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DNA taxonomy in morphologically plastic taxa: Algorithmic species delimitation in the *Boodlea* complex (Chlorophyta: Cladophorales)

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ABSTRACT

DNA-based taxonomy provides a convenient and reliable tool for species delimitation, especially in organisms in which morphological discrimination is difficult or impossible, such as many algal taxa. A group with a long history of confusing species circumscriptions is the morphologically plastic *Boodlea* complex, comprising the marine green algal genera *Boodlea*, *Cladophoropsis*, *Phyllodictyon* and *Struveopsis*. In this study, we elucidate species boundaries in the *Boodlea* complex by analysing nrDNA internal transcribed spacer sequences from 175 specimens collected from a wide geographical range. Algorithmic methods of sequence-based species delineation were applied, including statistical parsimony network analysis, and a maximum likelihood approach that uses a mixed Yule-coalescent model and detects species boundaries based on differences in branching rates at the level of species and populations. Sequence analyses resulted in the recognition of 13 phylogenetic species, although we failed to detect sharp species boundaries, possibly as a result of incomplete reproductive isolation. We found considerable conflict between traditional and phylogenetic species definitions. Identical morphological forms were distributed in different clades (cryptic diversity), and at the same time most of the phylogenetic species contained a mixture of different morphologies (indicating intraspecific morphological variation). Sampling outside the morphological range of the *Boodlea* complex revealed that the enigmatic, sponge-associated *Cladophoropsis* (*Spongocladia*) *vaucheriiformis*, also falls within the *Boodlea* complex. Given the observed evolutionary complexity and nomenclatural problems associated with establishing a Linnaean taxonomy for this group, we propose to discard provisionally the misleading morphospecies and genus names, and refer to clade numbers within a single genus, *Boodlea*.

1. Introduction

Despite the wide acceptance of the idea that species represent a fundamental unit of biological organization (Mayr, 1982), there has been a great deal of disagreement with regard to the criteria used to delimit species. This disagreement has led to a proliferation of different species concepts, followed by endless discussions on their respective value and applicability (Mayden, 1997). More recently, however, important conceptual progress has been made in thinking about species concepts (de Queiroz, 1998, 2007). A vast majority of evolutionary biologists now accepts that species are lineages. Coincidentally, and albeit being controversial at first, DNA sequences are being increasingly used to identify species (DNA barcoding, Hebert et al., 2003). With recent advances in methods for sequence-based species delimitation, formal analyses of species boundaries have become possible (Templeton, 2001; Sites and Marshall, 2003; Wiens, 2007; Zhang et al., 2008). Several methods for detecting species limits from DNA sequence data are based on diagnostic character variation. These methods, which are rooted in the phylogenetic species concept, aggregate *a priori* populations that lack discrete differences into a single species, which are distinguished from other species by unique nucleotide differences (Cracraft, 1983; Davis and Nixon, 1992; Wiens and Penkrot, 2002; Monaghan et al., 2005). Other procedures aim to detect discontinuities in sequence variation associated with species boundaries, assuming that clusters of closely related sequences that are preceded by long branches are suggestive for genetic isolated entities (Hudson and Coyne, 2002). One of these methods, statistical parsimony (Templeton et al., 1992), separates groups of sequences into different networks if genotypes are connected by long branches that are affected by homoplasy. Recently proposed, maximum likelihood approaches aim to determine species boundaries statistically from sequence data by analysing the dynamics of lineage

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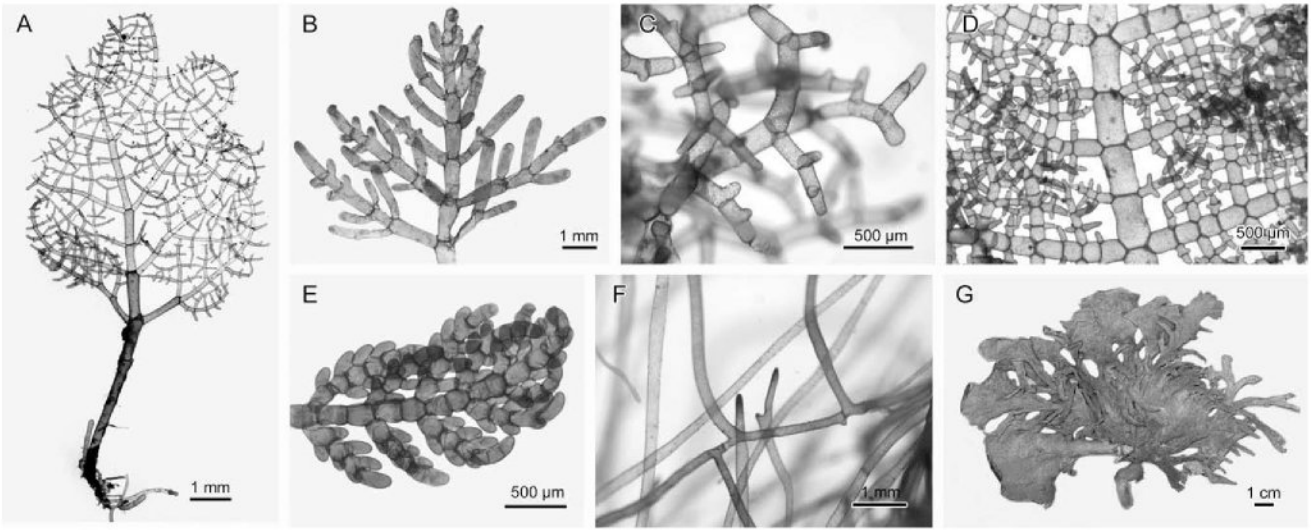

Fig. 1. Morphological diversity in the *Boodlea* complex. (A) *Phyllodictyon anastomosans*: stipitate reticulate blade composed of oppositely branching filaments, interconnected by tenacular cells, in a single plane (FL1109). (B) *Phyllodictyon* sp. from Kenya: detail of oppositely branching filaments characterized by very thick cells (HEC8669). (C) *Boodlea siamensis*: detail of a cushion-like thallus composed of irregularly branching filaments in three dimensions (TZ202). (D) *Boodlea montagnei*: reticulate blade composed of oppositely branching filaments, which are interconnected by tenacular cells (FL1128). (E) *Struveopsis siamensis*: blade with oppositely branching filaments, which are not interconnected (FL662b). (F) *Cladophoropsis membranacea*: unilateral branches without cross-walls (Csmem5). (G) *Cladophoropsis vaucheriiformis*: large, irregularly branching clump, composed of filaments associated with sponge tissue (HEC11394).

branching in phylogenetic trees, trying to determine the point of transition from species-level (speciation) to population-level (coalescent) evolutionary processes (Pons et al., 2006; Fontaneto et al., 2007).

Sequence-based species delimitation is particularly valuable in organisms in which morphological discrimination is difficult or impossible, such as in many algal groups (e.g., Saunders, 2005; Verbruggen et al., 2005, 2007; Harvey and Goff, 2006; Lilly et al., 2007; Vanormelingen et al., 2007). A group with a notorious long history of confusing species circumscriptions is the *Boodlea* complex, comprising the marine siphonocladalean green algal genera *Boodlea*, *Cladophoropsis*, *Phyllodictyon* and *Struveopsis* (Harvey,

Table 1
Survey of diagnostic features in the 13 recognized morphotypes.

Morphological group	Thallus architecture	Branches	Cross-wall at branches	Tenacular cells	Average diameter and length/width (l/w) ratio of apical cells
1. *Boodlea composita*	Cushions composed of tightly interwoven filaments	Opposite, older cells producing additional branches in three dimensions	Present	Rare	80 µm l/w: 3
2. *Boodlea montagnei* (Fig. 1D)	Reticulate blades without stipes	Opposite or single, regular, in a single plane	Present	Abundant	97 µm l/w: 3
3. *Boodlea siamensis* (Fig. 1C)	Cushions composed of tightly interwoven filaments	Opposite or single, older cells producing additional branches in three dimensions	Present	Abundant	92 µm l/w: 4
4. *Boodlea* sp. (Indonesia)	Cushions borne on thick (c. 600 µm), erect, branched filaments	Opposite or single, older cells producing additional branches in three dimensions	Present	Abundant	110 µm l/w: 3
5. *Cladophoropsis macromeres*	Mats composed of loosely entangled filaments	Single, unilaterally organized	Absent	Absent	320 µm l/w: 60
6. *Cladophoropsis membranacea* (Fig. 1F)	Cushions or mats composed of tightly interwoven filaments	Single, unilaterally or irregularly organized	Absent	Generally absent	185 µm l/w: 45
7. *Cladophoropsis philippinensis*	Cushions composed of loosely entangled filaments	Single or opposite, irregularly organized	Only present in older branches	Absent	510 µm l/w: 40
8. *Cladophoropsis sundanensis*	Cushions composed of tightly interwoven filaments	Single or opposite, irregularly organized	Occasionally present	Absent	90 µm l/w: 35
9. *Cladophoropsis vaucheriiformis* (Fig. 1G)	Clumps of variable morphology, associated with sponge tissue	Generally single, irregularly organized or filaments siphonous	Occasionally present	Occasionally present	105 µm l/w: 25
10. *Phyllodictyon anastomosans* (Fig. 1A)	Stipitate reticulate blades	Generally opposite, regular, in a single plane	Present	Abundant	100 µm l/w: 3
11. *Phyllodictyon* sp. (Kenya) (Fig. 1B)	Clustered stipitate blades	Generally opposite, regular to irregular, more or less in a single plane	Present	Rare	310 µm l/w: 5
12. Siphonous sp. (Florida)	Irregular cushion	Siphonous	Absent	Absent	87 µm l/w: –
13. *Struveopsis siamensis* (Fig. 1E)	Stipitate blades. Stipes and basal cells clavate with annular constrictions	Opposite, regular, more or less in a single plane	Present	Absent	160 µm l/w: 3

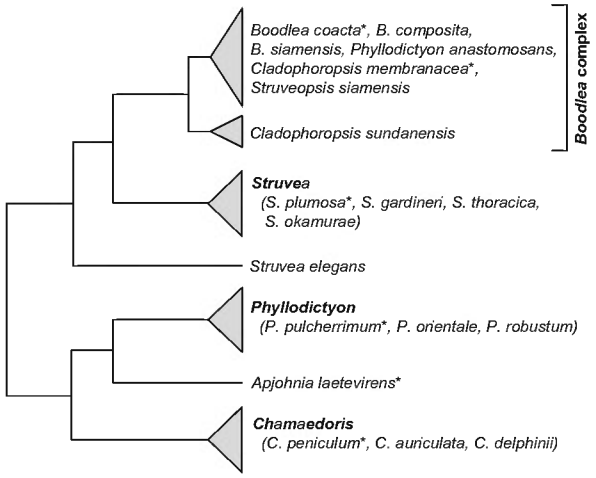

Fig. 2. Phylogenetic hypothesis of *Boodlea* and related genera based on Leliaert et al. (2007a,b, 2008). Taxa indicated by an asterisk represent generitypes.

1859; Egerod, 1975). These seaweeds are widely distributed along rocky coastlines and in coral reefs throughout the tropics and subtropics (Pakker et al., 1994). Previous taxonomic studies have attempted to delimit species based on the morphological species concept, seeking to recognize species by discontinuities in morphological characters such as thallus architecture, branching pattern, type of tenacular cells and cell dimensions (Fig. 1 and Table 1). More than 60 nominal species and infraspecific taxa have been formally described (14 in *Boodlea*, 36 in *Cladophoropsis*, 7 in *Phyllodictyon* and 5 in *Struveopsis*) (Index Nominum Algarum, 2008), but the relationships among these taxa were poorly understood. The number of morphospecies in the *Boodlea* complex was recently reduced to 13 by Leliaert and Coppejans (2006, 2007b). Analysis of morphological variation in this group was found to be problematic because many of the morphological features exhibit intraspecific variability to such an extent that generic boundaries are crossed.

Molecular phylogenetic studies have shown that most representatives of *Boodlea*, *Cladophoropsis*, *Phyllodictyon* and *Struveopsis* are closely related to the morphologically well defined genera *Chamaedoris*, *Struvea* and *Apjohnia* (Kooistra et al., 1993; Leliaert et al., 2003, 2007c). Within this clade, *Phyllodictyon* was shown to be non-monophyletic with *P. anastomosans* being more closely related to *Boodlea* than to the other *Phyllodictyon* species (including the type, *P. pulcherrimum*) (Leliaert et al., 2007a,b, 2008) (Fig. 2). Some other taxa are more distantly related; *Boodlea vanbosseae* Reinbold was found to be allied with *Cladophora catenata* (Linnaeus) Kützing, *Anadyomene* and *Microdictyon* (Leliaert et al., 2007b), while *Cladophoropsis herpestica* (Montagne) M.A. Howe falls within a clade of the *Cladophora* section *Longi-articulatae* (Leliaert et al., 2009).

Species boundaries in the *Boodlea* complex have remained uncertain because of low taxon sampling (Kooistra et al., 1993) or conservativeness of molecular markers (nuclear small and large subunit rDNA, Leliaert et al., 2007c). In a phylogeographic study, van der Strate et al. (2002) demonstrated that *Cladophoropsis membranacea* consists of at least three cryptic species with overlapping geographical distributions in the Atlantic Ocean, based on nrDNA internal transcribed spacer (ITS) sequence divergence, differential microsatellite amplification and thermal ecotypes. Biogeographic and systematic conclusions, however, were somewhat biased because only a single morphospecies was considered, and hence part of the genetic diversity within the species complex was overlooked.

In this study, we aim to elucidate species boundaries within the *Boodlea* complex based on nrITS sequences from 175 individuals sampled worldwide. Given the inherent difficulties of identifying species in this morphologically variable group of algae, we also sampled outside the known morphological bounds of the *Boodlea* complex, for example, including the sponge-associated *Cladophoropsis* (*Spongocladia*) *vaucheriiformis*. ITS sequences have been shown to provide good resolution at and below the species-level in a wide range of eukaryotic organisms, including siphonocladalean green algae (Bakker et al., 1992, 1995; van der Strate et al., 2002). Different methods of sequence-based species delineation were applied, including statistical parsimony network analysis, and a maximum likelihood approach, using the recently developed "general mixed Yule-coalescent" (GMYC) model, which detects species boundaries based on differences in branching rates at the level of species and populations.

2. Materials and methods

2.1. Taxon sampling

We sampled an extensive number of specimens (175) of the nominal species *Boodlea composita*, *B. montagnei*, *B. siamensis*, *Cladophoropsis macromeres*, *C. membranacea*, *C. philippinensis*, *C. sundanensis*, *C. vaucheriiformis*, *P. anastomosans* and *Struveopsis siamensis* from a broad geographical range (Table S1, online Supplementary material). Morphological species identification was based on differences in thallus architecture, presence of stipe cells, branching systems, timing of cross-wall formation, cell shape and dimensions, mode of thallus attachment and reinforcement, presence and morphology of tenacular cells, shape of crystalline cell inclusions, and cell wall thickness (Leliaert and Coppejans, 2006; 2007a,b). A number of plants could not be assigned to a described taxon: a siphonous *Cladophoropsis*-like specimen from Florida (designated as "siphonous sp."), three specimens from Indonesia with *Cladophoropsis philippinensis*-like basal filaments and terminal *Boodlea*-like branches ("*Boodlea* sp."), and a *Phyllodictyon*-like plant from Kenya with very large cells ("*Phyllodictyon* sp."). Collection of specimens and their preservation were carried out as described in Leliaert et al. (2007a). Published sequences from 42 isolates of *Cladophoropsis membranacea* (Kooistra et al., 1992; van der Strate et al., 2002) were also included. Voucher specimens from the latter study were kindly sent by Han van der Strate for morphological examination.

2.2. Gene sampling and phylogenetic analyses

Total genomic DNA was extracted from silica gel-dried specimens, herbarium material or from living plants in culture, and the target region, comprising nrDNA internal transcribed spacer regions (ITS1, ITS2) and the 5.8S rDNA, was amplified and sequenced as described in Wysor (2002) and Leliaert et al. (2007a,b). The Primer sequences are given in Table S2 (online Supplementary material). The 175 ITS sequences were aligned using MUSCLE (Edgar, 2004) via http://www.ebi.ac.uk/Tools/muscle/. The alignment (provided in a Supplementary online FASTA file) was straightforward and included a limited number of gaps. The amount of phylogenetic signal versus noise was assessed by calculating the I_{ss} statistic (a measure of substitution saturation in molecular phylogenetic datasets) with DAMBE v4.5.56 (Xia and Xie, 2001). Because nearly no variation was found within rDNA 5.8S sequences, and because data of this region were unavailable for several isolates (van der Strate et al., 2002), this region was excluded for further analysis.

The dataset (ITS1–ITS2) was analysed with Bayesian inference (BI) and maximum likelihood (ML), using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) and PhyML v2.4.4 (Guindon and Gascuel,

2003), respectively. The alignment was analysed under a general time-reversible model with and gamma distribution split into four categories (GTR + G4), as determined by the Akaike Information Criterion in PAUP/Modeltest 3.6 (Swofford, 2002; Posada and Crandall, 1998). BI consisted of two parallel runs each of four incrementally heated chains, and 3 million generations sampled every 1000 generations. The output was diagnosed for convergence using Tracer v.1.3 (Rambaut and Drummond, 2007a) and summary statistics and trees were generated using the last 2 million generations, well beyond the point at which convergence of parameter estimates had taken place. For the ML trees, the reliability of each internal branch was evaluated based on 1000 bootstrap replicates.

One of the species delimitation algorithms described below requires a chronometric phylogram (chronogram) in which branch lengths are roughly proportional to time. In order to obtain a chronogram, we applied molecular clock analyses to our data. First, the validity of a strict (uniform) molecular clock was tested using a likelihood ratio test by comparing the ML scores obtained with or without constraining a strict molecular clock in PAUP (Posada, 2003). A strict molecular clock was significantly rejected [ln$L$ without enforcing substitution rate constancy = −18379.89, compared to ln$L$ with enforcing substitution rate constancy = −18515.78; $-2\Delta\ln L = 271.78$, χ^2 statistic, d.f. (no. taxa − 2) = 174, $p = 0.0000$]. Due to the violation of the strict molecular clock in our data, a relaxed molecular clock was used to estimate divergence times. More specifically, we applied the uncorrelated lognormal (UCLN) model (Drummond et al., 2006) implemented in BEAST v1.4.6 (Drummond and Rambaut, 2007). Two independent Markov chain Monte Carlo (MCMC) analyses were run for 7 million generations, sampling every 1000. The output was diagnosed for convergence using Tracer v.1.3, and summary statistics and trees were generated using the last 5 million generations with TreeAnnotator (Rambaut and Drummond, 2007b). A logarithmic lineage-through-time plot of the ultrametric tree was generated using GENIE v3.0 (Pybus and Rambaut, 2002).

2.3. Sequence-based species delimitation

We applied two empirical methods for testing species boundaries. First, we aimed to detect discontinuities in sequence variation by using a statistical parsimony analysis, which partitions the data into independent networks of haplotypes connected by changes that are non-homoplastic with a 95% probability (Templeton et al., 1992). Statistical parsimony networks were constructed with TCS 1.21 (Clement et al., 2000), with calculated maximum connection steps at 95% and with alignment gaps treated as missing data. In the second procedure changes in branching rates were tested at the species boundary in our chronogram following Pons et al. (2006). The method exploits the differences in the rate of lineage branching at the level of species and populations, recognizable as a sudden increase of apparent diversification rate when ultrametric node height is plotted against the number of nodes in a lineage-through-time plot. The procedure uses waiting times between successive branching events on an ultrametric tree as raw data. A combined model that separately describes population (a neutral coalescent model) and speciation (a stochastic birth-only or Yule model) processes, i.e., a general mixed Yule-coalescent (GMYC) model, is fitted on the ultrametric tree. The method optimizes a threshold position of switching from interspecific to intraspecific events such that nodes older than the threshold are considered to be diversification events (i.e., reflect cladogenesis generating the isolated species) and nodes younger than the threshold reflect coalescence occurring within each species. The number of shifts and their location on the phylogenetic tree provides the number of species and their relative age. A standard log-likelihood ratio test (comparing the likelihood for the mixed model to that obtained assuming a single branching process for the entire tree) is then used to assess if there is significant evidence for the predicted shift in branching rates. A confidence interval for the number of shifts is defined by ±2 log likelihood units which is expected to be χ^2 distributed with 3° of freedom. Model fitting and phylogenetic tests were performed using a script provided by T.G. Barraclough (Imperial College London), implemented in *R* using functions of the APE library (Paradis et al., 2004).

3. Results

3.1. Morphological groups

Our dataset included 175 individuals distributed worldwide. We recognised 13 morphological entities based on differences in thallus architecture, branching system, cross-wall formation, cell dimensions, and presence or absence of tenacular cells (Table 1). These morphological groups correspond to 10 currently recognized species and three entities that could not be assigned to any previously described taxon. Most individuals could readily be assigned to one of the morphological entities. However, for a number of specimens unequivocal allocation to a single morphotype was problematic because of intermediate morphological features. These are indicated in Table S1 (online Supplementary material).

3.2. Sequence analysis and phylogeny

Visual inspection of the electropherograms of the nrITS sequences showed sequences with predominantly unambiguous peaks, indicating low intra-individual variation. The small number of ambiguities (or underlying peaks) constituted mainly single nucleotide polymorphisms that were not phylogenetically informative. Our observations are in agreement with cloning results in *Cladophoropsis membranacea* by van der Strate et al. (2002), who also found very low intra-individual polymorphism, including only autapomorphic point mutations. The ITS alignment of 175 sequences was 924 sites in total (ITS1: 439 sites, 5.8S: 157 sites and ITS2: 328 sites) and included 393 phylogenetic informative characters (ITS1: 226, 5.8S: 4 and ITS2: 163). For ITS1–ITS2 (excluding 5.8S), ML optimization carried out during the model selection procedure estimated nucleotide frequencies as $A = 0.24$, $C = 0.26$, $G = 0.27$ and $T = 0.23$. The best fit to the data was obtained with six substitution types and rates: $AC = 0.72$, $AG = 2.48$, $AT = 1.28$, $CG = 0.67$, $CT = 2.48$, and $GT = 1.00$, with among-site rate variation (gamma distribution shape parameter = 0.94) and no separate rate class for invariable sites. In total, 92 ribotypes were present. Substitution saturation test (Xia and Xie, 2001) showed that the ITS dataset did not suffer from saturation ($I_{ss} = 0.146 < I_{ss}.c = 0.694$, $p < 0.001$).

ML and BI yielded virtually identical tree topologies with comparable node support. The phylogenetic tree obtained from the ML analysis (ln$L$ = −4895.76), with indication of ML bootstrap values and BI posterior probabilities, is shown in Fig. 3. Four main clades (A–D), separated by long internal branches with high support were recovered. The relaxed molecular clock analysis under a UCLN model yielded a virtual identical tree topology as the ML and BI analyses (Fig. 4).

3.3. Sequence-based species delimitation

Two algorithmic methods of sequence-based species delimitation were applied. In the first method, patterns of sequence variation were investigated for the presence of species-level groups by identifying independent networks using statistical parsimony (Templeton et al., 1992). This network analysis separated the total

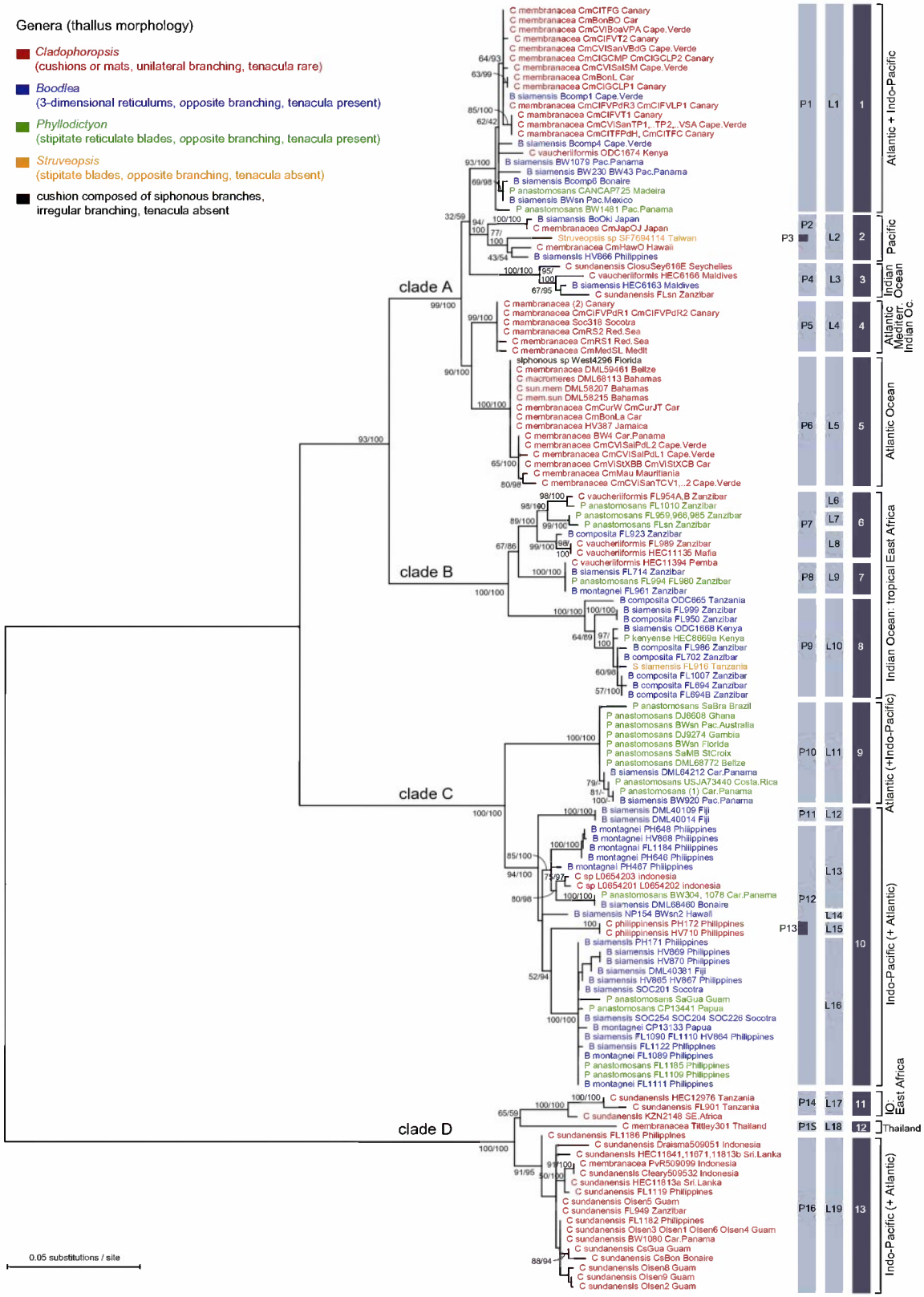

Fig. 3. Maximum likelihood phylogram of the *Boodleo* complex inferred from rDNA internal transcribed spacer sequences. ML bootstrap values and BI posterior probabilities are indicated at the branches. Terminal labels indicate morphospecies and sample region. The first column to the right of the tree indicates membership in statistical parsimony networks. The second column indicates species boundaries identified by the likelihood analysis. The third (dark grey) column shows monophyletic consensus groups of the former two analyses, representing putative phylogenetic species further discussed in this study. The fourth column indicates geographical distribution of the species. (1) includes the *P. anastomosans* samples BW95, BW747, BW847, BW1116, BW1426, BW1386 and BWsn; (2) includes the *C. membranacea* samples: CmClLZPdC3, CmClLZPdC4, CmClLZPdC2, CmClLZPM, CmClLZPdC5 and CmClLZPdC1.

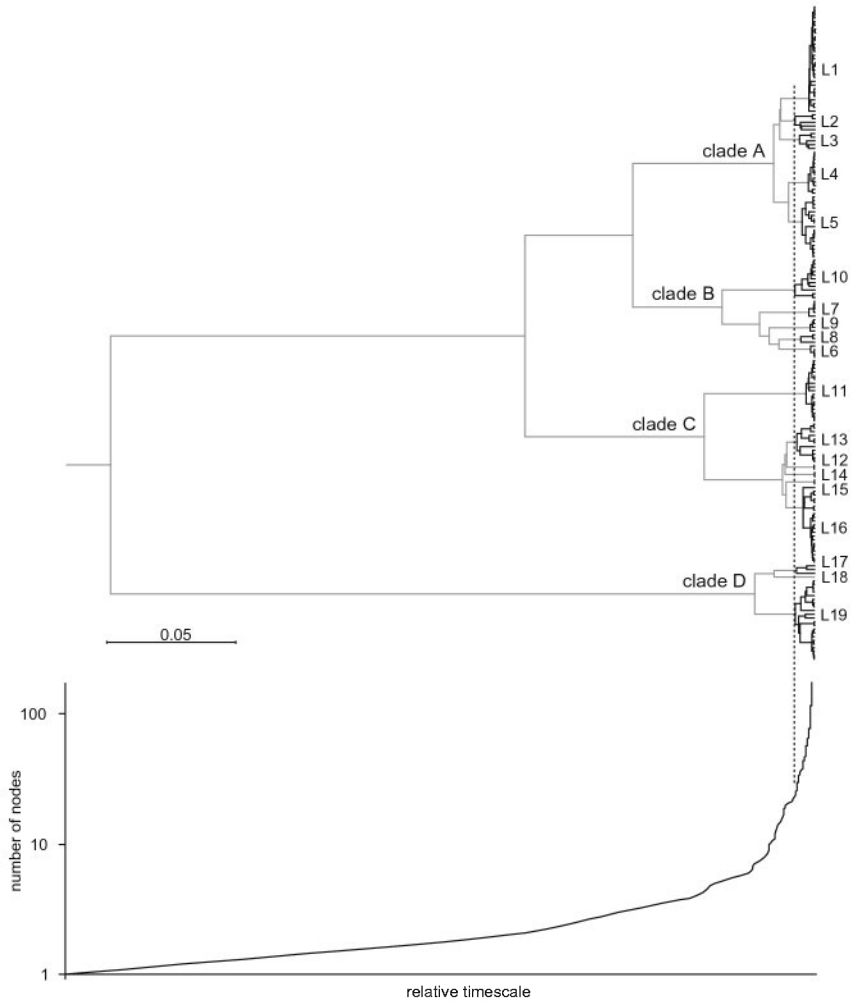

Fig. 4. Ultrametric tree of the *Boodlea* complex based on a Bayesian analysis of nrITS sequence data with divergence times estimated under a relaxed molecular clock using an uncorrelated lognormal (ULLN) model in BEAST. The graph below represents the corresponding lineage-through-time plot. The dotted vertical line indicates the maximum likelihood transition point of the switch in branching rates, as estimated by a general mixed Yule-coalescent (GMYC) model.

variation of ITS sequences into 16 groups based on a maximum connection limit of 12 steps (branches of 13 steps or more fell outside of the 95% confidence interval for non-homoplastic connections) (Fig. 3). Two groups were found to be nested within larger clades (p3 within p2, and p13 within p12). In the second method, we analysed branch length dynamics to detect putative species. A logarithmic lineage-through-time plot showed a gradual-to-exponential increase in branching rate towards the present (Fig. 4). Fitting of the position of the speciation-to-coalescence transition using the GMYC model resulted in the identification of 19 putative species, which by and large corresponded with the groups identified by the network analysis (Fig. 3). Only in two cases was a single network (p7 and p12) split into different clusters. However, confidence limits (defined by ±2 log likelihood units) were extremely broad, nearly spanning the entire tree and resulting in a range of estimated number of species clusters from 1 to 30. This high uncertainty was reflected in the test for significant clustering by comparing two models describing the likelihood of the branching pattern of the chronogram: (1) a null model that the entire sample derives from a single population following a neutral coalescence, and (2) a mixed (GMYC) model assuming a number of independently evolving species or populations joined by branching reflecting cladogenesis. The GMYC model was not favoured over the null model: $\log L = 1610.727$, compared to null model: $\log L = 1610.095$; $2\Delta\log L = 1.263$, χ^2 test, d.f. = 3, $p = 0.738$. These results indicated that there is no significant evidence for the predicted shift in branching rates from interspecific to intraspecific events.

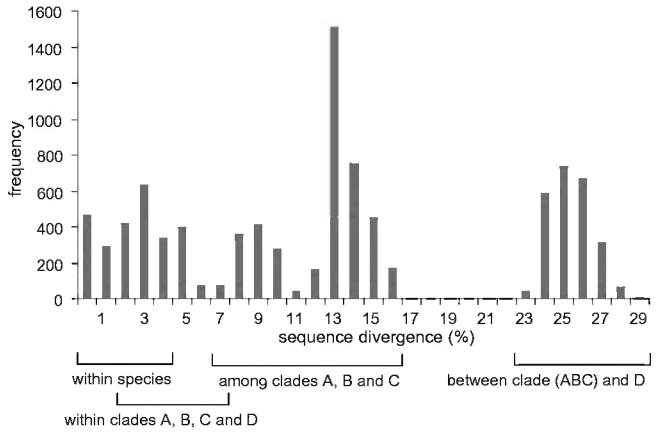

Fig. 5. Frequency distribution of pairwise genetic divergence (calculated as uncorrected *p*-distances) in the *Boodlea* complex.

We took a conservative approach towards reconciling the results of the two algorithmic species delimitation methods. More specifically, we recognized only clades that received high clade

Table 2
Distribution of the different morphotypes in the 13 phylogenetic species, defined in this study.

	Phylogenetic species												
Morphotype	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Boodlea composita*						•		•					
2. *Boodlea montagnei*							•			•			
3. *Boodlea siamensis*	•	•	•				•	•	•	•			
4. *Boodlea* sp. (Indonesia)										•			
5. *Cladophoropsis macromeres*					•								
6. *Cladophoropsis membranacea*	•	•		•	•							•	•
7. *Cladophoropsis philippinensis*										•			
8. *Cladophoropsis sundanensis*			•								•		•
9. *Cladophoropsis vaucheriiformis*	•		•			•	•						
10. *Phyllodictyon anastomosans*	•					•	•		•	•			
11. *Phyllodictyon* sp. (Kenya)								•					
12. Siphonous sp. (Florida)					•								
13. *Struveopsis siamensis*		•											

support and were compatible with both the statistical parsimony and the maximum likelihood solution as species (Fig. 3). By compatible, we mean that the recognized species comprises one or more of the species inferred by the algorithm. This resulted in 13 clusters, which we recognized as putative phylogenetic species. As a general rule, node support between species clades was markedly larger than within the species. Sequence divergence (calculated as uncorrected *p*-distances) within these putative species ranged from complete identity to 4%. Distances among species, within the main clades (A–D) ranged from 2% to 7% and among clades from 7% to 29% (Fig. 5, Table S3). A clear gap in sequence divergence was only noticeable between clade D and clade (A–C).

3.4. Morphology and biogeography

A striking observation was that isolates do not group based on their morphology. Most of the phylogenetic species include different morphotypes from a single or multiple genera, and at the same time, unique morphotypes are distributed in different clades of the phylogenetic tree (Fig. 3, Table 2). For example, representatives of *Boodlea* and *Phyllodictyon* are distributed in different species of clades A–C. Isolates with a *Cladophoropsis sundanensis* and C. *membranacea* morphology were recovered in the spp. 11 and 13 of clade D, as well as in sp. 3 within clade A. The sponge-associated C. *vaucheriiformis* turns up in four species in clades A and B. A number of morphotypes, such as C. *macromeres*, C. *philippinensis*, *S. siamensis* and *Phyllodictyon* sp. (Kenya) are embedded in species along with other morphological forms. Despite the mixture of morphologies, it should be noted that some apparent morphological trends are detectable in our phylogeny. For example, clade A mainly includes samples with a C. *membranacea* morphology, while this morphospecies seems to be absent in clades B and C. The latter mainly includes *Boodlea* and *Phyllodictyon* morphologies. Clade D only includes *Cladophoropsis*-type morphologies.

A loose geographic pattern could be detected in our phylogeny (Figs. 3 and 6). Most species are largely or entirely restricted to either the Atlantic (spp. 1, 5 and 9) or Indo-Pacific basin (spp. 2, 3, 10 and 13). Clade C is separated into a largely Atlantic and Indo-Pacific subclade (corresponding to spp. 9 and 10). Remarkably, the entire clade B (spp. 6, 7 and 8) is restricted to the tropical East African coast. However, several of the clades (spp. 1, 4, 10, and 13) also harboured isolates from distant localities resulting in a much wider tropical distribution. Several examples of identical ITS ribotypes from distant regions in different oceans were found; for instance Madeira and Pacific Mexico (sp. 1, ca. 8900 km); Canary Islands, Red Sea and Socotra (sp. 4, ca. 8500 km); Guam and Tanzania (sp. 13, ca. 11,800 km), and Philippines, Guam and Caribbean Sea (sp. 13, ca. 17,000 km via the Pacific Ocean). Many species were found to occur sympatrically in various areas. Regions with high diversity include the NE Atlantic (including the African coast, the Canary and Cape Verde Islands, harbouring spp. 1, 4, 5 and 9), the Caribbean Sea (including spp. 1, 5, 9, 10 and 13) and the tropical East African coast (Kenya and Tanzania, with spp. 1, 3, 6, 7, 8, 11 and 13).

4. Discussion

4.1. Sequence-based species delimitation

Several quantitative methods for delimiting species based on DNA sequence data have been recently proposed. A number of commonly used procedures, including population aggregation analysis (Davis and Nixon, 1992), cladistic haplotype aggregation (Brower, 1999) and the Wiens-Penkrot methods (Wiens and Penkrot, 2002) aggregate populations lacking discrete differences into a single species. In these procedures, species are recognized based on fixed nucleotide differences unique to sets of populations, which are defined a priori based on morphological, geographical or ecological information. In our wide dataset, circumscription of initial groups based on morphological features is problematic because cryptic diversity and morphological variability are known to be present in the species complex (Leliaert and Coppejans, 2007b). Likewise, defining populations based on geographical or ecological data is difficult because cryptic species of *Cladophoropsis membranacea* have been shown to occur sympatrically in the same environments (van der Strate et al., 2002). We therefore opted to apply species delimitation methods that rely solely on sequence variation and do not require prior assumptions of population boundaries.

A dataset of nrITS sequences from 175 isolates was subjected to different procedures that aim to detect discontinuities in sequence variation associated with species boundaries. The first method for estimating these shifts included Templeton's statistical parsimony analysis, which partitions the variation into homoplastic (i.e., long branches) and non-homoplastic (short branches) variation (Templeton et al., 1992; Templeton, 2001). This method has been shown to separate groups of sequences that correspond to species in a number of studies (e.g., Cardoso and Vogler, 2005; Ahrens et al., 2007). The second method, recently developed by Pons et al. (2006), exploits the dynamics of lineage branching in a phylogenetic tree, aiming to detect a change in the rate of branching associated with the species boundary in a likelihood framework. Although, this approach did not show a sharp shift between species diversification (phylogeny) and coalescent processes (genealogy within species), the species boundaries suggested by this method

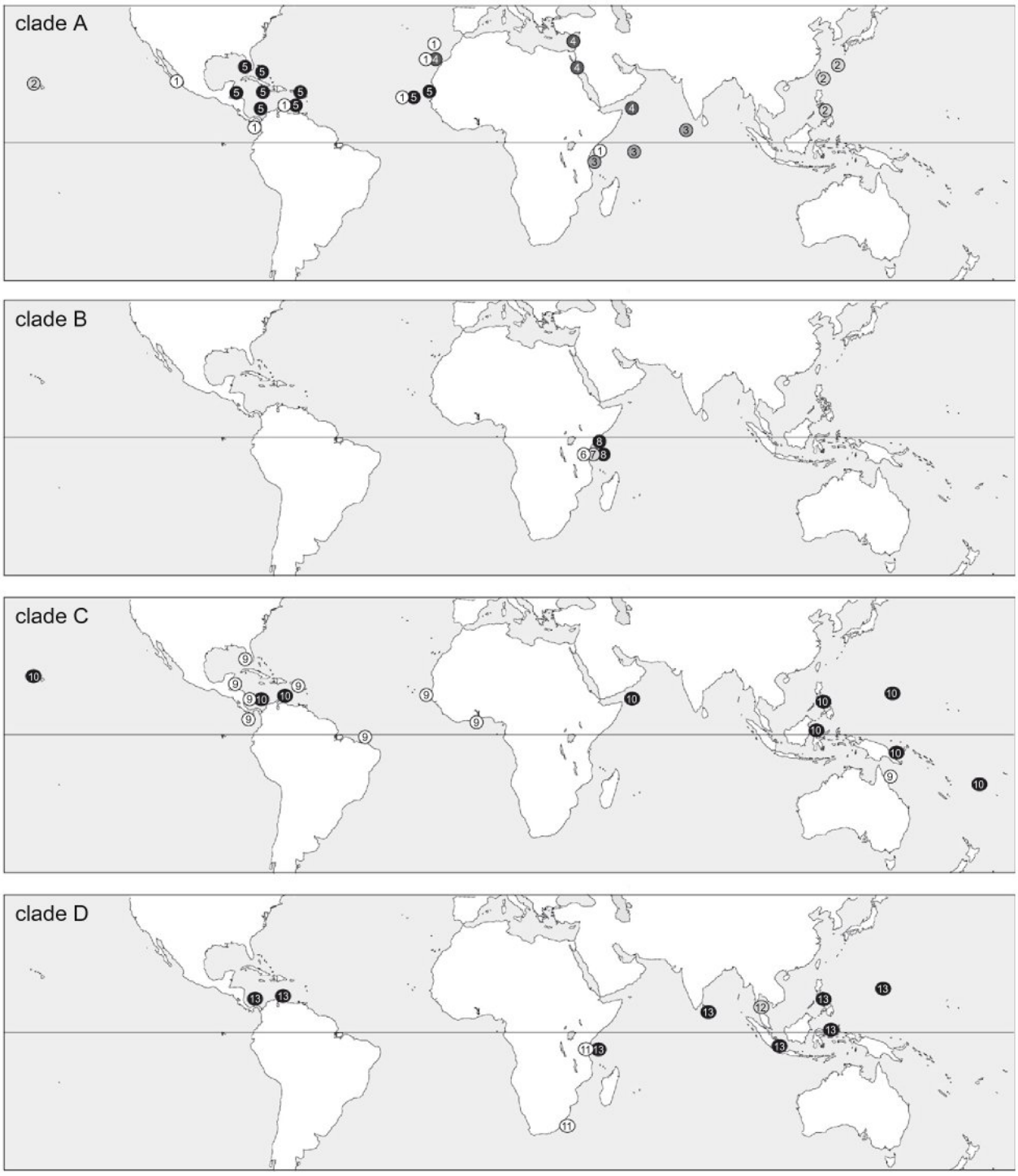

Fig. 6. Distribution maps showing sample sites of the 13 recognized phylogenetic species in the *Boodlea* complex. Each point may represent multiple samples; see Table S1 (online Supplementary material) for a complete listing.

were largely congruent with those of the statistical parsimony method (11 identical clusters). Where they differed, we identified clades that comprised one or more algorithmically defined species, that were separated using both methods and that received high support in the phylogeny. This resulted in the recognition of 13 species.

Failure to reject the null model (i.e., that of a single branching process for the entire tree) could have several interrelated explanations. First, the entire clade might represent a single, highly polymorphic species. A continuous, pantropical breeding population could be plausible given the high dispersal potential of *Boodlea* by thallus fragments that act as propagules (van den Hoek, 1987). However, this scenario is suspect, given that at least clades B and D have also been clearly segregated based on SSU and LSU nrDNA sequence data (Leliaert et al., 2007a). Moreover, ITS sequence divergence within the entire clade exceeds 30%, which is far outside the range of empirical levels of intraspecific divergence normally found in eukaryotes. Several studies have suggested low ITS variation (generally <8%) to be indicative of biological species (Coleman, 2005, 2009; Casteleyn et al., 2008). Similar values have

been implied in ulvophycean green algal species (Lindstrom and Hanic, 2005), including Cladophorales (Bakker et al., 1995). Within the morphospecies *Cladophoropsis membranacea*, species boundaries have been defined based on differential microsatellite amplification in combination with ITS sequence distances, which correlate with ecotypic differentiation in thermal tolerance (van der Strate et al., 2002). Intra-specific sequence divergence was found to be very low (<0.4%) whereas divergence between species was much larger (2–3%). Although, these levels of sequence divergence are similar to the ones found in this study, the discontinuity between intra- and interspecific divergence fades by increased taxon sampling. A second explanation for the lack of clear species limits is that the *Boodlea* complex comprises multiple incipient species, which form a continuum rather than discrete entities, because of incomplete reproductive isolation, or that formerly isolated species recently reverted to a continuous breeding population by hybridization (e.g., van Oppen et al., 2000). Incomplete reproductive isolation and hybridization events could account for the highly polymorphic ITS sequences in the *Boodlea* complex. This could be explained by the complex phylogeographic pattern in the *Boodlea* complex, showing a high degree of diversity in combination with genetic homogeneity (possibly as a result of occasional long distance dispersal events) over large spatial scales, resulting in a high degree of species co-occurrence. This hypothesis, however, remains to be tested.

Inability to detect a sharp species boundary based on ITS sequences might also be a direct consequence of intrinsic properties of the 18S-5.8S-26S nuclear ribosomal cistron, which is present in multiple copies in the nuclear genome. Various phenomena may generate intra-genomic variation of ITS sequences, creating problems for phylogenetic analysis and assumptions of orthology (reviewed in Alvarez and Wendel (2003); Nieto Feliner and Rosselló (2007)). van der Strate et al. (2002) and this study showed that intra-individual sequence variation was very low, and therefore did not affect phylogenetic reconstruction. The continuous variation of ITS sequences can also be a result of degradation of the ITS data at the species-level by recombination events between the multiple variants of interbreeding populations. The failure to reject the single branching process model for the entire tree may also be a result of violation of one of the assumptions in the GMYC model, namely, that of neutral coalescence of the nrITS sequences within species. Retained ancestral ITS polymorphism due to incomplete coalescence blur the transition between species-level and population-level branching processes. The possible lack of coalescence of the ITS sequences could be explained by the fact that speciation has been more rapid than concerted evolution of the multiple rDNA repeats. Species-level non-coalescence of ITS sequences has been demonstrated in various plants (e.g., in the Pine family, Wei et al., 2003; Campbell et al., 2005).

More loci, preferably from different genetic compartments will be needed to clarify species boundaries in the *Boodlea* complex. Several studies (e.g., Lane et al., 2007; Roy et al., 2009) have shown cases in which ITS failed to resolve clear species boundaries whereas non-recombinant DNA (e.g., plastid or mitochondrial markers) did give indications for genetic isolation. Analysis of multiple loci in siphonocladalean algae is problematic due to amplification problems of organellar DNA (as yet it has been impossible to amplify chloroplast or mitochondrial DNA in its members using standard primer combinations) and single-copy nuclear loci (as a consequence of the limited availability of genomic data for green algae and the omnipresence of large introns in their genes).

Various studies have emphasized the value of applying ITS2 RNA transcript secondary structure information for delimiting species. Coleman (2000) suggested that if two organisms differ with respect to a two-sided compensatory base pair change (CBC), they belong to different biological species. Although, a common core of secondary structure of the ITS2 has been found throughout the eukaryotes (Schultz et al., 2005), representatives of *Boodlea* seem to form an exception to this rule. Distinct hallmarks of the conserved eukaryotic ITS2 structure are a central loop with four helices (of which helix III is the longest), the presence of a U-U mismatch in helix II and a UGGU motif in the 5′ side near the apex of helix III. All these features are missing in the various foldings of the *Boodlea* sequences. Moreover, we were not able to find an unequivocally conserved structure for all sequences in the *Boodlea* complex, complicating analyses of CBC's.

4.2. Conflict between morphological and DNA-based species definitions

Species within the genera *Boodlea*, *Phyllodictyon*, *Struveopsis* and *Cladophoropsis* have had a long history of confusing circumscriptions (Murray, 1889; Harvey, 1859; Egerod, 1975; Kooistra et al., 1993). Previous taxonomic studies have attempted to delimit species according to the morphological species concept, in which species are recognized by discrete morphological characters. However, evidence from field studies indicated that traditionally employed distinguishing characters such as growth form, branching patterns and cell dimensions show high levels of intraspecific variability, sometimes crossing generic boundaries (Leliaert and Coppejans, 2007b). *Boodlea* for example is traditionally distinguished from *Phyllodictyon* in the formation of three-dimensional cushion-like thalli lacking a stipe, while *Phyllodictyon* is characterized by net-like, stipitate blades. Some mature thalli of *P. anastomosans* however, have been found to form a three-dimensional reticulum without stipes. Some *Boodlea* species (e.g., *B. montagnei* and *B. struveoides*) on the other hand, do form monostromatic blades, and *B. struveoides* has been described as stipitate blades. *Struveopsis siamensis* differs from *P. anastomosans* and *B. composita* only by the lack of tenacular cells. As many as 60 taxa have been described, but this number was drastically reduced by Leliaert and Coppejans (2006, 2007b).

Our phylogenetic analyses show considerable conflict between species boundaries based on morphology or DNA sequences in the *Boodlea* complex. Under the assumption that the DNA-based species delimitation is correct, high levels of intraspecific morphological variability as well as high prevalence of cryptic diversity have led to an inappropriate morphological taxonomy. Most morphotypes appear in different molecular phylogenetic species. Hence, the traditional, morphology-based species circumscriptions in this group are untenable. None of the nominal taxa are valid species according the molecular phylogenetic species delimitation (de Queiroz and Donoghue, 1988). Cryptic diversity in *Cladophoropsis membranacea* has previously been detected based on ITS sequence data (van der Strate et al., 2002). However, because only a single morphological entity was considered, this study revealed only partial phylogenetic relationships. This phylogeny shows that *C. membranacea* does not form a monophyletic group but that it intermingles with dissimilar morphotypes, confirming the earlier study of Kooistra et al. (1993). Cryptic diversity has been reported in a wide range of eukaryotes, particularly in morphologically simple organisms, such as unicellular or filamentous green algae (Kooistra, 2002; Šlapeta et al., 2006). Verbruggen et al. (2009) discussed a potential link between thallus complexity and the prevalence and profundity of cryptic diversity. Although, our results partially supports this link (i.e., *Boodlea* thalli are morphologically simple), the *Boodlea* phylogeny reveals a more complex mixture of cryptic diversity and morphological variability.

Various factors, including phenotypic plasticity, developmental variability and polymorphism, may account for the observed intraspecific morphological variability. Phenotypic plasticity in the *Boodlea* complex has been demonstrated in culture studies, where

changes in thallus architecture and branching pattern have be induced under different culture conditions. For example, the regularly opposite branching pattern of *Phyllodictyon anastomosans* has been found to change in a unilateral, *Cladophoropsis*-like branching pattern under low light and temperature conditions (Leliaert and Coppejans, 2006). The observation of intermediate morphologies between small *Phyllodictyon*-like plants and large *Boodlea*-like cushions during field studies are suggestive of developmental variability (Leliaert and Coppejans, 2007b). Polymorphism, possibly resulting from incomplete reproductive isolation and hybridization (as discussed above), offers another possible explanation for the observed infraspecific phenotypic variation.

4.3. Association with sponges, over and over again

Cladophoropsis vaucheriiformis, a species exclusively occurring in close association with sponges, is here revealed as an unusual member of the *Boodlea* complex. The species lives in association with halichondrine poriferans, resulting in tough, spongiose thalli ranging from prostrate mats to upright forms with finger-like processes (Leliaert and Coppejans, 2006; Kraft, 2007). The filaments and branches of this species often lack cross-walls, resulting in an apparently siphonous architecture (characteristic for bryopsidalean green algae). Because of this deviant morphology and anatomy, the species' systematic position has long been ambivalent. After being described as *Spongocladia* by Areschoug (1854), it was merged with *Spongodendron* by Murray and Boodle (1888) and finally transferred to *Cladophoropsis* by Papenfuss (1958). The data presented here show that various Indian Ocean isolates of *C. vaucheriiformis* belong to different clades along with specimens possessing typical *Boodlea*, *Cladophoropsis* or *Phyllodictyon* morphologies. This indicates that *C. vaucheriiformis* can be regarded as a growth form and that this algal–sponge association is not obligatory as previously thought (Papenfuss, 1950; Leliaert and Coppejans, 2006).

4.4. Taxonomic consequences

Our results call for radical taxonomic changes in this group of algae, including revised species definitions and a drastically different generic classification. As has been shown previously, *Phyllodictyon anastomosans* (including the taxonomic synonyms *Struvea multipartita*, *S. delicatula* and *S. tenuis*) is unrelated to the type of the genus (*P. pulcherrimum*) (Fig. 2) and should therefore be excluded from the genus (Leliaert et al., 2007a, 2008). *Phyllodictyon anastomosans* is clearly polyphyletic within the *Boodlea* complex and the taxon name is therefore untenable. Likewise, taxa of *Cladophoropsis* (type: *C. membranacea*) and *Struveopsis* (type: *S. chagoensis* C. Rhyne et H. Robinson) are distributed in different clades within the *Boodlea* complex. The obvious nomenclatural solution would be to transfer all species names to the oldest generic name, *Boodlea* (Murray, 1889).

The complexity of genetic variation (the failure to detect a sharp species boundary) and morphological diversity (prevalence of infraspecific variation and cryptic diversity) in *Boodlea* exemplifies the difficulty of establishing a Linnaean taxonomy for this group. Similar problems have been discussed in other taxonomically complex groups of organisms, such as rapidly radiating lineages (Ennos et al., 2005; Monaghan et al., 2006; Cardoso et al., 2009). Although, this DNA-based species delimitation approach provides a reliable estimate of species diversity in the *Boodlea* complex, we feel that assigning Linnaean names to the sequence-based groups at this stage would be inappropriate because of their ambiguous species status. Additional markers, when they become available, could provide stronger support for species boundaries. In addition, due to incongruence of the traditional morphologically defined taxa with the DNA-based species, nomenclatural changes are problematic because of uncertain correspondence of the observed sequence variation with existing Linnaean binomials. In other words, it is presently impossible to provide taxon names for the 13 delimited phylogenetic species, because these species cannot be readily linked with nomenclatural types. Moreover, the phylogenetic position of a number of taxa (e.g., *Cladophoropsis magna* Womersley) remain uncertain (Leliaert and Coppejans, 2006). Future studies should aim to acquire DNA sequences from original type or topotype material.

As a practical solutions while these problems are being sorted out, we propose to temporarily discard the misleading morphospecies names, and refer to clade or species numbers within the *Boodlea* complex (*Boodlea* sp. 1, 2, etc.). Similar solutions have been proposed in other algal species complexes (e.g., Coffroth and Santos, 2005; Lilly et al., 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.06.004.

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