Genes Genet. Syst.

Development and characterization of expressed sequence tag-simple sequence repeat markers for *Anaphalis margaritacea* var. *yedoensis* (Asteraceae)

Taishi Hoson[1,2*], Shoki Murakami[1,2], Takuro Ito[1,2] and Masuyuki Maki[1,2]

[1]*Botanical Gardens, Tohoku University, Sendai, Miyagi 980-0862, Japan*
[2]*Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-0862, Japan*

(Received 8 February 2023, accepted 31 May 2023; J-STAGE Advance published date: 26 September 2023)

RNA-sequencing was used to develop 16 microsatellite markers for the pearly everlasting, *Anaphalis margaritacea* var. *yedoensis* (Franch. et Sav.) Ohwi (Asteraceae), which inhabits gravel bars throughout the Japanese archipelago. The mean number of alleles for these 16 markers in two populations in the Hokkaido and Shizuoka Prefectures, was 3.5 and 4.0, respectively, while the mean expected heterozygosity was 0.525 and 0.560, respectively, with a significant genetic differentiation between the two populations. All markers could also be amplified in two conspecific taxa, *A. margaritacea* var. *margaritacea* and var. *angustifolia*, whereas 11 of the 16 markers were amplifiable in two congeneric species, *A. sinica* and *A. alpicola*. These newly developed microsatellite markers will support understanding of population genetics and mating systems in *A. margaritacea* var. *yedoensis*, and several will potentially be of use in similar studies in other *Anaphalis* species.

Key words: genetic diversity, microsatellite marker, pearly everlasting, spatial genetic structure, subdioecy

Flowering plants tend to show various gender expression patterns (Barrett, 2002; Ehlers and Bataillon, 2007). Subdioecy, where female, male, and hermaphrodite individuals co-occur within a population, is rare, being reported in <1% of flowering plant species (Rivkin et al., 2016). However, as subdioecious species are found in many families, this configuration may have independently evolved multiple times (Rivkin et al., 2016). Ehlers and Bataillon (2007) modeled subdioecy in flowering plants and found that selfing in hermaphrodites maintains inconstant males in a subdioecious population. Thus, estimating the selfing rate in hermaphrodite individuals in a subdioecious population can elucidate the evolution of subdioecy.

Anaphalis DC., the pearly everlasting, is a genus comprising approximately 110 species distributed mainly in Asia (Nie et al., 2013). Subdioecy is often reported in *Anaphalis* (Drury, 1970; Anderberg, 1991), and subdioecious *Anaphalis* species in Japan (Kadota et al., 2017) offer a good opportunity to examine the evolution of subdioecy.

Anaphalis margaritacea var. *yedoensis* (Franch. et Sav.) Ohwi is endemic to Japan and specifically inhabits gravel bars (Asami et al., 2012; Kadota et al., 2017; Ikeda et al., 2020). Recent river management projects to reduce river flooding have caused the local disappearance of gravel bars (Kalníková et al., 2018, 2021), and *A. margaritacea* var. *yedoensis* is now endangered on some riverbanks (Yoshioka et al., 2010; Yoshida et al., 2020). To successfully conserve such an endangered population, detailed knowledge of its genetic structure is required (Holsinger and Gottlieb, 1991; Escudero et al., 2003).

Simple sequence repeats (SSRs), or microsatellites, are co-dominant and normally show high levels of polymorphism (Hardy, 2003; Bouck and Vision, 2007). Thus, SSR markers can be used to estimate selfing rates (McCouch et al., 1997; Carlon, 1999) and examine genetic structures in wild populations (Bruford and Wayne, 1993; Abdul-Muneer, 2014). Expressed sequence tag (EST)-SSR markers are less likely to have null alleles and are well conserved among congeneric species (Bouck and Vision, 2007; Ellis and Burke, 2007), providing a powerful tool for population genetic studies. Herein, novel EST-SSR markers were developed based on RNA-seq of *A.*

Edited by Junko Kusumi
* Corresponding author. amb1t1oushoson@gmail.com
DOI: https://doi.org/10.1266/ggs.23-00037

margaritacea var. *yedoensis* and their cross-species transferability evaluated with congeneric taxa of *Anaphalis* distributed in Japan.

We collected an individual *A. margaritacea* var. *yedoensis* from a population beside the Eai River, Miyagi Prefecture (38°39′N, 140°53′E). Total RNA from the fresh leaves of this plant was extracted using an ISOSPIN Plant RNA kit (NIPPON GENE Co. Ltd., Tokyo, Japan). After poly-A purification, RNA libraries were constructed using an NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Libraries were sequenced on an Illumina NovaSeq 6000 platform that generated 150-bp paired-end reads. RNA library construction and sequencing was performed by Veritas Genetics (Danvers, MA, USA). Trimmomatic v 0.38 (Bolger et al., 2014) was used to trim sequence reads with avgqual 20 and minlen 36, and Trinity ver. 2.8.5 (Grabherr et al., 2011; Haas et al., 2013) was used to assemble trimmed reads. MISA Perl script (Thiel et al., 2003) was used to identify microsatellite regions and screen potentially useful microsatellite motifs with >10 repeats. Primer3 v 2.3.5 was used to design primers in the regions containing microsatellite loci (Rozen and Skaletsky, 2000). Primer sets were selected that reliably amplified products. Each amplification product was BLASTX searched using the threshold *E*-value < e^{-10} to indicate a significant hit (Table 1).

For genotyping, fresh leaves were collected from 20 individuals in each of two populations beside the Ken'ichi River in Hokkaido Prefecture (42°07′N, 140°01′E) and the Oi River in Shizuoka Prefecture (34°47′N, 138°15′E). Samples were collected from plants that were separated by several meters to avoid collecting more than one sample from the same genet. Total DNA was extracted using cetyltrimethylammonium bromide (Doyle and Doyle, 1987).

PCR amplification was performed in 3 μL containing 0.5 μL of genomic DNA (100 ng/μL), 2× Type-it Multiplex PCR Master Mix (QIAGEN, Venlo, Netherlands), 0.075 μM forward primer with a 5′ universal tail (Blacket et al., 2012), 0.25 μM reverse primer with the pigtail 5′-GTTCTT-3′ (Brownstein et al., 1996), and 0.1 μM of the fluorescent-labeled universal primers used by Blacket et al. (2012). PCR reactions were performed with an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 45–60 s, and a final extension of 10 min at 72 °C. PCR products were electrophoresed with a GeneScan 600 LIZ Internal Size Standard (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 Genetic Analyzer (Applied Biosystems).

For each amplified marker, GenAlEx 6.51b2 was used to calculate the number of alleles (N_A), number of effective alleles (N_E), observed heterozygosity (H_O), and expected heterozygosity (H_E) for both populations (Peakall and Smouse, 2006). The allelic richness (A_R, standardized by four individuals using rarefaction) was calculated with FSTAT 2.9.4 (Goudet, 1995; available from https://www2.unil.ch/popgen/softwares/fstat.htm) based on El Mousadik and Petit (1996). GENEPOP 4.7.5 was used to test for deviation from Hardy-Weinberg equilibrium and linkage disequilibrium of all pairwise combinations for each population (Raymond and Rousset, 1995; Rousset, 2008) after sequential Bonferroni corrections (Rice, 1989). Micro-Checker ver. 2.2.3 (van Oosterhout et al., 2004) was used to check for null alleles.

To test for cross-species transferability of the SSR markers, we collected 18 individuals from a population of *A. margaritacea* var. *margaritacea* (Naruko, Miyagi Prefecture; 38°45′N, 140°45′E) and four from a population of *A. margaritacea* var. *angustifolia* (Aso, Kumamoto Prefecture; 32°49′N, 130°57′E). In addition, we collected four individuals from populations of two congeners, *A. sinica* (Ochi, Kochi Prefecture; 33°32′N, 133°12′E) and *A. alpicola* (Mukawa, Hokkaido Prefecture; 42°51′N, 142°15′E). DNA extraction and PCR amplification were performed as described above.

To confirm the usefulness of the markers developed, we used STRUCTURE v2.3.4 to examine the genetic structure between populations of *A. margaritacea* with Bayesian clustering using (Pritchard et al., 2000). Ten independent runs were performed per K (K = 1–10) with 100,000 burn-in iterations followed by 100,000 Markov Chain Monte Carlo iterations. STRUCTURE HARVESTER was used to calculate ΔK values (Evanno et al., 2005; Earl and vonHoldt, 2012). To test the significance of the genetic differentiation, FSTAT 2.9.4 was used to calculate the F_{ST} values in all the pairs of the four populations of *A. margaritacea* (Goudet, 1995).

De novo assembly of 15,878,356 paired-end reads from RNA-seq produced 56,312 contigs. Raw data were deposited in the DNA Data Bank of Japan (DDBJ) (DRA accession DRA015110). Using these contigs, 2,540 microsatellite regions were found, and 124 primer sets were identified by using MISA Perl script. Sixteen PCR products were amplified from all individuals in the two populations (Table 1). A null allele was detected in one locus (AnaSSR_224; Table 2), suggesting that we erroneously genotyped the heterozygotes at this locus because null alleles were present (van Oosterhout et al., 2004). We excluded this locus from further analyses on var. *yedoensis*. No evidence of Hardy–Weinberg disequilibrium or linkage disequilibrium was present in any pairwise combination of loci. Among the 16 loci, two were monomorphic in the populations from the Ken'ichi River. For the populations from the Ken'ichi and Oi rivers, the N_A values ranged from 1 to 5 (mean 3.5) and from 2 to 6 (mean 4.0), the N_E values ranged from 1.000 to 4.103 (mean 2.448) and from 1.220 to 3.922 (mean 2.532), the A_R values ranged from 1.000 to 3.922 (mean 2.728) and from 1.607 to 3.932 (mean 2.857), the H_O values ranged

Table 1. Characterization of 16 microsatellite markers identified for *A. margaritacea* var. *yedoensis*

Locus Name	Primer Sequence (5'-3')	Repeat Motif	Size Range (bp)	BLASTX top hit description	E-value	GenBank Accession No.
AnaSSR_14	F: ACATTGCCGACACTGAAAGGA R: GTGGTTGGGTGGTCATCTCC	$(TCAC)_{10}$	279–291	no significant hit	–	LC746608
AnaSSR_15	F: GCAGTGGTTGGAGAAGAAGC R: TGGGCGACTTTCACCTGTTT	$(CT)_{11}$	299–317	Ricinus communis V-type proton ATPase subunit B 2 (LOC8267833), transcript variant X2	7.00E-14	LC746609
AnaSSR_20	F: GCAAGCAGGGGTAGATCGAA R: TGGAACGGACTTGTTTGGCA	$(ATG)_{11}$	274–289	no significant hit	–	LC746610
AnaSSR_23	F: CACCTAAATGCCATGATGGATTATGA R: TGTTGTGACTTGTGAGGAGGG	$(TAA)_{22}$	254–269	no significant hit	–	LC746611
AnaSSR_25	F: AGTTGCCTAAAAATCCGTTGCT R: CCTGCTCGTGCCATTTCTTG	$(CAT)_{15}$	153–186	Lates calcarifer BTB/POZ domain-containing protein KCTD15 (LOC108891737)	3.00E-14	LC746612
AnaSSR_28	F: AGTTTCCCAAATTGGAAGCCT R: AGTCCGCATCTTTGATCTTTCT	$(AC)_{12}$	124–138	no significant hit	–	LC746613
AnaSSR_154	F: ACTGATCCCACACCATTGCT R: TGGCCAAATGACACGTTTCC	$(TGG)_{14}$	179–188	Helianthus annuus formin-like protein 2 (LOC110878294)	1.00E-20	LC746614
AnaSSR_187	F: ACCTAAGAAAATTTTCATGCATTGCT R: CGACCCGGTTCTTTTTGAGC	$(TC)_{11}$	233–247	no significant hit	–	LC746615
AnaSSR_215	F: TCCATGCAGGAGGAGAAGGT R: TGCATGATTTTCCTTTTCGATTGC	$(AAT)_{10}$	240–249	Lactuca sativa monoacylglycerol lipase ABHD6 (LOC111894003)	1.00E-53	LC746616
AnaSSR_224	F: CCACTACATGGTGTGAAATGTGA R: TGGGAGTTGGAAGTCGGGTA	$(AG)_{10}$	194–200	Lactuca sativa protein NRT1/ PTR FAMILY 4.6 (LOC111891365)	5.00E-24	LC746617
AnaSSR_241	F: TGTTCGACCCTGTAAACTTGCT R: AGGCAGCTAACTTCGGACTG	$(TG)_{10}$	199–203	no significant hit	–	LC746618
AnaSSR_244	F: ACGGGTCGAATTTCACCCAA R: GCAGAAGCAGAACCAGTCCT	$(GA)_{11}$	257–285	Phoenix dactylifera secretory carrier-associated membrane protein 4 (LOC103720997)	1.00E-16	LC746619
AnaSSR_255	F: CCTCGATATCACCCACGACG R: TCGAAGCCGCCTTCTCTTTT	$(ATT)_{13}$	128–149	Simochromis diagramma solute carrier family 49 member 3 (slc49a3), transcript variant X2	4.00E-18	LC746620
AnaSSR_257	F: CACCGTACACCCCCTGTAAC R: GGCCCTGGACCAGTCATTG	$(GA)_{11}$	260–284	no significant hit	–	LC746621
AnaSSR_269	F: CCCCACGAAAATTAAACTGCCA R: TTTAGCTGTGCCTGGAGCTG	$(CA)_{17}$	269–317	no significant hit	–	LC746622
AnaSSR_274	F: TGGCTCGATTCAAAGCTCGT R: GCCCCATTATGAGCTCCCAA	$(TCA)_{11}$	309–321	no significant hit	–	LC746623

from 0.000 to 0.900 (mean 0.563) and from 0.100 to 0.750 (mean 0.517), and the H_E values ranged from 0.000 to 0.756 (mean 0.525) and from 0.180 to 0.745 (mean 0.560), respectively (Table 2).

In the cross-species transferability test, the fragment size ranges in congeneric species were similar to those in *A. margaritacea* var. *yedoensis* (Table 1; Table 3). Null alleles were not detected in any other species examined in this study, except for *A. margaritacea* var. *yedoensis*. All markers were amplifiable from all individuals of the two conspecific varieties, *A. margaritacea* var. *margaritacea* and var. *angustifolia* (Table 3).

Table 2. Genetic diversity parameters of 16 polymorphic microsatellite loci in two populations of *A. margaritacea* var. *yedoensis*

Population	Ken'ichi River (n = 20)						Oi River (n = 20)						All (n = 40)					
Locus	N_A	N_E	A_R	H_O	H_E	P-value (HWE)	N_A	N_E	A_R	H_O	H_E	P-value (HWE)	N_A	N_E	A_R	H_O	H_E	P-value (HWE)
AnaSSR_14	3	2.417	2.673	0.750	0.586	0.056	3	1.956	2.557	0.550	0.489	0.171	3	2.534	2.737	0.650	0.605	0.054
AnaSSR_15	5	2.909	3.527	0.900	0.656	0.187	5	2.712	3.363	0.650	0.631	0.836	8	4.178	4.184	0.775	0.761	0.446
AnaSSR_20	2	1.835	1.980	0.400	0.455	0.633	4	1.297	1.928	0.150	0.229	0.052	5	1.630	2.284	0.275	0.387	0.145
AnaSSR_23	5	4.103	3.922	0.800	0.756	0.748	4	3.587	3.531	0.600	0.721	0.236	5	3.936	3.722	0.700	0.746	0.482
AnaSSR_25	4	2.186	2.791	0.350	0.543	0.062	4	3.137	3.288	0.600	0.681	0.471	8	5.153	4.569	0.475	0.806	0.132
AnaSSR_28	4	1.444	2.196	0.300	0.308	0.298	5	3.113	3.417	0.700	0.679	0.348	7	2.273	3.223	0.500	0.560	0.339
AnaSSR_154	2	2.000	1.997	0.400	0.500	0.395	4	2.279	2.554	0.400	0.561	0.158	4	3.946	3.628	0.400	0.747	0.235
AnaSSR_187	1	1.000	1.000	0.000	0.000	–	5	3.252	3.472	0.500	0.693	0.117	5	2.568	3.100	0.250	0.611	–
AnaSSR_215	3	1.421	1.964	0.350	0.296	1.000	3	2.787	2.864	0.450	0.641	0.079	4	2.401	2.809	0.400	0.583	0.279
AnaSSR_224*	1	1.000	1.000	0.000	0.000	–	2	1.220	1.607	0.100	0.180	0.152	3	2.198	2.341	0.050	0.545	–
AnaSSR_241	3	1.946	2.180	0.400	0.486	0.326	2	1.280	1.694	0.250	0.219	1.000	3	2.090	2.438	0.325	0.522	0.691
AnaSSR_244	4	2.847	3.243	0.700	0.649	0.537	6	2.417	3.042	0.500	0.586	0.198	7	4.010	3.849	0.600	0.751	0.345
AnaSSR_255	4	3.941	3.678	0.900	0.746	0.884	3	1.728	2.262	0.350	0.421	0.290	6	4.678	4.375	0.625	0.786	0.605
AnaSSR_257	5	3.292	3.709	0.850	0.696	0.399	3	2.111	2.350	0.600	0.526	0.830	6	3.062	3.368	0.725	0.673	0.697
AnaSSR_269	3	1.985	2.442	0.600	0.496	0.560	6	3.922	3.932	0.700	0.745	0.383	7	3.946	4.038	0.650	0.747	0.544
AnaSSR_274	5	3.390	3.619	0.750	0.705	0.428	3	2.402	2.597	0.750	0.584	0.087	5	3.632	3.559	0.750	0.725	0.160
Average	3.5	2.448	2.728	0.563	0.525		4.0	2.532	2.857	0.517	0.560		5.5	3.336	3.459	0.540	0.667	

N_A, number of alleles; N_E, number of effective alleles; A_R, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; P-value, significance level for deviation from Hardy-Weinberg equilibrium (HWE); n, number of genotyped individuals.
*Locus contains null alleles.

Table 3. Cross-species transferability of 16 microsatellite markers for *Anaphalis* taxa in Japan

Species	*A. margaritacea* var. *margaritacea* (n = 18)						*A. margaritacea* var. *angustifolia* (n = 4)						*A. sinica* (n = 4)					*A. alpicola* (n = 4)				
Locus	N_A	N_E	A_R	H_O	H_E	Size Range (bp)	N_A	N_E	A_R	H_O	H_E	Size Range (bp)	N_A	N_E	H_O	H_E	Size Range (bp)	N_A	N_E	H_O	H_E	Size Range (bp)
AnaSSR_14	5	3.522	3.763	0.333	0.716	263–291	1	1.000	1.000	0.000	0.000	287	2	2.000	1.000	0.500	279–287	2	1.882	0.750	0.469	279–287
AnaSSR_15	7	4.629	4.457	0.889	0.784	301–325	4	3.556	4.000	1.000	0.719	298–318	2	1.280	0.250	0.219	317–337	3	2.133	0.500	0.531	303–329
AnaSSR_20	5	1.616	2.563	0.222	0.381	274–289	2	1.280	2.000	0.250	0.219	286–304			–					–		
AnaSSR_23	6	4.438	4.092	0.444	0.775	251–269	4	3.556	4.000	0.750	0.719	254–260			–			3	2.667	0.000	0.625	256–280
AnaSSR_25	5	3.071	3.473	0.389	0.674	153–162	3	2.667	3.000	0.000	0.625	165–174	2	1.280	0.250	0.219	156–159	2	1.882	0.250	0.469	132–153
AnaSSR_28	7	3.746	3.873	0.722	0.733	120–136	3	2.462	3.000	0.500	0.594	124–138	3	2.462	0.250	0.594	130–136	3	2.667	0.500	0.625	128–132
AnaSSR_154	6	4.291	3.946	0.111	0.767	179–188	1	1.000	1.000	0.000	0.000	179	1	1.000	0.000	0.000	176	1	1.000	0.000	0.000	182
AnaSSR_187	7	4.208	4.137	0.667	0.762	231–247	1	1.000	1.000	0.000	0.000	237	1	1.000	0.000	0.000	229	1	1.000	0.000	0.000	229
AnaSSR_215	3	2.111	2.218	0.722	0.526	240–249	2	1.280	2.000	0.250	0.219	240–246	1	1.000	0.000	0.000	246	2	1.280	0.250	0.219	240–246
AnaSSR_224	4	3.071	3.308	0.444	0.674	194–202	2	1.280	2.000	0.250	0.219	190–198	1	1.000	0.000	0.000	151	1	1.000	0.000	0.000	151
AnaSSR_241	5	3.208	3.472	0.833	0.688	199–209	4	2.909	4.000	0.250	0.656	199–205	4	2.909	0.500	0.656	197–213	1	1.000	0.000	0.000	201
AnaSSR_244	7	3.393	3.841	0.444	0.705	267–291	3	2.462	3.000	1.000	0.594	271–279			–			2	2.000	1.000	0.500	271–323
AnaSSR_255	7	5.355	4.694	0.278	0.813	134–152	4	2.909	4.000	0.500	0.656	128–146			–			3	2.667	0.750	0.625	130–145
AnaSSR_257	7	3.176	3.622	0.833	0.685	260–280	4	2.909	4.000	0.750	0.656	260–278	3	2.909	1.000	0.656	260–274	2	2.000	1.000	0.500	264–274
AnaSSR_269	4	2.757	3.156	0.333	0.637	295–313	4	2.909	4.000	0.500	0.656	295–313	2	1.882	0.750	0.469	291–299			–		
AnaSSR_274	4	2.757	3.244	0.444	0.637	309–318	2	1.280	2.000	0.250	0.219	306–309	3	2.133	0.750	0.531	309–321	2	1.600	0.000	0.375	315–318
Average	5.6	3.459	3.616	0.507	0.685		2.80	2.154	2.800	0.391	0.422		2.10	1.738	0.594	0.480		2.0	1.770	0.625	0.494	

N_A, number of alleles; N_E, number of effective alleles; A_R, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; n, number of genotyped individuals.
"–", locus not amplified.

The population of var. *margaritacea* exhibited significantly higher values of A_R and H_E than those of the other three populations, except for the A_R between var. *margaritacea* and var. *angustifolia* (Wilcoxon signed-rank tests with sequential Bonferroni correction; Rice, 1989; Supplementary Fig. S1). Bayesian clustering using STRUCTURE revealed that the genetic structures of *A. margariatacea* divided into three clusters; two of them comprised the two populations of var. *yedoensis* and another comprised the populations of var. *margaritacea* and var. *angustifolia* at the optimal cluster number of ΔK (K = 3) (Fig. 1). The genetic clusters at K = 4, which was next optimal, corresponded to all four populations of *A. margaritacea* (Fig. 1). Significant genetic differentiation between the four populations of *A. margaritacea* are indicated by F_{ST} values (Supplementary Table S1).

Several markers were not amplified in *A. sinica* or *A. alpicola*. AnaSSR_20 was not amplified in either *A. sinica* or *A. alpicola*, whereas AnaSSR_23, AnaSSR_244, AnaSSR_255, and AnaSSR_269 were amplified in one species but not the other (Table 3). This results is consistent with the genetic differentiation between these species and *A. margaritacea* as suggested by previous phylogenetic studies (Nie et al., 2013).

The 16 novel EST-SSR markers developed herein will be of value in assessing the population genetic structure and

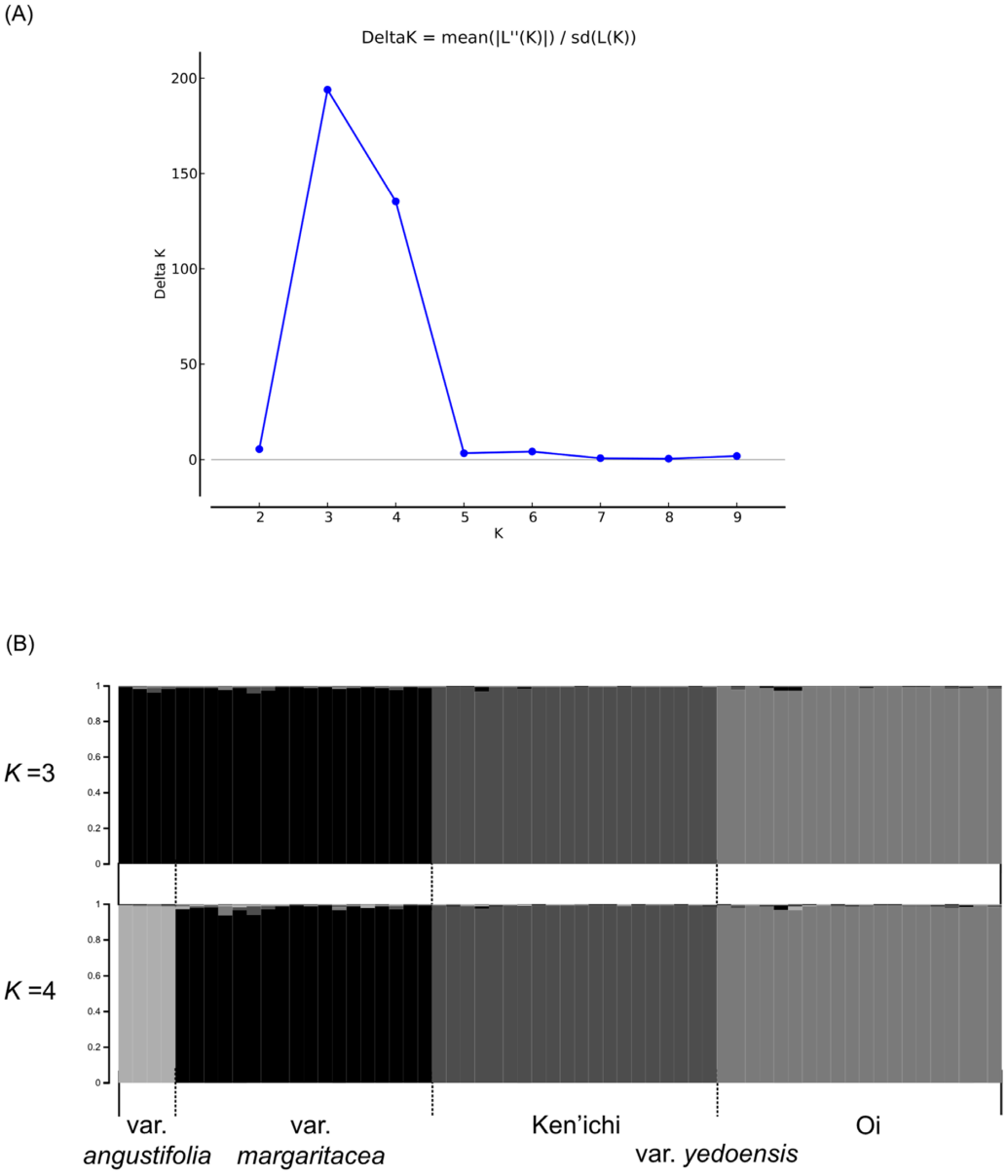


Fig. 1. STRUCTURE analysis of three varieties of *A. margaritacea*. (A) Optimal ΔK values based on the rate of change in log probability between consecutive K-values. (B) *A. margaritacea* genetic structure. Each bar represents the admixture proportion of parental populations per single individual.

mating system of *A. margaritacea* var. *yedoensis*. Furthermore, several markers developed in this study could be used in similar studies in congeneric species.

We thank Dr. Daiki Takahashi for great support for collection of congeneric species. We also thank all members of the Botanical Gardens, Tohoku University, for their support of this study. This work was partly supported by JSPS KAKENHI grants 20K21855 and 22H02366 to MM.

REFERENCES

Abdul-Muneer, P. M. (2014) Application of microsatellite markers in conservation genetics and fisheries management: recent advances in population structure analysis and conservation strategies. Genet. Res. Int. **2014**, 691759.

Anderberg, A. A. (1991) Taxonomy and phylogeny of the tribe Gnaphalieae (Asteraceae). Opera Bot. **104**, 1–195.

Asami, K., Akamatsu, H., Fukui, S., and Tamura, K. (2012) Morphological characteristics of flood refugia of cobble-bed vegetation. J. Hydro-Environ. Res. **6**, 127–136.

Barrett, S. C. H. (2002) The evolution of plant sexual diversity. Nat. Rev. Genet. **3**, 274–284.

Blacket, M. J., Robin, C., Good, R. T., Lee, S. F., and Miller, A. D. (2012) Universal primers for fluorescent labelling of PCR fragments—an efficient and cost-effective approach to genotyping by fluorescence. Mol. Ecol. Resour. **12**, 456–463.

Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**, 2114–2120.

Bouck, A., and Vision, T. (2007) The molecular ecologist's guide to expressed sequence tags. Mol. Ecol. **16**, 907–924.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996) Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. Biotechniques **20**, 1004–1010.

Bruford, M. W., and Wayne, R. K. (1993) Microsatellites and their application to population genetic studies. Curr. Opin. Genet. Dev. **3**, 939–943.

Carlon, D. B. (1999) The evolution of mating systems in tropical reef corals. Trends Ecol. Evol. **14**, 491–495.

Doyle, J. J., and Doyle, J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf 106 tissue. Phytochem. Bull. **19**, 11–15.

Drury, D. G. (1970) A fresh approach to the classification of the genus Gnaphalium with particular reference to the species present in New Zealand (Inuleae-Compositae). N. Z. J. Bot. **8**, 222–248.

Earl, D. A., and vonHoldt, B. M. (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. **4**, 359–361.

Ehlers, B. K., and Bataillon, T. (2007) 'Inconstant males' and the maintenance of labile sex expression in subdioecious plants. New Phytol. **174**, 194–211.

El Mousadik, A., and Petit, R. J. (1996) High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. Theor. Appl. Genet. **92**, 832–839.

Ellis, J. R., and Burke, J. M. (2007) EST-SSRs as a resource for population genetic analyses. Heredity **99**, 125–132.

Escudero, A., Iriondo, J. M., and Torres, M. E. (2003) Spatial analysis of genetic diversity as a tool for plant conservation. Biol. Conserv. **113**, 351–365.

Evanno, G., Regnaut, S., and Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. **14**, 2611–2620.

Goudet, J. (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. J. Hered. **86**, 485–486.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan., L., Raychowdhury, R., Zeng, Q., et al. (2011) Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. Nat. Biotechnol. **29**, 644–652.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B., Lieber, M., et al. (2013) *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. **8**, 1494–1512.

Hardy, O. J. (2003) Estimation of pairwise relatedness between individuals and characterization of isolation-by-distance processes using dominant genetic markers. Mol. Ecol. **12**, 1577–1588.

Holsinger, K. E., and Gottlieb, L. D. (1991) Conservation of rare and endangered plants: principles and prospects. *In* Genetics and Conservation of Rare Plants. (eds.: Falk, D. A., and Holsinger, K. E.), pp. 195–223. Oxford University Press, New York.

Ikeda, H., Iimura, K., Komura, S., Kawashima, C., and Sato, W. (2020) Vegetation transition and coarse sediment movement after gravel bar restoration with two meandering lanes in a steep river. J. Hydro-Environ. Res. **30**, 25–34.

Kadota, Y., Setoguchi, H., Soejima, A., Toma, T., Nakata, M., Morita, T., and Yonekura, K. (2017) Asteraceae. *In* Wild Flowers of Japan, Vol. 5. (eds.: Ohashi, H., Kadota, Y., Murata, J., Yonekura, K. and Kihara, H.), pp. 198–369. Heibonsha, Tokyo (in Japanese).

Kalníková, V., Chytrý, K., Biţa-Nicolae, C., Bracco, F., Font, X., Iakushenko, D., Kacki, Z., Kudrnovsky, H., Landucci, F., Lustyk, P., et al. (2021) Vegetation of the European mountain river gravel bars: a formalized classification. Appl. Veg. Sci. **24**, e12542.

Kalníková, V., Chytrý, K., and Chytrý, M. (2018) Early vegetation succession on gravel bars of Czech Carpathian streams. Folia Geobot. **53**, 317–332.

McCouch, S. R., Chen, X., Panaud, O., Temnykh, S., Xu, Y., Cho, Y. G., Huan, N., Ishii, T., and Blair, M. (1997) Microsatellite marker development, mapping and applications in rice genetics and breeding. Plant Mol. Biol. **35**, 89–99.

Nie, Z.-L., Funk, V., Sun, H., Deng, T., Meng, Y., and Wen, J. (2013) Molecular phylogeny of *Anaphalis* (Asteraceae, Gnaphalieae) with biogeographic implications in the Northern Hemisphere. J. Plant Res. **126**, 17–32.

Peakall, R., and Smouse, P. E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes **6**, 288–295.

Pritchard, J. K., Stephens, M., and Donnelly, P. (2000) Inference of population structure using multilocus genotype data. Genetics **155**, 945–959.

Raymond, M., and Rousset, F. (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. **86**, 248–249.

Rice, W. R. (1989) Analyzing tables of statistical tests. Evolution **43**, 223–225.

Rivkin, L. R., Case, A. L., and Caruso, C. M. (2016) Why is gynodioecy a rare but widely distributed sexual system? Lessons from the Lamiaceae. New Phytol. **211**, 688–696.

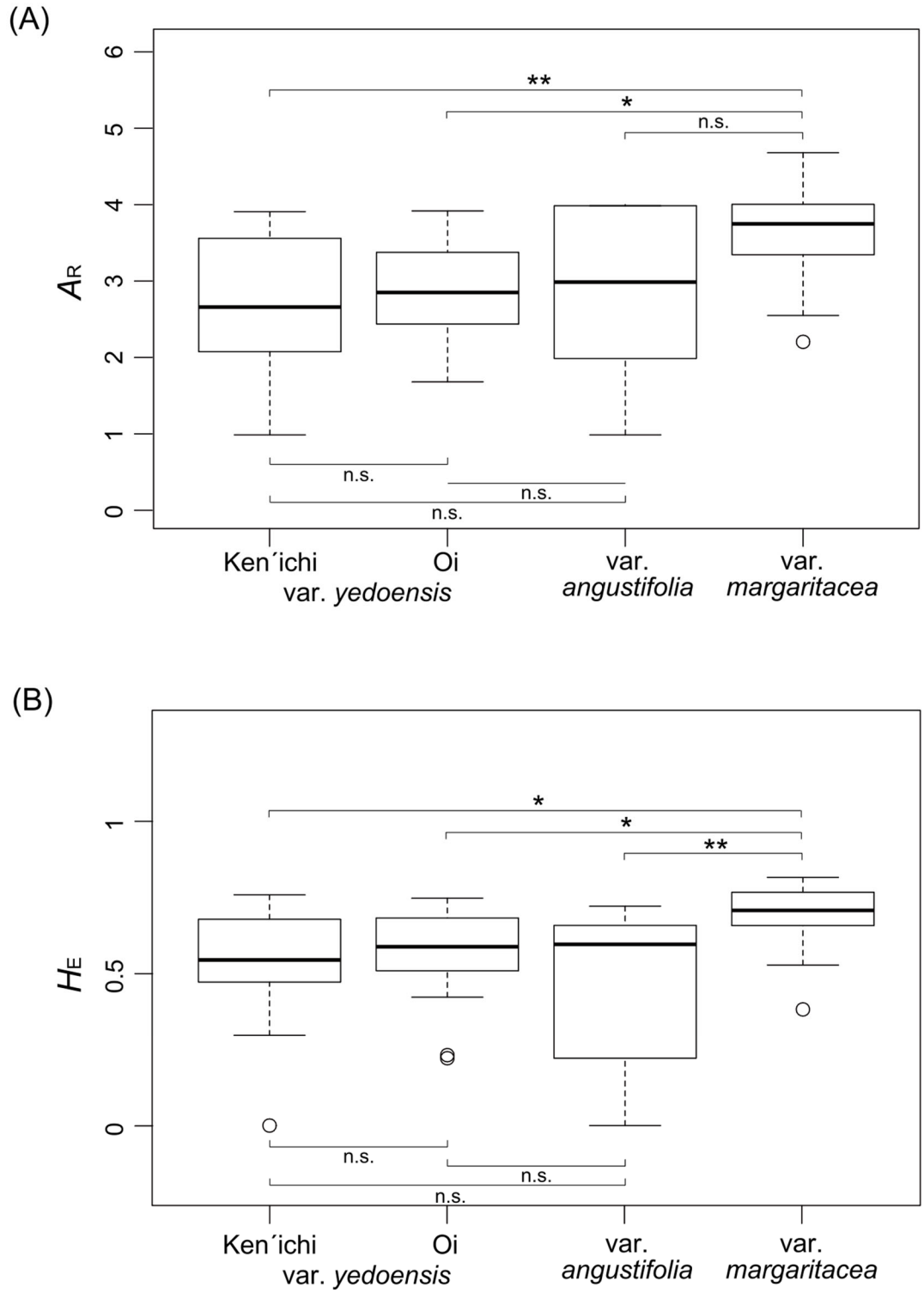
Rousset, F. (2008) genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol.

Resour. **8**, 103–106.
Rozen, S., and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. **132**, 365–386.
Thiel, T., Michalek, W., Varshney, R., and Graner, A. (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor. Appl. Genet. **106**, 411–422.
van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., and Shipley, P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes **4**, 535–538.
Yoshida, K., Maeno, S., Ogawa, S., Mano, K., and Nigo, S. (2020) Estimation of distributed flow resistance in vegetated rivers using airborne topo-bathymetric LiDAR and its application to risk management tasks for Asahi River flooding. J. Flood Risk Manag. **13**, e12584.
Yoshioka, A., Kadoya, T., Suda, S. I., and Washitani, I. (2010) Impacts of weeping lovegrass (*Eragrostis curvula*) invasion on native grasshoppers: responses of habitat generalist and specialist species. Biol. Invasions **12**, 531–539.

Supplementary Table S1. Pairwise genetic differentiation between populations (F_{ST})

		var. *angustifolia*	var. *margaritacea*	var. *yedoensis*	
				Ken'ichi	Oi
var. *angustifolia*		0.000			
var. *margaritacea*		0.250	0.000		
var. *yedoensis*	Ken'ichi	0.318	0.218	0.000	
	Oi	0.379	0.207	0.344	0.000

All populations were significantly differentiated ($p < 0.01$).



Supplementary Fig. S1. Genetic diversity parameters in four populations of *A. margaritacea* for (A) allelic richness (A_R) and (B) expected heterozygosity (H_E). Significant differences were assessed via Wilcoxon signed-rank test, **, $p < 0.01$; *, $p < 0.05$; n.s., not significant).