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Testing alternative mechanisms of evolutionary divergence in an African rain forest passerine bird

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Abstract

Models of speciation in African rain forests have stressed either the role of isolation or ecological gradients. Here we contrast patterns of morphological and genetic divergence in parapatric and allopatric populations of the Little Greenbul, *Andropadus virens*, within different and similar habitats. We sampled 263 individuals from 18 sites and four different habitat types in Upper and Lower Guinea. We show that despite relatively high rates of gene flow among populations, *A. virens* has undergone significant morphological divergence across the savanna–forest ecotone and mountain–forest boundaries. These data support a central component of the divergence-with-gene-flow model of speciation by suggesting that despite large amounts of gene flow, selection is sufficiently intense to cause morphological divergence. Despite evidence of isolation based on neutral genetic markers, we find little evidence of morphological divergence in fitness-related traits between hypothesized refugial areas. Although genetic evidence suggests populations in Upper and Lower Guinea have been isolated for over 2 million years, morphological divergence appears to be driven more by habitat differences than geographic isolation and suggests that selection in parapatry may be more important than geographic isolation in causing adaptive divergence in morphology.

Introduction

The evolutionary processes responsible for speciation in rain forests continue to engender debate and controversy (see reviews in Moritz *et al.*, 2000; Hill & Hill, 2001). Although numerous speciation mechanisms have been proposed, most researchers advocate allopatric speciation either in glacial refugia (Haffer, 1969; Mayr & O'Hara, 1986), on isolated mountains (Moreau, 1966; Fjeldså & Lovett, 1997; Roy, 1997; Garcia-Moreno & Fjeldså, 2000), or across major rivers and other topographic barriers (Patton & daSilva, in press; Moritz *et al.*, 2000). In contrast to this allopatric view are models that emphasize the role of natural selection along ecological gradients between parapatric populations (Endler, 1982a, b; Smith *et al.*, 1997; Schneider & Moritz, 1999; Schneider *et al.*, 1999). Endler (1982a, b) and Gentry (1989) emphasized the dominant role that natural selection in parapatry might potentially play in rain forest speciation; only recently have empirical studies demonstrated this role in a wide array of taxa (Rice & Hostert, 1993; Knox & Palmer, 1995; Orr & Smith, 1998; Lu & Bernatchez, 1999; Hendry *et al.*, 2000; Schluter, 2000; McKinnon & Rundle, 2002; Ogden & Thorpe, 2002). The results of these studies, suggesting parapatric speciation may be common, have been further bolstered by recent theoretical models showing that parapatric speciation can readily occur, even along habitat gradients of intermediate steepness (Doebeli & Dieckmann, 2003) and in parapatry with moderate levels of gene flow (Gavrilets, 2000; Gavrilets *et al.*, 2000).

Considerable attention has been focused on the role of Pleistocene glacial refugia in isolating previously contiguous rain forest populations and driving rain forest diversification (Crowe & Crowe, 1982; Endler, 1982b;

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Prance, 1982; Mayr & O'Hara, 1986; Patton & Smith, 1992; Colinvaux *et al.*, 1996, 2001; Haffer, 1997; Moritz *et al.*, 2000). But investigations attempting to distinguish the actual mechanism(s) of divergence and speciation (e.g. drift vs. selection) have seldom been undertaken (see Patton & Smith, 1992). Frequently, if divergence is found in allopatry, drift is assumed to be the cause (e.g. Roy, 1997), although selection may be an equally parsimonious explanation. Only through testing alternative mechanisms will the relative roles of selection and drift and the significance of parapatry and allopatry in divergence and speciation be understood (Moritz *et al.*, 2000).

Previously, we showed that populations of the Little Greenbul (*Andropadus virens*) from central and ecotone forests of Cameroon were morphologically as divergent as different species despite moderate levels of gene flow between them (Smith *et al.*, 1997). This result was found to be consistent with a major component of the divergence-with-gene-flow model of speciation, a model based on laboratory experiments with *Drosophila* (Rice & Hostert, 1993), and supported a role for ecotones in parapatric speciation (Smith *et al.*, 1997). Further work also suggests that populations of greenbuls surveyed in Upper and Lower Guinea, within or near hypothesized refugial areas, represented separate clades that had diverged from one another roughly 2 Ma (Smith, T.B. *et al.*, 2001). However, a thorough statistical morphological analysis was precluded in these earlier studies because sample sizes were small.

The vast ecotone surrounding the Congolese rain forest is over 1000 km wide in many regions and comprises over 3 million km^2. It is characterized by a mosaic of forest fragments embedded in savanna, with forest fragments becoming progressively smaller with distance from the central belt of equatorial rain forest until only savanna remains (Longman & Jenik, 1992). Ecologically, ecotone forest fragments differ from central forest habitats in numerous ways. The canopy layer is more open and reduced in stature, rainfall is lower and interannually more variable (Longman & Jenik, 1992), and species assemblages and available foods differ (Chapin, 1932, 1954), as does the prevalence of certain avian pathogens (Sehgal *et al.*, 2001). Phenotypic differences among populations of bird species in African ecotones were first described by Chapin (1932), who noted that ecotone populations had birds with a distinct morphology and that contact zones between species and subspecies were often concentrated between the forest–savanna transitional zone bordering the Congo forest. This observation was later confirmed quantitatively by Endler (1982b) who examined proportions of avian contact zones between hypothesized refugia and within and between habitats. Endler found that 52% of the avian contact zones occurred in the ecotone rather than between hypothesized locations of ancient refugia. The significance of ecotones in divergence was also further supported by Fjeldså (1994) who, using the avian phylogeny derived from the DNA–DNA hybridization studies of Sibley & Ahlquist (1990), found recently divergent taxa were concentrated in transition zones between major habitat types and on mountains.

In this paper, we use new morphological and genetic data collected over a 11-year period to test alternative mechanisms of diversification. The present study differs from previous ones (Smith, T.B. *et al.*, 1997, 2001) by expanding the populations surveyed from 12 to 18, more than doubling the number of individuals sampled (263 vs. 105), by including populations from mountains in Cameroon and the island of Bioko, and utilizing several new analytical approaches. These additional data and new analyses allow for a more comprehensive examination of the mechanisms causing phenotypic differentiation across habitats and geography. We assume that intraspecific divergence can, under certain circumstances, lead to speciation and that the magnitude of morphological divergence in fitness-related characters can be a reasonable index of the likelihood for speciation to eventually occur (Schluter, 2000). We contrast the magnitude of intraspecific variation in fitness-related traits of the Little Greenbul between populations that are allopatric and parapatric. The allopatric populations are located in Upper and Lower Guinea, areas believed to represent distinct refugia, and on the island of Bioko in the Gulf of Guinea. The parapatric populations are located along the forest–ecotone boundary of both Upper and Lower Guinea and along altitudinal gradients in the mountains of Lower Guinea (Fig. 1). Specifically, we examine whether geographically isolated populations are genetically and morphologically divergent from one another. If so, then geographic isolation should play an important role in diversification. In contrast, if populations from similar habitats are genetically divergent but show no morphological differences, while morphological differences across habitats are greater irrespective of genetic divergence, it would support a role for natural selection and suggest ecological speciation is important in diversification.

Materials and methods

Field sampling

Field research occurred over a 11-year period, between 1990 and 2001. We captured 263 individuals at 18 selected sites in Lower Guinea (Cameroon and Equatorial Guinea, including the island of Bioko) and Upper Guinea (Côte d'Ivoire) (Fig. 1).

Lower Guinea: locations and dates of field work for the forest sites are: (1) Ndibi (N 3°46′ E 12°12′), July 28 to October 20, 1990, (2) Nkwouak (N 3°52′ E 13°18′), August 7–8, 1990, June 29 to July 1, 1993, (3) Zoebefame (N 2°39′ E 13°23′), September 27–29, 1990, June 9–15, 1993, (4) Sakbayeme (N 4°2′ E 10°44′), May

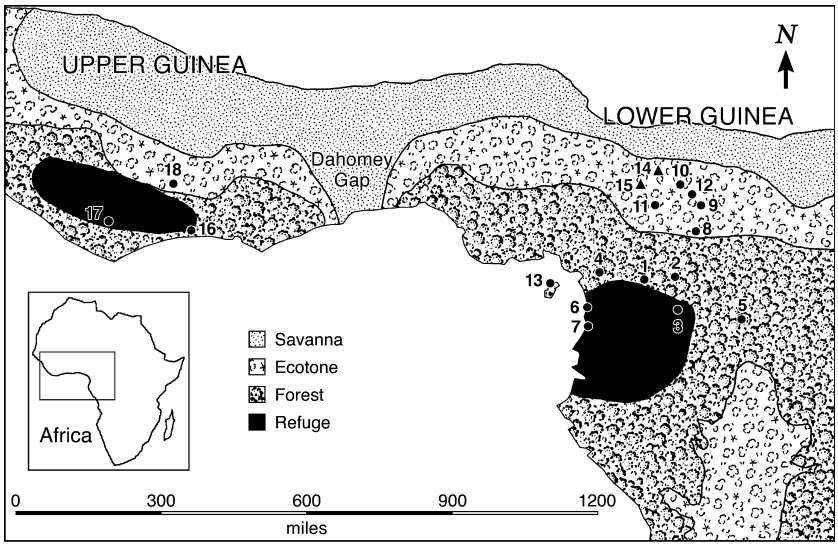

Fig. 1 Distribution of study sites in West Africa, showing forest and ecotone habitat and the locations of hypothesized Upper and Lower Guinea refugial areas (black). See numbers in 'Materials and methods' for names and localities of specific sites. Sites 14 and 15 are samples collected on mountains. Map modified from Endler (1982a); see also Maley (2001).

17–29, 2000, (5) Lac Lobeke (N 2°18′ E 15°45′), June 25–29, 1993, (6) Kribi (N 2°43′ E 9°52′), October 6–9, 1990, July 1–4, 1993, (7) Ellende (N 2°12.98′ E 9°47.57′) May 9–11, 1998.

Ecotone sites sampled included: (8) Bétaré Oya (N 5°34′ E 14°05′), August 11–13, 1990, May 5–8, 1995, (9) Meiganga (N 6°31′ E 14°18′), May 10–13, 1995, (10) Wakwa (N 7°16′ E 13°31′), June 19 to July 2, 1995, (11) Tibati (N 6°30′ E 12°35′), July 7–10, 1995, June 24–25, 1998, (12) Ngaoundaba (N 7°8′ E 13°42′), May 20–22, 1995. We also sampled one forest site on the island of Bioko: (13) (N 3°44′ E 8°43′), June 18–21 and 29, 1996. Forests on two mountains were sampled: (14) Mt Tchabal Gandaba (N 7°44′ E 12°42′), June 20–22, 1995 and (15) Tchabal Mbabo (N 7°15′ E 12°03′), July 16–18, 1995.

Upper Guinea sites in Côte d'Ivoire included the following forest sites: (16) CSRS (N 5°19′ W 5°01′), January 11–13 and 23–25, 2000, (17) Tai Entrance (N 5°49′ W 7°23′), June 25–26, 2001, and a single ecotone site, (18) Lamto (N 6°12′ W 5°01′), January 15–17, 2000.

The vegetational characteristics of the forest sites, which are summarized elsewhere (Letouzey, 1968; Louette, 1981; Smith *et al.*, 1997), include both secondary and mature forest and may generally be classified as lowland rain forest. The vegetational characteristics of the African ecotone are described by Letouzey (1968) and Louette (1981) as 'shrub–savanna with *Terminala glaucecens*' and 'forest–savanna mosaic', respectively. Further descriptions of Central African ecotone may be found elsewhere (Longman & Jenik, 1992; Smith *et al.*, in press-a). Characteristics of mountain vegetation for Mt Tchabal Mabo and Tchabal Gandaba have been detailed previously (Smith & McNiven, 1993; Smith *et al.*, 2000).

Between 15 and 20 mist nets (12 m, 30 × 30 mm mesh) were erected at each site. Netting took place between daybreak (06:00) and dusk (17:00). Captured birds were weighed, measured, banded with an aluminium numbered band for ongoing demographic and selection studies, bled, and released following methods described by Smith (1990a). Blood samples (50–100 μL) were collected from the brachial vein and stored in lysis buffer. All measurements were taken by TBS (author) using dial calipers, except mass, which was measured using a 50-g Pesola spring scale. Measurements were taken as follows: wing length, from carpal joint to the tip of the longest primary; tarsus length, from tibiotarsus joint to distal undivided scute; upper mandible length, chord length from point where culmen enters feathers of the head to tip; and bill depth in the vertical plane level at the anterior edge of the nares. Adult males were distinguished from females using a polymerase chain reaction based approach, which identifies a gene on the W chromosome (Ellegren, 1996). Juvenile birds were excluded from morphological analyses and were distinguished from adults on the basis of plumage characteristics (Keith *et al.*, 1992). We emphasized results of morphological analyses from adult male morphology, as sample sizes were larger than for females. Except where noted, results for each sex were similar.

Morphological and microsatellite analyses

To compare morphological differences among populations and domains we used both ANOVA and MANOVA on raw morphological data. As a second measure, we standardized all morphological traits by year (mean zero, unit variance) to account for seasonal variation in morphological traits by computing residual scores of measurements within years. This was carried out to account for possible seasonal differences in wear of some characters (see Gosler, 1986). We then performed the ANOVA and MANOVA on these residuals. Morphological

divergence between sites was computed as the multidimensional Euclidean distance between population means of normalized measurements following Smith *et al.* (1997). Principal components analysis was performed on log-transformed data, with components extracted from a covariance matrix. Statistical analyses were performed using the following programs: SPSS v. 10 (SPSS Inc., Chicago, IL, USA), Systat version 5.2.1 (Systat Software Inc., Point Richmond, CA, USA), and JMP 4 (SAS Institute Inc., Cary, NC, USA) for the Apple computer.

To quantify microsatellite variation we genotyped all individuals using 10 microsatellite loci (Bardeleben, 2004; Appendix I). 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, prior to performing analyses, we tested whether results should be based on *F*- or *R*-statistics following the methods of Hardy *et al.* (2002) and Hardy & Vekemans (2003). The results from this test indicate that seven of our 10 loci are better analysed with *F*-statistics. Appendix II shows the number of individuals genotyped, the sizes of alleles in each population, the inbreeding coefficient (F_{is}) for each locus by population, and expected and observed heterozygosities calculated using GENEPOP3.2a (Raymond & Rousset, 1995). We looked for the presence of nonrandom associations of alleles by calculating F_{is} values based on the methods of Weir & Cockerham (1984), and tested their significance using the exact test provided by GENEPOP3.2a. Significance values of F_{is}, pooled across all populations and for each locus by population, were also tested using FSTAT (Goudet, 1995). Heterozygote deficiencies were tested using the Hardy-Weinberg exact test option (Guo & Thompson, 1992) in GENEPOP3.2a. (http://wbiomed.curtin.edu.au/genepop/).

To quantify population genetic structure we tested for differences in allele frequencies among paired populations using a log-likelihood (*G*)-based exact test (Goudet *et al.*, 1996) performed using GENEPOP3.2a. 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://www.unil.ch/izea/softwares/fstat.html) to test the significance values of pairwise theta. To control for type I error we applied 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 (F_s) using the program by J. Brzustowski (http://biodb.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 ($F_{st}/1 - F_{st}$), as recommended by Rousset (1997). The significance of relationships was assessed using a Mantel test in Genepop3.2a (Raymond & Rousset, 1995). To examine population structure we also used the program STRUCTURE (Pritchard *et al.*, 2000), a model-based clustering method designed for multilocus genotypes. Using a range of values of *K* populations, from one to five (with burn in periods of 50 000 and 500 000 replications), we estimated the posterior probability [$P(K/X)$] to determine the most likely clustering of populations (Pritchard *et al.*, 2000). We estimated levels of gene flow using the maximum likelihood algorithm implemented in the software package MIGRATE (Beerli & Felsenstein, 2001). MIGRATE extends coalescent theory to include a per-locus mutation rate and migration rates to estimate Nm, the number of migrants per generation between populations of constant size. The results from the Markov-Chain Monte Carlo simulations reported here are based on 35 short chain searches and three long chain searches over 10 microsatellite loci.

Results

Patterns of morphological variation

Univariate and multivariate (two-way MANOVA, contrasting habitat and domain) analyses of five normalized morphological traits (cube root of mass, wing, tarsus, upper mandible length, and bill depth) revealed morphological differentiation between both domains and habitats for both adult males and females (Table 1). However, in each sex more characters were significantly different as a function of habitat type than domain. For example, among males, five characters were significantly different between habitats and two were significantly different by domain. Among females, four characters were significantly different between habitats and only one was significantly different by domain. The results of analyses using residual scores to correct for seasonal variation in morphology further supported this interpretation (Table 2). Residual values for all traits were significantly different by habitat, but no trait was different by domain. To investigate whether smaller samples from Upper than Lower Guinea may have contributed to the effect, we used a sampling test in which samples from Lower Guinea were truncated to match the sample size from Upper Guinea. The results were consistent, suggesting that habitat, not domain, was more important in driving morphological divergence (two-way MANOVA, habitat $F_{3,32} = 5.1$ $P < 0.01$, domain $F_{3,32} = 0.511$ $P =$ n.s., for adult males). The data are thus consistent with individual character differences being driven more by differences between habitat (forest vs. ecotone) than by isolation between Upper and Lower Guinea.

In Lower Guinea significant differences were found between forest and mountain populations (MANOVA Wilk's lambda = 0.35, $F_{3,63} = 19.1$, $P < 0.001$; Wilk's

Table 1 Results of two-way MANOVA between upper and lower domain and habitat (forest vs. ecotone) for adult males and females in six morphological characters. Significant *P* values are in bold.

	Whole model			Habitat			Domain		
Character	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Adult males									
Mass (g)	2	26.2	**0.0001**	1	42.5	**0.0001**	1	6.7	**0.01**
Wing length (mm)	2	52.0	**0.0001**	1	104	**0.0001**	1	0.88	0.35
Tail length	2	41.6	**0.001**	1	80.8	**0.0001**	1	0.69	0.41
Tarsus length	2	19	**0.0001**	1	35.7	**0.0001**	1	1.1	0.29
Upper mandible length	2	4.96	**0.01**	1	0.47	0.49	1	9.7	**0.002**
Bill depth	2	2.89	**0.06**	1	5.3	**0.022**	1	0.21	0.64
Wilk's lambda	12	10.67	**0.0001**						
Adult females									
Mass (g)	2	8.4	**0.001**	1	14.5	**0.0003**	1	0.59	0.44
Wing length (mm)	2	27.5	**0.0001**	1	51.9	**0.0001**	1	10.2	**0.002**
Tail length	2	34.2	**0.0001**	1	67.8	**0.0001**	1	0.68	0.41
Tarsus length	2	4.55	**0.01**	1	8.3	**0.005**	1	0.154	0.69
Upper mandible length	2	0.25	0.78	1	0.001	0.99	1	0.483	0.49
Bill depth	2	0.32	0.73	1	0.62	0.43	1	0.001	0.99
Wilk's lambda	12	6.81	**0.0001**						

lambda = 0.578, $F_{3,45} = 6.68$, $P < 0.001$ for males and females, respectively). Univariate comparisons also showed significant differences in all characters ($P < 0.01$) except for upper bill length in adult males and females and bill depth in females. However, there were no significant differences between ecotone and mountain populations in either sex (MANOVA Wilk's lambda = 0.89, $F_{6,48} = 0.55$, P = n.s.; MANOVA Wilk's lambda = 0.79, $F_{6,18} = 0.47$, P = n.s., for males and females respectively). The magnitude and direction of differences between sites were similar when we used residual scores. There were no significant differences between the two mountain populations for any morphological characters (ANOVA $F_{1,23} = 3.0$, P = n.s.).

Little Greenbul populations from the forests on the island of Bioko showed many significant morphological differences from Lower Guinea mainland populations. Overall, adult males from Bioko were larger in body size (as indexed by mass, wing, tail, and tarsus length) (MANOVA Wilk's lambda = 0.73, $F_{4,47} = 3.21$, $P < 0.01$) and had significantly longer and deeper bills ($F_{1,54} = 9.07$, $P < 0.01$; $F_{1,54} = 2.69$, $P < 0.05$, respectively) than Lower Guinea forest populations. Compared with the island populations, ecotone males were significantly different in morphological characters (MANOVA Wilk's lambda = 0.428, $F_{5,39} = 8.25$, $P < 0.001$) although the direction of change differed. Sample sizes, mean values, and standard deviations for each sample site are shown in Appendix III.

Based on the factor loadings, the first principal component (PC1) was primarily a size axis and the second (PC2) was primarily a shape axis (Fig. 2), a common pattern found in birds (Smith, 1990b). A principal component plot of the first two principal components showed that ecotone and mountain populations in Lower Guinea were similar in size and shape. Other populations differed in overall size with Lower Guinea forest showing the most divergence. Only the island population was strongly distinctive in shape from other populations (Fig. 2). This divergence in shape was apparently due in part to island birds having shorter wings and longer bills than mainland birds.

Table 2 Summary table of univariate ANOVA using residual values of morphological traits (corrected for seasonal variation in trait values) (Gosler, 1986). Note that all of the differences in morphology are because of differences in habitat (forest, ecotone, mountain and island) and none are because of domain (Upper vs. Lower Guinea). Data shown are for adult males only. Comparisons for females were nonsignificant.

Trait	Source	d.f.	Mean square	*F*-value	*P*-value
Mass	Habitat	3	52.64	12.05	0.0001
	Domain	1	0.62	0.14	0.7064
Wing length	Habitat	3	313.915	30.721	0.0001
	Domain	1	0.811	0.079	0.7786
Tail length	Habitat	3	356.616	21.813	0.0001
	Domain	1	1.813	0.111	0.7396
Tarsus length	Habitat	3	7.814	11.464	0.0001
	Domain	1	0.344	0.505	0.4783
Culmen length	Habitat	3	2.419	3.694	0.0134
	Domain	1	0.05	0.076	0.7835
Bill depth	Habitat	3	0.207	3.903	0.0102
	Domain	1	0.025	0.48	0.4896

Linkage disequilibrium, Hardy-Weinberg equilibrium, and genetic variation

No significant linkage disequilibrium was detected for any locus pair, with the exception of Avi26 and Avi30 in

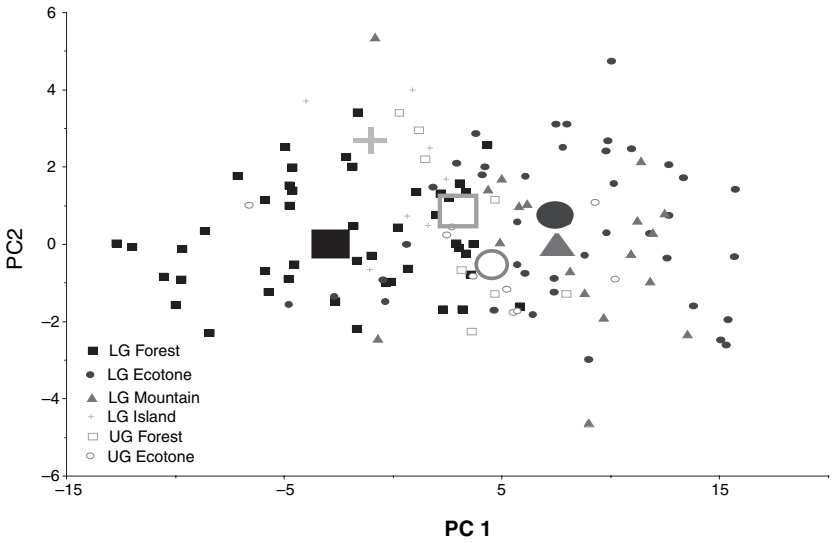

Fig. 2 Principal component plot of six morphological characters from adult males showing group centroids for the six regions in Lower Guinea (LG) and Upper Guinea (UG). Approximately 95% of the variance was accounted for by PC1 and PC2, with most (90.1%) accounted for by PC1. Factor loading suggest that PC1 is primarily a size axis and PC2 a shape axis, consistent with analyses with other bird species (Smith, 1990b).

the populations of Ndibi and Tibati (Appendix II). Therefore, we consider alleles at the 10 loci to be inherited independently. We found no evidence of the presence of null alleles for any of the loci. None of the loci showed a significant deficit of heterozygotes across populations (critical value following Bonferroni corrections for multiple comparisons). No significant differences among sites in the numbers of alleles, F_{is}, observed (H_0), or expected heterozygosity (H_e) were found (Appendix II).

Table 3 Inferring the value of the number of populations *K* for *A. virens*.

K	log *P*(*X*/*K*)	*P*(*K*/*X*)
1	−8663	∼0
2	−8406	∼0
3	−8382	0.0067
4	−8377	0.993
5	−8385	0.0003

Population genetic structure and gene flow

In a previous study (Smith, T.B. *et al.*, 2001), mtDNA (ND2) sequences were used to assess phylogenetic divergence between Upper and Lower Guinea. Two strongly supported lineages were identified corresponding to each domain whose corrected sequence divergence was 4.7%. Assuming a molecular clock, we estimated that the two populations split approximately 2 Ma. However, mtDNA haplotypes were not geographically partitioned within domains. In this study, we used a new array of 10 tetranucleotide microsatellite loci and increased localities and habitats sampled to include island, mountain, ecotone, and rain forest habitats in both refugia.

Analysis with the program STRUCTURE (Pritchard *et al.*, 2000) suggests division into four populations having the highest likelihood (posterior probability = 0.993). These populations included: two mainland populations (one in Upper and one in Lower Guinea, each including both forest and ecotone), a mountain population, and an island population (Bioko) (Table 3). This suggests that although there is evidence of considerable gene flow, four populations can be identified as genetically distinctive. The analysis did not distinguish ecotone and forest populations in either Upper or Lower Guinea as distinct genetic units even when these domains were analysed separately.

Based on the genetic divisions revealed using the program STRUCTURE, we estimated the magnitude and directionality of gene flow within and among domains using the program MIGRATE. Within Lower Guinea gene flow was highest in the direction from forest–ecotone to mountain. Gene flow from the island of Bioko was also higher towards the mainland (Table 4a). If selection pressures are comparable in island and mainland populations, then one might expect selection to play a more significant role in divergence for island populations, which receive fewer immigrants relative to mainland populations. Similarly, morphological divergence in mountain populations relative to forest populations has occurred despite the fact that mountains receive nearly twice as many emigrants from forest populations as they send immigrants to the forest, again suggesting that divergent selection may overcome the homogenizing force of gene flow. Gene flow between Upper and Lower Guinea was estimated to be as much as 18 times lower than between habitats within a domain (Table 4b).

There was a nonsignificant relationship between $F_{st}/1 - F_{st}$ and geographic distance for forest–forest or ecotone–forest population comparisons (Rousset, 1997) (Mantel's $r = 0.12$, $P =$ n.s.). However, a significant

Table 4 Results from our analysis using the maximum likelihood algorithm MIGRATE. (a) Migration rates (Nm plus 5% and 95% CIs) between study populations from Lower Guinea, the island, and the mountain. (b) Migration rates (Nm plus 5% and 95% CIs) for our study sites in Upper and Lower Guinea, further differentiated by habitat type (forest vs. ecotone). In both (a) and (b), Nm values in the dominant direction of gene flow are in italics.

(a)	Lower Guinea (forest and ecotone combined)	Island	Mountain
Lower Guinea (f and e)	–	*6.59* (*6.23–6.97*)	13.56 (13.04–140.9)
Island	5.32 (5.02–5.65)	–	1.25 (1.11–1.40)
Mountain	*20.93* (*20.02–21.85*)	*3.91* (*3.56–4.29*)	–

(b)	Upper Guinea forest	Upper Guinea ecotone	Lower Guinea forest	Lower Guinea ecotone
Upper Guinea forest	–	13.25 (12.57–13.96)	*4.64* (*4.23–5.06*)	*9.22* (*8.65–9.82*)
Upper Guinea ecotone	*18.30* (*17.47–19.15*)	–	2.90 (2.59–3.24)	*4.07* (*3.69–4.47*)
Lower Guinea forest	3.31 (3.03–3.60)	*4.43* (*4.11–4.76*)	–	*19.97* (*19.23–20.72*)
Lower Guinea ecotone	1.04 (0.91–1.18)	3.43 (3.19–3.67)	15.99 (15.46–16.53)	–

relationship was found between these variables for ecotone–ecotone pairs (Mantel's $r = 0.98$, $P < 0.05$). The latter result may reflect the geographic structure of ecotone forests that are distributed along rivers that run from savannas in the north to rain forests in the south. Consequently, dispersal may be constrained to movement along river drainages resulting in a pattern of isolation by distance.

Combined morphological and genetic analyses

Lower Guinea – bivariate plots of $F_{st}/(1 - F_{st})$ against normalized Euclidean distance of morphological characters showed that ecotone–forest and forest–mountain comparisons tended to be more divergent (average morphological divergence values 3.6 and 3.2, respectively) than forest–forest, ecotone–ecotone and mountain–ecotone comparisons (0.99, 1.17 and 0.9, respectively) irrespective of the magnitude of $F_{st}/(1 - F_{st})$ (Fig. 3a). The greatest morphological divergence was found between ecotone and forest site comparisons (Fig. 3a). The only between-habitat comparison to show little morphological divergence was between mountain and ecotone. Whether this was a result of the fact that the mountains we sampled were in the ecotone and, therefore, more similar in habitat characteristics, will require further investigation.

Genetic divergence between mountains tended to be high (Fig. 3b), while morphological divergence tended to be low. For example, sampling sites on the two mountains separated by only 91 km were genetically more

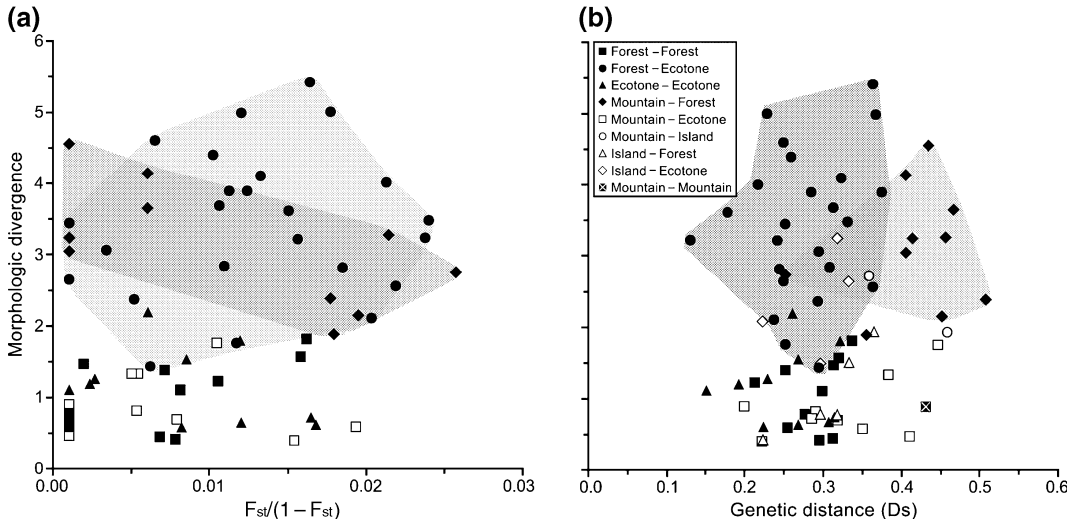

Fig. 3 Plot of normalized Euclidean distance of morphological characters divergence against (a) $F_{st}/(1 - F_{st})$ and (b) Nei's genetic distance for habitats in Lower Guinea. Shaded areas highlight the two habitats that exhibit the highest divergence between them (e.g. ecotone–forest and mountain–forest).

divergent ($D_s = 0.437$) than forest populations that were more than 800 km apart ($D_s = 0.252$; Appendix IV), but morphological divergence between mountains yielded some of the lowest values (0.94). This suggests that isolation on mountains may contribute to genetic but not morphological divergence.

Forest habitats on the island of Bioko showed levels of morphological and genetic divergence similar to that found between forest sites on the mainland (Fig. 3a,b). Despite Bioko being approximately 60 km from the mainland, there was little evidence of strong genetic isolation, although, as mentioned above, the model-based clustering method, STRUCTURE, did assign island individuals to a separate population, suggesting population substructure.

Upper Guinea – the level of morphological divergence among sites was lower and although sites were closer geographically, genetic distances (D_s) were as much as 52% larger between the ecotone and forest sites (Lamto vs. CSRS, 0.293 and Lamto vs. Tai, 0.347) than they were between the two forest sites (CSRS vs. Tai, 0.181). This result suggests that, unlike Lower Guinea, lower rates of gene flow may characterize this forest–ecotone boundary. Given that only two forest sites and one ecotone site were sampled, further sampling will be necessary to determine the generality of this observation.

Upper vs. Lower Guinea – forest–ecotone and ecotone–ecotone comparisons from Upper and Lower Guinea tended to show some of the highest values of morphological divergence (Fig. 4), while the Upper Guinea forest and Lower Guinea island comparisons tended to show the lowest morphological divergence. The greatest genetic divergence of any pair was between Upper Guinea ecotone and forest comparisons with Lower Guinea mountains. It should be emphasized that, in the present study, we sampled mountains from only mainland Lower Guinea and were, therefore, unable to contrast mountains between domains or between the mountains on the mainland and those on the island of Bioko.

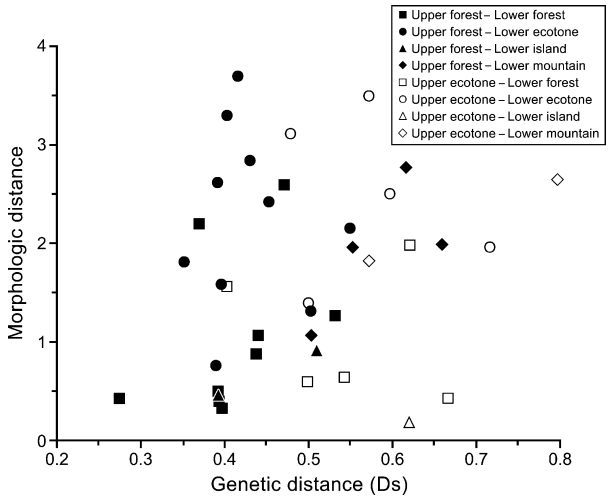

Fig. 4 Plot of normalized Euclidean distance of morphological characters divergence against Nei's genetic distance contrasting between habitats in Upper and Lower Guinea.

Discussion

Morphological divergence was found to be greater between ecotone and forest populations within Lower Guinea populations than between geographically isolated populations from the same habitats in Upper and Lower Guinea. Although Upper and Lower Guinea populations have been separated for approximately 2 million years (Smith, T.B. *et al.*, 2001), this period of isolation has resulted in little divergence in morphologic traits. While these results are consistent with morphological divergence being driven by natural selection they should nevertheless be interpreted with caution for two reasons. First, samples sizes for Upper Guinea were much smaller than for Lower Guinea. Thus it is possible that greater sample sizes would reveal differences between the two domains. Secondly, the larger number of molecular markers compared with quantitative traits analysed in this study may have increased the likelihood of finding significant genetic divergence relative to morphological divergence (Foulley & Hill, 1999). Finally, while we found no evidence for phenotypic plasticity in the traits we measured, plasticity cannot be completely ruled out as a causative agent. However, we believe that phenotypic plasticity is unlikely to be the major cause for the observed divergence.

With the exception of slight changes in traits because of seasonal wear (for which we have tested and excluded some individuals), there was little evidence for plasticity in the traits we measured. Furthermore, all of the traits are generally highly heritable in bird species and frequently correlated with performance and fitness, and are not typically determined by environmental factors (Grant, 1986; Schluter & Smith, 1986; Cook & Buckley, 1987). Previously, we have also shown that there is a significant inverse relationship between morphological divergence and gene flow between these habitat types (Smith *et al.*, 1997). In other words, gene flow in this case homogenizes the effect of selection on morphological divergence. The negative slope indicates that at least some of the variation in morphology that we measured in the field must have a genetic basis as gene flow would not homogenize variation in traits that were purely plastic. The inverse relationship between morphological divergence and gene flow is exactly the pattern predicted for genetically based traits under selection. While it is possible that some variation arises because of plasticity, our results to date substantiate our assertion that at least some of the variation arises because of variation at genetic loci influencing these traits.

Comparatively lower levels of morphological divergence between forest and ecotone populations in Upper Guinea than in Lower Guinea will require further investigation, although small sample sizes and the unique nature of ecotone forest fragments sampled in Upper Guinea may be factors. Only one ecotone site was sampled in Upper Guinea and this site (Lamto) included a large section of riverine forest contiguous with forest to the south. Consequently, Lamto may represent a mixture of selection regimes characteristic of both forest and ecotone habitats. Another difference is that the transition between forest and ecotone in Lower Guinea occurs along an elevational gradient of more than 800 m, a salient difference from Upper Guinea where the transition from forest to savanna does not occur along an elevational gradient. Nevertheless, this again underscores the need for additional sampling in Upper Guinea to increase sample size.

Morphological divergence per unit genetic distance was also high between mountain and forest populations in Lower Guinea, suggesting that divergent natural selection, when combined with genetic isolation, is important in driving morphological divergence. However, in contrast to studies suggesting that isolation on mountains promotes divergence and speciation (Moreau, 1966; Roy, 1997), our results indicate that mountain isolation alone is insufficient to promote morphological differences in this species. Populations from the two mountains, Tchabal Mbabo and Tchabal Gandaba, were found to be morphologically similar. However, significant morphological divergence does occur across forest–mountain gradients. Together, these data support a central component of the divergence-with-gene-flow model of speciation by suggesting that despite large amounts of gene flow, selection is sufficiently intense to cause morphological divergence.

The population on the island of Bioko was divergent from other populations primarily in morphologic shape characteristics, and was largely the result of island birds having shorter wings and longer bills than mainland populations. Previous studies showing island populations to have distinctive morphologies from mainland ones have attributed these differences to drift, reduced competition, or novel selective pressures (Mayr, 1963; Clegg *et al.*, 2002; Robinson-Wolrath & Owens, 2003). While further studies will be necessary to determine a cause, the relatively high levels of gene flow between Bioko and the mainland make drift an unlikely explanation. Interestingly, results from our analysis using the program MIGRATE suggest that the directionality of gene flow is primarily from the island to the mainland. Apparently, Little Greenbuls do not find the 60-km water barrier between the island and the mainland, which are in sight of each other, to be an obstacle to dispersal.

In contrast to Africa, where there is little doubt that lowland refugia existed, the notion that Pleistocene refugia existed in lowland South America has been questioned by some authors based on paleobotanical data (Colinvaux *et al.*, 1996, 2000, 2001). Other geographic barriers, such as rivers (Hall & Harvey, 2002) and mountains (Garcia-Moreno & Fjeldsa, 2000), may play important roles in vicariant divergence. South America also has dramatic ecological gradients. These include those found along the slopes of the Andes and lowland Cerrado of Brazil, which consists of a matrix of forest and savanna habitats (Oliveira & Marquis, 2002), and represents the second largest biome on the continent (da Silva & Bates, 2002). Although the evolutionary significance of these gradients is poorly understood, some recent studies point to their possible roles in diversification. For example, Smith, M.F. *et al.* (2001) tested models of diversification of mice in the *Abothix olivaceus/xanthorhinus* complex, and found evidence for both vicariant and gradient modes of speciation in Chile and Argentina. In addition, Spector (2002) suggested the Cerrado of eastern Bolivia might be an important driver of speciation in some insect species. Consistent with this idea are the high levels of endemism found in the Cerrado, estimated to be 44% for vascular plants and 30% for amphibians (da Silva & Bates, 2002). In contrast to African ecotones, da Silva & Bates (2002) report little intraspecific differentiation across the transition in bird species. In addition, a recent phylogenetic examination of woodcreepers found that sister taxa were not distributed across the Varzea-terra-firme ecotone in South America (Alexio, 2002). These results stand in contrast with the high intraspecific variation described in this study, and those recently described in sunbirds (Nectarinidae) from Central Africa, in which taxa in lowland and ecotone forests were found to be sister taxa, suggesting ecotone speciation (Smith *et al.*, in press-b).

Our results mirror those of Schneider & Moritz (1999) who found little morphological divergence in lizards separated by a major geographic barrier in the Wet Tropics of Australia. Although populations were isolated for approximately 5 million years, no morphological differences were found between populations from the same habitats in different refugia. In contrast, morphological differences were substantial between populations in adjacent but differing habitats, often separated by less than a few hundred metres. Similarly, two species in the lizard genus *Carlia* showed no morphological differences between similar habitats across the barrier, but showed substantial morphological divergence in parapatry between rain forest and neighbouring ecotone populations of the tall, open eucalyptus forest. In some comparisons these populations differed by as much as 25% in body size (Schneider *et al.*, 1999). Furthermore, Schneider *et al.* (1999) showed that increased predation intensity by birds in the more open ecotone habitat explained the presence of smaller individuals there. Mayr & O'Hara (1986) asserted that the difference in species assemblages between Upper and Lower Guinea bird species was consistent with isolation during the Pleistocene.

In the specific case of greenbuls, divergence occurred much earlier than the Pleistocene (Smith *et al.*, 2000) and we can reject the role of the Pleistocene in genetic divergence. The results presented here, based on patterns of intraspecific variation, suggest that habitat differences are important in driving adaptive divergence and ultimately diversification.

In conclusion, our study emphasizes the importance of testing alternative hypotheses in the study of divergence and speciation. We show that geographical isolation alone, even between ancient refugia, while potentially leading to genetic differentiation does not necessarily lead to substantive morphological divergence. Instead of isolation, the main driver of morphological divergence in populations of Little Greenbuls is likely to be because of differential natural selection caused by ecological differences in habitat. The specific factors that generate these differences will be the subject of future work. The results show that significant morphological divergence can occur between populations in different habitats even in face of high gene flow. The plausibility of speciation in the presence of gene flow has been receiving mounting empirical support (Schliewen *et al.*, 1994, 2001; Schluter, 1994; Johannson *et al.*, 1995; Knox & Palmer, 1995; Feder, 1998; Gilason *et al.*, 1999; Via, 2001; Spector, 2002) and recent theoretical work suggests that speciation in parapartry can occur even with moderate levels of gene flow if selection is sufficiently strong (Gavrilets, 2000; Gavrilets *et al.*, 2000; Doebeli & Dieckmann, 2003). According to the divergence-with-gene-flow model, such divergence may lead to speciation if traits important in reproductive isolation are associated or are the same traits as those under selection (Rice & Hostert, 1993). In a previous study Smith *et al.* (1997) found evidence of divergence with gene flow occurring between forest and ecotone habitats. Data presented here support these earlier findings and further suggest forest–mountain habitats also show strong patterns of divergence with gene flow. The data further suggest elevational gradients may be as important in diversification in African as they are purported to be in South America (Smith, M.F. *et al.*, 2001).

While we have not found evidence for reproductive isolation, we have shown that male Little Greenbuls sing structurally different songs in ecotone and forest habitats and that the vocalizations differ significantly in call frequency and rate (Slabbekoorn & Smith, 2002). Differences in vocalizations between ecotone and forest populations appear to arise from differences in ambient noise between the two habitats. Thus, the song of the male Little Greenbul has diverged in parallel with morphological traits important to fitness. If, as a consequence, male song provides cues that allow females to choose mates (i.e. positive assortative mating by habitat), reproductive divergence could follow. Whether females perceive these differences and use them in mate choice, and the extent to which song differs between Upper and Lower Guinea populations, remains the subject of future study.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/jeb/jeb825/jeb825sm.htm.

Appendix A1 Characterization of microsatellite loci for the Little Greenbul.

Appendix A2 Allelic variability of 10 microsatellite loci in 18 populations of Little Greenbul.

Appendix A3 Sample size for morphological comparisons for adult male *Andropadus virens* (*N*) and mean and standard deviation for six morphological characters.

Appendix A4 Summary table of Nei's D_s values and F_{st} values among study sites.

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