries, convergent morphological evolution, morphological stasis, phenotypic plasticity and polymorphism are common phenomena that limit the correspondence between how a species would be defined based on the morphological concept versus the evolutionary or biological species concepts (Verbruggen, 2014). Many algal species are known to exhibit substantial intraspecific morphological variation, either as a result of genetically controlled polymorphism or environmentally induced plasticity (e.g. de Senerpont Domis *et al.*, 2003; Lurling, 2003; Logares *et al.*, 2007). Ignoring this intraspecific morphological variation may result in an overestimation of species diversity (Trainor, 1998; Macaya & Zuccarello, 2010). On the other hand, morphological differences between two species will often only emerge after enough time has passed since lineage divergence, and therefore recently diverged species are likely to remain undetected (Fig. 1). Molecular phylogenetic data have indeed revealed numerous cases of (closely related) species that are morphologically indistinguishable (e.g. Zuccarello & West, 2003; De Clerck *et al.*, 2005; Fraser *et al.*, 2009; Fucikova *et al.*, 2011; Gutner-Hoch & Fine, 2011; Degerlund *et al.*, 2012; Kucera & Saunders, 2012; Moniz *et al.*, 2012; Soehner *et al.*, 2012; Payo *et al.*, 2013; Souffreau *et al.*, 2013). In addition, ancient cryptic lineages have been detected in algal groups that are morphologically depauperate, exhibit stabilizing selection, or are subject to convergent evolution towards reduced morphologies, such as planktonic unicells (Saez *et al.*, 2003; de Vargas *et al.*, 2004; Šlapeta *et al.*, 2006; Krienitz & Bock, 2012; Škaloud & Rindi, 2013) and seaweeds (Verbruggen *et al.*, 2009; Sutherland *et al.*, 2011).

To overcome difficulties associated with morphological data, the biological species concept has been applied to assess species boundaries in some algal groups, including diatoms (Mann, 2010), and some red, green and brown algae (e.g. Coleman, 1977; Zuccarello & West, 2003; Hiraoka *et al.*, 2011; Maggs *et al.*, 2011). The biological species concept is closely allied to the evolutionary species concept and focuses on reproductive isolation to distinguish species. Although this concept has been widely embraced, many taxonomists grew dissatisfied with

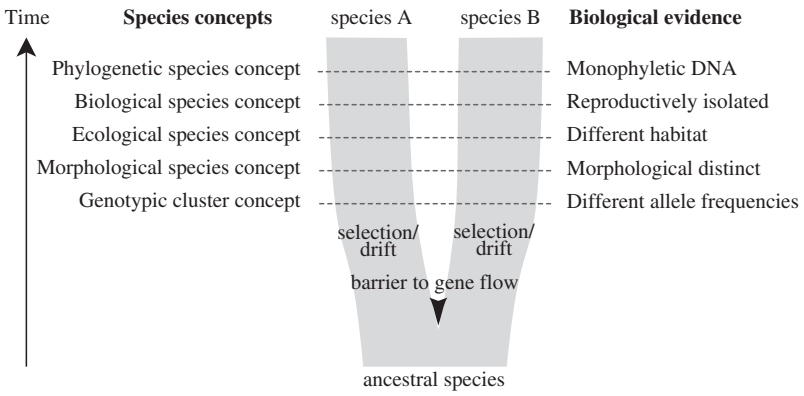


Fig. 1. Simplified diagram of speciation, species concepts and corresponding biological properties of species (after de Queiroz, 2007). As populations separate by a barrier to gene flow, independently acting selection and drift will result in two daughter lineages with separate evolutionary trajectories. Through time, these daughter lineages will acquire different properties, which have traditionally served as biological evidence for species delimitation, corresponding to different species concepts. During the process of speciation, these secondary properties do not necessarily arise at the same time or in a regular order, and therefore different species concepts may come into conflict, especially during early stages of speciation.

it because it did not give a clear indication of how strong reproductive isolation between populations had to be for them to qualify as different species. Reproductive barriers between sexually reproducing algal species are not always complete (Brodie *et al.*, 1993; Coyer *et al.*, 2002; Kamiya, 2004; Casteleyn *et al.*, 2009; Zardi *et al.*, 2011), and little is known about hybrid formation in most algal groups. Moreover, laboratory crosses also only take into account intrinsic reproductive barriers and not extrinsic barriers, such as differences in timing of sexual reproduction or ecological preferences. Finally, the application of the biological species concept is unfeasible or impractical in algae that reproduce only asexually, or for which sexual reproduction is difficult to induce under laboratory conditions.

The phylogenetic species concept was a strong rival to the biological species concept, and regards species as unit products of natural selection and descent that can be identified based on reciprocal monophyly and/or diagnosability (Mayden, 1997). Although monophyly and diagnosability can be based on any character (phenotypic or genotypic), it was the emergence of molecular data that made this species concept popular in algal systematics (Manhart & McCourt, 1992; John & Maggs, 1997).

Because the literature on species concepts is vast and outside the scope of this paper, we refer to Guiry (1992), Manhart & McCourt (1992), John & Maggs (1997) and Mann (2010) for comprehensive overviews of species concepts in algae. What is important here is that although many of the species concepts are related, none is completely satisfactory, and most are at least partially incompatible in that they can lead to different conclusions regarding species boundaries (Mallet, 1995).

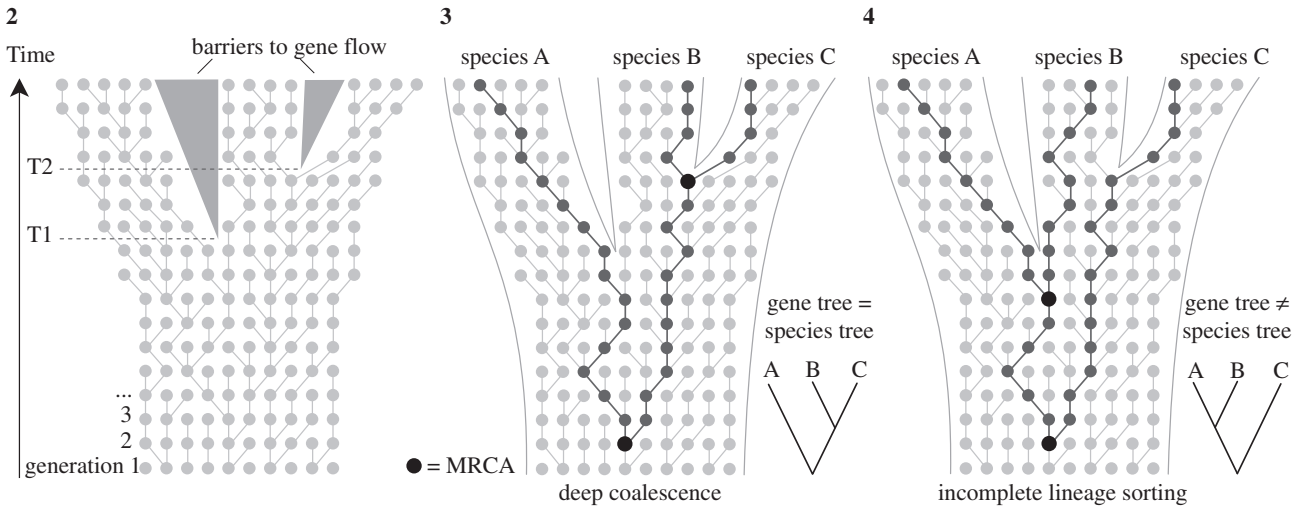
Debates on species concepts may be reaching some sort of consensus though. There is fairly general agreement that species are principal units in biology, and that there is a common evolutionary idea underlying them: speciation results from isolation of populations by interrupted gene flow, resulting in divergence due to selection and drift, and ultimately in separately evolving metapopulation lineages (Coyne & Orr, 2004). As such, species will show greater evolutionary independence from one another than do populations within a single species, and this independence will be subject to the amount of gene flow between lineages and the time of lineage separation (Hey & Pinho, 2012). Several authors have argued that many of the competing species concepts actually agree on the basic notion that species are separately evolving metapopulation lineages (Mayden, 1997; De Queiroz, 2007). In their view, traditional species concepts become secondary species properties, rather than necessary properties of species (Fig. 1). When two lineages separate, they will eventually acquire different properties (e.g. become sexually incompatible or morphologically distinct), which may serve as diagnostic evidence relevant to species delimitation. During the process of speciation these secondary properties do not necessarily arise at the same time or in a regular order, or may not arise at all, so conflict will occur between species concepts in many cases, especially between recently formed species.

It has become clear that species delimitation using morphological or breeding data is often problematic for the reasons discussed above. Molecular data are therefore particularly valuable for delimiting species and testing traditional species boundaries. Below, we provide an overview of recent developments in DNA-based sequence delimitation and discuss how these have shaped the current view on species boundaries in algae.

Gene genealogies and species phylogenies

Gene trees spanning intraspecific and interspecific evolution are vital to understanding the process of speciation (Templeton, 2001). Because the stochastic pattern of allelic coalescence can be modelled, it provides valuable information regarding lineage divergence, and hence species delimitation (Ence & Carstens, 2011). In phylogenetics, gene trees are often implicitly equated to species trees, but it is important to realize that population genetics processes are acting within the branches of a species tree (Pamilo & Nei, 1988; Avise & Wollenberg, 1997; Maddison, 1997) (Fig. 2). These processes, known as the coalescent (Hovmoeller *et al.*, 2013), can have three important effects.

First, DNA divergence times may be considerably older than speciation times (deep coalescence, Fig. 3). Second, deep coalescence in combination with incomplete fixation of gene lineages within species lineages may result in gene trees and species trees having different topologies (incomplete lineage sorting, Fig. 4). Third, because incomplete lineage sorting is associated with the persistence of ancestral alleles in recently diverging lineages, gene trees will inevitably go through phases where individuals of a species are polyphyletic and paraphyletic before becoming monophyletic as alleles become fixed through time (Avise & Ball, 1990; Klein *et al.*, 1998; Rosenberg & Nordborg, 2002) (Fig. 5). Clearly, this process has important consequences for delimitation of recently diverged species. Most importantly, it implies that reciprocal monophyly is not a necessary property of species, especially for recently diverged species (Kizirian & Donnelly, 2004; Knowles & Carstens, 2007). Simulation studies have indicated that a substantial amount of time (i.e. number of generations) may be required after lineage divergence before there will be a high probability of observing monophyly for a given locus, which is considered to depend on its



Figs 2-4. Illustrations of a Wright–Fisher process (a simplified version of the coalescence process), based on Degnan & Rosenberg (2009) and Mailund (2009). **Fig. 2.** Diagram showing a set of non-overlapping generations where each new generation is sampled from the previous at random with replacement. Each dot represents an individual gene copy and each line connects a gene copy to its ancestor in the previous generation. Starting with a set of individuals in a first generation, the second generation is created by randomly selecting a parent from the first population; the third generation is sampled from the second, and so on. During speciation events (T1 and T2), where populations become separated by a barrier to gene flow, gene copies will only be sampled from within the same population. This coalescence process, running inside the species tree, has two important consequences (Figs 3 and 4). **Fig. 3.** DNA divergence times may be much older than speciation times, known as 'deep coalescence'. As illustrated, the most recent common ancestor (MRCA) of two gene copy samples from species A, B and C is much older than the speciation event T1. **Fig. 4.** Deep coalescence in combination with incomplete fixation of gene lineages within species lineages (incomplete lineage sorting) may result in a gene tree and species tree with different topologies. For example, the gene tree based on three randomly sampled alleles would wrongly suggest a sister relationship between species A and B.

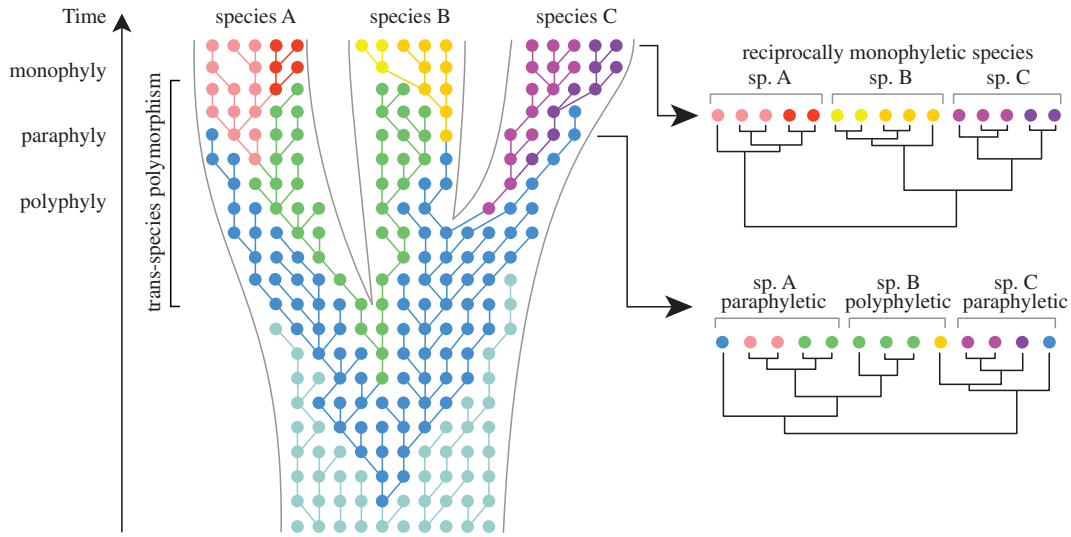


Fig. 5. Neutral coalescence process running within a species tree, based on Klein *et al*. (1998), Degnan & Rosenberg (2009) and Mailund (2009). Each dot represents an individual gene copy, each colour a different allele, and each line connects a gene copy to its ancestor in the previous generation. Within a population, selection and/or drift will result in changing allele frequencies over time. In the initial stages of lineage splitting, sister species will largely share identical alleles, which has important consequences for species delimitation. In this example, constructing a gene tree at an early stage of speciation would result in none of the three species being monophyletic. Only after sufficient time has gone by, will alleles be completely sorted in each lineage, resulting in reciprocal monophyly for the each of the three species.

effective population size (Avise, 2000; Hudson & Coyne, 2002; Hickerson *et al.*, 2006).

Thus, although within-species gene genealogies differ in nature from among-species phylogenetic relationships (Posada & Crandall, 2001), young species reside in a boundary zone where both processes meet. Therefore, species delimitation should ideally incorporate aspects of both population genetics and phylogenetics (Knowles & Carstens, 2007; Hey & Pinho, 2012).

DNA-based species delimitation methods and application in algae

The idea that species are separately evolving metapopulation lineages, along with theoretical progress in phylogenetic and population genetic analyses, has led to the development of new methods of species delimitation (Sites & Marshall, 2003; Camargo & Sites, 2013; Carstens *et al.*, 2013). As discussed above, gene trees contain vital information regarding the speciation process, and hence DNA sequences are increasingly being used for species identification (DNA barcoding), species discovery and delimitation (DNA taxonomy), and for testing traditional species-level taxonomies (Wiens, 2007). Although the terms DNA taxonomy and DNA barcoding originally had different meanings (see glossary), the distinction between the two is not always clear-cut as both methodologies use similar molecular data (Collins & Cruickshank, 2013). For example, short, standardized gene regions (DNA barcodes) are increasingly being used for species discovery and delimitation. Similarly, DNA barcoding studies are not always restricted to analysis of short DNA sequences, but increasingly make use of multi-locus data (Roe *et al.*, 2010).

For convenience, we subdivide DNA-based species delimitation methods into two categories, single-locus and multi-locus methods, although it should be noted that this subdivision is somewhat artificial as several methods can be applied to both single and multiple markers.

Single-locus species delimitation

Species delimitation methods based on single-locus data rely on the assumption that a single gene genealogy is representative of the species phylogeny. In general, single-locus methods impose a strict threshold of reciprocal monophyly for delimiting species and aim to detect discontinuities in sequence variation, assuming that interspecific divergence exceeds intraspecific variation.

Several single-locus methods for testing species boundaries are based on diagnostic character variation in DNA sequence data. These methods, which are rooted in the phylogenetic species concept, aggregate predefined populations with unique nucleotide differences into a single species (Davis & Nixon, 1992; Wiens & Penkrot, 2002). These methods have remained largely unexplored in algal studies because population boundaries in algae are often difficult to define *a priori* due to uncertainty regarding geographic ranges, and are therefore not further considered here.

Other methods do not require *a priori* definition of populations, and assess species boundaries based on molecular data alone. These methods rely on the assumptions that species are monophyletic for the gene under study and that there are discrete differences in sequence variation within and between species as a result of fixation of alleles in species lineages (Fig. 5). Distance approaches aim to detect a difference between inter- and intraspecific distances by analysing a frequency distribution of genetic distances between specimens. The transition between intra- and interspecific variation is visible as a distinct gap in the distribution, the so-called barcoding gap, and a corresponding distance threshold can then be applied to delimit species (Hebert *et al.*, 2004). Similarly, tree-based methods seek to detect discontinuities in sequence variation that are associated with species boundaries by identifying clades of closely related sequences that are preceded by long and well-supported branches. Distance and tree-based methods have been applied to identifying or delimiting species in several algal studies (e.g. Verbruggen *et al.*, 2007; Rybalka *et al.*, 2009; Freshwater *et al.*, 2010).

Although in some cases clear discontinuities between intra- and interspecific genetic distances have been observed (e.g. Saunders, 2005; Zimmermann *et al.*, 2011), the use of distance thresholds for species delineation may be problematic in some cases. First, among closely related species, levels of overlap between intra- and interspecific genetic distances are likely to be significant (Hamsher *et al.*, 2011; Hoef-Emden, 2012). Second, with increased geographic sampling, barcoding gaps usually blur (Meyer & Paulay, 2005; Bittner *et al.*, 2010; Bergsten *et al.*, 2012). As a result, distance and tree-based methods often rely on intuition to decide whether sequence divergence is high enough or branches are long enough to consider a clade a distinct species.

Automated methods offering the promise of making single-locus species delimitation more efficient and less subjective have been developed. Software packages such as 'Automatic Barcode Gap Discovery' (Puillandre *et al.*, 2011) and 'SPecies IDentity and Evolution' (Brown *et al.*, 2012) aim at automatically detecting barcoding gaps in large datasets (Table 1). Another approach, based on statistical parsimony, separates groups of sequences into different networks if the haplotypes are connected by long branches that are affected by homoplasy (Hart & Sunday, 2007). This method has been applied in some algal groups, including the dinoflagellate *Symbiodinium* (Correa & Baker, 2009) and the seaweeds *Boodlea* and *Padina* (Leliaert *et al.*, 2009; Silberfeld *et al.*, 2013).

Likelihood methods based on evolutionary models have been proposed to statistically determine species boundaries based on analysis of branch lengths in phylogenetic trees. The idea behind these methods is that differences between species-level evolutionary processes and population-level evolutionary processes result in different branching rates in a gene tree. In a method proposed by Pons *et al.* (2006), a

Table 1. A non-exhaustive list of algorithmic species delimitation methods and software packages.

Method	Data	Brief description	Reference
ABGD	Single-locus	'Automatic Barcode Gap Discovery'. Distance method that automates detection of a barcoding gap	Puillandre *et al.*, 2011
Spider	Single-locus	'SPecies IDentity and Evolution'. Distance method assessing specimen identification efficacy based on a set of *a priori* defined species. Implemented in R, requires 'ape' (ape-package.ird.fr)	Brown *et al.*, 2012
Statistical parsimony	Single-locus	Partitions sequence data into independent networks of haplotypes connected by changes that are non-homoplastic with a 95% probability	Templeton *et al.*, 1992; Hart & Sunday, 2007
GMYC model approach	Single-locus	Tree-based likelihood method fitting within- and between-species branching models to an ultrametric gene tree. Implemented in the R package SPLITS, requires ape	Pons *et al.*, 2006; Monaghan *et al.*, 2009
bGMYC	Single-locus	Bayesian implementation of the GMYC model aimed at quantifying uncertainty in species limits by accounting for uncertainty in the phylogenetic tree and in the parameters of the model. Implemented in R, requires ape	Reid & Carstens, 2012
'Species delimitation'	Single-locus	Takes *a priori* defined species and a user-specific gene tree to compute the probability of the observed monophyly or exclusivity having occurred by chance in a coalescent process. Implemented as a plugin to Geneious (geneious.com)	Masters *et al.*, 2011
BPP	Multi-locus	Bayesian method estimating posterior distribution for species delimitation models. Uses a fixed species tree and prior information on population sizes and divergence times	Yang & Rannala, 2010
SpeDeSTEM	Multi-locus	Maximum likelihood method estimating the species tree for different models of species boundaries, which are compared with Akaike information criteria	Carstens & Dewey, 2010; Ence & Carstens, 2011
O'Meara method	Multi-locus	Heuristic method that takes individual gene trees from multiple loci as input, and assesses differences between inter- and intraspecific branch length, and congruence among loci. Implemented in the Brownie package	O'Meara, 2010

model that combines a coalescent model of intraspecific branching with a Yule model for interspecific branching (general mixed Yule-coalescent or GMYC model) is fitted to a gene tree, resulting in an estimation of species boundaries and a statistical measure of confidence for the species boundaries. The GMYC model approach has been refined to allow for a variable transition from coalescence to speciation among lineages (Monaghan *et al.*, 2009), and to account for uncertainty in phylogenetic relationships and parameters of the GMYC model (Powell, 2012; Reid & Carstens, 2012; Fujisawa & Barraclough, 2014) (Table 1). The GMYC approach has been applied in a number of studies on macro- and microalgae (Leliaert *et al.*, 2009; Hoef-Emden, 2012; Tronholm *et al.*, 2012; Payo *et al.*, 2013; Silberfeld *et al.*, 2013).

Marker choice for single-locus species delimitation

Irrespective of the species delimitation algorithm used, the success of single-locus species delimitation methods will depend on the history of the species group under study (e.g. species delimitation will be more challenging in young lineages with some levels of inter-species gene flow than in older, well-diverged lineages), the sampling strategy, and the molecular marker used. DNA sequences should ideally be obtained for a sufficient number of specimens and geographic locations to obtain a good estimate of intraspecific variation (Bergsten *et al.*, 2012).

Apart from practical issues, such as ease of amplification, the effectiveness of a chosen neutral marker for molecular species discovery will depend on two criteria. First, the marker should contain sufficient variation within and among species. Second, interspecific variation should preferably exceed intraspecific variation to such a degree that a discontinuity in sequence variation, corresponding to the species boundary, is observable. This will depend on the coalescence time of the marker, which is determined by the effective population size and is independent of the mutation rate of the marker (Zink & Barrowclough, 2008).

Even though theoretically the mutation rate of a chosen marker will have no direct influence on its effectiveness to detect species, genetic variability is an important issue in DNA taxonomy. In principle, a single base pair difference could be sufficient for delimiting a species (e.g. Brodie *et al.*, 1996). However, species delimitation should ideally be based on more data because of the 'strong claims require strong evidence' principle in science. Describing or delimiting a new species differs from, and is a much stronger claim than, identifying a specimen: more evidence is expected when delimiting species than for identifying specimens. Thus, species delimitation requires markers with fast coalescence (which depends on effective population

size) and with sufficient variability (which depends on evolutionary rate and/or the length of the marker).

Given the prevalence of undiscovered and cryptic species in many algal groups, any marker chosen as a DNA barcode should preferably be suitable not only for species identification but also for species delimitation and discovery. For animals, the mitochondrial cytochrome oxidase I locus (COI or *cox*1) has been found to satisfy these criteria for most groups (Hebert *et al.*, 2003) (but see Meier *et al.*, 2006). However, COI has been shown to be less effective in many other eukaryotic groups (Pawlowski *et al.*, 2012).

Because algae form a phylogenetically heterogeneous group, the application of a single universal marker for species delimitation is unfeasible, and different markers are applied for species delimitation in different algal groups. Table 2 summarizes the main markers that are currently used for species delimitation and/or DNA barcoding in different algal groups. It should be noted, however, that the resolution of these markers may not be optimal to discriminate between closely related species in specific clades (e.g., Mattio & Payri, 2010; Zardi *et al.*, 2011; Janouškovec *et al.*, 2013).

Species diversity surveys of microbial eukaryotic communities have heavily relied on the nuclear small subunit ribosomal RNA gene (SSU or 18S rDNA) (de Vargas *et al.*, 1999; Moon-van der Staay *et al.*, 2001; Not *et al.*, 2012). Yet, comparison between genetic divergences of protein-coding vs. ribosomal RNA genes has indicated that the SSU rDNA generally underestimates the true number of species, especially in planktonic algae that have large population sizes and high turnover rates (Hall *et al.*, 2010; Piganeau *et al.*, 2011). Alternative universal markers have been proposed in various algal groups, including the nuclear and plastid rDNA (Sherwood & Presting, 2007; Liu *et al.*, 2009).

Substitution rates of the Internal Transcribed Spacer (ITS) of the nuclear rDNA are much higher than those of the rRNA genes, and this region has been advocated as a marker for species level phylogenetics for macro- and microalgae (e.g., van der Strate *et al.*, 2002; Lundholm *et al.*, 2006; Gile *et al.*, 2010; Pröschold *et al.*, 2011). Unfortunately, several

Table 2. Main markers currently employed for DNA-based species delimitation and/or barcoding in some of the principal algal groups. Recommended barcode markers are indicated in bold, although it should be noted that for none of the groups has a consensus been reached.

Algal group	Marker			References
	plastid	mitochondrial	nuclear	
Green algae	***tuf*A**, *rbc*L		SSU rDNA, LSU rDNA, rDNA ITS	Verbruggen *et al.*, 2007; Leliaert *et al.*, 2009; Hall *et al.*, 2010; Luo *et al.*, 2010; Saunders & Kucera, 2010; Škaloud & Peksa, 2010; Fucikova *et al.*, 2011; Rindi *et al.*, 2011; Škaloud & Rindi, 2013; Subirana *et al.*, 2013
Red algae	*rbc*L, Rubisco spacer	***cox*1**, *cox*2-3 spacer	Phycoerythrin, elongation factor, LSU rDNA	Robba *et al.*, 2006; Sherwood *et al.*, 2008, 2010; Le Gall & Saunders, 2010; Kucera & Saunders, 2012; Saunders & McDevit, 2012; Janouškovec *et al.*, 2013; Payo *et al.*, 2013
Brown algae	*psb*A, *rbc*L, Rubisco spacer	***cox*1**, *cox*3	rDNA ITS	Lane *et al.*, 2007; Kucera & Saunders, 2008; McDevit & Saunders, 2009; Mattio & Payri, 2010; Peters *et al.*, 2010; Tronholm *et al.*, 2010; Silberfeld *et al.*, 2013
Chrysophytes, Synurophytes	*psa*A, *rbc*L	*cox*1	SSU rDNA, **rDNA ITS**	Boo *et al.*, 2010; Kynčlová *et al.*, 2010; Škaloud *et al.*, 2012
Cryptophytes	Rubisco spacer	*cox*1	SSU rDNA, LSU rDNA, rDNA ITS	Lange *et al.*, 2002; Hoef-Emden, 2007, 2012
Diatoms	*rbc*L	*cox*1	SSU rDNA, LSU rDNA, rDNA ITS	Amato *et al.*, 2007; Evans *et al.*, 2007; Vanelslander *et al.*, 2009; Mann *et al.*, 2010; Moniz & Kaczmarska, 2010; Hamsher *et al.*, 2011; Kermarrec *et al.*, 2013
Dinoflagellates	*psb*A^{ncr}, 23S rDNA	*cox*1, cob	LSU rDNA, **rDNA ITS**	Litaker *et al.*, 2007, 2009; Lin *et al.*, 2009; Sampayo *et al.*, 2009; Stern *et al.*, 2010, 2012; LaJeunesse & Thornhill, 2011; Sato *et al.*, 2011; LaJeunesse *et al.*, 2012
Haptophytes	*tuf*A	*cox*1b-*atp*4	SSU rDNA, LSU rDNA, rDNA ITS	Saez *et al.*, 2003; de Vargas *et al.*, 2004; Bendif *et al.*, 2011; Edvardsen *et al.*, 2011; Hagino *et al.*, 2011; Sym *et al.*, 2011; Bittner *et al.*, 2013
Raphidophytes	*psa*A, *rbc*L	*cox*1	SSU rDNA, LSU rDNA, rDNA ITS	Demura *et al.*, 2009; Klopper *et al.*, 2013
Xanthophytes	*rbc*L, *psb*A-*rbc*L spacer		rDNA ITS	Zuccarello & Lokhorst, 2005; Rybalka *et al.*, 2009, 2013
Chlorarachniophytes			nuclear rDNA ITS, nucleomorph rDNA ITS	Gile *et al.*, 2010
Euglenophytes	SSU rDNA, LSU rDNA		SSU rDNA, LSU rDNA	Kim *et al.*, 2013

problems have been identified with the use of ITS for assessing species limits, including slow coalescence and intragenomic variation, potentially blurring the transition between species-level and population-level genetic distance (Álvarez & Wendel, 2003; Lane *et al.*, 2007; Vanormelingen *et al.*, 2008; Leliaert *et al.*, 2009).

Special emphasis has been given to a particular kind of genetic distance threshold, which is found in the secondary structure of the ITS RNA product. Several studies have suggested that compensatory base changes (CBCs) in specific helices of the ITS correlate with reproductive isolation and could thus be used for delimiting species (Müller *et al.*, 2007; Coleman, 2009; Wolf *et al.*, 2013). Investigations of the correlation between species boundaries and CBCs suggest that although the presence of CBCs does correlate well with reproductive isolation barriers, the inverse is not necessarily true: absence of CBCs should therefore not be interpreted as proof that individuals belong to a single species (Müller *et al.*, 2007; Alverson, 2008; Caisová *et al.*, 2011, 2013).

Species delimitation studies in many algal groups (macroalgae in particular) have largely relied on plastid and mitochondrial markers (Table 2). Besides a number of practical aspects that makes the amplification and sequencing of organellar loci relatively easy, their popularity for species delineation can be attributed to faster coalescence within species lineages compared with nuclear loci, resulting in clearer discontinuities between interspecific divergence and intraspecific variation (Palumbi *et al.*, 2001) (Fig. 6). This expectation is congruent with population genetic theory, which predicts that for diploid organisms, the effective population size of nuclear DNA is four times higher than that of the haploid and uniparentally inherited organellar DNA (Hudson & Coyne, 2002). Although mitochondrial or chloroplast genes have been shown to segregate earlier during speciation than most nuclear genes and hence detect earlier stages of speciation (Lane *et al.*, 2007; Birky, 2013; Payo *et al.*, 2013), some authors have highlighted potential problems with organellar DNA and suggested that inferences about species limits, especially in recently diverging lineages, are unwarranted unless corroborated by nuclear gene data (Zink & Barrowclough, 2008).

Multi-locus species delimitation

Although single-locus methods have proven effective for rapid and large-scale assessment of species diversity, concerns about the accuracy of species boundaries inferred from a single marker have been raised. Retention of ancestral polymorphism and incomplete lineage sorting, especially at early stages of speciation, will result in different neutral loci having their own gene trees that do not necessarily mirror the speciation process, with important consequences for assessing species boundaries (Hickerson *et al.*, 2006; Knowles & Carstens, 2007). Furthermore, reticulate evolutionary processes such as hybridization and introgression will remain unnoticed when using single gene data (e.g. Zardi *et al.*, 2011; Mols-Mortensen *et al.*, 2012). Although these criticisms have been vented ever since DNA taxonomy emerged, methods for multi-marker species delimitation have only recently been developed, mainly as a response to technical advances in sequencing technology that are making the collection of multi-marker datasets easier (Roe *et al.*, 2010; Harrington & Near, 2012; Niemiller *et al.*, 2012).

One approach uses genealogical concordance of unlinked, neutral loci to assess species boundaries. The rationale is that within species, the mixing effects of recombination between genes would cause unlinked loci to have different genealogies, but between isolated species, the extinction of ancestral alleles by genetic drift would lead to concordant genealogical histories. Hence, the transition between deep genealogical concordance (divergent genealogy) and shallow genealogical discordance (reticulate

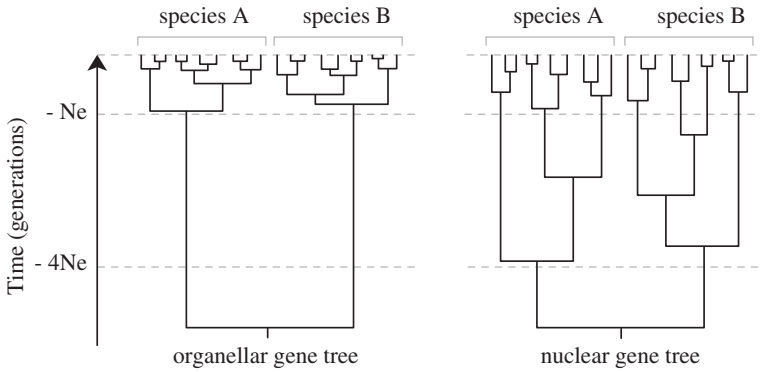


Fig. 6. Expected shapes of nuclear and organellar gene genealogies. Allelic coalescence of a neutral marker is expected to be about four times faster for organellar loci than for nuclear loci, resulting in a shorter time to arise at reciprocal monophyly and greater discontinuities between interspecific divergence and intraspecific variation (Hare, 2001; Palumbi *et al.*, 2001; Zink & Barrowclough, 2008).

genealogy) can be used to delimit species (Avise & Ball, 1990; Baum & Shaw, 1995). In practice, species can thus be identified if gene genealogies of multiple unlinked loci show congruent patterns of reciprocal monophyly (Taylor *et al.*, 2000; Dettman *et al.*, 2003). The criteria of reciprocal monophyly and genealogical concordance of unlinked loci has been widely used (although not always explicitly) to delimit algal species (Casteleyn *et al.*, 2008; Sherwood *et al.*, 2008; Fraser *et al.*, 2010; Škaloud & Peksa, 2010; Lundholm *et al.*, 2012; Schmidt *et al.*, 2012; Rybalka *et al.*, 2013; Škaloud & Rindi, 2013), even if some of these studies showed possible signals of hybridization and/or introgression, blurring species boundaries (Zuccarello *et al.*, 2005; Lane *et al.*, 2007; Niwa *et al.*, 2009; Destombe *et al.*, 2010; Hind & Saunders, 2013).

Genealogical concordance of unlinked, neutral loci is expected among well-diverged lineages. It should be noted that the majority of multi-locus species delimitation studies in algae show concordance between the different gene trees, and thus represent such well-diverged lineages. This is also suggested by molecular clock analyses, showing lineage divergences in the order of hundreds of thousands to tens of millions of years (Casteleyn *et al.*, 2010; Payo *et al.*, 2013; Souffreau *et al.*, 2013), which appeared to have been sufficient to reach reciprocal monophyly across markers used in these studies. Between younger lineages however, as discussed above, the criteria of reciprocal monophyly and strict congruence will probably fail to detect species boundaries. New methods that extend coalescent models to the interspecific level ('multispecies coalescent') offer perspectives for testing species boundaries in such shallow divergences (Table 1). The rationale of these methods is that despite species not necessarily being monophyletic in gene trees, a signal of species divergence may persist in gene trees of unlinked, neutral loci (Knowles & Carstens, 2007; O'Meara, 2010; Yang & Rannala, 2010).

Coalescence-based, multi-locus species delimitation methods are rapidly gaining popularity in studies on closely related species that are difficult to distinguish based on phenotypic features, and have been applied in various eukaryotic groups including animals (Carstens & Dewey, 2010; Leaché & Fujita, 2010; Yang & Rannala, 2010; Camargo *et al.*, 2012), fungi (Leavitt *et al.*, 2011) and land plants (Barrett & Freudenstein, 2011). Also in algae, phylogenetic and population genetic approaches have been combined to study boundaries between closely related species and assess confounding factors such as incomplete lineage sorting, trans-species polymorphism, hybridization and introgression.

For example, Payo *et al.* (2013) used three unlinked loci from the mitochondrial, plastid and nuclear genome, and a multispecies coalescent method (BPP, Yang & Rannala, 2010) to infer 21 cryptic species of the red seaweed *Portieria* in the Philippines. The number of species identified using the BPP analysis was considerably higher than under the strict criteria of reciprocal monophyly and genealogical concordance across loci, with differences in species boundaries mainly situated in a clade uniting taxa that diverged at small spatial scales, probably during the Plio-Pleistocene. Such shallow divergences were captured only by the mitochondrial locus, which featured more rapid coalescence within species lineages compared with the plastid and nuclear loci.

Species boundaries have also been under close scrutiny in *Fucus*, a brown algal genus in which the taxonomy has been confounded by significant intraspecific morphological variability. Species boundaries in one of the main clades have remained largely unresolved, even in analyses of variable markers such as the nuclear rDNA ITS and a mitochondrial intergenic spacer, which was attributed to hybridization and/or incomplete lineage sorting, in what was believed to be a recent and rapid radiation within the last 3.8 million years (Serrao *et al.*, 1999; Coyer *et al.*, 2006). A multilocus phylogenetic analysis of 13 nuclear loci (Canovas *et al.*, 2011) provided finer resolution, resolving nearly all currently recognized species, including *F. guiryi*, confirming its recent elevation to species level based on an integrative approach employing phylogenetic and population genetic analyses (based on 14 SNPs and two microsatellite loci), along with morphological, physiological and distributional data (Zardi *et al.*, 2011).

One of the diatom genera in which species boundaries have been thoroughly examined is *Pseudo-nitzschia*. In this genus, DNA sequence data are consistently used for species delimitation, which has resulted in the recent descriptions of numerous cryptic or pseudo-cryptic species (Amato *et al.*, 2007; Lelong *et al.*, 2012). In *P. pungens*, phylogenetic analysis has resulted in the recognition of three lineages that diverged recently, between 200 kya and 1.6 Mya (Casteleyn *et al.*, 2010). These lineages differ by only a few bases in their ITS and *rbc*L sequences, but they were consistently recovered in both gene trees. Although the two most closely related clades were found to interbreed in laboratory crosses and form natural hybrids (Casteleyn *et al.*, 2008, 2009), sympatric populations from both clades were highly differentiated for microsatellites (Adams *et al.*, 2009). Such differentiation independent of geography suggests the presence of some barrier to gene flow, and the presence of different species.

Future perspectives

Many taxonomists may feel uncomfortable with the idea of delimiting species based solely on DNA sequence data, and this is at least partially reflected in a reluctance to formally describe new algal species in many molecular studies (De Clerck *et al.*, 2013).

Several researchers have advocated that adequate delimitation of species should be based on additional lines of evidence, including morphological, ultrastructural, biochemical, geographic, ecological and/or breeding data (e.g. Pröschold *et al.*, 2001; Vanormelingen *et al.*, 2007; Walker *et al.*, 2009; Bendif *et al.*, 2011; McManus & Lewis, 2011; Neustupa *et al.*, 2011; Ni-Ni-Win *et al.*, 2011; Lam *et al.*, 2012; Milstein & Saunders, 2012; Škaloud & Rindi, 2013). Although comparisons of DNA-based species delimitation and species boundaries based on integrative taxonomy are useful in this respect, we argue that DNA sequence data serve as a reliable source of data for testing species boundaries even in the absence of additional phenotypic evidence. The hundreds of new species discovered using DNA sequences in the past 20 years have profoundly reshaped our ideas on algal diversity and present a telling case of our inability to accurately assess species diversity based on phenotypic characters alone. So far, the vast majority of those newly discovered lineages are not the result of recent speciation events that are difficult to detect due to a lack of variation in molecular markers and/or lineage sorting-related issues. Instead these species result from speciation events that pre-date the detection limit of most traditional molecular markers, often by millions of years.

The incessant flood of papers reporting new species based on DNA sequence data demonstrates that there are many more algal species remaining to be discovered and described. However, it would be naive to assume that the current (molecular) criteria to delimit species form an endpoint in the quest to describe the algal species diversity. On the one hand, our current procedures may result in species definitions that are too broad and encompass multiple separately evolving lineages. Conversely, they could lead to over-splitting if subclades within a species are erroneously considered to be separate species (Zachos & Lovari, 2013). These are both real issues, and further refinements in our understanding of algal diversity will require multimarker datasets, and integration of population genetic and phylogenetic methods to disentangle complications caused by processes like incomplete lineage sorting, trans-species polymorphism, hybridization and introgression (Fujita *et al.*, 2012; Camargo & Sites, 2013).

Because speciation is a process and not a single event in time (Hey & Pinho, 2012), uncertainty about species boundaries is inevitable in recently diverged lineages. One of the strengths of DNA-based species delimitation is that this uncertainty can be taken into account and quantified. While the methods applying strict thresholds to delimit species (e.g. reciprocal monophyly, genetic distance, CBCs, or a barcoding gap) do not do this, several of the newly proposed methods incorporate probabilistic tests for species boundaries that provide statistical support plus a level of uncertainty regarding species boundaries (Fujita *et al.*, 2012).

Carstens *et al.* (2013) have argued that although new DNA-based species delimitation methods hold great promise, it is important to be aware that they do make simplified assumptions about the process of speciation. Should these assumptions be violated in natural systems, suboptimal species limits may result. Because no model is ideal, Carstens *et al.* (2013) argue that species delimitation should preferably be based on a wide range of methods and a conservative consensus across methods. One important assumption made in coalescent-based species delimitation approaches is that data are sampled from neutral loci. However, it is well documented that divergent selection on ecological traits can lead to local adaptation and correlated reproductive isolation in a process of ecological speciation (Coyne & Orr, 2004; Camargo & Sites, 2013). In such cases, neutral markers would be uninformative for assessing rapid phenotypic divergence and speciation, necessitating the sampling of loci under selection that are potentially associated with selected traits (Nosil *et al.*, 2009). Another assumption made in current coalescent-based species delimitation methods (BPP and spedeSTEM) is that shared polymorphism is the result of incomplete lineage sorting. However, hybridization and gene flow between species may be prevalent in algae, decreasing the accuracy of species delimitation (Camargo *et al.*, 2012; Leaché *et al.*, 2014).

While it is now generally recognized that multi-locus data are essential for accurate species delimitation (Dupuis *et al.*, 2012), sampling of multiple unlinked genes using Sanger sequencing is challenging, especially because nuclear genes are essential. High-throughput sequencing methods are already facilitating the collection of multi-locus datasets for large numbers of individuals (McCormack *et al.*, 2012). These technologies also offer perspectives for sampling new types of informative markers. For example a restriction-site-associated DNA (RAD-tag) sequencing approach has been developed to genotype genome-wide single-nucleotide polymorphisms (SNPs) (Baird *et al.*, 2008), which are valuable for species delimitation studies in recent radiations and groups with occasional hybridization occurring between species (Shaffer & Thomson, 2007; Emerson *et al.*, 2010; Wagner *et al.*, 2013). Anchored hybrid enrichment or sequence capture is another development useful for producing large sets of nuclear loci across a wide range of samples (Lemmon *et al.*, 2012), which will be highly informative about species boundaries (McCormack *et al.*, 2013).

In conclusion, it is important to keep in mind the moving target that we are trying to circumscribe, a species. The question of species definition and delimitation is tied to the process of speciation, which has idiosyncratic components involving

multiple populations, individuals with different life cycles and reproductive successes, different selective histories, and which contains a large component of stochasticity. Elucidating the relative importance of these components in the speciation process defines the beauty of systematic biology. The use of molecular markers in species delimitation has pointed phycologists toward more realistic species boundaries. We anticipate that multispecies coalescent methods based on multi-locus data will further refine our view on algal species, especially in recently diverged lineages, in which factors such as incomplete lineage sorting, hybridization and introgression may confound species boundaries. These methods will increase the statistical rigour and objectivity of species delimitation, and are likely to result in the recognition of less inclusive entities, which in turn will have implications for estimates of algal species diversity. Resetting species boundaries towards a point where they truly reflect the biological reality will make the study of speciation processes more expedient. But quite probably, the question of 'what is a species?' will remain with us as long as we want to study the process of evolution that produces these apparent discontinuities that we call 'species'.

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Supplementary information

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2014.904524

Supplementary Table S1. Overview of papers published in *European Journal of Phycology* and *Phycologia* in 2012 and 2013, showing a high proportion of papers (68 out of 137) describing new taxa or making explicit statements on species boundaries. The table indicates for each paper whether or not species are delimited; if molecular, morphological and/or other sources of data are used for species delimitation; the applied species concept (if specified); the molecular marker(s) used; and the number of individuals analysed per species.

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