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ORIGINAL ARTICLE

Phylogeography of the fiscal shrike (*Lanius collaris*): a novel pattern of genetic structure across the arid zones and savannas of Africa

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ABSTRACT

Aim Savanna occupies a substantial part of Africa, being distributed around the two major tropical rain forest blocks in what is referred to as the Savanna Belt. Our current understanding of the genetic structure within species distributed across the Savanna Belt is primarily derived from mammalian taxa, studies of which have revealed a suture zone or transition between northern and east/southern Africa clades in south-western Kenya and north-western Tanzania. We conduct a phylogeographic study of the fiscal shrike (*Lanius collaris*), a polytypic species distributed across the Savanna Belt of Africa and for which morphological and vocal data are in agreement with the suture zone recovered for mammalian taxa, to test the hypothesis of a spatially congruent genetic break across several taxa, including birds.

Location Africa, south of the Sahara.

Methods We analysed DNA sequences recovered from four loci (one mitochondrial, two autosomal and one Z-linked) in 66 individuals, representing all recognized subspecies, as well as putatively closely related species. We make use of a combination of tree-building and population genetic methods to investigate the phylogeographic structure of the fiscal shrike across Africa.

Results The fiscal shrike consists of two primary lineages with a strong geographic component: a northern group distributed from southern Tanzania to Senegal, and a southern group distributed from Botswana/Zambia to South Africa with isolated populations in Tanzania and northern Malawi. Unexpectedly, Souza's shrike (*L. souzae*) was nested within *L. collaris*, as the sister group of the southern group. The positions of Mackinnon's shrike (*L. mackinnoni*) and that of the São Tomé shrike (*L. newtoni*) were variable, being either nested within the fiscal shrike or sister to the *L. collaris–L. souzae* clade. Our divergence time analyses suggest that the *Lanius collaris* species complex started to diversify around 2.2 Ma.

Main conclusions Our study reveals a distinct biogeographic pattern for a savanna distributed species in Africa, with the transition between the two primary genetic lineages occurring at a latitude of *c.* 15–16° S, 10° S further south than shown elsewhere for several mammalian species.

Keywords

Africa, divergence time, fiscal shrike, genetic transition, *Lanius collaris*, phylogeography, savanna belt, species trees, suture zone.

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INTRODUCTION

Savanna (open woodland with extensive grass cover) occupies a substantial part of Africa, being distributed around the two major forest blocks (Lower and Upper Guinea–Congolia), in what is often referred to as the Savanna Belt, but also extending into southern Africa. The extent and location of savanna on the continent has not been constant through time, with pollen and fossil data suggesting a gradual increase in grass abundance from about 16 Ma and widespread savanna in the north of the continent by the Late Miocene–mid Pliocene (8–2.6 Ma, deMenocal, 2004; Jacobs, 2004). This shift from forest to more open habitats may possibly be linked to the decrease in global temperature and increase in aridity that occurred just after the Mid-Miocene Climatic Optimum (Zachos *et al.*, 2001), with important step-like shifts in aridity occurring around 2.8, 1.7 and 1.0 Ma (deMenocal, 2004). Hence, the gradual but continuous increase in aridity over much of sub-Saharan Africa may have favoured an initial diversification of arid-adapted organisms through a shift in habitat preference, with later climatic cycles, as described for the Plio-Pleistocene, creating habitat heterogeneity throughout the continent, and thus providing opportunities for speciation (Jansson & Dynesius, 2002).

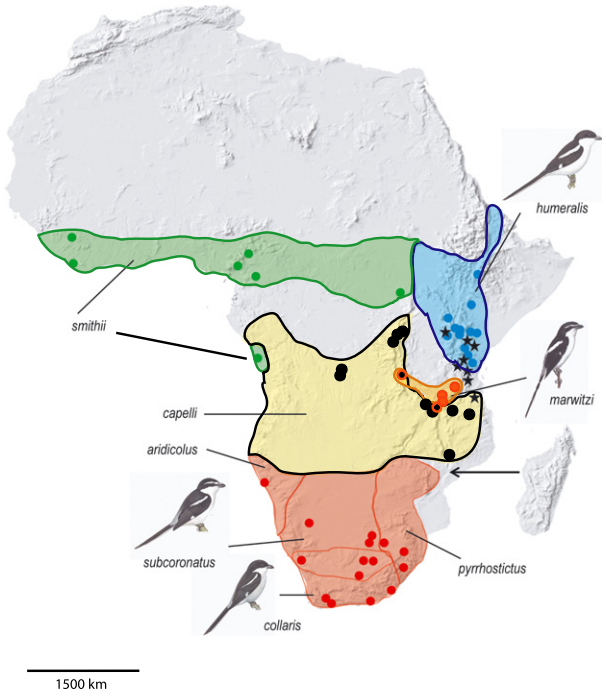

Figure 1 Distribution map depicting the subspecies of the fiscal shrike (*Lanius collaris*) in Africa. Subspecies ranges are based on Harris & Franklin (2000). Dots represent sampling localities in the present study. The two individuals with a mismatch between phenotype (*L. c. capelli)* and genotype (*L. c. marwitzi*) are indicated by circles with borders of different colours. Schematic genetic transition between northern and southern groups described in several mammalian studies are indicated by the stars (Flagstad *et al.*, 2001; Muwanika *et al.*, 2003; Rohland *et al.*, 2005; Lorenzen *et al.*, 2006, 2008; Brown *et al.*, 2007; Moodley & Bruford, 2007). The arrow indicates the genetic transition between the northern and southern lineages of fiscal shrike. Bird illustrations have been modified from Harris & Franklin (2000).

Studies that aim to understand geographic structure in species distributed across the Savanna Belt are scarce and have primarily focused on mammals (hartebeest: Flagstad *et al.*, 2001; warthog: Muwanika *et al.*, 2003; spotted hyena: Rohland *et al.*, 2005; bushbuck: Moodley & Bruford, 2007; Grant's gazelle: Lorenzen *et al.*, 2008). These studies have highlighted significant genetic differentiation between north/west (e.g. Sahel) and south/east distributed lineages, suggesting that gene flow is currently very limited between the two biogeographic regions. The two primary haplogroups from all these mammalian species are geographically close to each other or meet in north-western Tanzania/Kenya (Fig. 1), suggesting the occurrence of a congruent contact zone across several taxa (suture zone). Another transition zone has been highlighted a little further south, in southern Tanzania for the impala (Lorenzen *et al.*, 2006). However, despite this apparent geographic concordance, the time elapsed since the most recent common ancestor in these taxa appears to vary (0.5 Ma for hartebeest: Flagstad *et al.*, 2001; 0.45–1.62 Ma for giraffe: Brown *et al.*, 2007; 1.3 to 1.5 Ma for spotted hyena: Rohland *et al.*, 2005; 4.8 Ma for bushbuck: Moodley & Bruford, 2007), suggesting that this pattern may not be the result of a single vicariance event. Substantial genetic structure was further found within both of these biogeographic regions (south versus east: Muwanika *et al.*, 2003; Moodley & Bruford, 2007; Lorenzen *et al.*, 2009; within the Sahel region, Brouat *et al.*, 2009), with once again congruent genetic breaks among subgroups.

The genetic pattern recovered for mammals matches the hypotheses of speciation for savanna and arid-adapted birds proposed by Hall & Moreau (1970), with one taxon (species or subspecies) being distributed in the Sahel and the other lineage in the eastern and southern subregions of Africa, with a transition between the two taxa in north-western Tanzania/south-western Kenya. To date, no phylogeographic studies have been conducted at such broad geographic scales for African birds [but see Bowie *et al.* (2004, 2006) for widely distributed forest birds] and thus the 'super-species' hypothesis for open habitat birds proposed by Hall & Moreau (1970) remains to be tested.

The fiscal shrike, or common fiscal, (Laniidae: *Lanius collaris* Linnaeus, 1766) is a medium-sized (35–45 g) black and white predatory passerine that hunts small ground dwelling vertebrates and arthropods from an exposed perch. Fiscal shrikes are broadly distributed across Africa, from South Africa to Senegal (Lefranc & Worfolk, 1997; Harris & Franklin, 2000). They occupy a variety of habitats but avoid forested and very dry areas (e.g. rain forest blocks or Sahara). Substantial plumage differences exist among the eight currently recognized subspecies (Fig. 1), involving the colour

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of the belly (white or greyish), the colour of the rump (white or grey), the extent of white on the outer tail feather, the presence/absence of a white eyebrow, and the presence/absence of sexual dimorphism (chestnut flanks for females). Harris & Franklin (2000) recognized two primary subspecies groups, '*collaris*' (southern group) and '*smithii*' (northern group) that differ significantly in their song, with a putative transition between the two groups occurring at *c.* 5° S. The northern group includes two subspecies, *smithii* and *humeralis*, whereas the southern group includes subspecies *collaris* (including *predator*), *pyrrhostictus, capelli*, *marwitzi, aridicolus* and *subcoronatus* (Harris & Franklin, 2000). The subspecies *aridicolus/subcoronatus* and *marwitzi* are sometimes considered to be specifically distinct based on the presence of a distinctive white eyebrow (Lefranc & Worfolk, 1997; Harris & Franklin, 2000). There is substantial intergradation between subspecies *aridicolus/subcoronatus* and *collaris* in South Africa, as suggested by multiple specimens with intermediate white eyebrow stripes (Dean, 2005). *Lanius c. marwitzi* is restricted to grasslands in the Tanzanian highlands above 2000 m and is occasionally recorded on Nyika Plateau (northern Malawi, range of *L. c. capelli*), where individuals with intermediate or full eyebrows are sometimes reported (Harris & Franklin, 2000; Yosef, 2008). Finally, one species (*L. newtoni*), endemic to the island of São Tomé (Gulf of Guinea), is thought to be closely related to *L. collaris* but specifically distinct due to marked differences in habitat (interior of forest and not open habitat) and plumage (yellow underparts).

The fiscal shrike complex represents an ideal model taxon to test the hypothesis of concomitant patterns in genetic structure among African vertebrates adapted to open savanna habitats. Indeed, the distribution of fiscal shrike taxa, with their underlying acoustic and plumage differences, perfectly match the areas where genetically differentiated lineages of mammals have been described. Here, we aim to: (1) test the hypothesis of a northern lineage (subspecies *smithii* and *humeralis*) versus a southern/eastern lineage (subspecies *aridicolus*, *capelli*, *collaris*, *marwitzi, pyrrhostictus* and *subcoronatus*), and (2) compare the pattern observed for the fiscal shrike with the patterns previously highlighted for other vertebrates.

MATERIALS AND METHODS

Taxonomic sampling

We sampled 66 individuals from the fiscal shrike *sensu stricto* complex, representing all recognized taxa (Lefranc & Worfolk, 1997; Harris & Franklin, 2000; Dickinson, 2003; Dean, 2005) (Fig. 1; see Appendix S1 in the Supporting Information for details of exact localities). To increase our sample size for some subspecies, toe-pad samples from museum specimens collected between 1950 and 1975 ($n = 26$) were also used.

Outgroups were selected based on fresh tissue availability and information about the relationships among the primary shrike lineages (Olsson *et al.*, 2010; Per Alström, Swedish University of Agricultural Sciences, Uppsala and Urban Olsson, University of Gothenburg, Göteborg, pers. comm.). Trees were rooted with sequences from *Corvus corone* (Corvidae) and *Dicrurus adsimilis* (Dicruridae) (Fuchs *et al.*, 2004).

Laboratory procedure

DNA was extracted from tissue or blood using the Qiagen extraction kit (Valencia, CA, USA) following the manufacturer's protocol. We extracted the DNA from toe-pads in a room dedicated to historical DNA laboratory work. In this case, we used the same extraction protocols as for the fresh samples, but added 20 μL of dithiothreitol (DTT, 0.1 M) to facilitate digestion. We amplified and sequenced four loci, one mitochondrial (ATP6), one Z-linked (BRM intron-15) and two autosomal introns (MB intron-2, TGFb2 intron-5). The primer sequences used for polymerase chain reaction (PCR) amplification and sequencing are detailed in Appendix S2. The thermocycling conditions included a hotstart at 94 °C, an initial denaturation at 94 °C for 3 min, followed by 35–40 cycles at 94 °C for 40 s, 52–60 °C for 30–45 s, and 72 °C for 30–45 s, and was completed with a final extension at 72 °C for 10 min. Purified PCR products were cycle-sequenced using ABI Big Dye terminator chemistry (Applied Biosystems, Inc., Foster City, CA, USA) in both directions, with the same primers as used for PCR amplification, and run on an automated ABI 3730 DNA sequencer (Applied Biosystems, Inc.). Heterozygous sites in the nuclear loci (double peaks) were coded using the appropriate IUPAC code. All sequences have been deposited in GenBank (accession numbers HQ996672–HQ996961). Alignment was performed by eye and was straightforward owing to the low number of insertion and deletion events.

Phylogenetic analyses

Sexing

Individuals were sexed in order to determine the exact number of alleles for the Z-linked locus (two in males, one in females). Birds of the subspecies *collaris* and *subcoronatus* were sexed in the field using morphology (presence or absence of rufous flanks). Birds from the '*humeralis–smithii*' clade were sexed with the primer pair P2/P8 using the protocol described in Griffiths *et al.* (1998), as no sexual dimorphism in plumage or size has been described for these taxa. For the museum specimens, sex was inferred from the specimen labels (usually with the state of the gonads noted). We did not observe any discrepancy between molecular sexing and morphological sexing for individuals where both molecular and morphological data were available.

Phylogenetic reconstruction

Gene trees were estimated using maximum likelihood (ML) and Bayesian inference (BI), as implemented in

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RaxML v. 7.0.4 (Stamatakis, 2006; Stamatakis *et al.*, 2008) and MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003), respectively. The most appropriate models of nucleotide substitution were determined with TOPALi v. 2.5 (Milne *et al.*, 2009) and the Bayesian information criterion (BIC). For the Bayesian analyses, four Metropolis-coupled Markov chain Monte Carlo (MCMC) iterations (one cold and three heated) were run for ten million iterations with trees sampled every 100 iterations. We ensured that the potential scale reduction factor (PSRF) approached 1.0 for all parameters and that the average standard deviation of split frequencies converged towards zero.

We estimated the species tree for the *L. collaris* species complex using the species tree approach (*beast, Heled & Drummond, 2010) implemented in beast v. 1.5.4 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007). Species tree approaches implement the coalescent to estimate a species tree based on the different gene trees; this approach has been shown to outperform the traditional concatenation approaches in that incomplete lineage sorting is taken into account (Edwards, 2009), especially in cases where branch lengths are short and ancestral population sizes are large (Degnan & Rosenberg, 2006; Kubatko & Degnan, 2007). We assumed a strict molecular clock model for all loci and used the best fit model for each partition, as determined with TOPALi v. 2.5; each locus was specified with its own model and clock rate. We ran the chains for 50 million iterations.

We also used Tracer v. 1.5 (Rambaut & Drummond, 2007) to ensure that our sampling of the posterior distribution had reached a sufficient effective sample size (ESS) for meaningful parameter estimation.

Testing for selection and recombination

Prior to phylogenetic and population genetic analyses we established whether our data were in agreement with the expectations of neutral evolution and whether recombination hotspots were present in our sequenced intron loci. Violation of either of these assumptions is likely to alter the inferred phylogenetic topology and demographic parameter estimates and hence could lead to biased interpretations. We used the McDonald–Kreitman test (MK) (McDonald & Kreitman, 1991), as implemented in DnaSP v. 5.0 (Librado & Rozas, 2009) to test whether selection was acting on the mitochondrial protein-coding gene in the *Lanius collaris* species complex (that is including *L. mackinnoni*, *L. newtoni*, *L. souzae* and all taxa traditionally included in *L. collaris*, see Results). The stop codon was excluded from the analyses, leaving a total of 681 bp. We used sequences of *Lanius excubitor, L. collurio* and *L. senator* as outgroups. Significance was assessed using Fisher's exact test and a threshold value of $\alpha = 0.05$. We tested for selection acting on the nuclear loci by using the Hudson–Kreitman–Aguadé (HKA) test (Hudson *et al.*, 1987), as implemented in the software hka (http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm). We used sequences of *Lanius excubitor* as the outgroup for this test.

We used the GARD (Genetic Algorithm for Recombination Detection) and SBP (Single Break Point) algorithms, as implemented in HyPhy (Kosakovsky Pond *et al.*, 2005, 2006) to detect any evidence of recombination within the nuclear loci.

Determining the phase of alleles

We used phase v. 2.1.1 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003) to infer the association among heterozygous sites for each nuclear locus and individual. Three runs, using different seed values, were performed and results were compared across runs. We used the recombination model and ran the iterations of the final run 10 times longer than for the other runs. We used a threshold of 0.60 to consider the single nucleotide polymorphic sites (SNP) to be satisfactorily phased (see Harrigan *et al.*, 2008), and individuals that did not satisfy this threshold were removed from further analysis.

Population genetic analyses

Number of segregating sites (S), Haplotype diversity (Hd), nucleotide diversity (π), and Watterson's theta (θ) were estimated with DnaSP v. 5.0 for each subspecies or subspecies group. We used tcs v. 1.21 (Clement *et al.*, 2000) to reconstruct a 95% statistical parsimony network for each of the loci. We used Fu's F_S test (1000 replicates) and the Ramos-Onsins and Rozas R_2 statistic (Ramos-Onsins & Rozas, 2002), as implemented in DnaSP 5.0, to detect signatures of demographic change. The significance of the R_2 statistic was assessed using 1000 coalescent simulations. Insertion and deletion events were detected in some of the introns; we considered these characters as informative mutational events.

mtDNA divergence times

We applied a recently estimated neutral mutation rate from substitutions acting on four-fold degenerated sites, and its associated uncertainty [mean rate 0.073 substitutions/site/million years (s/s/Myr), 95% highest posterior density (HPD): 0.025–0.123 s/s/Myr; see Subramanian *et al.* (2009) for details], to the four-fold degenerated sites within our mitochondrial data set. We used beast v. 1.5.4 with a strict molecular clock model, Yule tree prior and a HKY model of sequence evolution, as in Subramanian *et al.* (2009). For the divergence time analyses, we only include members of the *Lanius collaris* species complex, as the neutral four-fold rate was estimated from intraspecific data. We compared the divergence time estimates obtained with this new molecular rate with estimates obtained with the 'standard' avian mtDNA rate of 2.1% divergence per million years (Weir & Schluter, 2008), using the complete mitochondrial data set. MCMC chains were run for 10^8 steps and were sampled every 1000 steps.

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Multi-locus network

We used POFAD v. 1.03 (Joly & Bruneau, 2006) and SplitsTree v. 4.0 (Huson & Bryant, 2006) to build a multi-locus network. We only included individuals for which: (1) all three nuclear loci could be satisfactorily phased, and (2) when sequences for all four loci were available ($n = 36$). We used uncorrected pairwise (p) distances as input for POFAD and made use of the standardized matrix for network reconstruction.

RESULTS

Selection and recombination

The MK test did not detect any evidence of selection within the *Lanius collaris* species complex (this includes *L. mackinnoni*, *L. newtoni* and *L. souzae*, see below); this result was not dependent on the outgroup used (*L. excubitor*, $P = 0.74$; *L. senator*, $P = 1.0$; *L. collurio*, $P = 0.47$). Likewise, the HKA test did not detect any evidence of selection acting on the nuclear introns (sum of deviations: 2.0160, d.f. = 4, $P = 0.72$).

No recombination was detected in the introns MB, TGFb2 and BRM using the GARD and SBP algorithms implemented in HyPhy.

Mitochondrial DNA

We obtained the complete ATP6 sequence (684 bp) for 56 individuals, including 45 individuals of the *L. collaris* species complex (see below). We gathered partial mitochondrial sequences (235 bp) from another 26 historical museum specimens to obtain a more precise picture of the distribution of the primary mitochondrial lineages. The Trn+Γ model of nucleotide substitution was selected for both the data set with complete data and for the partial sequence data set. Twenty-six haplotypes were detected within the *L. collaris* species complex (45 individuals with the complete ATP6 sequence; $S = 140$, $Hd = 0.932$, $\theta = 0.4681$, $\pi = 0.04828$).

The Bayesian and maximum likelihood analyses revealed that *L. collaris* is paraphyletic, as three different species (*L. mackinnoni*, *L. newtoni* and *L. souzae*) may be nested within it, although the relative position of the *L. mackinnoni–L. newtoni* clade is not well supported (complete sequences only, BI harmonic mean $-\ln = 3882.56$; individuals with partial sequences included, BI harmonic mean $-\ln = 4000.72$; Fig. 2). Instead, three primary groups emerged within this extended group (*L. collaris* species complex hereafter): *L. mackinnoni–L. newtoni* (ML: 83%, BI: 0.93), *L. collaris smithii–L. c. humeralis–L. c. capelli* (northern group, ML: 93%, BI: 1.0), and *L. souzae* together with *L. c. marwitzi–L. c. aridicolus–L. c. collaris–L. c. pyrrhostictus–L. c. subcoronatus* (southern group, ML: 60%, BI: 0.91). Hence, the mitochondrial tree was somewhat unexpected in that: (1) *L. souzae*, a species that has never been considered close to *L. collaris*, is actually nested within *L. collaris sensu lato* by being the sister taxon of the southern group, (2) *L. collaris capelli* is more closely related to the northern group than to the southern group, which contradicts vocal data (Harris & Franklin, 2000), (3) *L. c. marwitzi* is only slightly differentiated from other members of the southern group despite a large geographical distribution gap, and (4) haplotypes from *L. c. collaris/pyrrhostictus* and *L. c. aridicolus/subcoronatus* are intermingled and only weakly differentiated, in contrast to the marked plumage differences between the two groups. In contrast to the situation encountered in the southern group, the subspecies of the northern group were monophyletic (ML: 72–98%, BI: 1.0; with the exception of two individuals, see below), with *humeralis* being sister to the *smithii/capelli* clade (ML: 81%, BI: 0.59). However, two mismatches between taxonomy and mitochondrial haplotype assignment were observed for members of the northern group: one *capelli* (FMNH 4410161, no eyebrow) collected in Malawi was nested with the southern group and one *capelli* (ZMUC 74.739, no eyebrow) collected in Tanzania only differs by one substitution from individuals assigned to *L. c. marwitzi* (southern group). This result suggests that ZMUC 74.739 may be: (1) a hybrid or a back cross between *marwitzi* and *humeralis*, or (2) that the putative morphologically diagnosable eyebrow is very labile. Evidence for population expansion was only detected in the southern group (Fu's $F_S = 6.14$, $P = 0.001$; $R_2 = 0.0662$, $P = 0.007$).

mtDNA divergence time estimates

Our analyses revealed that the *Lanius collaris* species complex started to diversify about 2.2 Ma (95% HPD: 0.9–3.8 Ma). The northern group diversified into *L. c. humeralis*, *L. c. capelli* and *L. c. smithii* around 1.0 Ma (95% HPD: 0.4–1.7 Ma), an estimate that is very close to the estimate for the split between *L. souzae* and the southern group (1.2 Ma, 95% HPD: 0.5–2.2 Ma). The oldest divergence within the southern group, involving *L. c. marwitzi*, occurred *c.* 0.4 Ma (95% HPD: 0.14–0.8). The São Tomé Fiscal, *Lanius newtoni*, split from its closest continental relative *c.* 2.1 Ma (95% HPD: 0.9–3.7). The divergence times were older under the strict clock model and the 2.1%/Myr rate: the *L. collaris* species complex diversified around 6.4 Ma (95% HPD: 4.8–8.0), *L. newtoni* colonized São Tomé about 5.3 Ma (95% HPD: 3.4–7.5), *L. souzae* split from the southern group about 3.7 Ma (95% HPD: 2.5–5.0) and the northern group started to diversify 2.7 Ma (95% HPD: 1.9–3.6). Hence, the estimates obtained using the traditional clock were two to three times older than the ones obtained using the neutral four-fold rate. We consider the divergence time estimates using the four-fold neutral rate to be more reliable than the traditional 2.1% avian rate. Indeed, the use of this traditional rate has been criticized and compelling evidence suggests that this rate should not be broadly applied to birds due to large variation across lineages and loci (e.g. Warren *et al.*, 2003; Arbogast *et al.*, 2006; Pereira & Baker, 2006). In contrast, the four-fold substitution rate may evolve in a strictly neutral manner and thus be more reliable than the traditional rate, at least at the

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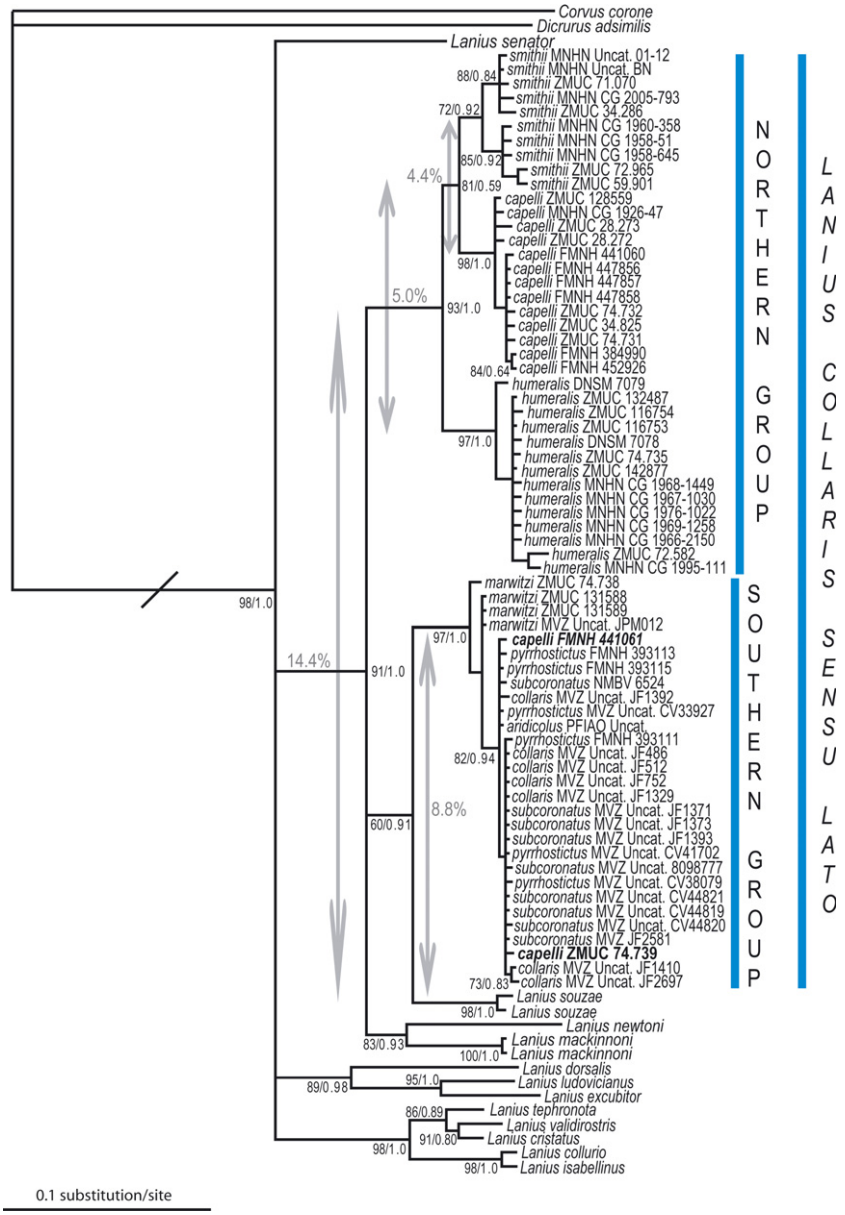

Figure 2 The 50% majority-rule consensus tree obtained from the Bayesian analyses of mitochondrial ATP6, including *Lanius collaris* individuals from which only partial sequences were obtained. Values close to nodes represent bootstrap values (if > 70%) and Bayesian posterior probabilities (if > 0.90). Mean uncorrected genetic distances among the primary clades are indicated by the grey arrows. Note the paraphyly of *L. collaris* as traditionally defined.

time scale relevant to divergence between recent species (0–2 Ma) where mutation saturation and selection may not be problematic (Ho & Larson, 2006).

Nuclear data

TGFb2 intron-5

We obtained complete TGFb2 intron-5 sequences (578 bp) for 45 individuals of the *L. collaris* species complex and six *Lanius* outgroups. This data set was complemented by partial sequences (220 bp) obtained from an additional 25 individuals of the *L. collaris* species complex. Three individuals (*L. c. collaris* MVZ uncat. JF1369 and JF1410, and *L. c. humeralis* ZMUC 116754) could not be phased at the 0.60 threshold and were thus excluded from further analyses. Thirty-six haplotypes were detected within the *L. collaris* species complex (individuals with the complete TGFb2 sequence; $S = 42$, $Hd = 0.97$, $\theta = 0.01485$, $\pi = 0.00954$). The phylogenetic affinities recovered by the TGFb2 intron (K81 + Γ model, harmonic mean −ln = 1868.86, see Fig. S1 in Appendix S3) were identical to that recovered by the mtDNA locus in that: (1) *capelli* (FMNH 441061) was more strongly related to the northern group, (2) *L. souzae* was nested within *L. collaris sensu stricto*, being closely related or nested within the southern group, and (3) there was no sharing of alleles among the northern and southern groups (considering FMNH 441061 to be part of the southern group). Within the northern and southern groups, alleles are shared among subspecies. Both the northern and southern groups have similar genetic diversity (northern group, $S = 14$, $Hd = 0.861$, $\theta = 0.00664$, $\pi = 0.00539$; southern group: $S = 16$, $Hd = 0.946$,

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$\theta = 0.00630$, $\pi = 0.00607$). There is evidence of population expansion in both groups (northern group Fu's $F_S = -3.263$, $P = 0.025$ $R_2 = 0.0635$, $P = 0.12$, southern group Fu's $F_S = -6.436$, $P < 0.01$, $R_2 = 0.1085$, $P = 0.53$).

MB intron-2

We obtained the complete myoglobin intron-2 sequence (694 bp) for 42 individuals of the *L. collaris* species complex and six *Lanius* outgroups. One individual could not be phased (ZMUC 116753) and was thus excluded. Twenty-six alleles were recovered within the *L. collaris* species complex ($S = 26$, $Hd = 0.801$, $\pi = 0.00427$, $\theta = 0.00753$). The pattern recovered by MB (K81, harmonic mean −ln = 1755.94; see Fig. S2 in Appendix S3) is identical to what was recovered for the other loci in that the southern group is recovered as monophyletic (ML: 71, BI = 0.98) and closely related to *L. souzae* (ML = 0.61, BI = 0.90). The northern group was not recovered as monophyletic as the relationships of *L. c. humeralis*, *L. c. capelli* and *L. c. smithii*, *L. newtoni* and *L. mackinonni* were unresolved. We found evidence of population expansion in both the northern group (Fu's $F_S = -3.981$, $P = 0.013$, $R_2 = 0.1226$ $P = 0.45$) and southern group (Fu's $F_S = -7.892$, $P < 0.01$, $R_2 = 0.0469$, $P = 0.037$).

BRM intron-15

We obtained the complete sex-linked BRM intron-15 sequence (360 bp) for 40 individuals (23 male and 16 female) from the *L. collaris* species complex and partial intron sequences (229 bp) for 15 further individuals. We could not sex and amplify the BRM locus or any other Z-linked locus for two fresh samples of *L. c. marwitzi* (ZMUC 131588 and 131589). We also could not sex *L. c. capelli* ZMUC 12899 using the P2/P8 primer combination; we conservatively considered this individual as a female in the analyses. Very low differentiation was found among *Lanius* species and some alleles were even shared among well-recognized species (e.g. one allele was shared between *L. ludovicianus* and *L. dorsalis*, another was shared between *L. excubitor*, *L. mackinnoni*, *L. newtoni* and the northern group of *Lanius collaris*). Furthermore, most haplotypes could be connected by a single mutation in the statistical parsimony network. Within the *L. collaris* species complex, thirteen alleles ($S = 15$) were recovered ($S = 15$, $Hd = 0.748$, $\theta = 0.00873$, $\pi = 0.00767$). There was no sharing of alleles between the northern and southern groups, and *capelli* FMNH 441061 was nested within the southern group (as also recovered with the mitochondrial and TGFb2 data) having the same allele as *L. marwitzi* (see Fig. S3 in Appendix S3). The most common allele in the northern group was also shared with *L. newtoni*, *L. mackinnoni* and *L. excubitor*. The southern group was monophyletic in both the Bayesian inference (K81uf model, harmonic mean −ln = 829.08, Fig. S3) and ML analyses with strong support (BI: 1.0, ML: 92), whereas the northern group formed a polytomy. No evidence of population expansion was detected in the BRM sequences (northern group Fu's $F_S = -0.753$, $P = 0.17$, $R_2 = 0.1195$ $P = 0.25$; southern group Fu's $F_S = -1.314$, $P = 0.12$, $R_2 = 0.089$ $P = 0.20$).

Multi-locus network and species tree analyses

The multi-locus network obtained using the mitochondrial and nuclear sequences revealed strong and congruent divergences among the northern and southern groups and the traditionally recognized species *L. mackinnoni*, *L. newtoni*, and *L. souzae* (Fig. 3). Based on the results of the individual gene trees, we defined seven lineages as terminal taxa ('species') for the species tree analyses: (1) *L. mackinnoni*, (2) *L. newtoni*, (3) *L. souzae*, (4) *L. collaris* – southern group including *L. c. collaris*, *L. c. aridicolus*, *L. c. capelli* FMNH 441061, *L. c. marwitzi*, *L. c. pyrrhostictus*, and *L. c. subcoronatus*, (5) *L. c. humeralis*, (6) *L. c. capelli* and (7) *L. c. smithii*. We used all individuals for which full phase information was available. The same topology as in our concatenated analyses were obtained using the species tree approach, although most of the relationships among these primary lineages did not receive significant posterior probability (PP) support. As in all individual loci, *L. souzae* grouped with the southern group lineage (PP = 1.0), rendering *Lanius collaris*, as traditionally defined, paraphyletic (Fig. 4).

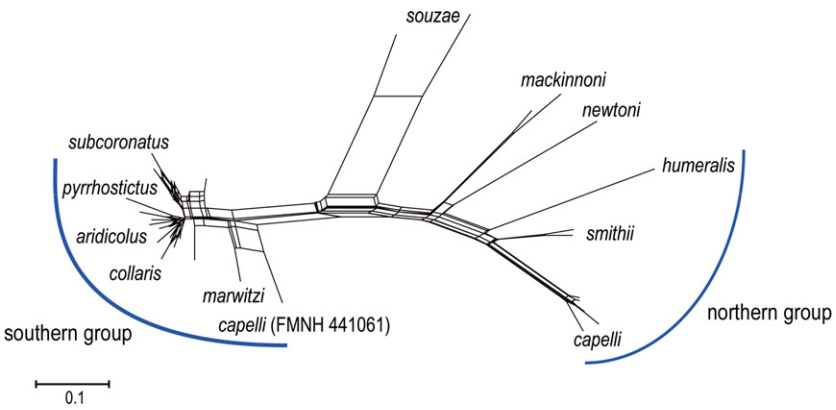

Figure 3 The multi-locus network obtained for the *Lanius collaris* species complex using standardized genetic distances from the four loci analysed. The scale is a relative distance measure between individuals.

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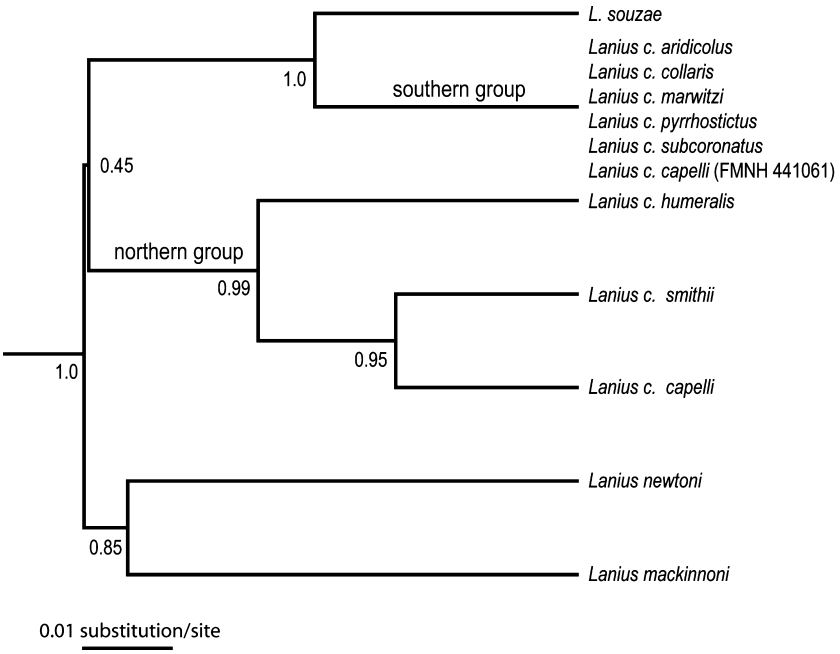

Figure 4 Species tree for the *Lanius collaris* species complex obtained using the coalescent approach implemented in *BEAST (Heled & Drummond, 2010). Values given next to nodes represent posterior probabilities.

DISCUSSION

Pan-African phylogeography

Our results recovered a surprising pattern with respect to relationships among fiscal shrike lineages in Africa. Although we recovered two primary lineages, as initially hypothesized, the composition, geographic patterns, and genetic divergence within and between these two clades differ strikingly from previous published work on morphology and vocalizations, a pattern that could be attributable to several different evolutionary processes including genetic (morphology) and cultural (song) drift during periods of isolation, selection on plumage colour and size, and sexual selection (morphology and song).

First, we found that the fiscal shrike *Lanius collaris* as traditionally defined does not form a monophyletic lineage, as Souza's shrike (*L. souzae*) was consistently nested within *L. collaris*, and sister to the southern group. This pattern was recovered in all four loci, as well as in the species tree analyses, where the stochastic nature of the coalescent process is explicitly taken into account. Various stages of paraphyly or polyphyly of species can be expected at an early stage of divergence (e.g. Funk & Omland, 2003). Yet the fact that the same pattern was recovered in each of the four loci we analysed, together with no allele being shared between the northern and southern groups, suggests that the topology we recovered is not a consequence of stochastic factors such as incomplete lineage sorting.

The phylogenetic placement of Souza's shrike is very intriguing as this species differs strikingly in plumage pattern from taxa traditionally attributed to the fiscal shrike complex. Souza's shrike is found in central Africa, in Miombo woodlands, a habitat that differs from that in which fiscal shrikes are usually found (savanna and grassland, arid areas with scattered trees/bushes). Hence, the striking plumage difference could be due to selective pressure as an adaptation to a different habitat (see Hoekstra *et al.*, 2006 for an example involving point mutations and adaptation to substrate colour).

The relationships of Mackinnon's shrike and São Tomé shrike with respect to Souza's shrike and the northern and southern groups of the fiscal shrike are not certain and vary among analyses and loci; *L. mackinnoni* and *L. newtoni* were generally recovered as sister to the *L. souzae*/*L. collaris* group, but in some cases they share similar alleles with some members of the northern group. The species tree analysis grouped these two taxa as the sister group of the clade formed by *L. souzae*/southern group and the northern group, but monophyly of the latter clade was weak (PP = 0.45). Hence, *L. mackinnoni*/*L. newtoni* may actually be nested within *L. collaris* as traditionally defined. The strong relationship between *L. mackinnoni* and *L. newtoni* was also unexpected based on traditional hypotheses. Indeed, whereas *L. newtoni* has traditionally been considered to be closely related to the fiscal shrike complex, *L. mackinnoni* has never been proposed to be related to this species complex. Yosef (2008) mentioned a possible link between *L. collaris* and *L. mackinnoni* but without specifying the characters he thought linked them. It is worth noting that *L. mackinnoni* and *L. newtoni* share similar habitat preferences: *L. newtoni* is endemic to São Tomé where it inhabits the interior of wet forest, whereas *L. mackinnoni* can be found at the edge of rain forest. In contrast, all other members of the *Lanius collaris* species complex are adapted to more open habitats. This result is in accordance with several other examples of where a vertebrate lineage colonizing an island or archipelago

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is able to exploit a new niche, often (but not always) accompanied by rapid morphological adaptation (e.g. Voelker *et al.*, 2009).

Our study revealed a completely new and unexpected pattern concerning the phylogenetic affinities of the subspecies *capelli*; our results indicate that the transition between the northern and southern group occurs at *c.* 15–16° S, which is 10° further south than previously thought. All published studies that have focused on the phylogeography of African vertebrates at the continental scale have suggested that a transition between the northern and southern clades occurs in NW Tanzania/SW Kenya at about 5° S, where the transition between the northern and southern group of fiscal shrikes was also previously thought to occur. This conclusion holds for a set of taxa with very different divergence times, from 0.5 to 4.8 Ma (Flagstad *et al.*, 2001; Rohland *et al.*, 2005; Brown *et al.*, 2007; Moodley & Bruford, 2007).

Our estimate for the split between the northern and southern groups of fiscal shrike is within this time frame when using the neutral four-fold substitution rate (2.2 Ma, 95% HPD: 0.9–3.8 Ma). However, when the traditional 2.1%/Myr rate is used, it places this divergence as far back as 6.4 Ma (95% HPD: 4.8–8.0). Our 95% HPD for the divergence time analyses are wide and thus it would be too speculative to link the first diversification event within the *Lanius collaris* species complex with any specific climatic oscillation that occurred during the Plio-Pleistocene. However, it is worth noting that the four primary lineages are each adapted to different types of habitat (Miombo, savanna/grassland, and forest edge). During the past 4 Myr (Middle to Late Pliocene), changes in plant communities have been substantial, abrupt, and occurred with high frequency as a result of climatic oscillations (deMenocal, 2004; Feakins *et al.*, 2005), and large expanses of C_4 plants (grasses) developed at the beginning of the Pleistocene (Cerling, 1992). This major change in plant community composition, around 2.5 Ma (when the *L. collaris* complex started to diversify), is also corroborated by changes in fossil mammal assemblages in north-east Africa (Hernández Fernández & Vrba, 2006). The climatic oscillations may have created a mosaic of heterogeneous habitats enabling each taxon to become locally adapted and hence to speciate in allopatry. For example, *L. souzae* (FMNH 441062 and 441063) and *L. collaris capelli* (FMNH 441060) were collected at the same locality but in different habitats, suggesting fine scale partitioning of habitat between these closely related taxa.

Northern group

There was strong divergence among the three lineages from the northern group, with the first diversification event dating back to 1 Ma (95% HPD: 0.4–1.7 Ma), with the isolation of the subspecies *L. c. humeralis* from the *L. c. capelli/L. c. smithii* group. The pattern we recovered for the fiscal shrike matches the one highlighted for giraffe (Brown *et al.*, 2007) and kob antelope (Lorenzen *et al.*, 2007), with a clear separation between populations found in West Africa and Northeast Africa (Kenya/Ethiopia). The divergence between the clade within each of the two mammalian taxa is estimated to have occurred 0.18–0.54 Ma, which marginally overlaps with the 95% HPD for the split between *L. c. humeralis* and *L. c. capelli/L. c. smithii* (1.0 Ma, 95% HPD: 0.4–1.7).

A greater number of mammalian studies have focused on the Sahelian part of North Africa, where extensive genetic structure has been recovered in several species of rodent (Mouline *et al.*, 2008; Nicolas *et al.*, 2008, 2009; Brouat *et al.*, 2009). We also detected some evidence of differentiation within the *L. c. smithii* clade, with the occurrence of two primary mtDNA clades, one restricted to Cameroon and the Democratic Republic of the Congo (DRC) and the other to Guinea, Liberia and Congo. The relationship of the Congo individual is surprising, as it is not related to the geographically closer individuals from Cameroon and the DRC, from the same subspecies. The fiscal shrike population from the Congo/Cabinda region has been associated with *L. c. smithii*, based on morphology (Harris & Franklin, 2000), where it appears to be surrounded by individuals from *L. c. capelli*. Such a pattern may be due to the existence of several areas of habitat persistence in West Africa during climatic cycles (Maley, 1996) coupled to complex patterns of population expansion and contraction for *L. c. capelli* and *L. c. smithii*. Our current sampling does not allow us to explicitly test this hypothesis, and increased sampling is warranted.

Southern group

Very little diversification was evident within the southern group despite: (1) widespread and disjunct ranges, with the subspecies *marwitzi* separated from other populations of the southern group by a population of the northern group (subspecies *capelli*), and (2) obvious morphological differences (absence or presence of a prominent white eyebrow) between the subspecies *collaris* and *pyrrhostictus* on the one hand, and *aridicolus*, *marwitzi* and *subcoronatus* on the other. The split between *L. marwitzi* and the other populations from the southern group is recent (0.4 Ma, 95% HPD: 0.14–0.8). There is no compelling evidence for hybridization between *L. c. marwitzi* and *L. c. capelli*, the adjacent representative of the northern group. Yet, two of the individuals we sampled constitute interesting cases. The first specimen is *L. c. capelli* FMNH 441061. This individual, with no eyebrow, collected on the Nyika Plateau (Malawi) was more closely related to *L. c. marwitzi* than to the northern group; this pattern was recovered in all four loci challenging the hypothesis that this individual is a hybrid. Interestingly, an individual with an intermediate white eyebrow has previously been described from the same area (Harris & Franklin, 2000; Yosef, 2008), but these occurrences seem to be irregular. The second specimen is *L. c. capelli* ZMUC 74.739, collected on Mount Mahale, on the eastern edge of Lake Tanganyika (Tanzania). This individual also lacks an eyebrow, but was associated with the southern group, bearing a mitochondrial haplotype that was very similar to *L. c. marwitzi*,

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and a partial TGFb2 sequence that was identical to another individual from the southern group. However, the northern and southern groups are not discernable when using only the 250 bp fragment of the TGFb2 intron sequenced from museum specimens, since fewer variable sites were sampled and as a consequence the most common haplotype was shared between the northern and southern group. Given these data, we consider hybridization to be absent or very limited. Hence, we have to consider an alternate hypothesis to hybridization to explain the mismatch between genotype and phenotype (lack of eyebrow) for FMNH 441061, and possibly also for ZMUC 74.739 (where our data are more limited).

The molecular divergence between the subspecies characterized by eyebrow markings (*aridicolus*, *marwitzi* and *subcoronatus*) and those not possessing such marking (*collaris*, *pyrrhostictus*) is very limited and there seems to be extensive gene flow among the southern African populations (J. Fuchs, T. Crowe & R. Bowie, unpublished data), despite marked biometric differences (Soobramoney *et al.*, 2005). The distribution of shrike populations with and without eyebrow patches seems to be stable in South Africa, although individuals with a white eyebrow are episodically observed within the range of other subspecies (Dean, 2005). The two forms are found in different habitats (birds that lack eyebrows occur in fynbos, karoo and grassland habitats, and those with white eyebrows occur in Kalahari broad-leaf woodland) with individuals with intermediate eyebrow occurring at the arid-savanna/grassland ecotone of central South Africa. Hence, one explanation for this pattern could be the environmentally driven expression of the eyebrow, i.e. phenotypic plasticity. The white eyebrow could offer an adaptation for foraging in more arid environments. Shrikes hunt from an exposed perch, looking downwards for moving prey. Hence, the white eyebrow may have some function with respect to reducing glare as a consequence of light reflectance.

How many species of fiscal shrike?

Our results clearly indicate the existence of two primary fiscal shrike lineages that correspond to our northern and southern groups, with an intervening well recognized species, *L. souzae*, being the sister-taxon of the southern group. No mitochondrial haplotypes or nuclear alleles were shared between the northern and southern fiscal shrike lineages. The mitochondrial genetic distance recovered between the northern and southern lineages (14.4%) is higher than most genetic distances recovered between well-recognized *Lanius* species in our data set (1% between *L. isabellinus* and *L. collurio*; 16% between *L. newtoni* and *L. dorsalis*; average across our *Lanius* outgroups: 11.3%). Preliminary knowledge about the visual and vocal displays of individuals from both groups indicates the presence of some differentiation (Harris & Franklin, 2000; Yosef, 2008). Finally, individuals of the northern group (*L. c. capelli*) and *L. souzae* (sister taxon to the southern group) occur in sympatry in northern Malawi, and were collected at the same site on Nyika Plateau (*capelli* FMNH 441060 and *souzae* FMNHN 441062 and 441063), suggesting complete reproductive isolation between members of the northern group and at least *L. souzae*. Given the existence of substantial genetic and morphological differentiation, and reproductive isolation between the northern group and the sister-taxon of the southern group, we advocate that the fiscal shrike *Lanius collaris*, as traditionally defined, be considered to consist of two species: northern common fiscal *Lanius humeralis* Stanley, 1814 (including the subspecies *capelli, humeralis* and *smithii*) and the southern common fiscal *Lanius collaris* Linnaeus, 1766 (including the subspecies *aridicolus*, *collaris*, *marwitzi* and *pyrrhostictus*). This distinction would hold using most species concepts, including the Biological Species concept (no alleles shared between the two groups, hence no gene flow) and Phylogenetic Species concept (identification possible at the molecular and morphological level: juvenile plumage, extent of sexual plumage dimorphism, paraphyly of the traditional 'species'). It is likely that this decision would also hold under the Recognition Species concept (Paterson, 1985) (distinctive songs between the northern and southern groups), although play-back experiments are needed to ascertain how birds from each group react to the song of the other group. Further sampling in the putative contact zones between the northern and southern lineages (northern Botswana and Mozambique, Malawi, Tanzania) may help to estimate the extent of gene flow/hybridization, if any, between the two lineages. Yet, even if some hybridization is taking place, it will very likely be localized and hence not invalidate recognition of distinct fiscal shrike taxa, as hybridization is a common phenomenon in birds (10% of all bird species are known to hybridize; Grant & Grant, 1992).

We also recovered strong genetic divergence among the three subspecies of the northern common fiscal, with no shared haplotypes in mtDNA, suggesting that more than one species may be involved in this group. Most of our geographic sampling from the northern common fiscal consisted of museum toe-pads. Consequently, the amount of nuclear DNA we were able to retrieve from those samples was limited (usually 220–250 bp per locus). Future research should focus on the sampling of both additional individuals within these three lineages and a larger number of loci if reasonable confidence limits of coalescent parameters are to be obtained (Hey, 2010).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix S1 List of all specimens, subspecies and localities included in the phylogeographic analyses.

Appendix S2 List of loci sequenced, location on the *Gallus gallus* and *Taeniopygia guttata* genomes, and primer sequences.

Appendix S3 The 50% majority-rule consensus trees obtained from the Bayesian analyses of the autosomal TGFb2 intron-5, myoglobin intron-2 and Z-linked BRM intron-15 alleles and the allele network obtained using the statistical parsimony algorithm implemented in TCS v. 1.21 (Figs S1–S3).

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