New genetic resources and a preliminary multi-locus assessment of species
boundaries in the *Batis capensis* species complex (Passeriformes: Platystoridae)

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Abstract

Species boundaries within the African Batises, small forest and savanna dwelling insectivorous birds, remain a challenge given complex distributions of plumage, call, and eye-color phenotypes throughout their distributional range. The relatively recent delineation of two species within *Batis mixta*: *B. mixta* and *B. crypta* based on morphological and mitochondrial sequence data, suggests that further evaluation of taxonomic limits is needed to fully enumerate the lineage diversity of these birds. Towards that end, we isolated, developed, and characterized new nuclear microsatellite loci to help provide clarification of species boundaries within the *Batis capensis* species group, and performed preliminary assessment of genetic structure for *B. crypta*, *B. mixta*, and *B. capensis*. We found that the new markers were highly polymorphic, and PCR-amplified across species. Using these markers we recovered three well-defined clusters of individuals, providing nuclear evidence that supports the distinctiveness of the three taxa. This suggests that these markers will have utility for phylogeographic and population genetic analyses for batises, a diverse radiation of African corvoid birds.

1 1.1 Introduction

2 Batises (*Batis* sp.) are small insectivorous birds, primarily restricted to forest and
3 savanna habitats across Africa. Individuals can be challenging to diagnose to species
4 due to plumage variation that is often subtle across many species, as well as
5 complex geographic variation within and between species in eye-color and song
6 (Erard & Fry, 1997; Louette, 2006). These features, together with overlapping or
7 parapatric distributional ranges, gaps in sampling, and a general lack of comparative
8 material for assessments of species diagnoses, highlight the need for continued
9 assessment of species boundaries among members of the genus *Batis*. For example,
10 in 2006, a new member of the Cape Batis (*B. capensis* Linnaeus 1766) species
11 complex, *Batis crypta* (Fjeldså, Bowie, Kiure, 2006), was described. The ability to
12 describe this new species was a direct consequence of material derived from new
13 collection efforts, that enabled the delineation of this species from another (*B. mixta*,
14 Shelley 1889) using both morphological and molecular evidence (mitochondrial
15 DNA). Prior to these results and the description of *Batis crypta*, *B. mixta* was
16 presumed to have had a much broader distribution (Figure 1).

17
18 Both *B. mixta* and *B. crypta* are patchily distributed among the sky island-like mosaic
19 of montane forest fragments in East Africa (Figure 1). Although the two taxa are
20 reciprocally monophyletic in mitochondrial DNA (Fjeldså et al., 2006) the
21 biogeographic pattern recovered, with individuals sampled from the Nguru
22 Mountains grouping with those from the northern Eastern Arc Mountains, is
23 unusual for Eastern Arc birds (Bowie et al., 2004; Kahindo et al., 2007; Fjeldså and

Bowie, 2008). Hence, additional analyses are warranted to determine if there is recurrent nuclear gene flow between these taxa (Figure 1). More broadly, the development of polymorphic nuclear DNA markers would greatly facilitate the determination of phylogeographic structure and taxonomic boundaries among *Batis* species and subspecies.

Here we isolate and develop microsatellite loci for *B. crypta*. We next evaluate and characterize these loci for a test panel of *B. crypta*, and then test if these loci can be PCR-amplified across two additional *Batis* species; the very closely related *B. mixta*, and the more distantly related species *B. capensis*. These data are used to make a preliminary assessment of genetic structure across these three species to demonstrate the utility of these new genetic markers and determine if the mtDNA break between *B. crypta* and *B. mixta* (Fjeldså *et al.*, 2006) is also reflected in the nuclear genome.

1.2 Methods

To develop and analyze microsatellites for *B. crypta*, we used the same methods and analyses as in Wogan *et al.* (2015a; 2015b) and Glenn and Schable (2005). We extracted genomic DNA using the DNeasy kit (Qiagen, USA). DNA from one individual was digested with the restriction enzyme *Rsa*I and *Xmn*I before SuperSNX24 linkers were ligated onto the fragments. We then hybridized the fragments to four biotin-labeled tetranucleotide probes [(ACAG)₈; (AAGT)₈; (AGAT)₈; (ACAT)₈] to form a dimer, which was attached to streptavidin-coated magnetic beads (Dynabeads M-270, Invitrogen) and

washed twice with 2X SSC, 0.1% SDS and four times with 1X SSC, 0.1% SDS at 52 °C before ethanol precipitation. We sequenced a total of 81 colonies, and selected colonies that contained a minimum of eight repeats for primer design. Primers were designed using Websat (Martins et al., 2009) which integrates Primer3 (Rozen and Skaletsky, 2000). The forward primer was 5'tagged with either a HEX or FAM flourophore.

To test the efficacies of our selected microsatellites, we used a panel of 16 individuals of *B. crypta* from a single population from the Misuku Hills of northern Malawi. We first ran a series of gradient PCRs with annealing temperatures ranging from 54-64 °C, and from this optimized the PCR-thermocycling conditions. All PCR reactions were carried out in a 10 µl volume consisting of 1 µl diluted DNA (1:10 dilution), 0.12 µl of Taq polymerase (Invitrogen), 1 µl 10X buffer, 0.3 µl 50 mM MgCl₂, 0.6 µl 10 µg/ µl BSA, 0.25 µl 10 mM dNTPs, 0.6 µl 10 mM of each primer, and dH₂O. The thermocycling profile was 94 °C for 3 minutes followed by 30 cycles of 94 °C for 45 s, annealing temperature (Table 1) for 30 s, and 72 °C extension for 45 s, followed by a final extension at 72 °C for 30 minutes. Genotyping was performed using LIZ500 size standard on an AB3730. All samples were PCR-amplified and genotyped twice to ensure consistency across replicates. We used Genemapper v. 4.0 (Applied Biosystems, USA) to bin alleles, and all binning was carried out by the same person. We subsequently tested cross-species PCR-amplification for *B. mixta* (n = 11) from the Nguru Mountains of Tanzania, and also for *B. capensis* (n = 10) from the Mulanje Forest Reserve in southern Malawi to assess the utility of the markers across species boundaries. Genotypes were generated using the same PCR-amplification conditions as used for *B. crypta*.

1.2.1 Microsatellite Analyses

We assessed the presence of null alleles, the likelihood of scoring errors due to allele stutter, and the probability of large allele dropout using Microchecker (van Oosterhout et al., 2004). We calculated the number of alleles, the polymorphism information content (PIC: Botstein et al., 1980), and the expected and observed heterozygosity (with 1000 bootstrap replicates). We used the exact test (with 1000 replicates) to check for deviations from Hardy-Weinberg Equilibrium for each marker using the R packages PopGenKit (Paquette, 2013) and pegas (Paradis, 2010). We also tested for linkage disequilibrium among the microsatellite loci using the log-likelihood ratio statistic in Genepop v. 4.2.3 (Raymond and Rousset, 1995; Rousset, 2008). Analyses were repeated using *B. mixta* and *B. capensis*.

1.2.2 Preliminary assessment of population structure and species boundaries

To undertake a preliminary assessment of genetic structure, and to assess the utility of our microsatellite loci in distinguishing among *B. crypta*, *B. mixta*, and *B. capensis*, we used the program Structure (Pritchard et al., 2000). We used the admixture model to test values of k from 2-5 each with 20 replicates. All runs used a 50,000 step burnin followed by a 150,000 step MCMC chain. The optimal number of clusters was determined using the Evanno method (Evanno et al., 2005) as implemented in the program Structure Harvester (Earl & vonHoldt, 2012). For the selected k-value we then used CLUMPP v. 1.1.2 (Jakobsson & Rosenberg, 2007) to combine the replicate runs and Distruct v. 1.1 (Rosenberg, 2004) to generate a barplot of population assignment.

93

94 1.3 Results

95 We designed and tested primers for 14 microsatellite loci. Of these, nine loci PCR-
96 amplified consistently. The number of alleles for the loci ranged from 6 – 11 (Table
97 1). We found that the PIC content of most loci was high, indicating that the loci are
98 informative. Analyses of the *B. crypta* test panel with Microchecker revealed that,
99 based on an excess of homozygous genotypes, null alleles might be present in BMI
100 32 and BMI 71; these same two loci as well as BMI 41 were found to be out of HWE.
101 Furthermore, tests for linkage disequilibrium among loci revealed that in *B. crypta*
102 all but one pair of loci are independent: BMI 17 and BMI 96 were found to be in LD
103 based on a statistically significant likelihood ratio test (p-value = 0.015).

104

105 Cross-species PCR-amplification for all nine microsatellite loci was successful in *B.*
106 *mixta* and *B. capensis* (Table 2). For *B. mixta*, an excess of homozygous genotypes
107 was recovered for two loci (BMI 16, BMI 71). For *B. capensis*, an excess of
108 homozygous genotypes was recovered for four loci (BMI 16, BMI 17, BMI 22, BMI
109 71). The excess of homozygous genotypes may occur as a consequence of the
110 presence of null alleles, or because of inbreeding or selection. Application of
111 statistical corrections (Brookfield, 1996; van Oosterhout et al., 2004) may be
112 required when using these markers more broadly across the genus *Batis*, and will be
113 of particular importance when using these loci for phylogeographic studies (Chapuis
114 & Estoup, 2007). There was no evidence of large allele drop out or scoring error due
115 to allele stuttering for any of the loci in any of these species. There was no evidence

of LD among loci in *B. dimorpha*, although we did find evidence of LD among several loci in *B. mixta*.

We found that among the three species we genotyped, there were numerous private alleles for each locus. For most loci heterozygosity was high, although in a few instances, the observed heterozygosity was lower than the expected (e.g. BMI 71). Our analyses using the program Structure supported the presence of three clusters that correspond to the three sampled species (Figure 2). This suggests that the microsatellite loci are informative for distinguishing among both closely and more distantly related species within the genus *Batis*. Because we selected samples from single localities within each species, we did not expect to find any signals of intraspecific population structure. Our analyses support our expectations, speaking to the utility of the markers for phylogeographic studies.

1.4 Discussion

Our preliminary analyses of phylogeographic structure across members of the *B. capensis* species complex provide support from the nuclear genome for the genetic distinctiveness of *B. crypta* and *B. mixta* (Fjeldså et al., 2006). In our sampling we found no evidence for admixture; however, there are important intervening populations that are as yet unsampled genetically. Additional sampling across the respective ranges of each species will shed more light on the extent of connectivity among populations and species.

139 The microsatellite loci isolated from *B. crypta* (Table 1, 9 loci, 2-11 alleles) are
140 comparable in variability to those isolated from other African montane passerine
141 bird species (*Modulatrix stictigula* Ripley 1952, 10 loci, 8 - 10 alleles, Sellas et al.,
142 2008; *Phyllastrephus flavostriatus* Sharpe 1876, 10 loci, 8 - 13 alleles, Lokugalappatti
143 et al., 2008; *Cinnyris stuhlmanni* Reichenow 1893, 10 loci, 5 - 13 alleles, Bowie et al.,
144 2010) as well as from more widespread southern African passerine bird species
145 (*Tychaedon coryphaeus* Vieillot 1817, 15 loci, 3 - 13 alleles, Ribeiro et al., 2009;
146 *Zosterops virens* Sundevall 1850, 10 loci, 2 - 13 alleles, Oatley et al., 2013; *Cossypha*
147 *natalensis* Smith A., 4 - 12 alleles, Wogan et al., 2015a; *Cossypha caffra* Linnaeus
148 1771, 10 loci, 3 - 8 alleles, Wogan et al., 2015a; *Sigelus silens* Shaw 1809, 13 loci, 2 -
149 11 alleles, Wogan et al., 2015b).

150

151 The availability of variable nuclear DNA markers will be especially useful for the
152 investigation of population structure and species boundaries among *Batis* species in
153 the Eastern Arc Mountains of Tanzania and SE Kenya. The Eastern Arc Mountains
154 comprise a series of “sky islands” spanning c. 450 km. Mitochondrial data indicate
155 that *B. mixta* spans the largest geographic gap (100 km) of unsuitable habitat,
156 separating the Usambara Mountains from the Nguru Mountains (Fjeldså et al.,
157 2006). This pattern of geographical structure has not been recovered among other
158 Eastern Arc bird species where the Nguru Mountains tend to either contain their
159 own endemic lineage, or be connected with the more geographically proximate
160 Ukaguru and Rubeho Mountains (e.g. sunbirds, Bowie et al., 2004; thrushes, Bowie
161 et al., 2005; greenbuls, Fuchs et al., 2011).

162

163 In summary, our findings suggests that these loci will be of utility in assessing
164 cryptic species boundaries, as well as phylogeographic structure, in the Cape Batis
165 species complex, and should be of wider utility within the genus.

166

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 266 (*Sigelus silens*) for use in phylogeographic and landscape genetics research.
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268

269

Figure Legend

Figure 1. Map depicting the distribution of *Batis crypta* (blue), *B. mixta* (green), and *B. capensis* (red) in eastern Africa. Photo of a male *B. crypta* from the Udzungwa Mountains, Tanzania. Photo by Rauri C.K. Bowie.

Figure 2. Results from population structure analyses depicting the three clusters supported by the deltaK method. The three clusters reflect the three species evaluated in this study and confirm earlier mitochondrial and morphological results suggesting that the distribution range of *B. mixta* comprises two species: *B. mixta* and *B. crypta* (Fjeldså et al. 2006).

Table 1

Characterization of nine microsatellite loci developed for *Batis crypta*. The listed annealing temperature was used to PCR-amplify the locus for all three batis species. The size range is for *B. crypta* only. The PIC is a value that ranges from zero to one, with values closer to one having higher information content. Hardy-Weinberg Equilibrium (HWE) was assessed using the exact test. A significant value indicates that the locus is not in HWE for the *B. crypta* population analyzed.

Locus	Primer sequence 5'-3'	Repeat motif	Ta (°C)	N _A	Allelic Size Range (bp)	PIC	HWE (p-value)	Genbank Accession
BMI 16	F: HEX-TGTAGTGGCTTCATTTGTCCAG R: GTGTGTTGTTCATGGAGGTGTC	(TATC) ₁₁	58	6	357 - 373	0.626	0.347	KU376476
BMI 17	F: FAM- ACCCCTTATTGTTGGAGGTTG R: TCTTTTCACGTCTCCTATGGTG	(TATC) ₁₁	58	6	151 - 187	0.772	0.300	KU376477
BMI 22	F: HEX- CAGAGGAGCTGATGTAGAAGCA AGGCTAAAATTCCTAAATGGG	(TCTA) ₁₂	60	8	242 - 286	0.828	0.192	KU376478
BMI 32	F: FAM- TTAGAAATGGCTGAAGGAGTGC R: GCTTAGGTCGGTCTGTCTGTCT	(TAGA) ₈	62	10	140 - 200	0.854	0.000	KU376479
BMI 41	F: FAM- TCCCATGTTCCAACCTTCTC R: ACTGTCAGGGGTGGTTTTTG	(TGGA) ₁₉	60	2	197 - 205	0.371	0.010	KU376480
BMI 44	F: FAM- GAACTACAAAGGATGACCCCAA R: CAAAGACCAGAAAGGACTGCTC	(AGAT) ₁₅	61	8	152 - 224	0.890	0.372	KU376481
BMI 71	F: HEX- GCAGTTTTGCTACAGGAGGAGT R: AAAATGGAGGTGCAGGGG	(TCTA) ₁₄	56	5	317 - 337	0.592	0.000	KU376482
BMI 96	F: HEX- CTGTTTTGCATGGTTACAGGAT R: CAAGTGGGAGTTGTAGGTCAC	(TAGGA) ₂₅	56	11	162 - 242	0.863	0.229	KU376483
BMI 98	F: FAM CATTGTAGTGTGTTGTGGTGTG R: TCCTGTGACTGCATCGTTTATC	(GATA) ₁₄	60	6	354 - 382	0.581	0.330	KU376484

Table 2

The expected heterozygosity (He) and observed heterozygosity (Ho) for each batis species analyzed.

Locus	<i>Batis crypta</i> n = 16	<i>Batis mixta</i> n = 11	<i>Batis capensis</i> n = 10
BMI 16	0.660 / 0.562	0.706 / 0.455	0.675 / 0.100
BMI 17	0.800 / 0.800	0.806 / 1.000	0.715 / 0.286
BMI 22	0.846 / 0.812	0.673 / 0.545	0.615 / 0.300
BMI 32	0.867 / 0.562	0.806 / 0.818	0.785 / 1.000
BMI 41	0.492 / 0.875	0.463 / 0.727	0.000 / 0.000
BMI 44	0.830 / 0.750	0.806 / 1.000	0.180 / 0.200
BMI 71	0.639 / 0.250	0.698 / 0.364	0.680 / 0.300
BMI 96	0.875 / 0.875	0.876 / 1.000	0.797 / 0.778
BMI 98	0.622 / 0.688	0.731 / 0.727	0.765 / 0.900

Figure 1

Figure 2