



TITLE:

Development and characterization of nuclear microsatellite markers in *Aphananthe aspera* (Thunb.) Planch. (Cannabaceae)

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CITATION:

Sakagami, Taishi ...[et al]. Development and characterization of nuclear microsatellite markers in *Aphananthe aspera* (Thunb.) Planch. (Cannabaceae). *Journal of Forest Research* 2020, 25(2): 120-123

ISSUE DATE:

2020-03

URL:

<http://hdl.handle.net/2433/251018>

RIGHT:

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Sakagami *et al* – *Aphananthe* SSR markers1 **Short communication**2 **Title**3 Development and characterization of nuclear microsatellite markers in *Aphananthe*4 *aspera* (Thunb.) Planch. (Cannabaceae)

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21 **Running head:** *Aphananthe* SSR markers

22 **Keywords:** *Aphananthe aspera*, Cannabaceae, genetic diversity, microsatellite markers

Sakagami *et al* – *Aphananthe* SSR markers23 **Abstract**

24 Nuclear microsatellite markers were developed for *Aphananthe aspera* (Thunb.)
25 Planch. (Cannabaceae), a deciduous canopy tree species distributed in East Asia, to
26 evaluate the genetic diversity and genetic structure of *A. aspera* populations in remnant
27 forest fragments in urbanized areas of Japan. A total of 94 primer pairs were designed
28 based on genomic sequence data. Of the 25 primer pairs which showed clear
29 microsatellite peaks, 20 pairs showed allelic polymorphisms in 57 individuals collected
30 from two distant populations. The length of PCR products ranged from 120 to 482 bp,
31 and expected heterozygosity for the 20 microsatellite markers ranged from 0.017 to
32 0.768. These newly developed SSR markers will be used in population genetic studies
33 of *A. aspera* to evaluate genetic diversity and the extent of genetic isolation of the
34 fragmented populations in urban areas.

35 Introduction

36 Habitat fragmentation can have major genetic and demographic consequences
37 on populations (Honnay *et al.* 1999; Oostermeijer *et al.* 2003; Fahrig 2017). It is
38 predicted that reduction in effective population size and the decreased population
39 connectivity can lead to negative effects such as greater inbreeding (Keller and Waller
40 2002), restricted gene flow (Browne and Karubian 2018), and reduced immigration
41 rates (Couvet 2002; Dubreuil *et al.* 2010). While the effects of habitat fragmentation on
42 tree species may be buffered by their longevity and greater capabilities of gene
43 dispersals, empirical studies showed the consequences vary by species and context
44 (Lowe *et al.* 2015), requiring further examinations in different study systems.

45 *Aphananthe aspera* (Thunb.) (Cannabaceae) is a temperate broad-leaved
46 deciduous tree species widely distributed in Japan (from subtropical Okinawa Island to
47 temperate Kanto region), extending to the Korean peninsula, China, and Taiwan. It is a
48 long-lived (in some cases up to > 500 years) and wind-pollinated canopy tree. Ripe
49 berries of *A. aspera* are black purple in color in autumn, and mainly dispersed by birds
50 (Yoshikawa and Kikuzawa 2009). It sometimes becomes dominant in warm-temperate
51 forests in alluvial lowlands, but currently forms small, scattered populations due to
52 human activities especially in urbanized area in Japan (Kimura *et al.* 2019). Although

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53 the species is ecologically important in terms of dominance and interaction with avian
54 and animal species in warm-temperate forests, there have been no studies on the
55 species' genetic diversity and genetic differentiation of the fragmented *A. aspera*
56 populations. Microsatellite markers (or simple sequence repeat, SSR) are highly
57 polymorphic, codominant, and useful to evaluate population genetic characteristics
58 (Jones and Ardren 2003). To investigate the negative effects on gene diversity and
59 genetic structure by fragmentation, microsatellite markers that can be applied to *A.*
60 *aspera* are needed. In this study, we developed nuclear microsatellite markers from *A.*
61 *aspera* using genomic sequence data and evaluated their polymorphisms.

62

63 **Materials and Methods**

64 We assembled the genomic sequence reads of *A. aspera* (GenBank accession
65 numbers of raw sequence reads; Bioproject: PRJDB5250, Submission: DRA005224,
66 Experiment: DRX069505 and Sample: DRS055811) (Sakaguchi et al. 2017), using
67 CLC Genomics Workbench 7.5.1 (CLC bio, Aarhus, Denmark) with the parameter
68 settings of word size = 19, bubble size = 161, and minimum contig length = 100. Then
69 the contigs including microsatellite regions, for ≥ 6 tri-nucleotide repeats, ≥ 8
70 di-nucleotide repeats and ≥ 15 mono-nucleotide repeats, were searched with

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71 MSATCOMMANDER (Faircloth 2008). In total, 4,220 microsatellite motifs were
72 found, and 94 of them were selected to design primer pairs using Primer 3 (Rozen and
73 Skaletsky 2000). For all the primer pairs, the forward primers were synthesized with
74 one of four different universal sequences (5'-CACGACGTTGTAAAACGAC3',
75 5'-TGTGGAATTGTGAGCGG-3', CGGAGAGCCGAGAGGTG-3', or
76 5'-CTATAGGGCACGCGTGGT-3' (Blacket *et al.* 2012)) and the reverse primers were
77 tagged with PIG-tail sequences (5'-GTTTCTT-3') (Brownstein *et al.* 1996). BLASTN
78 search for each contig was performed with a threshold of E-05 for significant hit. 57
79 individuals of *A. aspera* from each of the two populations [29 individuals from
80 Shimogamo Shrine, Kyoto, Japan (35°02'N/135°46'E), 28 individuals from
81 Kawaketa-mikabe Shrine, Shiga, Japan (35°06'N/136°13'E)] were used to evaluate the
82 polymorphisms of the target microsatellite loci. All sampled leaves were dried in silica
83 gel, polysaccharides removed with HEPES buffer (pH 8.0) (Setoguchi and Ohba 1995),
84 and then genomic DNA was isolated using the CTAB method (Doyle and Doyle 1987).
85 The PCR reaction was performed in a 10 μ L reaction volume containing 10–20 ng of
86 DNA, 5 μ L of Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.01 μ M of
87 forward primer, 0.2 μ M of reverse primer and 0.1 μ M of fluorescently labeled M13
88 primer. PCR amplification for all primer pairs started with 3 min at 95°C for initial

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89 denaturation, 35 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 3
90 min, and extension at 68°C for 1 min, and a final extension for 20 min at 68°C. PCR
91 product was loaded onto an auto sequencer (3130 Genetic Analyzer; Applied
92 Biosystems, Carlsbad, California, USA) to assess fragment lengths using GeneMapper
93 software (Applied Biosystems). To evaluate the utility of developed genetic markers,
94 genetic diversity indices (number of alleles, observed heterozygosity and expected
95 heterozygosity) were calculated by using GenAlEx ver. 6.5.2 (Peakall and Smouse
96 2012). Frequency of null alleles were estimated for each combination of locus and
97 population using CERVUS 3.0 (Marshall et al. 1998). Significance of Hardy-Weinberg
98 equilibrium and linkage disequilibrium between loci were tested by GenAlEx ver.
99 6.5.2.

100

101 **Results and discussion**

102 Of 94 primer pairs tested, 25 pairs showed clear microsatellite peaks (Table 1)
103 and 20 pairs displayed allelic polymorphisms in the two populations of *A. aspera*
104 (Table 2). The corresponding sequences of these regions were deposited in DDBJ
105 Center: accession numbers LC360729- LC360744 (Table 1). In the populations of *A.*
106 *aspera*, the number of alleles per locus ranged from 1 to 8, the observed heterozygosity

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107 ranged from 0.000 to 0.724 and the expected heterozygosity ranged from 0.000 to
108 0.783 (Table 2). One locus (Aa_105260) showed consistently significant deviations
109 from Hardy-Weinberg equilibrium in both populations ($P < 0.001$), and relatively high
110 frequencies of null alleles were estimated (0.563 for Shimogamo Shrine and 0.673 for
111 Kawaketa-mikabe Shrine, respectively). Hence, care must be taken when applying the
112 marker (Aa_105260) in any future genetic research. No locus pairs showed significant
113 linkage disequilibrium ($P > 0.05$). In conclusion, the novel 20 nuclear microsatellite
114 markers were polymorphic and reliably scorable in *A. aspera*. These markers will be
115 useful in future studies investigating the population genetics, and evolutionary history
116 and genetic interactions within populations of the species.

117

118 **Acknowledgements**

119 We are grateful to S. Mori, R. Nakamasu for collecting plant materials, and to
120 two anonymous reviewers and coordinating editor for their useful comments on this
121 manuscript. Research permissions were provided by Shimogamo Shrine and
122 Kawaketa-mikabe Shrine. This research was supported by the Japan Society for the
123 Promotion of Science Grant-in-Aid for Scientific Research (JSPS KAKENHI
124 16H04827, 16H04831 and 25291085) and the Environmental Research and
125 Technology Development Fund of the Ministry of the Environment (4-1702, 4-1902),

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126 and SICORP Program of the Japan Science and Technology Agency (grant no.

127 4-1403).

128

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- 189

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 190 **Table 1**

191 Nuclear microsatellite markers for *Aphananthe aspera* (Thunb.) Planch.. Shown for each primer pair are repeat motif, forward and reverse
 192 primer sequence (with tag sequence), the results of BLASTN database search with E-value and the accession number in DDBJ, and allele
 193 size range.

Locus	Repeat motif	Sequence	BLASTN	E-value	GenBank Accession No.	Allele size range (bp)
Aa_115573	(GT) ₁₂	F:CACGACGTTGTAAAACGACTAATGGAGCCGCATCACTTG R:GTTTCTTACAGCTAGCTATTTTCGTGGAAG	No significant hits	-	LC360719	122-128
Aa_84574	(AAT) ₁₀	F:CGGAGAGCCGAGAGGTGTCGGCACCTTACACATTCTTG R:GTTTCTTCGTTAAGATGGGCAGACAGG	No significant hits	-	LC360720	171-180
Aa_105260	(AC) ₁₄	F:CACGACGTTGTAAAACGACAAATTCACAGGAGCACATTTGG R:GTTTCTTGCTGCATGGCCATCTAACAG	No significant hits	-	LC360721	200-224
Aa_96067	(AG) ₁₂	F:CTATAGGGCACGCGTGGTCCGGCTCGTTTGAAGAAGAC R:GTTTCTTGCCGTTGACTCAGACTACCC	No significant hits	-	LC360722	250-262
Aa_103887	(GA) ₁₁	F:CACGACGTTGTAAAACGACTGCGGGTGAGAGATTCTAGC R:GTTTCTTGGAAGGTCAAAGCCCAAAGG	No significant hits	-	LC360723	356-366
Aa_95198	(TG) ₁₃	F:CGGAGAGCCGAGAGGTGCCACGTCCCCTAGCTTCTC R:GTTTCTTGAACATGGCCGTGGTGTAG	No significant hits	-	LC360724	425-437

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Aa_119647	(GA) ₁₀	F:TGTGGAATTGTGAGCGGGGCTGTCTTAAGATGGGAGAAG R:GTTTCTTGATTACAGCCCTGCACGAC	<i>Vitis vinifera</i> , whole genome shotgun sequence, contig VV78X118074.3, clone ENTAV 115	2.00E-12	LC360725	120-144
Aa_10258	(TCT) ₂₇	F:TGTGGAATTGTGAGCGGTGGCCAGAAGTACCCTTGTC R:GTTTCTTCTCCTCGTCCGTATCCTTGG	No significant hits	-	LC360726	421
Aa_27760	(CA) ₁₁	F:CGGAGAGCCGAGAGGTGACACACCGGAAGAAGAAAGC R:GTTTCTTGGACCACCTGCATACAAGAG	PREDICTED: <i>Ziziphus jujuba</i> myb-related protein Myb4-like (LOC107416961), mRNA	2.00E-15	LC360727	460-482
Aa_105963	(GA) ₁₂	F:CTATAGGGCACGCGTGGTACTTTGCACAGATGAACAGAAC R:GTTTCTTTATTGCTGCTGGAGGATGGC	PREDICTED: <i>Prunus avium</i> ultraviolet-B receptor UVR8 (LOC110758246), mRNA	1.00E-14	LC360728	127-143
Aa_104223	(GT) ₉	F:TGTGGAATTGTGAGCGGTGGGAATTTCAAATCCTGGCAG R:GTTTCTTACAAGAAGAGTCAAGCGCAG	No significant hits	-	LC360729	187-197
Aa_50376	(TC) ₁₁	F:CTATAGGGCACGCGTGGTGGTGAAC TTGTTGGGAGCAC R:GTTTCTTCATCCCACCCAATTTCACC	No significant hits	-	LC360730	225-231
Aa_104956	(GA) ₉	F:CACGACGTTGTAAAACGACGTCCTTGTCACGTGGCTTTC R:GTTTCTTTTTGCCAGAGATTGCAATTGG	No significant hits	-	LC360731	240-276
Aa_44791	(AC) ₁₀	F:CACGACGTTGTAAAACGACTCGTAGGAATTCGCACGTG R:GTTTCTTTCAGGATAATGGTGTCAAGCG	No significant hits	-	LC360732	423-439
Aa_104111	(AG) ₁₁	F:CTATAGGGCACGCGTGGTACCAAAC TCAACCACACCAG R:GTTTCTTGAAAGGATTGGCGTCGTTCC	No significant hits	-	LC360733	444-460
Aa_10240	(TC) ₁₁	F:CGGAGAGCCGAGAGGTGAGTTTGTTCCTTCTTTCTG R:GTTTCTTGTCATTATTGCCTAAGTTGCC	No significant hits	-	LC360734	145-159
Aa_13484	(AG) ₁₂	F:CTATAGGGCACGCGTGGTGATAAGGCGGGAGGAGTACG	No significant hits	-	LC360735	193-205

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		R:GTTTCTTCCCATTTGCCCGTTCTTTC				
Aa_17675	(A) ₁₉	F:CGGAGAGCCGAGAGGTGGTATGTGTTTATGACCTTGTGC	No significant hits	-	LC360736	250
		R:GTTTCTTTCGTAATGTTACCTCGCTAAGC				
Aa_13178	(AGT) ₇	F:CGGAGAGCCGAGAGGTGAAAGAAGGTTGAAGGCTGCG	No significant hits	-	LC360737	187-199
		R:GTTTCTTAGATCATCTTCTAATTCCGCCAC				
Aa_12869	(T) ₁₈	F:CTATAGGGCACGCGTGGTGCTAGGTCAAACCTATGGGCC	No significant hits	-	LC360738	221
		R:GTTTCTTTTTCCCTCGACGAGCTATGG				
Aa_13711	(AAT) ₆	F:TGTGGAATTGTGAGCGGATTGTGGGCCTCGACCTTAG	No significant hits	-	LC360739	327
		R:GTTTCTTAGCACCCAGTAACATCATGTG				
Aa_19942	(A) ₁₈	F:CGGAGAGCCGAGAGGTGAGGGTGAGCTGTCCTGTAAG	No significant hits	-	LC360740	195-196
		R:GTTTCTTTGAGATGGTTCGGAGGTCTG				
Aa_10150	(ATA) ₇	F:CGGAGAGCCGAGAGGTGGGCTGGATTGTTGCATTGC	No significant hits	-	LC360741	234-273
		R:GTTTCTTTACGAACAGAGACGTGGTGG				
Aa_4179	(AAT) ₆	F:CACGACGTTGTAAAACGACCCAGATTAGCTAGATGTCAC		2.00E-16	LC360743	191-197
		R:GTTTCTTAACCAGCAATCCGAGCATTC	<i>Cucumis melo</i> genomic chromosome, chr_1			
Aa_15273	(GA) ₉	F:CTATAGGGCACGCGTGGTACCAGGCCGATGAGAGTTTC	No significant hits	-	LC360744	244-246
		R:GTTTCTTGGCCCGTCATTTAACCAAGG				

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Table 2

Genetic diversity statistics for two populations of *Aphananthe aspera* (Thunb.) Planch. based on the newly developed polymorphic genomic microsatellite markers. Abbreviations are as follow; N_A : number of alleles, H_O : observed heterozygosity, H_E : expected heterozygosity, Null: null allele frequency estimate. Deviations from Hardy-Weinberg equilibrium are noted by asterisks (** $P < 0.001$).

Locus	Shimogamo Shrine (n = 29)				Kawaketa-mikabe Shrine (n = 28)				Total (n = 57)		
	N_A	H_O	H_E	Null	N_A	H_O	H_E	Null	N_A	H_O	H_E
Aa_4179	2	0.034	0.034	-0.002	1	0.000	0.000	-0.002	2	0.018	0.017
Aa_10150	5	0.556	0.719	0.201	5	0.704	0.783	0.125	6	0.630**	0.768
Aa_10240	4	0.586	0.612	0.050	4	0.714	0.631	0.050	4	0.649	0.647
Aa_13178	3	0.241	0.216	-0.060	2	0.500	0.497	-0.060	3	0.368	0.416
Aa_13484	4	0.483	0.594	0.141	4	0.607	0.578	0.141	4	0.544	0.606
Aa_19942	2	0.448	0.348	-0.274	2	0.464	0.357	-0.286	2	0.456	0.352
Aa_27760	4	0.655	0.631	-0.007	4	0.714	0.682	-0.007	4	0.684	0.663
Aa_44791	3	0.500	0.498	0.002	2	0.464	0.469	0.002	3	0.482	0.508
Aa_50376	3	0.172	0.161	-0.038	3	0.286	0.283	-0.038	3	0.228	0.223
Aa_84574	3	0.552	0.565	0.024	2	0.393	0.392	0.024	3	0.474	0.492
Aa_95198	4	0.448	0.521	0.057	3	0.571	0.579	0.057	4	0.509	0.554
Aa_96067	4	0.483	0.508	0.037	4	0.679	0.658	0.037	4	0.579	0.604
Aa_103887	4	0.310	0.278	-0.068	3	0.464	0.424	-0.068	4	0.386	0.357
Aa_104111	5	0.724	0.736	0.001	5	0.679	0.642	0.001	5	0.702	0.699
Aa_104223	3	0.379	0.355	-0.025	2	0.143	0.133	-0.026	3	0.263	0.263
Aa_104956	8	0.621	0.737	0.116	4	0.643	0.600	0.116	8	0.632	0.695
Aa_105260	7	0.345**	0.675	0.563	4	0.071**	0.226	0.673	7	0.211**	0.495
Aa_105963	3	0.517	0.450	-0.043	3	0.393	0.456	-0.043	3	0.456	0.453
Aa_115573	3	0.690	0.651	-0.019	3	0.607	0.548	-0.019	3	0.649	0.614
Aa_119647	3	0.414	0.424	0.044	3	0.536	0.506	0.044	4	0.474	0.474
Average	3.9	0.458	0.486	0.035	3.2	0.482	0.472	0.036	4.0	0.470	0.495

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