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Isolation and characterization of 10 microsatellite loci in *Cneorum tricoccon* (*Cneoraceae*), a Mediterranean relict plant[1]

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- *Premise of the study:* The main aim of this study was to isolate and characterize microsatellite loci in *Cneorum tricoccon* (Cneoraceae), a Mediterranean shrub relict of the early Tertiary, which inhabits western Mediterranean islands and coasts. Microsatellites will be useful for investigating biogeography and landscape genetics across the species distribution range, including current or past gene flow.
- *Methods and Results:* Seventeen microsatellite loci were characterized, of which 10 were polymorphic and amplified for a total of 56 alleles in three populations of *C. tricoccon*. The markers revealed average coefficients of expected heterozygosity (H_e = 0.425), observed heterozygosity (H_o = 0.282), and inbreeding coefficient value per population (F_{IS} = 0.408).
- *Conclusions:* These microsatellite primers will potentially be useful in the study of population and landscape genetics, conservation status of isolated populations, island-continental distribution, current or historical movements between populations, and in the investigation of the consequences of dispersal mechanisms of these plants.

Key words: Cneoraceae; *Cneorum tricoccon*; conservation genetics; landscape genetics; Rosidae.

Cneorum tricoccon L. (Rosidae, Cneoraceae) is a small evergreen shrub that inhabits western Mediterranean islands (the Balearics, Sardinia, and Giannutri [located offshore of the western Italian peninsula]), but populations also appear in some places on the Mediterranean coasts (Spain, France, and Italy). It occurs in Mediterranean shrubland communities, dominated by other species like *Pistacia lentiscus* L., *Olea europaea* L., *Rosmarinus officinalis* L., or *Quercus ilex* L. (Traveset, 1995a). Together, this species and *Neochamaelea pulverulenta* (Vent.) Erdtmann (endemic to the Canary Islands) constitute the entire family. *Cneorum tricoccon* has been categorized as Vulnerable (IUCN, 2001), with human activities and urban sprawl as the main threats for the species. *Cneorum tricoccon* is an andromonoecious, insect-pollinated species (Traveset, 1995a) with a long flowering period (from October through May). Although the species is not pollen limited, male flowers appeared to be more fertile than hermaphrodite flowers and xenogamous crosses were more productive than geitonogamous ones in hand-pollination (Traveset, 1995a). Seed dispersal is mediated by endemic lizards in the Balearics (*Podarcis lilfordi* Günther and *Podarcis pityusensis* Boscá) and on Giannutri Island (*Podarcis siculus* Raf.; Traveset, pers. obs.), whereas introduced carnivorous mammals (like *Martes martes* L. or *Genetta genetta* L.) are currently the main dispersers on islands where lizards are extinct (Traveset, 1995b). On the continent, both mammals and lizards (*Lacerta lepida* Daudin) are presumably responsible for dispersing this species (Traveset, unpublished data).

Microsatellites have been developed in *C. tricoccon* (diploid with $2n = 36$) to investigate the landscape genetics of the species and its dispersal capabilities, and also to characterize the main genetic parameters of each population, such as genetic variation or inbreeding coefficients, and to study its conservation status. The degree of inbreeding in each population is unknown and might play a relevant role in some low-density populations. This information would be useful in predicting whether inbreeding depression in the population could lead to extinction. The dynamics of the plant dispersal mechanism should be pinpointed, because in some populations lizards are extinct and the mammal presence is unknown. The existence of any type of gene flow (mediated by pollen and/or seed dispersal) between continent and island populations could also be interesting to examine and develop future conservation management plans.

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METHODS AND RESULTS

Eighty micrograms of total DNA were extracted from silica gel–dried young leaves of an individual pool formed by two individuals from the S'Arboçar population (see Appendix 1) using the DNeasy plant minikit (QIAGEN, Barcelona, Spain), following the manufacturer's protocol. Microsatellite sequences were isolated by Ecogenics GmbH (Zurich, Switzerland; http://www.ecogenics.ch) using the high-throughput genomic sequence approach described by Abdelkrim et al. (2009). Genomic DNA (0.8 μg) was analyzed on a Roche454 GS-FLX platform (Roche, Basel, Switzerland), using a 1/4 run and GS FLX titanium reagents. The total of 21 667 reads had an average length of 317 base pairs. Of these, 1104 contained a microsatellite insert with a tetra- or trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 214 reads, of which 33 were tested for polymorphism using Primer3 (Rozen and Skaletsky, 2000) for primer design. For this procedure, M13 was used as the tail (Schuelke, 2000). PCRs were performed in a 10-μL mix containing 4.1 μL of water, 1× *Taq* buffer (Biotools, Madrid, Spain), 2 nM $MgCl_2$, 0.2 μM of each dNTP, 0.04 μM of the unlabeled M13 tail forward primer, 0.16 μM of the reverse and the M13-labeled primer, 1 U of *Taq* polymerase, and 10 ng of template DNA. The PCR program consisted of one step of 4 min at 94°C followed by 30 cycles each of 30 s at 95°C, 45 s at locus-specific annealing temperature (see Table 1), and 45 s at 72°C. Another eight cycles were then performed, consisting of 30 s at 95°C, 45 s at 53°C, and 45 s at 72°C, followed by a final step of 10 min at 72°C. The products were run on an ABI 3730 automated sequencer (Applied Biosystems, Carlsbad, California, USA) using LIZ-500 as the internal lane size standard (Applied Biosystems), and the amplified fragment lengths were assigned to allelic sizes with GeneMarker version 1.85 software (SoftGenetics, State College, Pennsylvania, USA). After an initial screening, 10 of 22 loci were polymorphic (Ctric 0049, Ctric 02925, Ctric 05261, Ctric 06384, Ctric 7615, Ctric 09344, Ctric 10195, Ctric 14301, Ctric 15341, Ctric 19884), whereas seven loci were monomorphic (Table 1) and five loci that showed a multibanding pattern suggesting loci duplications or difficulties to interpretation were excluded (data not shown).

Genotypic data were obtained from three populations of *C. tricoccon* (S'Arboçar [Banyalbufar municipality], Cap Blanc, and Dragonera; Appendix 1) for 10 microsatellite loci. The number of alleles (*A*), observed heterozygosity (H_o), and unbiased expected heterozygosity (H_e) (Nei, 1978) were calculated using GENETIX version 4.02 (Belkhir et al., 1996–2004). Inbreeding coefficients (F_{IS}) and deviations from Hardy–Weinberg equilibrium and linkage disequilibrium between pairs of loci (using 1000 permutations) were calculated using GENEPOP version 4.0 software (Rousset, 2008).

The 10 polymorphic microsatellites had a total number of 56 different alleles in the 40 individuals analyzed of *C. tricoccon*. The number of alleles ranged from a minimum of four alleles for the loci Ctric 2925, Ctric 7615, Ctric 5261, and Ctric 9344 to a maximum of nine alleles for locus Ctric 6384 (Table 2). Observed heterozygosities ranged from 0.200 to 0.900, while expected heterozygosities ranged from 0.180 to 0.810 (Table 2). All loci (except Ctric 09344) showed significant departures from expected proportions under Hardy–Weinberg equilibrium in at least one population. The mean value of inbreeding coefficient value per population (F_{IS}) ranged from 0.208 to 0.661 (Table 2). Only one of 101 comparisons between loci showed significant linkage disequilibrium ($P < 0.05$). However, results were not significant when correction for multiple comparisons (Bonferroni correction) was considered.

CONCLUSIONS

The results obtained in this analysis of the genetic variability in *C. tricoccon* with 10 microsatellite primers specifically

TABLE 1. Main characteristics of 17 microsatellite loci developed in *Cneorum tricoccon*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
Ctric 00490	F: TGGTTTTGAGGTCCATAGGAGG	$(TG)_{16}$	237	56	JQ282779
	R: AGCACTACTACCTCGTCCAC				
Ctric 02925	F: TCTAGGAGGAACTGATGCCG	$(ATA)_7(ATG)_{16}$	174	56	JQ282780
	R: GCCAACAACAACTTTCCAGC				
Ctric 05261	F: GTTGAGTCCAAGATGGTGGTTC	$(AC)_{12}$	213	56	JQ282781
	R: CCTGCAACAAGGAAAAACAACAC				
Ctric 06384	F: TTACAATACCCGTCCCGTCC	$(TG)_{18}$	141	56	JQ282782
	R: CCACGAGATAAACACAAGACCG				
Ctric 07615	F: TCCGTTGTGGTATGTTCAGC	$(TG)_{17}$	100	56	JQ282783
	R: CTGTTGTGCGTCAATACAAGC				
Ctric 09344	F: TCTCTCCCGACCTCTCCAAG	$(AC)_{18}$	150	56	JQ282784
	R: TGTGTTTTGTCTCCACTGCG				
Ctric 10195	F: ACACTTGCACGAAATTGTCTTATG	$(GT)_{14}$	119	56	JQ282785
	R: TAACGGATGACGAACACCAC				
Ctric 14301	F: GGTTCAATCGCTCTTTTCGC	$(GA)_{15}$	129	56	JQ282786
	R: TTAGACGCGCCAATGCAATC				
Ctric 15341	F: ACGGTCCACACCTGATAAGC	$(CA)_{20}$	109	56	JQ282787
	R: ATTTTGTACGGGCCATGGAG				
Ctric 19884	F: ACAGTGAAAGCCAAGGTTGTAAG	$(AG)_{12}$	236	56	JQ282788
	R: TGCAGTCACTACTGATTTTACTTG				
Ctric 00438*	F: TCTTTCGGACTAGTTTCCCTTC	$(CA)_{17}$	231	56	JQ282789
	R: GCACTTCCACTCGCATTAGC				
Ctric 04087*	F: TCTTGACATACCCCATCCGC	$(CA)_{12}$	124	56	JQ282790
	R: GTTCGGTAGTTGAACGTGGC				
Ctric 06882*	F: CGTCGGGGAAGTTTATTAGCC	$(GT)_{11}$	249	56	JQ282791
	R: CCACACTTCTTACTCCAAGACAC				
Ctric 09984*	F: TACTGGAACGGAGGAACGTC	$(GT)_{17}$	250	56	JQ282792
	R: AGTAATGCTCTGCATCGACAG				
Ctric 09996*	F: AGCACTTACTCGAAATCGCC	$(GA)_{13}$	231	56	JQ282793
	R: CCGCCCACTCCTCATTTTAC				
Ctric 12062*	F: GTAGGTCAAACTCCCCCACC	$(CT)_{12}$	161	56	JQ282794
	R: TCGGTCCAAGAGAGGAAGAG				
Ctric 20970*	F: CGTGAGTCTTGCTTGATGGG	$(GA)_{15}$	151	56	JQ282795
	R: AAGACCACAGTCCACAGCAC				

Note: T_a = annealing temperature.

* Signifies monomorphic loci.

TABLE 2. Results for polymorphism genetic screening carried out with 10 microsatellite loci in three populations of *Cneorum tricoccon*.

		S'Arbossar (N = 30)				Cap Blanc (N = 5)				Dragonera (N = 5)			
Locus	Size range (bp)[a]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]
Ctric 00490	235–247	5	0.724	0.723	0.017$^{n.s.}$	4	0.200	0.700	0.765*	2	0.000	0.320	1.000$^{n.s.}$
Ctric 02925	175–181	2	0.240	0.365	0.361$^{n.s.}$	1	0.000	0.000	—	3	0.200	0.620	0.733*
Ctric 07615	100–106	3	0.345	0.606	0.446***	3	0.400	0.560	0.500$^{n.s.}$	2	0.200	0.180	0.000$^{n.s.}$
Ctric 14301	126–132	4	0.133	0.358	0.638**	3	0.400	0.460	0.238$^{n.s.}$	2	0.200	0.500	0.667$^{n.s.}$
Ctric 05261	213–219	4	0.623	0.467	0.267*	2	0.200	0.420	0.600$^{n.s.}$	1	0.000	0.000	—
Ctric 06384	141–163	6	0.448	0.756	0.421***	2	0.000	0.320	1.000$^{n.s.}$	1	0.200	0.180	0.000$^{n.s.}$
Ctric 10195	114–130	6	0.600	0.674	0.127$^{n.s.}$	4	0.200	0.580	0.714*	1	0.200	0.180	0.000$^{n.s.}$
Ctric 19884	231–247	4	0.267	0.293	0.108*	3	0.400	0.640	0.467$^{n.s.}$	1	0.000	0.000	—
Ctric 15341	102–122	6	0.593	0.810	0.286***	3	0.000	0.560	1.000*	3	0.200	0.460	0.636$^{n.s.}$
Ctric 09344	144–150	3	0.900	0.560	–0.596$^{n.s.}$	1	0.000	0.000	—	3	0.600	0.460	–0.200$^{n.s.}$
Mean		4.3	0.487	0.561	0.208***	2.6	0.180	0.424	0.661***	1.9	0.180	0.290	0.355***

Note: — = not available; A = number of alleles for each locus; F_{IS} = inbreeding coefficients; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals considered.

[a] Allele size range for each locus.

[b] Significant level for Hardy–Weinberg equilibrium tests: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s. = not significant.

designed for this species support their use for conducting population genetics and landscape genetics studies. Furthermore, the study of hypothetical gene flow between islands and continent populations, the isolation of each population, and the values of the inbreeding coefficient may be essential for understanding the demographic, reproductive strategies, and conservation status of this plant.

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APPENDIX 1. DNA accession number (individuals kept in Terrestrial Ecology laboratory, IMEDEA) and locality information for populations of *Cneorum triccocon* used in this study.

DNA accession no.[a]	Collection locality	Geographic coordinates	No. of individuals
AT-CT-01	S'Arbossar	39°40′47″N, 2°30′58″E	30
AT-CT-03	Cap Blanc	39°48′36″N, 2°44′22″E	5
AT-CT-04	Dragonera	39°59′34″N, 2°33′12″E	5

[a] AT = Anna Traveset.