Taiwania 65(2): 249–252, **2020**
DOI: 10.6165/tai.2020.65.249

NOTE

Development and characterization of EST-SSR markers in creeping mazus (*Mazus miquelii*), and cross-amplification in five related species

Masaya YAMAMOTO[1,*], Daiki TAKAHASHI[2], Chih-Chieh YU[3, 4], Hiroaki SETOGUCHI[2]

1. Hyogo University of Teacher Education, 942-1 Shimokume, Kato-city, Hyogo 673-1494, Japan.
2. Graduate School of Human and Environmental Studies, Kyoto University, Yoshida Nihonmatsu, Sakyo-ku, Kyoto 606-8501, Japan.
3. CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, China.
4. Center of Plant Ecology, Core Botanical Gardens, Chinese Academy of Sciences, Xishuangbanna 666303, China.
[*]Corresponding author's email: myamamo@hyogo-u.ac.jp; Tel: +81-(0)795-44-2201

(Manuscript received 5 February 2020; Accepted 12 April 2020; Online published 18 April 2020)

ABSTRACT: Simple sequence repeat (SSR) markers were developed from expressed sequence tags (ESTs) for *Mazus miquelii* (Mazaceae), one of the most widespread species of the genus found in mainland China to Japan and North America, with the goal of elucidating the hidden genetic diversity and pollination ecology of the species. Of the initial 48 EST-SSR markers designed based on transcriptome data, 36 loci were successfully amplified, 16 of which were polymorphic. Polymorphisms for these markers were tested on 72 individuals from three populations in Japan. Two to eleven alleles per locus were detected, and the levels of observed and expected heterozygosity ranged from 0.181 to 0.708 and 0.154 to 0.715, respectively. Most loci were amplified successfully in five related Japanese and Taiwanese species. These markers will empower ecological and evolutionary studies in the creeping mazus and facilitate the disentanglement of phylogenetic relationships with related species.

KEY WORDS: *microsatellite, genetic diversity, expressed sequence tag, Mazus, Mazaceae.*

INTRODUCTION

Genus *Mazus* Loureiro (Mazaceae) is consisted of annual or perennial herbs with distribution from Asia to Oceania and comprises approximately 30 species mainly from mainland China (Hong *et al*., 1998). *Mazus* species are small herbs characterized by a bilabiate corolla with a bilobed stigma. The behavior of a sensitive bilobed stigma has attracted the attention of many botanists and ecologists for nearly a century (e.g., Newcombe, 1924; Kimata, 1978; Jin *et al*., 2015). Recent ecological studies suggest that stigma closure and re-opening in *Mazus* is an evolutionary mechanism to enhance outcrossing via pollinator selection (Jin *et al*., 2015, 2017). However, the levels of selfing and/or outcrossing are still unknown in natural fields due to lack of high-resolution molecular markers for the plant group. As the stigma behavior may can control gene flow within and among populations, genetic insights can improve our understanding of how the unique pollination strategy has evolved and is being maintained in nature.

Simple sequence repeats (SSRs), also known as microsatellites, are useful molecular markers with many applications in plant genetic studies owing to their abundance, high level of polymorphisms, and ease of scoring (Bouck and Vision, 2007). Compared to genomic SSRs, expressed sequence tag (EST)-SSR markers show lower polymorphism, but are less susceptible to null alleles and better conserved across related species (Gupta *et al*., 2003; Bouck and Vision, 2007; Ellis and Burke, 2007). Therefore, we considered that novel EST-SSR markers in *Mazus* species would provide powerful molecular tools for the plant group.

Here, we developed EST-SSR markers for the well-studied diploid perennial herb *Mazus miquelii* Makino, one of the most widespread species in the genus found from mainland China to Japan and in North America as an introduced species. Additionally, we evaluated the transferability of these markers in five related Japanese and Taiwanese species.

MATERIALS AND METHODS

Total RNA was extracted from fresh leaf samples of *M. miquelii* (Table 1) using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, California, USA). The cDNA library was constructed and sequenced on an Illumina HiSeq 2000 sequencing system (Illumina, San Diego, California, USA; performed by BGI, Shenzhen, China) with 100 bp paired-end reads. Raw reads were quality trimmed using Trimmomatic 0.38 (Bolger *et al*., 2014). The clean reads were deposited in the DNA Data Bank of Japan (DDBJ; BioProject PRJDB9031), and assembled into 77,354 contigs using Trinity v2.8.4 (Haas *et al*., 2013). Microsatellites were detected using the Krait v0.10.2 (Du *et al*., 2018). To identity ideal microsatellites, the minimum repeat number was defined as 12 for mono-, 7

Table 1. Source localities of *Mazus* species used in this study.

Species	Population	Collection locality	Geographic coordinates	*N*
Mazus miquelii Makino*	Cultivated	Kyoto University, Kyoto City, Kyoto Pref., Japan	35°01′N, 135°45′E	1
M. miquelii†	A	Kamiukena-gun, Ehime Pref., Japan	33°31′N, 132°57′E	24
M. miquelii†	B	Kato City, Hyogo Pref., Japan	34°54′N, 135°00′E	24
M. miquelii†	C	Matsumoto City, Nagano Pref., Japan	36°10′N, 137°50′E	24
M. pumilus (Burn.f.) Steenis§	—	Kato City, Hyogo Pref., Japan	35°01′N, 135°45′E	2
M. goodenifolius (Hornem.) Pennell§	—	Amami Islands, Kagoshima Pref., Japan	—	2
M. quadriprotuberans N. Yonezawa§	Cultivated	Kyoto Botanical Gardens, Kyoto City, Kyoto Pref., Japan	35°02′N, 135°45′E	2
M. alpinus Masamune§	—	Taipingshan, Yilan, Taiwan	—	2
M. fauriei Bonati§	—	Pingxi, New Taipei, Taiwan	—	2

N, number of individuals sampled. *Samples used for cDNA library construction. †Samples used for initial PCR amplification trials and detailed evaluation for polymorphisms. §Samples used for transferability test.

for di-, 5 for tri-, and 4 for tetra-, penta-, hexa-nucleotide SSRs. A total of 1,597 markers were designed with Primer3 (Untergasser *et al*., 2012) implemented within Krait, of which 48 pairs were selected as candidate microsatellite markers for *M. miquelii* based on repeat number and product size. For all primer pairs, one of four different M13 universal sequences (5′-CACGACGTTGTAAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, 5′-CTATAGGG CACGCGTGGT-3′, or 5′-CGGAGAGCCGAGAGGTG-3′) and a pigtail sequence (5′-GTTTCTT-3′) were added to the 5′ end of each forward primer and the 3′ end of each reverse primer, respectively.

A total of 72 individuals of *M. miquelii* representing three populations (24 individuals per population) were used to evaluate the polymorphisms of the candidate microsatellite loci, and two individuals each of *M. pumilus*, *M. goodenifolius*, *M. quadriprotuberans*, *M. alpinus* and *M. fauriei* were used to test their transferability (Table 1). Genomic DNA was extracted from silica gel-dried leaf material using the cetyltrimethylammonium bromide method (Doyle and Doyele, 1987). PCR amplifications were conducted in 5-μL reactions containing approximately 0.5 ng DNA, 2.5 μL 2× Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.01 μM forward primer, 0.2 μM reverse primer, and 0.1 μM fluorescence-labeled M13 primer. The PCR thermal profile was set as follows: an initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 60°C for 3 min, and 68°C for 1 min; and then a final extension at 68°C for 20 min. PCR products were loaded onto an ABI 3130 autosequencer (Applied Biosystems, Carlsbad, California, USA) using the GeneScan 600 LIZ Size Standard (Applied Biosystems), POP7 polymer (Applied Biosystems), and a 36-cm capillary array. Fragment size was determined with GeneMapper software (Applied Biosystems). For each polymorphic locus, the number of alleles (A), number of effective alleles (A_E), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) were calculated using GenoDive (Meirmans and van Tienderen, 2004). The absence of linkage disequilibrium (LD) and deviation from Hardy-Weinberg equilibrium (HWE) were assessed in GENEPOP v4.2 (Raymond and Rousset, 1995) using the Markov chain method with default settings. Results were adjusted for multiple comparisons using sequential Bonferroni correction. The software MICRO-CHECKER v2.20 (van Oosterhout *et al.*, 2004) was used to detect the null alleles, stuttering, and allelic dropout.

RESLUTS AND DISCUSSION

Of the 48 candidate primer pairs tested, 16 loci displayed polymorphisms in *Mazus miquelii* (Table 2). Meanwhile, 12 of the remaining 32 pairs failed to amplify and 20 pairs showed monomorphic or unclear peaks (e.g., split peaks or a triallelic pattern). In three natural populations validated for polymorphism analyses, population B showed clear polymorphisms across all 16 loci, whereas a few loci (*maz17431*, *maz19506* and/or *maz49947*) were monomorphic in other populations (Table 3). Overall, the newly developed EST-SSR loci displayed relatively high levels of genetic diversity in *M. miquelii*; the average values for A, A_E, H_O, H_E and F_{IS} were 6.6, 2.1, 0.458, 0.501, and 0.086, respectively (Table 3). Although no paired loci consistently showed LD in all populations, HWE tests showed that the three populations significantly deviated from HWE at several loci (Table 3). However, with the exception of one locus, *maz290*, no loci deviated from Hardy-Weinberg equilibrium across the three populations. Six loci (*maz1523*, *maz47656*, *maz11580*, *maz49518*, *maz8188*, and *maz290*) exhibited evidence for the presence of null alleles in at least one population; however, the results of locus *maz290* were consistent across all the populations (Table 3). Although there was no evidence of allelic dropout in any of the loci, scoring problems due to stuttering were observed for *maz290* locus. These results suggest that deviation from HWE at *maz290* locus was most likely due to the presence of null alleles and/or genotyping errors. The results of cross-amplifications in five related species are shown in Table 4. Of the 16 EST-SSR primer pairs tested, 14 successfully PCR amplified in *M. fauriei,* and 13 in *M. pumilus*, *M. goodenifolius* and *M. alpinus*. In *M. quadriprotuberans*, all 16 primer pairs amplified successfully. Since the species delineation in the genus has been ambiguous and controversial due to its plastic morphological characteristics (Hong *et al*., 1998;

Table 2. Sixteen polymorphic EST-SSR markers developed for *Mazus miquelii*.

Locus	Primer sequences (5'–3')	Repeat motif	Allele size range (bp)	BLASTX top hit description [species]	*E*-value	GenBank accession no.
Maz 1523	F: CACGACGTTGTAAAACGACCAGCCTACACGCCACAAC R: GTTTCTTATGAGGCTGCGGAATCTAGG	$(AG)_8$	280–288	putative O-acyltransferase WSD1-like [*Davidia involucrata*]	0	LC511753
Maz 19506	F: CTATAGGGCACGCGTGGTTGTGTGAATGCCTGCAACTG R: GTTTCTTACCACGCTCATCTCCTTACC	$(TC)_7$	258–264	No significant hit	-	LC511754
Maz 27867	F: CTATAGGGCACGCGTGGTCGATGAACATGGACGAGCTTC R: GTTTCTTGTGGCCTGCTGATTGAATCC	$(ACC)_5$	425–440	hypothetical protein [*Davidia involucrata*]	0	LC511755
Maz 28563	F: CTATAGGGCACGCGTGGTGAGCAGATGTTGAGTGAGCG R: GTTTCTTTCTTCAAGTCTGCCTCTCCC	$(AC)_9$	156–166	No significant hit	-	LC511756
Maz 47565	F: TGTGGAATTGTGAGCGGTGTGAGAGATGCCGGAGATG R: GTTTCTTTCTGCTCTCTGACCAAGGAC	$(AC)_{10}$	280–298	hypothetical protein [*Davidia involucrata*]	2.00E-48	LC511757
Maz 49830	F: CACGACGTTGTAAAACGACCGGATGGTGCCTTTCTTGTG R: GTTTCTTTGTTCCTCTCTCCTTCCTTACC	$(TC)_{12}$	178–210	putative homeobox-leucine zipper protein MERISTEM L1-like [*Davidia involucrata*]	0	LC511758
Maz 59432	F: CGGAGAGCCGAGAGGTGCAATCCCAAGAAACCCACCG R: GTTTCTTCGGCATTCACATACCAGCTG	$(AAG)_6$	338–368	putative Caspase [*Davidia involucrata*]	0	LC511759
Maz 11580	F: TGTGGAATTGTGAGCGGTAACCCGTTTCCTGCCACAC R: GTTTCTTTTGGGATGTATGGCCGACAG	$(AG)_{11}$	162–212	polyU-specific endoribonuclease-B [*Rhizophora mucronata*]	5.00E-07	LC511760
Maz 17431	F: CTATAGGGCACGCGTGGTAATCTCTTCCAGGCCTTCGG R: GTTTCTTGCCATTGGTTGACTTGGTGG	$(AG)_{10}$	187–189	hypothetical protein [*Davidia involucrata*]	4.00E-103	LC511761
Maz 23141	F: CGGAGAGCCGAGAGGTGGATTTGTCGGCTAACTCGGC R: GTTTCTTTCACAAACTGAATCGCATCCC	$(AACTT)_5$	285–320	putative riboflavin biosynthesis protein PYRR, chloroplastic-like [*Solanum chacoense*]	1.00E-32	LC511762
Maz 49518	F: CTATAGGGCACGCGTGGTGATCAAACTTTGTCACTCCTGC R: GTTTCTTCCGTGTTGATCCATGTCGTG	$(AG)_9$	294–324	putative 1-deoxy-D-xylulose-5-phosphate synthase 1 [*Davidia involucrata*]	0	LC511763
Maz 49947	F: TGTGGAATTGTGAGCGGACGACGCTCTCCTCCATTAC R: GTTTCTTTCATCTTCTCGAACCTGGCC	$(CCG)_5$	261–285	hypothetical protein [*Davidia involucrata*]	0	LC511764
Maz 8188	F: TGTGGAATTGTGAGCGGTGCCCATTCTGACATTCACG R: GTTTCTTTTCCTTCGCCGTTACTAAGAC	$(AT)_{10}$	346–370	putative ADP-ribosylation factor GTPase-activating protein AGD11 [*Davidia involucrata*]	8.00E-92	LC511765
Maz 1458	F: CGGAGAGCCGAGAGGTGTCCTCACACTCAGACTGCAG R: GTTTCTTACCCTCCAAACCCACTTCTC	$(CCG)_5$	276–302	putative protein TIC 22-like, chloroplastic-like [*Solanum chacoense*]	4.00E-35	LC511766
Maz 27356	F: CGGAGAGCCGAGAGGTGGTCAATTCTGGCGGCCTTAC R: GTTTCTTCTTCTGCAAATTCCCTCCGC	$(CCG)_6$	321-–348	putative high mobility group B protein 14 [*Davidia involucrata*]	6.00E-46	LC511767
Maz 290	F: TGTGGAATTGTGAGCGGCGATGGTGATGTGGGAAGTG R: GTTTCTTAGACTCCACACAGCTAGCTC	$(AC)_{11}$	204–224	No significant hit	-	LC511752

Table 3. Characteristics of newly developed polymorphic EST-SSR markers in three *Mazus miquelii* populations.

	Population A (n = 24)					Population B (n = 24)					Population C (n = 24)					Total (n = 72)				
Locus	A	A_E	H_O	H_E	F_{IS}	A	A_E	H_O	H_E	F_{IS}	A	A_E	H_O	H_E	F_{IS}	A	A_E	H_O	H_E	F_{IS}
maz1523	2	1.9	0.667	0.493	-0.353	3	2.4	0.292	0.607	0.519*†	2	1.9	0.208	0.495	0.579†	4	2.1	0.389	0.531	0.268
maz19506	3	1.8	0.333	0.470	0.291	3	1.4	0.333	0.296	-0.125	1	1.0	0.000	0.000	NA	4	1.3	0.222	0.255	0.130
maz27867	4	1.8	0.458	0.453	-0.012	4	1.6	0.417	0.388	-0.075	2	1.4	0.375	0.310	-0.211	5	1.6	0.417	0.383	-0.087
maz28563	4	2.3	0.708	0.565	-0.253	3	2.2	0.750	0.559	-0.342	2	1.2	0.208	0.190	-0.095	5	1.8	0.556	0.438	-0.268
maz47565	3	2.7	0.833	0.642	-0.298	6	2.9	0.667	0.674	0.011	2	2.0	0.083	0.520	0.840*†	6	2.5	0.528	0.612	0.138
maz49830	3	2.2	0.542	0.551	0.016	5	3.5	0.917	0.722	-0.270*	3	2.9	0.667	0.669	0.004	8	2.7	0.708	0.647	-0.094
maz59432	4	3.1	0.875	0.687	-0.274	3	1.5	0.292	0.328	0.110	5	3.4	0.542	0.727	0.255*	6	2.3	0.569	0.581	0.019
maz11580	7	5.1	0.792	0.820	0.034	6	4.9	0.500	0.818	0.389*†	4	2.0	0.458	0.507	0.096	11	3.3	0.583	0.715	0.184
maz17431	1	1.0	0.000	0.000	NA	2	1.8	0.708	0.462	-0.533	1	1.0	0.000	0.000	NA	2	1.2	0.236	0.154	-0.533
maz23141	4	2.9	0.542	0.670	0.192	8	7.0	0.917	0.874	-0.049*	2	1.7	0.542	0.400	-0.353	9	2.7	0.667	0.648	-0.028
maz49518	3	2.1	0.458	0.538	0.148	6	4.6	0.583	0.803	0.274*†	2	1.8	0.333	0.457	0.270	7	2.4	0.458	0.599	0.235
maz49947	2	1.0	0.042	0.042	0.000	4	3.0	0.500	0.688	0.273	1	1.0	0.000	0.000	NA	4	1.3	0.181	0.243	0.257
maz8188	3	2.4	0.792	0.596	-0.328	6	3.4	0.542	0.724	0.252*†	3	2.3	0.250	0.578	0.567*†	8	2.6	0.528	0.633	0.166
maz1458	3	1.5	0.375	0.317	-0.183	3	2.1	0.375	0.533	0.296	3	1.4	0.333	0.289	-0.154	6	1.6	0.361	0.380	0.049
maz27356	4	3.0	0.917	0.680	-0.348	7	4.5	0.875	0.791	-0.107*	2	1.1	0.125	0.120	-0.045	11	2.1	0.639	0.530	-0.205
maz290	4	2.4	0.292	0.600	0.514*†	8	5.2	0.375	0.833	0.550*†	3	2.2	0.208	0.574	0.637*†	10	2.8	0.292	0.669	0.564
Mean	**3.4**	**2.3**	**0.575**	**0.537**	**-0.062**	**4.8**	**3.2**	**0.565**	**0.526**	**0.105**	**2.4**	**1.8**	**0.271**	**0.231**	**0.257**	**6.6**	**2.1**	**0.458**	**0.501**	**0.086**

A, number of alleles; A_E, number of effective alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; *significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.05$); †significant possibility of the presence of null alleles (95% confidence level).

Table 4. Cross-amplification of 16 polymorphic EST-SSR markers developed for *Mazus miquelii* in five other species of *Mazus*.

Locus	*M. pumilus*	*M. goodenifolius*	*M. quadriprotuberans*	*M. alpinus*	*M. fauriei*
Maz 1523	+	+	+	+	+
Maz 19506	+	+	+	+	+
Maz 27867	+	+	+	+	+
Maz 28563	—	+	+	+	—
Maz 47565	+	—	+	—	+
Maz 49830	+	+	+	+	+
Maz 59432	+	+	+	+	+
Maz 11580	+	+	+	+	+
Maz 17431	+	+	+	+	+
Maz 23141	—	+	+	+	+
Maz 49518	+	+	+	+	+
Maz 49947	—	+	+	+	+
Maz 8188	+	+	+	+	+
Maz 1458	+	—	+	—	—
Maz 27356	+	+	+	—	+
Maz 290	+	—	+	+	+

+, successful amplification in both two individuals;
—, unsuccessful amplification.

Hsieh, 2000; Deng *et al*., 2019), such a high transferability indicates that these EST-SSR markers could be useful for evaluating genetic variation and genetic relationship within and among species.

In this study, we developed 16 novel polymorphic EST-SSR markers for *M. miquelii*. These high-resolution molecular markers revealed substantial genetic variation harbored among *M. miquelii* populations in Japan, with most loci transferrable to related taxa. To our knowledge, the results presented herein represent the first analysis of SSRs and EST-SSR markers in the genus *Mazus*. Thus, these markers will empower future ecological and evolutionary studies involving this taxon and provide novel population genetic tools for other *Mazus* species.

ACKNOWLEDGMENTS

We are grateful to Dr. G. Kokubugata (National Museum of Nature and Science), Dr. T. Ito (Kyoto University), Mr. K. Ueno (Botanical Society of Nagano) and Kyoto Botanical Gardens for providing plant materials. This work was financially supported by Grants-Aid for Scientific Research from Japan Society for the Promotion of Science (16H04831 and 19K16219), and the Environment Research and Technology Development Fund (4-1702).

LITERATURE CITED

Bolger, A.M., M. Lohse and B. Usadel. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinform. **30(15)**: 2114–2120.

Bouck, A. and T. Vision. 2007. The molecular ecologist's guide to expressed sequence tags. Mol. Ecol. **16(5)**: 907–924.

Deng, T., N. Lin, X. Huang, H. Wang, C. Kim, D. Zhang, W. Zhu, Z. Yusupov, K.S. Tojibaev and H. Sun. 2019. Phylogenetic of Mazaceae (Lamiles), with special reference to intrageneric relationships within *Mazus*. Taxon **68(5)**: 1037–1047.

Doyle, J. and J.L. Doyle. 1987. Genomic plant DNA preparation from fresh tissue-CTAB method. Phytochem. Bull. **19**: 11–15.

Du, L., C. Zhang, Q. Liu, X. Zhang and B. Yue. 2017. Krait: an ultrafast tool for genome-wide survey of microsatellites and primer design. Bioinform. **34(4)**: 681–683.

Ellis, J.R. and J.M. Burke. 2007. EST-SSRs as a resource for population genetic analyses. Heredity **99(2)**: 125–132.

Gupta, P.K., S. Rustgi, S. Sharma, R. Singh, N. Kumar and H.S. Balyan. 2003. Transferable EST-SSR markers for the study of polymorphism and genetic diversity in bread wheat. Mol. Genet. Genomics. **270(4)**: 315–323.

Haas, B.J., A. Papanicolaou and M. Yassour, M. Grabherr, P.D. Blood, J. Bowden, M.B. Couger, D. Eccles, B. Li, M. Lieber, M.D. MacManes, M. Ott, J. Orvis, N. Pochet, F. Strozzi, N. Weeks, R. Westerman, T. William, C.N. Dewey, R. Henschel, R.D. LeDuc, N. Friedman, A. Regev. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. **8(8)**: 1494–1512.

Hong, D.Y., H. Yang, C.L. Jin and N.H. Holmgren. 1998. Scrophulariaceae. In: Wu, Z.Y. and P.H. Raven (eds.), Flora of China, vol. 18: 1–212. Science Press, Beiling.

Hsieh, T.H. 2000. Revision of *Mazus* Lour. (Scrophulariaceae) in Taiwan. Taiwania **45(2)**: 131–146.

Jin, X.F., Z.M. Ye, Q.F. Wang and C.F. Yang. 2015. Relationship of stigma behaviors and breeding system in three *Mazus* (Phrymaceae) species with bilobed stigma. J. Syst. Evol. **53(3)**: 259–265.

Jin, X.F., Z.M. Ye, G.M. Amboka, Q.F. Wang and C.F. Yang. 2017. Stigma Sensitivity and the Duration of Temporary Closure Are Affected by Pollinator Identity in *Mazus miquelii* (Phrymaceae), a Species with Bilobed Stigma. Front. Plant Sci. **8**: 783.

Kimata, M. 1978. Comparative studies on reproductive systems of *Mazus japonicus* and *M. miquelii* (Scrophulariaceae). Plant Syst. Evol. **129(4)**: 243–253.

Meirmans, P.G. and P.H. van Tienderen. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes **4(4)**: 792–794.

Newcombe, F.C. 1924. Significance of the behavior of sensitive stigmas II. Am. J. Bot. **11(2)**: 85–93.

Raymond, M. and F. Rousset. 1995. GENEPOP (Version1.2): population genetics software for exact tests and ecumenicism. Heredity **86(3)**: 248–249.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm and S.G. Rozen. 2012. Primer3 - new capabilities and interfaces. Nucleic. Acids Res. **40**: e115.

van Oosterhout, C., W.F. Hutchinson, D.P. Wills and P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes **4(3)**: 535–538.