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PRIMER NOTE

MICROSATELLITE MARKERS FOR *LEUCOBRYUM BONINENSE* (LEUCOBRYACEAE), ENDEMIC TO THE BONIN ISLANDS, JAPAN[1]

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- *Premise of the study:* Microsatellite primers were developed for *Leucobryum boninense*, endemic to the Bonin Islands, Japan, to investigate its level of genetic diversity and population genetic structure.
- *Methods and Results:* Using next-generation sequencing, 21 primer sets were developed, among which nine loci were polymorphic in the populations of the Bonin Islands. Among these polymorphic loci, the number of alleles per locus ranged from two to 10 (mean = 3.444) and the expected heterozygosity ranged from 0.066 to 0.801 (mean = 0.338).
- *Conclusions:* These results indicate the utility of the nine microsatellite markers that we developed for population genetic studies of *L. boninense*.

Key words: Bonin (Ogasawara) Islands; Leucobryaceae; *Leucobryum boninense*; microsatellite; Musci; next-generation sequencing.

The Bonin (Ogasawara) Islands are typical oceanic islands, located in the northwestern Pacific Ocean, approximately 1000 km directly south of Tokyo, Japan. The flora of the Bonin Islands is characterized by a high degree of endemism, including ca. 40% of native vascular plant species (Kobayashi, 1978) and ca. 5% of native bryophyte species (Furuki et al., 1991). *Leucobryum boninense* Sull. & Lesq. (Leucobryaceae, Musci) is one of the endemic bryophyte species in the Bonin Islands. This species is dioecious and does not produce dwarf male plants (Yamaguchi, 1993). Our preliminary molecular phylogenetic analyses revealed that mosses from the Ogasawara Islands (Chichi-jima, Haha-jima, and Ani-jima) and Kitaiwo-jima made a clade, although a 1-bp sequence difference was observed in the *rbcL* gene between mosses from these two island groups (Oguri et al., unpublished data).

Microsatellite regions are powerful molecular markers with high degrees of polymorphism and codominant inheritance. However, no microsatellite markers have been developed for the genus *Leucobryum* Hampe or for *L. boninense*, and there is no information on the genetic diversity of this species. In this study, a set of 21 microsatellite markers were developed for *L. boninense* using next-generation sequencing, which should provide a powerful tool for population genetic analyses of the species.

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METHODS AND RESULTS

Microsatellite primers were developed in *L. boninense* through Roche 454 GS Junior pyrosequencing of enriched DNA libraries. Total DNA was extracted from *L. boninense* gametophytes (voucher: EO294, Appendix 1) using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). DNA was shotgun sequenced with a Roche 454 GS Junior using a GS Junior Titanium Sequencing Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. A DNA sample was sequenced with one-fourth of the run using Multiplex Identifier (MID) adapters (Margulies et al., 2005). A total of 11,905 reads (average length = 431 bp) were generated from *L. boninense* genomic DNA. Microsatellites and primer designs from the large DNA sequence sets produced by pyrosequencing were identified using QDD version 2.1 (Meglécz et al., 2010). A total of 152 perfect microsatellite sequences with repeat motifs of 2–6-bp nucleotides were selected for further analysis from a total of 11,905 reads. Our selection criterion was a minimum of six repeats for all motifs. Sequence similarity detection and establishing of contigs followed the procedure described by Takayama et al. (2011). PCR primers were designed using Primer3 (Rozen and Skaletsky, 2000) implemented in QDD with the following criteria: (1) PCR product lengths ranged from 90 to 500 bp; (2) flanking regions contained ≤5 repetitions of di- to hexanucleotide motifs; and (3) length, annealing temperature, and the percentage of primer GC content were optimized between 18 and 27 bp, 57°C and 63°C, and 20% and 80%, respectively. A total of 35 primer pairs having >6 repeats were designed. For each primer set, the sequence of the forward primer was redesigned by adding a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) to its 5′ end using the method of Schuelke (2000).

A total of 16 moss samples from one population of *L. boninense* on Chichi-jima (27.0747°N, 142.2228°E), 16 samples from one population on Haha-jima (26.6794°N, 142.1570°E), nine samples from one population on Ani-jima (27.1220°N, 142.2125°E), and 20 samples from one population on Kitaiwo-jima (25.2620°N, 141.1660°E) were used in the amplification tests and for candidate marker characterization. Voucher specimens were deposited either in Makino Herbarium, Tokyo Metropolitan University (MAK), Tokyo, or the Herbarium of Hiroshima University (HIRO), Hiroshima (see Appendix 1). Total DNA samples from the gametophytes used for amplification tests were extracted using a cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). PCR amplification was performed in a final volume of 5 μL using the standard protocol of QIAGEN Multiplex PCR Kit (QIAGEN) with single-plex PCR (one primer pair per reaction). Three primers were used in

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each reaction: 0.2 μM reverse primer, 0.1 μM FAM-labeled M13 primer, and 0.01 μM forward primer. PCR amplification included initial denaturation at 95°C for 15 min, followed by 30 cycles of 94°C for 0.5 min, 57°C for 1.5 min, and 72°C for 1 min, and a final extension at 60°C for 30 min. PCR products were analyzed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). PCR product sizes were determined by comparisons with the GeneScan 500 LIZ Size Standard (Applied Biosystems) using GeneScan analysis software (Applied Biosystems). Results were analyzed using GeneMapper version 4.0 (Applied Biosystems).

Twenty-one of the 35 primer pairs that we developed above are shown in Table 1. The remaining 14 primer pairs did not amplify. For each locus, the number of alleles per locus (A) and the expected heterozygosity (H_e) were calculated using the Excel Microsatellite Toolkit (Park, 2001). H_e was calculated from haploid genotype data. These results are summarized in Table 2. For all 21 loci, except for locus Lb_34, DNA bands were successfully amplified using the samples from all four populations of *L. boninense*. For locus Lb_34, bands were amplified for most samples from the Ani-jima population and for half of the samples from the Chichi-jima population, but no DNA bands were amplified for any samples from the Haha-jima and the Kitaiwo-jima populations. Nine loci (Lb_10, Lb_11, Lb_17, Lb_20, Lb_25, Lb_27, Lb_31, Lb_34, and Lb_35) among these 21 loci were polymorphic, and the remaining 12 loci were monomorphic for the samples from all four populations of *L. boninense*. Nine, four, and five of the nine loci were polymorphic in the populations of Chichi-jima, Haha-jima, and Ani-jima, respectively. In contrast, only one locus, Lb_20, was polymorphic in the population of Kitaiwo-jima. For the polymorphic loci, A ranged from two to 10 (mean = 3.444) and H_e ranged from 0.066 to 0.801 (mean = 0.338).

CONCLUSIONS

Using next-generation sequencing, we developed a set of new PCR primers for 21 microsatellite loci for *L. boninense* (Leucobryaceae, Musci), which is endemic to the Bonin Islands. Nine of these loci were polymorphic in all four populations of the species we tested. Therefore, these nine polymorphic markers should be suitable for population genetic studies of *L. boninense*.

TABLE 1. Characteristics of 21 microsatellite markers developed for *Leucobryum boninense*.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
Lb_10*	F: CACGACGTTGTAAAACGACTTACAAGTGCGGTGCTCTGA R: CGTAGCGTCTACCAATTCCG	$(AT)_6$	367–369	57	AB738806
Lb_11*	F: CACGACGTTGTAAAACGACACGACTCTCACACCTCGCTC R: CTGTCAGTGTGGTTTGCTGC	$(AC)_6$	381–383	57	AB738807
Lb_17*	F: CACGACGTTGTAAAACGACCAAGATGCATGCAGGTTTGA R: GGCATCGGTAGTGAATCGTC	$(AG)_7$	299–301	57	AB738808
Lb_20*	F: CACGACGTTGTAAAACGACCAGCCACACCTCGTTATTCC R: CCATGGGTAGATCGAATGGA	$(AAG)_7$	367–382	57	AB738809
Lb_25*	F: CACGACGTTGTAAAACGACTTGCCAACCAATAAGTGCAA R: TCATCCATTACAAGCATGAATAA	$(AT)_8$	203–210	57	AB738810
Lb_27*	F: CACGACGTTGTAAAACGACTCCTGTCGACACAGTAGCCA R: GGTCGCATAACCTGCAAGA	$(AAT)_8$	205–212	57	AB738811
Lb_31*	F: CACGACGTTGTAAAACGACGCCTGCATCCACTTTGTAGG R: AACGAAGAGCAGCAGAAACG	$(TG)_{10}$	416–430	57	AB738812
Lb_34*	F: CACGACGTTGTAAAACGACTTTCAACCTACTTAAATCAACAAGC R: TTGGTTGCTTTGTCCATGCT	$(TCA)_{13}$	292–295	57	AB738813
Lb_35*	F: CACGACGTTGTAAAACGACTTTCAACCTTTGTTGATGAGGTT R: CCAATCTGAACCTTTACACATCA	$(ATT)_{16}$	277–317	57	AB738814
Lb_4	F: CACGACGTTGTAAAACGACTCGAGGTTGCAAGGTACCAG R: AGAGAACATTGTGGAGGCCA	$(GTG)_6$	161	57	AB738815
Lb_9	F: CACGACGTTGTAAAACGACATCAACTGGGTTGCTCCTCA R: ACAACTAGAGCACAAGCGGC	$(GA)_6$	212	57	AB738816
Lb_12	F: CACGACGTTGTAAAACGACAGCAAATCATCCGACTCCAA R: AAGGAAGGAGAAGGCGACAC	$(TCC)_6$	130	57	AB738817
Lb_13	F: CACGACGTTGTAAAACGACAAACAGTTTCAAGGGCAAAGC R: AAACCCTTAAGTCGAGGCCA	$(AT)_7$	117	57	AB738818
Lb_15	F: CACGACGTTGTAAAACGACACATCAACACCTTGTATGAGATAAAC R: TGTTTATCTTCACGCCGACC	$(AGA)_7$	114	57	AB738819
Lb_16	F: CACGACGTTGTAAAACGACATCGAGTGGGAAGACGGG R: TACGCTGCTGCACAAACACT	$(GA)_7$	222	57	AB738820
Lb_19	F: CACGACGTTGTAAAACGACCGTCCAGATAACACATACAAGTCAAA R: GCAGGACTGGGTATGTATCTGA	$(AT)_7$	158	57	AB738821
Lb_21	F: CACGACGTTGTAAAACGACTCTCAACAAGGTGGCCCTAA R: ATTTGGGAGGTGGGATTTGT	$(AC)_7$	108	57	AB738822
Lb_22	F: CACGACGTTGTAAAACGACTGGCCAAACGAAGCCTTTAT R: TCTAACCACAATTGCCTTGG	$(TA)_7$	362	57	AB738823
Lb_24	F: CACGACGTTGTAAAACGACCAAACTGCGCTACATCCCTC R: CATCTTGCATTGTAGATCCCTT	$(TA)_8$	383	57	AB738824
Lb_28	F: CACGACGTTGTAAAACGACCGTAATTCCAATCGCAGAGC R: CAACCAGACACGGTGATTGA	$(CAA)_8$	114	57	AB738825
Lb_32	F: CACGACGTTGTAAAACGACCGTGCAGCTTGTATTGCTTG R: TTATTAGACCTTGGCTGGAAAT	$(AT)_{11}$	241	57	AB738826

Note: T_a = annealing temperature.
* Indicates polymorphic loci.

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TABLE 2. Results of primer screening for 21 microsatellite markers in four populations of *Leucobryum boninense*.

	Chichi-jima			Haha-jima			Ani-jima			Kitaiwo-jima			Total		
Locus	*N*	*A*	H_e	*N*	*A*	H_e	*N*	*A*	H_e	*N*	*A*	H_e	*N*	*A*	H_e
Lb_10	15	2	0.133	16	2	0.125	9	1	0.000	20	1	0.000	60	2	0.066
Lb_11	16	2	0.233	16	2	0.125	9	1	0.000	20	1	0.000	61	2	0.095
Lb_17	14	2	0.264	15	1	0.000	9	1	0.000	20	1	0.000	58	2	0.070
Lb_20	15	2	0.248	15	2	0.133	9	1	0.000	19	3	0.205	58	5	0.473
Lb_25	14	2	0.495	16	1	0.000	9	2	0.222	20	1	0.000	59	3	0.189
Lb_27	14	2	0.538	16	1	0.000	9	3	0.750	20	1	0.000	59	3	0.367
Lb_31	14	2	0.527	16	1	0.000	9	2	0.500	20	1	0.000	59	2	0.508
Lb_34	8	2	0.250	—	—	—	7	2	0.571	—	—	—	15	2	0.476
Lb_35	14	4	0.692	15	2	0.248	9	5	0.722	20	1	0.000	58	10	0.801
Mean	14	2.222	0.376	16	1.500	0.079	9	2.000	0.307	20	1.250	0.026	54	3.444	0.338

Note: *A* = number of alleles per locus; H_e = expected heterozygosity; *N* = sample size.

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APPENDIX 1. Voucher specimens for samples of *Leucobryum boninense* used in this study. Voucher specimens of samples from Ani-jima are deposited in Makino Herbarium, Tokyo Metropolitan University (MAK), Tokyo, and samples from Chichi-jima, Haha-jima, and Kitaiwo-jima are deposited in the Herbarium of Hiroshima University (HIRO), Hiroshima.

Taxon	Collection locality	Voucher no.
L. boninense	Chichi-jima	SU11029, SU11030, SU11031, SU11034, SU11035, SU11037, SU11038, SU11039, SU11040, SU11041, SU11042, SU11043, SU11044, SU11045, SU11046, SU11047
	Haha-jima	TY29716, TY29717, TY29718, TY29720, TY29721, TY29722, TY29723, TY29724, TY29725, TY29727, TY29728, TY29729, TY29730, TY29731, TY29732, TY29734
	Ani-jima	EO293, EO295, EO296, EO297, EO298, EO299, EO300, EO301, EO302
	Kitaiwo-jima	SU11645, SU11646, SU11647, SU11648, SU11649, SU11650, SU11651, SU11652, SU11653, SU11654, SU11655, SU11656, SU11657, SU11658, SU11659, SU11660, SU11661, SU11662, SU11663, SU11664

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