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Authors: Wani, Gowher A., Shah, Manzoor A., Reshi, Zafar A., Atangana, Alain R., and Khasa, Damase P.

Source: Applications in Plant Sciences, 2(7)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300099

Applications in Plant Sciences 2014 2(7): 1300099

PRIMER NOTE

cpDNA MICROSATELLITE MARKERS FOR *LEMNA MINOR* (ARACEAE): PHYLOGEOGRAPHIC IMPLICATIONS[1]

GOWHER A. WANI[2], MANZOOR A. SHAH[2,4], ZAFAR A. RESHI[2], ALAIN R. ATANGANA[3], AND DAMASE P. KHASA[3]

[2]Department of Botany, University of Kashmir, Srinagar-190 006, Jammu and Kashmir, India; and [3]Centre for Forest Research and Institute for Systems and Integrative Biology, Université Laval, Quebec GIV0A6, Canada

- *Premise of the study:* A lack of genetic markers impedes our understanding of the population biology of *Lemna minor.* Thus, the development of appropriate genetic markers for *L. minor* promises to be highly useful for population genetic studies and for addressing other life history questions regarding the species.
- *Methods and Results:* For the first time, we characterized nine polymorphic and 24 monomorphic chloroplast microsatellite markers in *L. minor* using DNA samples of 26 individuals sampled from five populations in Kashmir and of 17 individuals from three populations in Quebec. Initially, we designed 33 primer pairs, which were tested on genomic DNA from natural populations. Nine loci provided markers with two alleles. Based on genotyping of the chloroplast DNA fragments from 43 sampled individuals, we identified one haplotype in Quebec and 11 haplotypes in Kashmir, of which one occurs in 56% of the genotypes, one in 8%, and nine in 4%, respectively. There was a maximum of two alleles per locus.
- *Conclusions:* These new chloroplast microsatellite markers for *L. minor* and haplotype distribution patterns indicate a complex phylogeographic history that merits further investigation.

Key words: Araceae; chloroplast DNA; haplotype; *Lemna minor*; microsatellites.

The duckweed family encompasses 38 monocotyledonous species in four genera (Landolt, 1986; Les et al., 2002). Duckweeds had long been classified in their own family, the Lemnaceae, but are now considered to be members of the arum or aroid family (Araceae); the name Lemnaceae is therefore rapidly falling out of favor among taxonomists, who treat it as the subfamily Lemnoideae (Cabrera et al., 2008). *Lemna minor* L., which is known as common duckweed, is a small, free-floating, and fast-growing aquatic plant with a chromosome count of 40 (Blackburn, 1933). The species is distributed worldwide and often grows as blanket-like mats on the surface of still or slow-moving, nutrient-rich, fresh and brackish waters. *Lemna minor* represents a potential source of bioethanol (Xu et al., 2011) and can extract organic pollutants and toxic metals from waters, which makes it useful in remediation efforts (Alvarado et al., 2008; Wang et al., 2010). The species reproduces quickly through vegetative budding and doubles its biomass in two to seven days, depending upon culture conditions. Conditions affecting growth include the availability of nutrients and water temperature (Landolt, 1986; Brain and Solomon, 2007; Kanoun-Boulé et al., 2009). Notwithstanding its wide geographic range, *L. minor* displays a characteristically sporadic local distribution (Savile, 1956). In some parts of the world, such as the Kashmir Himalayas, this species has tended to become more invasive (Shah and Reshi, 2014).

Because of its widespread distribution, a monographic account of the Lemnaceae by Daubs (1965) puts *L. minor* in a "catch-all" category, as many herbarium specimens have been inadvertently labeled with this binomial but are actually other species. Development of appropriate molecular markers has therefore attained a special significance for correct taxonomic delineation of the species. Moreover, a lack of genetic markers impedes our understanding of the population biology and dynamics of *L. minor*. The development of such markers promises to yield important insights into the biology and biogeography of this species, with useful implications for understanding its invasiveness. Although Wang and Messing (2011) recently sequenced the chloroplast genomes from species in three different genera within the Lemnoideae (i.e., *Spirodela polyrhiza* (L.) Schleid., *Wolffiella lingulata* Hegelm., and *Wolffia australiana* (Benth.) Hartog & Plas) for systematic analysis, there are no studies so far on duckweed species using simple sequence repeat (SSR) or microsatellite markers. Thus, our objective was to develop cpDNA-based SSR markers for *L. minor* because such markers could provide a wealth of information for evolutionary and population genetic studies.

[1]Manuscript received 26 December 2013; revision accepted 11 February 2014.

The authors thank Sonah Huma for help in designing the primers and Marie-Ève Beaulieu (Institut de Biologie Intégrative et des Systèmes [IBIS], Université Laval) for technical assistance in the laboratory. We also acknowledge funding from the Canadian Bureau for International Education (CBIE) to G.A.W. and a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to D.P.K. Thanks are due to Dr. Bill Parisan for help in language editing.

[4]Author for correspondence: mashah75@yahoo.com

doi:10.3732/apps.1300099

TABLE 1. Nine polymorphic microsatellite markers used for optimization on *Lemna minor*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
L6	F: CAGCAGCAATAACAGAAGCG R: TGTCTGTGATTGGGGATTGA	$(A)_{10}$	51	297–301	Pr032067216
L7	F: CATTTTTCCCCACACTTGCT R: TGCATCCCAAACAATTTTCA	$(A)_{11}$	51	281–282	Pr032067217
L14	F: TTGTTCTCATGATCGGTCAAA R: GCCTTACCATGGCGTTACTC	$(A)_{10}$	51	295–299	Pr032067209
L16*	F: CGCATCAATCGAGGATACAC R: TTCCGACAACTTCAGGAGAGA	$(T)_{10}$…$(A)_{15}$	51	216–220 Exp.: 224	Pr032067210
L19	F: TGCTGGAAAAATAAGGTGGG R: CCTTTTGATTTGAGACCGGA	$(A)_{10}$	51	282–288 Exp.: 289	Pr032067211
L20	F: TTAATCAGGACCCGAATCCA R: AAAGTCGACGGATTTTCCTC	$(T)_{10}$	51	288–293	Pr032067212
L25	F: GGTTGCGCCATACATATCAA R: TGGTGACATAAGTCCCTCCC	$(T)_{10}$	51	215–217	Pr032067213
L29	F: TCCAGGACTCCGAAAAGGTA R: CAATGGGGAATTGGCTTTATT	$(A)_{10}$	51	257–270	Pr032067214
L35	F: CAAGAAGAACGGGTTGATCC R: GGATTCGAGCCATAGCACAT	$(T)_{10}$	51	204–205	Pr032067215

Note: Exp. = expected size; T_a = annealing temperature.
* Diagnostic marker; Quebec: allele size = 241; Kashmir: allele size = 237.

METHODS AND RESULTS

Individuals of *L. minor* were collected from five populations in the Kashmir Valley, India, and from three populations in Quebec, Canada, using a panel of five to seven individuals per population. The date and site of collection within each region, together with geographic coordinates of the sites, are given in Appendix 1; voucher specimens could not be collected due to lack of availability of suitable specimens. To develop SSR markers for *L. minor*, the chloroplast genome of *L. minor* was downloaded from the National Center for Biotechnology Information (NCBI) database. The PerlScript MIcroSAtelitte (MISA; http://pgrc.ipk-gatersleben.de/misa/) was used to identify microsatellites in the *L. minor* chloroplast genome. The SSR information that was generated by MISA was used for designing primers flanking the repeats. To design primers that flanked the microsatellite locus, two PerlScripts were used as interface modules for the program-to-program data interchange between MISA and the primer-designing software Primer3 (Rozen and Skaletsky, 2000). Primer pairs were designed from the flanking sequences of SSRs using primer3_core in batch mode via the p3_in.pl and p3_out.pl PerlScripts (Sonah et al., 2011). The primer-designing conditions were: 100–300 bp amplicon size, 60°C optimal annealing temperature, 20 bp optimal primer length, and 50% optimal GC content (Sonah et al., 2011). Three sets of primer pairs were designed for each SSR to provide alternatives if amplification was unsuccessful.

Genomic DNA was extracted by grinding 0.25 g of fresh leaf tissue in liquid nitrogen and by using a prewarmed cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle and Doyle, 1987). Thirty-three primer pairs were designed initially, synthesized, and tested on seven individuals from Kashmir and Quebec by running the PCR products in 1.5% agarose gel in 1× Tris-acetate/EDTA (TAE). PCR amplifications were carried out in total reaction volumes of 15 µL containing 50 ng of template DNA, 0.2 µM forward primer, 0.5 µM reverse primer, 1.5 mM dNTPs (Applied Biosystems/Life Technologies, Grand Island, New York, USA), 1× PCR buffer including $MgCl_2$ (10 mM Tris [pH 8.0], 50 mM KCl, and 50 mM ammonium sulphate; Sigma Aldrich, St. Louis, Missouri, USA), 0.5 µM fluorochrome (Applied Biosystems/Life Technologies), and 1 unit of *Taq* DNA polymerase (Sigma Aldrich). The thermal cycling profile was 4 min at 94°C; followed by 35 cycles of 94°C for 1 min, 51°C annealing for 1 min, and 72°C for 1 min; followed by a final extension of 72°C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gels in 1× Tris-borate/EDTA (TBE) buffer and visualized by ethidium bromide staining. To check for variability in *L. minor*, five to seven individuals from each of the different populations were amplified for each primer set. Amplicons were aligned using BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, California, USA) to determine the possible identity of haplotypes, and fragments were measured using an ABI PRISM 3130xL Analyzer (Applied Biosystems, Carlsbad, California, USA) and scored using Peak Scanner version 1.0 software (Applied Biosystems).

DNA samples that were obtained from 26 individuals of five *L. minor* populations in Kashmir and from 17 individuals of three populations in Quebec were screened against 33 primer pairs. We found nine polymorphic loci (Table 1) and 24 monomorphic loci (Appendix S1), which allowed the identification of 11 haplotypes in Kashmir and one haplotype in Quebec (Table 2). Of these 11 haplotypes, one occurs in 56% of the genotypes, one in 8%, and nine in 4% each. The number of alleles and unbiased estimates of haploid diversity are shown in Table 3. One intraspecific diagnostic locus (L16*) showed discriminating alleles between Kashmir and Quebec, and could be useful to determine whether individuals introduced outside of the native range are from similar or different source populations in the native range.

CONCLUSIONS

For the first time, we have developed and characterized nine polymorphic and 24 monomorphic cpDNA microsatellite markers for *L. minor*. We expect these markers to be useful for population genetic studies and the reconstruction of introduction history, as well as to facilitate the understanding of other life history questions regarding *Lemna* and related species.

LITERATURE CITED

ALVARADO, S., M. GUÉDEZ, M. P. LUÉ-MERÚ, G. NELSON, A. ALVARO, A. C. JESÚS, AND Z. GYULA. 2008. Arsenic removal from waters by bioremediation with the aquatic plants water hyacinth (*Eichhornia crassipes*) and lesser duckweed (*Lemna minor*). *Bioresource Technology* 99: 8436–8440.

BLACKBURN, K. B. 1933. Notes on the chromosomes of duckweeds (Lemnaceae) introducing the question of chromosome size. *Proceedings of the University of Durham Philosophical Society* 9: 84–90.

TABLE 2. Haplotypes of cpSSRs at nine polymorphic loci of *Lemna minor*.[a]

			Locus								
Site	Plant no.	Haplotype	L6	L7	L14	L16	L19	L20	L25	L29	L35
Kashmir	LK1-226	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK1-228	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK1-229	HK1	—	301	—	—	—	313	237	277	225
Kashmir	LK1-231	HK1	317	301	—	—	308	—	237	277	225
Kashmir	LK1-239	HK1	317	—	—	237	—	—	—	—	225
Kashmir	LK2-251	HK2	317	301	315	237	302	308	NA	NA	NA
Kashmir	LK2-252	HK1	—	—	NA	NA	NA	NA	237	290	225
Kashmir	LK2-253	HK1	—	—	—	237	308	313	237	290	225
Kashmir	LK2-254	HK1	317	301	319	237	308	—	—	—	225
Kashmir	LK2-255	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK2-258	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK4-301	HK3	317	302	315	237	302	308	235	290	224
Kashmir	LK4-303	HK4	317	—	315	237	—	308	235	290	224
Kashmir	LK4-304	HK5	317	301	315	237	—	308	235	290	224
Kashmir	LK4-305	HK6	317	302	319	237	308	313	237	290	225
Kashmir	LK4-306	HK7	317	301	—	237	308	308	237	290	224
Kashmir	LK5-326	HK8	321	302	315	237	302	308	235	290	224
Kashmir	LK5-327	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK5-328	HK9	317	301	—	237	302	308	235	290	224
Kashmir	LK5-329	HK1	—	301	—	237	308	313	237	290	225
Kashmir	LK5-330	HK10	317	301	319	237	308	313	235	290	225
Kashmir	LK7-376	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK7-378	HK1	317	301	319	237	308	313	237	290	225
Kashmir	LK7-382	HK1	—	—	—	—	308	313	—	290	225
Kashmir	LK7-385	HK1	—	301	—	—	308	313	237	290	225
Kashmir	LK7-388	HQ1	—	301	—	—	—	313	237	290	225
Quebec	LQ1-07	HQ1	317	301	319	241	308	313	237	—	225
Quebec	LQ1-15	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ1-23	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ1-26	HQ1	317	301	319	241	308	313	237	290	225
Quebec	LQ1-30	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ2-1-1	HQ1	317	301	319	241	308	313	237	NA	NA
Quebec	LQ2-1-3	HQ1	NA	NA	NA	NA	NA	NA	NA	290	225
Quebec	LQ2-1-4	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ2-2-2	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ2-2-4	HQ1	317	301	319	241	308	313	237	290	225
Quebec	LQ2-4-1	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ3-2-2	HQ1	317	301	319	241	308	313	237	290	225
Quebec	LQ3-5-1	HQ1	—	301	319	241	308	313	NA	NA	NA
Quebec	LQ3-6-2	HQ1	NA	NA	NA	NA	NA	NA	237	290	225
Quebec	LQ3-7-2	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ3-10-3	HQ1	317	301	319	NA	308	313	237	NA	225
Quebec	LQ3-18-2	HQ1	317	301	319	241	308	313	237	290	225

Note: — = no peak; NA = not available.

[a] Number of haplotypes in Kashmir = 10; number of haplotypes in Quebec = 1.

TABLE 3. Chloroplast microsatellite genetic diversity values for nine polymorphic loci of *Lemna minor*.

	Kashmir (n = 26)		Quebec (n = 17)	
Locus	A	h_{unb}	A	h_{unb}
L6	2	0.1053	1	0.0000
L7	2	0.2571	1	0.0000
L14	2	0.4762	1	0.0000
L16	1	0.0000	1	0.0000
L19	2	0.3368	1	0.0000
L20	2	0.4545	1	0.0000
L25	2	0.4156	1	0.0000
L29	2	0.1660	1	0.0000
L35	2	0.3800	1	0.0000
Mean	1.709	0.2879	1	0.0000

Note: A = number of alleles; h_{unb} = unbiased haploid diversity; n = sample size.

BRAIN, R. A., AND K. R. SOLOMON. 2007. A protocol for conducting 7-day daily renewal tests with *Lemna gibba*. *Nature Protocols* 2: 979–987.

CABRERA, L. I., G. A. SALAZAR, M. W. CHASE, S. J. MAYO, J. BOGNER, AND P. DAVILA. 2008. Phylogenetic relationships of aroids and duckweeds (Araceae) inferred from coding and noncoding plastid DNA. *American Journal of Botany* 95: 1153–1165.

DAUBS, E. H. 1965. A monograph of Lemnaceae. Illinois Biological Monograph, vol. 34. University of Illinois Press, Urbana, Illinois, USA.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

KANOUN-BOULÉ, M., J. A. F. VICENTE, C. NABAIS, M. N. V. PRASAD, AND H. FREITAS. 2009. Ecophysiological tolerance of duckweeds exposed to copper. *Aquatic Toxicology* 91: 1–9.

LANDOLT, E. 1986. The family of Lemnaceae: A monographic study. Geobotanical Institute of the ETH, Stiftung Rübel, Zürich, Switzerland.

Les, D. H., D. J. Crawford, E. Landolt, J. D. Gabel, and R. T. Kimbell. 2002. Phylogeny and systematics of Lemnaceae, the duckweed family. *Systematic Botany* 27: 221–240.

Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

Savile, D. B. O. 1956. Known dispersal rates and migratory potentials as clues to the origin of the North American biota. *American Midland Naturalist* 56: 434–453.

Shah, M. A., and Z. Reshi. 2014. Characterization of alien aquatic flora of Kashmir Himalaya: Implications for invasion management. *Tropical Ecology* 55: 143–157.

Sonah, H., K. D. Deshmukh, A. Sharma, V. P. Singh, D. K. Gupta, R. N. Gacche, J. C. Rana, et al. 2011. Genome-wide distribution and organization of microsatellites in plants: An insight into marker development in *Brachypodium. PLoS ONE* 6: e21298.

Wang, Y., Y. Zang, B. Yang, and S. Chen. 2010. Characterization of *SSU5C* promoter of a *rbcS* gene from duckweed (*Lemna gibba*). *Molecular Biology Reports* 38: 2563–2568.

Wang, W., and J. Messing. 2011. High-throughput sequencing of three *Lemnoideae* (Duckweeds) chloroplast genomes from total DNA. *PLoS ONE* 6: e24670.

Xu, J., W. Cui, J. J. Cheng, and A. M. Stomp. 2011. Production of high-starch duckweed and its conversion to bioethanol. *Biosystems Engineering* 110: 67–72.

Appendix 1. Region, site, and date of collection of *Lemna minor* in India and Canada together with geographical coordinates of the sites.

Region	Collection date	Collection site	Population code	Altitude (m)	Geographic coordinates
Kashmir, India	27 May 2012	Forshore	LK1	1596.2	34.08°N, 74.51°E
Kashmir, India	27 May 2012	Ashaibagh	LK2	1578.9	34.06°N, 74.50°E
Kashmir, India	28 May 2012	Rangharstop	LK4	1583.4	34.06°N, 74.48°E
Kashmir, India	29 May 2012	Kuhumus Wullar	LK5	1580	34.20°N, 74.36°E
Kashmir, India	6 Feb. 2012	Shalimar	LK7	1583.4	34.08°N, 74.52°E
Quebec, Canada	18 Sept. 2012	Lac St. Francais	LQ1	46	45.01°N, 74.45°W
Quebec, Canada	5 Oct. 2012	Riv-sud	LQ2	99.2	46.74°N, 71.24°W
Quebec, Canada	5 Oct. 2012	Université Laval	LQ3	98	46.47°N, 71.17°W