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PRIMER NOTE

Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler

S. BENSCH,* T. PRICE and J. KOHN
Department of Biology 0116, University of California San Diego, La Jolla, CA 92093, USA

Microsatellites have been successfully used to identify differences between closely related species (Taylor *et al.* 1994), subspecies (Paetkau & Strobeck 1994) and populations of the same species (Bowcock *et al.* 1994). Roy *et al.* (1994) presented a thorough analysis at all three levels for wolves and their close relatives. Gotelli *et al.* (1994) were able to reveal hybridization between the Ethiopian wolf and domestic dogs, and others have used microsatellite variation to suggest recent population bottlenecks (Paetkau & Strobeck 1994). As more accurate models to analyse population structure data of microsatellite loci become available (Slatkin 1995) it appears likely that the study of microsatellite variation will contribute significantly to the fields of population genetics and conservation. In this paper we present information on the isolation and characterization of microsatellite loci in the warbler *Phylloscopus occipitalis*. Our research aims at investigating genetic structuring along an altitudinal gradient in its Himalayan breeding quarters and how this is related to morphological differentiation.

Genomic DNA isolated from blood of an adult female large crowned leaf warbler *Phylloscopus occipitalis* was digested with *Mbo*I and electrophoresed in a 1.5% agarose gel. DNA fragments in the size range of 300–600 bp were excised from the gel and isolated by electroelution. A size selected library was then constructed by ligating the DNA into the vector M13mp18 (opened with *Bam*HI), followed by transfection into *E. coli* DH5αF′ by electroporation (*E. coli* Pulser, Bio-Rad). According to the ratio of blue and white colonies 80% of the vectors contained inserted DNA. A total of ≈ 40 000 colonies were screened using the oligonucleotides CA_{15} and GA_{15} end-labelled with γ^{32}P-ATP. Nine positive clones were detected and sequenced (Table 1). The sequence of POCC3 happened to be identical to clone POCC2 although they were found on different plates. Primers were designed to have a Tm of 60 °C.

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*Present address: Department of Animal Ecology, Ecology Building, S-22362, Lund, Sweden. Fax: + 46-46-2224716. E-mail: Staffan.Bensch@zooekol.lu.se

The primers were used on putatively unrelated individuals of 16 *Phylloscopus occipitalis* sampled at Manali, Himachal Pradesh, India. In addition, we checked a family (male, female and four chicks) to look for inheritance patterns. Initial PCR amplifications were performed under the following conditions: 30 s at 94°C, 30 s at 55 °C, 30 s at 72 °C (28 cycles). Before the cyclic reactions the samples were incubated at 94°C for 3 min, and after completion at 72 °C for 5 min. Reactions of 10 μL included 50 ng of total genomic DNA, 0.2 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.4 μM of reverse primer, 0.2 μM of unlabelled forward primer, 0.02 μM of γ^{32}P-ATP labelled forward primer and 0.5 units of *Taq* DNA polymerase. The PCR products were resolved on a 8% denaturing polyacrylamide gel (long ranger, Bio-Rad). Gels were dried and exposed for 3–36 h.

The primers first used to amplify locus POCC1 failed to amplify one of the paternal alleles (see Callen *et al.* 1993). This was indicated by two of the chicks in the family not inheriting the father's allele and that several of the screened individuals appeared to be homozygotes for rare alleles. When one of the primers was redesigned a new allele appeared in the father and the two chicks. Primers for locus POCC4 failed to amplify readily scorable products, perhaps because of the long A-repeat adjacent to the CA-repeat (Table 1). For locus POCC9 we failed to amplify altogether. This clone was very difficult to sequence (with many stops on both sides of the repeat) and one can speculate that the PCR failed for the same reason that caused stops to occur in the sequencing reaction. In total, primers designed for five of the clones reliably amplified polymorphic loci (1, 2, 5, 6 and 8).

We also screened primers isolated from other species: four from loggerhead shrikes *Lanius ludovicianus* (N. Mundy MS), two from the pied flycatcher *Ficedula hypoleuca*, two from the barn swallow *Hirundo rustica* (Ellegren 1992) and six from reed buntings *Emberiza schoeniclus* (Hanotte *et al.* 1994). Of these, one loggerhead shrike primer pair (LS2), one pied flycatcher primer pair (PTC3) and one reed bunting primer pair (Escμ4) amplified variable microsatellite loci. All of the eight primer pairs found to be useful in *P. occipitalis* also showed to be variable in *P. reguloides* and for at least five of the loci in the willow warbler *P. trochilus* (Table 2). This supports earlier observations that microsatellite primers often work across species

Table 1 Characterization of eight microsatellite loci in *Phylloscopus occipitalis*. The number of different alleles were obtained from 16 unrelated individuals

Locus	Length of PCR product	Repeat motif	Number of alleles	Primer sequence (5′-3′)	Accession number
POCC1	229	$(CA)_{13}(CG)_3G(CA)_9$	8	F:TTCTGTGCTGCAATCACACA R:GCTTCCAGCACCACTTCAAT	U59113
POCC2	195	$(CA)_{10}$	6	F:AACCACACTGAGTAAGCTGCTG R:TTTAGCTCACCTTGCAAATGG	U59114
POCC4	198	$(TC)_7TGTCT(CA)_{11}(A)_{17}$	>4	F:AGCTTGATTTTTCTTTCTCTCGC R:GAAGGGTCAGTGTGTGCAGA	U59115
POCC5	109	$(AC)_{13}(AT)_6GG(AC)_4$	6	F:AGATGGCTGGGGGCATAT R:CCTTTAGCATTCACCTAGCACA	U59116
POCC6	174	$(CA)_{16}$	8	F:TCACCCTCAAAAACACACACA R:ACTTCTCTCTGAAAAGGGGAGC	U59117
POCC7	101	$(CA)_8$	1	F:TCCTTTTATCCATCTTTCCTCA R:AAATTGCCACTCTCCCAGG	U59118
POCC8	214	$(AC)_{15}$	6	F:GCATGTCTCTTCAGACATCTGC R:ATGTAGAGCTCCCATGGTGG	U59119
POCC9	188	$(CA)_8CT(CA)_7$ $CT(CA)_2$	?		U59120

Primer	*P. occipitalis* $n = 16$	*P. reguloides* $n = 23$	*P. trochilus* $n = 2$	*Acrocephalus arundinaceus* $n = 2$
POCC1	8 (211–229)	6 (211–221)	2 (205–209)	1 (194)
POCC2	6 (185–197)	7 (187–199)	3 (183–189)	1 (182)
POCC5	6 (95–109)	4 (99–105)	2 (95–97)	–
POCC6	8 (156–174)	7 (160–172)	1 (174)	1 (174)
POCC7	1 (101)	1 (101)	1 (101)	1 (101)
POCC8	6 (211–216)	7 (209–219)	3 (207–215)	–
LS2	3 (188–194)	3 (186–190)	1 (190)	–
PTC3	5 (133–147)	6 (141–163)	2 (135–139)	–
Escμ4	2 (146–152)	3 (144–148)	1 (148)	–

Table 2 Number of alleles (range of allele lengths) in cross-species amplification with six pairs of *Phylloscopus occipitalis* microsatellite primers, one primer pair from loggerhead shrikes (LS2), one from pied flycatchers (PTC3, Ellegren 1992) and one from reed buntings (Escμ4, Hanotte *et al.* 1994)

within a genus (e.g. Ellegren 1992). However, none of the primer pairs appeared to amplify polymorphic microsatellites in the great reed warbler *Acrocephalus arundinaceus*.

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