Microsatellite Primers in the Weedy Annual Herb Anacyclus clavatus (Asteraceae) and Four Closely Related Species

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Primer Note

Microsatellite primers in the weedy annual herb *Anacyclus clavatus* (Asteraceae) and four closely related species[1]

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- *Premise of the study:* Nuclear microsatellite primers were developed for the weedy herb *Anacyclus clavatus* to study the genetic structure of hybrid zones with closely related taxa in the western Mediterranean Basin, where different floral phenotypes are present.
- *Methods and Results:* We obtained two microsatellite libraries using next-generation sequencing and Sanger sequencing of cloned restriction fragments. A total of 13 polymorphic and 11 monomorphic loci were identified in three Iberian populations of *A. clavatus*. The primers amplified di- and trinucleotide repeats with 1–8 alleles per locus. Most primers also amplified in *A. homogamos*, *A. monanthos*, *A. radiatus*, and *A. valentinus*.
- *Conclusions:* These results indicate the utility of these markers in *A. clavatus* for population genetic and hybridization studies as well as their applicability across the genus.

Key words: *Anacyclus clavatus*; Asteraceae; hybridization; population genetics; weeds.

Anacyclus L. (Anthemideae, Asteraceae) is a Mediterranean genus of mostly weedy annual herbs with approximately 12 species distributed in North Africa, southern Europe, and the Middle East (Humphries, 1979; Oberprieler et al., 2007). This genus is characterized by an extraordinarily large variation in floral symmetry (Bello et al., 2013). This diversity is especially remarkable in areas where two to three species coexist. *Anacyclus clavatus* (Desf.) Pers. is present throughout the distribution area of the genus. The species cohabits with *A. homogamos* (Maire) Humphries, which is mainly restricted to inland areas of Morocco and Algeria, and *A. valentinus* L., which mostly occurs in coastal areas across all of the western Mediterranean Basin. Based on the phenotypes obtained by artificial crosses among these species, intermediate floral phenotypes were interpreted as hybrids (Humphries, 1981), although there are no molecular data supporting this hypothesis. We developed nuclear microsatellite markers for *A. clavatus* to investigate its genetic diversity, population structure, and gene flow among closely related species in hybrid zones.

METHODS AND RESULTS

Two different methods were used to obtain microsatellite libraries for *A. clavatus*. For the first microsatellite library, silica-dried leaves of 10 individuals of *A. clavatus* from Miraflores de la Sierra (40°47′34.53″N, 3°44′1.85″W) were sent to Genetic Identification Services (GIS; Chatsworth, California, USA) for DNA isolation and sequencing of cloned enriched restriction fragments following Jones et al. (2002). A voucher (*Álvarez 2173*) was deposited at the herbarium of the Royal Botanic Garden–Consejo Superior de Investigaciones Científicas (CSIC; MA). Recombinant plasmids were produced by ligating restriction fragments from *A. clavatus* DNA into the *Hin*dIII site of the pUC19 plasmid. The fragments were enriched for CA, GA, AAC and ATG microsatellite motifs, and ligation products were introduced into *E. coli* strain DH5α (ElectroMaxJ, Invitrogen, Carlsbad, California, USA) by electroporation. After transformation and recovery in super optimal broth with catabolite repression (SOC; Invitrogen), cells were incubated on Bluo-Gal/isopropyl-β-D-1-thiogalactopyranoside (IPTG)/ampicillin LB (BIA-LB) agar plates. To select insert fragments longer than 300 bp, white colonies were screened by PCR and subsequently sequenced. One hundred twenty-one sequences containing microsatellites were received from GIS, for which PCR primers were designed using DesignerPCR version 1.03 (Research Genetics, Huntsville, Alabama, USA). The second microsatellite library was prepared by Genoscreen (Lille, France) with the 454 GS FLX (Roche Diagnostics, Meylan, France) high-throughput DNA sequencer (Malausa et al., 2011). Total genomic DNA was extracted from silica-dried leaves of eight individuals of *A. clavatus* from Estación de Cártama (36°43′58.09″N, 4°39′37.02″W) using a modified cetyltrimethylammonium bromide (CTAB) method described in Doyle and Doyle (1987). A voucher (*Álvarez 2140*) was deposited at MA. Genomic DNA was fragmented and enriched with TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC motifs. A total of 27,006 high-quality sequences were obtained. Analysis of these sequences with QDD software (Meglécz et al., 2009) revealed 2341 sequences with microsatellite motifs, for which 115 primer pairs were designed.

A total of 83 primer pairs, of which 42 were obtained by GIS and 41 by Genoscreen, were tested by PCR using 90 individuals from three populations of *A. clavatus*, in which 30 individuals were collected from Antequera (37°02′34.00″N, 004°30′54.30″W), 30 from Cartagena (37°37′09.04″N, 001°04′58.04″W), and 30 from Los Escullos (36°48′04.02″N, 002°03′47.02″W). Vouchers (*Álvarez 2122*, *Álvarez 2152*, and *Álvarez 2161*) were deposited at MA. Total genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). PCRs were performed in a total volume of 20 μL, which contained 1× PCR Buffer, 2 mM $MgCl_2$,

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TABLE 1. Characteristics of 13 polymorphic microsatellite primers developed in *Anacyclus clavatus*.[a]

Locus[b]	Primer sequences (5′–3′)	Fluorescent dye	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
D3	F: GAAGGTGTGTCAAAAGGGTATT	NED	$(GAT)_8$	194–209	55	KF418743
	R: AAGCAACAAATGAAGAGAGAGG					
8	F: TCTTTACAAAGACCAGACGCC	6-FAM	$(AC)_7$	84–96	56	KF418722
	R: GCTAGGCACCTAGGTTAATCACTT					
9	F: CCATGAATATCATTCTTCCGTG	NED	$(CTT)_9$	77–96	56	KF418723
	R: CGCGGAAGGTGTAATAGAGTC					
15	F: TCATAATTACCCACCAACAGC	PET	$(AC)_{10}$	97–109	55	KF418724
	R: GCCATTTTTGTTTGATTTCAATAG					
16	F: ACTTGATAATTGATAAAACCACGGA	6-FAM	$(TG)_7$	86–96	56	KF418725
	R: CCTAGAACATCAGACGCCAA					
17	F: GAAGCTTTCTTAAGGTTTCTTCTTG	NED	$(TGT)_9$	129–153	55	KF418726
	R: TCATTTGAATCTCATCATAGGAAA					
18	F: TCACCAAATACTTCCCGAGC	VIC	$(TC)_8$	101–115	55	KF418727
	R: ACTTTTGATCGAGCAATCCG					
19	F: TTACCCGACTTGCTGAAAGG	PET	$(AAC)_6$	148–160	55	KF418728
	R: CCTTGCGTATTTGCACTCCT					
20	F: AGCTTACATTACAAGCCATGC	VIC	$(CA)_7$	89–97	55	KF418729
	R: GAGGGTTTGGTTTGATTTGC					
21	F: TCTTACCTGTTCCTTAGATCTTATTCA	6-FAM	$(CAA)_{11}$	120–137	55	KF418730
	R: TGATTTGAATTTTCTAATGCTGC					
24	F: CACGATCACTTTTCGATACTTACA	6-FAM	$(CT)_7$	89–105	56	KF418731
	R: AATTTGCGGCTGTGGTAAAG					
27	F: GGGTAGGTTTAACCATGGGG	NED	$(GA)_8$	185–191	55	KF418732
	R: TGACGATACATCCAAAGTATCCC					
28	F: AAAACACCTATCCACAATATGACC	VIC	$(AGA)_8$	263–278	56	KF418733
	R: AGTATCTTGTCTAGAGACACTCTTCCC					

Note: T_a = annealing temperature.

[a]All values are based on 90 samples from three South Iberian populations.

[b]Locus D3 was obtained by sequencing of cloned enriched restriction fragments and the remaining were obtained by next-generation sequencing.

0.2 mM each of dNTPs, 0.4 µM each of primers, 0.6 U of *Taq* DNA Polymerase (Bioline USA, Canton, Massachusetts, USA), and 40 ng of DNA template using the following thermocycler conditions: an initial denaturation step at 94°C for 2 min; followed by 35 cycles of 1 min at 94°C, 1 min at 54–56°C, 2 min at 72°C; and a final extension of 10 min at 72°C. The PCR products were separated by electrophoresis on a 3% agarose gel to select those primer pairs that amplify fragments of the expected sizes and that might show allelic variation. A total of 24 primer pairs were selected as candidates to evaluate polymorphic loci. Forward primers of each pair were marked with 6-FAM, VIC, NED, or PET fluorescent dyes (Table 1). PCR products were analyzed with Peak Scanner Software version 1.0 (Applied Biosystems, Foster City, California, USA).

A total of 13 loci were polymorphic (Table 1), whereas 11 were monomorphic (Appendix 1). We estimated the mean number of alleles per locus, observed and expected heterozygosities, and Hardy–Weinberg equilibrium (HWE; Table 2) with GenAlEx version 6.3 (Peakall and Smouse, 2006). Tests for linkage disequilibrium between markers in each population were performed using FSTAT version 2.9.3.2 (Goudet, 1995). In the Antequera population, the number of alleles per locus ranged from two to six, and the observed and expected heterozygosities were 0.316–0.667 and 0.278–0.745, respectively. In the Los Escullos population, the number of alleles ranged from one to seven, and the observed and expected heterozygosities were 0.000–0.826 and 0.000–0.631, respectively. Loci 15, 17, and 21 were monomorphic in this population. In the Cartagena population, the number of alleles ranged from one to eight, and the observed and expected heterozygosities were 0.000–0.955 and 0.000–0.774, respectively. Loci 17 and 21 were monomorphic in this population. Significant deviation from HWE ($P < 0.05$) was seen for loci 8, 9, 15, 18, and 21 in the Antequera population, for loci 20 and 24 in the Los Escullos population, and for loci 20 and 27 in the Cartagena population. No significant departures from linkage disequilibrium ($P > 0.05$) were detected for any pair of loci. Cross-amplification was performed for these 13 polymorphic loci in *A. homogamos*, *A. monanthos*

TABLE 2. Results of initial primer screening of polymorphic loci in three populations of *Anacyclus clavatus*.

	Antequera				Los Escullos				Cartagena			
Locus	*A*	H_o	H_e	HWE[a]	*A*	H_o	H_e	HWE[a]	*A*	H_o	H_e	HWE[a]
D3	3	0.630	0.510	0.535 ns	4	0.588	0.631	0.605 ns	8	0.600	0.591	1.000 ns
8	3	0.538	0.447	0.000 ***	2	0.250	0.219	0.450 ns	2	0.542	0.395	0.069 ns
9	3	0.320	0.574	0.009 **	2	0.308	0.260	0.354 ns	2	0.120	0.180	0.096 ns
15	5	0.346	0.700	0.002 **	1	0.000	0.000	–	2	0.034	0.034	0.925 ns
16	5	0.591	0.594	0.675 ns	4	0.560	0.566	0.420 ns	5	0.571	0.556	0.926 ns
17	4	0.500	0.515	0.912 ns	1	0.000	0.000	–	1	0.000	0.000	–
18	4	0.316	0.633	0.008 **	7	0.750	0.580	1.000 ns	4	0.800	0.580	0.898 ns
19	5	0.500	0.646	0.377 ns	7	0.462	0.478	0.303 ns	6	0.696	0.774	0.980 ns
20	2	0.333	0.278	0.327 ns	4	0.826	0.591	0.002 **	3	0.955	0.542	0.000 ***
21	6	0.667	0.745	0.001 ***	1	0.000	0.000	–	1	0.000	0.000	–
24	4	0.517	0.583	0.329 ns	4	0.273	0.550	0.008 **	3	0.083	0.081	0.997 ns
27	4	0.389	0.454	0.379 ns	4	0.667	0.584	0.713 ns	2	0.647	0.438	0.049 *
28	3	0.409	0.344	0.693 ns	3	0.091	0.088	0.997 ns	3	0.190	0.177	0.972 ns

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium probabilities.

[a]Deviations from HWE were not statistically significant (ns) and statistically significant at $*P < 0.05$, $**P < 0.01$, and $***P \leq 0.001$.

(L.) Thell., *A. radiatus* Loisel., and *A. valentinus*. All loci, except locus 15 in *A. radiatus*, amplified successfully within the expected allele size in all species.

CONCLUSIONS

Here we report on a set of polymorphic microsatellite markers for *A. clavatus*. Amplification success for most of these markers in almost half of the species of *Anacyclus* extends their potential usefulness to the entire genus. These markers will be useful for investigating the genetic structure, gene flow patterns, and mating system of *A. clavatus* across its distribution and especially in hybrid zones with closely related species.

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Appendix 1. Characteristics of 11 monomorphic microsatellite primers developed in *Anacyclus clavatus*.[a]

Locus[b]	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
A9	F: TCAGTGACTTTAGAAAGGTAGTAAGGA	$(GT)_{13}AT(GT)_8$	170	54	KF418744
	R: CTCATGTGGGGTGTTCCTCT				
A121	F: TCTCGCTACTCCCGCTTTAC	$(ACA)_{14}$	237	54	KF418738
	R: GCAGGATCACTTAAAGGATATCAG				
A123	F: TCAGTGACTTTAAAAGGTAGTAAGGA	$(GT)_{15}$	165	54	KF418739
	R: TAAGTGCTCCACACCCATGT				
C101	F: GCATAAACCTTCGGAATCTCA	$(TTG)_9$	119	54	KF418740
	R: ATGGTGACAATCGTGGTAACC				
D8	F: TTCCTTTGCCTCTTTCTTGG	$(ATC)_7$	193	55	KF418745
	R: GTTCCCGACTGTGGTCTCTC				
D101	F: ACTCCATGACCGAAGAGGTG	$(TCA)_2TCT(TCA)_5$	238	54	KF418741
	R: GACACTTGTGGTCCCTCGAT				
D103	F: ATGGTGGTGGAGCATAGG	$(TCA)_{19}$	289	54	KF418742
	R: GAGGACGAGGATGATGAGA				
4	F: TTCTCCATTTTCTTTGATCTTGG	$(TTG)_7$	145	56	KF418734
	R: GGGACGTATGTACTCACCTTCG				
23	F: CCATGTTATGGATTCACTTAGTAAAAG	$(AG)_7$	141	55	KF418735
	R: CCATATGTTGGAAGGGGTGT				
25	F: GGAGGGGTTGGATTCTCATA	$(CT)_8$	94	55	KF418736
	R: GAGGAGTTCTTAGTGAGATGTTGG				
30	F: GGTGGTCTTGGTAAATGAAAGA	$(GA)_7$	122	55	KF418737
	R: TGAGGGGTTGAGGTTCTTGT				

Note: T_a = annealing temperature.

[a]All values are based on 90 samples of three South Iberian populations.

[b]Loci in italics are those obtained by sequencing of cloned enriched restriction fragments and the remaining are those obtained by next-generation sequencing.