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Diversification across major biogeographic breaks in the African Shining/Square-tailed Drongos complex (Passeriformes: Dicruridae)

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Surprisingly, little is known about the extent of genetic structure within widely distributed and polytypic African species that are not restricted to a particular habitat type. The few studies that have been conducted suggested that speciation among African vertebrates may be intrinsically tied to habitat and the dynamic nature of biome boundaries. In the present study, we assessed the geographic structure of genetic variation across two sister-species of drongos, the Square-tailed Drongo (*Dicrurus ludwigii*) and the Shining Drongo (*D. atripennis*), that are distributed across multiple sub-Saharan biogeographic regions and habitat types. Our results indicate that *D. ludwigii* consists of two strongly divergent lineages, corresponding to an eastern–southern lineage and a central-western lineage. Furthermore, the central-western lineage may be more closely related to *D. atripennis*, a species restricted to the Guineo-Congolian forest block, and it should therefore be ranked as a separate species from the eastern–southern lineage. Genetic structure is also recovered within the three primary lineages of the *D. atripennis–D. ludwigii* complex, suggesting that the true species diversity still remains underestimated. Additional sampling and data are required to resolve the taxonomic status of several further populations. Overall, our results suggest the occurrence of complex diversification patterns across habitat types and biogeographic regions in sub-Saharan Africa birds.

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Introduction

Patterns of spatial distribution among African vertebrates have long-puzzled evolutionary biologists (e.g. Hall & Moreau 1970; Crowe & Crowe 1982). Genetic data have allowed scrutiny of the composition of superspecies (Pearson 2000) and enabled the explicit testing of the timing of origination of biogeographic patterns in response to the formation of putative barriers, with a particular emphasis on the montane forests of the Eastern Arc Mountains (e.g. Bowie *et al.* 2006; Fjeldså & Bowie 2008; Fuchs *et al.* 2011b; Fjeldså *et al.* 2012), the Upper/Lower Guinea Forest Blocks (Marks 2010; Fuchs & Bowie 2015) and the Savannah zones of Africa (Fuchs *et al.* 2011a; Voelker *et al.* 2012, 2014).

Relatively little is known about the structure of genetic diversity within widely distributed and polytypic African species that are not restricted to a particular habitat type. The few studies that have been conducted suggest restricted gene flow among populations across ecotones (Smith *et al.* 1997) or strong association of genetic clusters with habitat (Moodley & Bruford 2007; Oatley *et al.* 2011, 2012), suggesting that speciation among African vertebrates may be intrinsically tied to habitat diversity and boundaries. Illustrative of this is the phylogeographic pattern recovered for the Fiscal Shrike (*Lanius collaris*; Laniidae), which is distributed across Africa's savanna zones (Fuchs *et al.* 2011a). Not only was the focal species of this study (*L. collaris*) recovered as polyphyletic, with *L. souzae* being phylogenetically nested, and parapatrically distributed in Miombo habitat (Fuchs *et al.* 2011a), but also the closest relative of that lineage (*L. mackinnoni*) is distributed in the primary forest blocks. Hence, this study of a widely distributed bird species across its geographical range in Africa suggested a diversification pattern with a more complex interaction between geography (northern vs. eastern–southern savannas) and habitat (savanna, Miombo woodland and forest) than was implied by traditional taxonomy. Whether this example reflects a general misconception of the processes driving the formation of biodiversity in Africa combined with misleading taxonomy, or whether this represents an exceptional case, requires the study of additional lineages.

The Shining (*Dicrurus atripennis*) and Square-tailed (*D. ludwigii*) Drongos are sister-species in the Dicruridae (Pasquet *et al.* 2007), a monogeneric family of corvoid passerines endemic to the Old World. The two species are very similar in shape: medium-sized black songbirds (18–24 cm) with a slightly forked tail. They differ in that the Shining Drongo has steel-green reflections, whereas the Square-tailed Drongo is less shiny and is slightly smaller, with a less forked tail, and a shorter and more broad-based bill with longer gape bristles (Vaurie 1949; Rocamora & Yeatman-Berthelot 2009). The Shining Drongo is monotypic and restricted to mature evergreen forests in the Upper and Lower Guinea Forest Blocks, with a discontinuity in distribution corresponding to the savanna zone extending from the interior to reach the coast in Ghana, Togo and Benin, known as the Dahomey Gap. In contrast, the Square-tailed Drongo occurs in a wide variety of wooded habitat (e.g. mostly gallery forest and dense wooded savanna) across Africa with five subspecies typically recognized (Fig. 1). Its distribution is patchy, especially in eastern Africa.

The present study aims to better understand the diversification patterns within these two species of drongo and establish, using DNA sequence data, the relative placement and timing of formation of any major biogeographic breaks across Africa within this superspecies complex.

Material and methods

Sampling

We included several closely related species of drongos (Pasquet *et al.* 2007) both to test the sister-species relationships between the Shining and Square-tailed Drongos and to compare the level of genetic divergence among any recovered intraspecific lineages with the level of divergence observed among traditionally recognized drongo species. Our genetic sampling for the two species covers most of their distributional ranges (n = 78: Fig. 1 and Table S1); this was supplemented by examination of 68 specimens of *D. ludwigii* and >100 of *D. atripennis*, including voucher specimens corresponding to many of the tissue samples used in this study. Trees were rooted using sequences from the Fiscal Shrike (*Lanius collaris*) and the Carrion Crow (*Corvus corone*).

Laboratory protocols

DNA was extracted from tissue or blood using the Qiagen extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We sequenced one mitochondrial protein-coding gene (ATP6), three nuclear introns (myoglobin intron-2, MB; beta fibrinogen intron-5, FGB; transforming growth factor beta 2 intron-5, TGFb2) and one Z-linked intron (Brahma Protein intron-15, BRM). The PCR-amplification protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 54–60°C for 30 s and 72°C for 75 s, and was terminated by a final elongation step at 72°C for 15 min. Sequences from historical specimens were obtained by performing several overlapping PCR amplifications (size 200–350 bp) using specific primers designed in this study (Table S2). Individuals were sexed by PCR using the primer pair 2550F and 2718R under standard PCR-amplification conditions (Fridolfsson & Ellegren 1999). We did not detect any conflict between sex determination with molecular techniques and inspection of gonads. Newly generated sequences have been deposited in GenBank (Accession Number: KX133844-KX134193).

Phasing of nuclear alleles and testing for selection and recombination

We used PHASE v2.1.1 (Stephens *et al.* 2001), as implemented in DNASP 5.0 (Librado & Rozas 2009), to infer the alleles for each nuclear locus. Three runs 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 initial runs. We considered the output of the long final PHASE run as the best estimate of allele. We assessed the impact of incorporating alleles with phasing probability lower than 0.6 (see Harrigan *et al.* 2008) by performing the gene tree analyses with and without the concerned individuals.

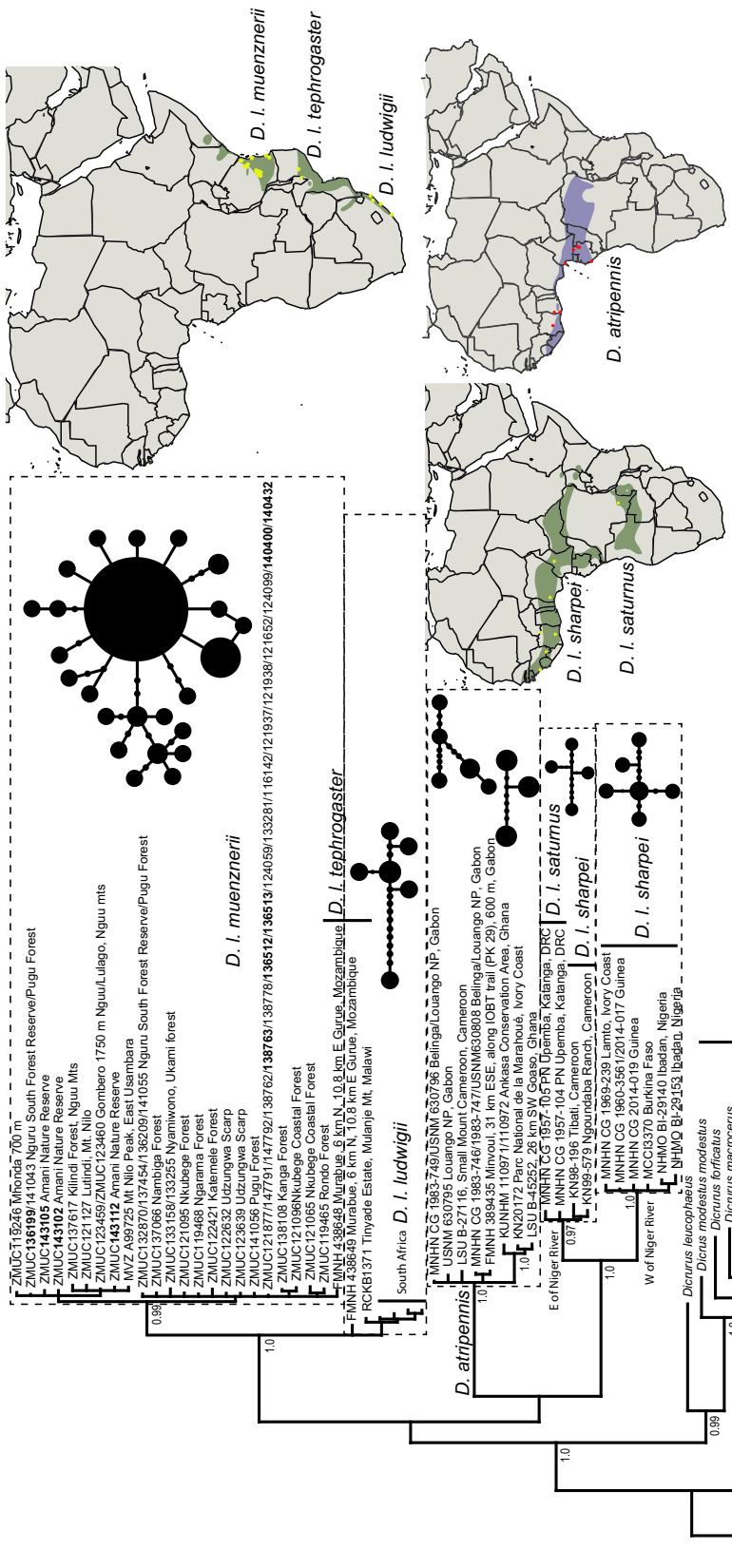

Fig. 1 The 50% majority rule consensus rule tree resulting from the Bayesian analysis of the ATP6 gene (only unique haplotypes are included). The two outgroups were removed for graphical purposes. Numbers close to nodes refer to posterior probabilities greater than 0.95. The primary lineages are each delineated by boxes with dashed lines. The 95% statistical parsimony networks obtained using TCS (Clement *et al.* 2000) are represented within each box. Small black circles represent unsampled or extinct haplotypes. Distributions of *Dicrurus ludwigii* (green; yellow dots indicate sampling localities) and *Dicrurus atripennis* (blue; red dots indicate sampling localities) (BirdLife International & NatureServe 2013). Maps were made using R (R Core Team 2013) libraries *maps* and *mapdata* (Becker & Wilks 2013), *maptools* (Bivand & Lewin-Koh 2014) and *scales* (Wickham 2014). The distribution maps for both species were not identical between BirdLife International, NatureServe (2013), and Rahbek *et al.* (2012); we encourage the reader to only consider the present distribution maps as coarse estimates and that areas such as SW Congo and NW Angola require additional survey effort to accurately delineate species distributional ranges. Specimen numbers in bold indicate the large-sized birds from the Eastern Arc Mountains mentioned in the Discussion.

We used the McDonald–Kreitman test (MK test; McDonald & Kreitman 1991) in DNASP 5.0 (Librado & Rozas 2009) to test for evidence of selection acting on ATP6. Significance was assessed using Fisher's exact test and a threshold of 0.05. We performed three MK tests on the *D. atripennis–D. ludwigii* clade using sequences from three different proximate outgroups (*D. leucophaeus*, *D. macrocercus* and *D. modestus*). We tested for selection acting on the nuclear loci using the Hudson–Kreitman–Aguadé test (HKA; Hudson *et al.* 1987), as implemented in the software HKA (https://bio.cst.temple.edu/~hey/software/software.htm#HKA). Sequences from *D. leucophaeus* were used as the outgroup.

We used the GARD algorithm (Genetic Algorithm for Recombination Detection, Kosakovsky Pond *et al.* 2005, 2006) as implemented on the DATAMONKEY webserver (www.datamonkey.org; Delport *et al.* 2010), to detect evidence of recombination within each nuclear locus.

Population genetic analyses and demographic history

Haplotype diversity (Hd), nucleotide diversity (π) and Watterson's theta (θ) were estimated with DNASP 5.0 for each subspecies or clade recovered in our Bayesian inference topology (see below). We used Fu's *Fs* test (1000 replicates) and Ramos-Onsins and Rozas R^2 statistic (Ramos-Onsins & Rozas 2002) to detect signatures of demographic change. We used TCS 1.21 (Clement *et al.* 2000) to reconstruct a 95% statistical parsimony network for each of the loci.

We used POFAD v1.03 (Joly & Bruneau 2006) and SPLITSTREE v4.0 (Huson & Bryant 2006) to reconstruct a multilocus network. For this, we only included the individuals from the *D. atripennis–D. ludwigii* complex for which sequences from all five loci were available ($n = 65$). We used uncorrected *p*-distances as input for POFAD and made use of the standardized matrix for network reconstruction.

Phylogenetic reconstruction

Gene tree reconstructions of the haplotypes and alleles were performed using Bayesian inference (BI), as implemented in MRBAYES 3.2 (Ronquist *et al.* 2012). We used the *nst = mixed* option such that model uncertainty is taken into account during the phylogenetic reconstruction, and incorporated rate variation using the *gamma* setting. Four Metropolis-coupled MCMC chains (one cold and three heated) were run for 5×10^6 iterations with trees sampled every 10^3 iterations. We tried several prior distributions for the branch-length parameters (exp: 10 to exp: 500). We used the CIPRES 3.1 gateway server (www.cipres.org by: https://www.phylo.org/; Miller *et al.* 2010) to run MRBAYES 3.2 (Ronquist *et al.* 2012).

Species trees were reconstructed using the coalescent-based model implemented in *BEAST (Heled & Drummond 2010). All primary lineages could be included in the species tree analyses, even though some individuals could not be sequenced for some loci (e.g. nuclear loci for the historical samples). The substitution model for each locus was selected using TOPALI (Milne *et al.* 2009) under the Bayesian information criterion. Each locus had its own substitution rates matrix and clock model. We used a Yule process for the tree prior. We used a normal prior distribution for ATP6 (0.026 substitution/site/lineage/million year –s/s/l/myr-; 95% HPD: 0.021–0.031 s/s/l/myr) and TGFb2 (0.0017 s/s/l/myr-; 95% HPD: 0.0013–0.0022 s/s/l/myr) rates that correspond to those obtained by Lerner *et al.* (2011); rates for the other nuclear loci were estimated in relation to ATP6 and TGFb2. Two runs were conducted for 5×10^8 iterations, with trees and parameters sampled every 5×10^3 iterations. The first 25×10^6 iterations were discarded as the burn-in period.

We used TRACER v1.6 (Rambaut *et al.* 2014) to ensure that our effective sample size for all Bayesian analyses of the underlying posterior distribution was large enough (>200) for meaningful estimation of parameters.

Estimating divergence times

We estimated the Times to Most Recent Common Ancestor (TMRCA) among the *Dicrurus* haplotypes using BEAST 1.8. We performed analyses with the strict and uncorrelated lognormal molecular clock models enforced with a Yule tree prior. MCMC chains were run for 5×10^7 steps and were sampled every 10^3 steps. Inferring divergence times within species is a challenging task as internal fossil calibration is seldom available. To circumvent this problem, we used two substitution rates, and their associated uncertainties to calibrate the trees. Lerner *et al.* (2011), using complete mtDNA genomes from the honeycreepers (Passeriformes, Drepanidinae) and calibration points based on the age of volcanic islands in the Hawaiian archipelago, proposed a new substitution rate for ATP6 (0.026 s/s/l/myr; 95% HPD: 0.021–0.031 s/s/l/myr). Subramanian *et al.* (2009) suggested that the time dependency phenomenon (Ho & Larson 2006) could primarily be attributed to nonsynonymous substitutions. They estimated the rate of evolution at fourfold degenerated sites from complete mtDNA sequences of Adelie Penguins (*Pygoscelis adeliae*) to be 0.073 s/s/l/myr (95% HPD: 0.025–0.123 s/s/l/myr); we also made use of this rate to estimate divergence times among *Dicrurus* taxa. It should be noted that the rate estimated by Subramanian *et al.* (2009) is a proxy of the mutation rate and hence independent of variation in body size or other life history traits.

We also performed divergence time analyses on the data set used for the species tree analyses (see above). We used a combination of the ATP6 rate (0.026 s/s/l/myr; 95%

HPD: 0.021–0.031 s/s/l/myr) and a substitution rate for TGFb2 (0.0017 s/s/l/myr; 95% HPD: 0.0013–0.0022 s/s/l/myr; Lerner *et al.* 2011).

We used TRACER v1.6 (Rambaut *et al.* 2014) to ensure that our effective sample size of the underlying posterior distribution was large enough (>200) for meaningful estimation of parameters.

Molecular species delimitation methods

We used a Bayesian implementation of the general mixed Yule-coalescent model (bGMYC 1.0; Reid & Carstens 2012) to delimit species with our molecular data. This implementation is an extension of the GMYC model (Pons *et al.* 2006) that incorporates gene tree uncertainty by sampling over the posterior distribution of sampled gene trees. We obtained a posterior distribution of ultrametric gene trees of the unique *D. atripennis–D. ludwigii* mitochondrial haplotypes using BEAST v1.8 (Drummond & Rambaut 2007) under an uncorrelated lognormal clock model (0.026 s/s/l/myr, standard deviation = 0.0025). We ran MCMC for 10^7 iterations with sampling of parameters and trees every 10^3 iterations. The first 10% of the samples were removed as the burn-in period. We analysed 100 trees sampled randomly from the posterior distribution and used the default setting in bGMYC. We ran the MCMC chains for 5×10^4 iterations, with a burn-in of 4×10^4 iterations, and sampled parameters every 100 iterations.

We also used the software BPPv3.1 (Rannala & Yang 2003; Yang & Rannala 2010; Yang 2015) to estimate the joint probability of the species tree and the speciation probability (model A11, Yang & Rannala 2014). A speciation probability of 1.0 on a node indicates that every species delimitation model visited by the rjMCMC algorithm supports the hypothesis that the two lineages descending from a particular node represent distinct taxa (species). We consider speciation probability values >0.95 as strong support for a putative speciation event. We made use of a gamma prior on the population size parameters (θ) and the age of the root in the species tree (τ_0), while the other divergence time parameters were parameterized with a Dirichlet prior (Yang & Rannala 2010). We restricted the analyses to eight taxa, the six lineages within the *D. ludwigii–D. atripennis* complex as well as two outgroup species (*D. aeneus* and *D. leucophaeus*). We evaluated the influence of the priors on the posterior probability distribution by changing the priors for θ and τ_0, assuming either small or large ancestral population size with G set to (2, 2000) and (1, 10), respectively, and shallow or deep divergence with G set to (2, 2000) and (1, 10), respectively. We allowed the loci to have different rates (locus rate = 1 and used a Dirichlet distribution) and took into account the differences in heredity scalar (heredity = 2). We ran the rjMCMC analyses for 4×10^5 generations with a burn-in period of 4×10^4 and different starting seeds. Each analysis was run twice.

Results

Phylogenetic relationships

Mitochondrial data. We obtained the complete ATP6 sequence for 78 individuals (48 haplotypes) of the *D. atripennis–D. ludwigii* complex. The MK tests on the *D. atripennis–D. ludwigii* clade using sequences from three different proximate outgroups were all non-significant (Fisher's exact test: *D. leucophaeus*, $P = 1.0$, *D. macrocercus*, $P = 0.39$; *D. modestus*, $P = 0.41$).

Phylogenetic reconstructions of the different haplotypes recovered three primary clades within the *D. atripennis–D. ludwigii* complex (Fig. 1). The Square-tailed Drongo (*D. ludwigii*), as currently defined, was not monophyletic as the savanna populations from central (*D. l. saturnus*) and western Africa (*D. l. sharpei*) were more closely related to *D. atripennis*, a species restricted to the interior of lowland forests, than to populations from eastern and southern Africa (*D. l. muenznerii*, *D. l. tephrogaster* and *D. l. ludwigii*). Substantial genetic divergences, that does not match subspecies delimitation was recovered between populations distributed on both sides of the Niger River for the *D. l. sharpei-saturnus* clade. Yet, small genetic divergences were recovered among individuals sampled within *D. l. sharpei* West of the Niger River (distributed from Nigeria to Guinea) and within *D. l. sharpei—saturnus* East of the Niger River (from Katanga Province DRC to Cameroon; hereafter referred to *sharpei* East of the Niger River), although the distribution of the latter lineage appears to be disjunct, with a break in north-western Angola (Fig. 1). There was also strong genetic differentiation between east and southern African populations, with populations from South Africa and Malawi (*D. l. ludwigii*) being divergent (average number of nucleotide substitution per site, Dxy = 3.8%) from populations distributed in Eastern Africa (*D. l. muenznerii*). The two individuals sampled from Mozambique (*D. l. tephrogaster*) belonged to two different mitochondrial subclades, one (FMNH438648) being nested within the Eastern African clade, whereas the phylogenetic placement of the haplotype from the second individual (FMNH438649) was unresolved between *D. l. ludwigii* and *D. l. muenznerii*.

Nuclear gene trees. We could not obtain any nuclear sequences from the toe-pad samples. We did not detect any evidence of recombination across our Dicruridae sequences for any of the four introns. We did not detect any evidence of selection on the nuclear loci based on the HKA test ($P = 0.69$). Incorporating the alleles with phasing

probability lower than 0.6 had no (MB, TGFB2, BRM)-to-very limited effect (FGB) on the topology as the number of sites per individual with phasing probability lower than 0.6 was never more than one for species from the *D. atripennis–D. ludwigii* clade. This lack of statistical power stems from a mutation being present in only a single individual in the data set, which most present probabilistic algorithms would find difficult to resolve.

The gene trees from the four loci generally were poorly resolved, with only a few nodes receiving strong support (Figs S1–S4). However, several patterns emerged consistently across the nuclear loci. First, the eastern and southern lineages of *D. ludwigii* (*D. l. ludwigii–muenznerii–tephrogaster*) formed a clade in all three autosomal introns; each of the three taxa possessed several unique alleles and allele sharing among the three lineages usually involved the two most common alleles. Second, the central (*D. l. sharpei* E of Niger River) and western lineages (*D. l. sharpei* W of Niger River) were monophyletic for two of three autosomal introns (FGB and MB) and paraphyletic for the third locus (TGFb2). Third, *D. atripennis* had very high allelic diversity at all nuclear loci and the alleles attributed to this taxon never formed a monophyletic group in any analyses for the three autosomal introns. Alleles from *D. atripennis* were usually found to be paraphyletic to *D. ludwigii sensu lato* (FGB, TGFb2) but these were polyphyletic in MB (four alleles were related to *D. balicassius–D. bracteatus*). Finally, there was a tendency for the alleles from *D. atripennis* and *D. ludwigii* to form a clade (FGB: 0.82, TGFb2: 1.0); the MB gene tree is characterized by a large polytomy at the base.

Allele sharing across species was common for the Z-linked locus (e.g. between *D. adsimilis* and *D. atripennis* or between *D. forficatus* and *D. ludwigii*), possibly due to the short intron length (about 360 bp) and hence limited number of informative characters. Noticeably, the two alleles that were shared across species were in a central position in the allele network, as would be expected under the ancestral condition; however, most of the primary lineages within the *D. ludwigii–D. atripennis* clade possessed derived (private) haplotypes. For example, *D. l. sharpei* had private alleles, whereas for *D. l. muenznerii* or *D. atripennis*, all but one allele were restricted to these taxa.

Multilocus network and species tree

The multilocus network obtained using the mitochondrial and nuclear sequences revealed well-differentiated groups corresponding to *D. atripennis*, *D. sharpei* (*D. l. sharpei–*

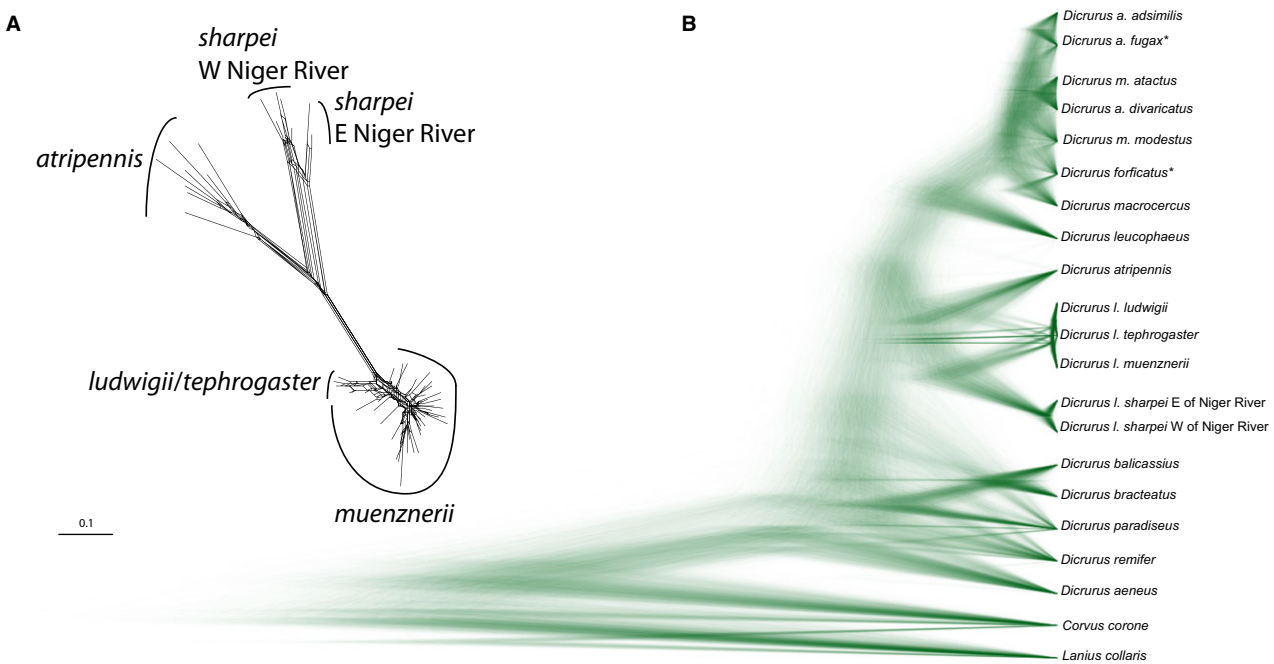

Fig. 2 —A. Multilocus network obtained using standardized genetic distances from the five loci for all individuals from the *Dicrurus atripennis–D. ludwigii* complex for which all loci were available (n = 65). —B. Species trees obtained using the algorithm implemented in *BEAST (Heled & Drummond 2010) with sequences from all loci: one mitochondrial and four nuclear loci. All primary lineages were included in the species tree analysis despite some individuals not being sequenced for all loci (e.g. nuclear loci for the historical samples). The species tree obtained using the nuclear data only was very similar; the asterisks indicate the two taxa that were inverted in the mitochondrial-nuclear vs. nuclear only topologies. The cloudogram was obtained using DENSITREE v2.2.1 (Bouckaert 2010) and reflect the uncertainty in the posterior distribution of sampled topologies.

Table 1 Estimates of divergence times within the *Dicurus ludwigii–D. atripennis* complex.

Clade	ATP6 clock (mtDNA only)	ATP6 uncorrelated lognormal (mtDNA only)	ATP6 fourfold (mtDNA only)	ATP6 clock, TGFb2 clock (species tree – *BEAST)	ATP6 uncorrelated lognormal, TGFb2 clock (species tree – *BEAST)
D. ludwigii/D. atripennis	4.0 (3.0–5.0)	3.3 (1.8–5.0)	4.2 (2–7.4)*	3.4 (2.4–4.3)	3.3 (2.4–4.2)
D. l. ludwigii/D. l. muenznerii	0.9 (0.6–1.3)	1.6 (0.6–2.8)	1.2 (0.4–2.2)	0.15 (0.06–0.3)	0.14 (0.07–0.24)
D. l. sharpei–D. l. saturnus/D. l. ludwigii/D. l. muenznerii	NA	NA	NA	2.9 (1.8–3.9)	2.7 (1.8–3.7)
D. atripennis/D. l. sharpei–D. l. saturnus	3.7 (2.8–4.8)	2.9 (1.5–4.5)	3.2 (1.3–5.7)	NA	NA
D. atripennis	0.6 (0.5–1.1)	1.0 (0.3–2.0)	0.8 (0.2–1.5)	NA	NA
D. l. sharpei W of Niger River/*D. l. sharpei* E of Niger River–*D. l. saturnus*	1.5 (1.0–2.0)	1.6 (0.6–2.7)	1.6 (0.6–3.0)	0.3 (0.1–0.5)	0.3 (0.1–0.5)

*Not monophyletic.

saturnus) and *D. ludwigii* (*D. l. ludwigii–muenznerii–tephrogaster*) (Fig. 2A). The topology from the species tree analyses (nuclear and all loci combined) recovered the monophyly of the *D. atripennis–D. ludwigii* clade (PP_{Nuc}: 0.96/ $PP_{Nuc\text{-}Mt}$: 0.99; Fig. 2B) and the monophyly of the two primary lineages within *D. ludwigii* (*D. l. sharpei* E of the Niger River–*D. l. sharpei* W of the Niger River, as well as *D. l. ludwigii–muenznerii–tephrogaster*). Unlike in the mitochondrial analyses, *D. ludwigii* was found to be monophyletic, although without statistical support (PP_{Nuc}: 0.68/ $PP_{Nuc\text{-}Mt}$: 0.58). Two other species, *D. adsimilis* and *D. modestus*, were not recovered as monophyletic in the species tree, as the populations of the northern savanna regions (*D. modestus* and *D. adsimilus divaricatus*) appear to be separated from *D. a. adsimilis* and *fugax* of the southern savannas (Fig. 2B), but nodes in the *D. macrocercus–D. adsimilis* clade are poorly supported with the present five locus data set.

Divergence times

Our divergence time analyses using the mitochondrial data set revealed that the split between the two primary clades (*D. atripennis–D. l. saturnus—sharpei* and *D. l. ludwigii–muenznerii–tephrogaster*) occurred between 3.3 and 4.2 mya (Table 1), depending on the substitution rate used. This distinction implies a split between central and western lineages, and between eastern and southern lineages. The split between *D. atripennis* and the central and western subspecies of *D. ludwigii* occurred shortly after the initial split (between 2.9 and 3.7 mya). The split between the central and western populations of *D. ludwigii* occurred about 1.5 mya (1.5–1.6 mya), and the split between eastern and southern lineage was estimated to have occurred between 0.9 and 1.6 mya. The split between the *D. atripennis* populations distributed east and west of the Dahomey Gap/ Niger River area was estimated to have occurred 0.6–1.0 mya. The estimates using the different substitution rates and data partitions were very similar, although the estimates using the uncorrelated lognormal clock model yielded estimates that were slightly different (Table 1), including for the relative order of lineage divergence.

The divergence times obtained using the species tree algorithm were very similar to those estimated using the unique mitochondrial haplotypes (Table 1), although strong discrepancies occurred towards the tips of the tree for estimates of the Times to Most Recent Common Ancestor between some of the lineages we recognize (e.g. *D. l. sharpei* E Niger River/*D. l. sharpei* W Niger River: 1.6 mya in the mitochondrial analyses vs. 0.3 mya for the species tree).

Statistical tests for species delimitation

There was strong agreement between the two methods we used to delimitate species using molecular data.

The molecular species delimitation method bGMYC, using the mitochondrial haplotypes only, indicated that the current diversity at the species level in the *D. atripennis–D. ludwigii* clade is underestimated as five species were recognized at the 0.05 threshold; the five lineages that may represent biological species correspond to: *D. l. muenznerii*, *D. l. ludwigii* (including *D. l. tephrogaster*), *D. atripennis*, *D. l. sharpei* East of the Niger River (including *D. l. saturnus*) and *D. l. sharpei* West of the Niger River (Figs 3 and 4).

The analyses performed with BPPv3.1 were consistent with the bGMYC analyses as four lineages (*saturnus–sharpei*, *muenznerii*, *ludwigii–tephrogaster*, *atripennis)* had speciation probabilities of 1.0, a result that was not sensitive to any combination of values for tree depth ($G = 1$, 10 or $G = 2$, 2000) or effective population size ($G = 1$, 10 or $G = 2$, 2000). Concerning the possible split of *D. l. sharpei* East of the Niger River (including *D. l. saturnus*) and *D. l. sharpei* West of the Niger River, the posterior probabilities for the two lineages being considered different species varied between 0 (combination of tree depth $G = 1$, 10 and effective population size $G = 2$, 2000) and 0.99–1.0 (combination of tree depth $G = 1$, 10 and effective population size $G = 1$, 10 and combination of tree depth $G = 2$,

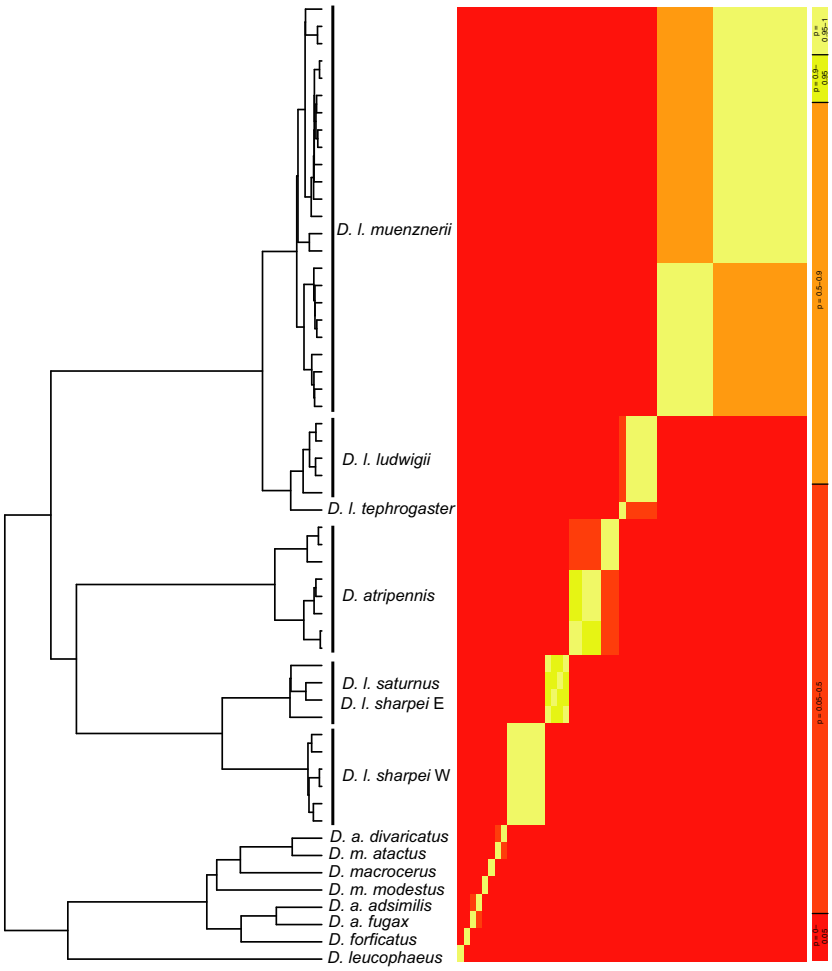

Fig. 3 Summary of the bGMYC species delimitation method using the mitochondrial data set. The tree is the maximum clade credibility tree from BEAST within the *D. adsimilis–D. ludwigii* clade. The heat map is a sequence-by-sequence matrix in which cells are coloured by the posterior probability such that the corresponding sequences are conspecific, with increasing probability represented by light yellow to red colours. The scale is given on the right of the tree. The analyses would support the recognition of five species within the *D. atripennis–D. ludwigii clade*.

2000 and effective population size G = 1, 10), depending on prior combinations. Hence, the most crucial parameter regarding the two *sharpei* lineages (E and W of the Niger River) is effective population size which is very variable across the different loci (as inferred from Watterson's theta; Table 2). Only one prior combination (tree depth G = 2, 2000; effective population size G = 1, 10) made the distinction between *tephrogaster* and *ludwigii* significant (P = 0.97); for all other prior combinations, the posterior probabilities for the split varied between 0.43 and 0.87.

Hence, both algorithms would recognize at least four species: *atripennis*, *sharpei muenznerii* and *ludwigii–tephrogaster*, with strong support towards splitting *sharpei* into two species separated by the Niger River.

Discussion

Diversification at the savanna-forest ecotone

Our analyses revealed a novel pattern of genetic variation in a broadly distributed sub-Saharan lineage of birds, implying complex patterns and processes of diversification across the savanna belt and the two primary lowland forest blocks of Africa.

Hall & Moreau (1970) identified sets of closely related species, including the *D. atripennis–D. ludwigii* superspecies, that differ in habitat use. Several previous studies investigating phylogeographic patterns in Afrotropical birds have tended to be restricted to a particular habitat or subregion (e.g. Upper/Lower Guinea Forest Blocks: Marks 2010; Fuchs & Bowie 2015; Eastern Africa Bowie *et al.* 2004, 2006; Fuchs *et al.* 2011b; Southern Africa: Ribeiro *et al.* 2011; Oatley *et al.* 2012; Ribeiro *et al.* 2014). Consequently, the superspecies hypotheses proposed by Hall & Moreau (1970) has remained largely untested using molecular data. Although Hall & Moreau (1970) did not explicitly propose superspecies status for members of the Fiscal Shrike (*L. collaris*) species group that occupy different habitats, the molecular phylogeny of Fuchs *et al.* (2011a) recovered a pattern of a forest-associated lineage (*L. mackinnoni–L. newtoni*) being sister to a species adapted to open savanna (*L. collaris sensu lato*). These results suggest that

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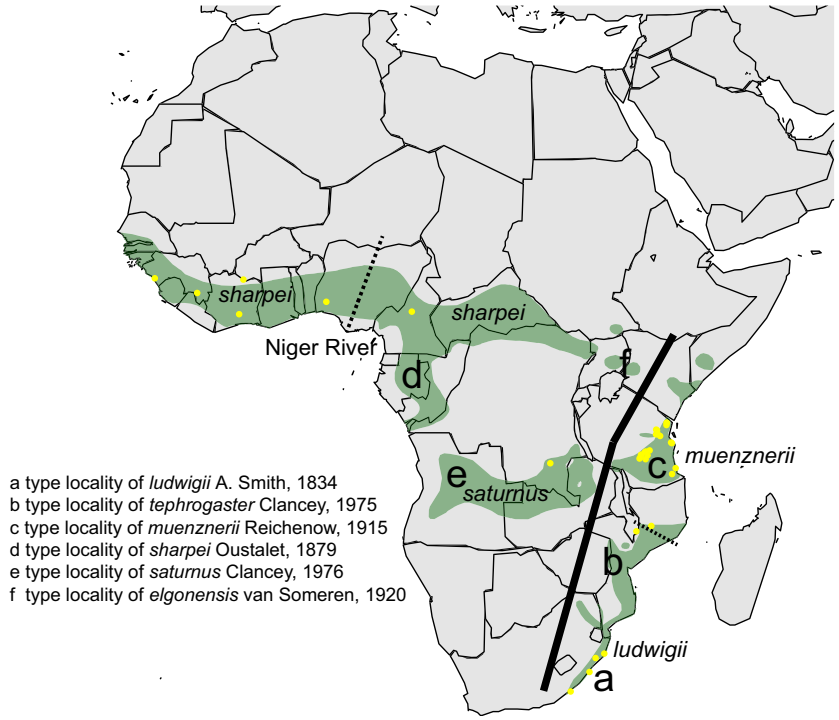

Fig. 4 The distribution of the primary lineages within *D. ludwigii sensu lato* as inferred from our analyses. Yellow dots indicate sampling localities in the present study. The black line indicates the split between the two primary lineages within *D. ludwigii sensu lato* (*D. l. saturnus–D. l. sharpei–D. l. elgonensis* to the west and *D. l. ludwigii–D. l. muenznerii–D. l. tephrogaster* to the east), whereas the dashed lines indicate the geographic locations of the putative breaks within each of the two primary lineages. Approximate locations of the type localities for every known available name are indicated by the letters *a* to *f*. There is no available name for the lineage distributed west of the Niger River and we refrain from proposing a name until further individuals are examined.

population divergence could have taken place across ecological gradients (i.e. a parapatric mode of divergence, Smith *et al.* 1997; Moritz *et al.* 2000) although a period of allopatry followed by secondary contact of lineages cannot be excluded. Strong genetic structure was also recovered across the savanna-adapted shrike complex, with three primary lineages, *L. souzae* (found in the Miombo woodlands), *L. collaris* (South Africa, Namibia to Malawi, Tanzanian highlands) and *L. humeralis* (Malawi, Tanzania, Kenya to western Africa).

The biogeographic pattern recovered for the *D. atripennis–D. ludwigii* superspecies is very similar to that described for the *Lanius* shrikes (Fuchs *et al.* 2011a), with three primary lineages: (i) a forest-associated species distributed in the Lower Guinea Forest Block (*L. mackinnoni* for *Lanius*, *D. atripennis* in *Dicrurus*), (ii) a lineage distributed in West Africa (*L. smithii* in *Lanius* and *D. sharpei–D. saturnus* in *Dicrurus*) and (iii) a lineage distributed in southern and eastern Africa (*L. collaris–L. souzae* in *Lanius* and *D. ludwigii–muenznerii–tephrogaster* in *Dicrurus*). In both cases, the relationships among the three primary savanna-open forest lineages received limited support in the species tree analyses (PP: 0.45 for *Lanius*, Fuchs *et al.* 2011a; PP: 0.58 for *Dicrurus*), suggesting that the three primary lineages could have diverged within a short time period. Despite these broad-scale similarities, noticeable differences also exist between the patterns recovered in the two species complexes. First, the relationships of the eastern lineages vary between *Lanius* (subspecies *humeralis* being related to the western African subspecies *smithii*) and *Dicrurus* (subspecies *muenznerii* being related to the southern African lineage and not to *saturnus–sharpei*). Second, and despite the fact that both lineages diversified over a short period of time, the timing of divergence among members of each species complex differed when using the same mitochondrial locus and substitution rate (neutral fourfold rate): the earliest divergence was found to be 2.2 mya (0.9–3.8 mya; Fuchs *et al.* 2011a) for *Lanius* and 4.2 (2–7.4 mya) for *Dicrurus*, suggesting that different Earth history events likely shaped the diversification patterns of these two species complexes.

The most surprising aspect of our results involves the lack of genetic differentiation between individuals sampled in south-eastern DRC (subspecies *saturnus*) and Cameroon (*sharpei*), because of the geographical distance involved and the apparent existence of distribution gaps (Figs 1 and 4). Tectonic uplift along the equatorial western Africa margin has given rise to edaphic variation and formation of savanna ridges, especially in the Ogooué-Kwanza section, which could explain the continuous distribution of *D. ludwigii* across the rainforest zone (which is otherwise occupied by *D. atripennis*). However, there appears to be a true distribution gap in the savanna zone of south-western Congo and adjacent northern Angola. This pattern of a lack of genetic divergence in spite of a biogeographic discontinuity has also been highlighted for *Xenopus* frogs (Furman *et al.* 2015).

Table 2 Genetic diversity values (*N*, number of potential alleles; *S*, number of segregating sites; Hd, Haplotype diversity; π, nucleotide diversity; Θ, Watterson's theta) for the primary lineages with the *Dicrurus atripennis–D. ludwigii* complex. *F*u's *F*s and R^2 were not performed for sample sizes <5 alleles

	ATP6	MB2	FGB5	TGFb2	BRM
	684	718	564	568	355
D. atripennis					
N	12	18	18	18	14
S/Haplotypes/Hd	33/8/0.924	10/9/0.758	23/16/0.987	15/14/0.967	8/6/0.802
π/Θ	0.01715/0.01598	0.00212/0.00405	0.00962/0.01184	0.00701/0.00768	0.00494/0.00703
Fs/R^2	1.154/0.1592	−4.948*/0.0755*	−9.521*/0.101	−7.629*/0.1138	−1.299/0.1299
D. l. ludwigii (South Africa)					
N	7	14	12	14	11
S/Haplotypes/Hd	6/4/0.81	2/3/0.56	1/2/0.41	2/3/0.56	0/1/0
π/Θ	0.00362/0.00358	0.00119/0.00088	0.00072/0.00059	0.00133/0.00111	0/0
Fs/R^2	0.281/0.1815	0.535/0.2143	0.735/0.2045	0.292/0.1896	NA/NA
D. l. tephrogaster (Malawi/Mozambique)					
N	3	6	6	6	3
S/Haplotypes/Hd	29/3/1	2/3/0.80	1/2/0.333	2/3/0.60	0/1/0
π/Θ	0.02875/0.2827	0.00149/0.00122	0.00059/0.00078	0.00153/0.00154	0/0
Fs/R^2		−0.082/0.2667	−0.003/0.3727	−0.427/0.1896	
D. l. muenznerii (Tanzania)					
N	45	82	86	88	71
S/Haplotypes/Hd	34/23/0.866	12/13/0.792	16/17/0.509	19/18/0.75	6/8/0.00287
π/Θ	0.00491/0.01138	0.00182/0.0037	0.00254/0.00563	0.00201/0.00663	0.00287/0.00347
Fs/R^2	−14.254*/0.12	−6.201*/0.0295*	−10.612*/0.445	−14.795*/0.0322*	−2.381/0.0855
D. l. sharpei (E of Niger River)–*D. l. saturnus*					
N	4	4	4	4	3
S/Haplotypes/Hd	14/4/1	2/2/0.50	5/3/0.833	3/4/1	0/1/0
π/Θ	0.01048/0.01116	0.00139/0.00152	0.00531/0.00483	0.00323/0.00288	0/0
D. l. sharpei (W of Niger River)					
N	7	10	10	10	7
S/Haplotypes/Hd	13/6/0.952	4/4/0.644	7/6/0.778	9/8/0.956	1/2/0.286
π/Θ	0.00627/0.00776	0.00155/0.00197	0.00397/0.00438	0.00587/0.0056	0.0008/0.00114
Fs/R^2		−0.657/0.0295*	−1.533/0.1345	−3.202*/0.167	−0.095/0.3488

Asterisks indicate $P < 0.05$.

Biogeographic patterns in Eastern Africa and Southern Africa

The phylogeographic pattern revealed in the eastern and southern African clade indicates a geographic divergence between the eastern and southern groups in southern Malawi, a pattern mirroring subspecies boundaries (*muenznerii* vs. *ludwigii–tephrogaster*) in accordance with current taxonomy (Dickinson & Christidis 2014). This geographic transition is in accordance with the geographic placement of the division between the two major lineages of Fiscal Shrike (northern and southern lineages; Fuchs *et al.* 2011a), suggesting that southern Malawi constitutes an important region of faunal turnover between eastern (N Malawi, Tanzania, Kenya) and southern (S Malawi Mozambique, Zimbabwe, South Africa) African lineages. The splits among the savanna-adapted lineages occur at slightly different times (0.9–1.6 mya ago for *Dicrurus* and 2.2 mya for *Lanius*), indicating that different Earth history events may have promoted genetic divergence of these two taxa. However, the 95% HPD intervals (0.6–1.3 and 0.6–2.8 depending on the molecular clock assumption for *Dicrurus* and 0.9–3.8 for *Lanius*) do overlap and given the inherent uncertainty in dating, the possibility of a single period of divergence due to a common vicariant event cannot be rejected. Regardless of the number of divergence events, the divergence of southern and eastern lineages occurred during the Pleistocene, suggesting that the divergence of avian lineages in this part of Africa may have been caused by the increased amplitude of climatic oscillations, orbital insulation and the progressive drying of eastern Africa due to tectonic uplift (Blome *et al.* 2012; Prömmel *et al.* 2013; Lyons *et al.* 2015).

We found evidence of possible past or current hybridization in both *Lanius* (one individual collected on Nyika Plateau, northern Malawi, had the southern lineage haplotype) and *Dicrurus* (one individual collected in Mozambique –

FMNH 438648 – theoretically in the range of *tephrogaster* had a *muenznerii* haplotype). All nuclear alleles from FMNH 438648 were shared and usually involved the most common allele from the eastern–southern lineage, so it is not possible at present to conclude whether this haplotype is the result of ancient or recent gene flow, or even the retention of ancestral polymorphism.

It should be noted that northern Mozambique remains a significant collecting gap (in general, for fauna and flora), and we therefore do not know whether the distribution gap that appears there (in Figs 1 and 4) is real or not. Tissue samples from Rondo Plateau and Nkubege Forest in the Lindi District in south-eastern Tanzania grouped with *muenznerii*, but other voucher specimens from Lindi and Mtwara districts (not used for genetic samples due to lack of tissues) show phenotypic traits resembling subspecies *tephrogaster*, with generally rather slight and greenish blue gloss and dull slate-grey rump and underparts below the breast, and generally small size (wing: 92–104 and tail: 76.5–88 mm). Hence, the mosaic of savanna scrubland and semi-evergreen woodlands along the coast of south-eastern Tanzania and northern Mozambique could be a zone of contact between *muenznerii* and *tephrogaster–ludwigii*.

The Eastern Arc Mountains of Tanzania represent habitat configurations where animals and plants could persist throughout the Pleistocene, putatively because of predictably high precipitation (Fjeldså & Bowie 2008; Fjeldså *et al.* 2012). Here, *D. l. muenznerii* is patchily distributed, with local strongholds in places where a habitat continuum exists from the semi-evergreen lowland forests to evergreen montane forest (Fjeldså *et al.* 2010), and the drongos inhabiting these forest tracts are phenotypically quite variable. Half of the birds resemble those of the coastal lowlands of Tanzania and Kenya, but nine (see Fig. 1) of 19 (47%) voucher specimens of birds used for the phylogenetic analysis were rather large (wing 103–114, tail 89.5–93.5 mm), with a stronger tendency towards a forked and lyre-shaped tail, more prominent stiff and forward-directed forehead feathers and generally more shiny blue plumage, approaching *D. atripennis* phenotypically. However, this dichotomy in plumage traits is found along the entire mountain range and is not reflected in the variation in mitochondrial DNA. Variation in the expression of nuclear genes involved in the control of feather structure and melanization could be a potential explanation (e.g. Poelstra *et al.* 2014).

Within the southern group (South Africa to Malawi), very little genetic differentiation was detected among populations sampled on either side of the Limpopo River, where Clancey (1976) suggested a transition between the subspecies *ludwigii* and *tephrogaster*.

Evolution in the Guineo-Congolian forest blocks

Our analyses revealed that the split between the *D. atripennis* populations distributed east and west of the Dahomey Gap/Niger River area is of comparable age (0.6–1.0 mya) with the estimates of divergence times within two species of woodpecker (*Campethera caroli* and *C. nivosa*; 0.6 and 1.2 mya, respectively; Fuchs & Bowie 2015). Hence, our data suggest that the two primary biogeographic barriers that separated the Upper and Lower Guinea Forest blocks appeared between 0.5 and 1.2 mya, possibly playing a significant role by facilitating allopatric divergence and thereby elevating the number of endemic species attributed to each of these forest blocks.

One further noticeable feature found within the forest species *D. atripennis* is the high haplotype and nucleotide diversity in the nuclear introns. Such a high level of genetic diversity without phylogeographic structure was also recovered in the Buff-spotted Woodpecker (*Campethera nivosa*), similarly distributed across the Lower Guinea Forest Block (Fuchs & Bowie 2015). This pattern of molecular variation would support either the hypothesis of high effective population size throughout the evolutionary history of *D. atripennis* and hence habitat stability in the tropical forests of Africa (e.g. Fjeldså & Bowie 2008; Fjeldså *et al.* 2012) or several episodes where populations persisted in different refugia during dry and cold periods before re-emerging during population expansion. Hence, a pattern of high genetic variability and low levels of genetic structure could potentially be expected for birds found in the Lower Guinea Forest Block. Yet, current genetic data also suggest that strong phylogeographic structure exists in some understorey birds distributed in the Lower Guinea Forest Block (Voelker *et al.* 2013). Consequently, differences in the levels of genetic structure between lineages could be explained by differential dispersal capacities among lineages, where understorey birds are more sensitive to fragmentation of habitat than mid-storey (e.g. woodpeckers) or canopy birds (e.g. drongos) (Burney & Brumfield 2009 for an example on Neotropical birds).

Taxonomy and nomenclature

Our study revealed that the diversity at the species level within the *D. atripennis–D. ludwigii* species complex has been underestimated, with the number of lineages that could be considered species varying between three and five.

The Square-tailed Drongo (*D. ludwigii sensu lato*) consists of two primary lineages (central-western *saturnus–sharpei* and eastern–southern *ludwigii–muenznerii–tephrogaster*). The monophyly of *D. ludwigii sensu lato* remains uncertain as mitochondrial data suggested that the central-western subspecies are more closely related to *D. atripennis* than to the eastern and southern subspecies, whereas the species tree approach suggested *D. ludwigii sensu lato* to be

monophyletic, although with low support. The two primary lineages do not share any mitochondrial or nuclear alleles suggesting complete lineage sorting. Furthermore, the two molecular species delimitation methods (bGMYC and BPP) also suggested that the two lineages are distinct at the species level. Finally, the two lineages are characterized by differences in plumage: individuals of the subspecies *saturnus* and *sharpei* have white tips (or at least some traces) on the axillaries and on the small feathers along the ventral edge of the metacarpus, whereas they are generally absent in the subspecies *ludwigii*, *muenznerii* and *tephrogaster* (Vaurie 1949). The few cases of individuals of *ludwigii–muenznerii–tephrogaster* with white feather tips on these feathers have dull greyish plumage as a sign of immaturity. The two primary lineages also differ in the gloss of the mantle feathers, which is dark greenish blue in *ludwigii–muenznerii–tephrogaster*, against more ultramarine blue in *sharpei* (Vaurie 1949, and our own morphological notes). Consequently, there are diagnosable molecular and morphological characters that enable differentiation of the central and western lineage from the eastern and southern lineage. We acknowledge that these morphological characters are subtle, but given the high degree of morphological similarity across drongo species, they appear to be diagnostic. We propose that *D. ludwigii sensu lato* be split into at least two species: Square-tailed Drongo *D. ludwigii* (A. Smith, 1834; including the subspecies *tephrogaster* Clancey, 1975 and *muenznerii* Reichenow, 1915) and Sharpe's Drongo *D. sharpei* Oustalet, 1879 (including *saturnus* Clancey, 1976, and *elgonensis* Van Someren, 1920). Although both molecular species delimitation methods may suggest that *D. l. ludwigii–D. l. tephrogaster* and *D. l. muenznerii* could be distinct at the species level, we recommend further studies be performed to document the extent of gene flow between the two lineages in Malawi and Mozambique as a *tephrogaster* haplotype was nested in *muenznerii*.

The nomenclatural situation of clades encompassing the newly proposed *D. sharpei* is very complex as two primary lineages are recovered, which may themselves warrant species status according to the bGMYC analyses. Hence, our molecular data are not in accordance with the traditional taxonomy: *D. l. sharpei* is thought to occur from Senegal–Gambia to southern Sudan and western Kenya and south to the lower Congo River and north-western Angola, whereas *D. l. saturnus* occurs in central Angola, northern Zambia and southern Malawi (Dickinson & Christidis 2014). Our mitochondrial data suggest a very close relationship between individuals sampled in Katanga, south-eastern DRC (corresponding to the range of *saturnus*) and individuals sampled in northern Cameroon, that is in the theoretical range of *D. l. sharpei*, whereas individuals sampled from western Nigeria to Guinea (also in the theoretical range of *sharpei*) are more distantly related to these individuals. The type locality for *D. l. sharpei* Oustalet, 1879 is the Upper Ogoué River, Gabon. Hence, the name *D. l. sharpei* should apply to the lineage including the localities from Katanga and northern Cameroon, and by extension to Uganda and north-western Kenya, potentially also including *D. l. elgonensis* Van Someren, 1920. No names have been proposed for the populations distributed west of the Niger River to Senegal, and the substantial molecular differentiation between the two lineages across the Niger River may warrant the recognition of a distinct lineage in the West (Fig. 4). We refrain from describing this taxon until further material can be examined, including individuals sampled in Angola and Gabon, to determine whether geographical populations are morphologically diagnosable.

New classification

***Dicrurus ludwigii* A. Smith 1834**

Dicrurus ludwigii ludwigii A. Smith, 1834, which includes *tephrogaster* Clancey, 1975 (South Africa to Malawi, Central and Southern Mozambique).

Dicrurus ludwigii muenznerii Reichenow, 1915 (Northern Mozambique, Tanzania, Kenya).

***Dicrurus sharpei* Oustalet, 1879**

Dicrurus sharpei sharpei Oustalet, 1879, which includes *elgonensis* Van Someren, 1920; see Vaurie 1949 regarding the difference in gloss colour and intensity (N DR Congo, Uganda, S Sudan, W Kenya, to NW Angola and Nigeria east of the Niger River).

Dicrurus sharpei saturnus Clancey, 1976 (we recognize this subspecies until further individuals from Gabon and Angola are sampled to enable a broader comparison).

Dicrurus sharpei unnamed subspecies (tentatively Nigeria west of the Niger River to Senegal).

***Dicrurus atripennis* Swainson, 1837** Monotypic (Sierra Leone to NE DR Congo).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The majority rule (50%) consensus trees resulting from the Bayesian analyses of FGB5.

Fig. S2. The majority rule (50%) consensus trees resulting from the Bayesian analyses of MB.

Fig. S3. The majority rule (50%) consensus trees resulting from the Bayesian analyses of TGFb2.

Fig. S4. The majority rule (50%) consensus trees resulting from the Bayesian analyses of BRM.

Table S1. List of the *Dicrurus* samples used in this study.

Table S2. (a) Primers sequences used to PCR-amplify and sequence the DNA. (b) Primers sequences/combination used to PCR-amplify and sequence the DNA from historical samples.