

Molecular Markers for use in Megapodes - unpublished report -

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Abstract

Several galliform microsatellite primers were tested for amplification in members of all six megapode genera. Mitochondrial D-loop and sex-linked primers were also tested for the first time in megapodes. Results show that chicken and Australian brush turkey microsatellite loci can be amplified in a number of megapode species. Further work is required to establish the level of polymorphism each of these loci displays in megapodes. Sequence differences within the D-loop sequence of *A. arfakianus* individuals were also detected. Amplification of the CHD locus in several megapodes reconfirms the belief that megapode gender is genetically determined.

Introduction

Megapodes are an unusual group of Galliformes that use environmental sources of heat to incubate their eggs. There are 22 species of megapode, nine of which are listed as endangered by IUCN (Dekker *et al.*, 2000). Megapodes, such the Maleo, are threatened by destruction of their communal nesting grounds and populations may become genetically isolated (Baker & Butchart, 2000). In order to understand the population structure of megapodes, molecular methods are needed. A microsatellite library has been designed for the Australian Brush Turkey, but no molecular markers have been developed for any of the endangered megapodes. The production of microsatellite libraries for specific species can be very costly and time consuming. An alternative to preparation of new libraries, is the screening of markers developed for other species in related taxa. The objective of this project was to screen microsatellite primers designed for chicken and the Australian Brush Turkey for use in megapodes. In order to determine whether polymorphisms are detectable in these loci several individuals of each species are required. Recent changes in regulations governing the export of genetic material from countries, such as Indonesia, where the majority of megapodes exist, has prevented me from examining the extent of polymorphism within each locus amplified. Thus, only data on amplification of megapode microsatellite loci are reported here. The utility of the mitochondrial D-loop (Randi & Lucchini 1998) and CHD-W genes (Griffiths *et al.* 1998) as molecular markers for use in megapodes is also described.

Materials and Methods

Genomic DNA was provided by Dr S. Birks (Burke Museum, USA) for *A. arfakianus* (Aa1); *T. fuscirostris* (Tf1); *M. decollatus* (Md1); *M. emerita* (Me1/Me2); *M. layardi* (Ml1); *M. tenimberensis* (Mt1); *M. reinwardt* (Mr1); *M. pritchardii* (Mp1); *M. cumingi* (Mc1) and *Eulipoa wallacei* (Ew1/Ew2). DNA was also extracted from muscle tissue provided by AMNH, USA for *M. maleo* (Mm1) and *M. freycinet* (Mf1),

from eggshell membranes, collected at *M. maleo* nesting grounds at Tambun (Mm2, 4, 5, 6) and Muara Pusian (Mm3, 7, 8), and *Gallus gallus* (Gg1) from a commercial hatchery and from *A. arfakianus* (Aa2) feathers provided by Amsterdam Zoo and from *A. lathami* (A11) feathers provided by Ann Göth.

DNA Extraction

Tissue: tissue preserved in alcohol was blotted on sterile paper and a 2mm piece of tissue was incubated in 0.5 ml STE (100 mM NaCl; 10 mM Tris; 1 mM EDTA, pH 8.0) for 1 hour at room temperature and then washed twice with fresh aliquots of STE. *Egg membranes*: 1cm² samples were excised from membranes stored in DMSO storage buffer and washed twice in 0.5 ml STE. Dry egg membranes were cut into 10 x 1 mm² and washed in 0.5 ml STE for 10 mins. *Feathers*: the quills of three feathers were dipped in 70 % alcohol, then in STE for 10 minutes. The samples were then cut into <1 mm pieces.

The samples were briefly centrifuged and the STE removed carefully by pipette and replaced with 800 μ l Chelex 100® (5 % in water). Proteinase K was added to a final concentration of 125 g/ml and incubated @ 55 °C for 100 minutes. The Protease was denatured by incubation at 95 °C for 10 minutes and samples were vortexed for 1 minute and centrifuged for 1 minute at full speed. A 400 μ l aliquot of the supernatant was transferred to a fresh tube containing 400 μ l Chelex 100® (5 %) and re-spun for 1 minute. A 2 μ l sample of the supernatant was used for amplification. A further 400 μ l sample was precipitated with in 0.3 M sodium acetate (pH 5.3) and 1.8 volumes ethanol and resuspended in 50 μ l Tris 10 mM for long term storage at -20 °C.

PCR of Microsatellite Loci

DNA samples were amplified in 10 μ l aliquots containing 1 μ M primers, 125 mM δ NTPs and 0.5 units of Red Hot Taq. Primer sequences, their annealing temperatures and Mg⁺⁺ concentrations are given in Table 1. Cycling parameters were: 3 minutes initial denaturation at 95 °C, 30 seconds at 94 °C, 30 seconds at the annealing temperature and 30 seconds at 72 °C over 40 cycles followed by a 5 minute extension at 72 °C. Aliquots of PCR amplicons were loaded onto a 2% agarose gel in 0.5x TBE stained with EtBr and bands were photographed on a UV transilluminator.

Measurement of amplicon fragment lengths

PCR amplicons labelled with Hex were loaded onto an ABI lightcycler against a known marker by Dr Neil Leat in the Department of Biotechnology, University of the Western Cape.

PCR of mitochondrial and scn markers

The CHD-W locus was amplified from A11, Aa1, Md1 and Mr1 using primers P2 and P8 (1 μ M), 200 μ M dNTPs and 2.5mM MgCl₂ catalysed by Red Hot Taq polymerase following Griffiths *et al.*, 1998. Amplification products were electrophoresed on a 3% agarose gel in 0.5X TBE and on 9% acrylamide (39:1) in 1x TAE.

The d-loop of the mitochondrial genome was amplified from Aa1 and Aa2 to determine within species sequence variation using primers PHPH/PHDL, following Randi & Lucchini, 1998. Amplicons were purified by GFX PCR clean up kit (Amersham) and sequenced using the PHDL primer.

Results & Discussion

DNA was extracted and amplified from all megapode samples available. Table 2 details the samples for which DNA was amplified and by which method it was successfully extracted.

Microsatellite cross-species amplification

Microsatellite primers designed on the basis of chicken and Australian brush turkey sequences amplified other megapode DNA with varying degrees of success (see Table 3). LEI160 produced clear amplicons for all species. Length polymorphisms were detected between genera, but there were no observed length polymorphisms within the *Megapodius* species (Md, Mf and Me) or within *Eulipoa* (Ew), see Table 4. Chicken locus LEI0096 was amplified in several species of *Megapodius*, whilst LEI0065 and LEI0085 were amplified in several different megapode genera.

The majority of Australian Brush Turkey primers tested amplified Wattled brush turkey (Aa) and Black-billed Talegalla (Tf) DNA. This is not surprising as they are the most closely related species tested. Locus BrT01 and BrT12 were also amplified successfully in a number of *Megapodius* species.

DNA from *M. decollatus* amplified well with chicken and brush turkey primers, although classical taxonomy (Jones *et al.*, 1995), previous sequencing studies (Birks *et al.*, 2002) and length polymorphisms of the LEI160 fragment suggest that it is closely related to the other *Megapodius* species and not to Brush turkeys. These amplifications may have been non-specific due to high template concentration, but dilutions of 10 and fold did not reduce amplification efficiency.

In the absence of data on polymorphism of these loci within species it is impossible to make conclusions as to which microsatellite loci are most useful for megapodes. Further investigation into the use of BrT12 and LEI0070 is recommended.

Sex determination in Megapodes

DNA from A11, A12, Md1 and Mr1 was successfully amplified using CHD-W sex determination primers. Size polymorphism between the ZW (female) and ZZ (male) amplicons was very small and could not be detected on an agarose gel. Separation on a 9% acrylamide for 5 hours at 45 amps in 1x TAE gel did clearly resolve two distinct bands in DNA samples known to be extracted from female megapodes. This confirms that although incubation temperature may have an effect on the sex ratio of megapode chicks (Goth & Booth, in press), gender is genetically determined in megapodes in the same way as other bird species.

D-loop polymorphism in Aepypodius arfakianus

159 bases of unambiguous d-loop sequence of Aa1 and Aa2 were compared. There were three polymorphisms in this partial sequence, suggesting that d-loop sequences may be a useful marker in population studies of megapodes. Further work is required. The partial d-loop sequences of *A. aepypodius* 1 and 2 were submitted to the Genbank with Acc. No: AY789454 and AY789455 respectively.

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References

- Baker, GC & Butchart, SHMB (2000) Threats to maleo and solutions for their conservation. *Oryx*. 34:255-261.
- Birks, S & Edwards, S (2002) A phylogeny of the megapodes (Aves: Megapodiidae) based on nuclear and mitochondrial DNA sequences. *Phylogenetics and Evolution* 23: 408-421
- Dekker, RWRJ & Baker, GC (eds) *Megapodes – Status Survey and Conservation Action Plan 2005-2009*. WPA/Birdlife/SSC Megapode Specialist Group, IUCN, Switzerland.
- Griffiths, R, Double, MC, Orr, K and Dawson, RJ (1998) A DNA test to sex most birds *Molecular Ecology* 7: 1071-1075
- Jones, DN, Dekker, RWRJ & Roselaar, CS (1995) *The Megapodes*. Oxford University Press, UK.
- Randi, E & Lucchini, L (1998) Organisation and evolution of the mitochondrial DNA control region in the avian genus *Alectoris*. *J. Molecular Evolution* 47:449-462

Table 1: Microsatellite loci primers tested

primer	Repeat motif	Primer sequences (5' – 3')	Ta ° used	[Mg ⁺⁺]
BrT 01 AJ347718	(TG) ₄ CAAG(TG) ₆	F=ACGCACACCTACACACTGTAC R=TCCAACGTTTATGATTCTCTGA	55	1.5
BrT 02 AJ347719	(GT) ₁₆	F=TCTTTTTTGTATGTATGAGCGTG R=AGTTTTTTCGAAATGGTGTTAGT	55	1.5
BrT 04 AJ347721	(CT) ₉	F=GGTGAGGAACACTGAGTATCTT R=CAGTCTCATGAAGAAGGAAGAT	55	1.5
BrT 08 AJ347723	(TTCC) ₂₅ & (TC) ₇ & (T/C) rich regions	F=AGGCATATCAATTTCAAAGCA R=TGATACCATGCAATAACAGACA	55	1.5
BrT 11 AJ347726	(CA) ₁₁	F=CCATTATGAATGGACTTAAAATGAGTTAG R=CAGCGACCTGAATCAGACAG	55	1.5
BrT 12 AJ347727	(CA) ₁₁	F=GATCTTCAATAGCTAGATATCCATCAG R=AATGTTGCATACCTTTGGCTG	55	1.5
LEI160 X85523	(VG) ₁₂ (AG) ₁₃	F=GCAGACAGCCGTTAATATATGCG R=AACCAAAACACAAGCTCTTGCA	63	1.5
LEI70 X82869	(AC) ₁₇ AT(AC) ₄ (G C) ₄	F=GCGGAGAGCAATTAGTCTGCAA R=CGGCTCGGGAAAACAATCAC	63	1.5
LEI31 X83980	(GT) ₁₀ TT(GT) ₃	F=CTATGGGCAGTGGTGGAGAA R=CAATGGAGGGCACTGTTAGA	63	1.5
LEI65 X82812	(AC) _{21.5}	F=TGAAACATGTATGGAGTCTCAGCA R=GACAGCTAAATGCCAGTTCATGG	62	2.0
LEI85 X82800	(AC) _{14.5}	F=GGATGAAGTGCCACCATCAGG F=ATGCGTGCTTAGAGGCCAGTG	58	2.5
LEI96 X83257	(AC) ₈ GC(AC) ₅ (AT) _{4.5}	F=CTACAAATGGGTGAAGTTTCCTCG R=TCCAAAGTGAGAGCTGCAAGG	63	2.0
LEI100 X82859	(AC) ₁₃	F=TTGTGAAGACAGGCAGATGC R=GCCTGGTATTATTTCCCTCTGTC	60	2.0
LEI154 X85515	(CA) ₇ CG(CA) ₄ TA(CA) _{6.5}	F=AGGCACCGAGCTACGTCTGG R=ACCTCCCAGCAGCCACAGTC	63	2.0

Table 2: DNA samples from which PCR amplicons could be obtained.

Code	Tissue Supplied	DNA Extraction	Specimen from:
Aa1	DNA	S. Birks (use 1/10)	<i>Aepyodius arfakianus</i> , MV E147, Burke Museum *
Aa2	Feathers	GCB chelex 18/12/03(use neat or EtOH use 1/10)	<i>Aepyodius arfakianus</i> , B02098, M van Hees, A'dam Zoo
Tf1	DNA	S. Birks	<i>Talegalla fuscirostris</i> , MV E651, Burke Museum *
Mm1	Tissue	GCB chelex 27/10/03 (use neat or EtOH use 1/2)	<i>M. maleo</i> PRS 1204, Paul Sweet – AMNH
Mm2	Eggshell membrane	GCB chelex 09/12/03	<i>M. maleo</i> Membrane 4 Tambun – M. Christy 30 days
Mm3	Eggshell membrane	GCB chelex 09/12/03	<i>M. maleo</i> Membrane 5a Muara Pusian – M. Christy 10 days
Mm4	Eggshell membrane	GCB chelex 06/01/04	<i>M. maleo</i> Membrane 1 Tambun – M. Christy 2-3 days
Mm5	Eggshell membrane	GCB chelex 06/01/04	<i>M. maleo</i> Membrane 2 Tambun – M. Christy 10 days
Mm6	Eggshell membrane	GCB chelex 06/01/04	<i>M. maleo</i> Membrane 3 Tambun – M. Christy 20 days
Mm7	Eggshell membrane	GCB chelex 06/01/04	<i>M. maleo</i> Membrane NN Muara Pusian – M. Christy
Mm8	Eggshell membrane	GCB chelex 06/01/04	<i>M. maleo</i> Membrane X1 Muara Pusian – M. Christy
Md1	DNA	S. Birks	<i>Megapodius decollatus</i> , MDS, Burke Museum
Mf1	Tissue	GCB chelex 27/10/03 (use neat or EtOH use 1/2)	<i>Megapodius freycinet</i> MKL67, Paul Sweet, AMNH
Me1	DNA	S. Birks	<i>Megapodius emerita</i> , AMNH MKL 67, Burke Museum
Me2	DNA	S. Birks	<i>Megapodius emerita</i> , MV KNG 16, Burke Museum *
Mc1	DNA	S. Birks	<i>Megapodius cumingii</i> (Birks MCM) R Sinclair, Burke Mus
Ml1	DNA	S. Birks	<i>Megapodius layardi</i> (Birks MLD) Sharon Birks, Burke Mus.
Mp1	DNA	S. Birks	<i>Megapodius pritchardii</i> (Birks MPI) E. Curio, Burke Mus.
Mr1	DNA	S. Birks	<i>Megapodius reinwardt</i> (Birks MCM) MV C561, Burke Mus.
Mt1	DNA	S. Birks	<i>Megapodius tenimberensis</i> (Birks MTM) Burke Museum
Ew1	DNA	S. Birks	<i>Eulipoa wallacei</i> , RM 9997-00262, Burke Museum
Ew2	DNA	S. Birks (use 1/10)	<i>Eulipoa wallacei</i> , EUL3, Burke Museum
Gg1	Eggshell membrane	KG chelex	<i>Gallus gallus</i> membrane from Country Fair Farm, SA

- samples originally supplied by Les Christidis, Victoria Museum, Australia.

Table 3: Positive amplification of megapode DNA samples using chicken and Australian brush turkey microsatellite markers

Primer	Aa1	Tf1	Mm	Md1	Mf1	Me1	Mp	Mt	MI	Mc	Mr	Ew1	Gg1	Ann. temp	Mg+
BrT01	+++	+++	+	-	-	+	++	-	++	++	-	-	++	55	1.5
BrT02	+++	++	-	+++	-	+	+++	-	-	-	-	+	-	55	1.5
BrT04	+++	+++	+	-	-	-	-	-	-	-	-	-	-	55	1.5
BrT08	++	-	+	+++	+	-	-	-	-	-	-	-	-	55	1.5
BrT11	+++	+++	++	++	-	-	-	-	-	-	-	+	-	55	1.5
BrT12	-	+++	+++	+++	-	-	-	+++	+++	+++	+++	+++	-	55	1.5
LEI96	++	-	-	+++	++	-	+++	+	+++	+++	+	-	+++	63	2.0
LEI100	-	-	+	-	+	-	-	-	-	-	-	-	+++	60	2.0
LEI54	+	-	-	-	-	+	+	-	-	-	++	-	+++	63	2.0
LEI65	+++	++	++	+	+	+++	-	-	-	+++	+	+	++	62	2.0
LEI85	+	-	+	+++	+	+	+	+++	-	+	+++	-	+	58	2.5
LEI160	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	63	1.5
LEI70	+++	-	+++	+++	-	+++	-	-	+++	-	+++	-	+++	63	1.5
LEI31	++	-	-	++	-	-	-	-	-	-	-	-	++	63	1.5

+++ good amplification; sub-standard amplification; poor amplification +/- multiple bands; - no amplification.

Table 4: Fragment sizes of megapode DNA amplified with LEI160-Hex

DNA SAMPLE	FRAGMENT 1	FRAGMENT 2
Gg1	157.00	179.37
Tf	136.55	157.05
Mm1	136.64	156.73
Mm3	137.70	157.11
Md1	139.00	160.00
Me1	139.00	160.00
Me2	139.00	160.00
Mf	139.00	160.00
Ew1	142.48	163.64
Ew2	142.46	162.63