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Establishment of microsatellite markers to assess the mating system of the fan-tailed gerygone (*Gerygone flavolateralis*) for studying cuckoo–host arms race

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We developed a set of microsatellite markers for the fan-tailed gerygone (*Gerygone flavolateralis*), host of a brood parasite, the shining bronze-cuckoo (*Chalcites lucidus*). We used 454 pyro-sequencing to establish 17 polymorphic microsatellite markers. The number of alleles per locus ranged from 6 to 18, the expected heterozygosity from 0.328 to 0.931 and the polymorphism information content from 0.306 to 0.916. The developed set of microsatellites will allow us to determine the gerygone's mating system, which is crucial to understand inheritance of dichromatism in skin colour of their chicks.

Introduction

Coevolution of avian brood parasites and their hosts is extensively studied (Feeney *et al*. 2014). Lotem (1993) theoretically excluded the possibility that the arms race between avian brood parasites and their hosts would extend to the nestling stage, but recent studies showed that some host species have evolved the ability to discriminate against or eject parasite nestlings (Langmore *et al*. 2003, Sato *et al*. 2010, Tokue & Ueda 2010), which resulted in the evolution of chick mimicry in some species of cuckoos (Sato *et al*. 2010, Langmore *et al*. 2011). The endemic New Caledonian subspecies of the fan-tailed gerygone (*Gerygone flavolateralis flavolateralis*) is the exclusive host of the native subspecies of the shining bronze-cuckoo (*Chalcites lucidus layardi*). Nestlings of the fan-tailed gerygone are dimorphic in skin colour, they occur in a bright and a dark form (Sato *et al*. 2015), which has not previously been found in any other host species. The chick dichromatism might have evolved as a defence against brood parasites, in which case

a mismatching skin colour should be the cue for ejection of cuckoo chicks by the host. This would help host parents in recognising brood parasites if their own chicks always have the same skin colour. Skin colour might be inherited by both parents (e.g. Mendelian inheritance), by the female (e.g. maternal effects, epigenetic effects), or by the male (in this case, extrapair copulation could lead to different skin colour of chicks). Therefore, mixed broods (chicks with dark and bright colour in the same nest), which we observed, could be caused by Mendelian inheritance but also by extrapair copulations.

However, to provide insight into the genetic basis of skin pigmentation, we first need to uncover the mating system of the fan-tailed gerygone. Microsatellite markers would allow us to determine parentage of each chick, and then to assess which parent contributes to inheriting the skin pigmentation. In this paper, we report the identification and characterisation of microsatellite loci for the fan-tailed gerygone, developed using 454 pyro-sequencing. Knowledge of the mating system will allow us to identify candidate genes for pigmentation and further explore the genetic mechanisms underlying skin colour polymorphism.

Methods

We collected 44 blood samples and 6 tissue samples (extracted from dead chicks) from wild fan-tailed gerygones on mainland New Caledonia during three breeding seasons in October–December of 2011–2013. The samples came from three areas: 42 from the main study area, the Parc des Grandes Fougères and close surroundings (21.6°S, 165.8°E), 3 from approximately 40 km west (21.6°S, 165.4°E), and 5 from approximately 130 km northwest (20.7°S, 165.0°E) of the main study area. We stored the samples at 4 °C in 95% ethanol (except for 9 blood samples stored in Queensbuffer). We isolated DNA from blood samples using the NucleoSpin Quick Pure Blood Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. To improve the efficiency of DNA isolation from dried blood, we incubated the dried blood samples with Proteinase K and buffer during two hours at 50 °C (with occasional whirling) before DNA isolation. For tissue samples, we followed the procedure by Hogan *et al.* (1994). To establish microsatellite markers, we sequenced DNA isolated from tissue of four individuals with a GS-FLX LAB (Eurofins MWG Operon, Ebersberg, Germany). The 454 pyrosequencing generated 92 234 reads (59.38 Mb) with an average length of 609 bp.

For further analysis, we used clipped sequences, following the recommendations by Eurofins. Quality control showed no need for trimming. We screened the FASTA file containing reads of each individual for dinucleotides (minimum 6 repeats), trinucleotides (minimum 4 repeats) and tetranucleotides (minimum 4 repeats) using MSATCOMMANDER (Faircloth 2008) and PRIMER3 (Rozen & Skaletsky 2000) with default settings implemented in MSATCOMMANDER. We screened all possible microsatellites markers in ACCESS for the same repeats (the same designed primers). We selected potential primers as described by Opgenoorth (2009) (e.g. excluding extremely long loci, Short Tandem Repeat (STR) stretch too close to the vector). Out of 1379 potential STR markers (484 dinucletides, 680 trinucletides, 211 tetranucleotides), we randomly ordered 40 primer pairs (17 dinucleotides, 15 trinucletides, 8 tetranucletides) from Biomers (Ulm, Germany). To make the design more cost effective, we labelled primers with a universal M13 Primer (according to Schuelke 2000). We tested primer pairs Gego001–Gego040 with gradient polymerase chain reaction (PCR) protocol (50–60 °C) to check optimal annealing temperature for each primer. We ran PCRs in a total volume of 15 μl with the following components: 25 ng of genomic DNA, 0.2 μmol of each reverse primer, and the M13 universal primer (fluorescently labelled with 6-FAM), 0.05 μmol of each forward primer, 0.2 mmol each dNTP (Solis BioDyne, Tartu, Estonia), 2 mmol $MgCl_2$, 1× PCR buffer (Solis BioDyne), and 0.5 U Taq DNA Polymerase (FIREPol, Solis BioDyne). For all PCRs, we used a thermal cycler and checked for contamination with negative controls (Mastercycler Gradient, Eppendorf, Germany).

In case of successful amplification, we tested polymorphism of the primers by PCR, with 10

DNA samples isolated from tissue (6 samples) or blood (4 samples). We used the following PCR profile: initial denaturation at 94 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at the primer annealing temperature 58 °C, 30 s at 72 °C, followed by eight cycles of 30 s at 94 °C, 45 s at 53 °C, 45 s at 72 °C for M13 annealing, and a final elongation step at 72 °C for 10 min. Finally, we combined 17 functional primers into three multiplex PCR systems (*see* Table 1 for details). For all 50 individuals, we ran PCRs in a total volume of 15 μl in reaction with Qiagen Multliplex PCR Kit (Qiagen, Mississauga, Canada) following the manufacturer's recommendations: each 10 μl of multiplex PCR contained 15–40 ng of DNA, 5 μl of the 2× Qiagen Multiplex PCR Master Mix and 1 μl of the primers mix. We used the following multiplex PCR profile: initial denaturation at 94 °C for 15 min, 35 cycles of 30 s at 94 °C, 90 s at the primer annealing temperature 58 °C, 60 s at 72 °C, and a final elongation step at 60 °C for 30 min. Afterwards, we separated PCR products on 6% polyacrylamide gels on an ABI Prism 377 automated sequencer (Perkin Elmer, Forster City, USA) and scored in reference to a ROX standard (79–362 bp) by GENESCAN 3.1.2 and GENOTYPER 2.5 software (Applied Biosystems, Foster City, CA, USA). To ensure consistent scoring, we included a reference individual with known genotypes on each genotyping run. We genotyped all samples three times to detect and correct genotyping errors.

We used GENEPOP 4.0 (Rousset 2008) to calculate the number of alleles, allele frequencies, expected (H_e) and observed (H_o) heterozygosity, linkage disequilibrium, and deviation from the Hardy-Weinberg equilibrium. We used MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004) to detect genotyping errors and to test for the presence of null alleles. To calculate the polymorphism information content (PIC), we used the EXCEL MICROSATELLITE Toolkit (Park 2001).

Results and conclusion

Out of 40 potential microsatellites tested initially on 10 individuals, we chose 17 (polymorphic, no scatter bands and well-shaped peaks) for further analysis on 50 individuals from different geographic regions. The chosen polymorphic loci all had at least six alleles, and the average number of alleles per locus was 10 (Table 1). The H_o values ranged from 0.250 to 0.851, and those of H_e from 0.328 to 0.931. Following the classification of Botstein *et al.* (1980), 16 microsatellites were highly informative (PIC > 0.5) and one reasonably informative (0.5 > PIC > 0.25) (Table 1). In all 50 individuals together, we detected 177 alleles across 17 loci. MICRO-CHECKER did not detect any genotyping errors or null alleles. After a Bonferroni correction ($p < 0.0029$), we detected linkage between 3 of 136 compared pairs of loci, and 9 loci significantly deviated from the Hardy-Weinberg equilibrium. It is unlikely that the linkage between some markers was due to these markers being located in proximity on the same chromosomes, because we chose the microsatellite markers randomly from the pyrosequencing data set. Linkage disequilibrium and deviation from the Hardy-Weinberg equilibrium rather point at a population structure in the 50 tested individuals. The population structure could be caused by geographic distance or acclimatisation/adaptation to different habitat types as the fan-tailed gerygone uses a variety of habitat types from open grassland to rainforest. We conclude that the established highly polymorphic microsatellite markers are suitable to determine the mating system of the fan-tailed gerygone, which is an important step to study skin colour inheritance in this unique cuckoo–host system.

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Table 1. Characteristics of 17 microsatellite loci of the fan-tailed gerygone (*Gerygone flavolateralis*): Locus designation, GenBank accession number, repeat motif, primer sequence, multiplex group number, number of observed alleles (N_a), range of allele length, polymorphism information content (PIC) values, level of observed heterozygosity per locus (H_o), and expected heterozygosity per locus (H_e).

Locus	GenBank	Repeat motif	Primer sequence (5´–3´)	Multiplex group	N_a	Allele length (bp)	PIC	H_o	H_e
Gego002	KP869849	ATCC	F: GATCCCACGACAGAGCTACA R: TAGTGTCTTCCAGCAGCTCC	1	11	223–267	0.786	0.5652	0.8187
Gego003	KP869850	ATC	F: AGCAGAATGAAATAGCAAAAGGT R: GCCCAGTTAAGGACAAATGCT	2	9	251–281	0.805	0.7609	0.8349
Gego008	KP869851	AGAT	F: TGTCACAGGGTCTCTTCTAAGG R: AACCCCTTCCATCCTAGGTG	3	15	146–218	0.829	0.7391	0.8555
Gego011	KP869852	AC	F: GGGAAAAGAGGCACAGCTCT R: CTATTTGGGGAGGCTTGGGT	3	9	109–131	0.520	0.4694	0.5929
Gego012	KP869853	GT	F: CTCTGGAAGAAGGCCTGTCT R: GCCCTGCATATCCTTTGCTG	2	11	155–175	0.752	0.5417	0.7842
Gego014	KP869854	ATCT	F: GTGTTCTAGAGCTCCAAGGGT R: GCATGGCAAGTTTCACATCAG	1	8	145–181	0.733	0.6531	0.7734
Gego016	KP869855	ATCT	F: GCCAAGTGACATTGGAGCAA R: ACAAGGGAAGGGTCATCTGA	2	10	178–214	0.835	0.8511	0.8609
Gego017	KP869856	AAC	F: CCTCCTGCAGCCCTAACTTA R: TGAAGGCAAGTCTTTAGTGTGT	1	10	247–280	0.562	0.2766	0.5987
Gego019	KP869857	GT	F: AGGAAATGAGGTGGGGTCTG R: GGAGCCAACTGACAAGGAAA	3	11	233–255	0.717	0.5116	0.7466
Gego021	KP869858	GT	F: CATTGAAACCCTCCCCTTGC R: TCCCAATCCAGTTTTACATGTGG	2	8	228–242	0.650	0.4783	0.6861
Gego023	KP869859	AGAT	F: GGACAGACGGATGGATGGAT R: CGGTGAGGTTCCCAGATGAA	2	12	152–196	0.843	0.7551	0.8660
Gego029	KP869860	GAT	F: CACCTGACCTGACAAGTCCT R: TGGTCCTTGTCTGTGGTGAG	1	12	142–178	0.833	0.6000	0.8570
Gego032	KP869861	ATCT	F: ATATCCACCGCCAATGCCTA R: CCCATGCTCAAACCACAACA	3	8	180–208	0.745	0.7273	0.7863
Gego033	KP869862	ATC	F: GTACGAGTCCCTCACCTCTG R: CCCTGTAACTACCCAGCCAT	1	9	192–216	0.560	0.5000	0.5879
Gego034	KP869863	ATCC	F: CACGTTCATCTTGGCAGCTT R: CTGGCAAGTGGATGGATGGA	3	10	215–251	0.856	0.5238	0.8801
Gego035	KP869864	AC	F: CAGGACAGCTTCACAATAGGC R: AATCTGGGGTTGCTGGGTTT	2	6	157–171	0.306	0.2500	0.3285
Gego038	KP869865	AC	F: ATCCGCACTAACTCTCCAGC R: GCTTTCATCGCTGACCAGAC	1	18	164–208	0.916	0.6170	0.9311

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