Isolation and Characterization of Microsatellites Markers in *Centaurium Grandiflorum* ssp. *Boissieri*

Vania Jiménez-Lobato (vania.lobat@gmail.com)
Universidad Autonoma de Guerrero/university of Seville https://orcid.org/0000-0003-2050-9026
Marcial Escudero
University of Seville: Universidad de Sevilla
Zoila Díaz Lifante
University of Seville: Universidad de Sevilla
Cristina Andrés Camacho
University of Seville: Universidad de Sevilla
Alejandra de Castro
University of Seville: Universidad de Sevilla
Juan Arroyo
University of Seville: Universidad de Sevilla

Short Report

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Abstract

Estimation of outcrossing/selfing rates and characterization of genetic diversity with microsatellite markers are crucial to understand the evolution of mating system in plant species. We developed, optimized and characterized eight new primers pairs for *Centaurium grandiflorum* ssp. *boissieri* and transferred them to three subspecies of *Centaurium quadrifolium*. Two SSR loci were transferred from *Sabatia campestris* to the four mentioned taxa of *Centaurium*. Polymorphisms, H_e, H_o and H-W deviations were estimated in two populations of *C. grandiflorum* ssp. *boissieri*, and in seven individuals of *C. quadrifolium* ssp. *barrelieri*, *C. quadrifolium* ssp. *parviflorum* and *C. quadrifolium* ssp. *quadrifolium*. A total of 80 individuals were used in these experiments. The number of polymorphic loci varied among species from one to ten. A total number of 127 alleles were scored. The average number of alleles per locus was 12.7. H_e was higher than H_o in all sampled populations. Hardy-Weinberg equilibrium was found for some loci in different species. This is the first report of microsatellites successfully amplified in the whole *Centaurium* genus. They will be valuable for estimation of mating system parameters, genetic diversity and explore its relationship with the wide flower morphology, especially anther-stigma separation, found along the genus.

Introduction

The genus *Centaurium* Hill (Gentianaceae) comprises ca. 27–30 species [1, 2], and is a sub-endemic genus in the Mediterranean basin where most of the diploid taxa occur [3]. The life form vary from annual, biannual to short-lived perennial herbs. Species inhabit dry, open and, in some cases, disturbed habitats for what are considered pioneer species. The flowering season occurs from April to August, depending on species, but generally during warm and dry periods of the year in a typical Mediterranean climate. Only during the morning or at the dusk, when temperatures are milder, pollinators are active otherwise they are null or scarce, reducing the opportunity for cross-pollination. The short and unpredictable daily period of pollinator activity suggest the selection of traits to favors the ability of individuals to self-fertilize, facilitating the establishment of predominant selfing populations (i.e. “reproductive assurance” hypothesis) [4, 5, 6, 7]. However, the observed wide range of flower features displayed by this genus associated to geological and climatic history of the Mediterranean basin [8, 9] point toward a wide variation in mating system among and within species. To determine the level of selfing in natural populations and its relation to flower traits it is necessary to dispose of appropriate molecular markers unequivocally detecting heterozygosity.

We characterized eight new SSR polymorphic markers for *C. grandiflorum* subsp. *boissieri* and tested them for cross-amplification in other three subspecies of *C. quadrifolium* which is the sister species of *C. grandiflorum* ssp. *boissieri*: *C. quadrifolium* ssp. *barrelieri*, *C. quadrifolium* ssp. *parviflorum* and *C. quadrifolium* ssp. *quadrifolium*) [9]. This clade has shown a great phenotypic variation in flower size, flower display and herkogamy [2, 9], traits related to pollination biology. Further, we describe genetic diversity of three populations of *C. g. boissieri* across its distribution range in Andalousía region. The loci will be mainly used to estimate parameters of mating system and its consequences on genetic diversity within and among species of *Centaurium*. Further, the data also will be used to analyze the relationship between flower morphology and mating system within species. These are the first microsatellite loci developed for the whole *Centaurium* genus, which is most characteristic of short-lived flora of the Mediterranean, and thus may serve to disentangle crucial questions on the evolution of reproductive strategies in harsh dry and hot seasons.

Materials And Methods

Genomic DNA was obtained using Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) and was extracted from three individuals of *C. grandiflorum* subsp. *boissieri* belonging to three different populations of Andalucía Province in Spain (“Universidad Pablo de Olavide”, Seville [37º21'18.04"N – 5º56'16.56"O]; “El Bosque”, Cadiz [36º45'55.14"N – 5º29'48.05"O]; and “Río Frío”, Granada [37º09'36.77"N – 41º11'07.93"O], whose vouchers are located in SEV herbarium). Microsatellite libraries were developed by Ecogenics Company.

The design of potential microsatellites loci was outsourced to the company Ecogenics (Schlieren-Zürich, Switzerland, https://www.ecogenics.ch), which combined enrichment for microsatellite motifs in a C. g. boissieri genomic DNA library with 454-sequencing on a Illumina Miseq system (Illumina, San Diego, California, USA) with an average read length of 80–400 bp. Using a pipeline property of Ecogenics, a total of 640 microsatellites loci were found, including 263 di-, 352 tri- and 25 tetranucleotides (Table 1).

Table 1
Characteristics of 10 microsatellite loci for *Centaurium g. boissieri* based upon genotyping three populations.

Locus	Primer sequences (5'-3')	Repeat motif	Allele size range (bp)
Boi8	F: GAGATGCAACGAGTCGAACC	(GAA)	161–167
	R: TCGTAGCCTGAGCCATCTTC		
Boi9	F: GCTACCCGAAGTTTTCCGAC	(AGG)	238–277
	R: CGAGTTTGACCGAGCCATTC		
Boi16	F: ACATGTACGTGCCTCCTAGC	(TA)	215–233
	R: TTGGGAGCCAAAAACGCATC		
Boi19	F: AATAATCATGGTGGCGCACG	(GTAT)	156–252
	R: TGCATACAAGAATTCGCAAAAGC		
Boi23	F: TGTGTTGGAAACCGCTAATATCC	(ATGT)	230–282
	R: GTGCAAGGCTCACAATCTCC		
Boi32	F: GTTAAGATCACACAGCCCGC	(AAT)	199–238
	R: GTATGGCTCGTTTCACCTGC		
Boi38	F: TGTTCCTACATATACGAGTAAAAGC	(AT)	238–272
	R: AATAGGTTCTCAAGAGCCATAAAC		
Boi39	F: AATGCAAGGCAAGTTCTCGG	(GA)	225–253
	R: TCACGAGAATGGATTGGGGC		

We selected 40 primer pairs within a size range from 100 to 250 bp to be tested on 59 individuals of *C. grandiflorum* ssp. *boissieri* collected from three different populations. Also, we tested another eight microsatellites designed for *Sabatia campestris* from the same Tribe Chironieae (Gentianaceae) [10]. The two loci that amplified for *C. g. boissieri* were also tested for cross-amplification *Centaurium quadrifolium*, using 7 individuals per subspecies (Table 1). A total of 80 individuals were used. PCR's were performed in 20μL of reaction mixture containing 60 ng/μl of template genomic DNA, 0.5 U taq polymerase, 1 x My Taq Red Reaction Buffer (Bioline, London, UK), 0.01% bovine serum albumin (BSA) (Promega, Madison, WI, USA), 0.04 μM M13-tailed forward primer, 0.40 μM PIG-tailed reverse primer and 0.40 dye-labelled M13 primer (FAM, VIC, NED or PET dyes; Invitrogen, Madrid, Spain), following the methods of Boutin-Ganache et al.[11]. PCR were undertaken using a touchdown PCR protocol on a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems, Foster city, CA, USA). The thermal profile consisted of initial denaturalization at 94°C for 2 min; 17 cycles with denaturalization at 92°C for 30 s, annealing at 60–44°C for 30 s (1°C decrease in each cycle), and extension at 72°C for 30 s; 25 cycles at 92°C for 30 s, 44°C for 30 s, 72°C for 30 s; and a final extension of 5 min. at 72°C. PCR products were analyzed in ABI prism 3130 and 3730 systems (STAB VIDA, Portugal) and sized using Gene Marker 2.4 (SoftGenetics, State College, PA, USA) and Gene Scan 500 LIZ size standard.

We estimated: 1) number of observed alleles (*A*), 2) observed (H_o) and expected (H_e) heterozygosity, and 3) deviations from Hardy-Weinberg equilibrium (PHW_{Eq}) using Marcov chain with 100,000 permutations. All analyses were done using Arlequin software v.3.5.2.2 [12].

Results And Discussion

A total of eight new polymorphic primers pairs plus two cross-amplification loci (Sc205, Sc249) transferred from *S. campestris* were amplified in four taxa of *Centaurium* (Table 1). The rest of loci tested were monomorphic for all 80 samples or did not amplified clearly, producing multiple peaks. We scored 127 alleles among all four taxa. The number of alleles per locus varied from 3 (sc249) to 23 (Boi39) with an average of 12.7. In *C. g. boissieri* all 10 loci were polymorphic with mean A = 7.2 (range 2–14), H_o = 0.46 (range 0.133–0.867) and H_e = 0.704 (range 0.140–0.931). In *C. q. barrelieri*, six loci were polymorphic with mean A = 3.833 (range 2–6), H_o = 0.238 (range 0.0-0.571) and H_e = 0.619 (range 0.264–0.868). Further, two loci were monomorphic and other two did not amplify. In *C. q. quadrifolium* six loci were polymorphic with mean A = 4.167 (range 2–6), H_o = 0.495 (range 0.0-0.857) and H_e = 0.691 (range 0.264–0.890); other two loci were monomorphic and two loci did not amplify. Finally, in *C. q. parviflorum*, only one locus was polymorphic with A = 2, H_o = 0.0 and H_e = 0.533.

Other six loci amplify but were monomorphic and other three loci did not amplify (Table 2). Significant deviations for Hardy-Weinberg equilibrium were found for some loci in different species (Table 1) which can be the result of small sample sizes used, particularly in *C. q. barrelieri*, *C. q. quadrifolium* and *C. q. parviflorum.*

Table 2
Number of individuals (*N*), number of alleles (A), observed (H_o) and expected (H_e) heterozygosity, and *P* value for deviations from Hardy-Weinberg equilibrium (PHW_{Eq}) tested for 10 microsatellites in four taxa of *Centaurium*. *C. g. boissieri* was tested in three localities.

	C. g. boissieri														
	El Bosque					UPO					Río Frío				
Locus	*N*	*A*	H_o	H_e	PHW_{Eq}	*N*	*A*	H_o	H_e	PHW_{Eq}	*N*	*A*	H_o	H_e	PHW_{Eq}
Boi38	36	11	0.278	0.886	0	□	□	□	□	□	12	5	0.167	0.803	0.002*
Boi19	52	7	0.538	0.762	0.069	30	9	0.667	0.869	0.029*	26	6	0.231	0.708	< 0.001*
Boi32	52	13	0.731	0.901	0.088	30	7	0.467	0.832	0.027*	30	10	0.667	0.839	0.019*
sc205	48	10	0.583	0.845	0.085	28	6	0.571	0.757	0.269	30	11	0.667	0.880	0.038*
Boi9	56	12	0.286	0.773	0	20	5	0.200	0.716	< 0.001*	30	5	0.267	0.694	0.001*
Boi16	58	6	0.586	0.553	1	28	3	0.143	0.500	0.003*	28	4	0.214	0.267	0.219
Boi8	54	3	0.630	0.561	0.002*	30	3	0.667	0.559	0.029*	30	3	0.600	0.577	0.045*
Boi23	54	14	0.704	0.895	0.020*	30	8	0.867	0.816	0.531	30	10	0.467	0.885	< 0.001*
sc249	54	2	0.148	0.140	1	30	2	0.133	0.405	0.020*	30	2	0.133	0.405	0.021*
Boi39	58	11	0.517	0.840	< 0.001*	30	12	0.667	0.931	0.007*	30	9	0.533	0.825	0.092
	C. q. barrelieri					***C. q. parviflorum***					***C.q. quadrifolium***				
	Jumilla					Peralta					Lillo				
Locus	*N*	*A*	H_o	H_e	PHW_{Eq}	*N*	*A*	H_o	H_e	PHW_{Eq}	*N*	*A*	H_o	H_e	PHW_{Eq}
Boi38	14	2	0.571	0.527	1	14	1	□	□	□	14	2	0	0.264	0.077
Boi19	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Boi32	14	6	0.429	0.846	0.015*	14	1	□	□	□	14	6	0.857	0.890	0.379
sc205	10	2	0	0.356	0.112	□	□	□	□	□	10	4	0.2	0.778	0.010*
Boi9	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Boi16	□	□	□	□	□	4	1	□	□	□	10	2	0.2	0.556	0.365
Boi8	14	2	0.286	0.264	1	14	1	□	□	□	14	□	□	□	□
Boi23	12	5	0	0.848	< 0.001*	14	1	□	□	□	14	5	0.857	0.824	0.819
sc249	14	□	□	□	□	14	1	□	□	□	14	□	□	□	□
Boi39	14	6	0.143	0.868	< 0.001*	10	2	0	0.533	0.048*	14	6	0.857	0.835	0.256

* Locus with significant deviations from Hardy-Weinberg Equilibrium (P < 0.05)

Eight new polymorphic loci characterized in this study and two cross-amplification loci transferred from *Sabatia campestris*, are the first microsatellites amplified and reported for *Centaurium* species. These ten loci showed high number of alleles and can be used to characterize mating system in the four species of *Centaurium* analyzed. However, they also can be tested for other species of the genus.

The fact that in *C. q. parviflorum* just one out of seven loci was polymorphic, might indicate a low genetic diversity in this species, which itself could be related to a predominant selfing reproductive strategy that characterizes this species. In this sense, these markers will be crucial to estimate accurately outcrossing/selfing rates in the genus, which in previous studies has been inferred just from flower features and/or the capacity of autonomous selfing of species [6, 7, 9]. Estimation of outcrossing/selfing rates will help to understand the evolution of mating strategies in annual and pioneer species and its implications on the genetic diversity and genetic structure. Particularly in *Centaurium*, these analyses will enable to explore its relationship with the wide variation of flowers traits found in different species along the phylogeny, and the reproductive mechanisms that allow them to adapt to new disturbed and open habitats.

Declarations

Consent to participate

Informed consent was obtained from all individuals participants included in the study.

Consent to publish

The participants have consented to the submission of the case report to the journal. And declare that the manuscript has not been submitted to more than one journal for simultaneous consideration.

Author contribution

JA and ME conceived the research topic. VJ-L, ZD-L, CAC and AC performed the field work and gathered the vegetal tissue. VJ-L, ME and AC performed the laboratory work. VJ-L performed the analyses. ME, JA and VJL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflicts of interest/Competing interests

Authors declare no conflict of interest.

Availability of data and material

Raw Data

Code availability

Not applicable

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