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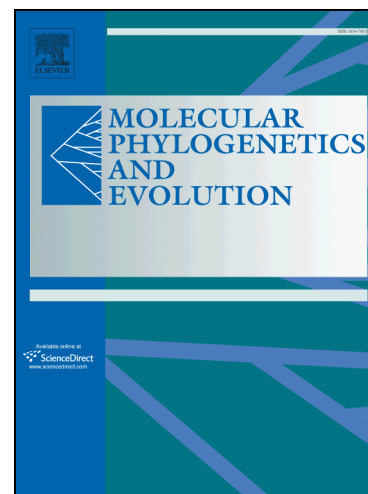Please cite this article as: Fuchs, J., Bowie, R.C.K., Concordant genetic structure in two species of woodpecker distributed across the primary West African biogeographic barriers, *Molecular Phylogenetics and Evolution* (2015), doi: http://dx.doi.org/10.1016/j.ympev.2015.03.011

Concordant genetic structure in two species of woodpecker distributed across the primary West African biogeographic barriers

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ABSTRACT

The lowland forests of western and central tropical Africa are separated by several potential biogeographic barriers to dispersal for forest adapted vertebrates. The two primary barriers are 1) the Dahomey Gap, a savanna corridor that reaches the coast of southern Ghana, Togo and Benin, and separates the West African rainforest into the Upper (Ghana west to Guinea) and Lower Guinea (Nigeria to Uganda and Angola) forest blocks, and 2) the Lower Niger River, a large delta that separates Western and Eastern Nigeria. Previous studies on terrestrial vertebrates (lizards, mammals and birds) have highlighted a genetic break in the Dahomey Gap/Lower Niger River area although the relative importance of each barrier has not been assessed due to limitations in geographic sampling or properties of the markers used. We compared the phylogeographic history of two co-distributed sister-species of woodpeckers (*Campethera caroli* and *C. nivosa*) using data from three loci representing all inheritance modes. Our analyses revealed that both the Dahomey Gap and possibly the Lower Niger River acted as strong biogeographic barriers for the two woodpecker species, with the Lower Niger River being the first barrier to have formed, leading to three distinct populations of *C. nivosa.* Our divergence time analyses revealed that both these biogeographic barriers formed during the Pleistocene, supporting the Pleistocene refuge hypothesis, with the Dahomey Gap likely appearing about 0.5 myr BP. No genetic structure was recovered among sampled populations in either the Upper or the Lower Guinea Forest Block for both species, despite the considerable geographic area covered.

KEYWORDS: Lowland forest, Dahomey Gap, Lower Niger River, phylogeograpy, Pleistocene, *Campethera*

INTRODUCTION

The most prominent distributional disjunction for lowland forest taxa in west Africa is the Dahomey Gap, a broad savanna corridor that extends from the arid interior of the Sahel to reach the coast of southern Ghana, Togo and Benin, thereby interrupting the West African lowland rainforest (Moreau 1966, Hall and Moreau 1970, Crowe and Crowe 1982). The Dahomey Gap, delimited by the Volta and Wémé rivers (Booth 1958), breaks the Guineo-Congolian Forest into the Upper and Lower Guinea forest blocks. The spatial extent of the gap has varied in response to glaciation with Africa becoming more arid and the Dahomey Gap wider during glacial periods at higher latitudes. This dynamic process has led several authors to suggest that the high species diversity across the Guineo-Congolian rainforest might have arisen as a consequence of the formation of two primary refugia located in the Upper and Lower Guinea forest blocks, enabling population differentiation in allopatry, followed by putative secondary contact as forests expanded during interglacial periods (Mayr and O'Hara 1986). This basic premise has been expanded upon by other authors, leading to a more nuanced view of the number of refugia distributed across the Guineo-Congolian rainforest. For instance, Endler (1982) suggested the existence of one refugium in the Upper Guinea Forest block and two refugia in the Lower Forest Block. Comprehensive syntheses of the number and location of putative lowland refugia across Guineo-Congolia are provided by Grubb (2001) and Plana (2004).

Consistent with the refugium paradigm, molecular studies have recovered significant genetic differentiation among sister-lineages that are distributed across the Dahomey gap (e.g. Nesi et al. 2013, Leaché et al. 2014). For example, no haplotypes were shared between morphologically similar populations sampled across the Dahomey gap for two songbird species, *Stiphrornis erythrothorax* (Beresford and Cracraft 1999; Schmidt et al. 2008) and *Hylia prasina* (Marks 2010). The biogeographic pattern is likely even more complex as the populations from

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the Upper Guinea Forest block were paraphyletic in *Hylia* (Marks 2010), suggesting that further phylogeographic structure is apparent within the Upper Guinea Forest block, a result in conflict with Mayr and O'Hara's (1986) hypothesis of only one refugium west of the Dahomey Gap. Whereas the Dahomey Gap is consistently hypothesized to be a barrier for forest vertebrates, the Volta River (East of Ghana) and Niger River (Nigeria) may also act as barriers to dispersal for vertebrates. However, few recent authors investigating distribution patterns of diverse taxa have distinguished the area between the Volta and Niger rivers (east of the Dahomey Gap and West Nigeria) as a distinct faunistic zone or area of endemism, with Booth (1958; 'Western Nigeria') being an exception.

In accordance with Booth's (1958) hypothesis, some recent molecular studies on the phylogeographic structure of forest rodents, have demonstrated that populations sampled west of the Niger River and east of the Dahomey Gap are consistently differentiated from other Upper and Lower Guinea Forest Block populations (Nicolas et al. 2008, 2010) forming a discrete entity.

The Brown-eared (*Campethera caroli*) and Buff-spotted Woodpeckers (*C. nivosa*) are two sister-species of small African woodpeckers (14-18 cm) that share a similar distribution across the Upper and Lower Guinean forest blocks, with a break in distribution across the Dahomey Gap (Winkler and Christie 2002; Figure 1). The two species mostly occur in lowland primary or dense secondary forests where they feed on ants and termites. Two subspecies are currently recognized for *C. caroli*: *arizela* distributed west of the Dahomey Gap and *caroli* distributed east of the Dahomey Gap (Dickinson 2003). Three subspecies are currently accepted for *C. nivosa*: *nivosa* extending from Guinea to the western parts of the Democratic Republic of the Congo (DRC), *herberti* extending from Central and Northern DRC to Uganda and Kenya, and *poensis* restricted to Bioko Island of the west coast of Cameroon (Dickinson 2003). The present

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taxonomic arrangement for *C. nivosa* is not consistent with the distribution of primary biogeographic barriers that have been proposed for Guineo-Congolia (Dahomey Gap and or Niger Delta). Thus, it is plausible that despite a very similar present-day distribution pattern, the current taxonomy of these two sister-species of woodpeckers is reflective of different underlying evolutionary histories. Alternatively, several phylogeographic studies on African birds have demonstrated that geographic variation in plumage pattern is not necessarily correlated with spatial patterns of genetic variation (Marks 2010, Fuchs et al. 2011, Oatley et al. 2012). Thus, a full understanding of the evolutionary history of these Guineo-Congolian woodpeckers awaits the analysis of molecular data.

In the present study, we aim to 1) test the relative importance of each biogeographic barrier (Dahomey Gap, Niger River) in shaping the phylogeographic structure observed in these two co-distributed sister-species of Guineo-Congolian forest woodpeckers and (2) assess the extent to which groups of alleles delineated by DNA-based species delimitation methods correspond to described morphological diversity.

MATERIAL AND METHODS

Sampling and laboratory protocols

We sequenced 19 individuals of *Campethera caroli* and 68 individuals of *C. nivosa* that were sampled across the breeding range of the two species (Supplementary Table 1).

DNA was extracted from tissue or blood using the Qiagen extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocol. We sequenced three loci: a mitochondrial protein coding gene (ATP6), an autosomal intron (MB intron-2) and a Z-linked intron (BRM

intron-15). The PCR-amplification and cycle-sequencing conditions were standard for these loci (Supplementary Table 2). Newly generated sequences have been deposited in Genbank (Accession Number XXXXX-XXXXX). We used the primer pair 2550F and 2718R to sex individuals when no anatomical information was available (Fridolfsson and Ellegren 1999).

Determining the phase of alleles

We used PHASE V2.1.1 (Stephens et al., 2001), as implemented in DNASP 5.0 (Librado and 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.

Testing for selection

We used the McDonald-Kreitman test (McDonald and Kreitman, 1991) in DNASP 5.0 (Librado and Rozas, 2009) to test for evidence of selection acting on ATP6. We used sequences of *Campethera caillautii* and *Campethera abingoni* as outgroup taxa. Significance was assessed using Fisher's exact test and a threshold of 0.05. We tested for selection acting on the nuclear loci by using the HKA test (Hudson et al., 1987), as implemented in the software HKA (https://bio.cst.temple.edu/~hey/software/software.htm#HKA). Sequences from *Campethera abingoni* were used as an outgroup taxon.

Phylogenetic reconstruction

Gene tree reconstructions of the haplotypes were performed using Bayesian inference (BI), as implemented in MRBAYES 3.2 (Ronquist et al. 2012). For the substitution models, we

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made use of the *nst=mixed* option, such that model uncertainty is taken into account during the phylogenetic reconstruction, and incorporated rate variation using the *invgamma* setting. Four Metropolis-coupled MCMC chains (one cold and three heated) were run for $5*10^6$ iterations with trees sampled every 1000 iterations. We used default prior distributions for the substitution models and tried several prior distributions for the branch-length parameters. We used TRACER v1.5 (Rambaut and Drummond, 2007) to check that our effective sample size of the underlying posterior distribution was large enough for a meaningful estimation of parameters.

Population genetic analyses and demographic history

Haplotype diversity (Hd), nucleotide diversity (π) and Watterson's theta (θ) were estimated for each subspecies or clade recovered in our BI topology with DNASP 5.0. We used Fu's *Fs* test (1000 replicates) and Ramos-Onsins and Rozas R_2 statistic (Ramos-Onsins and Rozas, 2002) to detect signatures of demographic change. We considered insertion and deletion events in introns as informative mutational events. Sequence files were modified to take into account this form of genetic variation by replacing the missing sequence with a nucleotide that would induce a mutation.

We generated Extended Bayesian Skyline Plots (EBSP) using the mitochondrial and the phased nuclear data to explore differences in demographic history between groups (Heled and Drummond 2009); these graphs plot effective population size through time, providing a temporal reference to demographic events such as bottlenecks and expansions. Extended Bayesian Skyline Plots explicitly incorporate multiple, independent loci, and estimate the number of coalescent intervals of population size change. Each marker was assigned the best-fit model of nucleotide

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substitution as estimated by the BIC. We enforced a strict molecular clock for each locus to help the analyses converge. We ran three independent analyses, each of $25*10^6$ generations, for each of the three well-sampled clades (*C. caroli* Lower Forest Block, *C. nivosa* East of Niger River, *C. nivosa* West of Niger River), as implemented in BEAST version 1.7.4 (Drummond and Rambaut 2007). We used the rate proposed by Lerner et al. (2011) for ATP6 (0.026 s/s/l/myr; 95% HPD: 0.021-0.031 s/s/l/myr). We assessed convergence by comparing the resulting replicate plots, making sure each replicate recovered the same demographic patterns.

mtDNA divergence times

We estimated the Time to Most Recent Common Ancestor (TMRCA) among the *Campethera* haplotypes using BEAST 1.7.4 with a strict molecular clock model enforced, a HKY + I substitution model and a Yule tree prior. MCMC chains were run for 10^7 steps and were sampled every 10^3 steps. Inferring divergence times within species is a challenging task as internal fossil calibrations are rarely available. To circumvent this problem, we used three substitution rates, and their associated uncertainties, to calibrate the trees. Lerner et al. (2011), using complete mtDNA genomes from the honeycreepers (Passeriformes, Drepanididae) and calibration points based on the age of volcanic islands in the Hawaiian archipelago, proposed new substitution rates 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 could primarily be attributed to non-synonymous substitutions. They estimated the rate of evolution at four-fold degenerated sites from complete mtDNA sequences of Adelie Penguins (*Pygoscelis adeliae*) to be 0.073 (95% HPD: 0.025-0.123 s/s/l/myr). Finally, we also made use of the 'traditional' mitochondrial DNA substitution rate (2.1%/myr, 0.0105 s/s/l/myr, Weir and Schluter 2008). We

compared the divergence time estimates among the primary *C. caroli*/*C. nivosa* lineages obtained using these three independent substitution rates with estimates obtained using a calibration point of 2.8 myrs (normal distribution 95% HPD: 1.8-4.2 mya) for the *C. caroli*/*C. nivosa* split (Fuchs et al. 2007). The latter estimate was obtained using a secondary calibration point based on an initial biogeographic assumption (see Fuchs et al. 2006, 2007 for details). We acknowledge that we will not be able to ascertain the exact absolute timing at which the diversification within *Campethera* occurred. Yet, using these different plausible rates and calibrations will enable us to: 1) propose a time frame for the observed diversification events (Pliocene, Pleistocene, both) and 2) determine whether intra-specific diversification events occurred at similar times within the two species.

We made use of the program TRACER v1.5 (Rambaut and Drummond 2005) to help ensure that our effective sample size of the underlying posterior distribution was large enough (>200) for a meaningful estimation of parameters.

Establishing species limits: Coalescent-based analyses using the Isolation with Migration model and molecular species delimitation methods

We used the Markov chain Monte Carlo method implemented in the program IMa2 (Hey 2010) to fit the data to a model that included both isolation and migration. IMa2 estimates six parameters scaled to the neutral mutation rate (μ): θ_{pop1} ($4Ne_{pop1}\mu$), θ_{pop2} ($4Ne_{pop2}\mu$), θ_{popA} ($4Ne_{popA}\mu$), t (T/μ, where T is the time since population divergence in years before present), m1 ($2M/\theta_{pop1}$, where M is the effective number of migrants moving into population 1) and m2 ($2M/\theta_{pop2}$, where M is the effective number of migrants moving into population 2). We defined

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inheritance scales to reflect the difference in modes of inheritance among the loci used: 0.25 for the mtDNA locus, 0.75 for the Z-linked locus, and 1.0 for the autosomal locus. We used an HKY model of nucleotide substitution for all loci. We used a geometric heating scheme (h1= 0.9, h2 = 0.75) coupled with 60 chains. For each data set, upper bounds for the prior of the final run were adjusted based on preliminary runs with large uniform priors. Parameters and genealogies were sampled every 10 steps until we sampled 10^5 genealogies. The fit of 25 demographic models involving different combinations of population sizes and migration rates were then determined using likelihood ratio tests implemented in the L-mode module of IMa2 (Hey and Nielsen 2007). To assess convergence, we monitored the extent of autocorrelation and parameter trend lines throughout the run and we also compared the results between two independent runs.

For the two nuclear loci, the longest region without observed recombination was determined by using the program IMgc (Woerner et al. 2007). IMgc removes sites and /or haplotypes to produce the most information-rich contiguous DNA sequence segment that passes the four-gamete test.

We only estimated the parameters of the isolation with migration model for the comparison between *nivosa* East of Niger River versus *nivosa* West of Niger River, that is, the two primary clades in *nivosa*, as this was the only comparison for which we had enough sampled individuals to meaningfully estimate the parameter values of the model.

We also used a Bayesian implementation of the general mixed Yule-coalescent model (bGMYC 1.0; Reid and Carstens 2012). This implementation is an extension of the GMYC model (Pons et al. 2006) that incorporates gene tree uncertainty by sampling over the posterior

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distribution of sampled gene trees. We obtained a posterior distribution of ultrametric gene trees from the 42 unique *Campethera* mitochondrial haplotypes using BEAST v1.7.4 (Drummond & Rambaut 2007) under a strict clock model (0.026 s/s/l/myr, standard deviation = 0.0029). We ran MCMC for 10^7 iterations with sampling of parameters and trees every 1000 iterations. The first 10% of the samples were removed as the burn-in period. We analyzed 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 used BPP v2.0 (Rannala and Yang 2003, Yang and Rannala 2007) to estimate the speciation probability for the populations of each species distributed across the Dahomey gap. The method implemented in BPP v2.0 accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. 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 greater than 0.95 as strong support for a putative speciation event. A gamma prior is used 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 and Rannala 2007). We ran the rjMCMC analyses for $5*10^5$ generations with a burn-in period of $1*10^4$ and different starting seeds. We ran each analysis twice and 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

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performed analyses to estimate the combination of genetic diversity/split times necessary to render the first speciation even below PP = 0.95.

RESULTS

The MK test did not detect any evidence of selection within the *Campethera caroli*/*C. nivosa* clade; this result was not dependent on the outgroup taxon chosen (*C. caillautii*, p=0.17; *C. abigonii*, p=0.52). Likewise, the HKA test did not detect any evidence of selection acting on the nuclear introns for *Campethera caroli* (sum of deviations: 1.6766, df=2, p=0.43) or *C. nivosa* (sum of deviations: 1.8325, df=2, p=0.40).

Phylogeographic structure of Campethera caroli *and* C. nivosa.

The analyses performed on the mitochondrial locus revealed a genetic break between populations distributed across the Dahomey gap/Lower Niger River break for *C. caroli* (Fig. 2). For *C. nivosa,* the biogeographic barrier between the two primary clades appears to be the Niger River. The Dahomey Gap acts a strong secondary biogeographic barrier for the *nivosa* populations distributed west of the Niger River, with populations East and West of the Dahomey Gap being differentiated (Fig. 2). Hence, our results are consistent with the Niger River becoming established before the formation of the Dahomey Gap.

For the nuclear introns, no alleles were shared between *caroli* and *nivosa*, although each species was not necessarily monophyletic (Fig. 3). Within species, some nuclear alleles were shared between the two primary intraspecific clades for both *caroli* and *nivosa* but most alleles were restricted to a particular geographically localized lineage (Fig. 3).

Genetic diversity and demographic parameters

Nucleotide diversity (π) and Watterson's theta (θ) values are greater in *C. nivosa* than in *C. caroli* at every locus, and within *C. nivosa* MB and BRM had greater diversity in the Lower Guinea Block than in the Upper Guinea Block (Supplementary Table 2).

The Extended Bayesian Skyline Plot analyses indicated that the likely number of population changes for the *caroli* Lower Guinea Forest Block population was almost equally distributed between k=1 and k=2 (mean k=1.74). The skyline profile is consistent with a population expansion over the last 10 kyrs (Fig. 4). Traditional summary statistics that are able to detect population changes (*Fs*, R2) recovered mixed results; the mitochondrial ATP6 reflected a signal of population expansion but the nuclear introns (BRM and MB) did not (Supplementary Table 3). The *nivosa* Upper Guinea Forest Block population was found to encompass a recent population expansion (mean k=1.027), although the posterior distribution included k=0, whereas populations from the Lower Guinea Forest block were found to increase steadily (mean k= 1.819). Summary statistics for the *nivosa* Lower Guinea Forest block populations were in strong agreement with a population increase as Fu's *Fs* was significant for the three loci. In contrast, summary statistics are not consistent with a model of population expansion for the *nivosa* Upper Guinea Forest block populations, with only one locus (MB) recovering a significant negative value for Fu's *Fs* (Supplementary Table 3).

Timing of diversification

Analyses of the mitochondrial data set suggest that the first diversification events within *C. caroli* and *C. nivosa* differ in time and space (Table 1). The first split for *nivosa* involved the

Niger River and occurred between 1.2 (95% HPD: 0.8-1.6 mya, 0.026 s/s/l/myr mitochondrial rate) and 2.8 mya (95% HPD: 1.9-3.9 mya, 0.0105 s/s/l/myr rate). The first split for *caroli* likely developed across the Dahomey Gap between 0.6 mya (95% HPD: 0.2-1.2 mya, neutral fourfold degenerated rate) and 1.6 mya (95% HPD: 1.0-2.3 mya, 1.05% mitochondrial rate). Hence it seems that phylogeographical structure resulting from the reduction in gene flow among spatially distinct populations of *nivosa* occurred earlier than within *caroli*. The second split within *nivosa,* involving the Dahomey Gap, occurred between 0.5 mya (95% HPD: 0.1-0.9 mya, neutral fourfold degenerated rate) and 1.1 mya (95% HPD: 0.6-1.7 mya: 1.05% mitochondrial rate), an interval that is very similar to the divergence time recovered among lineages of *caroli.* The Time to Most Recent Common Ancestor for the haplotypes sampled in the Lower Guinea Forest block was identical for both species (0.2-0.5 mya). In summary, these results suggest that: 1) genetic structure within *caroli* and *nivosa* occurred during the Pleistocene, 2) that the initial phylogeographic subdivision appeared earlier and in a different location in *nivosa* than for *caroli*, 3) that the divergence times between lineages distributed across the Dahomey Gap are very similar for both species and, 4) that the TMRCA for haplotypes distributed across the Lower Guinea Forest Block are similar for both species.

Establishing species limits: Coalescent-based analyses using the Isolation with Migration model and molecular species delimitation methods

Satisfactory mixing of chains in the IMa2 model was achieved for all parameters with the exception of *t* (time), which did not converge back to zero. The IMa2 model suggested that asymmetric levels of gene flow occurred between the *nivosa* populations distributed across the Niger River. The likelihood ratio tests rejected all models where: 1) extant population sizes were

equal (highest p-value=0.007, 2LRR=10.04, df = 2), and 2) or asymmetric migration rates were recovered indicating the lack of movement of alleles from the population west of the Niger River to the population East of the Niger River (highest p-value=0.02, 2LRR=7.66, df = 2). Models that assume no gene flow were not rejected as long as the West of the Niger River population size was allowed to vary relative to either the population East of the Niger River or the ancestral population size (lowest p-value=0.062, 2LRR=5.548, df =2). Most models assuming differential gene flow inferred non-zero gene flow from populations West of the Niger River to populations East of the Niger River and no gene flow from populations East of the Niger River to populations West of the Niger River.

In summary, the analyses suggest that: 1) population size is significantly larger East of the Niger River, and 2) that the hypothesis of no gene flow between populations distributed on either side of the Niger River could not be statistically rejected. The best model (different population sizes and coalescent migration rate zero from East of the Niger River to West of the Niger River) favoring limited gene flow from populations West of the Niger River to populations East of the Niger River (m_{10}=0.1164) but no gene flow in the opposite direction (m_{01} = 0).

Analyses performed under the Generalized Mixed Yule Coalescent (Pons et al. 2006) and a random set of a hundred trees from the posterior distribution of the mitochondrial haplotypes (100 trees) using bGMYC indicated that the number of species recognised could be as many as four (probability threshold 0.05): *caroli* Upper Guinea Forest block, *caroli* Lower Guinea Forest block, *nivosa* West of the Niger River, and *nivosa* East of the Niger River (Fig. 5). The probability for distinguishing between the *nivosa* populations distributed to the West and East of the Dahomey Gap was low (p=0.10.)

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The analyses performed using the species delimitation rjMCMC algorithm implemented in BPP with a guide tree reflective of five species (*caroli*_{Upper}, *caroli*_{Lower}, *nivosa*_{Upper}, *nivosa*_{Nigeria}, *nivosa*_{EastNigerRiver}), the splitting scheme with the highest number of putative taxa, indicated that all models visited are consistent with five discrete lineages (PP=0.99-1.0). This result was not dependent on the assumption of a large or small effective population size, or a shallow or deep divergence time, as all prior combinations resulted in the same posterior probability distribution.

DISCUSSION

Our analyses provided new insight into the patterns and processes driving the diversification of forest vertebrates across the lowland rainforest of Africa. We focus below on the timing of the formation of the biogeographic barriers, before comparing the evolutionary history of the primary clades in both woodpecker taxa, and finally provide perspective on a taxonomic revision of these taxa in light of the molecular DNA results.

The Dahomey Gap and Lower Niger River acted as biogeographic barriers during the Pleistocene

Our analyses revealed a very similar phylogeographic history between the two sister *Campethera* species. The older intra-specific split occurred between 1.2 and 2.8 mya fragmenting *C. nivosa* populations distributed across the Lower Niger River. We did not have access to samples of *C. caroli* collected between the Lower Niger River and the Dahomey Gap, as this species is very rare in this area (Winkler and Christie 2002). The Dahomey Gap separated populations from the Lower and Upper Guinea Forest block for both species between 0.5 and 1.6

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mya (0.5-1.1 mya *C. nivosa*; 0.6-1.6 mya *C. caroli*), depending on the calibration used, with substantial overlap in the posterior distribution for the estimates of this split between the two taxa (Table 1) suggesting that the same event promoted the divergence of the *C. caroli* and *C. nivosa* populations distributed across the Dahomey Gap. Interestingly, the divergence across the Dahomey Gap for chimpanzee populations was estimated at 0.5 myr before present (Stone et al. 2010, Bjork et al. 2011, Gonder et al. 2006, 2011), which is very similar to the dates of divergence obtained for the two *Campethera* species using three of our independent calibration points (0.5-0.8 myr, Table 1).

Two primary conclusions can be drawn from the pattern recovered by our analyses: 1) the Lower Niger River formed before than the Dahomey Gap, and 2) the two barriers appeared and shaped the genetic structure of lowland forest birds at the onset of the Pleistocene or even well into the Pleistocene (depending on the calibration points or substitution rates used, Table 2).

Previous studies on African lowland rainforest birds suggest that haplotypes are rarely shared between populations sampled across the Dahomey gap (Beresford and Cracraft 1999; Schmidt et al. 2008, Marks 2010) and our analyses are consistent with this result. The phylogeographic relationships of the populations inhabiting the forests west of the Lower Niger River and east of the Dahomey Gap (Western Nigeria, Benin, hereafter referred to as wLNR) have not been assessed for volant vertebrates (e.g. Marks 2010, Nesi et al. 2012) due to the lack of genetic samples from the wLNR population. Our analyses revealed that populations sampled from this area are more closely related to, yet divergent from, populations of the Upper Guinea Forest Block than to populations east of the Lower Niger River. Highly distinctive mitochondrial lineages from the wLNR have also been recovered for forest dwelling rodents (Nicolas et al.

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2008) and chimpanzees (Gagneux et al. 2001, Gonder et al. 2006). The phylogeographic relationships of these wLNR populations to populations distributed in either the Upper and Lower Guinea Forest blocks were variable. Rodents from the wLNR are related to the populations sampled in the Lower Guinea Forest Block (Nicolas et al. 2008) whereas chimpanzees from the wLNR have mitochondrial haplotypes that are closer to populations sampled from the Upper Guinea Forest Block. In his zoogeographic analysis of mammal distribution patterns, Booth (1958) suggested the existence of three areas where differentiation among taxa occurred during glacial cycles: one refugium west of the Volta River, the western limit of the Dahomey Gap (Upper Guinea Forest Block), one refugium east of the Niger River (Lower Guinea Forest Block), and one potential refugium distributed between the Volta and Niger Rivers ('Western Nigeria' *sensu* Booth 1958) that has stronger taxonomic affinities with the Upper Guinea Forest Block. Hence, current genetic data are largely in agreement with the hypothesis of Booth (1958) regarding the existence of three refugia, although the phylogeographic relationships among those refugia are more lineage-specific than postulated by Booth (1958).

Several authors have suggested alternative biogeographic barriers to be important in structuring African lowland forest terrestrial vertebrates. The Sanaga River is, for example, a biogeographic barrier to gene flow between Chimpanzee populations distributed on either side of it, although occasional hybridization occurs (Gonder et al. 2011). A phylogeographic study based on genome-wide single nucleotide polymorphims of *Hemidactylus* geckos also suggested a role for the Sanaga River acting as a barrier to gene flow (Leaché et al. 2014). Other studies revealed no impact of the Sanaga River on the broad scale phylogeographic structure of terrestrial vertebrates, with individuals sampled on both side of the river being part of the same terminal

clade (Nicolas et al. 2008, 2010). In the present study, the individual we sampled north of the Sanaga River is undifferentiated from the individuals sampled south of the Sanaga River, suggesting that the river does not constitute a biogeographic barrier for *Campethera* woodpeckers. Results from molecular dating analyses suggest that the Sanaga River formed more recently than the Dahomey Gap. Hence, although the Sanaga River may play a role in limiting gene flow among vertebrates with limited dispersal relative to birds and chimps, its impact in shaping the broad-scale genetic structure of terrestrial vertebrates at deeper times, or those with greater dispersal abilities, is much more limited than the impact of the Dahomey Gap or Niger River, which appear to be more significant biogeographic barriers.

Here, we propose a biogeographic scenario involving the appearance and disappearance of biogeographic barriers where: 1) lineages of *C. nivosa* restricted to either side of the Niger River were first separated between 1.2 and 2.8 myrs BP, leading to two lineages, 2) next lineages occupying either side of the Dahomey Gap were isolated, leading to three well differentiated lineages (Upper Forest Block, wLNR, Lower Forest Block), and 3) directional gene flow from the wLNR to the Lower Guinea Block was initiated after the Niger River became less effective as a biogeographic barrier. This scenario is indirectly supported by our coalescent-based analyses that identified recurrent asymmetric gene flow from west to east across the Lower Niger River.

Evolutionary history within the Upper and Lower Forest blocks sensu stricto

Our analyses revealed concomitant divergence times between populations of two sister-species of woodpeckers distributed across the Dahomey Gap. However, other population genetic parameters (genetic diversity, demographic history) differ between the two species. Below we

outline the differences in the context of the two forest blocks *sensu stricto,* with the wLNR population excluded.

Both woodpecker species are characterized by a lack of obvious genetic structure within either the Lower or Upper Guinea Forest Blocks (no conclusion should be drawn for the *caroli* Upper Block population due to the low sample size). This result is consistent with several vertebrate studies that did not recover phylogeographic structure among sampled sites for Upper Guinea Forest species (*Myonycteris* and *Megaloglossus*, Nesi et al. 2013; *Pan*: Gonder et al. 2011, *Lissonycteris*: Nesi et al. 2013), but are at odds with studies where substructure or deep breaks were recovered among sampled populations (*Hylia*: Marks et al. 2010, *Lemnomys*: Nicolas et al. 2008, *Crocidura*: Jacquet et al. 2014). Such variation in phylogeographic patterns, ranging from deep genetic breaks among populations to no or little genetic variation and structure, despite high standing variation, highlights the need for additional phylogeographic studies of Upper Guinea Forest block taxa if any generalities are to be drawn.

Traditional hypotheses suggested the existence of two refugia in the Lower Guinea Forest Block (Endler 1982). Some data sets support this general hypothesis (Marks 2010, Schmidt et al. 2008). In all cases, current data support the hypothesis that the Lower Guinea Forest populations are coherent entities that are genetically structured at fine spatial scales by large rivers (e.g. Congo River, Sanaga River) that act as efficient barriers to dispersal for vertebrates. This holds not only for chimpanzees (e.g. Gonder et al. 2011) but also for understorey birds (Voelker et al. 2013) and geckos (Leaché et al. 2014). Hence, it is surprising that we did not find any evidence of genetic structure in the Lower Guinea Forest Block within the two *Campethera* species, despite broad geographic sampling. Our focal *Campethera* taxa are part of the same avian

community as *Hylia* and to a lesser extent *Stiphrornis*, yet diversification among populations of *Stiphrornis* is rather ancient, 2-9-3.6 myr BP (Schmidt et al. 2008), especially when compared to *Campethera*. Most haplotypes within *nivosa* and *caroli* only differ from each other by one substitution over considerable geographic distances (e.g. between Angola and Uganda, c. 2400 km). This result is consistent with more recent population expansion from a single refugial source in both taxa. The Time to Most Recent Common Ancestor for the Lower Guinea Forest haplotypes for *caroli* and *nivosa* are similar, and varied between 0.2 and 0.5 myr BP depending on the calibration used; this time frame is much older than the currently reconstructed or postulated refugia (Maley 1987, 1996, Endler 1982) suggesting the time frame of the paleobotanical reconstructions of the refugia locations are not necessarily applicable to most data sets as coalescent times of haplotypes tend to be much older. A similar case of no obvious genetic structure across such a large geographic area as the Lower Guinea Forest Block has to our knowledge only been recovered to date in one other bird species, the Olive Sunbird *Nectarinia obscura* (Bowie et al. 2004), and the bat *Myonycteris torquata* (Nesi et al. 2013).

Species delimitation in Campethera caroli *and* C. nivosa *using molecular DNA data*

Delimiting species using genetic data can be challenging as the methods bridge several different fields (systematics, phylogeography, and population genetics; Carstens et al. 2013) and the philosophical basis of such approaches remains controversial (Fujita et al. 2013). We made use of two commonly used methods (BPP, bGMYC) to test whether different algorithms provide the same signal in delimiting taxa that could be considered putative species. We observed some discrepancy in the number of putative taxa that each method delineated: bGMYC favoured four species corresponding to the four primary clades we recovered (Fig. 5), whereas BPP support

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five lineages thereby also delineating the wLNR population as specifically distinct. These results are robust to different combinations of ancestral population size and divergence time. These two methods are among the most liberal algorithms used to delineative species with molecular data (Miralles and Vences 2013, Satler et al. 2014). Although species delimitation methods using molecular data are useful guides, we argue that other characters (e.g. plumage, song, biometrics) should be investigated before species splits are accepted. Following this framework it seems consistent to recognize the populations distributed to the West and East of the Dahomey Gap as different species for both *C. caroli* (*C. arizela* - Upper Guinea Forest Block and *C. caroli* - Lower Guinea Block) and *C. nivosa* (*C. nivosa* - Upper Guinea Block and *C. herberti* - Lower Guinea Block). These monophyletic molecular clades are supported by diagnostic plumage differences between *C. arizela* and *C. caroli* and possibly between *C. nivosa* and *C. herberti*, although a re-assessment of plumage variation is needed for the latter two taxa.

The available data on the *nivosa* population from the wLNR suggests that it is most appropriate to provisionally associate this population with *Campethera nivosa sensu stricto* (that is the population distributed in the Upper Guinea Block) based on the mitochondrial gene tree and non-significant values in the bGMYC analyses. Additional data needs to be collected to determine which stage of the speciation continuum this population occupies (e.g. Webb et al. 2011) before being able to robustly determine its taxonomic status. The relationships of the Bioko endemic subspecies that we were not able to sample (*C. nivosa poensis*) in our study, clearly awaits molecular analysis. Yet, knowledge of the biogeographic history and origins of the terrestrial vertebrates on Bioko are generally concordant, and suggests that lowland taxa are of recent origin and most closely related to populations distributed east of the Niger River (e.g.

Corythornis leucogaster: Melo and Fuchs 2008; *Zosterops stenocricotus*: Melo et al. 2011, *Hemidactylus* geckos, Leaché et al. 2014) and hence we suggest that the Bioko subspecies is genetically very closely related to populations distributed east of the Niger River (*C. herberti*).

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Table 1. Time to Most Recent Common Ancestor for the *Campethera caroli*/*C. nivosa* haplotypes

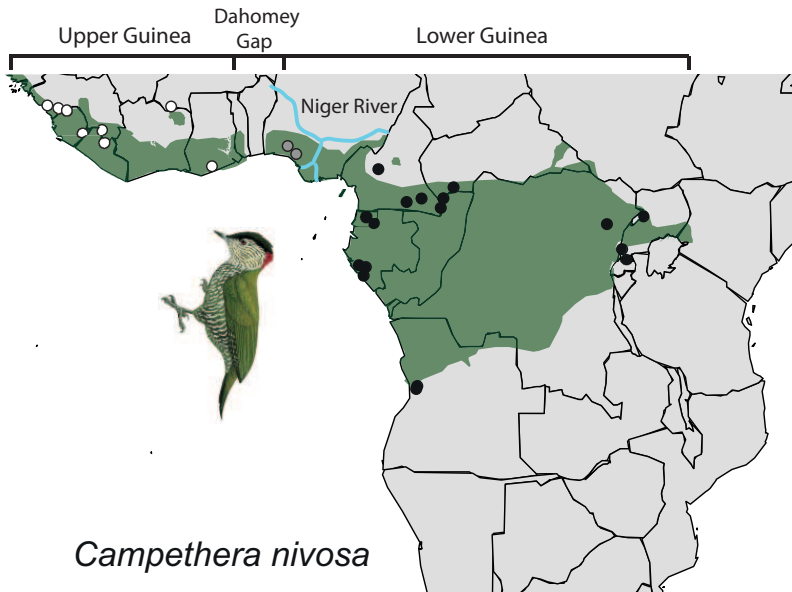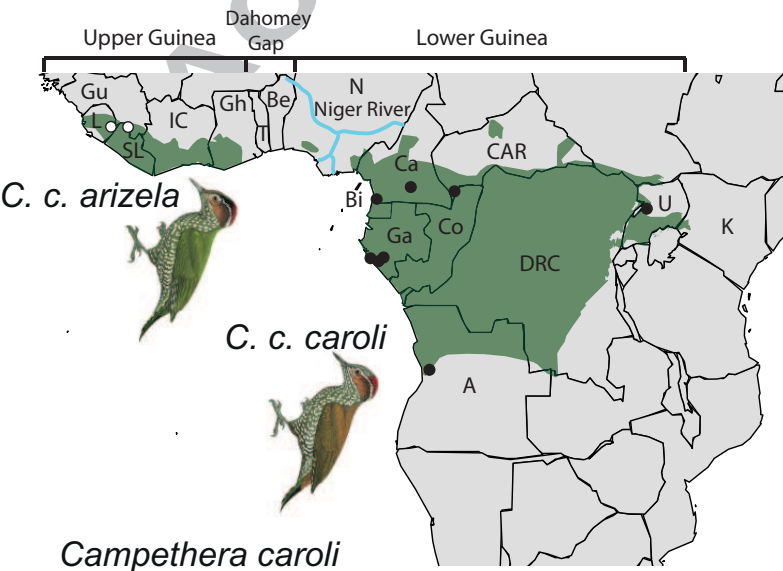| Clade	Weir and Schluter (2008)	Fuchs et al. (2007)	Lerner et al. (2011)	Four fold
caroli/*nivosa*	3.6 (2.5-4.8)	2.5 (1.4-3.6)	1.5 (1.0-1.9)	1.3 (0.5-2.4)
nivosa	2.8 (1.9-3.9)	2.0 (1.0-3.0)	1.2 (0.8-1.6)	1.3 (0.5-2.3)
nivosa Nigeria/Upper Guinea Forest block	1.1 (0.6-1.7)	0.8 (0.3-1.2)	0.5 (0.3-0.7)	0.5 (0.1-0.9)
nivosa Upper Guinea Forest Block	0.3 (0.1-0.5)	0.2 (0.1-0.4)	0.1 (0.05-0.2)	0.2 (0.05-0.3)
nivosa Lower Guinea Forest block	0.5 (0.3-0.8)	0.3 (0.1-0.6)	0.2 (0.1-0.3)	0.3 (0.1-0.5)
caroli	1.6 (1.0-2.3)	1.1 (0.5-1.8)	0.6 (0.4-0.9)	0.6 (0.2-1.2)
caroli Lower Guinea/Forest block	0.5 (0.3-0.8)	0.3 (0.1-0.6)	0.2 (0.1-0.3)	0.2 (0.1-0.5)

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Figure 1. Distribution of *Campethera caroli* and *C. nivosa* based on Winkler and Christie (2002). Dots represent sampling localities (details in Supplementary material Table 1). The two primary biogeographic barriers (Dahomey Gap and Lower Niger River) as well as the longitudinal extent of the two forest block are indicated. Codes for countries are: B, Benin; Ca, Cameroon; CAR, Central African Republic; Co, Republic of the Congo; DRC, Democratic Republic of Congo; Ga, Gabon; Gh, Ghana; Gu, Guinea; IV, Ivory Coast; K, Kenya; L, Liberia; SL, Sierra Leone; T, Togo; U, Uganda. The abbreviation Bi indicates the island of Bioko (Equatorial Guinea).
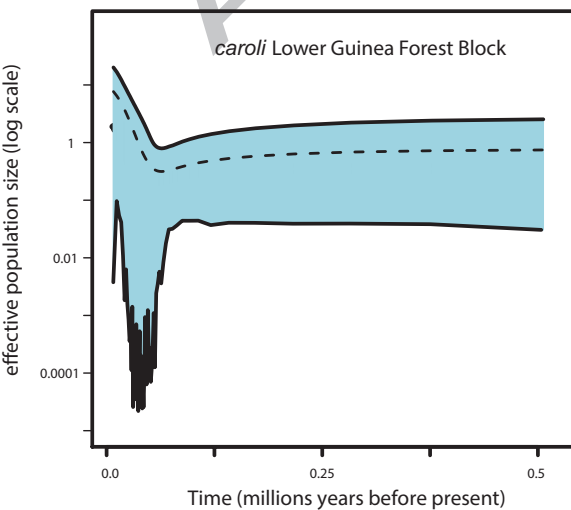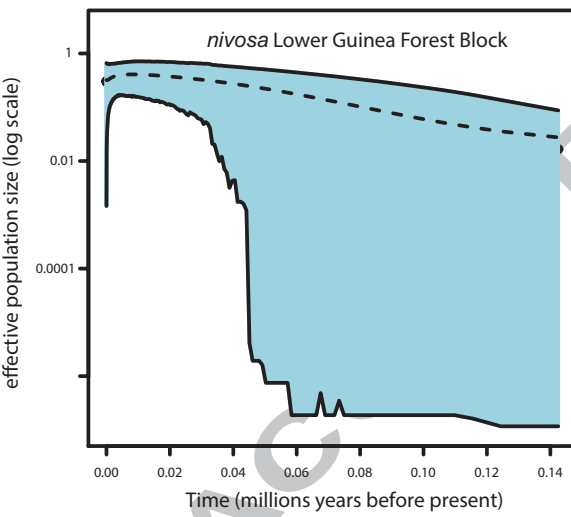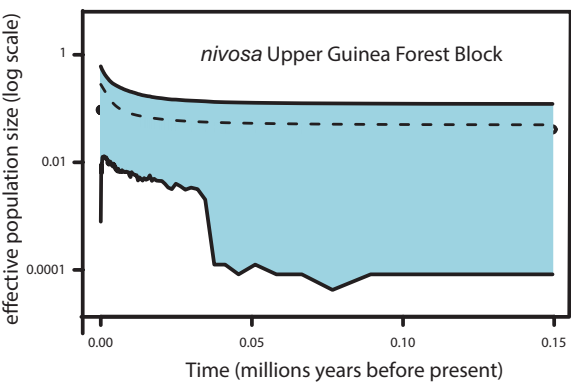
Figure 2. Fifty-percent majority rule consensus tree from the MRBAYES analyses obtained using unique mitochondrial haplotypes. Numbers close to branches are posterior probabilities. Next to the primary clades are the minimum spanning networks depicting the relationships among the haplotypes obtained using the statistical parsimony algorithm implemented in TCS with circle size proportional to the number of individuals. Color codes for the networks are: black (Lower Guinea Forest Block), grey (Western Nigeria), and white (Upper Guinea Forest Block).

Figure 3. Fifty-percent majority-rule consensus tree obtained from the Bayesian Inference analyses of the (A) Myoglobin intron-2 (autosomal) and (B) BRM intron-15 (Z chromosome). Next to the primary clades are the minimum spanning networks for the relationships among the haplotypes obtained using the statistical parsimony algorithm implemented in TCS with circle size proportional to the number of individuals. Color codes for the networks are black (Lower Guinea Forest Block), grey (Western Nigeria), and white (Upper Guinea Forest Block).
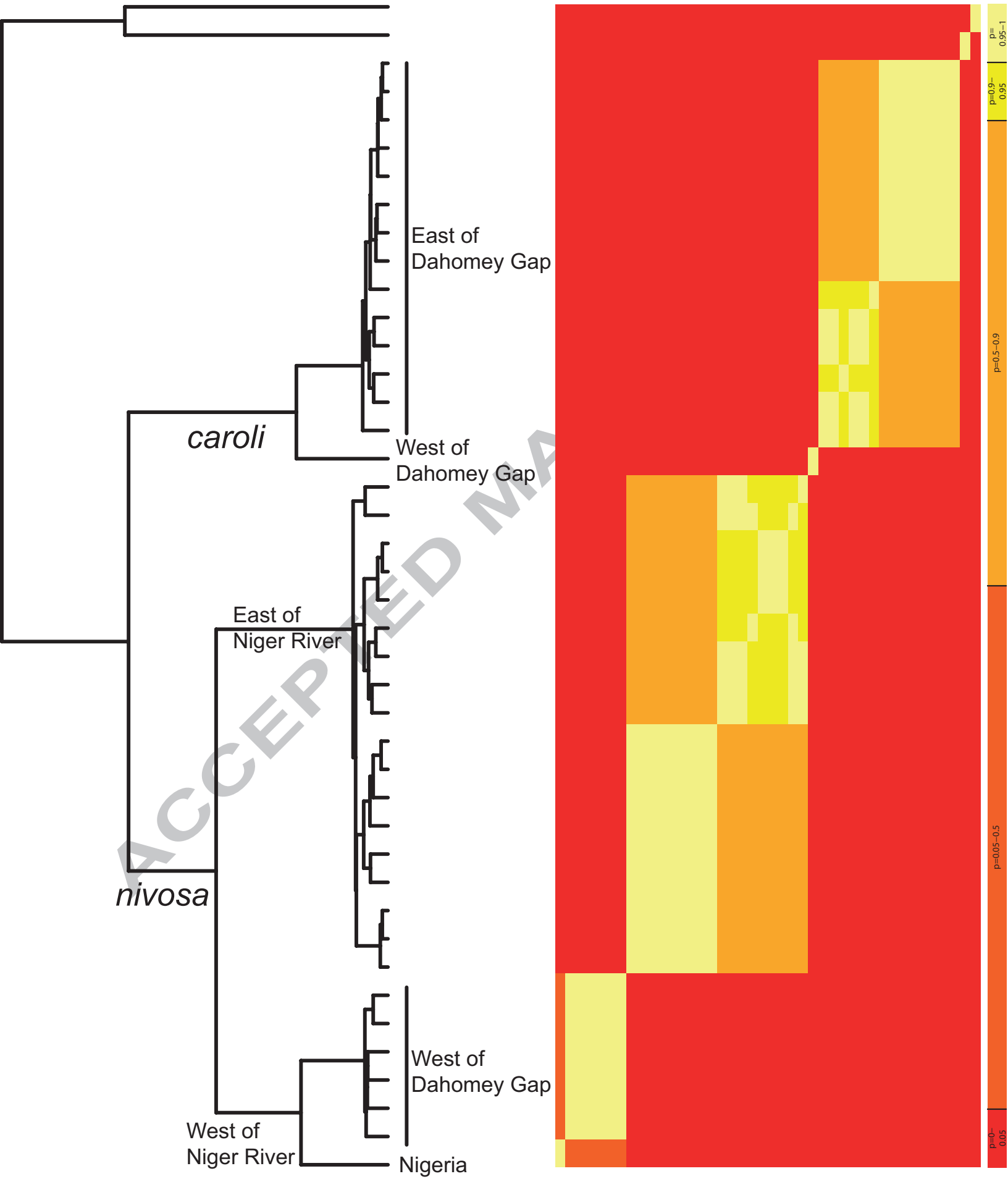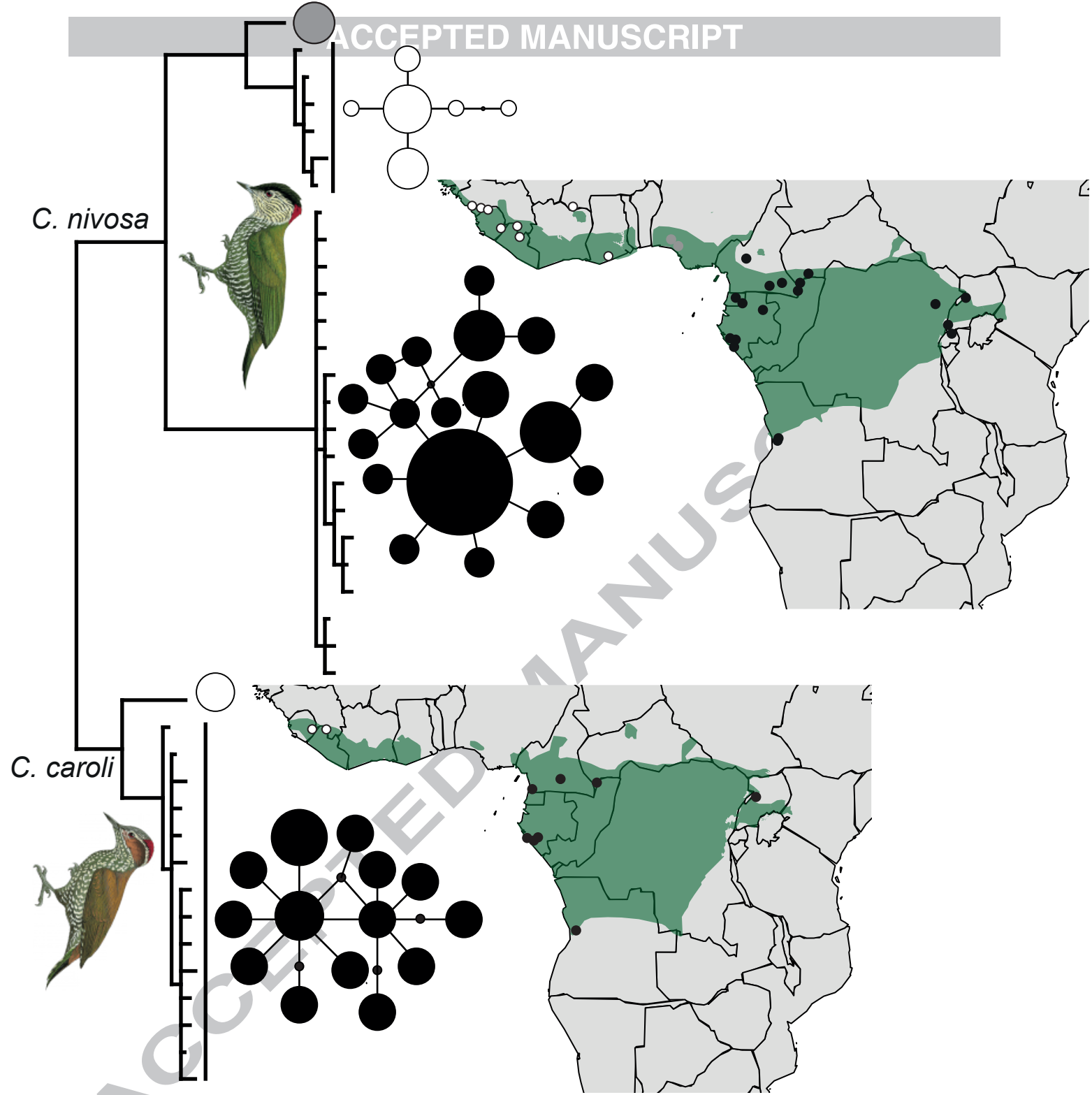
35

Figure 4. Extended Bayesian Skyline Plots for the Lower Guinea Forest Block population for *C. caroli* and *C. nivosa* and for the Upper Guinea Forest Block population for *C. nivosa*. No EBSP was reconstructed for the Upper Guinea Forest block population for *C. caroli* due to low sample size. Support for population expansion was found for all taxa although the magnitudes of the expansions were variable.

Figure 5. Summary of bGMYC species delimitation using the mitochondrial data set. The tree is the maximum clade credibility tree from BEAST with ingroup clade. The heat map is a sequence-by-sequence matrix in which cells are colored by the posterior probability that the corresponding sequences are conspecific, with increasing probability represented by light yellow colors. The scale is given on the right of the tree. The analyses would support the recognition of four species.

Upper Guinea   Dahomey Gap   Lower Guinea

Gu
L
SL
IC
Gh
Be
T
N
Niger River
Ca
CAR
Bi
Ga
Co
U
K
DRC
A

C. c. arizela

C. c. caroli

Campethera caroli

Upper Guinea   Dahomey Gap   Lower Guinea

Niger River

Campethera nivosa

Campethera abingoni

Campethera caillautii

Nigeria — Ni

West of Niger River

West of Dahomey Gap

SL, Cap

Li | Li, Gu, SL, Gh | Li — Gu

Gh, SL BF

nivosa

EGu

EGu, Ga, Ca | Ga

EGu

Ga | Rca | Ga

An | An | Ca | Ga

Rca | Ga

East of Niger River

Ca | RCA, Ug, Rw, Ca | Ga

Ca

Rca | RDC

West of Dahomey Gap — Li, SL

caroli

East of Niger River

East of Dahomey Gap

Rca | Ca

Ca | Ga

Ca | Rca | An | Ug | Ga

Rca | Rca | Ug

Ca | Ga

0.1

Figure 5

C. nivosa

C. caroli

Highlights

- *Campethera caroli* and *C. nivosa* are genetically structured
- Both species have a similar phylogeographic history
- Dahomey Gap acts as a biogeographic barrier in both taxa
- Lower Niger River is a biogeographic barrier for *C. nivosa*
- No genetic structure in both species within the Upper and Lower Guinea Forest block