The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2011, **103**, 821–835. With 4 figures

Patterns of divergence in the olive sunbird *Cyanomitra olivacea* (Aves: Nectariniidae) across the African rainforest–savanna ecotone

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Received 11 October 2010; revised 29 January 2011; accepted for publication 1 February 2011

In the debate over modes of vertebrate diversification in tropical rainforests, two competing hypotheses of speciation predominate: those that emphasize the role of geographical isolation during glacial periods and those that stress the role of ecology and diversifying selection across ecotones or environmental gradients. To investigate the relative roles of selection versus isolation in refugia, we contrasted genetic and morphologic divergence of the olive sunbird (*Cyanomitra olivacea*) at 18 sites (approximately 200 individuals) across the forest–savanna ecotone of Central Africa in a region considered to have harboured three hypothesized refugia during glacial periods. Habitats were characterized using bioclimatic and satellite remote-sensing data. We found relatively high levels of gene flow between ecotone and forest populations and between refugia. Consistent with a pattern of divergence-with-gene-flow, we found morphological characters to be significantly divergent across the gradient [forest versus ecotone (mean ± SD): wing length 60.47 ± 1.81 mm versus 62.18 ± 1.35 mm; tarsus length 15.51 ± 0.82 mm versus 16.00 ± 0.57 mm; upper mandible length 21.77 ± 1.09 mm versus 23.19 ± 0.98 mm, respectively]. Within-habitat comparisons across forest and ecotone sites showed no significant differences in morphology. The results show that divergence in morphological traits is tied to environmental variables across the gradient and is occurring despite gene flow. The pattern of divergence-with-gene-flow found is similar to that described for other rainforest species across the gradient. These results suggest that neither refugia, nor isolation-by-distance have played a major role in divergence in the olive sunbird, although ecological differences along the forest and savanna ecotone may impose significant selection pressures on the phenotype and potentially be important in diversification. *Biological Journal of the Linnean Society*, 2011, **103**, 821–835.

ADDITIONAL KEYWORDS: allopatry – diversification – ecological speciation – ecotone – parapatry – remote sensing.

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INTRODUCTION

The modes of vertebrate diversification in tropical rainforests have been debated for decades (Haffer, 1969; Stebbins, 1974; Prance, 1982; Endler, 1982b; Moritz *et al*., 2000). In tropical Africa, a dominant but controversial hypothesis is the forest refugia hypothesis (Endler, 1982a, b; Prance, 1982; Mayr & O'Hara, 1986; Nicolas *et al*., 2011). As described by Mayr & O'Hara (1986) for some bird species, it asserts that isolation in forest refugia during glacial periods was the main driver of speciation. To support their hypothesis, Mayr & O'Hara (1986) used biogeographical data to show that present day contact zones between forest species and subspecies tend to concentrate between hypothesized refugia. However, their analysis excluded savanna species, making it impossible to reject the possibility that peripatric or parapatric speciation might have occurred between the forest and savanna within a hypothesized refugial area. An analysis by Endler (1982a, b) including both forest and savanna species showed that 52% of the contact zones between sister taxa occurred roughly in the ecotone between forest and savanna, and not between the hypothesized locations of ancient refugia. This work, as well as subsequent research (F'jeldså, 1994), showed that recently diverged avian taxa are found along habitat gradients and in mountainous regions, which also are often contact zones between species and subspecies (Chapin, 1932). Consequently, these findings suggest that ecotones play an important role in diversification and speciation.

The ecotone between rainforest and savanna in Central Africa comprises a vast region, characterized by a mosaic of forest fragments, often associated with rivers, embedded in savanna. Ecologically, the ecotone habitats differ from central forest ones in several important aspects: habitats are more open, rainfall is lower and more variable (Longman & Jenik, 1992), and species assemblages and the types of available foods differ (Chapin, 1932, 1954; Smith *et al*., 1997, 2005c). Recent work suggests that diversifying selection across the rainforest–savanna ecotone may be important for promoting diversification (Smith *et al*., 1997, 2005a, c; Freedman *et al*., 2010). Studies of some African passerines, the little greenbul (*Andropadus virens*) (Cassin, 1858) and the black-bellied seedcracker (*Pyrenestes ostrinus*) (Smith, 1840), suggest divergent selection across the forest–ecotone boundary has led to divergence in morphological and behavioural traits such as song that are important in reproductive isolation (Smith *et al*., 1997, 2005a, c; Slabbekoorn & Smith, 2002). The extent to which these patterns of divergence occur in other avian taxa is unknown. In addition, little is known about potential selective forces that may act along the forest–ecotone gradient.

Many speciation events purportedly driven by refugial isolation are relatively old, frequently pre-dating the Pleistocene. Because reconstructing the ecological context in which they took place is rarely possible, direct tests of refugia and gradient hypotheses are not feasible. An alternative approach involves contrasting contemporary patterns of variation along gradients and between refugia that have been shaped by the most recent climate cycle. Accordingly, the reasonable assumption is made that the relative importance of refugial isolation and selection along the rainforest-ecotone gradient since the Last Glacial Maximum reflects their relative importance across previous climate cycles. In the present study, we contrast genetic and morphological divergence in the olive sunbird (*Cyanomitra olivacea*) across the forest–ecotone boundary of Cameroon and Equatorial Guinea, and investigate the roles of selection versus isolation in hypothesized forest refugia (Maley, 1996) in generating observed divergence. The olive sunbird is a medium-sized sunbird occurring across Central Africa in both primary and secondary forest, and ranges into the larger forest fragments of the ecotone. Olive sunbirds feed on both nectar and insects and are one of the more common forest sunbirds (Mackworth-Praed & Grant, 1973; Fry, Keith & Urban, 1988; Cheke, Mann & Allen, 2001). The objectives of the present study were: (i) to relate patterns of variation with hypothesized refugia and habitat characteristics based on bioclimatic and satellite remote sensing data; (ii) to examine the pattern of genetic and morphological differentiation among forest and ecotone populations to determine whether it is consistent with a pattern of divergence with gene flow; and (iii) to contrast the patterns found in the present study with others, aiming to evaluate the importance of the African forest–savanna ecotone in promoting diversification.

MATERIAL AND METHODS

Field sampling

Fieldwork took place between 1990 and 2005 at 18 sites in Cameroon and Equatorial Guinea that span three hypothesized refugial areas and the rainforest–savanna gradient (Fig. 1, Table 1). The vegetation of the forest sites is varied but includes both secondary and mature forest and is generally characterized as lowland rainforest (Letouzey, 1968). The vegetation of the ecotone region is characterized as 'forest–savanna mosaic' (Letouzey, 1968). A more quantitative classification of habitat was carried out using remote sensing (see below).

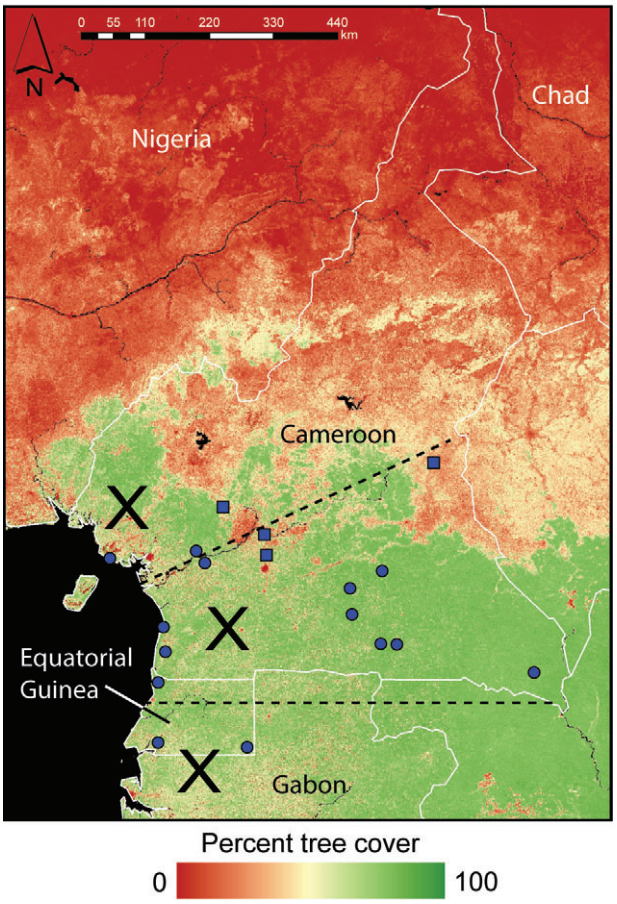

Figure 1. Sampling sites for olive sunbirds superimposed on a map of western Central Africa showing percent tree cover. Field work was conducted in the countries of Cameroon and Equatorial Guinea. Blue circles correspond to forest sites; blue squares correspond to sites in the forest–savanna ecotone. The approximate locations of hypothesized forest refugia (Maley, 1996) are indicated with an 'X'. Dashed lines indicate barriers corresponding to savanna habitat that likely existed in between forest refugia during glacial periods. These barriers were implemented in generalized dissimilarity models to test the relative contribution of forest refugia in explaining genetic and morphological differentiation among sampling sites. Sampling sites were assigned to each forest refugium based on proximity: two sites in southern Equatorial Guinea; three sites in northwestern Cameroon, north of the barrier indicated; and the remaining 13 sites in central and southern Cameroon and northern Equatorial Guinea.

At all sites, between 15 and 20 standard mist nets (12 m long and 30 × 30 mm mesh size) were erected to capture birds. Netting took place between daybreak (06.00 h) and dusk (17.00 h). Captured birds were weighed, measured, banded with an aluminum numbered band, bled, and released. Because the present study was part of a larger effort to study fitness components and selection pressures in marked populations of olive sunbirds, whole specimens were not collected. Blood samples (one or two drops) were collected from the brachial vein and stored in lysis buffer. All measurements were taken using dial calipers except mass, which was measured using a 50-g Pesola spring scale. Morphological data was collected from all sites, except during 1990 and from the sites of Mokula and Nchoho in Equatorial Guinea. Measurements were taken from a total of 76 males: wing length, from carpal joint to the tip of the longest primary; tarsus length, from tibiotarsal joint to distal undivided scute; upper mandible length, chord length from the point where culmen enters feathers of the head to the tip; and bill depth in vertical plane level at the anterior edge of nares. Adult males were distinguished by their yellow pectoral tuffs, and juvenile males were distinguished from females using a polymerase chain reaction-based approach for sexing which identifies a gene on the W chromosome (Ellegren, 1996). Morphological analyses for adults and juveniles were performed separately. All raw measurements are available upon request.

Bioclimatic and remote sensing data

A series of bioclimatic metrics were obtained from WORLDCLIM, version 1.4 (Hijmans *et al.*, 2005). These climate metrics are derived from monthly temperature and rainfall values and represent biologically meaningful variables for characterizing habitats (Hijmans *et al.*, 2005). The bioclimatic data layers included 11 temperature and eight precipitation metrics, expressing spatial variations in annual means, seasonality, and extreme or limiting climatic factors. The climate metrics were developed using long time series of a global network of more than 4000 weather stations from various sources such as the Global Historical Climatology Network, the United Nations Food and Agricultural Organization, the World Meteorological Organization, the International Center for Tropical Agriculture, R-HYdronet, and additional country-based stations. The station data were interpolated to monthly climate surfaces at 1-km spatial resolution by using a thin-plate smoothing spline algorithm with latitude, longitude, and elevation as independent variables (Hijmans *et al.*, 2005).

Remote sensing data from satellite observations included a suite of environmental variables to characterize the landscape and the vegetation. Among these variables, we focused on the vegetation continuous field product as a measure of the percentage of tree canopy cover (Hansen *et al.*, 2002) as well as the normalized difference vegetation index (NDVI), enhanced vegetation index (EVI), and estimated leaf area index (LAI) to characterize vegetation greenness

Table 1. Sample locations and study periods

Habitat	Site name	Location		Study period
Forest	Kribi	N 2°43′	E 9°52′	6–9 October 1990, 1–4 July 1993
	Bouamir	N 3°11′26″	E 12°48′42″	28 April 1995, 20–21 June 1995, 8–11 June 1996, 20–23 July 1999
	Elende	N 2°12.98′	E 9°47.57′	9–11 May 1998
	Mokula	N 1°02.86′	E 11°09.78′	14–22 May 1998
	Ncoho	N 1°14.20′	E 9°57.11′	26–31 May 1998
	Ndibi	N 2°43′50′	E 9°52′19″	28 July–20 October 1990, 4–5 July 1993, 27 June to 4 July 2005
	Zoebefame	N 2°39′	E 13°23′	27–29 September 1990, 9–15 June 1993, 24–27 July 2005
	Bobo Camp	N 2°39′	E 13°28′	15–20 June 1993, 19–22 July 2005
	Lac Lobeke	N 2°18′	E 15°45′	25–29 June 1993
	Nkwouak	N 3°52′	E 13°18′	7–8 August 1990, 29 June to 2 July 1993, 7–12 July 2005
	Kompia	N 3°32′	E 12°50′	5–6 March 1997, 26–27 July 1999
	Etome	N 4°02′	E 9°06′	13–14 July 1999
	Sakbayeme	N 4°02′	E 10°44′	17–19 May 2000
	Sangmbengue	N 4°04′	E 10°33′	May 25–26, 2000
Ecotone	Ndikinimeki	N 4°46′	E 10°50′	16–17 July 1997
	Kousse	N 4°27′	E 11°33′	18–19 July 1997
	Obala	N 4°10′	E 11°32′	20–21 July
	Bétaré Oya	N 5°34′	E 14°05′	11–13 August 1990, 5–8 May 1995

and seasonality, and canopy density. These variables were derived from NASA's Terra MODIS (Moderate Resolution Imaging Spectroradiometer) sensor at 1 km spatial resolution (Justice *et al*., 1998). For seasonality, we developed several metrics for NDVI, EVI, and LAI that included annual mean, minimum, maximum, and mean of driest and wettest quarters. These metrics were important to separate the forest and ecotone vegetation types in the study area as the degree of vegetation seasonality and deciduousness increase along the gradient from rainforest to ecotone and woodland savanna. For landscape features, we included the digital elevation and surface roughness derived from Shuttle Radar Topography Mission (http://www2.jpl.nasa.gov/srtm/) and aggregated to 1-km resolution.

To improve the accuracy of the data we checked for covariance among variables, and only included those that contributed substantial unique variance (e.g. Pearson's correlations < 0.9). Various criteria were used to decide which layers of correlated pairs were retained for further analysis. These included keeping layers that are more commonly used in distribution modelling (within the WORLDCLIM dataset) or that exhibit larger variance over the study area, as well as have the best data quality (NDVI, EVI, LAI). To extract the values of the climate and remote sensing variables for nonspatially explicit analyses, we created polygons of nine to 25 pixels around each sample site. For spatial analyses (generalized dissimilarity modelling; see below), we used variable layers at 1-km resolution.

Morphological and genetic analysis

Morphological divergence between sites was computed as the multidimensional Euclidean distance between population means of normalized measurements *sensu* Smith *et al*. (1997). We used a multivariate analysis of variance (MANOVA) to compare morphological traits across habitat types, and principal components analysis (PCA) to examine the clustering of populations in multivariate space. Statistical analyses were performed using both SYSTAT, version 5.2.1 (Systat Software, Inc.) and JMP, version 7 (http://www.jmp.com/software/). Before PCA was performed on multiple geographically distinct populations, we tested for the proportionality of covariance matrices (Flury, 1984) using CPC software (http://www.uoregon.edu/~pphil/programs/cpc/cpc.htm).

To quantify microsatellite variation, we genotyped all individuals using eight microsatellite loci (see Supporting information, Table S1). R_{ST}, a measure based on variation in allele size rather than frequency, may be a more appropriate means of estimating population differentiation where F_{ST} is biased. Thus, before performing the analyses, we tested whether results should be based on *F*- or *R*-statistics *sensu* Hardy *et al*. (2003). The test, which was nonsignificant, revealed that an allele identity rather than an allele size base statistic was appropriate indicating that F_{ST} was a suitable statistic. The number of individuals genotyped for each population, number of alleles, the inbreeding coefficient (F_{is}) for each locus by population, and expected and observed heterozygosities

were calculated using GENEPOP, version 3.2a (Raymond & Rousset, 1995) (http://genepop.curtin.edu.au/; see also Supporting information, Table S2). We searched for the presence of nonrandom associations of alleles by calculating F_{is} values *sensu* Weir & Cockerham (1984), and tested their significance using the exact test provided by GENEPOP, version 3.2a. Significance values of F_{is}, pooled across all populations and for each locus by population, were also tested using FSTAT, version 2.9.3.2 (Goudet, 1995) (http://www2.unil.ch/popgen/softwares/fstat.htm). Each microsatellite locus was tested and verified to not be heterozygote deficient using the Hardy–Weinberg exact test option (Guo & Thompson, 1992) in GENEPOP, version 3.2a. To control for Type I error, a Bonferroni correction was applied to significant results (Rice, 1989).

We tested for differences in allele frequencies among paired populations to quantify population genetic structure using a log-likelihood (G)-based exact test performed using GENEPOP, version 3.2a. As an estimate of population structure, we calculated theta, an F_{ST} analog developed by Weir & Cockerham (1984), which assumes an infinite alleles model of mutation (Kimura & Crow, 1964). We used the program FSTAT, version 2.9.3.2 (Goudet, 1995) (http://www2.unil.ch/popgen/softwares/fstat.htm) to test the significance values of pairwise theta, applying a Bonferonni correction (Rice, 1989). Because relative measures of differentiation, such as estimates of F_{ST} can be difficult to compare (Hedrick, 1999), we also estimated Nei's standard genetic distance (D_S) using software provided by J. Brzustowski (http://www.biology.ualberta.ca/jbrzusto/). D_S has been found to be one of the least biased estimators of genetic distance (Paetkau *et al*., 1997). We tested for isolation-by-distance by comparing ln (geographic) distance and genetic distance ($F_{ST}/1 - F_{ST}$). The significance of relationships was assessed using a Mantel test in GENEPOP, version 3.2a (Raymond & Rousset, 1995). To examine population structure we also used the program STRUCTURE (Pritchard, Stephens & Donnelly, 2000), a model-based clustering method designed for multilocus genotypes. Using a range of values of K populations, from one to nine (with burn-in periods of 50 000 and 500 000 replications), we estimated the posterior probability [Pr(K/X)] to determine the most likely clustering of populations (Pritchard *et al*., 2000).

Spatial autocorrelation

Spatial autocorrelation statistics measure the effect of proximity of sampling sites on the variable of interest measured at those sites. This effect could either be positive, in which nearby sites are more similar to each other than expected by chance, or negative, in which nearby sites are more divergent than expected by chance. Spatial autocorrelation in biotic variation may be the result of several different processes, and the mere presence of spatial autocorrelation does not necessarily indicate a problem. If biological variation is related to environmental variation, spatial autocorrelation may be the result of spatial dependence on inherently spatially autocorrelated environmental variables. It is this dependence that we wish to test for, and so it is important not to overcorrect for spatial autocorrelation because the effect that we are looking for might be removed. Nevertheless, the absence of spatial autocorrelation in a priori tests provides confidence that it will not pose a potential problem in subsequent analyses. To assess the potential influence of spatial proximity on genetic and morphological variation, we calculated the autocorrelation coefficient r (Peakall, Ruibal & Lindenmayer, 2003) in GENALEX, version 6 (Peakall & Smouse, 2006) using 999 permutations to test for significance and 1000 bootstrap replicates to estimate the 95% confidence interval.

Spatial modelling of population divergence

As an additional way to contrast the roles of refugia, isolation-by-distance and environmental heterogeneity in population divergence, and to spatially project heterogeneity in the traits quantified, we used generalized dissimilarity modelling (GDM; Ferrier *et al*., 2007). GDM is a matrix regression technique that predicts biotic dissimilarities (turnover) between sites based upon environmental dissimilarity and geographical distance. A major advantage of GDM over other modelling methodologies is that it can fit nonlinear relationships of environmental variables to biological variation through the use of I-spline basis functions (Ferrier *et al*., 2007). It is a two-step method: first, dissimilarities of a set of predictor variables are fitted to the dissimilarities in the response variable. The contributions of predictor variables to explaining the observed response variation are tested by permutations, and only those variables that are significant are retained in the final model. These procedures result in a function that describes the relationship between predictor and response variables. Second, using the function resulting from the first step, a spatial prediction is made of the response variable patterns. For visualization purposes, classes of similar response are colour coded, where larger colour differences between two localities represent larger phenotypic or genetic differences.

GDM analyses were carried out at the site level, using population averages to compute population pairwise differences. Nei's D_S was used for

microsatellites, and Euclidean distances were calculated for morphological characters and pathogen prevalence. To take into account differences in within-site variation, pairwise Euclidean distances (d) were divided by the sum of the standard deviation of the two sites i and j concerned: $d = |\bar{X}_i - \bar{X}_j| / (\sigma_i - \sigma_j)$.

The relative importance of predictor variables in a GDM can be assessed by means of response curves. Thus, the influence of geographical distance and refugia relative to other variables in explaining phenotypic variation can be assessed. We examined the potential effects of refugial isolation by means of least-cost-paths, calculated in PATHMATRIX, version 1.1 (Ray, 2005). Least-cost-paths are computed from friction surfaces in which the values of each grid cell represent the cost of travelling through that grid cell. The least-cost-path computed from the friction surface represents the distance travelled at the same time as minimizing the costs. We first calculated the distances between all pairs of sites assuming a homogeneous cost surface, which is equivalent to the geographical distances between sites. We then assessed the role of refugia by assuming that dispersal between two adjacent refugia would be two orders of magnitude more costly than the distance between the two most distant study sites. Thus, if refugia were important in divergence in olive sunbird populations, we would expect to see a signature of high levels of divergence between refugia but more similar levels of population divergence within refugia, and a strong correlation of this signature with the least-cost-paths.

To further evaluate the extent to which geographical distance is potentially correlated with environmental differences, for each region and for each response variable we ran independent tests with the following sets of predictor variables: (1) environmental variables and geographical distance; (2) only geographical distance; and (3) only environmental variables. Comparisons of the results from these three runs provided an indication of the correlation between geographical distance and environmental differences. To the extent that environmental heterogeneity and geographical distance are correlated, it is not possible to distinguish between the effects of the two on patterns of variation.

Because no formalized significance testing has yet been developed for GDM, to assess the significance of the level of variation that was explained by our models, we ran additional models in which the environmental layers were substituted for layers with random values for each grid cell. The resulting percentage of variation explained was compared against that of the full model. We considered the performance of the full model not significant if it explained an equal amount or less of the total variation than a model with random environmental variables. Although it is desirable to compare the full model against a null distribution of a large number of random models rather than a single one, we ran into the limitations of the current version of the software, which does not allow for batch processing. Nevertheless, the large differences in variation explained between random models and those considered significant provided confidence that our results are unlikely to change if a null distribution of random models would be generated.

RESULTS

Habitat and bioclimatic classifications from remote sensing

No evidence of spatial autocorrelation was found for any of the traits studied, and we therefore continued analyses without corrections for autocorrelation. As expected, the ecotone and forest sites were significantly different in the percentage of tree cover ($\chi^2 = 16.6$, d.f. = 1, $P < 0.0001$) and NDVI magnitude ($t = 5.2$, d.f. = 6, $P < 0.003$). Tree cover of ecotone sites was in the range 11.5–35.6% (mean ± SD: 26.3 ± 8.35%), whereas forest sites were in the range 56–80.8% (76.76 ± 4.5%) and showed no overlap. Other bioclimatic and remote sensing data revealed significant differences (after Bonferroni correction) between ecotone and forest sites, with forest sites generally being wetter and with greater canopy cover than ecotone sites. These included: higher precipitation in the driest month in the forest ($t = –4019$, d.f. = 15, $P < 0.003$); greater coefficient of variation of seasonality in the ecotone ($t = 4.288$, d.f. = 13, $P < 0.01$); higher precipitation of the warmest quarter in the forest ($t = –3.58$, d.f. = 15, $P < 0.01$); higher maximum leaf area index in the forest ($t = –9.12$, d.f. = 14, $P < 0.001$); higher year round NDVI in the forest ($t = –212$, d.f. = 15, $P < 0.05$); and higher mean EVI in the dry season in the ecotone ($t = 2.45$, d.f. = 7.4, $P < 0.04$). With these results, we were able to have spatially refined (1-km resolution) environmental variables that map the gradient between forest and ecotone vegetation types across the landscape. Using these variables, we then examined how genetic and morphological characteristics correlate with environmental variables.

Morphological and genetic differentiation across habitats

Rainforest and ecotone populations from Cameroon and Equatorial Guinea were found to be significantly divergent from one another based on a MANOVA of four morphologic characters (Table 2; see also Supporting information, Table S3). Adult males from ecotone habitats tended to have significantly longer

Table 2. Results of a multivariate analysis of variance between habitats (forest versus ecotone) for adult males in four morphological characters

Character	d.f.	*N*	Adult males: Forest (mean ± SD)	Adult males: Ecotone (mean ± SD)	*F*	*P*
Mass (g)	1	81	10.75 ± 0.88	10.99 ± 0.70	13.5	0.15
Wing length (mm)	1	79	60.47 ± 1.81	62.18 ± 1.35	13.55	**0.0004**
Tarsus length	1	79	15.51 ± 0.82	16.00 ± 0.57	5.7	**0.019**
Upper mandible length	1	79	21.77 ± 1.09	23.19 ± 0.98	25.7	**0.0001**
Wilk's lambda = 0.719	1				7.41	**0.001**

Significant *P*-values are shown in bold.

wings, tarsi, and bills than those in the forest. By contrast, within-habitat comparisons among adult males across both forest and ecotone sites showed no significant differences in morphology [analysis of variance (ANOVA): $P > 0.1$].

To examine differences as a function of habitat, we contrasted principal components (PC) 1 and 2 between forest and ecotone habitats. Because the matrix of eigenvectors may differ across geographically disparate populations, we first tested whether principal components were different among forest and ecotone populations. The results showed both covariance matrices were proportional ($\chi^2 = 1.893$, d.f. = 1, $P > 0.16$), allowing ecotone and forest populations to be directly compared. Both PC1 (primarily a size axis based on factor loadings) and PC2 (shape axis) were significantly different (one-way ANOVA: $F = 5.57$, d.f. = 61, $P < 0.0001$ and $F = 2.938$, d.f. = 32.5, $P < 0.006$, respectively). The relationships between the two habitats are further illustrated in Figure 2, which shows how PC1 and PC2 vary with respect to percentage tree cover across the gradient.

To examine the variability of morphological characters within and between habitats we estimated coefficients of variation (CV). The CVs varied among characters within habitats: wing 3.1 (0.32), 2.3 (0.36); tarsus 4.1 (0.43), 3.4 (0.54); and upper mandible length 5.2 (0.54), 5.2 (0.54), for forest and ecotone populations, respectively. However, there were no significant differences between habitats [*t*-tests: $P > 0.1$; for an estimation of SE and tests, see Sokal & Rohlf (1981)].

Two lines of evidence suggest that olive sunbird ranges expanded and population size increased since the Last Glacial Maximum (LGM). First, we used an ecological niche modelling approach to predict current and paleo species distributions. A comparison of the predicted distributions suggests that olive sunbird ranges have expanded considerably since the LGM (see Supporting information, Fig. S1). Second, we estimated the magnitude, direction, and timing of population size change in a hierarchical Bayesian framework. All simulations provide strong evidence for population expansion (see Supporting information, Fig. S2, Table S4), in line with the species distribution modelling results.

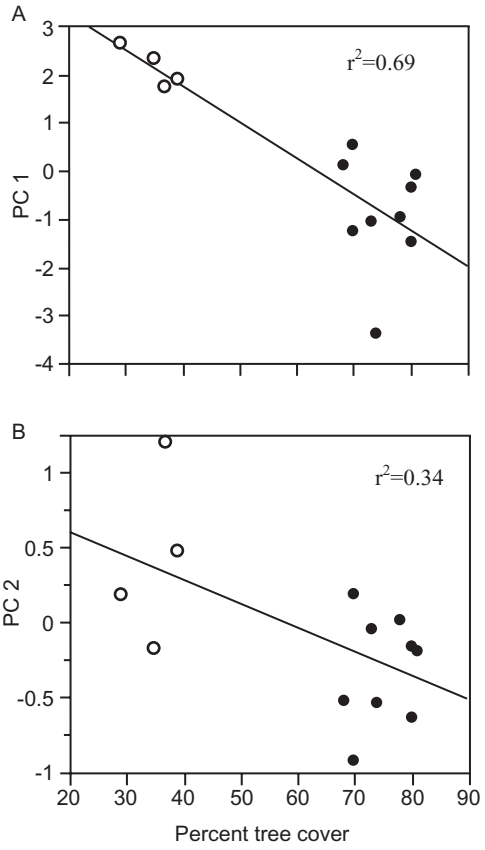

Figure 2. Principal components (PC) 1 (A) and 2 (B). Open circles are ecotone sites and closed circles are forest sites. Percentage tree cover was estimated from remote sensing data. Slopes of PC1 and PC2 on percentage tree cover were significantly different ($F = 12.6$, d.f. = 75, $P < 0.001$ and $F = 10.8$, d.f. = 76, $P < 0.01$, respectively).

Table 3. Results of generalized dissimilarity modelling analyses on morphological data

	Number of sites	Percentage of total variation explained				Selected variables*
		Full	Environmental	Distance	Random environmental	
Microsatellites	14	23.5	23.5	0.1	6.7	1, 3, 5, 12, 13, 16
Mass	13	38.4	38.4	7.1	3.5	2, 4, 8, 11, 12, 13, 17
Wing l	13	69.4	69.4	2.3	34.6	4, 5, 6, 8, 13, 16
Tarsus l	13	50.9	50.9	0	7.6	3, 4, 6, 8, 12, 13, 17
Upper mandible l	13	46.4	46.4	4.8	3.0	3, 4, 6, 9, 13, 16

*1, geographical distance; 2, elevation (SRTM); 3, elevation standard (SRTMstd); 4, surface moisture or canopy roughness (QSCATMean); 5, seasonality in surface moisture (QSCATStd); 6, Treecover; 7, normalized difference vegetation index during greenest month (NDVIgr); 8, maximum annual normalized difference vegetation index (NDVImax); 9, mean annual normalized difference vegetation index (NDVImean); 10, annual mean temperature (Bio1); 11, mean diurnal temperature range (Bio2); 12, temperature seasonality (Bio4); 13, maximum temperature of the warmest month (Bio5); 14, minimum temperature of the coldest month (Bio6); 15, annual precipitation (Bio12); 16, precipitation seasonality (Bio15); 17, precipitation of the warmest quarter (Bio18); 18, precipitation of the coldest quarter (Bio19).
Results are shown for models in which the following variables were entered: geographical distance and environmental variables (full); only environmental variables; only geographical distance; only random environmental variables. Least-cost-paths representing barriers between forest refugia were also entered in the models, although they were never selected as significantly contributing to explaining the observed variation.

Generalized dissimilarity models explained more of the total observed microsatellite and morphological variation than expected at random (Table 3). Five to seven variables contributed significantly to the full models. Given this number of predictor variables in relation to the number of sites, there is the potential for overfitting. However, each of the variables included was found to contribute to the accuracy of our predictive map. Although overfitting could remain an issue and the results obtained should therefore be interpreted with caution, overfitting is more of a concern when examining small-scale patterns of variation, and not the broad-scale patterns examined in the present study. Geographical distance was a significant explanatory variable only in the model for microsatellite variation (Table 3; see also Supporting information, Fig. S3), although its relative contribution was low, and a model with only distance explained very little of the microsatellite variation (0.1% of total variation versus 23.5% for the full model and a model that included only environmental variables). These results suggest that isolation-by-distance is not an important factor in the divergence of olive sunbird populations. Moreover, least-cost-paths, used to evaluate isolation in forest refugia, were not selected in the model, suggesting that refugia did not play a significant role in genetic divergence. Tree cover and seasonality in moisture levels were the most important variables in explaining microsatellite variation (Table 3; see also Supporting information, Fig. S3), although the total variation explained was relatively low, and the projected pattern did not show a clear differentiation between forest and ecotone sites (Fig. 3). Variables that contributed most to explaining the observed morphological variation were related to differences among ecotone and forest habitats (see above), and included precipitation of the warmest quarter for body mass; tree cover and maximum NDVI for wing length; maximum NDVI for tarsus length; and mean annual surface moisture/canopy roughness and precipitation seasonality for upper mandible length (see Supporting information, Fig. S3). Least-cost-paths were not selected in any of the models for morphological variation.

The projected patterns of variation in wing length show particularly strong separation between forest and ecotone (Fig. 3). The patterns of body mass also show separation between forest and ecotone, but not as strongly, because the pattern is smoothed by the relatively large-scale influence of precipitation of the warmest quarter. Finally, the patterns for tarsus and upper mandible lengths appear to be most strongly related to a separation between lowland and mountainous areas (Fig. 3), yet moderate separation between forest and ecotone is also apparent.

Contrasting morphological and genetic divergence

All genetic measures of population structure and divergence were low and nonsignificant, suggesting

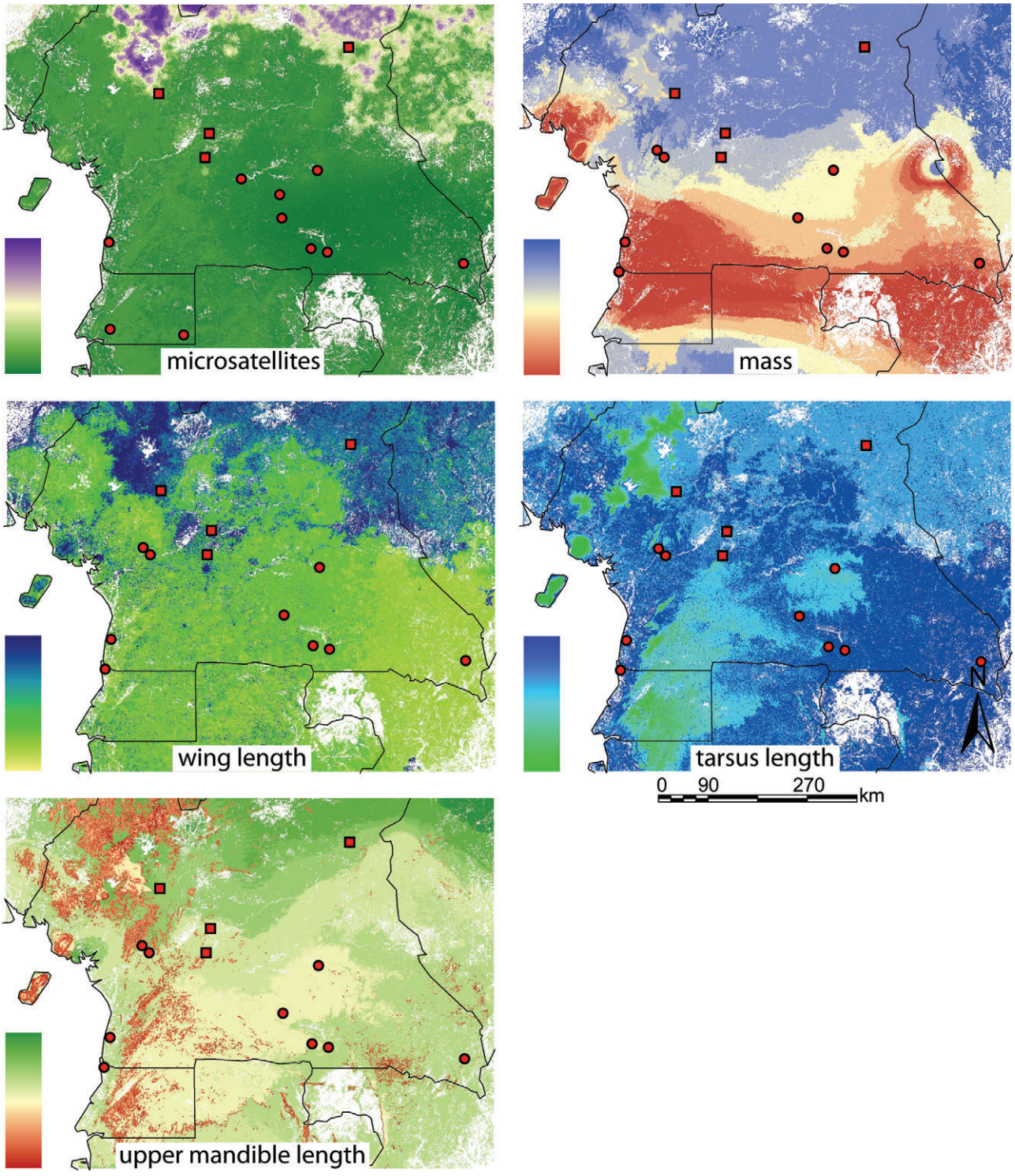

Figure 3. Projected patterns of genetic and morphological variation by generalized dissimilarity modelling. Pairwise comparison of colours between any two points in the landscape indicates the differentiation between those two points: larger colour differences correspond to larger genetic or morphological differences (see colour bars). Red squares indicate ecotone sampling localities and red circles indicate forest sites.

high levels of gene flow among populations. There was no evidence of genetic structure based on values of F_{ST} and D_S among study sites (Table 4). In addition, we found nonsignificant relationships between $F_{ST}/(1 - F_{ST})$ and geographical distance for forest–forest, ecotone–forest, and ecotone–ecotone population comparisons (Mantel's $r = 0.22$, $P > 0.8$), which suggests that isolation-by-distance does not play a role in genetic population divergence on this scale. Bayesian clustering analysis (STRUCTURE) for clusters ranging in number from $K = 1$ to 9 revealed no support for any population structure among or between forest and ecotone populations.

A plot of D_S against normalized Euclidean distance of morphological characters showed that ecotone–forest comparisons are more divergent ($\chi^2 = 1.39$, SD = 0.38) than either forest–forest or ecotone–ecotone comparisons (D_S values 0.87, 0.28, and 0.47, 0.21, respectively), irrespective of the magnitude of genetic divergence (Fig. 4). However, forest–forest comparisons tended to be more morphologically divergent than ecotone–ecotone comparisons.

DISCUSSION

Patterns of differentiation across the forest–savanna ecotone

The results show evidence of marked phenotypic differentiation in olive sunbirds across the forest–savanna ecotone in Central Africa. By contrast, we did not find any evidence for a role of forest refugia in generating divergence in olive sunbirds. As opposed to earlier avian studies of forest–ecotone morphological and genetic divergence, the present study is the first to use bioclimatic and remote sensing data to quantify habitat characteristics. Morphological differences are clearly associated with percent forest cover (in the range 60–85% for forest and 25–45% for ecotone sites) and other environmental variables. A multimodel comparison using generalized dissimilarity modelling suggested that divergence is unlikely to be driven by geographical distance or isolation in forest refugia, but rather by spatial heterogeneity in environmental variables. If forest refugia would have had a major role in divergence, genetic divergence between forest–forest and ecotone–ecotone sites in different refugia was expected. However, sunbirds show significant gene flow among all populations and differences in size and shape across the gradient, tending to be larger in the ecotone than in the forest. Larger size could have multiple causes; for example, longer wings could be a function of more open habitats and the need to fly faster to avoid predators (Schluter, 1988). Longer bills have also been shown to evolve in response to flower length in many nectarivorous species (Temeles & Roberts, 1993). However, because characters such as wing and bill length are typically positively correlated in birds (Boag & van Noordwijk, 1987), additional life-history data, especially on diet characteristics and predation levels in ecotone and forest habitats, will be necessary to fully address what factors are driving morphological differences.

Despite considerable morphological divergence between forest and ecotone sites, measures of genetic divergence in neutral markers were low, suggesting considerable gene flow between forest and ecotone populations. The absence of genetic differentiation despite marked phenotypic divergence suggests a strong pattern of divergence with gene flow. High levels of gene flow are consistent with two recent studies suggesting olive sunbirds may exhibit higher levels of dispersal than some other forest species. A mark–recapture study of olive sunbirds in fragmented landscapes of Tanzania found olive sunbirds exhibited higher dispersal rates than the other six species studied (Lens *et al.*, 2002) and a continental-wide study of *Cyanomitra olivacea* using mitochondrial DNA found very low levels of divergence (1.0–2.4%) across approximately 9000 km, from Ghana to eastern South Africa (Bowie *et al.*, 2004).

Evidence for diversification

Patterns of morphological and genetic divergence found here are consistent with expectations under the divergence-with-gene-flow model of speciation (Rice & Hostert, 1993; Smith *et al.*, 1997; Nosil, 2008). Although the lack of neutral differentiation might appear to conflict with an important role for selection across the forest–ecotone gradient in diversification, it is consistent with expectations during the early stages of diversification, where isolated regions of neutral divergence are linked to loci under divergent selection (Michel *et al.*, 2010). Given the modest number of microsatellite loci used in the present study, the probability of any of them falling within such a genomic island is quite low. Neutral genetic and morphological variation are clearly decoupled, with morphology expressing a higher rate of change; between-habitat (ecotone–forest) morphological divergence is greater per unit of neutral genetic divergence than within-habitat comparisons (either ecotone–ecotone or forest-forest). These patterns of morphological divergence and gene flow are similar to those found previously in the little greenbul (*Andropadus virens*), an African rainforest passerine, from the same region (Smith *et al.*, 1997, 2005c). Although these patterns alone, without additional data on reproductive isolation and genes under selection, are insufficient to suggest olive sunbirds are speciating in parapatry, the results are consistent with a growing

Table 4. Summary table of F_{ST} values (lower triangle) and Nei's D_S values (upper triangle) for the study sites

Site	Ndibi	Nkwouak	Zoebe-fame	Lac Lobeke	Kribi	Elende	Bobo Camp	Bouamir	Kompia	Ncoho	Mokula	Etome	Bétaré Oya	Kousse	Obala	Ndiki-nimeki
Ndibi	–	3.34694	3.41302	3.34375	3.34286	3.48214	3.30733	3.35440	3.44643	3.43527	3.35516	3.37500	3.26488	3.34722	3.42347	3.37782
Nkwouak	0.00051	–	3.46544	3.35714	3.32500	3.39732	3.42011	3.40659	3.41582	3.39509	3.35317	3.41071	3.26786	3.29563	3.40816	3.38910
Zoebefame	0.00303	0.00650	–	3.42692	3.34516	3.44355	3.42699	3.43114	3.53226	3.51613	3.44265	3.53763	3.30376	3.37814	3.39977	3.47538
Lac Lobeke	0.00119	–0.00699	–0.00292	–	3.26875	3.34766	3.35938	3.37500	3.40848	3.45508	3.35764	3.46875	3.30729	3.27951	3.35045	3.39556
Kribi	**0.02222**	0.00448	–0.00696	–0.01093	–	3.28750	3.33684	3.35577	3.26071	3.35938	3.33333	3.40000	3.12500	3.19444	3.25000	3.33158
Elende	**0.05344**	0.01657	0.01151	0.00386	0.00945	–	3.40132	3.35096	3.44643	3.40625	3.40278	3.72917	3.30208	3.32639	3.25893	3.43092
Bobo Camp	–0.00148	**0.01970**	0.00508	0.00357	0.01790	0.02835	–	3.35121	3.47556	3.43257	3.42105	3.39474	3.32675	3.30848	3.37594	3.38850
Bouamir	–0.00798	–0.00460	–0.01369	–0.01229	0.00279	–0.00850	–0.01114	–	3.47527	3.41587	3.37607	3.48077	3.33654	3.32479	3.36264	3.41700
Kompia	**0.02555**	0.00424	0.02044	0.00384	–0.01905	0.02630	**0.03155**	0.01050	–	3.40179	3.38492	3.46429	3.30357	3.32143	3.37245	3.41541
Ncoho	**0.02938**	0.00541	**0.02285**	**0.02434**	0.01823	0.02265	**0.02631**	0.00063	0.00291	–	3.47917	3.43750	3.27604	3.35417	3.36161	3.41941
Mokula	0.00808	–0.00499	0.00424	–0.00197	0.01244	0.02373	**0.02490**	–0.00903	0.00006	**0.03433**	–	3.51852	3.32870	3.32099	3.42063	3.44006
Etome	0.02570	0.02212	**0.03819**	0.04010	0.04511	**0.12132**	0.02859	0.02987	0.03283	0.03337	**0.05759**	–	3.25000	3.29630	3.46429	3.42105
Bétaré Oya	0.01446	0.00231	–0.00532	0.01560	–0.01699	0.03110	**0.02973**	0.01147	0.00976	0.00894	0.02635	0.01935	–	3.17130	3.19643	3.31579
Kousse	0.02098	–0.00697	0.00013	–0.01024	–0.01424	0.01828	0.00699	–0.00911	–0.00307	0.01405	0.00612	0.01210	–0.00481	–	3.25000	3.32602
Obala	**0.03588**	0.01901	–0.00079	0.00386	–0.00414	–0.01000	0.01982	–0.00522	0.00454	0.00880	0.02785	**0.05220**	–0.00425	–0.00682	–	3.35902
Ndikinimeki	0.00474	–0.00392	0.00497	–0.00034	0.00132	0.02083	0.00572	–0.00618	–0.00073	0.00759	0.01551	0.01953	0.01105	–0.00292	–0.00036	–

Matrix of Nei's D_S (uncorrected) above the diagonal and F_{ST} [bold indicates significant F-values ($P < 0.05$)] below the diagonal.

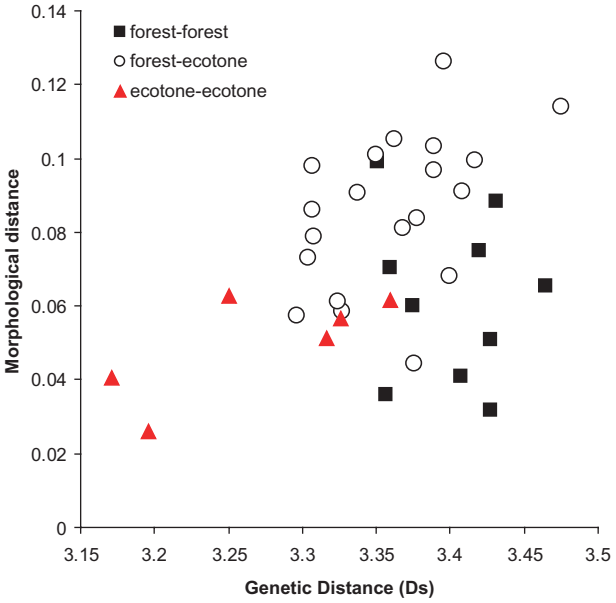

Figure 4. Plot of normalized Euclidean distance of morphological character divergence against Nei's D_S genetic distance for habitats in Lower Guinea. Overall ecotone–forest habitat comparisons show the greatest divergence per unit of genetic distance.

number of studies suggesting that ecological differences may drive divergence and may ultimately lead to speciation (Schneider *et al*., 1999; Moritz *et al*., 2000; Schluter, 2000; Ogden & Thorpe, 2002; Hendry & Taylor, 2004; Jordan *et al*., 2005; Senar *et al*., 2006; Fitzpatrick *et al*., 2008; Niemiller, Fitzpatrick & miller, 2008; Nosil, 2008; Milá *et al*., 2009; Nosil, Harmon & Seehausen, 2009; Cabanne *et al*., 2011). Future work will be needed to assess evidence for prezygotic isolation, although recent models of sympatric speciation suggest that reproductive isolation may occur rapidly, even in the face of moderate levels of gene flow (Gavrilets, 2000; Gavrilets, Li & Vose, 2000). Although empirical studies showing divergence leading to reproductive isolation are lacking, research on the little greenbul shows divergence in both morphology and song across the forest–ecotone boundary (Smith *et al*., 1997, 2005a; Slabbekoorn & Smith, 2002). In addition, recent playback experiments also show that males respond more strongly to male songs from their own habitat (Kirschel, in press). By contrast to little greenbuls that sing a complex song, sunbirds have simple vocalizations and it is unclear to what extent vocal differences could be important in mate choice and reproductive isolation. Studies of the sunbird vocal differences across the gradient will be the subject of future work. Additional markers will also be necessary to determine whether the lack of genetic differentiation found in this species is a result of high dispersal rates or possibly the result of shared ancestral polymorphism and incomplete lineage sorting (Funk & Omland, 2003). Furthermore, advances in population genomics and the advent of full-genome sequence data for non-model species will make it feasible to detect genes under selection and thus evaluate relative rates of gene flow between neutral and selective loci across habitat gradients and the extent of the genome that may be under selection across the gradient (Michel *et al*., 2010; Thibert-Plante & Hendry, 2010). Recently, the African rainforest lizard *Trachylepis affinis* from Cameroon showed evidence of adaptive genetic diversification across the forest–ecotone gradient, with refugial isolation augmented by divergent adaptation to different rainforest environments playing a less significant role (Freedman *et al*., 2010). Finally, a recent study of blood parasites in olive sunbirds, involving many of these same populations investigated in the present study, shows differences in parasite prevalence along the forest–ecotone gradient, suggesting the possibility of differential selection because of differences in disease pressures (Sehgal *et al*., 2011). In summary, the data reported in the present study add to the growing number of studies suggesting that the forest–ecotone boundary in sub-saharan Africa is an important region promoting population divergence (Chapin, 1932, 1954; Smith *et al*., 1997, 2001a, 2005b, c; Smith, Schneider & Holder, 2001b; Freedman *et al*., 2010).

ACKNOWLEDGEMENTS

We thank the governments of the Republic of Cameroon and Equatorial Guinea for permission to conduct the field research. This research was supported by grants from the National Geographic Society, National Environmental Research Council, and the National Science Foundation DEB-9726425 and IRCEB9977072 to T.B.S. T.B.S. conceived the study and collected and took the lead in analyzing the data and writing the manuscript; H.A.T. conducted the GDM analysis; A.M. performed the demographic analysis; R.N.M.S. and G.V. assisted with the field work; J.P., B.M., D.P., and R.K.W. assisted with the molecular genetic analysis; and W.B. and S.S. compiled the remote sensing data. All authors contributed to the writing of the manuscript.

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SUPPORTING INFORMATION

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

Figure S1. Ecological niche models of olive sunbird under current and paleo (LGM, Last Glacial Maximum) climate conditions. Warmer colours indicate higher, and cooler colours lower habitat suitability. A comparison of the two maps suggests range expansion since the LGM. Crosses indicate current species occurrence localities used in MAXENT (version 3.3.3a) niche models.

Figure S2. Posterior distributions for current and ancestral population size (A, C, E, G, I) and time since population expansion in years (B, D, F, H, J), derived from coalescent simulations implemented with MSVAR. Each row consists of a separate Markov chain Monte Carlo simulation, using different hyperpriors. In all simulations, population expansion is predicted. Timing of expansion falls close to the Last Glacial Maximum for three simulations (1, 4, 5). Exceptions are the second simulation, as a result of estimation problems consistent with the flat posterior on ancestral population size, and the third, which suggests a more recent expansion event, approximately 1000 years ago.

Figure S3. Response curves for variables that contributed significantly to explaining variation in microsatellites, morphology and parasite prevalence in the olive sunbird in Cameroon. The maximum reached by each curve indicates the relative importance of that variable in explaining the total variation. The slope of the curve represents the rate of change in the trait studied.

Table S1. Microsatellite loci from *Cyanomitra olivacea*. Primer sequences, repeat motifs, and the number of repeat units in the sequenced clones are indicated.

Table S2. Allelic variability of eight microsatellite loci in 18 populations of *Nectarinia obscura*. *N*, number of individuals genotyped; No. alleles, number of differently sized alleles; F_{IS}, coefficient of relatedness; H_{exp} and H_{obs}, expected and observed heterozygosities calculated using GENEPOP.

Table S3. Sample size for morphological comparisons for adult male olive sunbirds (*N*) and mean ± SD for four morphological characters for sampling sites. Sample sizes for genetic analyses are given by (N_G) and include individuals of all sex and age classes.

Table S4. Parameter settings used in MSVAR demographic analyses.

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