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Primer Note

Development of EST-derived microsatellite markers in the aquatic macrophyte *Ranunculus bungei* (Ranunculaceae)[1]

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- *Premise of the study:* Microsatellite or simple sequence repeat (SSR) markers were developed to investigate the influence of ecological factors on gene flow and spatial genetic structuring of the submerged plant *Ranunculus bungei* (Ranunculaceae), which is regarded as an important species for understanding how plants adapt to an aquatic environment.
- *Methods and Results:* Twenty-two microsatellite loci were identified from an expressed sequence tag (EST) library. The number of alleles per locus ranged from one to five, and the expected heterozygosity varied from 0.0 to 0.5 in four Chinese populations of *R. bungei*. Fourteen loci were polymorphic and significantly deviated from Hardy–Weinberg equilibrium. All of the loci were found to be amplifiable in two other species of *Ranunculus* section *Batrachium*, and cross-amplification in six riparian and aquatic species of Ranunculaceae was also partially successful.
- *Conclusions:* These novel EST-SSR markers will be useful for ecological and evolutionary studies of *R. bungei* as well as related species.

Key words: aquatic plant; genetic diversity; microsatellite; Ranunculaceae; *Ranunculus bungei*.

Ranunculus bungei Steud. (section *Batrachium* DC., Ranunculaceae) is a perennial submerged plant that can proliferate vegetatively through rhizomes or sexually via selfing or outcrossing (Cook, 1966). *Ranunculus bungei* is widely distributed in heterogeneous environments within the temperate and alpine regions of China and is significant for studies of the adaptation to aquatic habitats in angiosperms (Chen et al., 2015). In recent years, investigations have been carried out to examine genetic variation and population structure in *R. bungei* with intersimple sequence repeat (ISSR) markers or chloroplast noncoding spacers (Wang et al., 2010; Chen et al., 2014), but these markers are less powerful in studies on reproductive system, hybridization, patterns of gene flow, and fine-scale population structure. The development of suitable markers can provide a better understanding of the evolutionary progress and the underlying ecological factors of *R. bungei* and its related species.

Microsatellite or simple sequence repeat (SSR) markers are molecular markers with many desirable genetic attributes (e.g., codominant inheritance and hypervariability), which have been used to reveal genetic patterns in a wide variety of species (Kalia et al., 2011). For clonal plants, estimation of genetic variation is often biased with markers of low discriminatory ability (Arnaud-Haond et al., 2005); therefore, genetic studies on aquatic macrophytes, which are characterized by limited sexual proliferation (Barrett et al., 1993), should be assessed using appropriate polymorphic markers. Although a large number of SSR loci for *Ranunculus* L. species have been identified (e.g., Noel et al., 2005; Matter et al., 2012), we found that cross-species amplification was rarely successful in *R. bungei* based on preliminary experiments. Therefore, we developed 22 novel EST-SSR markers from *R. bungei* for use in investigations of population and landscape genetics of this widely distributed submerged species.

METHODS AND RESULTS

A mixture of tissues from roots, stems, and leaves was used for the transcriptome sequencing of *R. bungei*, conducted by Chen et al. (2015) using the Illumina HiSeq 2000 sequencer (Illumina, San Diego, California, USA). A total of 5,312,841 clean reads of *R. bungei* deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession no. SRR1822529; Chen et al., 2015) were used for de novo transcriptome assembly using Trinity version 2.1.0 with default parameters (Grabherr et al., 2011). The longest sequence was chosen for transcripts with several isoforms, as identified with a perl script (available at https://github.com/jinweiwu/perl/blob/master/extract). The MIcroSAtellite identification tool (MISA) Perl script (Thiel et al., 2003) was then used to screen for microsatellite motifs from total unigenes, and the minimum number of each type of repeat was set to six. MISA recovered a total of 9903 SSR motifs for *R. bungei*, and 50 unigenes were randomly chosen for the EST-SSR development.

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PCR primer design for the targeted unigenes was performed with Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). An initial evaluation of the primers was facilitated in 20 individuals randomly selected from four Chinese populations of *R. bungei* (Appendix 1), and 22 primer pairs showing unique products ranging from 100–500 bp were individually labeled with the fluorescent dyes 6-FAM or HEX (Table 1). Characterization of the EST-SSR loci was estimated in the four populations of *R. bungei*, with 22, 20, 15, and 16 individuals, respectively (Appendix 1). Genomic DNA was extracted from the freeze-dried leaves of *R. bungei* individuals using the DNAsecure Plant Kit (Tiangen Biotech, Beijing, China). PCR amplifications were performed in 20-μL reaction mixtures containing 1.5 μL of genomic DNA (~30 ng/μL), 0.5 μL of each primer (10 μM), and 10 μL 2× Master PCR Mix (Tiangen Biotech). Microsatellites were amplified under the following PCR conditions: a 5-min initial denaturation step at 95°C; followed by 30–35 cycles of 30 s at 95°C, 30 s at 50–58°C (Table 1), and 1 min at 72°C; and a final extension at 72°C for 7 min. PCR products differed in fluorescent label or length (>80 bp) and were multiplexed and analyzed on the ABI 3730XL sequencer (Applied Biosystems, Foster City, California, USA) with GeneScan 500 LIZ Size Standard (Applied Biosystems). Microsatellite genotyping was performed using GeneMarker version 1.5 software (SoftGenetics, State College, Pennsylvania, USA). The number of alleles, observed and expected heterozygosities, and deviations from Hardy–Weinberg equilibrium (HWE) at each locus were estimated using GenAlEx 6.5 (Peakall and Smouse, 2012). Linkage disequilibrium of locus pairs was tested using Arlequin version 3.5.1.3 (Excoffier et al., 2005).

Cross-species amplification was conducted in two other species of *Ranunculus* section *Batrachium* (*R. aquatilis* L. var. *eradicatus* Laest. [n = 11] and *R. trichophyllus* Chaix ex Vill. [n = 14]), as well as six riparian and aquatic species of Ranunculaceae (*R. cheirophyllus* Hayata [n = 5], *R. natans* C. A. Mey. [n = 8], *Halerpestes tricuspis* (Maxim.) Hand.-Mazz. [n = 12], *H. ruthenica* (Jacq.) Ovcz. [n = 6], *Caltha palustris* L. [n = 3], and *C. natans* Pall. [n = 5]) (Appendix 1).

The characteristics of 22 EST-SSR loci are presented in Table 1. Fourteen loci were polymorphic, two of which were fixed for different alleles in multiple populations (Table 2). The loci BatrB6, BatrB9, and BatrB12 showed the highest number of alleles (five), and eight loci were monomorphic among all individuals (Table 2). The expected and observed heterozygosity ranged from 0.0 to 0.5 and 0.0 to 1.0 per locus, and all polymorphic loci showed significant deviation from HWE (Table 2). Significant linkage disequilibrium (P < 0.05) was observed among seven locus pairs in four *R. bungei* populations (BatrB5 and BatrB6, BatrB6 and BatrB10, BatrB6 and BatrB12, BatrB9 and BatrB15, BatrB10 and BatrB13, BatrB10 and BatrB15, and BatrB13 and BatrB15). The deviation from

Table 1. Characteristics of 22 EST-SSR markers developed in *Ranunculus bungei*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye	GenBank accession no.	Putative function [Organism]	*E*-value
BatrB1	F: GCAGTTGCCATAGATACC R: CAGGGAATGGAAATAGG	$(TC)_7$	418–450	54	HEX	KY748028	—	—
BatrB2	F: GCAAAGGGTAAGACTGCTAT R: ATCAAGTTCCGATTCTGGTT	$(GT)_7$	408–410	52	6-FAM	KY748029	ABC transporter G family member 6-like [*Vitis vinifera*]	8e-06
BatrB3	F: TCCATTTCCACGGCTC R: AACGCCAGAGCATCCAAA	$(TTC)_6$	385	52	6-FAM	KY748030	—	—
BatrB4	F: AAGGCATACAAATCAAACTC R: TTTCACCATCATCCACCT	$(AAT)_6$	465	52	6-FAM	KY748031	—	—
BatrB5	F: AATTCTGCTGCCCCTAT R: TACTTCTTCTGCCTTGCTT	$(GA)_7$	465–472	58	6-FAM	KY748032	—	—
BatrB6	F: CAGGGACTGGACAGATACAC R: CTCATAGGAGACGGTTGGT	$(CAG)_6$	345–366	56	HEX	KY748033	Spt20 domain-containing protein [*Cephalotus follicularis*]	1e-19
BatrB7	F: CAGAGATAAGCCTGTGAAT R: ATCCATCTAAAGCCCACT	$(TG)_6$	394	50	HEX	KY748034	Protein NRT1/PTR FAMILY 4.5-like [*Nelumbo nucifera*]	1e-43
BatrB8	F: AGAAGAGGAGTGACGGAGAT R: GCAAGAAACATGCCAAAA	$(AAG)_6$	490	54	HEX	KY748035	Dyskerin-like protein [*Cynara cardunculus*]	2e-63
BatrB9	F: ACCTGGTGATCTTGAAGTAAA R: CTAATCCGAAACAGTGTATCTAA	$(GA)_9$	322–349	51	HEX	KY748036	—	—
BatrB10	F: GCCAAGCTCTTCTGCTCT R: GTGTCTTTGATTGATTTACCG	$(AG)_{10}$	297–313	54	6-FAM	KY748037	Hypothetical protein CICLE_v10031149mg [*Citrus clementina*]	4e-80
BatrB11	F: TAGATGAAGAACTAGGGCAAA R: GCAAGCGAAGAAACCA	$(GA)_7$	143–171	50	6-FAM	KY748038	Plastidic pyruvate kinase beta subunit 1 isoform 3 [*Theobroma cacao*]	5e-57
BatrB12	F: GCAGCGGAGTAAAACCT R: CATTACAAACATACCAGCAT	$(TAT)_{11}$	172–193	54	HEX	KY748039	MFP1 attachment factor 1 [*Beta vulgaris*]	2e-35
BatrB13	F: GCTTCTATTCTACCCTTGTTC R: GCAGCACCTCCTACTTCG	$(AG)_7$	107–109	56	6-FAM	KY748040	40S ribosomal protein S15-like [*Asparagus officinalis*]	6e-97
BatrB14	F: ATTCCAAAGAGCCAGCG R: TGAACAAGCGAAAGGTAGC	$(AG)_6$	350	58	6-FAM	KY748041	3-ketoacyl-CoA synthase [*Eranthis hyemalis*]	4e-15
BatrB15	F: CAGATGGGTACGAGGTTGG R: CAGATTGTATGGGATTTGTGAA	$(ACC)_6$	288–300	56	HEX	KY748042	Heterogeneous nuclear ribonucleoprotein H2 [*Malus domestica*]	2e-104
BatrB16	F: GGAAATGGCTGGCTGATA R: GATTCGGGAAGAGGTGGT	$(CTG)_{13}$	453–459	54	HEX	KY748043	Protein-tyrosine sulfotransferase [*Amborella trichopoda*]	5e-61
BatrB17	F: CCAAGGCACCAGTTTCAG R: TTGTTGTGGAGATGGACGA	$(TGG)_6$	430–445	54	6-FAM	KY748044	RNA-binding protein 38 [*Fragaria vesca*]	3e-65
BatrB18	F: ATCGCATCTCCATCGTTA R: GCAGTAGACATCCCAGC	$(TCA)_7$	426	54	6-FAM	KY748045	Zeta-carotene desaturase, chloroplastic/chromoplastic [*Ziziphus jujube*]	3e-106
BatrB19	F: CGAGAAGGAAACCCGTCAT R: AACATTGTGGAGCACCAGATT	$(CAC)_7$	380	56	HEX	KY748046	Heterogeneous nuclear ribonucleoprotein 1-like [*Elaeis guineensis*]	2e-84
BatrB20	F: CCCTTCCCTTGTGCTTG R: GAATGCCCAGTTAGCCC	$(CAC)_6$	163–172	54	HEX	KY748047	—	—
BatrB21	F: CAAAAGGACTTGGAGACG R: GTGGTGTTCAGAGCGATT	$(TC)_{13}$	466–471	52	6-FAM	KY748048	Ethylene-responsive transcription factor 4-like [*Nelumbo nucifera*]	7e-08
BatrB22	F: TACATCACCCTGTCTGAATAA R: ACAAGACCCTTTGGAAAT	$(ATT)_6$	321	51	6-FAM	KY748049	Hypothetical protein PHAVU_007G198500g [*Phaseolus vulgaris*]	2e-105

Note: T_a = annealing temperature.

TABLE 2. Results of initial primer screening in four populations of *Ranunculus bungei*.[a]

	Maduo (N = 22)			Dingri (N = 20)			Baishan (N = 15)			Tongliao (N = 16)			Total	Mean	
Locus	A	H_e[b]	H_o	A	H_e[b]	H_o	A	H_e[b]	H_o	A	H_e[b]	H_o	A	H_e	H_o
BatrB1	3	0.654***	0.363	1	0.000	0.000	3	0.353*	0.100	3	0.553	0.429	4	0.390	0.223
BatrB2	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.000	0.000
BatrB3	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB4	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB5	2	0.406	0.273	1	0.000	0.000	2	0.521**	0.900	2	0.349	0.429	3	0.319	0.401
BatrB6	1	0.000	0.000	2	0.358	0.450	3	0.574*	1.000	2	0.516**	0.929	5	0.362	0.595
BatrB7	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB8	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB9	1	0.000	0.000	2	0.142	0.150	2	0.521**	0.900	2	0.519***	1.000	5	0.295	0.513
BatrB10	2	0.359***	0.000	1	0.000	0.000	2	0.526**	1.000	2	0.519***	1.000	4	0.351	0.500
BatrB11	3	0.246	0.273	2	0.492*	0.700	1	0.000	0.000	1	0.000	0.000	3	0.185	0.243
BatrB12	3	0.280*	0.136	2	0.513	0.500	2	0.526**	1.000	2	0.495*	0.786	5	0.453	0.606
BatrB13	1	0.000	0.000	1	0.000	0.000	2	0.526**	1.000	2	0.519***	1.000	2	0.261	0.500
BatrB14	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB15	2	0.500***	1.000	2	0.500***	1.000	2	0.500**	1.000	2	0.500***	1.000	2	0.500	1.000
BatrB16	1	0.000	0.000	1	0.000	0.000	2	0.526**	1.000	2	0.519***	1.000	3	0.261	0.500
BatrB17	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.000	0.000
BatrB18	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB19	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
BatrB20	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	3	0.606**	1.000	3	0.151	0.250
BatrB21	3	0.534	0.364	1	0.000	0.000	1	0.000	0.000	2	0.516**	0.929	3	0.262	0.323
BatrB22	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals of each population.

[a] Locality and voucher information are available in Appendix 1.

[b] Significant deviations from Hardy–Weinberg equilibrium: * represents significance at the 5% nominal level; ** represents significance at the 1% nominal level; *** represents significance at the 0.1% nominal level.

HWE and significant linkage disequilibrium could be explained by the small population size, inbreeding, and clonal reproduction in *R. bungei*. All of the loci were amplified successfully in two *Ranunculus* species from section *Batrachium*, and four loci could be amplified successfully in all eight species (Table 3). The allele size ranges were similar among *Ranunculus* section *Batrachium* species, but greatly differed among the taxa from different genera in loci BatrB1, BatrB9, BatrB11–13, BatrB17, and BatrB21.

CONCLUSIONS

Fourteen polymorphic and eight monomorphic microsatellite loci were developed in *R. bungei*. The polymorphism observed for the SSRs in *R. bungei* is moderate when compared with other aquatic plants (Nies and Reusch, 2004; Wu et al., 2013).

TABLE 3. Cross-amplification of 22 EST-SSR markers developed in *Ranunculus bungei* across eight other species of Ranunculaceae. The number of alleles in populations of each species is presented for the loci that could be successfully amplified.[a]

Locus	*Ranunculus aquatilis* var. *eradicatus* (N = 11)	*Ranunculus trichophyllus* (N = 14)	*Ranunculus cheirophyllus* (N = 5)	*Ranunculus natans* (N = 8)	*Halerpestes tricuspis* (N = 12)	*Halerpestes ruthenica* (N = 6)	*Caltha palustris* (N = 3)	*Caltha natans* (N = 5)
BatrB1	3	1	—	3	—	1	—	—
BatrB2	2	1	—	1	—	—	—	—
BatrB3	1	2	—	—	—	—	—	3
BatrB4	2	2	—	—	2	—	—	2
BatrB5	3	1	1	2	3	2	3	3
BatrB6	3	4	3	3	2	2	3	1
BatrB7	2	2	—	—	—	—	—	—
BatrB8	1	1	—	—	2	—	—	—
BatrB9	3	4	2	3	2	1	2	1
BatrB10	3	3	1	2	1	3	1	1
BatrB11	4	2	3	—	—	—	2	—
BatrB12	3	1	—	—	—	2	—	2
BatrB13	1	2	—	—	—	3	—	—
BatrB14	1	2	—	—	—	—	—	—
BatrB15	2	1	—	—	1	1	—	2
BatrB16	2	3	3	—	3	—	2	2
BatrB17	2	3	—	—	2	—	—	—
BatrB18	1	1	—	—	—	—	—	—
BatrB19	1	1	—	—	—	—	—	—
BatrB20	2	2	3	1	1	1	—	2
BatrB21	1	2	—	—	—	—	3	2
BatrB22	1	2	—	—	—	—	—	—

Note: — = primers could not be amplified in any individual; N = number of individuals.

[a] Locality and voucher information are available in Appendix 1.

Cross-species amplification also indicates that these markers may be widely used in related Ranunculaceae species. We conclude that the EST-SSRs described here will facilitate ecological and evolutionary studies of *R. bungei* as well as related species.

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Appendix 1. Geographic information of *Ranunculus*, *Halerpestes*, and *Caltha* populations in this study.[a]

Species	Location	Geographic coordinates	Voucher specimen accession no.	*N*
Ranunculus bungei Steud.	Maduo, Qinghai	34.8619°N, 97.4919°E	15072202	22
R. bungei	Dingri, Tibet	28.5936°N, 86.8331°E	15080502	20
R. bungei	Baishan, Jilin	41.9949°N, 127.6250°E	15082204	15
R. bungei	Tongliao, Neimenggu	44.9290°N, 120.4876°E	15082601	16
R. aquatilis L. var. *eradicatus* Laest.	Ruoegai, Sichuan	33.5356°N, 103.1276°E	Xu2372	11
R. trichophyllus Chaix ex Vill.	Hejing, Xinjiang	43.0369°N, 86.0483°E	Xu4332	14
R. cheirophyllus Hayata	Arongqi, Neimenggu	47.9993°N, 123.0647°E	Xu6079	5
R. natans C. A. Mey.	Menyuan, Qinghai	37.7917°N, 101.1616°E	15081102	8
Halerpestes tricuspis (Maxim.) Hand.-Mazz.	Maqin, Qinghai	34.3675°N, 100.2547°E	15071803	12
H. ruthenica (Jacq.) Ovcz.	Wuwei, Gansu	36.9762°N, 103.0154°E	Xu6597	6
Caltha palustris L.	Luobei, Heilongjiang	47.7099°N, 130.9365°E	Xu0282	3
C. natans Pall.	Genhe, Neimenggu	50.7675°N, 121.4955°E	Xu0502	5

Note: *N* = number of individuals.

[a]All specimens are deposited in the herbarium of Wuhan University, Wuhan, China.