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PRIMER NOTES

Polymorphic microsatellite markers in the ostrich (*Struthio camelus*)

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The ostrich (*Struthio camelus*), the only member of the *Struthionidae*, is the largest living bird. There are four subspecies of ostrich in Africa which differ slightly in size and in the colour of the bare skin of the thighs and neck. *Struthio camelus molybdophanes* and *Struthio camelus massaicus* occur in eastern Africa, *S. c. camelus* in northern and western Africa and *Struthio camelus australis* in southern Africa (Brown 1980). The northern subspecies are separated from *S. c. australis* by a belt of woodland, although Freitag & Robinson (1993) suggest that this has not always been an effective barrrier. The three northern subspecies have overlapping ranges but *S. c. molybdophanes* has clear phenotypic and behavioural differences from both *S. c. massaicus* and *S. c. camelus*.

Apart from their importance as the only member of a highly specialized family, the ostrich has also become commercially significant as a result of the growth of ostrich farming in Africa, Australia and, more recently, in Europe. Genetic markers in the ostrich would assist in the design of breeding programs aimed at maintaining genetic diversity. Markers would also be useful in identifying individuals and in establishing parentage in these extremely valuable birds. They would, in addition, be tools for the study of the relationships between the four subspecies and the various more-or-less isolated domesticated populations.

Microsatellites have become the genetic markers of choice for these purposes because of their high levels of polymorphism and ease of use. These markers have been used in a very wide variety of organisms including birds (Ellegren 1992; Crooijmans *et al*. 1993), but in the ostrich only one such marker has been described to date (Ward *et al*. 1994). Here, we report the isolation and characterization of 14 ostrich microsatellite markers.

Ostrich (*S. c. massaicus*) genomic DNA was digested with *Sau*3AI, size-selected fragments in the range of 470–570 bp were cloned into pUC19, and the resulting library was screened with a $(GT)_{10}$ synthetic oligonucleotide probe as described previously (Kemp *et al*. 1995). Positive clones were subjected to a secondary screening and selected clones were sequenced. Out of a total of 23 positive clones subjected to sequencing, 20 contained microsatellites; two could not be sequenced and one sequenced clone had no microsatellite. In addition to the $(GT)_n$ repeat motif, $(AG)_n$, $(GC)_n$ and $(T)_n$ repeat structures were detected.

PCR primers were designed on the basis of sequence flanking the microsatellite regions by a computer program (S. J. Kemp, unpublished results). PCR conditions were optimized for all 20 primer pairs and, on agarose gel electrophoresis, they each showed a single band from ostrich genomic DNA. PCR was performed in a 5 µl volume as described previously, except that the enzyme used was Amplitaq Gold (Perkin Elmer) and the reaction buffer was PARR (Cambio). The thermal cycling (on a PTC-100 V thermocycler, MJ Research, USA) comprised 10 min at 95 °C, followed by 5 cycles of 2 min at 94 °C, 1 min at the selected annealing temperature and 1 min at 72 °C. This was followed by 20 cycles in which the denaturing temperature was reduced to 90 °C. The annealing temperature selected for each primer pair is shown in Table 1.

To distinguish the microsatellite alleles, PCR amplification was carried out with one primer of each pair end-labelled with [γ^{33}P]-dATP and the product was analysed on a 6% polyacrylamide gel (Sequagel, National Diagnostics) as described previously (Kemp *et al*. 1995). The degree of polymorphism was estimated by applying each marker to a panel of 18 genomic DNA samples from ostriches of two subspecies; *S. c. massaicus* (n = 6) and *S. c. molybdophanes* (n = 12). DNA was extracted from blood samples which were collected from ostrich farms in Kenya (*S. c. masaicus* and *S. c. molybdophanes*) and Zimbabwe (*S. c. molybdophanes*).

Fourteen of 20 microsatellite loci tested for polymorphisms revealed useful allelic polymorphism and numbers of alleles varied from four to 13 per locus. The results showed strikingly different allele distribution amongst the two small samples of each subspecies. LIST002 and LIST004, with six and seven alleles, respectively, showed no alleles common between the two subspecies and all 14 loci showed some alleles unique to either *S. c. massaicus* or *S. c. molybdophanes*. The difference between the two populations are significantly different ($P < 0.001$) by the exact test (using GENEPOP software, Raymond & Rousset 1995). Although the number of individuals sampled here is small and the degree of relatedness of individuals from each population is unknown, these results suggest a significant degree of isolation between *S. c. massaicus* and *S. c. molybdophanes* and support their status as subspecies rather than races. Previous studies of restriction-site polymorphism in mitochondrial DNA of ostrich have shown evidence of deep divisions between *S. c. molybdophanes* and the other three subspecies in their mtDNA sequence (Freitag & Robinson 1993), and our observations indicate that this is probably also true of the nuclear genome.

The polymorphic microsatellite loci were designated from LIST001 to LIST0014 and their sequences have been submitted to the GenBank database (accession numbers U858080 to U85821).

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Table 1 Microsatellite loci in the ostrich

Locus	T_a	Repeat structure	Size	No. of alleles	Primer sequences 5′–3′
LIST001	55	(TG)3 (TA)3GG(TG)18	201	9	CATGACCTCAATCAGATACC CGCTTTGCAATATTCCAAGC
LIST002	55	(AC)11C4 (AC)3	123	6	CCTCAACAAATCCAGTATCC GCTGTAGTTCTGTAATCTGC
LIST003	55	(TG)19AGTA(TG)3	201	5	CTACACACAAAGGAATTAGAAG GGAGATAGCGTAAATCCACC
LIST004	58	(TG)13 (CG)4 (TG)4 (TG)4T2 (TG)2	242	7	CTGTTATTACCACTGAGAATAGTAAC TGTGAAACTGTAGCCTGACC
LIST005	55	(TG)2CG(TG)10	197	10	ATGGTGCTTTCCAGTGGTGTGC CATTGACCCAGGCAAGAAATCC
LIST006	58	(TG)2AG(TG)2GG(TG)6CG(TG)16	111	7	GTACCAGACCACACAGACC GGGACAACATCACAAAAGGC
LIST007	58	(AG)16	140	5	TGAGCATGTATTCCCAGTAAGC TGTCTGTGTTGAGCTGTACGG
LIST008	55	(T)28	160	5	ATGGTAAGATGGGATATGCC GCTGTCTCTCATCAGTATCG
LIST009	55	(CA)14CG(CA)3CG(CA)3	199	13	CATTGCAAACACTCTGCTGC TGAACGACAGGGTTATTGGC
LIST0010	58	(CA)5 A2 (CA)19	154	7	CAACTCATCACATTTCCAGG TTTAGTGGTCTTTTAGGGGG
LIST0011	58	(GT)24	135	10	ACTGAAGTTTCCTTCTCCCC TTCCTGAAGCAACCACAC
LIST0012	58	(GT)16TT(GT)14	128	5	GATAGAAAAGGAGCAGTGGC CTTCATATTCCAGCAGTACAGC
LIST0013	55	(GT)8 (ATGT)5 (GT)5	298	4	AAGCTCTTTGTACTCTCCAC TACGTTATTACTTCACTTTAT
LIST0014	55	(GT)16GC(GT)4AT(GT)9	318	5	ATCATCCCAGTCAGGAGCACC TCTGTGGAAGCAGGTCTTGG

T_a, annealing temperature.

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Isolation and characterization of microsatellite loci in the Komodo dragon *Varanus komodoensis*

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The Komodo dragon *Varanus komodoensis* is a monitor lizard that was endemic, until the late 1970s, to five south-eastern Indonesian islands of the Lesser Sunda region (Auffenberg 1981). Poaching of prey species caused its extinction on the island of Padar, and less than 3000 individuals remain on four islands within the Komodo National Park.

As part of a conservation genetics survey, samples were collected from a total of 78 wild specimens. Microsatellite primers have been developed to analyse the extent of genetic variability and degree of gene flow among islands. This information is particularly important for the management of the extant populations of the Komodo dragon. It will allow, for instance, the identification of distinctive genetic units that

might provide the source material for both augmentation and reintroduction.

A partial genomic library was first constructed and screened for the presence of CA, GA, and AAT sequence repeats as described in Rassmann *et al*. (1991). Approximately 0.25% of the colonies gave a positive hybridization signal. To increase the efficiency of the process, a library enriched for CA and GA repeats was constructed using a modification of the methods of Armour *et al*. (1994) and Kandpal *et al*. (1994), also described by Hammond *et al*. (in press). A total of 10 µg of genomic DNA was digested with *Mbo*I and the resulting DNA fragments were selected in the 200–700 bp size range. Approximately 200 ng of DNA fragments was ligated to 2 µg of linker sequences with compatible overhangs. The sequences of the linker oligonucleotides are as follow: SAULA (5′-GCGGTACCCGGGAAGCTTGG-3′) and SAULB (5′-GATCCCAAGCTTCCCGGGTACCGC-3′). The ligation reaction produced blunt-ended molecules which were then amplified by PCR in a 25 µl reaction containing 1× PCR buffer (MBI Fermentas: 100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40, pH 8.8), 1.5 mM $MgCl_2$, 80 µM of each dNTP, 25 pmoles of SAULA as the PCR primer, and 1–3 U of *Taq* polymerase. An initial 5 min extension step at 72 °C allowed the *Taq* polymerase to heal the nick between the genomic DNA and the linker sequence (Hammond *et al*., in press). The following cycle parameters were used: 30 × 94 °C, 30 s, 67 °C, 30 s, 72 °C, 120 s. The PCR products were denatured by boiling for 10 min and then hybridized to 5 µg of biotinylated $(CA)_{22}$ and $(GA)_{22}$ probes in 500 µl of sodium phosphate buffer (0.4 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.5% SDS, pH 7.4) for 15–18 h at 50 °C. The entire mixture was incubated in 10 mL of buffer A (150 mM NaCl, 100 mM Tris, pH 7.5) containing 50 mg of Vetrex–avidin D (Vector Laboratories) for 40 min at ambient temperature to allow the binding of the biotinylated probe/genomic DNA hybrids to the avidin matrix. Unbound fragments were removed by washing the avidin/DNA mix with 10 mL of buffer A at ambient temperature, followed by centrifugation for 2 min at 600 ***g***. This process was repeated once, and nonspecific probe/genomic DNA duplexes were then removed by carrying out a third wash in 4 mL of 0.1× buffer A at 55 °C for 30 min. The targeted genomic DNA was then recovered by resuspending the pellets in 4 mL of dH_2O at 65 °C for 30 min and then concentrated and purified using Centricon-100 concentrators (Amicon). The retained fragments were then amplified in a second PCR as above without the initial extension step. The PCR products were digested with *Mbo*I to remove the linkers and then ligated into pUC18 cut with *Bam*HI (Pharmacia 'Ready-to-go' kit). Clones were hybridized with oligonucleotide probes labelled at the 3′ terminus with [α-^{32}P]-dCTP using a terminal deoxynucleotidyl transferase (Rosenberg *et al*. 1990).

Approximately 5% of the enriched clones gave a positive hybridization signal. Although this value is more than an order of magnitude higher than the number of positive clones obtained in the conventional library, the efficiency of the biotin capture-enrichment method used for the Komodo dragon was much lower than that reported for other taxa. For instance, 80% of the clones of a library enriched for CA repeats in loggerhead shrikes contained microsatellites (Mundy & Woodruff 1996), while Ostrander *et al*. (1992),

Table 1 Characteristics of 10 microsatellite loci in the Komodo dragon *Varanus komodoensis* ($n = 78$). H_o is the average observed heterozygosity in four island populations where samples were obtained from 23, 32, 12, and 11 individuals, respectively. T_a is the optimal annealing temperature. Cloned sequences have been registered in the GeneBank database under the accession numbers AF005428–AF005437

Locus	Type of repeat of the sequence cloned	Number of amplified alleles	Allele size range (base pairs)	H_o (± SE)	T_a (°C)	Primer sequences (5′–3′)
K1	CA_{25}	5	175–183	(0.66 ± 0.12)	54	K1F: TGTCGCCAAGTTTTCTGTGCAG K1R: TCGCAGAGATCCAGTCCTTTCC
K2	CA_{12}	2	142–144	(0.20 ± 0.14)	52–53	K2F: TTCTTTTCATCTGCCCTCGCTCC K2R: CAACAGCAAGCAAGCAAGCA
K3	CA_{12}	2	117–119	(0.18 ± 0.09)	52	K3F: GCACACACATTCACAGACAAATCAAG K3R: CTGATCTTCTTTTACTGGAGAGGGTG
K4	AC_{13}	2	103–105	(0.25 ± 0.08)	53	K4F: ACCGCAGCTCCTTCTACAAG K4R: CCAACAGTGTAAATCCCAGCAC
K5	TAA_{11}	2	179–182	(0.16 ± 0.09)	50	K5F: ATGTTGTAAGCCGCCCCAAG K5R: CGAGCCACATTTCCCTCTGTTATT
K6	$CA_{18}CCACAA(CA)_3$	5	117–131	(0.27 ± 0.14)	56	K6F: GCAGCACACCTGAGTAGAGCAAG K6R: AGGCTGAAGTCTGGGGAGCAGA
K7	$TC_{19}AC_{18}$	8	189–205	(0.51 ± 0.08)	54	K7F: TCACAATGACTTCAGTGCTATCCTG K7R: AACCAACTGTGCTACGCCCTC
K8	$C_{10}TC_4AC_{13}$	2	135–138	(0.20 ± 0.11)	56	K8F: CAGCACTGAAAGCGATACCAGAG K8R: ATGAGGACCTTGTGGTGCCAAGAC
K9	$TC_5CC(TC)_{23}(AC)_{15}$	9	175–199	(0.49 ± 0.10)	55	K9F: CTCCTATATTCCCTCCTCTGACTGG K9R: GGCACCTACAAATGAAAAGCTGG
K10	$(TG)_2A(TG)_3GTCGCA(AC)_{19}$	3	137–147	(0.13 ± 0.03)	55	K10F: TGAACCAGAGGCAGTGGAGAAGAC K10R: GCAGCAACCAGTGACACAGAAAAG

using canine genomic DNA, found that 40–50% of the clones hybridized to a CA_n probe.

Twenty-eight and 67 clones were isolated from the standard and the enriched library, respectively. After isolation of the recombinant DNA molecules and sequencing of the DNA insert, approximately 70–75% of the clones were found to contain a simple sequence repeat (20 and 50 clones from the standard and the enriched library, respectively). In the conventional library, eight DNA inserts came from the same clone, while 10 fragments obtained with the enrichment protocol had an identical sequence. Eight and 20 PCR primer pairs were designed on sequences cloned from the standard and the enriched library, respectively. Primers were tested on DNA extracted from tissue samples (Bruford *et al*. 1997) in a 10 µl PCR volume containing 2.5 pmol of forward primer labelled at the 5′ terminus with [γ-^{32}P]-ATP using a polynucleotide kinase (Sambrook *et al*. 1989), 2 pmol of unlabelled reverse primer, 0.1 mM of each dNTP, 1–2.5 mM $MgCl_2$, 0.7 U of *Taq* polymerase, 1 µl of 10× PCR buffer (MBI Fermentas). A Hybaid thermal cycler was used with the following cycle parameters: 1 × 93 °C, 3 min; 35 × 93 °C, 60 s, 50–56 °C 90 s, 72 °C, 120 s; 1 × 72 °C, 7 min.

Out of the eight microsatellites obtained from the conventional library, four were polymorphic, and three were monomorphic, while for two of them the gel autoradiography did not give a clear pattern. Six of the 20 loci identified with the enrichment protocol were polymorphic, six were monomorphic, and for eight other loci either nonspecific amplification or no clear pattern was obtained (Table 1). Overall, the number of alleles varied from two to nine. High values of the standard error calculated for the average observed heterozygosity reflect the differences in genetic variability between islands. The smallest of the four islands had four loci fixed for one allele, while in the others the allele frequency distribution was more heterogeneous.

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Isolation and characterization of microsatellite loci in the brown long-eared bat, *Plecotus auritus*, and cross-species amplification within the family Vespertilionidae

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Species belonging to the order Chiroptera display a wide diversity of behavioural and physiological adaptations, many of which are unique to the order. Direct studies of bat populations are often hampered by an inability to observe individuals all year round, hence genetic studies (for example, McCracken & Bradbury 1977) have proven more useful for determining social structure and geneflow. Here, we describe the isolation and characterization of microsatellite loci from a palaearctic bat species, the brown long-eared bat, *Plecotus auritus* (family: Vespertilionidae), and investigate their suitability for use in other bat species.

Two methods were used in the construction of microsatellite libraries, a fuller description of which can be found in Rassmann *et al.* (1991) and Hammond *et al.* (1998). A non-

Table 1 Characteristics of six microsatellite loci cloned from *Plecotus auritus*. F = forward primer, R = reverse primer. Allele number, size ranges and heterozygosities were obtained by screening 662 individuals from north-eastern Scotland. The clone sequences from which the primers were designed have GenBank accession numbers AF006681–AF006686

Locus	Clone repeat	Primer sequences (5´-3´)	Annealing temperature (°C)	No. of alleles	Allele size range (bp)	Observed *H*
Paur01	$(GT)_{25}$	F: CAATTTCAAGGCAGTGCTC R: TGC TGT CCC TGC ATG CTG	55	10	145–165	0.73
Paur02	$(TC)_{27}$	F: GGATTCAATTCTGGTCAAGGAC R: AACCACCCATTCTCTGTAGTGTAG	52	19	200–240	0.90
Paur03†	$(TG)_{17}$	F: CTGGAGTGTTGTTTTGCCCTTC R: GCTGATGGTGGAGTCTCCTTTTTC	55	7	244–264	0.57
Paur04	$(CT)_{17}C\ (CA)_6$	F: GATCACAACCCTGAAAGG R: TGCATGGACTCAGGAAAC	$60–56^{TD}$	13	219–261	0.45
Paur05	$(GT)_{10}$	F: GGACAGTATGCCATGTTATGCTG R: GCACTTTCACAAACCTAGATGG	$60–56^{TD,PARR}$	12	226–248	0.82
Paur06	$(AC)_{18}\ (AG)_{18}$	F: GATCAGATTTCCAAACAGAG R: AGGTTCTTTCTTCAGCTATG	52	24	168–226	0.93

†Locus Paur03 is located on the X chromosome (see text), and Observed *H* is for females only.
^{TD}Touchdown program of decreasing annealing temperatures used 60 °C for five cycles, 58 °C for five cycles, and 56 °C for 20 cycles.
^{PARR}The reaction is enhanced when the *Taq* buffer is replaced by PARR™ buffer (Cambio).

Table 2 Number of alleles from cross-species amplification within the family Vespertilionidae with *Plecotus auritus* microsatellite primers. Numbers in parentheses are numbers of individuals tested per species. The two mitochondrial clades of *Pipistrellus pipistrellus* identified by Barratt *et al*. (1997) were regarded separately

Locus Annealing temperature (°C)	Paur01 52	Paur02 48^{PARR}	Paur03 48^{PARR}	Paur04 $56–52^{TD}$	Paur05 $56–52^{TD/PARR}$	Paur06 50
Plecotus austriacus (8)	5	1	4	2	5	5
Corynorhinus townsendii (4)	1	1	2	1	6	–
Pipistrellus pipistrellus CLADE I (6)	–	3	2	–	5	–
Pipistrellus pipistrellus CLADE II (4)	–	1	1	–	4	–
Pipistrellus kuhli (5)	–	1	4	–	6	–
Pipistrellus hesperus (2)	–	–	–	–	1	–
Pipistrellus subflavus (2)	–	1	–	–	3	–
Pipistrellus mimus (1)	–	2	–	–	2	–
Pipistrellus stenopterus (1)	–	1	–	–	2	–
Myotis daubentonii (3)	–	–	2	–	1	3
Myotis nattereri (2)	–	–	2	4	2	4
Nyctalus noctula (1)	–	1	–	–	–	–

'–-' denotes multiple bands, a smear or no product.
^{TD} and ^{PARR} as described in Table 1.

enriched library was constructed by ligating *P. auritus* DNA restricted with *Hae*III, *Alu*I and *Rsa*I into pUC18. A microsatellite-enriched library was constructed by ligating *Mbo*I-digested DNA to annealed linkers. This was PCR amplified, and the product hybridized with 5´ biotinylated $(GA)_{22}$ and mixed with Vetrexavidin (Vector Laboratories). Repeat-containing DNA fragments were then eluted and ligated into pUC18. In each case, transformation was carried out by electroporation, and transformants screened with GA and CA dinucleotide repeats. Two rounds of screening were performed for the non-enriched library, whereas the enriched library only required one due to prior selection for repeats. DNA from positive colonies was sequenced either manually or by cycle sequencing using AmpliTaq®DNA Polymerase FS (Perkin Elmer) and an ABI 373 sequencer.

Genomic DNA was either extracted from wing biopsies as described in Worthington *et al*. (1996), or from muscle and skin using a standard phenol–chloroform procedure. Individuals were screened for each microsatellite locus separately in a 10 µL PCR reaction containing 10–50 ng of DNA,

1× *Taq* buffer (IGI), 1.5–2.5 mM Mg^{2+}, 1.5 pmol each primer, 1 μL of DMSO, 0.15 mM dNTPs and 0.5–1 U of *Taq* polymerase (IGI). One primer from the pair was end-labelled with [γ-^{32}P]-ATP using T4 Polynucleotide Kinase at 37 °C for 1 h prior to the PCR reaction. Cycling was carried out in a Hybaid Omnigene thermal cycler using thin-walled microtitre plates and the following PCR conditions: 3 min at 94 °C; 30 cycles of 94 °C for 1 min, primer-specific annealing temperature (see Table 1) for 2 min, 72 °C for 2 min; 10 min at 72 °C. PCR products were separated on a 6% polyacrylamide gel and sized using an M13 sequencing ladder.

Twenty-two and 80 positive clones were sequenced for the non-enriched and enriched libraries, giving eight and seven microsatellites, respectively. The majority of positives from the enriched library were large repeats created during library construction, a situation which is preventable by 'blocking' the 3′ end of the biotin-labelled probe (Hammond *et al*. 1998). In total, 12 primer pairs were tested, from which one gave no pattern and five gave multiple bands. The remaining six, detailed in Table 1, were highly polymorphic. For locus Paur03, 343 out of 344 males tested were homozygous (expected number of heterozygotes = 189, $P < 10^{-3}$). It is therefore proposed that this locus is located on the X chromosome; the heterozygote male may have been misidentified, or may have been carrying a genetic abnormality.

Cross-species amplification within the order Chiroptera was investigated using 55 individuals from 20 species spanning the two suborders. The results for the family Vespertilionidae are outlined in Table 2. As expected, the most universal amplification was found in the two additional *Plecotus* species, although amplification was extensive within the family as a whole. Further species tested, not detailed in Table 2, are listed below, with the number of individuals in parentheses. Suborder Megachiroptera: *Pteropus livingstonii* (2), *P. seychellensis* (2), *P. rodricensis* (2), *Rousettus aegyptiacus* (2). Suborder Microchiroptera: *Macroderma gigas* (2), *Rhinolophus ferrumequinum* (3), *Rhinonyctersis aurantius* (2), *Carollia perspicillata* (2). The only amplification observed within these species was at locus Paur03 for Megachiropteran species, where all individuals were fixed for one allele (238 bp).

Vespertilionid bats were rare at 35 Ma (Sigé 1968), suggesting a common ancestor close to this time. Amplification of orthologous loci among species with similar coalescence times has been observed in other groups, for example cetaceans (20–40 Myr, Schlötterer *et al*. 1991). In contrast, coalescence times with other Microchiropteran species may date back to the Eocene. As such, the lack of amplification observed for these species is as expected. Evolutionary relationships between the two chiropteran suborders remain unclear.

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Highly polymorphic microsatellite markers in the great cormorant *Phalacrocorax carbo*

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Two races of the great cormorant (*Phalacrocorax carbo*) occur within Europe: a continental race (*P. c. sinensis*) which breeds in north-central Europe from Belgium and The Netherlands east to the Baltic and Black Seas, and a north Atlantic race (*P. c. carbo*) which breeds on British, Norwegian and northern French coasts (Jonsgard 1993). Over the past 10–20 years numbers of both species have increased following the introduction of protective legislation (van Eerden & Gregersen 1995).

In Britain there has also been an increased tendency for birds to forage and breed inland, a habit usually associated with the continental race (Gibbons *et al*. 1993). Whether this movement inland represents *P. c. carbo* changing its habit because of population pressure on coastal colonies, or is an invasion of *P. c. sinensis* from European sites is unclear. Either way, the increased use of inland freshwaters by great cormorants has highlighted the potential for conflict with fisheries interests (Marquiss & Carss 1994; Carss & Marquiss 1996). Any effective management program for the species must be underpinned by information on the dynamics of

inland colonies, data which has proven difficult to acquire using traditional ringing and observational analyses.

Here we describe the isolation and characterization of several highly variable di-, and tetra-nucleotide microsatellite loci, which will be suitable for examination of colony formation, integrity, isolation and gene flow.

Microsatellite loci were isolated using an enrichment cloning approach similar to that of Armour *et al*. (1994). High-molecular-weight DNA was extracted from muscle tissue of three cormorant individuals using the salting-out procedure of Bruford *et al*. (1992), and subsequently pooled. The DNA was digested using *Sau*3AI restriction endonuclease and fragments of 300–600 bp in length were isolated. This fraction was ligated to a SAU linker molecule made by annealing equimolar amounts of SAU-L-A (5'-GCGGTACCCGGGAAGCTTGG-3') and SAU-L-B (5'-GATCCCAAGCTTCCCGGGTACCGC-3') oligonucleotides. Resultant fragments were denatured and hybridized (at 60 °C in 2× SSC, 0.1% SDS) to pieces of Hybond N+ membrane saturated with synthetic microsatellite polymers $(CA)_n$, $(GA)_n$ $(GAAA)_n$ or $(GATA)_n$. The filters were washed at low stringency (three washes of 2× SSC, 0.1% SDS) to remove nonrepetitive DNA, then heated to 95 °C in sterile water for 5 min to remove the microsatellite-enriched fraction. DNA from the enriched wash was precipitated, then complementary strands reformed in a PCR reaction (20 cycles at 94 °C denaturation and 55 °C annealing) using the SAU-L-A linker oligonucleotide as a primer. The SAU linkers were removed from the enriched fraction by digestion with *Sau*3AI, then fragments ligated into a pUC18/*Bam*HI plasmid vector (Pharmacia Ltd). Vector molecules were transformed into highly competent INVαF′ One Shot™ *Escherichia coli* then grown overnight at 37 °C on Luria–Bertani (LB) medium. Colonies were lifted onto Hybond N+ membranes and screened for the presence of the appropriate microsatellite using standard hybridization procedures (Sambrook *et al*. 1989). Positive colonies were dye-terminator cycle-sequenced using an ABI 377 automated sequencer (Perkin Elmer) following the manufacturer's instructions, and *Phalacrocorax*-specific PCR primers were designed according to the criteria used in Piertney & Dallas (1997).

DNA was extracted from the feathers of 100 unrelated, adult great cormorants from colonies in Scotland, England and Holland according to Ellegren (1991). PCR amplifications were performed in a total volume of 10 μL using an MJ Research PTC-100 thermal cycler. The reaction mix comprised 10 ng of template DNA, 2.5 mM $MgCl_2$, 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween-20, 0.2 mM of each nucleotide, 5 pmoles of each primer (forward primer end-labelled with [γ^{32}P]-dATP) and 0.5 U of *Taq* polymerase. PCR profile followed a 'touchdown' (Don *et al*. 1991) procedure to a final annealing temperature of 55 °C or 52 °C depending on the GC content of the primer sequence (see Table 1). Fragments were resolved by electrophoresis on 6% denaturing polyacrylamide gels (Sambrook *et al*. 1989), and sized by comparison with an M13 mp18 DNA standard.

Table 1 Description of seven microsatellite loci for *Phalacrocorax carbo*. Primer sequences are given in 5′–3′ orientation together with respective clone fragment length, optimal PCR annealing temperature, array length/type, number of alleles resolved, and observed heterozygosity (direct count). The number of alleles resolved in cross-species amplification is also given, where: species 1 = *P. aristotelis*; species 2 = *P. auritus*; species 3 = *P. atriceps*; and species 4 = *P. c. novahollandiae*. The clone sequences from which the primers were designed have EMBL accession numbers Y12766 to Y12771

Primer	Sequence (5′–3′)	Length (bp)	Temp (°C)	Repeat array	No. of alleles	H_O	Species 1 (n = 20)	Species 2 (n = 20)	Species 3 (n = 5)	Species 4 (n = 8)
PcD 2F	GATGGAACTGAATAAAAGTTGG	170	55	$(GA)_{20}$	16	0.61	8	4	4	4
PcD 2R	TTATGCAGAACTGAATTTTCC									
PcD 4f	CAGAGGTTTTCAGCACACG	138	52	$(GT)_{14}$	10	0.62	0	0	0	5
PcD 4R	GAGTGTGTATTTGAACCTGTTGC									
PcD 5F	CCACTATTCTACACTCACTTCG	196	52	$(AC)_{20}$	22	0.77	2	2	2	4
PcD 5R	GAACTGTTAGCAAATAAATCCTG									
PcD 6F	GCACACACATAGAATGACCAG	165	52	$(CA)_{14}$	9	0.51	2	2	3	4
PcD 6R	AGCAAGAGGTTAACTCAGC									
PcT 1F	CCTCGTAAACTCCTAAATCTGG	296	55	$(CTTT)_{19}$	44	0.84	6	9	3	4
PcT 1R	GAAGCCTAGAAACTCACAAGC									
PcT 3F	CTTCCTGCTATGTCTATGCTTG	237	55	$(GATA)_{28}$	49	0.90	7	12	5	8
PcT 3R	ACAGCAAACAGCATCTATTCC									
PcT 4F	GGAGTCAGAGAACAACACAACC	197	55	$(GATA)_{25}$	44	0.89	11	10	6	9
PcT 4R	CAGCAGAGCGAGTTCTTTTAAC									

Approximately 1% of clones contained microsatellite arrays. A previous unenriched cloning methodology (following the procedure of Piertney & Dallas 1997) had identified four dinucleotide microsatellite regions in 50 000 clones (0.008%), suggesting that the enrichment procedure described here had increased the microsatellite yield considerably. Thirteen clones were sequenced and, from these, primer pairs were designed to amplify seven different loci (see Table 1). Each locus proved to be extremely polymorphic, with a mean observed heterozygosity of 0.73 and a mean number of alleles per locus of 28. Alleles assorted according to Mendelian expectations with no evidence of linkage disequilibrium or a high incidence of null alleles. As such, these loci will be invaluable in the examination of colony integrity, dispersal, population structuring and gene flow.

To examine the suitability of these primers in other pelicaniform species, the primers were also tested on the double-crested cormorant (*P. auritus*), Antarctic blue-eyed shag (*P. atriceps*), European shag (*P. aristotelis*), and the black shag (*P. c. novahollandiae*). The same PCR conditions and electrophoretic methodology were used as previously described. All primer pairs except PcD4 gave a single PCR product of expected size that was invariably polymorphic (Table 1), suggesting that these primers could be useful for population studies in related species.

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