

Development and characterization of expressed sequence tag-simple sequence repeat markers for the near-threatened halophyte *Limonium tetragonum* (Thunb.) A. A. Bullock (*Plumbaginaceae*)

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Next-generation RNA sequencing analysis was performed to develop 13 novel expressed sequence tag-simple sequence repeat markers to evaluate the genetic variation in the near-threatened halophyte *Limonium tetragonum* (Thunb.) A. A. Bullock. In the four populations examined, the total number of alleles at each locus ranged from two to seven, with an average of 3.1. The average observed and expected heterozygosity ranged from 0.00 to 0.13 and 0.28 to 0.78, respectively. Three of the 13 loci had the same homozygous alleles within populations, but different alleles among populations. Compared to other halophytes, relatively low genetic diversity was observed in this species. Further studies are necessary to determine the population demography of *L. tetragonum* and to clarify the cause of its low genetic diversity.

Key words: halophyte, *Limonium tetragonum*, EST-SSR marker, near-threatened plant, genetic variation

Halophytes are salt-tolerant plants found in inland semi-saline deserts, marshes, wetlands and tidal flats along coastal and brackish areas that possess special physiological mechanisms and traits to adapt to the saline environment (Flowers and Colmer, 2008, 2015; Aslam et al., 2011). Substantial natural and anthropogenic disturbances are observed in this environment. Over the years, the coastal habitat of halophytes has decreased due to the overexploitation and habitat transformation of salt marshes and tidal flats in seashores and estuaries (Lotze et al., 2006; Japanese Association of Benthology, 2012). In recent years, many Japanese rivers have also experienced

extreme physical geographic alterations due to severe floods and strong typhoons, which have destroyed river embankments and sandbars. Recently, the frequency of heavy rains has increased and is predicted to rise further in the future (Ministry of Land, Infrastructure, Transport and Tourism, 2020, <https://www.mlit.go.jp/hakusyo/mlit/r01/hakusho/r02/html/n1115000.html>; Intergovernmental Panel on Climate Change, 2021). Consequently, the number of organisms living in these areas has decreased drastically.

Limonium tetragonum (Thunb.) A. A. Bullock is a semelparous perennial plant distributed in the Korean Peninsula and the Honshu region of Japan in the sandbars and tidal mud of coastal and brackish waters (Yonekura, 2017; Nakanishi, 2020). It is predominantly found in salt marshes in Japan (Ueda and Kamihogi, 2013). Despite its importance as coastal vegetation, *L. tetragonum* is listed as a near-threatened species in the Japanese Red List (Ministry of the Environment, 2020, https://www.env.go.jp/en/press/107905_00001.html).

To investigate the genetic variety in organisms, the population genetics approach using molecular techniques

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is useful. These techniques reveal the genetic discrepancies in a particular species, but they can be difficult to use due to the need for species-specific genetic markers. The expressed sequence tag-simple sequence repeat (EST-SSR) method is useful for genetic marker development. Various genetic markers for plants, such as *Carex angustisquama* Franch. (Nagasawa et al., 2018) and *Anaphalis margaritacea* (Hoson et al., 2023), have been developed using this method. In this study, we used next-generation sequencing to develop variable EST-SSR markers for *L. tetragonum* for population genetic analyses.

Total RNA was extracted from young leaves of *L. tetragonum* (collected in Ako-Shi, Hyogo, Japan; 34° 4'19"N, 134° 23'37"E) using the ISOSPIN Plant RNA and Assist Buffer for ISOSPIN Plant RNA (Nippon Gene, Tokyo, Japan). cDNA library construction and sequencing were performed by Macrogen Japan (Tokyo, Japan) using the NovaSeq 6000 system (100 bp, paired-end; Illumina, San Diego, CA, USA). After low-quality sequences and adaptors were removed using Trimmomatic (version 0.39, Bolger et al., 2014), *de novo* assembly of 40,523,408 cleaned reads using Trinity (version 2.13.2, Grabherr et al., 2011) produced 108,260 contigs (N50: 1,205 bp).

Microsatellite regions (≥ 10 dinucleotide repeats and ≥ 7 trinucleotide repeats) were explored using Krait (ver 1.3.3, Du et al., 2018). Primer pairs with an optimal melting temperature of 60 ± 2 °C, GC content of 30–70% and product size range of 100–500 bp were designed. From the 144 generated primer sets with 10 trinucleotide repeats, 96 primer sets were selected. The forward primer for each locus was synthesized with one of four different universal primers and with one of four fluorescent dyes (Thermo Fisher Scientific, MA, USA) (6FAM: 5'-GCCTCCCTCGCGCCA-3'; VIC: 5'-GCCTTGCCAGCCCGC-3'; NED: 5'-CAGGACCAAGGCTACCGTG-3'; or PET: 5'-CGGAGAGCCGAGAGGTG-3'; Blacket et al., 2012). The reverse primer was tagged with a PIG-tail (5'-GTTTCTT-3') to promote full adenylation (Brownstein et al., 1996).

DNA samples were amplified in 10- μ l reaction mixtures containing 1 μ l (20–30 ng/ μ l) template DNA, 2 $\times$ Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.01 μ M forward primer, 0.2 μ M reverse primer and 0.1 μ M fluorescent dye-labeled universal primer. The PCR protocol was as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 3 min, 68 °C for 1 min; and a final extension at 68 °C for 20 min. The amplified products were analyzed using the ABI 3500 Genetic DNA analyzer (Applied Biosystems, Foster City, CA, USA). Amplified samples (up to 1 μ l) were added to 10 μ l Hi-Di formamide (Thermo Fisher Scientific) with 0.1 μ l GeneScan 500 LIZ Size Standard (Applied Biosystems). Fragment sizes were determined using the Peak Scanner (Thermo Fisher Connect Platform).

To prepare the DNA samples, leaves collected in the

field were put into 2-ml sterilized tubes with RNALater solution (QIAGEN) for four days in a refrigerator and then placed in a freezer until DNA extraction. DNA was extracted using ISOSPIN Plant DNA (Nippon Gene) according to the manufacturer's instructions. To check the efficacy of the designed primers, two individual DNAs (Pop B and Pop C) were mixed, adjusted to 20 ng/ μ l concentration, and subjected to PCR amplification using the 96 primer pairs. Of the 78 primers, 55 exhibited single peaks (monomorphic; Supplementary Table S1) and 23 exhibited polymorphic peaks. The utility of these 23 primers was tested using two individuals each from Pops A, B and D in Japan. In total, 13 primers exhibited clear polymorphic peaks. Subsequently, 32 individuals from each of four populations (Pops A–D) were genotyped to estimate their genetic diversity indices (Pop A, Kagawa prefecture, Shikoku district; Pop B, Mie prefecture, Chubu district; Pop C, Aichi prefecture, Chubu district and Pop D, Fukushima prefecture, Tohoku district; detailed localities are not shown for conservation). Pops A and B individuals were collected from river mouths, Pop C individuals from the edge of tidal flats, and Pop D individuals from the tidal flats left as a protected area in the restoration project after the Great East Japan Earthquake (Mabuchi et al., 2017; Kurosawa, 2021).

GeneAlex 6.5 (Peakall and Smouse, 2012) was used to estimate the number of alleles per locus (N_A), expected heterozygosity (H_E) and observed heterozygosity (H_O). Hardy–Weinberg equilibrium (HWE) within a locus was tested using the online version of GENEPOP 4.2 (Raymond and Rousset, 1995). Micro-Checker (Brookfield 1 estimator; Van Oosterhout et al., 2004) was used to calculate the estimated null allele frequency. For the selected markers, a similarity search for each contig was conducted against the National Center for Biotechnology Information non-redundant protein database using the default BLASTX algorithm (Table 1).

We observed that the 13 primer pairs amplified clear peaks (Table 2). In the four pooled populations, N_A was 2–7, H_O was 0.00–0.13 and H_E was 0.28–0.78. In each population, significant departures from HWE (significant heterozygosity deficit) after Bonferroni correction were detected at loci LMTG_5, 8, 28, 57, 82, 85, 86 and 87 in Pop A and LMTG_57, 69 and 82 in Pop B. The estimated null allele frequencies were statistically significant at LMTG_5, 8, 28, 57, 82, 85, 86 and 87 in Pop A, LMTG_57, 69 and 82 in Pop B and LMTG_57 in Pop C. Average number of alleles was highest in Pop A and lowest in Pop C and D in all four populations. Moreover, three of the 13 loci had the same homozygous alleles within populations but different alleles among different populations.

EST-SSR markers exhibit lower levels of polymorphism than SSR markers obtained by scanning whole-genomic regions (genomic SSR markers) (Pinto et al., 2006; Parthiban et al., 2018). Relatively low genetic variation was

Table 1. Thirteen novel EST-SSR markers for *Limonium tetragonum*

Locus	Primer sequences (5'-3')	Repeat motif	Repeat number	Fluorescent label	Fragment range (bp)	BLASTX top hit description	E-value	DDBJ
LMTG_3	F: TTGGACTTGGAGTTTCCCCG R: GGAGAAATCACCATTGAAAGAGTCG	TGA	8	NED	89-101	No significant similarity found	-	LC812166
LMTG_5	F: GTTGCCTGGAAGTTAGCTGG R: TGGTCTAGCTAGTCACTCCCC	GTA	7	6FAM	100-109	No significant similarity found	-	LC812167
LMTG_8	F: CTAAGATGCAGCTGCGCTCC R: GATACATCTGAGAGCCCGGC	TAT	8	PET	144-153	<i>Persicaria maculosa</i> genome assembly, chromosome: 5	2E-12	LC812168
LMTG_28	F: TCCTATGTGGAGTCCCAGGC R: GCATCGTCATTATCATGGGAAGG	CTG	7	PET	133-142	No significant similarity found	-	LC812169
LMTG_30	F: GCTCCTAAATTTCAAGACGACGG R: TGATGTTGCTGTTGTTGAGGG	CAT	7	VIC	134-149	PREDICTED: <i>Brassica rapa</i> uncharacterized LOC103840659 (LOC103840659), mRNA	0.0002	LC812170
LMTG_47	F: AGGACCATTCACCAACAGC R: ACTGCTGATCGGATGCACC	ATG	8	NED	146-155	PREDICTED: <i>Nicotiana sylvestris</i> transcription factor bHLH145-like (LOC104238834), mRNA	1E-36	LC812171
LMTG_55	F: GCGTTCACCACTGCAAAGC R: CCCAACTCTTAAAACTAGAACTTCTCG	TAC	7	NED	162-168	No significant similarity found	-	LC812172
LMTG_57	F: GGTATGTTGCAAGCCATTCTACC R: CCTCTTGCTGGTTCTCTGC	AGT	8	6FAM	169-175	<i>Arabidopsis thaliana</i> genome assembly, chromosome: 3	1E-48	LC812173
LMTG_69	F: TCTGATAACAGGTCTGCCGC R: ATGTGCAGGAGTGAGCTTGG	TGA	16	6FAM	208-277	No significant similarity found	-	LC812174
LMTG_82	F: TGGTGATGGCCTTGCAGG R: GCAGATGGTGAAGAGACGG	TGA	7	VIC	142-148	No significant similarity found	-	LC812175
LMTG_85	F: TCCAAAGCTGTTGGTTGAGC R: ACAACTTCACACTCGCCTCC	GAA	7	6FAM	138-141	<i>Toona sinensis</i> AT1G64770 mRNA, partial sequence	5E-93	LC812176
LMTG_86	F: ACAGCCTTGGGTAGATGAACC R: CCAAAATCCAAAGAAGTCGTCGG	TTC	7	VIC	139-145	PREDICTED: <i>Lotus japonicus</i> transcription factor MYB8-like (LOC130745487), mRNA	8E-57	LC812177
LMTG_87	F: TGATCTGCTTCGTCTCATACTCC R: GAGAAAAGAAAGCAGGGGGG	TAC	7	NED	146-149	PREDICTED: <i>Lupinus angustifolius</i> heme oxygenase 1, chloroplastic-like (LOC109328549), mRNA	2E-54	LC812178

Table 2. Characteristics of 13 polymorphic EST-SSR markers in four populations of *L. tetragonum* in Japan

Locus	Pop A ($N = 32$)				Pop B ($N = 32$)				Pop C ($N = 32$)				Pop D ($N = 32$)				All ($N = 128$)			
	N_{GI}	N_A	H_O	H_E	N_{GI}	N_A	H_O	H_E	N_{GI}	N_A	H_O	H_E	N_{GI}	N_A	H_O	H_E	N_{GI}	N_A	H_O	H_E
LMTG_3	32	1	0.000	0.000	32	2	0.000	0.061	32	1	0.000	0.000	32	1	0.000	0.000	128	3	0.000*	0.387
LMTG_5	32	3	0.125*	0.571	30	1	0.000	0.000	32	1	0.000	0.000	32	2	0.063	0.061	126	3	0.048*	0.480
LTMG_8	29	2	0.034*	0.479	32	2	0.406	0.496	32	2	0.063	0.061	32	1	0.000	0.000	125	4	0.128*	0.598
LMTG_28	32	2	0.000*	0.117	32	1	0.000	0.000	32	1	0.000	0.000	32	2	0.063	0.061	128	2	0.016*	0.499
LMTG_30	32	2	0.031	0.031	32	1	0.000	0.000	32	1	0.000	0.000	32	1	0.000	0.000	128	3	0.008*	0.623
LMTG_47	31	1	0.000	0.000	32	1	0.000	0.000	31	1	0.000	0.000	32	2	0.094	0.089	126	2	0.024*	0.500
LMTG_55	32	2	0.031	0.031	32	1	0.000	0.000	32	1	0.000	0.000	32	1	0.000	0.000	128	2	0.008*	0.500
LMTG_57	32	2	0.063*	0.482	32	3	0.031*	0.532	32	2	0.000*	0.061	32	1	0.000	0.000	128	4	0.023*	0.652
LMTG_69	32	1	0.000	0.000	31	4	0.097*	0.551	32	1	0.000	0.000	32	1	0.000	0.000	127	7	0.024*	0.783
LMTG_82	32	3	0.125*	0.495	32	2	0.000*	0.264	32	1	0.000	0.000	32	1	0.000	0.000	128	3	0.031*	0.465
LMTG_85	32	2	0.063*	0.404	29	1	0.000	0.000	32	1	0.000	0.000	32	1	0.000	0.000	125	2	0.016*	0.493
LMTG_86	32	3	0.156*	0.474	32	1	0.000	0.000	32	1	0.000	0.000	31	1	0.000	0.000	127	3	0.039*	0.503
LMTG_87	32	2	0.125*	0.451	31	1	0.000	0.000	32	2	0.031	0.031	32	1	0.000	0.000	127	2	0.039*	0.281
Average		2.0	0.058	0.272		1.6	0.041	0.146		1.2	0.007	0.012		1.2	0.017	0.016		3.1	0.031	0.520
N , number of assayed individuals; N_{GI} , number of successfully genotyped individuals; N_A , number of alleles per locus; H_O , observed heterozygosity; H_E , expected heterozygosity; $[H_O]^*$, significant deficit of heterozygosity.																				

observed at all loci in the four populations of *L. tetragonum* (Table 2). The lack of genetic variation is caused by inbreeding, bottlenecks or genetic drift (Frankham et al., 2002). In the genus *Limonium*, SSR markers have been developed for the closely related and critically endangered sea lavender *L. dufourii* (Girard) Kuntze (Palop-Esteban and González-Candelas, 2002). It has been reported that three of the 13 markers (loci) fixed homozygosity in 120 individuals. The results of EST–SSR marker analysis in the perennial sedge *Carex angustisquama* Franch., which lives in solfatara fields in northern Japan, showed a mean N_A of 3.14, mean H_O of 0.06 and mean H_E of 0.54 (Nagasawa et al., 2018). These primers were checked with a closely related species, *C. doentizii*, and the primers worked well for this species (mean N_A = 4.85, mean H_O = 0.452 and mean H_E = 0.587), from which the authors concluded that the low genetic variation of *C. angustisquama* is a species demographic history (Nagasawa et al., 2018). Therefore, further study will be needed to assess the cause of low genetic diversity and to determine the population genetic structure of *L. tetragonum* in Japan.

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