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Genetic data support reproductively isolated species in the endemic Cladophoraceae (Chlorophyta) of Lake Baikal, Russia

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ABSTRACT

Ancient lakes are centres of biological diversification that hold many examples of adaptive radiation and species flocks. The recently discovered species flock of Cladophoraceae in Lake Baikal is a group of green algae that exhibit low genetic divergence in ribosomal markers (LSU, SSU, and ITS), but wide morphological differentiation. Microsatellite markers showed evidence of polyploidy in this group, requiring alternate data scoring methodologies. In this study, we use two clustering methods (STRUCTURE and Gaussian Clustering) to delineate species within 15 distinct morphotaxa of the cladophoralean Baikal clade. The two cluster analyses produced comparable results, although subtle differences in the assignment of individuals were observed. Our results indicate that many morphologically distinguishable species are discrete genetic clusters supporting reproductive isolation. This is the case for *Chaetomorpha* (= *Ch.*) *baicalensis*, the attached form of *Ch. curta, Ch. moniliformis, Cladophora* (= *Cl.*) *compacta*, and *Cl. kursanovii*. The unattached form of *Ch. curta* and a species of *Rhizoclonium* are recovered as growth forms of *Ch. moniliformis* and the attached form of *Ch. curta*, respectively. The remaining morphotaxa were not clearly delimited. While we have evidence for polyploidy within this species flock, it was not possible to determine the ploidy level of each individual with accuracy as no correlation in the number of alleles was observed between loci. A more detailed study including other sources of data, such as nuclear DNA content or chromosome counts, is required to demonstrate the ploidy changes and their role in speciation in these species.

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INTRODUCTION

Ancient lakes, lakes older than 1 million years (mya), are well known centres of biological diversification that contain some of the most remarkable examples of endemism, speciation, species flocks, and adaptive radiations (Cristescu *et al.* 2010; Wilke *et al.* 2008). Lake Baikal, located in south-eastern Siberia, Russia, is the oldest lake in the world (about 30 mya; Mats 1993). Like other ancient lakes, it is considered an evolutionary laboratory due to its unique biological diversity. The fauna contains approximately 2600 species, more than half are endemic, and many taxonomic groups are considered the products of adaptive radiations (Barluenga *et al.* 2006; Fitzpatrick *et al.* 2009; Herder *et al.* 2008; Keller *et al.* 2013; Salzburger & Meyer 2004; Seehausen 2006; Sherbakov 1999; Wilke *et al.* 2008). However, the filamentous green algae of the lake have not been extensively studied (for examples, see Boedeker *et al.* 2018; Volkova *et al.* 2018). The species flock of Baikalian Cladophoraceae (Ulvophyceae, Chlorophyta) is a group of freshwater green algae that includes taxa endemic to Lake Baikal. The taxonomy of the species in this group was based mainly on morphology and recognised 16 distinct taxa (4 genera, 14 species, and 2 varieties; Boedeker *et al.* 2018; Izhboldina 2007) with a morphological range as broad as the entire family Cladophoraceae, which includes filamentous genera such as the branched *Cladophora* Kützing and the unbranched *Chaetomorpha* Kützing and *Rhizoclonium* Kützing (Boedeker *et al.* 2016). Species within Cladophoraceae usually have a haplodiplontic and isomorphic life cycle (Škaloud *et al.* 2018). The actual reproduction mode of Baikalian species is unknown, but at least some asexual reproduction is likely, as it has been observed in the common freshwater *Cladophora glomerata* (Linnaeus) Kützing (van den Hoek *et al.* 1995; Zulkifly *et al.* 2013).

Recent studies using nuclear ribosomal markers (SSU, LSU, and ITS) have revealed that the mostly endemic Baikalian Cladophoraceae are a monophyletic group ('Baikal clade') with low levels of genetic differentiation, nested within the genetically diverse genus *Rhizoclonium* (Boedeker *et al.* 2018). This was a striking result, considering the group's morphological diversity. Phylogenetic analyses of ribosomal markers resolved the Baikal clade into two major groups (A and B; Fig. S1; Boedeker *et al.* 2018) but were not able to support most of the morphotaxa.

The low genetic differentiation based on rDNA markers, the wide morphological differentiation between the taxa, and the co-occurrence of many taxa in the same localities across the lake suggest that this group could be a species flock that has undergone sympatric speciation (Boedeker *et al.* 2018). In addition, the recent development and use of simple sequence repeat (SSR) markers indicates that polyploidy is likely in these taxa (Díaz-

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Martínez *et al.* 2020). Therefore, the clade of Baikalian Cladophoraceae is an interesting group for studying speciation in green algae. However, a better understanding of the species limits is required to determine the underlying evolutionary processes (i.e. possible sympatric speciation).

Species delimitation is crucial for many aspects of biology. Although there is still debate about the definition of species and how they should be delimited, there is general agreement that species are independently evolving metapopulation lineages (De Queiroz 2007; Wiens 2007). This allows for a set of operational criteria to support and delimit species, such as reproductive isolation, phylogenetic patterns, and genetic clustering (De Queiroz 2007). The use of these criteria, combined with new methods, has improved our understanding in recognising species and how to delimit them (Carstens *et al.* 2013; Hausdorf & Hennig 2010; Leliaert *et al.* 2014; Zhang *et al.* 2013).

Many current phylogenetic species delimitation methods are based on DNA sequence data that are used in barcode gap determination (Puillandre *et al.* 2012), coalescent-based groupings (Fujisawa & Barraclough 2013), or tree-based methods (Zhang *et al.* 2013). These approaches provide a good overview of species diversification and are widely used in taxonomic assessments and diversity exploration (e.g. Díaz-Martínez *et al.* 2016; Muangmai *et al.* 2014). Although these methods are useful, they are not free of caveats. Problems can arise when the patterns recovered do not reflect the speciation process, for example due to hybridisation or incomplete lineage sorting, or when the phylogenetic signal of the selected genes is too low due to low mutation rates or recent divergence (Carstens *et al.* 2013; Edwards & Knowles 2014).

Delimitation methods based on population genetics, on the other hand, can provide more accurate species delimitation in cases where DNA sequence methods fail (Carstens *et al.* 2013; Hausdorf & Hennig 2010; Shaffer & Thomson 2007). Compared to DNA sequence-based methods based on coalescence (Fujisawa & Barraclough 2013) or genetic distances (Puillandre *et al.* 2012), this approach requires more extensive sampling and more markers to capture the genetic diversity of the populations, but can reveal clusters of genetically similar genotypes that have evolved independently with little or no ongoing gene flow (Hausdorf & Hennig 2010). The resulting clusters could be interpreted as putative species following the Genotypic Cluster Criterion (Mallet 1995), that considers a species as a monotypic or polytypic cluster of biological entities identified by morphology or genetics, and the Intrinsic Reproductive Isolation criterion (Mayr 2000) where reproductively isolated populations accumulate genetic differences over time. However, this method is limited by stage of the speciation process, and populations in early stages of speciation may be difficult to delimitate (Hausdorf & Hennig 2010). On the other hand, it has been demonstrated that intraspecific genetic variation could lead to an overestimation of species if the genetic clusters are not interpreted carefully (Sukumaran & Knowles 2017).

The most commonly used methods in population genetics include clustering algorithms using model-free multivariate analysis such as Gaussian clustering or discriminant analysis of principal components (Hausdorf & Hennig 2010; Jombart *et al.* 2010), or explicit models like STRUCTURE or Structurama (Huelsenbeck *et al.* 2011; Pritchard *et al.* 2000) using different types of genetic markers, such as single nucleotide polymorphisms (SNPs) and SSRs. SSRs are repetitive short DNA sequences (*c.* 2–6 base pairs) scattered in the genomes of many organisms. Other markers such as single nucleotide polymorphism have gained popularity due to recent advances in sequencing technology (Defaveri *et al.* 2013; Nielsen *et al.* 2011), easy genotyping, and low homoplasy levels (Shaffer & Thomson 2007). Regardless, some advantages of using SSRs over other markers are their codominance, neutrality, and high levels of polymorphism (Selkoe & Toonen 2006). In addition, advances in next-generation sequencing make obtaining a large number of potential SSRs rapid and cost effective (Schoebel *et al.* 2013). Being based on polymerase chain reaction techniques, SSRs are especially useful when biological material is poorly preserved or in small quantities, conditions occurring in the Baikal clade.

In this study, we explored the species boundaries of the species flock of Cladophoraceae of Lake Baikal by genetic clustering analyses for species delimitation using recently developed putatively neutral SSR markers (Díaz-Martínez *et al.* 2020). We also determined whether the genetic clusters are consistent with the currently recognised morphotaxa *sensu* Izhboldina (2007) and the results obtained by Boedeker *et al.* (2018).

MATERIAL AND METHODS

Taxon sampling

A total of 727 samples of 15 taxa were analysed (Table S1). The initial identification was done using morphological characters (Boedeker *et al.* 2018; Izhboldina 2007; see Table S2). In this work we considered each morphotype independently as a preliminary morphotaxon, making a total of 15 distinct units (Table 1).The samples included: two distinct morphotypes of *Ch. curta* (attached and unattached form) and two dubious identifications of *Cl. globulus/Cl. compacta* and *Cl. globulus/Cl. pulvinata*. Samples of *Chaetocladiella pumila* (K.I.Meyer) K.I.Meyer

Table 1. Endemic morphotaxa of Cladophoraceae from Lake Baikal analysed in this study. The groups correspond to the two major clades (A and B) found in Boedeker *et al.* (2018; Fig. S1) using rDNA sequences.

Taxa	Group
Chaetomorpha baicalensis K.I.Meyer	B
Chaetomorpha curta (Skabitschevsky) Skabitschevsky, attached form	A
Chaetomorpha curta, unattached form	A
Chaetomorpha moniliformis Skabitschevsky	A
Cladophora compacta (K.I.Meyer) K.I.Meyer	B
Cladophora floccosa K.I.Meyer var. *floccosa*	B
Cladophora floccosa var. *irregularis* Skabitschevsky	B
Cladophora globulus (C.Meyer) C.Meyer/*Cladophora compacta*	B
Cladophora globulus/Cl. pulvinate (K.I.Meyer) K.I.Meyer	B
Cladophora kursanovii Skabitschevsky	A
Cladophora meyeri Skabitschevsky var. *meyeri*	B
Cladophora meyeri Skabitschevsky var. *gracilior* (Meyer) Hollerbach	B
Cladophora pulvinata (K.I.Meyer) K.I.Meyer	B
Gemmiphora compacta Skabitschevsky	B
Rhizoclonium sp. Kützing	A

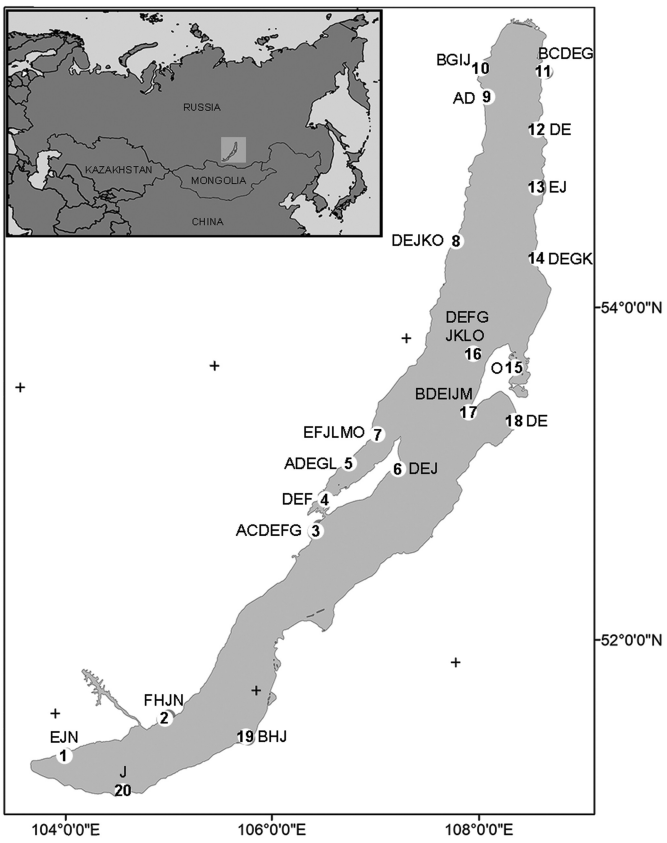


Fig. 1. Map of Lake Baikal indicating the collection localities and morphotaxa found at each location. Morphotaxa: A, *Chaetomorpha* (= *Ch.*) *baicalensis*; B, *Ch. curta* attached form; C, *Ch. curta* unattached form; D, *Ch. moniliformis*; E, *Cladophora* (= *Cl.*) *compacta*; F, *Cl. floccosa* var. *floccosa*; G, *Cl. floccosa* var. *irregularis*; H, *Cl. globulus/Cl. Compacta*; I, *Cl. globulus/Cl. pulvinata*; J, *Cl. kursanovii*; K, *Cl. meyeri* var. *gracilior*; L, *Cl. meyeri* var. *meyeri*; M, *Cl. pulvinata*; N, *Gemmiphora compacta*; O, *Rhizoclonium* sp. Localities and approximate coordinates in Table S3.

& Skabitschevsky and *Chaetocladiella litoralis* (Skabitschevsky) Meyer & Skabitschevsky were not included because sample amplification failed (Díaz-Martínez *et al.* submitted). Samples were collected in 20 localities around Lake Baikal during September 2014 by C. Boedeker using scuba diving (Fig. 1; Table S3). A target number of 10 individuals per morphotaxon per locality were collected and dried in silica gel for DNA extractions.

DNA extractions and SSRs amplification

Total genomic DNA was extracted using the Chelex protocol (Goff & Moon 1993) to avoid losing material during the extraction. The primers and amplification conditions followed Díaz-Martínez *et al.* (2020). Eleven SSR markers were used in samples of morphotaxa belonging to clade A, while only nine were used in samples in clade B, similar to previous results (Díaz-Martínez *et al.* 2020).

PCR products were mixed in 96-well plates, combining up to four different dyes per well, and sent to Macrogen Inc. (Seoul, Korea) for fragment analysis on an ABI3730XL Genetic Analyser (Life Technologies Corporation, Carlsbad, California, USA.) with GeneScan 500 LIZ as a size standard. Chromatograms were analysed using GeneMarker 2.0.2 (SoftGenetics, LLC, State College, Pennsylvania, USA).

A previous study showed that individuals of the species flock of Cladophoraceae of Lake Baikal are likely polyploid as they showed more than two alleles (signal peaks) per locus per individual (Díaz-Martínez *et al.* 2020). Common statistics designed for microsatellites such as F_{st}, expected heterozygosity, or linkage disequilibrium, are based on diploid individuals and cannot be used in polyploids if the allele dosage is uncertain or if the ploidy level is high (Bruvo *et al.* 2004; Obbard *et al.* 2006; Pfeiffer *et al.* 2011; García-Verdugo *et al.* 2013). To overcome this problem, alleles were coded into a binary presence (1)-absence (0) matrix as 'allele phenotypes', an approach successfully used in population genetics of many taxa (Andreakis *et al.* 2009; Cidade *et al.* 2013; López-Vinyallonga *et al.* 2015; Samah *et al.* 2016; Sampson & Byrne 2012). In spite of the reduction of informative power, this approach allows analysis of polyploids and the combination of individuals with different ploidy levels (Andreakis *et al.* 2009; Bruvo *et al.* 2004). Other statistics, such as total number of alleles per locus and number of private alleles are not affected (López-Vinyallonga *et al.* 2015). Loci for which alleles failed to amplify in particular samples were coded as missing data. For loci 15, 515–35, and 515–46 in samples of group B, alleles were coded as true absences as we considered them informative for cluster analysis as they correlate with the morphotaxa.

Summary statistics for each SSR were calculated, including total number of alleles and alleles per individual. The polymorphic information content for dominant markers (PIC), a good measure of genetic diversity (Roldan-Ruiz *et al.* 2000), was calculated in GDdom (Abuzayed *et al.* 2017). Statistics for all morphotypes were calculated in GenAlEx 6.5 (Peakall & Smouse 2012) including the number of alleles (*a*), number of alleles with a frequency < 5% (*aF*), mean value of unbiased diversity (*uh*), and number of private alleles (*Pa*). The number of unique genotypes was calculated for the whole dataset and for each morphotaxon independently. The overall power of the loci to discriminate between individuals was calculated with an accumulation curve using the *poprr* package (Kamvar *et al.* 2015) implemented in R 3.2.2 (R Core Team 2015) with the function *genotype_curve*.

Clustering analyses

Species delimitation was performed using two different clustering methods: a model-based Bayesian clustering analysis as implemented in STRUCTURE (Pritchard *et al.* 2000), and the Gaussian clustering (GC) as implemented in the R package *prabclus* (Hausdorf & Hennig 2010).

STRUCTURE uses a model-based algorithm which assigns individuals to clusters with similar genetic patterns and assuming a Hardy-Weinberg equilibrium (Pritchard *et al.* 2000). The analyses were set up supporting dominant markers by adding a row of recessive alleles according to the manual (Pritchard *et al.* 2000). Missing data were coded as '-9'. Other options used were 'correlated allele frequencies' and 'no admixture', as it is regarded that these last parameters can detect subtle population structure (Falush *et al.* 2007; Porras-Hurtado *et al.* 2013). All individuals

of the full dataset were considered a single population and morphotaxon assignment was not considered as a prior to avoid bias (Porras-Hurtado *et al.* 2013). An exploratory analysis was run to calculate the number of clusters (K) that best fit the data. This consisted of 10 independent runs, K = 2 to K = 18 and 50,000 Markov chain Monte Carlo (MCMC) iterations with a burn-in of 50,000. The best K was determined calculating the Ln(K) and Delta K values (Evanno *et al.* 2005) in STRUCTURE HARVESTER (Earl & vonHoldt 2012). A more extensive analysis was done to improve cluster assignment performance using the selected K, 10 independent runs, each of 250,000 MCMC iterations with 250,000 iterations of burn-in. A consensus Q matrix was obtained in CLUMPAK (Kopelman *et al.* 2015) using the greedy algorithm and plotted using STRUCTURE PLOT (Ramasamy *et al.* 2014).

Gaussian clustering algorithm constructs clusters according to the normal distribution that can best explain the differences between individuals (Carstens *et al.* 2013; Hausdorf & Hennig 2010; Mrinalini *et al.* 2015; Sauer *et al.* 2013). The data matrix was transformed into pairwise distance matrices using the Jaccard index. As *prabclust* cannot deal with missing data, they were transformed into absences ('0'). The number of clusters was inferred with the function *prabclus* using the 'kruskal' nonmetric multidimensional scaling (NMDS), without 'noise component', with the number of clusters ranging from 2 to 18, and 99 permutations. The number of dimensions was calculated using the *stressvals* function with dimensions ranging from 2 to 10, selecting a value below 10% as suggested by the authors of the program (*prabclus* documentation).

Finally, the resulting assignments were compared, looking for clusters consistent with each morphotaxon and for agreement between both clustering methods. The proportion of individuals assigned to only one cluster was calculated and heat map-like bidimensional plots were created using the R package *adegraphics* to visually compare the number of individuals assigned to each cluster.

RESULTS

SSRs statistics

Marker analyses revealed that individuals had more than two alleles for most loci (Table 2), except for locus 515–35 which had up to two. Although the number of alleles per individual in each locus was up to 8 (i.e. loci 14 and 34), PIC values for each locus ranged from 0.09 to 0.41 (average of 0.22). The number of missing data in all alleles was relatively low (< 1%), except for loci 14 (6.6%) and 34 (36.0%). In spite of the missing data, we decided to include these loci in the analysis, as missing data occurred mainly in *Cl. floccosa, Cl. meyeri*, and *Cl. globulus*, and the loci were informative for the remaining morphotaxa.

The number of alleles found among all 11 loci was 170 and the number of alleles per morphotaxon ranged from 31 to 101 (Table 3). The overall unbiased diversity value (*uh*) was 0.068, ranging from 0.029 to 0.118. The proportion of alleles with a frequency < 5% (*aF*) ranged from 21.1% to 52.5%. A total of 21 private alleles (*Pa*) were detected, ranging from 0 to 4 per morphotaxon. A total of 600 different genotypes were detected. The majority of morphotaxa showed a large proportion of unique genotypes, except for *Ch. moniliformis* (only 34% unique) and the unattached form of *Ch. curta* (57%; Table 3). The cumulative curve plot of loci versus number of genotypes showed that the markers had sufficient power to discriminate each individual as the curve plateaus (Fig. S2).

Species delimitation by cluster analysis

The results of the exploratory analysis in STRUCTURE revealed that K = 12 was the number of clusters that best fit the data [mean Ln P(K) = – 12,517.6] based on the ΔK plot (Fig. S3). The number of individuals by cluster ranged from 16 to 133. Most morphotaxa were recovered as independent clusters containing a large proportion of the pre-identified individuals (Figs 2, 3; Table S4). One hundred percent assignment of morphotaxa to clusters was found in *Ch. baicalenesis* (cluster = 3_{strc}); the attached form of *Ch. curta* (6_{strc}); *Ch. moniliformis* (5_{strc}); and *Cl. kursanovii* (2_{strc}). In addition, a high value of 76% occurred for *Cl. compacta* (1_{strc}). A good proportion of individuals of both varieties *Cl. meyeri* var. *meyeri* and *Cl. meyeri* var. *gracilior* (66.67% each) was assigned to the same cluster (4_{strc}). For the two varieties of *Cl. floccosa,* the assignment to independent clusters was not clear. Instead, they appeared in different clusters mixed with samples of *Cl. compacta* and *Gemmiphora compacta* (7_{strc}) and *Cladophora pulvinata* (9_{strc}), respectively.

Table 2. Characteristics and number of alleles by locus for studied SSR loci. Groups according to Boedeker *et al.* (2018).

Locus name	SSR motif	Total number of alleles	Alleles per individual	Average number of alleles	Polymorphic information content	Cross-amplification
5a	TAC	12	1 to 5	2.5	0.24	All
6	TA	23	1 to 6	2.1	0.26	All
14	ATA	27	1 to 8	2.6	0.14	All
15	AAT	4	1 to 4	2.4	0.33	Group A only
16	GTA	21	1 to 7	2.7	0.16	All
34	TCA	31	1 to 8	2.2	0.09	All
46	TA	16	1 to 5	1.8	0.09	All
50–15	TTA	40	1 to 7	2.5	0.09	All
515–35	ATT	2	1 to 2	1.4	0.37	Group A only
515–46	TTGT	3	1 to 3	2.3	0.41	Group A only
515–64	TAT	4	1 to 3	1.7	0.25	All
Range or means	–	16.6	1 to 5	2.2	0.22	–

Table 3. Characteristics and genetic diversity of the SSRs in each morphotaxon. Total number of individuals (N), number of alleles (*a*), alleles with a frequency < 5% (*aF*), mean value of unbiased diversity (*uh*), number of unique genotypes and the number of private alleles (*Pa*) are shown. *Ch.* = *Chaetomorpha*, *Cl.* = *Cladophora*.

Morphotaxa	N	*a*	*aF*	*uh*	Unique genotypes	*Pa*
Chaetomorpha baicalensis	30	39	6	0.029	25	1
Ch. curta attached	41	50	12	0.049	39	0
Ch. curta unattached	21	34	2	0.048	12	0
Ch. moniliformis	122	38	16	0.012	41	0
Cladophora compacta	127	101	53	0.084	126	4
Cl. floccosa var. *floccosa*	65	86	34	0.098	62	3
Cl. floccosa var. *irregularis*	65	73	19	0.118	65	2
Cl. globulus/Cl. Compacta	31	72	18	0.105	31	2
Cl. globulus/Cl. pulvinata	21	63	19	0.076	21	1
Cl. kursanovii	100	55	16	0.042	76	3
Cl. meyeri var. *gracilior*	30	72	11	0.115	30	3
Cl. meyeri var. *meyeri*	30	72	8	0.096	30	2
Cl. pulvinata	20	60	0	0.072	20	0
Gemmiphora compacta	20	42	0	0.040	20	0
Rhizoclonium sp.	4	31	0	0.034	4	0

Rhizoclonium sp. was assigned to cluster 6_{strc} which included the attached form of *Ch. curta* and some individuals of the unattached form. The samples of *Cl. globulus/Cl. compacta* and *Cl. globulus/Cl. pulvinata* were assigned mostly to cluster 12_{strc} with the inclusion of a few individuals of other morphotaxa (Fig. 3). The cluster 8_{strc} contained only a few individuals of various morphotaxa. The cluster assignment for each individual is shown in Table S5.

The Gaussian clustering was used with NMDS = 4, which had an optimum imprecision of 8.18%. Fourteen clusters were recovered. Although there was no clear distinction of each cluster in the 2D plot, this was a false impression because two other dimensions are not displayed (Fig. S4). The clusters contained from 14 to 112 individuals. The overall assignment was similar to STRUCTURE but slight differences were observed (Fig. 4). For example, *Ch. moniliformis* and *Cl. kursanovii*, previously recognised in STRUCTURE as single clusters, were further divided (7_{GC} and 9_{GC} and 2 $_{GC}$ and 3_{GC}, respectively), and the assignment of a few individuals also differed (Table S6).

DISCUSSION

Our results indicate that many morphologically distinguishable species are discrete genetic clusters. While traditional molecular markers based on ribosomal DNA sequences are not able to resolve species in the clade of Baikalian Cladophoraceae, population genetics using SSRs provided evidence to delimit these species. Species delimitation is fundamental in several biological disciplines but remains a difficult task due to the complexity of the speciation process and to uncertainty of which species concepts and criteria are most appropriate to distinguish between species (Carstens *et al.* 2013; Duminil & Di Michele 2009; Leache & Fujita 2010). Under the 'species as lineages framework' (De Queiroz 2007) the use of different data sources is crucial to correctly delimit species (Carstens *et al.* 2013).

Most loci had more than two alleles (Table 2) supporting the occurrence of polyploidy in this group and complicating population genetic analyses (Díaz-Martínez *et al.* 2020). It was not possible to determine the ploidy level of each individual (including the haploid stages) as no correlation was observed in the number of alleles between loci. Two loci had up to eight alleles suggesting up to octoploidy (loci 14 and 34; Table 2). An analysis of chromosome numbers (Hinson & Kapraun 1991) or total DNA content (Kapraun 2007) may elucidate ploidy level changes. However, this was not possible with our samples and will need further work using new collections.

The PIC values showed that many of the SSRs used have a relatively high diversity (the maximum value expected for dominant markers is 0.5; Roldan-Ruiz *et al.* 2000) and are therefore adequate for the analyses. Noteworthy, loci 14 and 34 were the SSRs that showed the highest number of alleles and also had the largest proportion of missing data. However, this appears to have had no significant effects on the genetic clustering methods (see below). We speculate that these results could be related to mutations in flanking regions.

The proportion of unique genotypes is high in most morphotaxa. However, this is not the case for *Ch. moniliformis*, where only 34% of samples had unique multi-locus genotypes and had a low unbiased diversity value (*uh* = 0.012), even though this species had one of the largest sample sizes. This suggests that clonal reproduction, by either fragmentation or zoospores (as observed in freshwater species *Cl. glomerata*; van den Hoek *et al.* 1995; Zulkifly *et al.* 2013) may be prevalent in this species, although the actual mechanism is still unknown. This finding contrasts with *Cl. compacta* and *Cl. kursanovii* (*uh* = 0.084 and *uh* = 0.042, respectively), morphotaxa also with large distribution ranges which exhibit a much higher proportion of unique genotypes (99% and 76%, respectively). To our knowledge, the life cycle of these species is still unknown (Izhboldina 2007), but the different diversity values may indicate other reproductive strategies such as sexual reproduction. Further analyses will be performed to explore the genetic diversity and reproductive strategies of these species in the lake.

STRUCTURE has been used widely in studies where polyploidy is present and the alleles are coded as allelic phenotypes. This may be attributed to its extensive use among population geneticists and the relatively easy and explicit input of binary data matrices obtained from dominant markers (Meudt *et al.* 2009). However, it is reported that STRUCTURE can fail in obtaining the 'correct' K (clusters) if deviations from Hardy-Weinberg equilibrium are present (Hausdorf & Hennig 2010; Huelsenbeck *et al.* 2011). For this reason, the use of complementary and independent methods is recommended (Hausdorf & Hennig 2010; Kamvar *et al.* 2014). Gaussian clustering uses mixtures of normal allele frequency distributions and therefore is regarded as a good alternative to model-based methods for dominant data (Hausdorf & Hennig 2010; Sauer & Hausdorf 2012; Sauer *et al.* 2013).

Other potential bias to species delimitation by genetic clustering might be the decoupling of the life cycles (dominance of sporophyte or gametophyte in populations) (Krueger-Hadfield *et al.* 2016) and the isolation of asexual lineages from the original populations during the transition

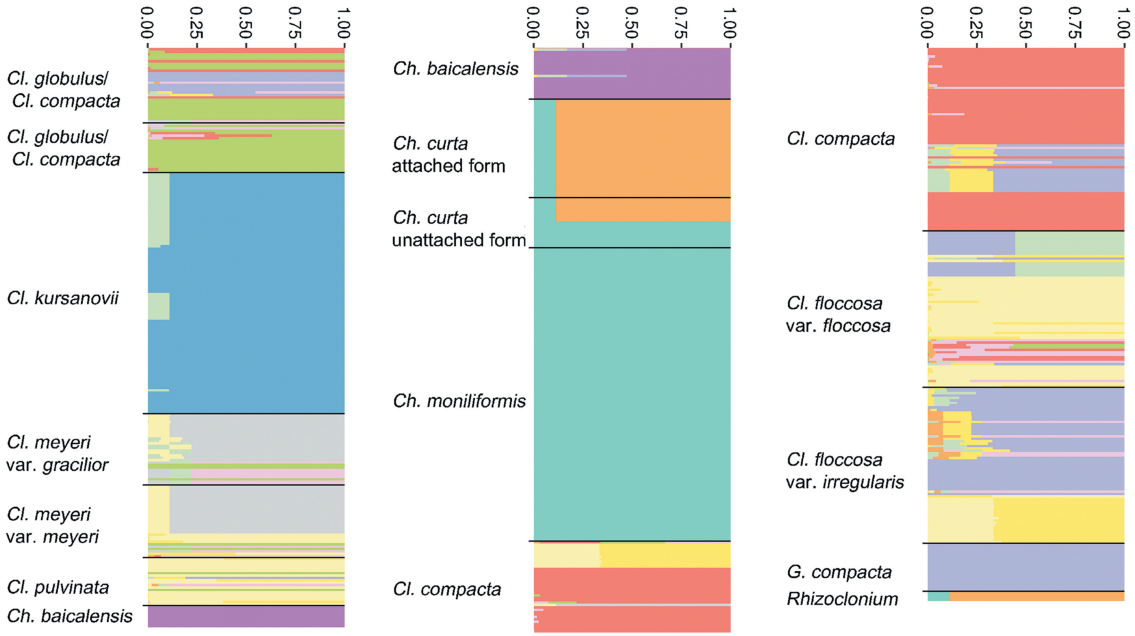
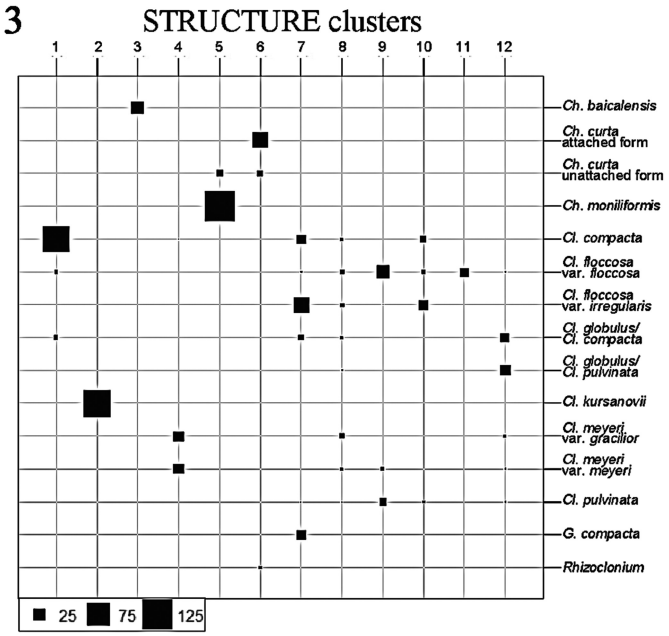


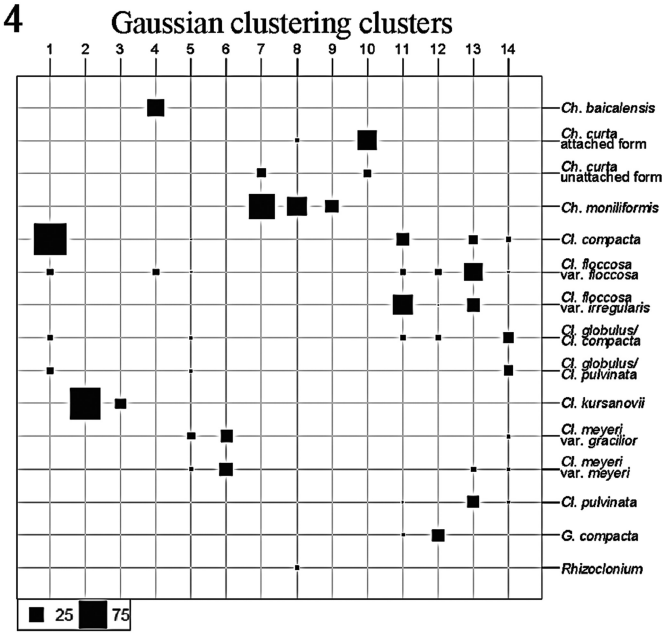
Fig. 2. Assignment of individuals by STRUCTURE (K = 12). Bar plot indicating the probability of assignment of each individual to a given cluster (by colour).

from sexuality to asexuality (Dudgeon *et al.* 2017). This is a possible scenario for those morphotaxa that were divided into two or more genetic clusters (e.g. *Ch. moniliformis* and *Cl. kursanovii*; Fig. 3). However, this effect appears to be negligible because of the low genetic differentiation observed in most of these clusters. Finally, the combination of samples of unknown ploidy level may have influenced the species delimitation, particularly for morphotaxa samples that could not be assigned properly to any genetic cluster or for morphotaxa assigned to more than one cluster (e.g. both varieties of *Cl. floccosa*). If sexual reproduction and alternation of haploid and diploid phases occur, the genetic diversity could be underestimated (Krueger-Hadfield & Hoban 2016), and the genetic structure might not be well determined (Wang *et al.* 2008). Future research is needed to determine the life cycle of the Baikalian species and their population structure.

The two cluster analyses (STRUCTURE and GC) produced comparable results, although GC recovered two more clusters than STRUCTURE, and other subtle differences in the assignment of individuals were observed. These genetic clusters likely correspond to reproductively isolated lineages as the assignment did not lump individuals based on locality or geographic regions, but mostly by morphotaxa. The cluster delimitation supports many of the morphotaxa identified in this study, such as *Ch. baicalenesis, Ch. curta* (attached form), *Ch. moniliformis, Cl. compacta*, and *Cl. kursanovii*. Some individuals of the unattached form of *Chaetomorpha curta* and *Ch. moniliformis* were recovered in the same cluster in both methods, supporting their conspecificity. The two types of thalli appear similar in shape and size, with the only difference being the attached or unattached habit. However, another group of samples of unattached *Ch. curta* was assigned to the same cluster as the attached form, which may indicate that the unattached morphology is influenced by environment and can arise in different species. In spite of their morphological differences, the attached form of *Ch. curta* and *Rhizoclonium sp*. were placed in the same cluster by STRUCTURE (6_{strc}) supporting the ITS phylogeny obtained by Boedeker *et al.* (2018). The GC placed the *Rhizoclonium* samples in a *Ch. moniliformis* cluster (8_{GC}) indicating slight genetic differences from *Ch. curta*. A more detailed analysis is required to confirm if this is evidence of hybridisation or an artefact of the data. For *Cl. meyeri*, the analysis suggests no genetic distinction between either variety, similarly to the ITS phylogeny (Boedeker *et al.* 2018). Our results also show that both *Cl. globulus/Cl. compacta* and *Cl. globulus/Cl. pulvinata* may actually belong to the same species. The remaining morphotaxa, namely *Cl. foccosa* var. *irregularis, Cl. floccosa* var. *floccosa, Cl. pulvinata*, and *Gemmiphora compacta*, have unclear assignments with both methods (clusters $7–11_{strc}$ and $4–12_{GC}$), and a re-examination of their morphological characteristics is needed. Furthermore, our results indicate that a reassessment of the taxonomy and nomenclature of the Cladophoraceae species of Lake Baikal is required. Unfortunately, this requires a more intensive taxonomic review for not only these species but for the entire order, as shown by previous phylogenetic analyses of Cladophoraceae (Boedeker *et al.* 2016, 2018).

Polyploidy is likely to have occurred in this group, making it interesting for the study of speciation and evolution. Polyploidy is regarded as a form of instant speciation as the offspring becomes reproductively incompatible with their parents (Albert & Schluter 2005; Schluter 2001). This may also explain the morphological variation in the group, because polyploidy is also often related to morphological changes (Comai 2005; Herben *et al.* 2017). The formation of polyploids via genome doubling (autopolyploids) or hybridisation (allopolyploids) could have led to the diversification of Baikalian Cladophoraceae, as it has been reported that





Figs 3-4. Bidimensional plots showing the number of individuals of each morphotaxa (rows) assigned to a given cluster (columns). For total number of individuals assigned to each cluster see Tables S4 and S6.

Fig. 3. Bidimensional plot from STRUCTURE data with 12 clusters recovered.
Fig. 4. Bidimensional plot from Gaussian clustering data with 14 clustersre covered.

polyploidisation favours the establishment of species in new environments (Soltis *et al.* 2010; Te Beest *et al.* 2012). The role of polyploidy was not solved and studies including other sources of data (i.e. DNA content or chromosome counting) will be necessary to reveal ploidy changes and their influence in speciation and adaption in these species.

Lake Baikal has undergone several geological (Logatchev 1993; Mats 1993) and climatic changes (Sherstyankin & Kuimova 2006). The transition of the region into a subarctic climate (*c.* 2.4 mya) coincides with a major change in the diversification of many species in the lake (Karanovic & Sitnikova 2017; Schön & Martens 2012; Sherbakov 1999; Stelbrink *et al.* 2015; Yokoyama & Goto 2005). An accurate timing of the diversification of these algal morphotaxa would indicate whether or not this diversification correlates with these environmental changes.

The Baikalian Cladophorales species flock shows a complex evolutionary history that remains to be fully understood. One of the most intriguing questions about this group is if the species have diversified in sympatry. A finer analysis of population genetics may help to understand the population structure, the genetic diversity within species, and provide further evidence of speciation in sympatry such as gene flow between populations and lack of isolation by distance (Coyne & Orr 2004).

The species delimitation performed on the Baikalian Cladophoraceae allowed us to delimit many of the species as genetic clusters that support reproductive isolation of lineages. This is further supported by the cluster analyses which did not lump individuals based on locality or geographic regions, but mostly by morphotaxa. It was possible to delimit five out of 15 morphotaxa with 100% confidence (Figs 3, 4; Table S5); two morphotaxa pairs seem to be conspecific; and two other morphotaxa are growth forms of other species. The remaining morphotaxa could not be fully resolved and need deeper review and additional data. The endemic Cladophoraceae species of Lake Baikal are a recently discovered group with many properties of a species flock (Boedeker *et al.* 2018) that may have evolved in sympatry or via polyploidisation. Therefore, it might represent one of the first examples of sympatric speciation in algae.

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