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Microsatellite loci isolated from *Chamaeleo chamaeleon*

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Introduction

The common chameleon, *Chamaeleo chamaeleon* (Linnaeus, 1758) is the northernmost representative of Chamaeleonidae family and the one with the largest distribution area. It occurs in southern Europe (Portugal, Spain and Greece), in some Mediterranean islands (Cyprus, Malta, Sicily and Crete), in North Africa (Morocco, Algeria, Tunisia, Egypt and Libya), and the Middle East (Turkey, Lebanon, Israel, Saudi Arabia, Syria and Yemen) (Blasco *et al.* 1985; Miraldo *et al.* 2005; Miraldo and Paulo 2008). The study of 16S ribosomal mitochondrial DNA in this species showed very low variability in the Iberian populations compared to the North African populations (Paulo *et al.* 2002). It also suggested that populations of existing chameleons in Iberian peninsula were founded by chameleons from North Africa, through two separate colonizations, one from Mediterranean North African populations which originated the Mediterranean Malaga population; and the other from the Atlantic coast of Morocco which originated the Atlantic populations of Cadiz, Huelva and Algarve. These colonizations could have been recent (< 200 kya) and eventually human mediated (Paulo *et al.* 2002). However, the mitochondrial diversity was generally low and did not allow disentangle the relative timings of colonization. More variable markers are needed to assess in more detail the colonization patterns and genetic structure of these populations, that will be important for defining conservation measures. Here, we describe six polymorphic microsatellite markers developed for this species.

Materials and methods

Isolation of microsatellites was carried out based on the protocol by Hammond *et al.* (1998) and Glenn and Schable (2005). DNA extraction from the muscle tissue of the phalanges was done using the NucleoSpin Tissue (Macherey-Nagel, Duren, Germany) extraction kit. One genomic library was prepared from DNA, from a single individual (who was chosen between eight extracted), after confirming in 1% agarose gel and stained with ethidium bromide that the DNA was of high molecular weight and in sufficient quantity by comparison with standard values (lambda standards). DNA was digested with *Rsa*I (Promega, Madison, USA) and selected fragments between 500 and 1000 bp were isolated from 1.2% agarose gel. Fragments were enriched for CA repeats using biotinylated probes captured with streptavidin beads (Dynabeads, Invitrogen, Carlsbad, USA). The enriched DNA was amplified by PCR and then cloned using TOPO TA-Cloning Kit for sequencing (Invitrogen) with the vector pCR® 4-TOPO®, which is incorporated in the bacteria *Escherichia coli* One Shot Top 10 (Invitrogen). Colonies were screened following the polymerase chain reaction (PCR)-based screening method of Lunt *et al.* (1999). The amplified fragments were purified and sequenced using a Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Norwalk, USA), following the cycle sequencing conditions: 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The fragments were separated on a sequencer ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, USA) and chromatograms were analysed in the program Sequencher 4.05 (GeneCodes, Ann Arbor, USA). The design of the primers for the fragments that contained microsatellites was done using Perl Primer v.1.1.17 software (http://perlprimer.sourceforge.net/).

The designed primers were tested in 30 individual samples of chameleons that were already stored in our lab from previous mitochondrial DNA studies (Paulo *et al.* 2002) from four locations in the Iberian peninsula (Algarve, $N = 10$; Huelva, $N = 4$; Cadiz, $N = 4$; Malaga, $N = 4$) and three locations in Morocco (El Jadida, $N = 3$; Essaouira, $N = 3$; Erfoud, $N = 3$). Two distinct methods were used for

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labelling the amplified fragments with fluorescence: a more traditional, where each of the forward primer was labelled with fluorescence, and another method using the M13-tailed primer protocol for fluorescence labelling of PCR fragments (Schuelke 2000). Each of the forward primers were 5′ tailed with the M13 (uni-43) tail sequence 5′-AGGGTTTTCCCAGTCACGACGTT-3′ (Venkatesan *et al*. 2007) which hybridize with a fluorescence labelled M13 (uni-43) primer. The latter labelling method allows saving in the number of primers ordered with fluorescence marking, since the same tail fluorescently labelled can be used for marking different loci. The fluorescent labelling was done in 5′ end of the primer (first method) or of the tail (second method) with 6-carboxy-fluorescine (FAM) or hexachloro-6-carboxy-fluorescine (HEX). For the first method (loci *mCCH1* and *mCCH7*), the PCR mixes consisted of: 1x *Taq* Buffer (Promega, Madison, USA), 1 mM of $MgCl_2$ (Promega), 0.15 mM of dNTPs (Bioline, London, UK), 0.1 μM of each primer, 0.025 U of Go*Taq* (Promega) and 10 ng of DNA in a final volume of 10 μL. The amplification conditions were: 2 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at the specific annealing temperatures of the primer (table 1) and 45 s at 72°C, with a final extension step of 72°C for 10 min at 72°C. For the tail method (remaining eight loci), the PCR mixes consisted of: 1x *Taq* Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs (Bioline), 0.5 μM of the tail (labelled with HEX or FAM), 0.2 μM of primer forward (containing the 5′ tail sequence), 0.5 μM of primer reverse, 0.025 U of GoTaq (Promega) and 10 ng of DNA in a final volume of 10 μL. The amplification conditions were: 5 min at 94°C, followed by 10 cycles of 30 s at 94°C, 1 min at the primer-specific annealing temperature (table 1) and 30 s at 72°C, followed by 25 cycles of 30 s at 94°C, 1 min at the 55°C and 30 s at 72°C with a final extension step of 72°C for 10 min. PCR products were checked on 0.5% agarose gels, stained with ethidium bromide. The alleles were then separated by capillary electrophoresis in a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Carlsbad, USA) together with GeneScan ROX Size Standard (Applied Biosystems). Different loci could be run in multiplex if they had different sizes and/or colours. Electropherograms were analysed with GeneMapper 3.7 (Applied Biosystems). The genetic variability of microsatellite loci was analysed as the number of alleles in each locus, and observed and expected heterozygosity using the program Genetix ver. 4.05.2 (Belkhir *et al.* 1996–2004). Deviations from Hardy–Weinberg equilibrium were tested in the program GenePop (Rousset 2008).

Results and discussion

In this study we isolated 82 colonies that were amplified by PCR and 28 of them were sequenced, of which 20 had microsatellite repeats (71% enrichment): 18 with a GT repeat, one with CT and one with GA. Primer pairs were possible to design from the flanking regions of the microsatellites in 15 of these sequences, and 11 pairs were tested. Ten of these were successfully amplified and genotyped.

Table 1. Characterization of microsatellites in *C. chamaeleon*.

Locus	GenBank accession number	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	$MgCl_2$ (mM)	N	Size range (bp)	N_a	H_o	H_e	F_{IS}
mCCH1	KJ126775	F: TCTTGAAACCATCCTTCCTC R: AAATAATTCCCTTCCTGCCT	$(CA)_{23}$	56 – 53	1	17	216–262	11	0.647	0.888	0.277**
mCCH2	KJ126776	F: TTTGAAGAGCACCTGAGTGG R: CTACGCTAACCCAAAGGAGG	$(CA)_{11}$	57	1.5	23	225–233	4	0.522	0.569	0.085**
mCCH4	KJ126777	F: ATGGAAAGCCTTAGAACGG R: CTCGCAGAACTCACACC	$(GT)_9$	57	1.5	15	107	1	0.000	0.000	–
mCCH5	KJ126778	F:CAATGTGGTTTGCTTACTG R: ATCTTCTGTCAAATGCCC	$(TG)_2TT(TG)_6$	57	1.5	25	270	1	0.000	0.000	–
mCCH6	KJ126779	F: AACCATGGCTCAAATATCC R: ACCCACCTAAGTTAGCAG	$(TG)_{35}$	57	1.5	27	227–229	2	0.074	0.391	0.814***
mCCH7	KJ126780	F: GGGCTGAATTCCTTTCC R: TGTTGATGACGCTTGAC	$(GT)_3$	56–53	1	23	207–239	4	0.130	0.279	0.538**
mCCH8	KJ126781	F: TGATCTTTACAGTCTTTGGG R: GTCCAGTGTCTTAACAGC	$TGT(TG)_5$ $TT(TG)_2T$	57	1.5	24	325–327	2	0.000	0.082	1.000*
mCCH9	KJ126782	F: TCTGCCAAGCGTAGGAC R: TAAACACAAAGCCCTAGCGA	$(GT)_4GA(GT)_2$	57	1.5	30	233	1	0.000	0.000	–
mCCH10	KJ126783	F: TAGCTCAGACCATAACCACC R: ACCTTCACTGCACAGCA	$(CA)_5AT(CA)_3$	57	1.5	25	193	1	0.000	0.000	–
mCCH11	KJ126784	F: TGCTAATGGGTTTGGGAAGG R: TTTGTGGGTGGGTGCTG	$(CA)_{10}CCA$	57	1.5	19	310–324	4	0.210	0.530	0.610***

Primer sequences, repeat motif, T_a, annealing temperatures and $MgCl_2$ concentrations are given for each locus. N, sample size, allele size ranges, N_a, number of alleles, H_e, expected heterozygosity; H_o, observed heterozygosity; F_{IS} values are also indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2. Allele frequencies for each locus found in each population of *C. chamaeleon* analysed.

	Algarve	Huelva	Cadiz	Malaga	El Jadida	Essaouira	Erfoud
mCCH1							
N	3	3	4	3	3	0	1
216	0	0	0.125	0	0	–	0
218	0.3333	0.3333	0.125	0	0	–	0
220	0	0.6667	0	0	0.1667	–	0
224	0	0	0	0	0.1667	–	0
226	0	0	0	0	0.3333	–	0
228	0	0	0	0	0.1667	–	0
230	0.6667	0	0.5	0	0	–	0
232	0	0	0	0.6667	0.1667	–	0
236	0	0	0	0.3333	0	–	0.5
238	0	0	0	0	0	–	0.5
262	0	0	0.25	0	0	–	0
mCCH2							
N	4	4	4	3	3	3	2
225	0.25	0	0.125	0.3333	0.5	0.6667	0.5
227	0	0	0	0.3333	0	0	0.25
229	0.75	1	0.875	0	0.5	0.3333	0.25
233	0	0	0	0.3333	0	0	0
mCCH6							
N	8	4	4	3	3	3	2
227	1	1	0.875	1	0	0	0.75
229	0	0	0.125	0	1	1	0.25
mCCH7							
N	4	4	4	3	3	3	2
207	0	0	0.25	0	0	0	0
233	0.875	1	0.75	1	1	0.6667	0.5
237	0	0	0	0	0	0.3333	0
239	0.12	0	0	0	0	0	0.5
mCCH8							
N	6	4	4	3	3	2	2
325	0	0	0.25	0	0	0	0
327	1	1	0.75	1	1	1	1
mCCH11							
N	2	3	4	3	3	3	1
310	0	0	0	0	0.5	0.5	0
314	1	1	1	0	0.5	0.5	0.5
320	0	0	0	0.8333	0	0	0.5
324	0	0	0	0.1667	0	0	0

N, number of individuals analysed.

Amplification of loci *mCCH1* and *mCCH7* were done with the method of forward primer labelled with fluorescence and the remaining loci were amplified with the method of labelled tail. Four loci were monomorphic and the remaining six were polymorphic (table 1). In these latter, the allele number ranged from 2 to 11 and the expected and observed heterozygosity ranged from 0.082 to 0.888 and from 0.000 to 0.647, respectively (table 1). All loci had high and significant F_{IS} values resulting from a lower number of heterozygotes than expected. Several causes are possible that cannot be discarded in the current work: allelic dropout, considering that the DNA quality of the older samples was low; null alleles, since some samples did not amplify any product for some of the loci but amplified well in others; population structure since individuals from different geographical origins were used; high levels of localized inbreeding, since these are fragmented and small populations. Even with a low number of alleles (two), locus *mCCH6* allowed the discrimination between some populations (table 2). The other locus with only two alleles (*mCCH8*) did not allow any discrimination with this small sample size. Despite the reduced number of individuals, some trends are visible in the distribution of allele frequencies, that deserve to be further investigated with increased sampling (table 2): southwest Iberian peninsula has very low variability considering it has the largest sample size ($N = 10$); higher variability in Moroccan populations, with some alleles that are not detected in the Iberian peninsula (loci *mCCH1*, *mCCH7* and *mCCH11*); also the population of Malaga has unique alleles in some loci as well as the population of Cadiz. So far, these results are in agreement with our previous research and conclusions (Paulo *et al.* 2002).

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