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Characterization of microsatellite markers for the duckweed *Spirodela polyrhiza* and *Lemna minor* tested on samples from Europe or the United States of America

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Abstract: Microsatellite primers are a valuable tool to use for both observational and experimental studies in numerous taxa. Here, we develop 18 and 16 microsatellite markers for the widespread duckweeds *Lemna minor* L. and *Spirodela polyrhiza* (L.) Schleid, respectively. All 18 *L. minor* primers and 12 of the 16 *S. polyrhiza* primers amplified polymorphic loci when tested on samples from Europe or Western Pennsylvania, USA.

Keywords: Lemnaceae, simple sequence repeats, genotyping, genetic identification, molecular markers

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Introduction

The globally distributed duckweed family (Lemnaceae) or subfamily (Lemnoideae) is composed of 36 species (Bog *et al*, 2020) of very small floating or submerged aquatic plants (Landolt, 1986; Sree *et al*, 2016). Duckweeds have a long history of scientific study given their highly specialized morphology, widespread distribution, high abundance and production of the world's smallest flowers (Jacobs, 1947; Hillman, 1961; Landolt, 1986, 1992). More recently, there has been an explosion in research interest given their potential applied uses including for agricultural feed (Cheng and Stomp, 2009), bioremediation (Gupta and Prakash, 2013; Ekperusi *et al*, 2019) and biofuel production (Cui and Cheng, 2015). Furthermore, their use as a model system to experimentally study numerous topics in ecology and evolutionary biology is quickly expanding (Laird and Barks, 2018). This growing basic and applied interest stems from their ability to reproduce clonally very quickly with population doubling times in as little as 1.5 days (Ziegler *et al*, 2015). In addition, they are amenable to large-scale manipulative experiments in both the lab and field mesocosms (Armitage and Jones, 2019; Hart *et al*, 2019; Tan *et al*, 2021; O'Brien *et al*, 2022), and have growing genomic data and tools (Wang *et al*, 2014; Ho *et al*, 2019; Xu *et al*, 2019; Cao *et al*, 2020) and characterization of their microbiome and herbivore communities (Acosta *et al*, 2020; Subramanian and Turcotte, 2020). Finally, duckweed express variation in numerous traits across species and among genotypes (clonal lineages) within species (Van Steveninck *et al*, 1992; Hart *et al*, 2019; Chen *et al*, 2020; Hitsman and Simons, 2020; Anneberg *et al*, 2023). Therefore, being able to identify genotypes may also be beneficial in many

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ecological studies to assess differences in traits among genotypes and to determine how these genotypes may respond to different environmental conditions.

Genetic markers, such as microsatellite markers, are important tools to study population genetics. Microsatellites, also known as simple sequence repeats (SSR), are tandem repeats two to ten base pairs in length, that are flanked by conserved sequences and occur ubiquitously throughout eukaryotic genomes (Tautz and Renz, 1984). They are highly informative as locus-specific genetic markers due to their high abundance, high reproducibility, co-dominance, and polymorphic nature (Morgante and Olivieri, 1993; Powell *et al*, 1996). The length of the sequence repeats can be determined through PCR amplification using primers specific to their flanking regions; variation in PCR product length is a function of the number of repeated sequences. The high levels of polymorphisms observed in SSR markers (Tautz, 1989; Schlötterer and Tautz, 1992) and the relative ease of detection of these polymorphisms by PCR amplification have led to the wide applications of microsatellites as genetic markers (Vieira *et al*, 2016). Such within-species markers have numerous applications including quantifying biogeographic distributions, population genetic structure, evolutionary history, and mating systems.

Moreover, a growing number of experimental evolution studies use SSR markers to track changes in genotypic composition of asexually reproducing populations over multiple generations (Turcotte *et al*, 2011; Hart *et al*, 2019; Agrawal *et al*, 2013) in large replicated experiments for which genotyping-by-sequencing remains too costly. These cost savings are magnified when several loci can be multiplexed and genotyped in the same reaction (Markoulatos *et al*, 2002).

With the growing interest in duckweed, microsatellite markers have been developed for a few duckweed species. Wani *et al* (2014) developed nine polymorphic and 24 monomorphic haplotype chloroplast DNA-based microsatellite primers for L. minor. Xu *et al* (2018) developed 60 microsatellite primers for *Spirodela polyrhiza*, 19 of which were polymorphic within three populations of *S. polyrhiza* from China. Feng *et al* (2017) developed three microsatellite primers for the identification of *S. polyrhiza* and *Landoltia punctata* (G. Mey.) Les & D. J. Crawford haplotypes. More recently, Fu *et al* (2020) developed 70 microsatellite primers within coding regions for *L. gibba* L. It is important to continue developing and reporting new microsatellite markers as populations can differ in which markers function (e.g. due to null alleles) and are polymorphic Chapuis and Estoup (2007).

Here, we report on the successful development of 18 and 16 new microsatellite markers, respectively, for two commonly studied and widespread duckweed species: the common duckweed *Lemna minor* L. and the greater duckweed *Spirodela polyrhiza* (L.) Schleid. A small subset of these microsatellite primers was used to differentiate genotypes in our experimental studies on evolutionary coexistence (Hart *et al*, 2019). In addition, we report genotyping results using these markers on individuals sampled in Europe and the United States of America (USA). We thus provide new tools and evidence that they function, which can be utilized by the growing community of duckweed researchers (Laird and Barks, 2018).

Materials and methods

Sample collection

Our objective when sampling was not to genetically characterize duckweed populations, but instead find genotypes that differ in ecologically relevant traits to use in various experiments. Thus, we genotyped few individuals from numerous bodies of water in various locations. Primers were developed at ETH Zurich (Europe) and the University of Pittsburgh (USA), and thus were tested on different collections of duckweeds. We collected duckweeds from numerous still bodies of water (e.g. ponds, lakes, wetlands) primarily in Switzerland and Western Pennsylvania (USA); however, a few samples were also collected from the Netherlands and Germany. In addition, some European duckweed samples included in the set were obtained from the Landolt Duckweed Collection (formerly in Zurich, Switzerland, see Supplemental Tables S1 and S2 for collection locations). Given the two-part development of the primers, some duckweed samples were only tested on the primers developed in that country (as noted in Supplemental Tables S1 and S2).

Duckweeds mostly reproduce clonally via meristematic pockets from which clonal daughters emerge, creating clonal clusters of one to eight individuals that eventually split into smaller clusters (Landolt, 1986). We sampled single duckweed clusters and established isofemale laboratory colonies from these clusters. We then sterilized each colony using sodium hypochlorite following a method adapted from Barks *et al* (2018). From each colony, we put single individuals into individual sterile petri dishes (one individual per dish) containing sterile 0.5 strength Schenk and Hildebrandt growth medium (Schenk and Hildebrandt, 1972) supplemented with sucrose (6.7g/L), yeast extract (0.067g/L), and tryptone (0.34g/L) for 24 hours to encourage algal and bacterial spore germination. Then each individual was exposed to one of an array of concentrations of sodium hypochlorite (0.3% or 0.5%) for varying amounts of time (3 or 6 minutes for *L. minor*, 4 or 7 minutes for *S. polyrhiza* respectively), then rinsed with autoclaved distilled water and allowed to grow (Barks *et al*, 2018). Sterile colonies were maintained in sterile 0.25 strength Schenk and Hildebrandt media (Schenk and Hildebrandt, 1972) without the additional supplements in room temperature laboratories or growth chambers under plant grow lights. These collections do not reproduce sexually under lab conditions.

Table 1. *Lemna minor* microsatellite markers and motifs including the location of initial primer development (USA or Europe), optimized $MgCl_2$ concentrations, and annealing temperatures (T_A). In addition, we report marker success rate, which is the number of samples successfully genotyped divided by those attempted, the number of unique alleles, and number of unique genotypes for each primer. Average heterozygosity (H) is the fraction of individuals that are heterozygotic for each primer. See Supplemental Table S1 for specific allele values. Allele lengths with an * denote that these lengths include the M13 tail sequence.

Primers	Forward Primer (5'-3') Reverse Primer (5'-3')	Motif	Location of Development	$MgCl_2$ (mM)	T_A (°C)	Observed Product Length (bp)	Marker Success Rate	Unique Alleles	Unique Genotypes	Average H
LmR.1.A	F: GTTCCTAAGGATTCATCACC R: TACGAGGAGGGACACGAG	AAG	Europe	2.0	60	178–185*	75/81	2	2	0
LmR.4.A	F: AGTGGCTACGAACGGAAGAG R: AGAGGAACGTTGTGTCTGGG	AAG	Europe	0.9	63	219–234	28/28	5	5	0.036
LmR.4.B	F: CTTATTGGATCTTCGCGCCG R: AAGATATCTGACGGCGTTGG	AG	Europe	1.2	63	366–392	28/28	6	6	0.071
LmR.5.C	F: GATGCCAGTAGATCCGGC R: ACGCCTGAACACGATTGATG	AGAT	Europe	2.0	60	320–444	104/109	25	41	0.846
LmR.8.B	F: TGTACTCATCTGTGGGCGAG R: AACAATTTGGCCACCGTCAG	AGAT	USA	1.2	63	306–376	28/28	10	9	0.036
LmR.8.C	F: GACAACTTAGGGTGCACGC R: GGAGTGAGAGCTGAGGACTG	AGG	USA	1.2	60	435–450	28/28	3	3	0
LmR.10.A	F: TCCTTTCTCGTGTCTCCCAG R: ATGCCCGACCTAGTCC	AG	Europe	2.0	60	222–254*	31/81	4	5	0.032
LmR.10.C	F: CTCTCCTTTCTCCTCCACGG R: ATCGCAACCCTCTAGCCG	AGAT	Europe	2.0	60	179–254*	79/81	4	4	0.278
LmR.12.B	F: TCTCTGCTGACCGACTCAAG R: GCCGTTGGATCTTTCTCACG	AT	USA	1.2	60	274–320	27/28	8	9	0.111

Continued on next page

Table 1 continued

Primers	Forward Primer (5'-3') Reverse Primer (5'-3')	Motif	Location of Development	$MgCl_2$ (mM)	T_A (°C)	Observed Product Length (bp)	Marker Success Rate	Unique Alleles	Unique Genotypes	Average H
LmR.14.A	F: TCGCACTAGAGAGATGGGTG R: TCCCATTACCAGGATGCGAG	AAT	USA	1.2	60	261–270	24/28	3	3	0.042
LmR.14.B	F: CATGCCAGGTAAATGCCCTC R: TCGAGCTCCTTCTCCAAACC	ATC	USA	0.9	63	430–440	28/28	3	3	0
LmR.14.C	F: TTCGTCGAGGGTATGAGCTG R: TCTCTTATTTGACACGCGCG	AG	USA	0.9	63	162–178	28/28	7	7	0.036
LmR.15.A	F: GTGACAGCGTATCCTTGTGC R: CAGCGGCAAGATCATCAAG	ATC	Europe	1.2	60	222–285	109/109	13	15	0.578
LmR.15.B	F: TCGAGCTAATCAGTGGAGCC R: GAGTGCTCGGCTTGACTTTC	AG	Europe	1.2	60	170–210	104/109	13	25	0.692
LmR.15.C	F: CATGTTCCCACCCACTTGAC R: AAGGAAGAGGGAGCAAGGG	AT	Europe	1.2	60	368–400	109/109	14	26	0.743
LmR.26.B	F: GTGTCTCCGAGAGCCTACAG R: TTTAAAGCTCGGTGGGTCCC	AG	USA	1.2	63	283–329	28/28	10	7	0.964
LmR.31.A	F: GGTGATCTCAGGTAGCCGAG R: TGAGATCACCACTGTCTGCC	AAG	USA	0.9	63	402–432	26/28	5	6	0.077
LmR.31.B	F: AGTCGGCATAGTACTTCCCG R: CTTCTTCAAGACCGTTCCGC	AAG	USA	1.2	63	155–239	28/28	7	9	0.071

Table 2. *Spirodela polyrhiza* microsatellite markers and sampling results as described in Table 1 with allele calls in Supplemental Table S2.

Primers	Forward Primer (5'-3') Reverse Primer (5'-3')	Repeat Motif	Location of development	$MgCl_2$ (mM)	T_A (°C)	Observed Product Length (bp)	Marker Success Rate	Unique Alleles	Unique Genotypes	Average H
Sp.1035	F: TGCTTGGTCACTCTTGTCTG R: CGATTCCTAGCTCCTCTGC	AT	Europe	1.2	60	361–369	42/42	4	5	0.381
Sp.1467	F: AGTTGAGGAAGCTTCATGG R: ATTACCTCCAGCACCTCTCC	AG	Europe	2.0	58	386–411*	9/20	5	4	0.444
Sp.2597	F: TCCCATTCACCACAGTCTCC R: TCATTCCACCACGTCCCAC	AT	Europe	2.0	58	397–399*	14/20	2	2	0.071
Sp.5050	F: ATTAACCTTGGGCGCAGAG R: TAGCAGCAGAGTGTGAGGG	AAT	Europe	2.0	58	287*	14/20	1	1	0
Sp.5250	F: AAACGAGACCTCCTACGCC R: GCCTGCGAGTAATATGTGC	ATGCCC	Europe	2.0	58	385*	19/20	1	1	0
Sp.7286	F: CGAATATGCCGAGGAATGC R: TCCTCGATCTGCCGCTTTAG	CG	Europe	1.2	60	386–394	42/42	5	7	0.310
Sp.7688	F: AATGGTTGACTCGACGCTG R: TCACACCGCCATAATTTCGC	AGC	Europe	2.0	58	199–211*	19/20	2	2	0.158
Sp.7814	F: AGTGTAGGGTGCAGCTGTG R: TTCGTGAAAGGCCTAGCAC	AG	Europe	1.2	60	220–228	42/42	5	6	0.095
Sp.7908	F: GAGACACATCATTGCCAGC R: TAATGCAGGCCACACAACC	AG	Europe	2.0	58	234–236	20/20	2	2	0.850
Sp.8563	F: GTATTGGGTGGGCAAATCG R: AAGGGATAGGGTCGTGTCC	AG	Europe	2.0	58	350–354*	14/20	3	4	0.071

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Table 2 continued

Primers	Forward Primer (5'-3') Reverse Primer (5'-3')	Repeat Motif	Location of development	$MgCl_2$ (mM)	T_A (°C)	Observed Product Length (bp)	Marker Success Rate	Unique Alleles	Unique Genotypes	Average H
Sp.8910	F: CCTTCCCTACGTTGACTCCC R: GCGTTTCTCTGATCAGCACC	ACG –> CGT	Europe	2.0	58	358	20/20	1	1	0
Sp.9307	F: GGGAGCGAGCTGTATGAAG R: TTTCAACACCCTCACCATGC	AG	Europe	2.0	58	450–452*	9/20	2	3	0.444
Sp.9311	F: GTGAGAAAGGAAAGGTGGC R: TGCTCAGGATTCTATGGGCC	AG	Europe	2.0	58	253–255*	10/20	2	3	0.400
Sp.Pso27	F: AAGGGTTTCAGTGCGGACG R: CTCGCCTTCTCGTACATCATC	AAG	Europe	2.0	58	133*	9/20	1	1	0
Sp.Pso31	F: TCCACCGTCTCCCTGTAATG R: CCACTCCCTCGTCGTGAAG	AAG	Europe	1.2	60	240–270	32/42	7	7	0.406
Sp.Pso32	F: TGCTGGCGATGTCAATGTTG R: CTTCAGCACCAAGAGAGCTC	ATC	Europe	2.0	58	377–380*	19/20	2	3	0.895

Microsatellite marker development

A total of 18 *L. minor* and 16 *S. polyrhiza* microsatellite markers were developed across Europe or the USA. We downloaded the whole genome shotgun sequence data for *S. polyrhiza* strain 7498 from the National Center for Biotechnology Information's GenBank database (accession ATDW01000001.1) deposited by Wang *et al* (2014). For *L. minor*, a draft genome (strain 8627) was downloaded from www.lemna.org on 16 October 2015 (genome draft lm8627.ASMv0.1). A recent study using tubulin-based polymorphism suggests that this lineage is in fact an interspecific hybrid of *L. japonica* Landolt and *L. turionifera* Landolt both closely related to *L. minor* (Braglia *et al*, 2021). The species identity for most samples on which we report below has been confirmed using morphology and/or barcoding (Fazekas *et al*, 2012; Barks *et al*, 2018). While some microsatellite markers are known to amplify across more than one duckweed species (Xu *et al*, 2018), we have not yet explicitly tested these markers against other species. Using msatcommander (version 1.0.8, Faircloth 2008), we identified microsatellite loci using the default settings, avoiding mononucleotide repeat motifs. We then selected loci that would produce products of different lengths, had different motif lengths and were found on different contigs. The 5' end of forward primers were labelled with one of several fluorescent dyes from various suppliers.

Primers developed at ETH Zurich were M13-tailed to reduce cost during development (Boutin-Ganache *et al*, 2001). This entailed adding the full or a partial M13 sequence of TGTAAAACGACGGCCAGT for the *S. polyrhiza* primers and GGAAACAGCTATGACCAT for *L. minor* primers to the 5' end of the forward primer. The M13-labelled forward primers were used in combination with an M13 primer that had the same sequence but was fluorescently dye-labelled at its 5' end. Some primers amplified loci that were fully or mostly monomorphic or did not amplify as consistently as others. For these primers, we only have fragment lengths that include the M13 tail (see Table 1 and Table 2), and we estimate that this lengthens the PCR product by 12–19 base pairs. For most primers, however, following initial testing with M13, we ordered new labelled primers that did not include the M13 tail.

At least 20 duckweed samples were tested using each primer. European duckweed samples were tested across 7 *L. minor* and 16 *S. polyrhiza* primers, and USA duckweed samples were tested across 15 *L. minor* and 4 *S. polyrhiza* primers (see Supplemental Tables S1 and S2 for details). Each duckweed sample was tested with each primer using at least two independently extracted DNA samples. We only report allele lengths that were consistent in both samples.

Microsatellite amplification and optimization

All duckweed collections were extracted and genotyped at least twice by first sampling four to ten individuals from each monoclonal collection and lyophilizing them for 24 hours. We then extracted DNA using a modified CTAB-based method by Healey *et al* (2014). For primers developed in Europe, the conditions were the following: PCR amplification was conducted in 15μL volume reactions containing 3μL of template DNA, 3μL of 5X Colorless GoTaq Flexi buffer (Promega, USA), 2.0mM $MgCl_2$, 0.2mM dNTP mix, 0.05μM of forward primer, 0.2μM of reverse primer, 0.2μM of M13 primer tagged with a fluorescent probe (e.g. 5' 6-FAM or 5' HEX), and 1 unit of GoTaq G2 Flexi DNA Polymerase (Promega, USA). DNA concentrations were rarely quantified as amplification was successful across a range of values (e.g. 2–40ng/μL). Thermocycling conditions for both *S. polyrhiza* and *L. minor* from Europe that were M13 tagged were: initial denaturing at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, followed by eight M13 cycles consisting of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C, followed by a final extension at 72°C for 10 min. For all primers developed in the USA, the conditions were the following: PCR amplification was conducted in 15μL volume reactions containing 3μL of template DNA, 3μL of 5X Colorless GoTaq Flexi buffer (Promega, USA), 1.2mM $MgCl_2$, 0.2mM dNTP mix, 0.08μg/μL of Bovine Serum Albumin (BSA), 0.2μM of each forward and reverse primer, and 1 unit of GoTaq G2 Flexi DNA Polymerase (Promega, USA). Thermocycling conditions for *S. polyrhiza* were: initial denaturing at 94°C for 5 min, followed by 34 cycles of: 1 min of denaturing at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C, followed by a final extension at 72°C for 10 min. For *L. minor*, touchdown PCR was employed with an initial denaturation of 94°C for 5 min, followed by five cycles of denaturation (94°C, 1 min), annealing (67°C, 1 min; decreasing by 1°C per cycle), and extension (72°C, 1 min). Then 25 cycles of 1 min at 94°C, 1 min at 63°C, and 2 min at 72°C, followed by a final extension at 72°C for 15 minutes. Primers were then optimized for annealing temperatures and $MgCl_2$ concentration (Table 1 and Table 2).

Fragment length analyses for all primers were conducted on ABI 3730 Genetic Analyzers (Applied Biosystems) at either the ETH Zurich Genetic Diversity Center (Switzerland), Keck DNA Sequencing Lab at Yale University (USA), or the University of Pittsburgh Genomics Research Core (USA), using either GeneScan™ 500 or 600 LIZ™ Dye Size Standards (Applied Biosystems). Allele calls were made using either Geneious (version 9.1.6, Kearse *et al* (2012)) or GeneMarker software (version 3.0.0, SoftGenetics, State College, Pennsylvania).

Results and discussion

We successfully developed 18 *L. minor* and 16 *S. polyrhiza* microsatellite primers (Table 1 and Table 2) which were tested on samples of duckweeds from Europe or Western Pennsylvania (USA). Some markers were more successful than others (Table 1 and Table 2). All markers amplified in some samples; of these, all

18 *L. minor* primers and 12 of the 16 *S. polyrhiza* primers amplified polymorphic loci, having more than one allele. Moreover, these polymorphic loci differed in product length and can be used in multiplex reactions to increase efficiency and lower genotyping costs. We also found that some loci were much more polymorphic than others. For *L. minor*, these included loci amplified by primers LmR.5.C, LmR.8.B, LmR.15.A, LmR.15.B, LmR.15.C and LmR.26.B, some of which showed high allele richness even when tested on only 28 samples (Table 1). For *S. polyrhiza* these included loci amplified by primers Sp.1467, Sp.7286, Sp.7814, and Sp.Pso31 (Table 2). Monomorphic loci may still be useful in different duckweed populations (Chapuis and Estoup, 2007). Many microsatellite loci also showed heterogeneity (Supplemental Tables S1 and S2), which helps make the primers more informative to distinguish genotypes. We note that some primers developed in one continent were not tested on samples from the other continent (see caption in Supplemental Tables S1 and S2); we suspect these primers will work across continents given patterns observed in the others, but this remains to be tested.

Comparing between species, we saw that *S. polyrhiza* has lower allelic and genotypic richness across most primers, although we also tested fewer samples of this species. This is consistent with our own recent large-scale sampling (Hobble et al. In preparation) as well as other studies using different genotyping methods, that similarly found low genetic diversity in *S. polyrhiza* (Bog *et al*, 2015; Xu *et al*, 2015; Feng *et al*, 2017). It has been hypothesized that this low genetic variation in *S. polyrhiza* is due to its low mutation rate (Xu *et al*, 2019). In addition, primers differed greatly in average observed heterozygosity, but species had similar mean heterozygosities (0.256 for *L. minor* and 0.283 for *S. polyrhiza*). Given that our sampling was designed to find unique genotypes (shallow and widespread) and not characterize populations, we limit our discussion of population genetic indices. The primers we developed can help researchers address various ecological and evolutionary questions as well as better identify and catalogue genotypes for the expanding applied uses of duckweed in bioremediation, biofuel production and as a forage crop.

Supplemental data

Supplemental Table S1: *Lemna minor* sample collection sites and allele lengths.
Supplemental Table S2: *Spirodela polyrhiza* sample collection sites and allele lengths.

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Author contributions

All authors contributed to testing, optimizing, and evaluating marker data, and contributed to reviewing the manuscript. JEK and MMT wrote the initial draft of the manuscript.

Conflict of interest statement

The authors have no conflicts of interest to report.

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