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# Microsatellite design for species delimitation and insights into ploidy for the Lake Baikal Cladophoraceae species flock

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**ABSTRACT**

Ancient lakes are centres of adaptive radiation and speciation. The Cladophoraceae endemic to ancient Lake Baikal is a morphologically diverse group nested within *Rhizoclonium* that may represent a case of sympatric speciation. Recent research using ribosomal DNA markers indicates that these taxa form a monophyletic group but was not able to resolve boundaries between all of the investigated morphospecies due to very low genetic diversity. For this reason, a population genetics approach using more variable markers was investigated. In this study, we developed a set of microsatellites (SSRs) using high-throughput sequencing (HTS) data obtained from three morphospecies of Cladophoraceae from Lake Baikal. To increase amplification rate of the microsatellites across taxa, we performed an *in silico* cross-validation step comparing the microsatellites retrieved from three HTS datasets and tested the most promising loci on 14 of the mostly endemic morphospecies. We obtained 11 SSRs that cross-amplified among morphospecies, eight SSRs in 12 taxa and three in only four taxa. Our results showed that most loci had more than two alleles, but also displayed variation between and within morphospecies. These results indicate that this group may have gone through polyploidisation. Polyploid systems require a different approach from standard population genetic analyses. We produced 'allelic phenotypes' (presence/absence matrices) to analyse genetic diversity. We showed that similarity indices mostly grouped morphospecies, suggesting that this scoring method will be useful in species delimitation, but further work is needed to elucidate the speciation process in this algal species flock in Lake Baikal.

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## INTRODUCTION

Ancient lakes are centres of great diversity which can be viewed as natural evolutionary laboratories containing examples of ecological diversification, speciation, and adaptive radiations (Cristescu *et al.* 2010; Wilke *et al.* 2008). Lake Baikal in south-eastern Siberia, Russia, is more than 25 million years old and the oldest lake in the world (Mats 1993). The diversity of the lake is extraordinarily high with more than half of the animal species being endemic and considered examples of adaptive radiations (e.g. Schön & Martens 2012; Sherbakov 1999; Yokoyama & Goto 2005). However, studies on the ecology, evolution, and speciation of the algal flora are limited (for example, see Boedeker *et al.* 2018; Kulikovskiy *et al.* 2012; Volkova *et al.* 2018).

Among the algae of Lake Baikal, the family Cladophoraceae is highly represented (Boedeker *et al.* 2018; Izhboldina 2007). The family Cladophoraceae is a cosmopolitan group of algae that can be found in fresh, brackish, and marine waters. The life cycle is generally haplo – diplontic and isomorphic, but asexual reproduction occurs in several species (Škaloud *et al.* 2018). Thalli are filamentous, containing multiple nuclei and chloroplasts per cell, and have relatively simple morphologies consisting of uniseriate branched or unbranched filaments (Škaloud *et al.* 2018; Van den Hoek *et al.* 1995). The morphological simplicity of the species within the order, phenotypic plasticity in many species, and convergent evolution of characters have created confusion in the taxonomy and classification of this group. During the last decades, molecular markers have solved many of the taxonomic problems as well as helping in species delimitation (i.e. Boedeker *et al.* 2012, 2016; Hanyuda *et al.* 2002; Zhu *et al.* 2018).

The traditional taxonomy of the endemic Baikalian Cladophoraceae is based on morphology (Izhboldina 2007) and reflects their wide morphological diversity with 16 distinct taxa (14 species and 2 varieties) placed in four genera: *Chaetocladiella* C.Meyer & A.P.Skabichevsky, which branches only near its holdfast; the unbranched *Chaetomorpha* Kützing (= *Ch.*); the abundantly branched *Cladophora* Kützing (= *Cl.*); and the diminutive *Gemmiphora* Skabichevsky (= *G.*) (Boedeker *et al.* 2018; Izhboldina 2007). However, recent molecular studies (Boedeker *et al.* 2018) using nuclear ribosomal markers (SSU, LSU, and ITS) revealed that the endemic Baikalian Cladophoraceae are actually a monophyletic group with low genetic differentiation having a maximum of 2.8% pairwise differences in the ITS region (Boedeker *et al.* 2018). These phylogenies also showed that this clade is nested within *Rhizoclonium* Kützing, a genus which shows little

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morphological differentiation (simple unbranched or sparsely branched filaments), but high genetic diversity (Boedeker *et al.* 2018). The phylogenetic signal of the ribosomal markers differentiated the Baikalian Cladophorales into two major clades (A and B; Table S1), but lacked support for many other clades and showed little or no genetic difference between some morphospecies.

These results suggest that this group fulfils many of the criteria of a species flock such as monophyly, endemism, and high morphological diversity (Greenwood 1984), but also questions whether the morphospecies are reproductively isolated and whether this group represents a case of sympatric speciation. To address these questions, a population genetics approach using more variable markers is needed. Among the markers available for population genetics, microsatellites have been considered a good option to assess genetic diversity and population structure of animals, plants, and algae (Selkoe & Toonen 2006). Microsatellites, also referred to as simple sequence repeats (SSRs), consist of repeated motifs of two to six nucleotides that occur in all eukaryotic genomes. Although other genetic markers are used in population genetics (e.g. amplified fragment length polymorphisms, random amplified polymorphic DNAs, inter simple sequence repeats, and single nucleotide polymorphisms), SSRs have several advantages that make them ideal for studying population genetics. These include co-dominance (enabling differentiation of homozygotes and heterozygotes), putative neutrality, and high levels of polymorphism. In addition, they can be applied to small and preserved tissues (Guichoux *et al.* 2011; Selkoe & Toonen 2006). Furthermore, technologies of high-throughput sequencing (HTS) and high computational power provide an easy way to obtain many potential SSRs for low cost and short development time while exploring large portions of an organism's genome (Gardner *et al.* 2011; Schoebel *et al.* 2013).

The number of studies using SSRs in algae has increased over the last years, showing their reliability to assess many questions, such as identification of individuals belonging to a particular population (Henrichs *et al.* 2013; Krueger-Hadfield *et al.* 2017); assessment of population structure (Kostamo *et al.* 2012; Krueger-Hadfield *et al.* 2011; Sjøtun *et al.* 2017); analysis of hybridisation (Montecinos *et al.* 2017; Zardi *et al.* 2011); and life cycle dynamics (Couceiro *et al.* 2015; Guillemin *et al.* 2008; Krueger-Hadfield *et al.* 2016). However, only a few studies have used SSRs in Cladophorales. These investigations involve population structure of *Cladophoropsis membranacea* (Hofman Bang *ex* C.Agardh) Børgesen (Van der Strate *et al.* 2000, 2002a, 2002b, 2003) and revealed fine-scale structure, and even possible cryptic species at larger scales.

One of the main difficulties in the study of population genetics in algae is the occurrence of isomorphic haplodiplontic life cycles (Valero *et al.* 2001). In some cases, it is possible to overcome this by distinguishing reproductive structures, measuring nuclear DNA content, using chemical tests, or counting the number of alleles in each locus (Couceiro *et al.* 2015; Engel *et al.* 2004; Guillemin *et al.* 2008; Krueger-Hadfield *et al.* 2011; Van der Strate *et al.* 2002b). Species of Cladophorales have a mostly isomorphic alternation of generations (van den *et al.* 1995) and reproductive structures are similar between sporophytic and gametophytic phases, making it impossible to distinguish morphologically between haploids and diploids. Another potential challenge is the occurrence of polyploidy which has been frequently reported (Boedeker *et al.* 2012; Hinson & Kapraun 1991; Kapraun 2007), making it even more difficult to determine the actual ploidy level of individuals. Little is known about the effects of polyploidization in algae, although it is generally assumed that it modifies the life cycle and expression of morphology (i.e. Kapraun 2007; Kapraun *et al.* 2007; Parodi & Cáceres 1991; Wik-sjöstedt 1970). Most of the studies on Cladophoraceae are based on *Cladophora glomerata* (Linnaeus) Kützing, where it is hypothesised that polyploidy has modified its life cycle leading to obligate asexual reproduction, and aided the transition from marine to fresh water (see Graham 1982). It has also been suggested that polyploidy is involved in the great morphological variation in *Cladophora* species (Wik-sjöstedt 1970). Another study on *Cladophora* suggested that populations of different ploidy levels may show differences in the number of apical ramifications, although other characteristics, such as cell size, remain highly similar (Parodi & Cáceres 1991).

The aim of this is study was to design a set of microsatellite markers that cross-amplifies across the species flock of Cladophoraceae of Lake Baikal. SSRs were developed using HTS data of three morphospecies. We calculated statistics and similarity clusters to provide a preliminary overview of their utility in providing diversity and genetic signals to address evolutionary questions of this group (e.g. species boundaries and population structure).

## MATERIAL AND METHODS

### HTS data and assembly

Total DNA was extracted from samples of three morphospecies: *Cl. kursanovii* Skabitschevsky, *Ch. baicalensis* K.I.Meyer and *Ch. moniliformis* Skabitschevsky from Lake Baikal collected during September 2014 (collection numbers 475, 50, and 515, respectively). Many individuals from the same locality were combined to obtain sufficient DNA for HTS. These species were selected based on ease in identification based on morphology, with each morphospecies likely to belong to a genetically distinct lineage, and include species of both clades A and B from Boedeker *et al.* (2018). DNA extractions were done using a CTAB lysis protocol (Zuccarello & Lokhorst 2005). A minimum of 50 ng of DNA was sequenced (Novogene, Beijing, China) following the protocols suggested by the company. The respective genetic libraries for HTS were created by digesting the genomic DNA with the restriction enzyme *EcoR*I, ligation of fragments with adapters, fragment recovery (200 ~ 400 bp and 400 ~ 600 bp) and sequenced in an Illumina HiSeq PE250 sequencer. The resulting reads were cleaned by removing adapters and low-quality reads (Phred score < Q20). Finally, the reads of the three morphospecies were assembled independently using Velvet 1.2.07 (Zerbino & Birney 2008) and Velvetopt 2.2.5 with

the parameters starting with *k*-mers of 23 to 31 base pairs with subsequent increments of three base pairs per *k*-mer (–s 23 – e 31 – x 3).

## Microsatellite detection and primer development

The first search for SSRs was done using all assembled sequences. We used MSATCOMMANDER (Faircloth 2008) to create a list of potential SSRs including their sequence, microsatellite motif type, and the respective forward and reverse primers. This helped us to determine the frequency and motif types of SSRs in the genomes.

To increase the probability of amplifying the same loci between morphospecies of Baikalian Cladophoraceae, we performed a cross-validation protocol *in silico* using the sequences from the three samples. All the sequences listed by MSATCOMMANDER as containing a microsatellite region were imported into Geneious R8.1 (www.geneious.com; Kearse *et al.* 2012) and assembled using the *de novo* option at High Sensitivity/Medium. All contigs were examined by looking for those containing the same sequences in at least two of the three datasets/morphospecies. Contigs of less than 100 bp were discarded. New primers were designed using Primer3 2.3.7 plug-in (Rozen & Skaletsky 2000) favouring conserved flanking regions.

## Microsatellite testing

A total of 44 samples from 14 endemic morphospecies of Baikalian Cladophoraceae plus several ambiguously identified individuals and two *Rhizoclonium* sp. samples were tested (Table S1). The samples were collected from different localities around Lake Baikal (Fig. S1, Table S2). DNA was extracted using a Chelex method (Goff & Moon 1993). For specimens forming entangled mats, each individual was examined using a stereoscope, and parts of the thallus arising from the same basal portion were used. For unbranched filamentous forms, only a single filament was used.

The primers were tested in two steps: first to check if the primers produced fragments of expected size, and second, to check if the loci were polymorphic. First, a standard PCR was conducted using the protocol: 94 °C for 4 min for initial denaturation, followed by 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR mix had a volume of 20 µl and consisted of 1 µl of diluted genomic DNA (1:10), 200 µM of each dNTP, 0.4 µM of each primer, 1.5 mM of $MgCl_2$, 0.05% BSA, 1X reaction buffer, and 0.7 units of BIOTAQ *Taq* polymerase (Bioline, London, UK). The PCR products were electrophoresed in 1.5% agarose, and post-stained with ethidium bromide to check for products.

All primers that produced amplicons were used for the next step to check fragment read quality. A three primer method for fluorescently labelling fragments (Schuelke 2000) was used for almost all the primer combinations, except for loci 5a and 6. The method consists of the addition of a third primer with the M13 (5′-TGT AAA ACG ACG GCCAGT-3′) sequence ligated on its 5′ end to one of the four fluorescent dyes (6FAM, PET, VIC, or NED), and a modified microsatellite forward primer with an M13 tail at its 5′ end. A two-cycle PCR program used consisted of 94 °C for 4 min for initial denaturation, followed by 25 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 120 sec at 65 °C for extension; followed by 8 cycles at 94 °C for 30 sec, 30 sec at 53 °C, and 120 sec at 65 °C for extension, followed by a final extension at 65 °C for 15 min. The extension temperature was modified to reduce the occurrence of stutters (Seo *et al.* 2014). The PCR mix had a volume of 15 µl and consisted of 1 µl of diluted genomic DNA, 200 µM of each dNTP, 0.4 µM of each M13 labelled and reverse primers, 0.1 µM of forward primer, 1.5 mM of $MgCl_2$, 0.05% BSA, 1X reaction buffer, and 0.7 units of *Taq* polymerase. For loci 5a and 6, the forward primers were directly labelled with FAM and PET, respectively. A standard PCR program and mix concentrations were followed. We performed repeated PCRs on random samples using DNA polymerase with proofreading exonuclease activity (RANGER DNA Polymerase, Bioline, London, UK), which has an increased fidelity, to compare and confirm the chromatogram profiles of the selected loci (i.e. 5a and 6), and check if noticeable differences occurred between repeats and polymerases.

The labelled PCR products were combined with up to four different loci per vial ('poolplexed') and analysed commercially (Macrogen Inc., Seoul, Korea) on an ABI3730XL Genetic Analyser (Life Technologies Corp.) with GeneScan 500 LIZ as a size standard. The resulting chromatograms were analysed and scored in GeneMarker 2.0.2 (SoftGenetics, LLC, State College, Pennsylvania, USA) using the default parameters for SSRs. Each chromatogram was reviewed manually and compared with chromatograms of the same and different morphospecies, looking for repeated profiles and identical fragment sizes to assign and correct the alleles. In difficult profiles showing stutters the allele scoring followed the method of the manual routine of Pfeiffer *et al.* (2011) with slight modifications. This approach is considered reliable over automated scoring to reduce noise in difficult loci due to polyploidy (Bhandawat *et al.* 2016; Cărăbuș *et al.* 2015; Könyves *et al.* 2016; Pfeiffer *et al.* 2011). Therefore, depending on their putative quality, the peaks in the chromatograms were considered as alleles. For this study, the highest main peaks were coded as alleles and peaks directly before or after the main peak with no less than 85% of the height of the main peak were considered as different alleles. An increase of intensity after a putative allele was coded as a different allele, as it is assumed that the intensity of stutters decreases after the 'true' allele, and stutter bands tend to occur before the 'real' peaks. Peaks with different intensities but far enough to be discarded as stutter were coded as allele if the peak was present in other profiles. Context information, such as morphospecies and locality, was also considered, for example, alleles that were found multiple times in the same morphospecies or location. The occurrence of chromatograms showing identical peaks and profiles in different samples was also used to corroborate the allele scoring.

All alleles were scored as 'allelic phenotypes'. This method consists of coding each allele independently in a presence/absence matrix, similar to DNA fingerprints (Andreakis *et al.* 2009; Besnard *et al.* 2008; Dufresne *et al.* 2014; García-Verdugo *et al.* 2013; Pfeiffer *et al.* 2011). For each locus, total number of

Table 1. Number and proportion of SSRs obtained in the three HTS datasets. Group designation follows Boedeker *et al.* (2018)

| Motif repeat | No. of SSRs | | |
|---|---|---|---|
| | *Chaetomorpha baicalensis* (Group B) | *Chaetomorpha moniliformis* (Group A) | *Cladophora kursanovii* (Group A) |
| 2 | 131 | 392 | 265 |
| 3 | 1735 | 3859 | 3367 |
| 4 | 49 | 168 | 406 |
| 5 | 21 | 85 | 191 |
| 6 | 14 | 20 | 21 |
| Total | 1950 | 4524 | 4250 |

alleles, and maximum and minimum number of alleles per individual were calculated, as well as the number of distinct multi-locus genotypes. Other genetic diversity statistics at the morphospecies level were calculated in GenAlEx 6.5 (Peakall & Smouse 2012) such as the number of alleles, the mean value of unbiased diversity (*uh*), and the number of private alleles (*Pa*). Allelic phenotypes of all loci were combined in a single matrix. The information contained in the SSR markers was explored by creating a UPGMA tree considering all genotypes independently and using a Jaccard similarity index in NTSYSpc 2.1 (Applied Biostatistics, Inc.).

## RESULTS

### HTS data

The HTS retrieved more than 16 million reads in each of the three samples. Other genomic features are reported in Table S3. The total cleaned reads amounted to 10.75 gigabases (Gb) in *Ch. baicalensis*, 10.1 Gb in *C. moniliformis*, and 9.35 Gb in *Cl. kursanovii*. The total numbers of contigs obtained after assembly were considerably different between species: 135,578 in *Ch. baicalensis*, 318,757 in *Ch. moniliformis*, and 105,464 in *Cl. kursanovii*. Microsatellite number differed between species and appeared to have no relation to the number of contigs generated. In *Ch. baicalensis*, we found 1950 SSRs, in *Ch. moniliformis* 4524, and in *Cl. kursanovii* 4250. Three base pair repeat motifs were dominant in all species, followed by dinucleotide repeats (Table 1).

### Microsatellite cross-amplification

After filtering dubious and short assemblies, 52 SSRs were cross-validated in at least two datasets. To complement the potential markers, another 24 SSRs present only in one dataset were selected, making a final SSRs list of 76 potential markers. From the total potential markers, 38 SSRs amplified in all morphospecies, 18 amplified only in morphospecies from group A, and 20 SSRs did not produce amplicons.

From the total of 56 SSRs (38 that amplified in all samples and 18 only in group A), 22 SSRs produced unusable chromatograms and 23 SSRs were not polymorphic. The remaining 11 SSRs (Table 2) showed variation and were used with all 44 individuals exploring their variability and characteristics. Three of these loci are markers that amplified only in morphospecies of group A (Table 2). Unfortunately, the samples of *Chaetocladiella* (= *C.*) *pumila* (K.I.Meyer) K.I.Meyer & Skabitschevsky and *C. litoralis* (Skabichevsky) Meyer & Skabichevsky did not produce good amplicons and were not included in subsequent analyses. Although primers were successful in most samples, some individuals did not produce amplicons at loci 14 (4 individuals), 34 (20 individuals), and 515–35 (6 individuals). These loci were not discarded as they appeared to be informative for other morphospecies, particularly those from group A.

Table 2. Primer sequence and characteristics of the selected 11 SSRs markers. The ability to cross-amplify in all species in clade A (Boedecker *et al.* 2018) is shown.

| Locus name | Primer sequence 5′-3′ | Motif | Total number of alleles | Allele range | Alleles per individual | Mean number of alleles | Labelling strategy | Cross-amplification |
|---|---|---|---|---|---|---|---|---|
| 5a | F: GTTGTCTGATCCGTTCTATTGCT<br>R: CTTCGCTACCAAGTAACCAAGAA | TAC | 9 | 112–148 | 1 to 4 | 2.55 | Direct fwd. primer | All |
| 6 | F: CGTTGATCATTATCTTGAGCACG<br>R: ATAGCTACTTGTACAGCGGTTAT | TA | 9 | 152–182 | 1 to 6 | 2.84 | Direct fwd. primer | All |
| 14 | F: AGCTACTTTGTGTCATAACGGC<br>R: GGTGTCTGTCCCTATCCAGG | ATA | 19 | 155–188 | 0* to 7 | 2.24 | M13 fwd. primer | All |
| 15 | F: GCACTTTATACTGCCTGTTATCG<br>R: AATAACTCTCGTTTATAATGGCCTGG | AAT | 4 | 151–166 | 1 to 4 | 2.50 | M13 fwd. primer | Only in A |
| 16 | F: ATACCCCGCCAGAAGAGTGC<br>R: ACGGCCACAAAACTACCTAAACT | GTA | 17 | 117–168 | 1 to 5 | 2.81 | M13 fwd. primer | All |
| 34 | F: CATTCGTATACAACAACTCCCAG<br>R: GGTGAGTCTTCCACTCTGACTCT | TCA | 13 | 120–168 | 0* to 7 | 1.44 | M13 fwd. primer | All |
| 46 | F: AATAAACTCGTGGAGGTATGCAA<br>R: GTAAAAAGCGCACAAGGAACTT | TA | 11 | 130–162 | 1 to 4 | 1.89 | M13 fwd. primer | All |
| 50–15 | F: GCCAGAAATGGCCAATCG<br>R: AGGCCAGCATGAGGTACAG | TTA | 12 | 191–257 | 1 to 3 | 2.44 | M13 fwd. primer | All |
| 515–35 | F: ATCGTTGGTAGAGCGCTGG<br>R: AGCTTGCAGAAAGGTGCG | ATT | 3 | 242–287 | 1 to 3 | 1.33 | M13 fwd. primer | Only in A |
| 515–46 | F: ATGCTTCATTGGCCCTTGG<br>R: GGTACAACAGGGACTTGGC | TTGT | 3 | 200–224 | 1 to 3 | 2.33 | M13 fwd. primer | Only in A |
| 515–64 | F: ACCTCGCTATTGGTTTGGC<br>R: TGACCGTATGGACCTTGGC | TAT | 3 | 271–283 | 1 to 2 | 1.58 | M13 fwd. primer | All |

* indicates the presence of potential null alleles.

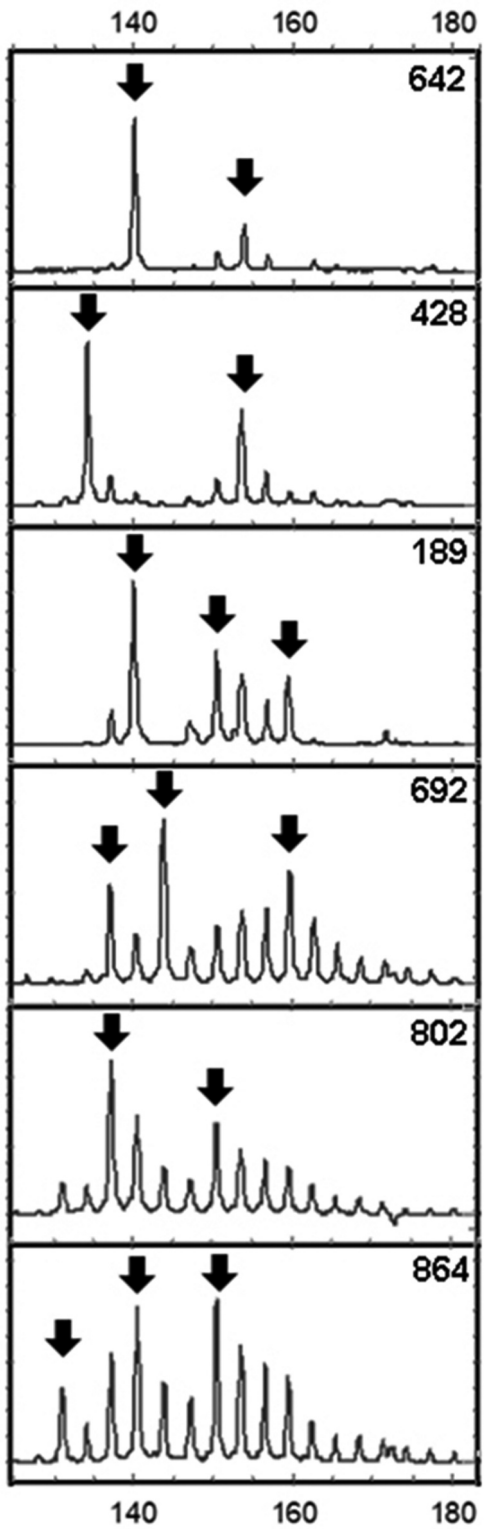


Fig 1. Chromatograms of locus 5a showing alleles scored. Arrows indicate alleles, based on peak height and position. Numbers as sample number for different morphospecies (Table S1).

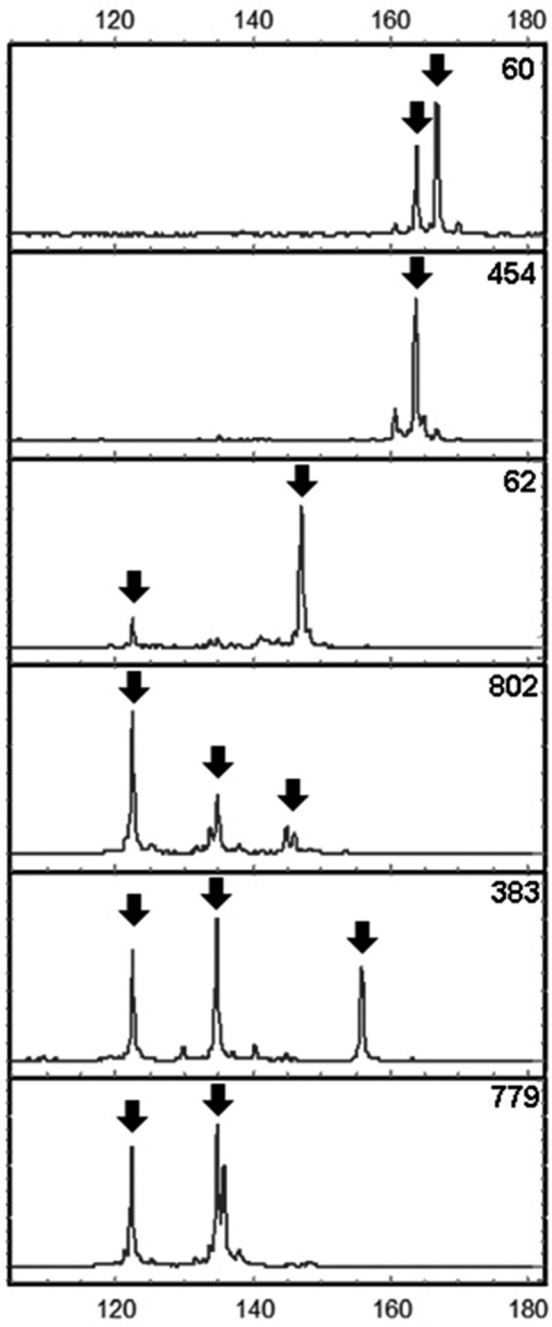


Fig 2. Chromatograms of locus 16 showing alleles scored. Arrows indicate alleles, based on peak height and position. Numbers as sample number for different morphospecies (Table S1).

## Microsatellite diversity

The scoring revealed consistent patterns of multiple alleles per locus per individual, indicative of polyploidy (Figs 1, 2). All loci except for locus 515–64 had more than two alleles (Table 2). Number of alleles per individual was up to seven in loci 14 and 34. Total number of alleles found in all 11 loci combined was 103 where the total number of alleles for each morphospecies ranged from 19 to 37 (Table 3). The unbiased diversity value (*uh*) had a mean value of 0.136, and a total of 30 private alleles (*Pa*) were detected (Table 3). All samples had a unique genotype differing in at least one allele. It is worth noting that chromatogram profiles of some loci were identical in samples of the same morphospecies, and that fragments amplified with proof-reading polymerase did not show significant differences from those obtained with the standard *Taq* polymerase. Therefore, these chromatograms were reproducible, and the

Table 3. Characteristics and genetic diversity of alleles for each morphospecies of the Baikalian Cladophorales clade. N, number of individuals; Alleles, total number of alleles; *uh*, mean value of unbiased diversity; and *Pa*, the number of private alleles.

| Taxa | N | Alleles | *uh* | *Pa* |
|---|---|---|---|---|
| *Chaetomorpha baicalensis* K.I.Meyer | 3 | 27 | 0.091 | 3 |
| *Chaetomorpha curta* (Skab.) Skabitschevsky | 3 | 30 | 0.123 | 2 |
| *Chaetomorpha moniliformis* Skabitschevsky | 3 | 18 | 0.026 | 0 |
| *Cladophora compacta* (K.I.Meyer) K.I.Meyer | 4 | 37 | 0.172 | 4 |
| *Cladophora floccosa* K.I.Meyer var. *floccosa* | 3 | 24 | 0.097 | 0 |
| *Cladophora floccosa* var. *irregularis* Skabitschevsky | 2 | 31 | 0.233 | 3 |
| *Cladophora globulus* (C.Meyer) C.Meyer/*Cladophora compacta* | 2 | 36 | 0.252 | 1 |
| *Cladophora globulus/Cladophora pulvinata* (K.I.Meyer) K.I.Meyer | 2 | 28 | 0.136 | 2 |
| *Cladophora kursanovii* Skabitschevsky | 4 | 36 | 0.107 | 8 |
| *Cladophora meyeri* Skabitschevsky var. *meyeri* | 3 | 31 | 0.175 | 4 |
| *Cladophora meyeri* var. *gracilior* (Meyer) Hollerbach | 2 | 34 | 0.272 | 2 |
| *Cladophora pulvinata* (K.I.Meyer) K.I.Meyer | 3 | 25 | 0.071 | 0 |
| *Gemmiphora compacta* Skabitschevsky | 2 | 25 | 0.087 | 1 |
| *Rhizoclonium* sp. | 2 | 28 | 0.068 | 0 |
| Total | 38 | 103 | 0.136* | 30 |

* indicates mean value.

profiles obtained from both types of polymerase were considered to be equally informative.

Jaccard (J) pairwise distances among all individuals retrieved similarity values ranging from 0.04 to 0.94 with a mean of 0.26. The similarity tree built using this index (Fig. 3) shows the grouping of many individuals belonging to the same morphospecies: *Ch. baicalensis* (J = 0.5); the attached form of *Ch. curta* (J = 0.61); *Cl. kursanovii* (J = 0.55); *Cl. pulvinata* (J = 0.67); *G. compacta* (J = 0.64); and *Rhizoclonium* sp. (J = 0.75). *Ch. moniliformis* was included in a group with high similarity (J = 0.84); however, this also included a sample of the unattached form of *Ch. curta* 638. The remaining individuals, with overall low levels of similarity (J < 0.5), were not grouped in exclusive branches according to their respective morphospecies.

## DISCUSSION

The HTS approach for the development of SSRs in non-model organisms allowed us to obtain a set of microsatellites that cross-amplified between morphospecies. These results showed that the markers designed displayed variation that can address evolutionary questions of these endemic algae such as their population structure and species delimitation of the morphotaxa. Microsatellites also revealed that the species flock of Baikalian Cladophoraceae consists of a clade that could be shaped by polyploidisation events and therefore needs a different approach to analyse its diversity. In addition, some preliminary insights into the partial genome of the Baikalian Cladophoraceae were obtained.

The total number of SSRs discovered from the HTS datasets differed between morphospecies. Although *Ch. baicalensis* and *Cl. kursanovii* had a similar number of contigs, the number of SSRs in *Ch. baicalensis* was nearly 60% fewer (Table 1). The dominance of trinucleotide SSRs in Baikalian Cladophoraceae appears to be similar to other green algae, such as *Chlamydomonas communis* and *Volvox reinhardtii* (Zhao *et al.* 2014), and the brown alga *Saccharina japonica* (Li *et al.* 2015; Zhang *et al.* 2016). In contrast, dinucleotide repeats are reported as dominant in the green alga *Ulva prolifera* (Ulvophyceae; Li *et al.* 2016), red algae (Ayres-Ostrock *et al.* 2016; Couceiro *et al.* 2011), and land plants (see Schoebel *et al.* 2013). The reason for trinucleotide SSRs dominance in Baikalian Cladophoraceae is unclear.

The *in silico* cross-validation process allowed us to detect and design markers that cross-amplified among the Baikalian Cladophoraceae, either in all morphospecies or in species of group A (56 combined). However, only 11 were selected for the final tests, as many were not polymorphic. The number of non-polymorphic markers (23) and the high proportion of primers that produced amplicons between Baikalian Cladophoraceae species may result from low genetic variation between these species, in spite of their morphological differences, possibly due to a recent radiation as has been suggested (Boedeker *et al.* 2018).

The results provided an overview of genetic differentiation and diversity in morphospecies. The UPGMA tree (Fig. 3) often grouped morphospecies, indicating that the scoring methodologies are useful in delimiting species. Unbiased diversity values (Table 3) also differed between each morphospecies. However, these results should not be considered conclusive until more data are gathered and more individuals per species and per population are included.

The number of alleles per locus indicates that polyploidy occurs in this group. Although this could be attributed to duplicated loci across the genome, the occurrence of more than two alleles in different loci indicates that polyploidy is most likely, as observed in other algal studies (Andreakis *et al.* 2009; Varela-Álvarez *et al.* 2017ba, b). Contamination of multiple individuals in DNA extracts could also be discarded as some loci with more than two alleles occurred in extractions where clearly a single filament was used, such as in individuals of *Chaetomorpha*. In addition, the occurrence of repeated profiles in different samples of the same species suggests that random amplifications of fragments are also unlikely. Karyological and flow cytometry studies using fresh

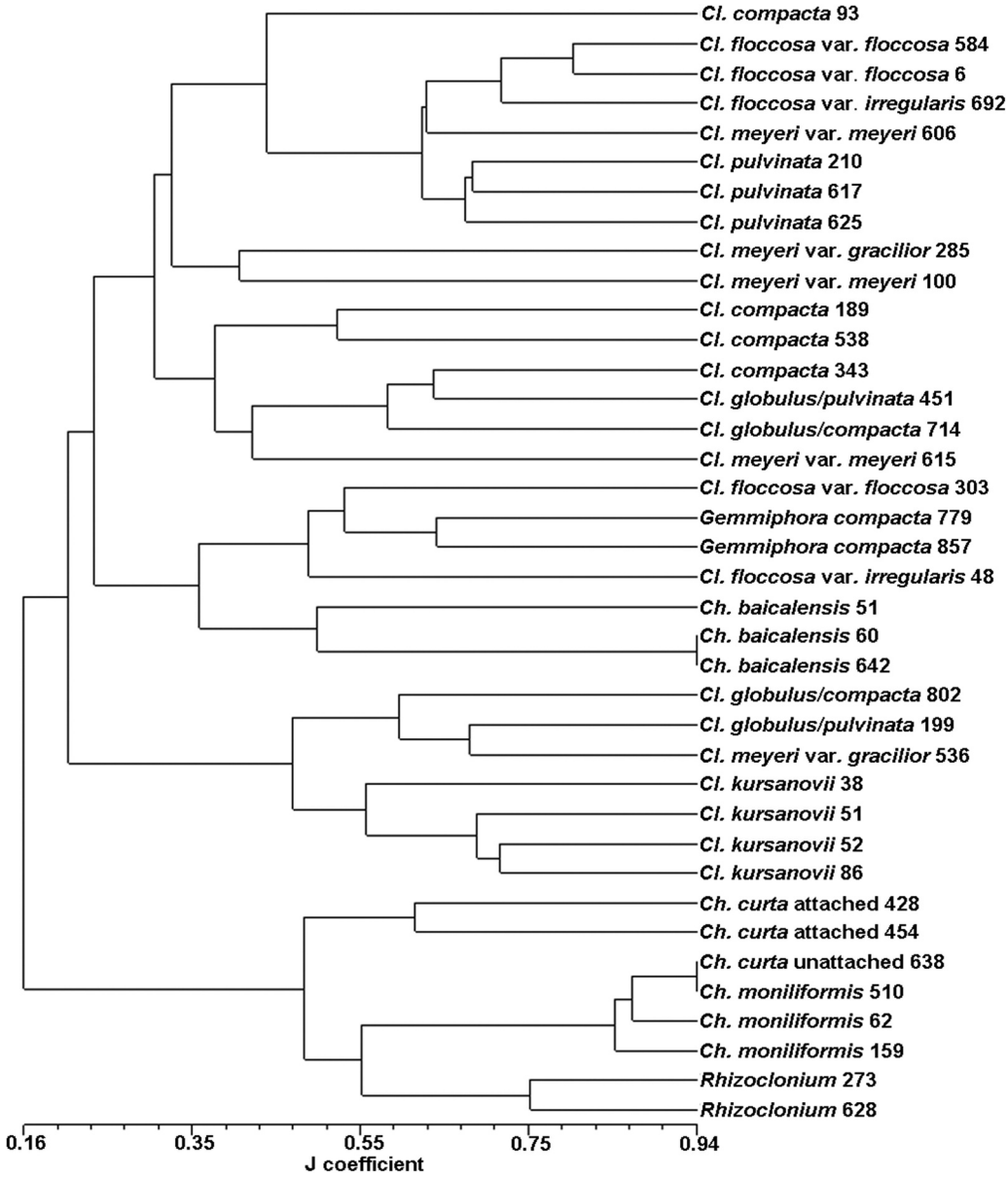


Fig 3. UPGMA tree based on the Jaccard similarity coefficient from allelic phenotypes of 11 loci. See Table S1 for taxon names.

or cultured samples may contribute to confirm the ploidy levels.

Polyploidy complicates the use of standard statistics for population genetic analysis (e.g. $F_{st}$, expected heterozygosity). In cases of low ploidy levels, such as in tetraploids, it is possible to determine allelic dosage using peak ratios of chromatograms (Esselink *et al.* 2004) or by computational algorithms (e.g. Clark & Jasieniuk 2011; Van Puyvelde *et al.* 2010). However, these methods require previous knowledge of sample ploidy level (i.e. chromosome counts or total DNA content per nucleus) and knowledge of whether polyploidy is due to alloploidy (product of hybridisation between different species) or autoploidy (genome doubling), in order to sort allele dosage correctly (Dufresne *et al.* 2014).

Coding the SSRs as dominant data in 'allelic phenotypes' is a simple way for dealing with polyploids, as no complex assumptions are made about the data (Cidade *et al.* 2013) and have the advantage of allowing the inclusion of organisms with different ploidy level in the same dataset. Although this reduces the potential informative power of SSRs, it is a convenient solution when the ploidy number is unknown and when the sorting of alleles cannot be resolved (García-Verdugo *et al.* 2013; Obbard *et al.* 2006; Pfeiffer *et al.* 2011). In addition, some other properties of the population such as the total number of alleles and number of private alleles within populations are not affected (López-Vinyallonga *et al.* 2015). This approach proved to be informative for Baikalian Cladophoraceae as our analyses showed diversity within the microsatellite markers, and the number of private alleles confirms that these markers have enough power to discriminate between most morphospecies regardless of the unknown ploidy level. Likewise, the J-similarity tree, which considers all genotypes independently, retrieved clusters that in many cases were consistent with morphospecies (e.g. *Cl. curta, Cl. kursanovii, Cl. pulvinata, Ch.*

*baicalensis*, and *Ch. moniliformis*). These results are similar to phylogenetic trees based on ribosomal markers (Boedeker *et al.* 2018). However, broader sampling and a more in-depth analysis are required to perform proper species delimitation and test for reproductive isolation of species.

Polyploidy could be playing an important role in the evolution of the Baikalian Cladophoraceae species flock. Polyploid speciation occurs when the progeny is reproductively isolated from their parents ('instant speciation'; Albert & Schluter 2005; Schluter 2001). Furthermore, polyploidy often results in phenotypic changes and life cycle modifications in plants (Comai 2005; Soltis *et al.* 2010). Therefore, this process could explain some of the wide morphological diversity found in this group.

In conclusion, the HTS data combined with the *in silico* cross-validation procedure allowed us to explore the genome of these taxa and design a set of cross-amplifying SSRs to study population genetics and reproductive isolation of this mostly endemic Baikalian Cladophoraceae, a group recently discovered to represent a species flock. The markers designed here proved to be promising for studying reproductive isolation and genetic differentiation, as information was sufficient to cluster several morphospecies. Finally, our data revealed evidence for polyploidy, a process that could have shaped the evolution of this group.

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