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Development of 12 EST-SSR markers in *Aquilegia* (Ranunculaceae) and cross amplification in three Chinese species

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In order to investigate the population genetic structure of three sympatric Chinese *Aquilegia* species, we developed a novel set of 12 SSR markers from the EST unigene database. The number of alleles per locus ranged from one to five. The observed heterozygosity (H_o) and the expected heterozygosity (H_e) varied from 0.067 to 1.000 and from 0.064 to 0.791, respectively. Most of the SSR loci in the three *Aquilegia* species did not significantly deviate from random mating expectation and the Hardy-Weinberg equilibrium (HWE). Because of their high level of polymorphism, the twelve EST-SSR markers will be valuable for mating system, population genetics and conservation studies of *Aquilegia* resources in the future.

Introduction

Aquilegia (Ranunculaceae), including more than 70 species, is becoming a new model plant in ecological and evolutionary research due to its floral diversity, variable habitats and unique phylogenetic status (Kramer & Hodges 2010). The floral diversity is generally related to the specialized pollinators that prefer different floral traits. Tang *et al*. (2007) investigated the flower phenology in three sympatric *Aquilegia* species (*A. incurvata*, *A. yabeana* and *A. ecalcarata*) in Qinling Mountains, China and found that their different pollinators played important roles in reproductive isolation. The spurless *A. ecalcarata* was pollinated by the syrphid fly *Metasyrphus latifasciatus*, while the spurred *A. incurvata* and *A. yabeana* were mainly pollinated by different bumblebees (*Bombus trifasciatus* and *B. religious*, respectively). We aimed to know whether there is any interspecific gene flow and how the behaviors of the different pollinators influence the genetic structure of each species. In this study, we developed a set of novel microsatellite markers to facilitate population genetic studies of the three *Aquilegia* species.

Material and methods

Perfect microsatellites were obtained by screening through the EST database of *A. formosa* × *A. pubescens* available from Unigene Microsatellite Database (http://veenuash.info/web1/index.htm)

with microsatellite length greater than ten repeats for dinucleotides, eight for trinucleotides, and six for tetra-, penta- and hexanucleotides. Totally 386 unique EST sequences containing SSR were derived, of which 30 EST-SSRs were randomly selected for primer design using the software Primer3 (Rozen & Skaletsky 1999).

EST-SSR markers were evaluated in three Chinese *Aquilegia* species (*A. ecalcarata*, 33.623°N, 107.756°E; *A. incurvata*, 33.589°N, 107.790°E; *A. yabeana,* 34.074°N, 107.692°E). The genomic DNA was extracted from silica-dried leaves using CTAB method (Doyle & Doyle 1990) and 15 individuals per species were tested for PCR. Amplification was carried out in a volume of 10 μl containing 1× Taq PCR Mix (Xian Runde Biotechnology Co., Ltd., China), 0.2 μM of each primer, and 40 ng of DNA template. PCR was performed with the Touch Down program in MJ Research PTC-200 Thermal Cycler: 94 °C for 5 min; 20 cycles of denature at 95 °C for 30 s; annealing temperature at 59 °C for 45 s with 0.5 °C touchdown per cycle and extension at 72 °C for 60 s; then additional 9 cycles of the similar protocol but with the annealing temperature at 50 °C; followed by a final extension of 7 min at 72 °C for 6min. The amplified products were separated in 10% denaturing polyacrylamide gels, and visualized by silver staining. Fragment sizes of each locus were weighted with a 50 bp ladder using Quantity One (Bio-Rad).

Results

Out of 30 microsatellite loci, 12 produced reliable polymorphic products (Appendix 1). Possible null alleles were not found in the 12 loci by using MICRO-CHECKER 2.2.3 (Van Oosterhout *et al*. 2004). The program Genepop (Rousset 2008) was used to estimate population genetic parameters. In *A. ecalcarata*, the observed (N_a) and the effective (N_e) numbers of alleles per locus varied from 2 to 5, and from 1.142 to 4.787, respectively (Appendix 2). The observed heterozygosity (H_o) and the expected heterozygosity (H_e) ranged from 0.133 to 1.000, from 0.124 to 0.791, respectively. In *A. incurvata*, N_a and N_e ranged between 1–4, and 1–2.941; H_o and H_e ranged between 0–0.867 and 0–0.660, respectively. In *A. yabeana*, N_a and N_e ranged between 2–5 and 1.557–3.103; H_o and H_e ranged between 0.267–0.933 and 0.358–0.678, respectively. Several loci (A1 and A6 in *A. ecalcarata*, A10 in *A. incurvata*, A1 and A10 in *A. yabeana*) presented potential inbreeding. Two loci (A1 and A10) significantly deviated from Hardy-Weinberg equilibrium in *A. yabeana*. Linkage disequilibrium was detected among some pairs of EST-SSRs loci (A1 and A9, A8 and A9, A9 and A11, A10 and A11, A5 and A12).

The phylogenetic tree constructed using the UPGMA method indicated that individuals of each species clustered into one group and the relationship between the two *Aquilegia* species with spurs (*A. incurvata* and *A. yabeana*) was much closer (data not shown). These novel EST-SSR markers with high polymorphism in the three Chinese *Aquilegia* species provided a particularly useful tool for investigating the population structure, phylogenetic relationships and conservation genetics in *Aquilegia*.

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Appendix 1. Characterization of EST-SSRs derived from three *Aquilegia* species.

Locus	GenBank number	Repeat motif	Forward primer/reverse primer (5´–3´)	Expected size (bp)	Size range (bp)	Potential function
A1	S27716232	(AT)9	F: ACCTCCCTCCCTGTTGG R: GCTGGTGGCTATGATGATGC	165	155–204	unknown
A2	S27702138	(AG)12	F: CGAGAGCGGAGAGTCACACCAGT R: ATCCGTCTCCAAGCCATCTACCC	165	165–171	ftsH-like protease
A3	S27730007	(TC)11	F: AGCGAACAAAGCGAGAAC R: TAGGGCAAATTAAAGAACAAGT	210	191–220	pyruvate kinase
A4	S27703621	(TC)9	F: CCCGGTCCCCAGTGTAAAGT R: CTAGAAGAGGCGACGACGAGAAT	262	260–266	zinc finger A20 and AN1 domain-containing stress-associated protein
A5	S27726706	(ATG)7	F: CCACGTCATCTTCCCCAAAAT R: TGACGACGACGACGAAGAAAAT	237	232–238	EID1-like F-box protein 3
A6	S27718411	(GCT)9	F: GCCCCGTCCTCCACCACT R: CCCCCATCAGCTCCCACAG	172	163-174	protein SEUSS-like 2
A7	S27716239	(ACT)7	F: AACTCAGCCGGATAAAAGACAGC R: GGCCATCCCAAGAGCAGTTC	178	154–186	NAC domain containing protein 35
A8	S27695852	(AAG)7	F: CGAAATCACTTAATAACGGACAAC R: GATGGCAAAAGCAGCAATAAGC	392	362–392	zinc finger (MYND type) family protein/F-box family protein
A9	S27766206	(ACA)7	F: AGACAACAAGCTCGGCAAAAATG R: GCCACCACAACTTCACCACCTT	368	354–369	transcription factor bHLH64
A10	S27747128	(AGT)8	F: AACTGAATATGGATAGGGGAGAT R: ATTGCCAGGGTACCATAAGACT	416	416–431	dehydrin COR47
A11	S27706925	(TGC)8	F: CAATCAACGACCCATCAGGACCAG R: GATGCAGCAGCTTCTATTACAAA	478	450–499	transcriptional corepressor LEUNIG
A12	S27767864	(AAAG)6	F: CTGTTTGCGTGAGGGTGAGA R: CAGCCAAGAAAAGACGACAAACAT	251	163–259	LUC7 related protein

Appendix 2. Parameters of twelve EST-SSRs in three Chinese *Aquilegia* species. N_a = observed number of alleles; N = effective number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; F = inbreeding coefficient; P_{HWE} = Chi square p value for Hardy-Weinberg equilibrium test.

Species	Locus	N_a	N_e	H_o	H_e	F	P_{HWE}
A. ecalcarata	A1	4.000	2.542	0.533	0.607	0.121	0.329
	A2	4.000	3.659	0.800	0.727	–0.101	0.747
	A3	2.000	1.724	0.800	0.420	–0.905	1.000
	A4	2.000	1.471	0.400	0.320	–0.250	1.000
	A5	4.000	2.866	0.733	0.651	–0.126	0.721
	A6	2.000	1.471	0.267	0.320	0.167	0.464
	A7	3.000	2.074	0.667	0.518	–0.288	0.952
	A8	3.000	1.495	0.400	0.331	–0.208	1.000
	A9	2.000	1.800	0.800	0.444	–0.800	0.899
	A10	2.000	1.142	0.133	0.124	–0.071	1.000
	A11	3.000	2.018	1.000	0.504	–0.982	1.000
	A12	5.000	4.787	0.800	0.791	–0.011	0.473
A. incurvata	A1	3.000	2.018	0.733	0.504	–0.454	1.000
	A2	4.000	2.239	0.800	0.553	–0.446	0.974
	A3	2.000	1.991	0.800	0.498	–0.607	0.9985
	A4	2.000	1.965	0.600	0.491	–0.222	0.903
	A5	3.000	2.632	0.867	0.620	–0.398	0.861
	A6	2.000	1.069	0.067	0.064	–0.034	na
	A7	3.000	2.228	0.867	0.551	–0.573	0.998
	A8	1.000	1.000	0.000	0.000	na	na
	A9	4.000	2.941	0.667	0.660	–0.010	0.413
	A10	2.000	1.385	0.200	0.278	0.280	0.326
	A11	2.000	1.642	0.800	0.391	–1.045	1.000
	A12	2.000	1.867	0.733	0.464	–0.579	1.000
A. yabeana	A1	3.000	2.778	0.267	0.640	0.583	0.002
	A2	3.000	2.663	0.667	0.624	–0.068	0.546
	A3	2.000	1.991	0.867	0.498	–0.741	0.999
	A4	2.000	1.965	0.600	0.491	–0.222	0.899
	A5	5.000	3.103	0.733	0.678	–0.082	0.346
	A6	3.000	1.590	0.400	0.371	–0.078	0.602
	A7	2.000	1.557	0.533	0.358	–0.491	1.000
	A8	3.000	2.866	0.667	0.651	–0.024	0.311
	A9	3.000	2.663	0.933	0.624	–0.495	0.911
	A10	3.000	2.273	0.333	0.560	0.405	0.005
	A11	2.000	1.800	0.533	0.444	–0.200	0.896
	A12	3.000	2.074	0.600	0.518	–0.159	0.742