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Characterization of microsatellite loci in *Erysimum mediohispanicum* (Brassicaceae) and cross-amplification in related species[1]

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- *Premise of the study*: We have developed and optimized microsatellite loci from a genomic library of *Erysimum mediohispanicum.* Microsatellites were also tested for cross-amplification in 31 other *Erysimum* species.
- *Methods and Results*: A total of 10 microsatellite loci were successfully amplified. They were polymorphic for 81 *E. mediohispanicum* individuals from two locations in Sierra Nevada (southeastern Spain), which showed similar patterns of genetic diversity. On average, microsatellites had 8.6 alleles per locus and an expected heterozygosity of 0.69. Only one locus significantly departed from Hardy–Weinberg equilibrium in both locations. Most of the markers successfully amplified in other *Erysimum* species.
- *Conclusions*: The genetic attributes of microsatellite loci will allow their application to population genetic studies in *Erysimum*, such as genetic differentiation and structure, gene flow, pollinator-mediated speciation, and hybridization studies.

Key words: Brassicaceae; cross-amplification; *Erysimum mediohispanicum*; microsatellites.

Erysimum (Brassicaceae) is a genus comprising approximately 223 species (Al-Shehbaz et al., 2006) mainly distributed in the northern hemisphere. *Erysimum mediohispanicum* Polatschek is a biennial to perennial monocarpic herb endemic to the Iberian Peninsula, where it is distributed in two geographically distinct areas: one in northeastern and the other in southeastern Spain. *Erysimum mediohispanicum* typically exhibits high within- and among-population variation in floral traits, in particular corolla shape and size (Gómez et al., 2009a). It has been shown that different pollinators discriminate among different flower shapes, suggesting that phenotypic evolution and diversification can occur in this species (Gómez et al., 2008, 2009b). We have characterized 10 new polymorphic microsatellite loci for *E. mediohispanicum* and tested their cross-amplification in 31 other *Erysimum* species from different locations across Europe and Africa. These microsatellite loci will be primarily used for population genetics and gene flow studies in *E. mediohispanicum* populations characterized by different patterns of variation in floral shape and size as well as in the species' pollinator community.

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METHODS AND RESULTS

Microsatellite libraries were developed by Genetic Identification Services (Chatsworth, California, USA; http://www.genetic-id-services.com) following Jones et al. (2002). Genomic DNA was extracted using the DNeasy Plant Extraction kit (QIAGEN, Venlo, Netherlands) from one individual. Approximately 100 µg of DNA were digested with *Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, and *Eco*RV (New England Biolabs, Ipswich, Massachusetts, USA), and subsequent fragments were enriched in four motifs: CA-, AAC-, ATG-, and GA- using Biotin and Steptavidin magnetic beads for reversible capture (CPG, East Bank Demerara, Guyana). Resulting fragments were ligated into pUC19 plasmid and cloned into an *E. coli* strain DH5alpha (Invitrogen, Carlsbad, California, USA). After incubation, a total of 100 randomly chosen recombinant clones were selected, purified, and sequenced. Primer pairs were designed for 26 clones showing tandemly repeated motifs flanked by high quality sequence regions. Primer design was conducted using Designer PCR 1.03 (Research Genetics, Huntsville, Alabama, USA). Such primers were tested on a total of 81 *E. mediohispanicum* individuals from two locations in southeastern Spain: El Dornajo (37°7.67′N, 3°25.77′W; $N = 30$, UGR herbarium specimen: GDAC17407-1-2) and La Cortijuela (37°4.66′N, 3°28.29′W; $N = 51$, UGR herbarium specimen: GDAC17421-1-2).

DNA was isolated from silica-dried leaf samples using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, Missouri, USA). PCR was performed in 15 µL of reaction mixture containing 0.17 ng/µL of template genomic DNA, 1× buffer (ref. M0273S, New England BioLabs), 0.16 mM each dNTP (Sigma-Aldrich), 0.33 µM each forward (fluorescently tagged; Applied Biosystems, Foster City, California, USA) and reverse primer, and 0.02 U/µL *Taq* polymerase (ref. M0273S, New England Biolabs). PCR was conducted in a Gradient Master Cycler Pro S (Eppendorf, Hamburg, Germany) with an initial 30 s of denaturation at 94°C, 35 cycles at 94°C for 15 s, annealing temperatures (T_a; Table 1) for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 3 min. PCR products were diluted 1:15 and analyzed by MACROGEN analyzers (Geumchun-gu, Seoul, Korea; http://www.macrogen.com) using 400HD ROX (Applied Biosystems) as standard. Alleles were called using Peak Scanner Software version 1.0 (Applied Biosystems).

TABLE 1. Characteristics of 10 microsatellite markers in *Erysimum mediohispanicum*. GenBank accession number, repeat motif, forward (F) and reverse (R) primers, F primer fluorescent tag, allele size ranges, and optimal annealing temperatures (T_a) are given.

Locus (GenBank Accession No.)	Repeat motif	Primer sequence (5′–3′)	Fluorescent label	Product size (bp)	T_a (°C)
C5 (JF766210)	CCA_8	F: TCTTTCTTCTGCGGTTTATTC R: CGTTTTTGTTGTTGTTCTGG	6-FAM	164–182	56
D2 (JF766211)	CAT_{23}	F: ACGGAAGATGACGATGATCGACTG R: CAATGTCCCTAATTGGTCAATGG	HEX	117–189	54
D4 (JF766212)	ATC_7	F: TAAGGTGTTACCGGATTGTC R: GTGACGATTCGCTCCTTG	NED	200–215	57
E4 (JF766213)	CT_{20}	F: CCTTCCTCCGACTACTCTCC R: TGAGCGACTGATGATGATTC	HEX	145–178	57
E8 (JF766214)	CT_{50}	F: AGCTCACAGCCGTCGATGTTTGC R: GAGGTGAAATACACGTAGAACCT	6-FAM	157–229	50
D11 (JF766215)	TCA_{14}	F: TCCAGGGTCTGAGTCAATATG R: TTACCACTCCTTGCTTCTGAA	HEX	179–197	53
E6 (JF766216)	TC_{14}	F: CTTGTAACCGAGCCACTCA R: ATACGGAGAAGAAAGCGAATC	NED	131–159	53
D10 (JF766217)	TCA_{12}	F: ACTGCCATCAAACGACCTC R: TTGGTTGGAAAAGGGATTG	NED	166–185	53
E5 (JF766218)	GA_{13}	F: TCCATTTACACAATCCGTTCAT R: CCAACCTGACATCTTTGCTTC	6-FAM	167–195	50
E3 (JF766219)	GA_{17}	F: TTCCTCCAGATGAAACTACACAGG R: ACTTACATCGGATCGGTTGAG	HEX	215–253	56

TABLE 2. Genetic diversity of two populations of *Erysimum mediohispanicum*. Sample size (N), number of observed alleles (N_a), allelic richness (R_s), observed heterozygosity (H_o), expected heterozygosity (H_e), and P values for departure from Hardy–Weinberg equilibrium (HWE) are given for each microsatellite marker and population.

	El Dornajo						La Cortijuela					
Locus	N	N_a	R_s	H_o	H_e	HWE	N	N_a	R_s	H_o	H_e	HWE
C5	27	7	7.00	0.74	0.737	0.984 n.s.	50	6	5.94	0.76	0.778	0.766 n.s.
D2	29	19	18.50	0.72	0.925	0.057 n.s.	50	17	16.87	0.78	0.891	0.041*
D4	30	7	6.60	0.50	0.629	0.277 n.s.	51	6	5.92	0.59	0.640	0.567 n.s.
E4	29	9	8.92	0.62	0.844	0.141 n.s.	51	13	12.83	0.71	0.854	0.001**
E8	29	10	9.93	0.31	0.861	0.000***	47	14	14.00	0.45	0.851	0.000***
D11	27	3	3.00	0.52	0.562	0.600 n.s.	48	5	4.98	0.46	0.564	0.003**
E6	29	7	6.93	0.72	0.664	0.920 n.s.	50	8	7.87	0.68	0.696	0.993 n.s.
D10	29	5	5.00	0.59	0.645	0.734 n.s.	51	5	5.00	0.47	0.481	0.221 n.s.
E5	28	12	11.89	0.71	0.870	0.798 n.s.	51	12	11.68	0.75	0.797	0.131 n.s.
E3	28	2	2.00	0.11	0.278	0.001**	49	4	3.96	0.14	0.155	1.000 n.s.

Note: For HWE significance, n.s. = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Seven of the 26 tested primers failed to amplify, nine were monomorphic or showed complex patterns (primer sequences available upon request), and 10 were polymorphic (Table 1). We estimated the number of observed alleles per locus (N_a), allelic richness (R_s), observed heterozygosity (H_o), and expected heterozygosity (H_e) using FSTAT version 2.9.3 (Goudet, 1995). Linkage disequilibrium was tested with FSTAT using Bonferroni correction, and departures from Hardy–Weinberg equilibrium (HWE) were performed with GenAlEx version 6.3 (Peakall and Smouse, 2006).

For the El Dornajo population, the number of alleles per locus varied between two and 19, allelic richness between 2.0 and 18.5, observed heterozygosity between 0.11 and 0.74, and expected heterozygosity between 0.28 and 0.93 (Table 2). For the La Cortijuela population, the number of alleles per locus varied between four and 17, allelic richness between 4.0 and 16.9, observed heterozygosity between 0.14 and 0.78, and expected heterozygosity between 0.16 and 0.89 (Table 2). All loci showed no linkage disequilibrium ($P > 0.004$ in all cases; nominal level = 0.0011). For El Dornajo and La Cortijuela, two and four out of 10 loci significantly departed from HWE, respectively (Table 2). However, only one out of 10 loci significantly departed from HWE in both locations (Table 2).

We analyzed the cross-amplification success of the 10 polymorphic microsatellite loci in a total of 31 *Erysimum* species (Appendix 1). Most of the loci amplified in several species. On average, each loci amplified in 79.1% (range = 50.0–96.9%) of the species (Appendix 1).

CONCLUSIONS

Genetic diversity parameters indicate that these microsatellite loci can be a useful tool to study neutral genetic variation in *E. mediohispanicum* populations. Questions like the relationship between genetic diversity and phenotypic diversity, the effects of geographical variation in pollinator abundance and diversity on genetic diversity, differentiation, and structure, or the extent of pollinator-mediated gene flow within and among populations can now be addressed. Given that most of the primers successfully amplified a band of the expected size in several *Erysimum* species, these microsatellites also have the potential to become an efficient molecular tool to address similar questions in other *Erysimum* species, as well as to explore speciation

processes and contact zones commonly found in this highly diverse genus across its distribution range.

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APPENDIX 1. Amplification of 10 highly polymorphic microsatellite markers in 31 *Erysimum* species (one individual per species).

	Locus									
Species	C5	D2	D4	E4	E8	D11	E6	D10	E5	E3
E. baeticum Polatschek subsp. *baeticum*	+	−	+	+	+	−	+	+	+	+
E. bicolor DC.	+	−	+	+	+	+	+	+	+	+
E. bonnanianum C. Presl	+	+	+	+	−	−	+	+	−	+
E. collisparsum Jord.	+	+	+	+	−	+	+	+	−	+
E. corinthium (Boiss.) Wettst.	+	+	+	+	+	+	+	+	+	−
E. crepidifolium Rchb.	+	+	+	+	−	+	+	+	−	+
E. duriaei Boiss.	+	+	+	+	+	−	+	+	+	+
E. fitzii Polatschek	+	+	+	+	+	+	+	+	+	+
E. gomezcampoi Polatschek	+	+	+	+	−	−	+	+	+	+
E. gorbeanum Polatschek	+	+	+	+	−	+	+	+	+	+
E. incanum Kunze subsp. *mairei* (Sennen & Mauricio) Nieto Fel.	+	−	−	+	+	+	+	+	−	+
E. jugicola Jord.	+	+	+	+	−	+	+	+	+	+
E. merxmuellieri Polatschek	+	−	+	+	+	+	+	+	+	+
E. metlesicsii Polatschek	+	+	+	+	−	+	+	−	−	−
E. myriophyllum Lange	+	−	+	−	+	−	−	−	+	+
E. myriophyllum var. *cazorlense* (Heyw.) Polatschek	+	+	+	+	+	+	+	+	+	+
E. nervosum Pomel	+	−	+	+	+	−	−	−	+	−
E. nevadense A. Heller	+	+	+	+	+	+	+	+	+	+
E. odoratum Ehrh.	+	+	+	+	−	+	+	+	−	+
E. penyalarense Polatschek	+	+	+	+	+	+	+	+	+	+
E. popovii Rothm.	+	−	+	+	+	−	+	+	+	+
E. pseudorhaeticum Polatschek	+	+	+	+	−	−	+	+	−	+
E. rhaeticum DC.	+	+	+	+	−	+	+	+	−	+
E. riphaeanum Lorite, Abdelaziz, Muñoz-Pajares, Perfectti & J. M. Gómez	+	+	+	+	−	+	+	+	+	−
E. rondae Polatschek	+	+	+	+	−	+	+	+	+	+
E. ruscinonense Jord.	+	+	+	+	−	−	+	+	+	+
E. scoparium Wettst.	+	+	+	+	−	+	+	+	+	+
E. seipkae Polatschek	+	+	+	+	−	+	+	+	+	+
E. semperflorens Wettst.	+	−	+	+	+	−	+	+	+	−
E. sylvestre Scop.	+	−	+	+	−	−	+	−	−	+
E. wilczekianum Braun-Blanq. & Maire	−	−	−	−	+	−	+	−	−	−

Note: Plus and minus signs represent successful and unsuccessful amplifications, respectively.