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TECHNICAL NOTES

Isolation of tetranucleotide microsatellite loci in the burrowing parrot (*Cyanoliseus patagonus*)

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Abstract We isolated seven novel polymorphic microsatellite DNA loci from the burrowing parrot (*Cyanoliseus patagonus*) and optimised them for future studies of population differentiation and genetic variation. The loci were screened for polymorphism using 38 samples from wild individuals from three neighbouring colonies in Argentina. The primers amplified highly variable loci characterised by 3–10 alleles per locus and their observed and expected heterozygosities ranged from 0.15 to 0.78 and 0.15 to 0.81, respectively. When we analysed 52 samples across Argentina and Chile, we found strong genetic differentiation between the Chilean and the Argentinean subspecies as well as significant differentiation between two geographically separated subspecies within Argentina. Our results indicate the suitability of these microsatellites for investigating further questions regarding the population genetics in this species.

Keywords *Cyanoliseus patagonus* · Patagonian Conure · Primer · Psittaciformes · Tetranucleotide microsatellites

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Introduction

Burrowing Parrots *Cyanoliseus patagonus* are colonial Psittaciformes. In Argentina, they mainly inhabit the phytogeographical province of "Monte", a semi-desert scrubland characterised by bushy steppes and xerophyte forests (see López et al. 2006). In Chile, they are distributed in the semiarid slopes of the Andes (Galaz Leigh 2005). Three subspecies are proposed for Argentina (Darrieu 1980; Nores and Yzurieta 1983; Bucher and Rinaldi 1986): *C. p. andinus* (north-western Argentina), *C. p. conlara* (western central Argentina) and *C. p. patagonus* (central to south-eastern Argentina), and one in central Chile *C. p. bloxami* (Olson 1995). In general, the abundance of Burrowing Parrots is highly variable (Bucher and Rinaldi 1986). In some parts of its range in Argentina, this species is common or abundant (particularly the subspecies *patagonus*; see Masello et al. 2006), but elsewhere it is rare or occasional (Bucher and Rinaldi 1986). This species has suffered a clear retraction since the early nineteenth century (Bucher and Rinaldi 1986). The situation is particularly worrying in Chile, where Burrowing Parrots have undergone a dramatic decline and are listed as "threatened" species in the vertebrate Red List of Chile (Glade 1993, see also Galaz Leigh 2005). Microsatellite markers will allow us to investigate the genetic structures of different populations and identify distinct management units, as well as to study the ecology and behaviour of the species in more detail.

Materials and methods

Fieldwork was carried out from November to December 2007 (Argentina) and in February 2008 (Chile). Most of the known colonies of the four previously proposed subspecies (Darrieu

Table 1 Microsatellite loci in Burrowing Parrots *Cyanoliseus patagonus*, including GenBank accession number, primer sequence, repeat motif, size of cloned allele in bp, number of alleles (*k*); expected heterozygosity (H_E), observed heterozygosity (H_O), polymorphic information content (*PIC*) and frequency of null alleles (estimated by CERVUS)

Locus Accession number	Primer sequence (5′–3′)	Repeat motif	Size (bp)	*k*	H_O (H_E)	PIC	Null allele frequency
Cyanp1	F: CGGACAGTTTATGTCCATGTT	$(CTAT)_{16}$	114	7	0.78 (0.79)	0.75	0.00
EU732667	R: CCCAGGTGTTTTCTTTGGAA						
Cyanp2	F: ACCTGTCCACACACAAACTT	$(TAAA)_3$ $(TAGA)_6$ $(TAAA)_6$	175	3	0.15 (0.15)	0.14	0.00
EU732668	R: TCATAAAAACATTAAATAACCGATAGA						
Cyanp3	F: TTTGAGCTGCAACAATTAAAGC	$(TATC)_2$ ATC $(TATC)_{11}$	158	7	0.63 (0.68)	0.62	0.03
EU732669	R: TGAAGATGCTTATGTGTTATTTGC						
Cyanp4	F: GAGGGCATGTTCCTAACAACTC	$(ATAG)_{14}$	194	10	0.78 (0.76)	0.71	0.00
EU732670	R: TATGCCCACATTGGAGAGC						
Cyanp5	F: TTCCAATCACATTGGCTGTC	$(ATCT)_{12}$	179	6	0.68 (0.73)	0.68	0.02
EU732671	R: TAAGCTCTTGCCAGGAATGC						
Cyanp6	F: GCAAAGGCTGGCTTTTACTG	$(TATC)_{14}$	188	8	0.78 (0.79)	0.77	0.00
EU732672	R: CCTGAACAGTGATGGGTTTG						
Cyanp7	F: TGCAGATGCAGAGCAAGATG	GATA $(GGTA)_2$ GCTA $(GATA)_3$ GAT $(GATA)_{11}$	162	6	0.34 (0.81)*	0.77	0.41
EU732673	R: TGACAGCGCTCTACTTATTTCAG						

1980; Nores and Yzurieta 1983; Bucher and Rinaldi 1986) were visited by two of us (JFM and PQ) in order to sample naturally moulted feathers as a source of DNA for this and further studies. Burrowing Parrots start moulting their primary feathers at the beginning of their breeding season, i.e. from November onwards (Bucher et al. 1987). These feathers tend to accumulate at the bottom of the cliffs or "barrancas" (gorges or ravines) with the colonies, where it is possible to sample them. Taxonomic assignment was conducted following Darrieu (1980) and Nores and Yzurieta (1983).

Genomic DNA was extracted from naturally moulted feather samples using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). PCR amplifications were performed in a 10 µl volume consisting of 1× QIAGEN PCR buffer (containing TrisCl, KCl and $(NH_4)_2SO_4$ at unspecified concentrations), 0.5 µl of each of 10 µM forward and reverse primers, 1.5 mM $MgCl_2$, 0.40 mM of each dNTP and 0.5 U Taq DNA polymerase (Qiagen) and 1 µl template, using an Eppendorf Mastercycler Gradient. A Touchdown thermal cycling programme encompassing a 10°C span of annealing temperatures ranging between 60 and 50°C was used for the amplification. Following an initial denaturation step of 95°C for 3 min, the cycling parameters were: 20 cycles at 95°C for 30 s, an annealing temperature of 60°C (decreasing by 0.5°C per cycle) for 30 s, 72°C for 40 s, 15 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 40 s, and a final extension step of 72°C for 5 min. PCR products were run on Elchrom Spreadex EL 400 gels in an Elchrom SEA 2000 apparatus and sized with the M3 size standard (Elchrom, Cham, Switzerland).

Microsatellites were isolated using magnetic bead capture enrichment following Glenn and Schable (2005). A genomic library was made after double enrichments for the tetranucleotide motifs $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$ and $(AGAT)_8$. Total DNA was digested with Rsa I (New England Biolabs, Ipswich, MA, USA), and fragments were ligated to double-stranded SuperSNX24 linkers. Fragments were hybridised to biotinylated oligonucleotides and captured with magnetic streptavidin beads (Invitrogen, Carlsbad, CA, USA). Enriched DNA was amplified by PCR using SuperSNX24 as the forward primer. Cloning was conducted using a TOPO-TA cloning kit (Invitrogen). Forty clones, with inserts between 300 and 700 bp in length, were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced. Sequences from both strands were assembled and edited in Bioedit (Hall 1999), and microsatellites were located using TandemRepeatsfinder (http://tandem.bu.edu/trf/trf.html) and confirmed by eye. Primers were designed from the flanking sequences of twenty clones containing repeats using Primer 3 software (http://frodo.wi.mit.edu/primer3/input.htm) and tested for amplification on 1.2% agarose gels. Ten of the tested primer pairs amplified high-quality PCR products that showed

polymorphism across ten selected individuals and were further genotyped in a larger sample.

Each locus was tested for polymorphism and heterozygosity using 38 individuals from three neighbouring colonies in Argentina. The characteristics of the seven unique working primer pairs are given in Table 1. We estimated the number of alleles per locus (k), observed and expected heterozygosities (H_O and H_E), polymorphic information content (PIC), frequency of null alleles and parentage exclusion probabilities using CERVUS version 3.0 (Marshall et al. 1998). GENEPOP (version 3.4, Raymond and Rousset 1995) was used to detect significant deviations from Hardy–Weinberg equilibrium (HWE).

The adequacy of our microsatellite markers for working on questions relating to population genetics was tested by determining the degree of genetic differentiation among 52 individuals from the four different subspecies. Pairwise F_{ST} values were estimated for all four populations of Burrowing Parrots (*C. p. patagonus* $n = 15$; *C. p. andinus* $n = 14$; *C. p. conlara* $n = 12$; *C. p. bloxami* $n = 11$) using FSTAT 2.9.3 (Goudet 2001; as per Weir and Cockerham 1984). Significance was tested by 6,000 permutations and Bonferroni correction. Visualisation of genetic differences between individuals was assessed by factorial component analyses (FCA) calculated with GENETIX (Belkhir et al. 2001), which projects individuals into a two-dimensional space according to their allele frequencies for all loci.

Results and discussion

Locus cyanp7 deviated significantly from HWE. Overall, the high numbers of alleles per locus, the high PIC values and heterozygosity and the paternity exclusion probabilities of 0.99 demonstrate the potential of these Burrowing Parrot microsatellite primers to address a variety of questions, like kinship analysis and population differentiation (Table 1).

Pairwise F_{ST} values showed a moderate differentiation between the Chilean subspecies *C. p. bloxami* and Argentinean subspecies (Table 2). This differentiation could be explained by limited gene flow due to the geographical distance among colonies and the highest region of the Andes (up to approx. 6,900 m in the studied region), which

Table 2 Pairwise F_{ST} values between populations of subspecies

	C. p. conlara	*C. p. patagonus*	*C. p. bloxami*
C. p. andinus	0.007	0.018*	0.061*
C. p. conlara		0.012	0.074*
C. p. patagonus			0.077*

Significance (*) after Bonferroni correction with corrected P value of $P < 0.0083$

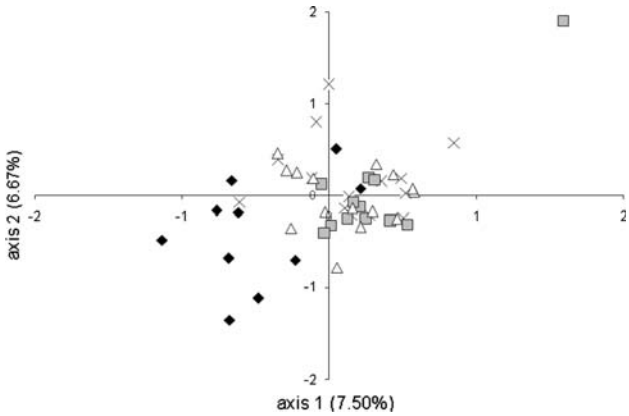

Fig. 1 Factorial component analyses. Distribution of individuals ($n = 52$) by means of allele frequencies of seven microsatellite loci. *Squares*, *C. p. conlara*; *triangles*, *C. p. patagonus*; *crosses*, *C. p. andinus*; *diamonds*, *C. p. bloxami*

presents a barrier to migration. Within Argentina only the most geographically separated subspecies (*C. p. patagonus* and *C. p. andinus*) showed significant (but low) differentiation, while differentiation of the subspecies *C. p. conlara* could not be detected (Table 2), indicating gene flow between geographically neighbouring subspecies. Likewise, graphical representation of genetic differentiation by FCA analysis reflects the separation of the Chilean subspecies distributed mainly along the negative parts of axes 1 and 2 (Fig. 1). These results highlight the suitability of these microsatellite markers for investigating fine-scale population structure within this species.

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