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Evolutionary consequences of human disturbance in a rainforest bird species from Central Africa

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Abstract

Relatively little attention has been directed towards understanding the impacts of human disturbance on evolutionary processes that produce and maintain biodiversity. Here, we examine the influence of anthropogenic habitat changes on traits typically associated with natural and sexual selection in the little greenbul (*Andropadus virens*), an African rainforest bird species. Using satellite remote-sensing and field survey data, we classified habitats into nonhuman-altered mature and human-altered secondary forest. Mature rainforest consisted of pristine rainforest, with little or no human influence, and secondary forest was characterized by plantations of coffee and cacao and high human impacts. *Andropadus virens* abundance was higher in secondary forest, and populations inhabiting mature rainforest were significantly larger in wing and tarsus length and bill size; characters often correlated with fitness. To assess the extent to which characters important in sexual section and mate choice might be influenced by habitat change, we also examined differences in plumage colour and song. Plumage colour and the variance in plumage luminance were found to differ between forest types, and song duration was found to be significantly longer in mature forest. The possible adaptive significance of these differences in traits is discussed. Despite relatively high levels of gene flow across habitats, amplified fragment length polymorphism analysis revealed that a small proportion of high-F_{ST} loci differentiated mature from secondary forest populations. These loci were significant outliers against neutral expectations in a simulation analysis, suggesting a role for divergent selection in differentiation across habitats. A distance-based redundancy analysis further showed that forest type as defined by remote-sensing variables was significantly associated with genetic dissimilarities between habitats, even when controlling for distance. The observed shifts in morphology, plumage and song were consistent with divergent selection on heritable variation, but a role for plasticity cannot be ruled out. Results suggest that anthropogenic habitat changes may have evolutionary consequences, with implications for conservation and restoration.

Keywords: AFLP, genetic structure, human disturbance, plumage divergence, rainforest diversification, song divergence, speciation

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Introduction

Tropical rainforests comprise only 7% of the Earth's land surface but are estimated to contain over half of all species (Myers 1986). Consequently, the loss of rainforest has a greater proportional effect on the world's total biodiversity than any other ecosystem (Foley *et al*. 2005). Each year, approximately 1.4 million hectares of tropical rainforest are lost (Achard *et al*. 2002). In addition, the alteration of tropical forests by agriculture, logging, fires and road building is causing profound changes in the biotic

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communities that occupy them (Vitousek *et al*. 1997; Foley *et al*. 2005; Tyliankis *et al*. 2007). For example, it is now estimated that over 30% of the Amazon basin is in various stages of pasture abandonment or secondary forest growth (Houghton *et al*. 2000). Over the last two decades, a great deal of attention has focused on ways of mitigating rainforest loss and slowing the rate of extinction (Myers *et al*. 2000; Myers 2002). While these efforts are essential for the preservation of species and should be expanded, more attention should be directed towards understanding how anthropogenic changes affect evolutionary trajectories of species that persist in degraded habitats (Seehausen *et al*. 1997; Smith *et al*. 2001a). Highly sensitive species, with narrow niches, may simply go extinct in response to habitat degradation, while others, with wider environmental tolerances, may persist in human-altered landscapes and adapt to the changes. Human alterations may have a profound influence on the direction and strength of natural and sexual selection (Smith *et al*. 1995; Both *et al*. 2006; Hendry *et al*. 2006; Seehausen 2006b). In human-altered habitats, native predators, pathogens, competitors and food resources may be reduced or absent and may be replaced by novel ones (Foley *et al*. 2005). Habitat changes that alter the signalling environment may disrupt mate choice/recognition processes, shift the direction of selection on secondary sexual characters, and/or cause extinction through hybridization (Seehausen *et al*. 1997; Seehausen 2006a; Slabbekoorn & den Boer-Visser 2006; Smith & Grether in press). Previously, there have been few studies to examine evolutionary change in human-altered habitats in which multiple characters important in both natural and sexual selection have been examined simultaneously.

Here, we examine evidence for evolutionary effects of anthropogenic habitat alteration on a species of passerine rainforest bird from Central Africa, the little greenbul (*Andropadus virens*). The little greenbul is a common equatorial rainforest bird species found in both mature rainforest and human-dominated landscapes (Louette 1981) and has been the subject of long-term ecological and evolutionary study (Smith *et al*. 1997; Smith *et al*. 2001b; Smith *et al*. 2005). We contrast populations living in non human-altered mature (pristine) and human-altered secondary forest, characterized by plantations of coffee and cacao. To evaluate evidence for possible evolutionary changes on multiple traits typically under natural and sexual selection, we (i) classify forest habitats using remote sensing data; (ii) estimate differences in relative abundance to evaluate the potential of density-dependent natural selection (see Svensson & Sinervo 2000); (iii) examine patterns of divergence in morphological traits that have been shown to be important in performance and fitness in other species (e.g. bill size, wing and tarsus length); (iv) assess the potential for differences in sexual selection by examining differences in plumage colouration and song; and (v) assess patterns of genetic differentiation, gene flow and evidence for loci under selection between populations living in different environments using a genome-wide scan with amplified fragment length polymorphisms (AFLP).

Materials and methods

Remote sensing and ground surveys

To characterize and quantify differences between primary and secondary forest, we used data gathered from satellite remote sensing. Remote-sensing data from the recently launched Moderate Resolution Imaging Spectroradiometer (MODIS) sensor (Justice *et al*. 1998) were used to quantify differences between the two habitats. Specifically, three different vegetation satellite products at 1-km spatial resolution were utilized: (i) monthly global MODIS Normalized Difference Vegetation Index (NDVI) product (MOD13A2; Huete *et al*. 2002) from the year 2001. The NDVI is computed through the normalized difference in surface reflectances at near-infrared and red wavelengths, and is sensitive to photosynthetic activity (Huete *et al*. 2002). (ii) Monthly global MODIS leaf area index (LAI) data (MOD15A2) is derived from visible and near-infrared reflectances and by a model that simulates the radiative transfer in vegetation canopies (Knyazikhin *et al*. 1998; Myneni *et al*. 2002). The LAI is defined as the one-sided projected green leaf area per unit ground area (Myneni *et al*. 2002), and in this study, we used monthly averages based on 5 years of MODIS LAI data (2000–04). (iii) MODIS vegetation continuous field (VCF) product which is a measure of the percentage of tree canopy cover (Hansen *et al*. 2002) in the year 2001. Similar to NDVI and LAI, satellite reflectances at visible and near-infrared wavelengths were used in a regression tree framework that solves for basic vegetation characteristics including tree, herbaceous and bare ground cover (Hansen *et al*. 2002).

For the monthly NDVI and LAI data, we computed several vegetation metrics expressing spatial and temporal variations. These include annual mean, maximum and minimum NDVI/LAI, as well as NDVI/LAI during the driest and wettest quarters. Finally, ground surveys of sites were used to confirm the degree of human disturbance of each site.

Field sampling

Fieldwork took place between 1990 and 2005 at five mature and seven secondary forest sites in Cameroon and Equatorial Guinea (see Smith *et al*. 2005 for further details). Locations and dates of fieldwork for each secondary forest sites included: Kribi (2°43′N, 9°52′E), 6–9 October 1990, 1–4 July 1993; Nkwouak (3°52′N, 13°18′E), 7–8 August 1990, 29 June–2 July 1993, 7–12 July 2005; Kompia (3°32′N, 12°50′E), 5–6 March 1997, 26–27 July 1999; Etome (4°02′N, 9°06′E), 13–14 July 1999; Sakbayeme (4°2′N, 10°44′E), 17–19 May

2000; Sangmbengue (4°04′N, 10°33′E) May 25–26, 2000; Ndibi (2°43′50″N, 9°52′19″E), 28 July–20 October 1990; 4–5 July 1993, 27 June–4 July 2005. Mature forest sites included: Zoebefame (2°39′N, 13°23′E), 27–29 September 1990, 9–15 June 1993, 24–27 July 2005; Bobo Camp (2°39′N, 13°28′E), 15–20 June 1993, 19–22 July 2005; Lac Lobeke (2°18′N, 15°45′E), 25–29 June 1993; Bouamir (3°11′26″N, 12°48′42″E), 28 April 1995, 20–21 June 1995, 8–11 June 1996, 20–23 July 1999; and Elende (2°12.98′N, 9°47.57′E), 9–11 May 1998. For further information see previous work (Smith *et al*. 2005).

Between 15 and 20 mist nets (12 m, 30 × 30 mm mesh) were erected at each site. Netting generally took place between daybreak (0600 h) and dusk (1700 h), except during periods of rain or high temperatures. Relative abundance of individuals at each site was calculated by multiplying the number of net metres run by the number of hours nets remained open. We first examined data for all years combined regardless of season, and second, to control for season and year, we examined two secondary and two mature sites sampled in 2005 during the month of July.

Captured birds were weighed, measured, banded with a numbered aluminium band for ongoing demographic and selection studies, bled, and released (Smith 1990). Blood samples (50–100 μL) were collected from the brachial vein and stored in lysis buffer. Four feathers (left and right wing coverts and left and right outer retrices) were collected for plumage colour analysis. All measurements were taken by T.B. Smith 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 at the anterior edge of the nares. Adult males were distinguished from females using a polymerase chain reaction (PCR)-based approach, which identifies an intron of different length on the Z and W chromosomes (Ellegren 1996). Juvenile birds were excluded from morphological analyses and were distinguished from adults on the basis of plumage characteristics (Keith *et al*. 1992).

Morphological analyses

To compare morphological differences among populations, we used both ANOVA and MANOVA on raw morphological data. Principal components analysis (PCA) was performed on log-transformed data, with components extracted from a covariance matrix. Before PCA was performed on multiple geographically distinct populations, we tested for the proportionality of covariance matrices (Flury 1984) using the program available at http://www.uoregon.edu/~pphil/programs/cpc/cpc.htm. Previous work has showed no evidence of morphological differences being caused by seasonal wear (Smith *et al*. 2005).

Plumage colour analysis

We measured plumage colour variation 'through the eyes of the birds' using an avian colour vision model. Interested readers who are unfamiliar with this approach may wish to read the excellent paper by Endler & Meilke (2005). Here, we mainly provide information specific to our application.

Reflectance spectra (300–700 nm) of collected feathers were measured with a diode-array spectrometer (Ocean Optics S2000) using a reflectance probe (Ocean Optics R-400) and xenon strobe (Ocean Optics PX-2). Feathers were scanned by holding them up to a 1.3-mm diameter aperture in a razor-thin steel plate with the reflectance probe orientated 45 degrees relative to the upper surface of the plate at the distance where the aperture edges matched the acceptance angle of the detector optical fibre. All reflectance measurements were taken on the dorsal surface of the feathers. On outer retrices, the outer and inner sides of the feather were treated as separate regions, with 'inner' defined as the side closer to the midline of the bird. On wing coverts, the middle and edge of the outer side were treated as separate regions (the inner side was not scanned because it is normally covered). Each of the four feather regions was scanned with the feather barbs perpendicular to the light path and also with the barbs parallel to the light path. Replicate measurements (i.e. same feather type, region and orientation) were taken near the base, middle and tip of the feather and averaged.

Reflectance spectra were converted into bird-specific cone excitation estimates (E_j) using typical cone λ_{max} values for higher passerines (Endler & Meilke 2005). E_j were used to calculate relative cone contrasts and coordinates in tetrahedral colour space (see Endler & Meilke 2005). The sum of E_j, across the four cone classes (U, ultraviolet; S, short; M, medium; L, long) provides an estimate of luminance (perceived brightness). E_j calculations require that the ambient light (irradiance) spectrum be specified and this was an important consideration because the altered light environment of secondary forest is a potential source of divergent selection on colouration. We compared the colour of the feathers between habitat types, and between the sexes, using two light environments that are expected in both mature and secondary forest sites: the light spectrum that prevails under cloudy conditions ('open/cloudy') and the light spectrum that prevails at low sun angles ('early/late'; see Endler 1993). The results for these light environments did not differ at $P = 0.05$ and thus, for brevity, we present only the open/cloudy results. J. A. Endler kindly provided 300–700-nm data for most of the light environments characterized in Endler (1993) and 350–700-nm data for early/late. To use the early/late spectrum in our calculations, we assumed that light intensity drops linearly to zero from 350 nm to 300 nm, which is approximately what occurs in the other light environments.

To test for any overall difference in tetrahedral colour space between the sexes and between birds from mature and secondary forest sites, we employed the nonparametric statistics program LSED-MRPP (Endler & Meilke 2005). All eight measurement classes (see above) were entered in a single run of the model, with individual birds treated as subgroups. LSED-MRPP reports an effect size or disparity (K) as well as a P value. According to Endler *et al*. (2005), colour patterns can be considered different if $K > 0.01$, easily distinguished if $K > 0.05$, and completely different if $K > 0.2$. LSED-MRPP detects differences in dispersion as well as location (i.e. centroid) of points in colour space. To test specifically for differences in location, one feather measurement class at a time, we used the analysis of distance (AOD) method, which evaluates the squared distance between groups (AOD module in STATA 9.2, Stata Corporation). AOD is conceptually similar to MANOVA but does not rely on distributional assumptions (Gower & Krzanowski 1999; Fenty 2004). For AOD tests, we report unsquared distances (d) and P values based on 1000 permutations. Significant differences in location between groups of points in colour space indicate that the groups differ in colour but do not reveal how they differ. Thus, where significant differences were found using AOD, we examined the nature of these differences at the level of pairwise cone contrasts (e.g. M vs. U) and tetrahedral chroma (defined as the Euclidian distance from the centre of colour space) using Wilcoxon rank sum tests. Because this hierarchical procedure helps guard against type II errors, no multiple test correction procedure was employed. To test for variance differences between groups, we used the Robust Test for Equality of Variances (robvar W50 in STATA 9.2). Wilcoxon and variance equality tests were also employed to examine differences in luminance between groups.

Vocalization analysis

Little greenbuls are continuous singers, producing sequences of four categories of song types, labelled as song type I–IV (Slabbekoorn & Smith 2002b). The song types differ in their relative complexity: having either few or many different notes in a song; and stereotypy: with each other song exhibiting exactly the same or similar set of song notes. Song type I is the simplest and is made up of a repetitive train of 'chuck' calls, which can also be uttered separately in another context than singing such as alarm calling. Song type II is a short and stereotypic song with relatively moderate frequency changes and often ending with low emphasis in a brief repetition of simple notes. Song type III is also short and stereotypic, but typically covers a wider frequency range with abrupt alternation between low and high notes and high emphasis to the end. Song type IV is the longest and most variable song with each rendition being a different selection and sequence of notes, often ending with a prominent whistle. Individuals vary in the predominant sequence of producing these four song types and individuals at the same location are more similar in their sequence than individuals at different locations (Slabbekoorn & Smith 2002b).

The variation in relative stereotypy suggests that song type IV may be especially important for mate attraction, and the others may be more potent signals in competition among males (Collins 2004). However, we have no data that address function, and when little greenbuls are singing, they use all song types without any obvious dependency on context or season. Therefore, we assume that all song types play a role in inter- or intrasexual selection. In an earlier study, comparing songs of rainforest and ecotone forest, the sequence of song types revealed geographical variation on a large scale but did not correspond to specific habitat features (Slabbekoorn & Smith 2002b). This may be because order is likely to be environmentally neutral, that is with no selective advantages for specific sequences under particular environmental conditions. In contrast, spectral and temporal features may be shaped by environmental selection pressures such as sound transmission properties and ambient noise profiles (Wiley & Richards 1982; Slabbekoorn & Smith 2002a; Slabbekoorn 2004). Here, we analysed the two most prominent song types, III and IV (having either the most pronounced rapid frequency modulations or being the longest and most variable one). Both song types were previously found to show significant habitat-dependent divergence in several of the parameters (Slabbekoorn & Smith 2002b). See Supplementary materials for details on acoustic equipment and measurement procedures.

Genetic analyses

Using available samples, we conducted genome scans for a subset of 84 individuals from four mature forest sites (Bouamir, Bobo Camp, Lac Lobeke and Zoebefame) and four secondary forest sites (Kompia, Kribi, Ndibi and Nkwouak), using AFLP. We generated AFLP profiles using a protocol modified from (Vos *et al*. 1995). Whole genomic DNA was extracted from blood samples using a QIAGEN kit and digested with restriction enzymes *Eco*RI and *Mse*I. Fragments were ligated to oligonucleotide adapters with T4 DNA ligase. A random sample of fragments was obtained through a preselective amplification using primers E-t and M-c, followed by two selective amplifications using primer pairs E-tag and M-cgt, and E-tgc and M-cga, with each E primer fluorescently labelled with dye 6-FAM. Selectively amplified fragments were run in an ABI 3700 genetic analyser with a LIZ-500 size standard. Peaks were visualized using GENEMAPPER 3.7 and scored manually, with individuals and populations randomized to avoid observer bias. Only unambiguously scorable loci and individuals were included in the analysis, and peaks found in less than 2%

Table 1 F_{ST} values from AFLP loci (below diagonal) and geographical distances (kilometres) between localities (above diagonal). Mature/pristine forest sites are shown in bold

	Bouamir	**Bobo Camp**	**Zoebefame**	**Lac Lobeke**	Kompia	Kribi	Ndibi	Nkwouak
Bouamir	0.0	94	88	342	39	331	93	93
Bobo Camp	0.1182	0.0	8	258	120	400	189	135
Zoebefame	0.1469	0.1920	0.0	266	115	392	181	134
Lac Lobeke	0.0798	0.1196	0.0530	0.0	351	657	427	322
Kompia	0.0372	0.0376	0.2373	0.1291	0.0	343	75	63
Kribi	0.1617	0.0404	0.2145	0.1548	0.1028	0.0	285	403
Ndibi	0.1373	0.0388	0.2524	0.2068	0.0561	0.0429	0.0	123
Nkwouak	0.1063	0.0181	0.2172	0.1151	0.0197	0.0049	0.0280	0.0

of individuals were excluded. Methodological error rate was assessed by running a subset of four individuals twice from the preselective amplification step. The average per-locus error rate for the AFLP data was 1.9%, a rate comparable to that of other AFLP studies in birds (Busch *et al*. 2000; Spaulding *et al*. 2006). To assess genetic structure among samples, we conducted a principal coordinate analysis (PCO) on a genetic distance matrix generated from the binary presence–absence matrix as implemented in GENALEX 6.0 (Peakall & Smouse 2006). We also examined patterns of population structure using the assignment probability test in the program STRUCTURE 2.1 (Pritchard *et al*. 2000). This program uses Bayesian inference to generate posterior probabilities of assignment of individuals to each of a given number of populations (K). As recommended for dominant markers, we applied a model of no admixture with correlated allele frequencies (Pritchard & Wen 2004) and the optimal value of K was calculated using STRUCTURE as well as the method by Evanno *et al*. (2005). We calculated pairwise F_{ST} values across habitats using ARLEQUIN 3.1 and tested for significance through 1000 random permutations of the data set (Excoffier *et al*. 1992). Detection of loci potentially under selection was conducted with the program DFDIST (Beaumont & Nichols 1996), which plots F_{ST} against heterozygosity under the assumption of Hardy–Weinberg equilibrium to identify significant outlier loci. Significance values at the 95% level for outlier loci were obtained by generating a null distribution of F_{ST} values based on 50,000 simulated loci with a mean F_{ST} equivalent to the 'neutral' mean F_{ST} of the empirical distribution, which was obtained by trimming the 10% highest and lowest F_{ST} values (Beaumont & Nichols 1996; Bonin *et al*. 2006).

Analysis of genetic diversification on habitat-related variables

To examine how habitat and environmental variables could explain genetic variation, we used a distance-based redundancy analysis (dbRDA) (Anderson 2001). The dbRDA method performs a multivariate regression on a distance response matrix. We examined two genetic response matrices based on AFLP results, in conjunction with a number of environmental predictor variables from remote sensing. We utilized the program DISTLM version 5 (Anderson 2003) and performed both: (i) marginal tests on predictor variables, and (ii) conditional tests where population geographical distances are included as covariables (to allow for the evaluation of whether any of the predictor variables explains genetic variation above that explained by geographical distance alone). P values were obtained by running 9999 unrestricted simultaneous permutations of the rows and columns of the multivariate residual matrix for both the marginal and conditional models. The predictor variables used in the dbRDA analysis included one categorical variable — 'habitat' (mature vs. secondary forest), and four continuous variables — 'distance' (latitude and longitude), and the MODIS-based remote-sensing environmental variables 'LAI annual mean', 'percent tree cover' and 'NDVI annual maximum'. In addition, we combined the three remote-sensing variables into a predictor matrix labelled 'combined environmental variables'. We evaluated two response matrices: (i) a genetic distance response matrix based on Nei's genetic distance (D_s) from AFLP data; and (ii) a genetic distance response matrix utilizing AFLP principal coordinate analysis axes 1 and 2-values transformed into a Euclidean distance matrix with the sites used shown in Table 1.

Results

Habitat classification and relative abundance

Using MODIS NDVI, LAI and percent tree cover metrics, we developed small polygons of 9–25 pixels around each sample site to extract the remote-sensing measurements. Mature and secondary forest sites were significantly different in per cent tree cover ($t = -3.69$, d.f. = 8.5, $P < 0.01$), annual mean leaf area index ($t = 2.6$, d.f. = 11.8, $P < 0.02$) and nearly significant for NDVI ($t = -2.1$, d.f. = 9.65, $P < 0.06$). Mature (pristine) forest sites were located at least 30 km

from the nearest road or human settlement and based on ground surveys showed little or no signs of human disturbance. Ground surveys revealed secondary forest sites to consist mainly of cacao and coffee plantations adjacent to human settlements, with significant disturbance associated with wood harvesting, burning and various forms of cultivation.

The relative abundance of *Andropadus virens* differed significantly between mature and secondary forest (ANOVA, $F = 6.108$, d.f. = 10, $P < 0.03$). Abundances were nearly five times greater in secondary than in mature habitats (mean ± sd = 5.39 ± 1.02 individuals/net-hour and 1.18 ± 1.35, respectively). To control for possible interyear differences and seasonality which might influence abundance, we contrasted two mature sites (Zoebefame and Bobo Camp) with two secondary sites (Ndibi and Nkwouak) during July 2005. When these were compared, differences remained highly significant between forest types (ANOVA, $F = 84.54$, d.f. = 3, $P < 0.01$).

Morphological differentiation

Adult males from mature and secondary forest were found to differ significantly in three morphologic characters. Individuals from mature forest had larger wings, tarsi, and bill depths than those in secondary forest (Fig. 1). In contrast, within habitat comparisons, contrasting adult males showed no significant differences in morphology (ANOVA, $P > 0.1$). In addition, consistent with previous studies (Smith *et al*. 2005), we found no evidence of seasonality driving morphological differences.

To examine morphological differences in multivariate space, we contrasted principal components 1 and 2 between habitats. As the matrix of eigenvectors may differ across geographically disparate populations, we first tested whether principal components differed between forest types. We found that both covariance matrices were proportional allowing habitats to be directly compared (Smith *et al*. 2005). Based on factor loadings (see Supplementary materials), PC1 represents primarily a size axis and accounted for 90% of the explained variance, and PC2 represented a shape axis accounting for 9.7% of the variation. Both PC1 and PC2 were significantly different between forest types (Fig. 1), suggesting the existence of differences in both size and shape between forest types.

Plumage colour variation

The feathers of *A. virens* were nearly sexually monochromatic ($K = 0.0036$; $P = 0.01$). Although statistically significant, this between-sex disparity value is well below the $K > 0.01$ threshold set by Endler *et al*. (2005). AOD detected no significant sex differences in location in colour space (all feather measurement classes: $d \geq 0.003$, $P \geq 0.2$). Thus, the

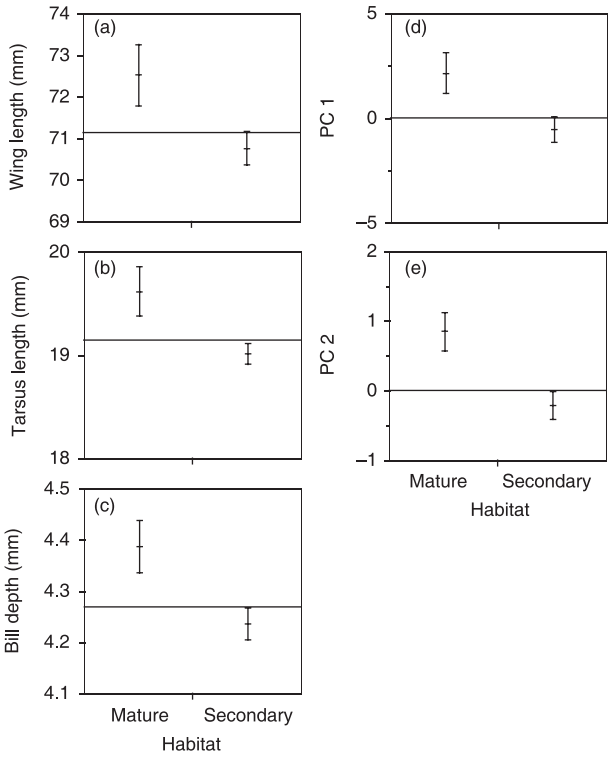

Fig. 1 Differences in morphological characters between mature and secondary forest sites showed significant differences (MANOVA Wilk's Lambda = 0.84, $F_{1,53} = 3.37$, $P < 0.02$) for (a) wing length (ANOVA, $F_{1,60} = 4.19$, $P < 0.04$), (b) tarsus length (ANOVA, $F_{1,60} = 6.8$, $P < 0.01$), (c) bill depth (ANOVA, $F_{1,60} = 5.46$, $P < 0.02$), D. PC1 (ANOVA, $F_{1,55} = 3.8$, $P < 0.05$) and F. PC 2 (ANOVA, $F_{1,55} = 6.93$, $P < 0.01$). Horizontal line represents the grand mean. As in previous studies of habitat variation in this species, bill length showed no significant difference (Smith *et al*. 2005).

statistically significant disparity value probably reflects a sex difference in the dispersion (not location) of points in colour space. No significant sex difference in tetrahedral chroma variance was detected, however (for all measurement classes, $W50_{1,79} \leq 0.41$, $P \geq 0.5$).

Birds from mature and secondary forests differed significantly and detectably in colouration ($K = 0.0118$; $P =$ 7.95E-6), although not to the extent that the differences would be easy for the birds to distinguish (because K was < 0.05). AOD detected significant habitat differences in two of the eight measurement classes: the outer side of the outer retrix in parallel orientation ($d = 0.0067$, $P = 0.04$) and the wing covert edge in parallel orientation ($d = 0.0065$, $P = 0.05$). For both feather types, birds from secondary forest tended to have higher chroma (i.e. greater colour saturation) than birds from mature forest (Wilcoxon tests; outer retrix: $z = 1.92$, $P = 0.054$, $n = 81$; wing covert: $z = 1.60$, $P = 0.11$, $n = 81$). The variance in chroma did not differ significantly between habitats (all $W50_{1,79} \leq 0.3$,

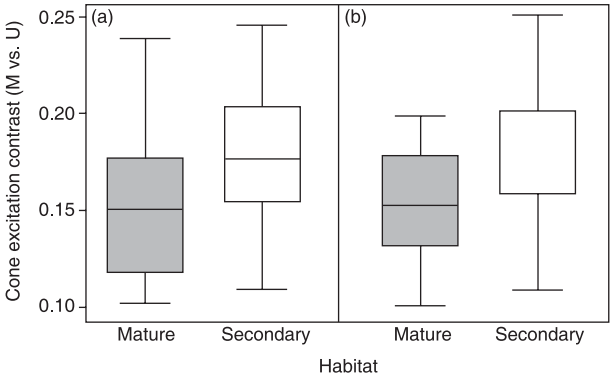

Fig. 2 Habitat differences in plumage colour (M vs. U cone contrasts) for two feather measurement classes. (a) Outer retrix, outer side in parallel orientation. (b) Wing covert edge in parallel orientation. Horizontal lines represent medians, boxes represent interquartile ranges, and vertical lines represent ranges. See text for Wilcoxon test results.

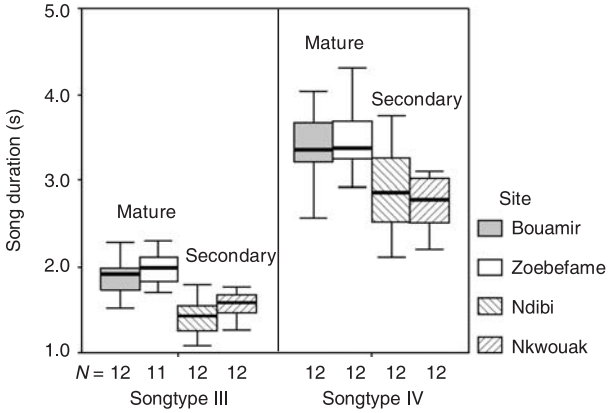

Fig. 3 Song duration of little greenbul song for the relatively short and stereotypic song type III and the relatively long, complex, and variable song type IV. Bouamir and Zoebefame are two primary forest sites and Ndibi and Nkwouak are two secondary forest sites. We recorded 12 individuals at each site and analysed a replicate target set of 12 songs for each song type. One exceptional individual at Zoebefame did not sing songs that we could classify as song type III. The song duration is significantly longer in primary forest sites than in secondary forest sites (ANOVA, $F = 232.48$, $P = 0.004$, with habitat and song type as fixed factors and sites as random factor nested within habitat).

$P \geq 0.5$). At the level of pairwise cone contrasts, M vs. U cone contrasts showed the greatest differentiation between mature and secondary forest birds (Fig. 2; outer retrix: $z = 2.52$, $P = 0.012$, $n = 81$; wing covert: $z = 2.68$, $P = 0.007$, $n = 81$). S vs. U contrasts were also significant for both feather types (outer retrix: $z = 2.29$, $P = 0.022$, $n = 81$; wing covert: $z = 2.67$, $P = 0.008$, $n = 81$). The L vs. U and M vs. S contrasts were significant for outer retrices (L vs. U: $z = 2.00$, $P = 0.046$; M vs. S: $z = 2.16$, $P = 0.031$) but not for wing coverts (L vs. U: $z = 1.74$, $P = 0.082$; M vs. S: $z = 1.92$, $P = 0.054$). Thus, the between-habitat component of plumage colour variation primarily involved differences in the relative excitation of the U (ultraviolet) cone. Humans probably would not be capable of detecting these colour differences (because our eyes are insensitive to ultraviolet light), but they should be visible to the birds.

Plumage luminance did not differ, on average, between the sexes (Wilcoxon tests, all $P \geq 0.2$, $n = 81$). The variance in luminance was consistently higher in females but significantly so for only one of the eight measurement classes (inner side of the outer retrices in perpendicular orientation; $W50_{1,79} = 4.83$, $P = 0.03$). There were no significant habitat differences in average plumage luminance (all $P \geq 0.2$, $n = 81$), but the variance in luminance was consistently higher among birds from secondary forest and significantly so for seven of the eight measurement classes (outer side of outer retrix in perpendicular orientation: $W50_{1,79} = 2.41$, $P = 0.12$; all others: $W50_{1,79} > 4.0$, $P < 0.05$).

Vocalizations

An analysis of song from two primary rainforest sites (Bouamir and Zoebefame) and two secondary forest sites (Ndibi and Nkwouak) shows significant differences (Fig. 3). All parameters, except maximum frequency and delivery rate for songtype III, were normally distributed (Kolmogorov-Smirnov test) and we used general linear models (ANOVA) with habitat (mature vs. secondary forest) and song type (III and IV) as fixed factors, and site as a random factor nested within habitat (SPSS for Windows 2002, Release 11.5.0). While none of the spectral measures (minimum and maximum frequency, and a general measure of frequency use based-on-peak frequency measurements) showed differences, there was a consistent divergence in some temporal parameters. Song duration was significantly longer in mature than in secondary forest (Fig. 3, ANOVA, $F = 232.48$, $P = 0.004$), leading to a consistently faster delivery rate in secondary forest (ANOVA, $F = 36.26$, $P = 0.026$): 8.7 ± 1.1 notes per second in the secondary site and 6.9 ± 0.8 in the primary forest sites for song type III, and 9.3 ± 0.8 notes per second in the secondary site and 8.2 ± 0.7 in the primary forest sites for song type (IV).

The number of notes in a song did not differ by habitat (ANOVA, $F = 3.99$, $P = 0.18$; 13.0 ± 2.1 in the secondary sites, and 13.0 ± 1.5 in the mature forest sites for song type III, and 25.9 ± 5.2 in the secondary sites and 28.0 ± 3.4 in the mature forest sites for song type IV).

Population genetic structure

The genome scan using AFLP markers yielded 63 polymorphic loci (50 from primer pair E-tag/M-cgt, and 13 from primer pair E-tgc/M-cga) in 84 individual little

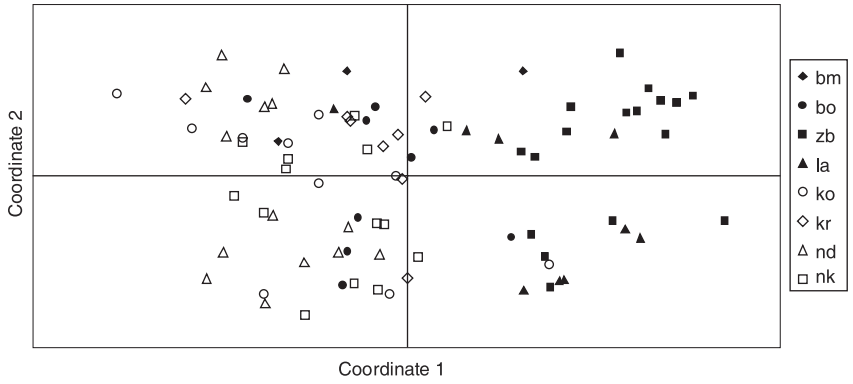

Fig. 4 Principal coordinate analysis (PCO) plot of 84 *Andropadus virens* individuals, based on a genetic distance matrix from 63 AFLP loci, showing genetic differentiation between undisturbed forest sites (filled markers) and human-disturbed secondary forest (empty markers). Populations included in the analysis, with samples sizes, are Bouamir (bm, $n = 3$), Bobo Camp (bo, $n = 9$), Zoebefame (zb, $n = 17$), Lac Lobeke (la, $n = 9$), Kompia (ko, $n = 10$), Kribi (kr, $n = 8$), Ndibi (nd, $n = 14$) and Nkwouak (nk, $n = 14$). The two first coordinates explained 47% of the variance.

greenbuls from four mature forest populations (Bouamir, Bobo Camp, Zoebefame and Lac Lobeke) and four secondary forest populations (Kompia, Kribi, Ndibi and Nkwouak). A principal coordinate analysis of the genetic distance matrix among individuals revealed marked differentiation between habitats along the first principal coordinate (Fig. 4). Individuals from the Bobo Camp population, however, clustered mostly with secondary forest individuals. Results from the STRUCTURE analysis produced a similar pattern of across-habitat differentiation. The optimal value of K was 2, and each of the clusters corresponded generally to the mature and the secondary forest populations, respectively (Fig. 5). Again, however, individuals from Bobo Camp, a mature-forest site, had an average posterior probability of assignment to the secondary forest cluster of 69.4% against 30.6% to the mature forest cluster.

While the average F_{ST} value for the pairwise across-habitat comparison across all 63 AFLP loci was 0.12, four loci had F_{ST} values above 0.30 (loci number 5, 42, 53 and 58, Table 1). Simulation analysis using DFDIST revealed that two of these loci (42 and 53), were significant outliers on a plot of F_{ST} against heterozygosity (Fig. 6), and are therefore likely to be either under directional selection or linked to a locus under selection (Beaumont & Nichols 1996; Beaumont & Balding 2004). The average F_{ST} value for the pairwise across-habitat comparison excluding outlier loci was 0.06, indicating high rates of gene flow in loci unlikely to be under selection.

The results of the multivariate distance-based redundancy analysis (dbRDA) marginal tests show that both measures of AFLP genetic distance (individual pairwise genetic distances D_s and PCO axes 1 and 2 individual Euclidean pairwise distances) showed highly significant relationships with all environmental predictor variables of habitat and distance (Table 2). Habitat, the combined remote-sensing variables, and distance each explain approximately the same amount of genetic variation (~12–14% for D_s and ~25–28% for PCO Euclidean distance). When geographical distance was taken into account in the form of a covariable in the dbRDA analyses (conditional tests), habitat and combined environmental variables remained significantly correlated with D_s and PCO Euclidean distance (Table 2). In the conditional tests, the three combined remote-sensing variables explain about three to four times the amount of genetic variance as habitat, with the NDVI annual maximum metric accounting for most of the remote sensing correlated genetic variation. Thus, habitat, as quantified by remote-sensing variables, appears to play a role in genetic dissimilarities, lending further evidence in support of genetic differences between mature and secondary forest.

Discussion

Our results suggest that conversion of mature pristine rainforest to degraded secondary forest may carry evolutionary consequences for rainforest species that persist. Here, we show genetic differentiation between mature and secondary forest and evidence for divergence in both morphological (including wing, tarsus, and bill size and plumage colouration) and behavioural (song) traits. While the exact time frame for these habitat changes are uncertain, the conversion of mature to secondary forest was well underway 100 years ago (Bates 1930; Merfield 1957).

Rather than *de novo* evolution, could greenbuls have always existed in secondary forest and simply spread to new secondary forest when it became available? While this is possible, we think it is unlikely. While large tracts of secondary forest are common today, few large regions of secondary forest would have existed before human activities. In addition, many tracts of secondary forest today are entirely surrounded by mature rainforest—making dispersal of secondary forest adapted birds into them unlikely. Further study is needed, but our findings that greenbuls show concordant patterns of divergence at multiple sites, varying in isolation, suggests that these changes are

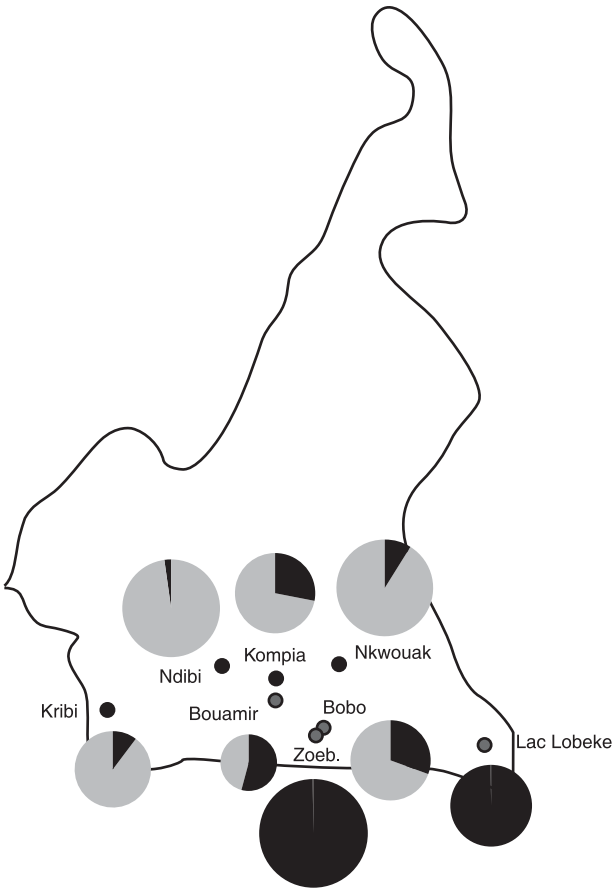

Fig. 5 Map of Cameroon showing sampling localities used in AFLP analysis and percent posterior assignment probabilities of 84 individuals of *Andropadus virens* to an optimal number of clusters ($K = 2$) using 63 AFLP loci in the program STRUCTURE. Black dots represent secondary forest sites, and grey dots depict mature forest sites. Each colour in each pie diagram represents the percent posterior probability of assignment to each of the two clusters, averaged across all individuals in that population, and the size of each pie is proportional to the sample size. Black colour corresponds to high assignment probability to largely mature forest cluster, whereas gray colour represents high probability of assignment to a mostly secondary forest cluster. Individuals from Bobo Camp showed a higher average assignment probability to the secondary forest cluster despite being from a mature forest location (see text).

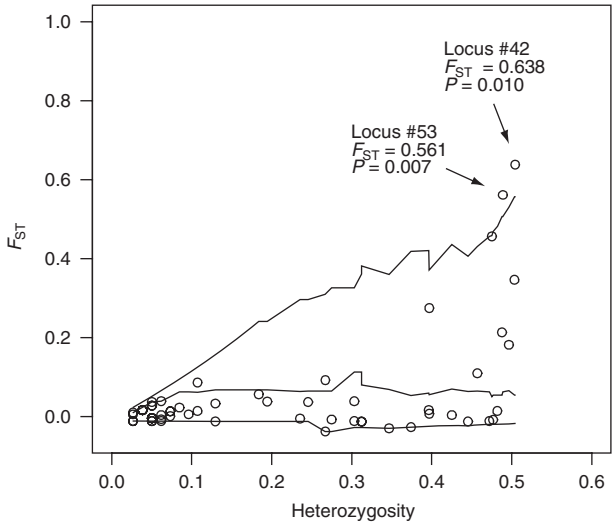

Fig. 6 Outlier loci on a plot of F_{ST} against heterozygosity generated with DFDIST. Each data point corresponds to an AFLP marker, lower and higher lines represent 5% and 95% confidence intervals, and middle line represents the median. Outlier loci found above the 95% confidence interval are indicated by arrows, with F_{ST} values and corresponding P values.

occurring independently and in a parallel fashion. While direct responses to the environment (i.e. phenotypic plasticity) could account for this pattern, the morphologic traits examined here have been shown to have a heritable basis and to be under selection in other species (Grant 1986; Schluter & Smith 1986; Cooke & Buckley 1987; Schluter 1988). Ultimately, common garden experiments would be required to evaluate the relative importance of plasticity and genetic differentiation. Another potentially confounding issue is that sampling was conducted over multiple years, which raises the possibility that some differences could have resulted from temporal variation. We think that temporal variability is unlikely to have played a major role, for two reasons. First, sampling of mature and secondary sites was interspersed among years and seasons. Second, in several pairwise comparisons of secondary and mature sites sampled within a 2-month interval, the pattern of variation among traits was consistent with that found among sites overtime.

Morphology and abundance

Morphological traits were larger in mature forest than in secondary forest. Previous studies have shown that longer wings are favoured in more open habitats (Schluter 1988), which is consistent with the relatively open understorey of mature forests in comparison to secondary growth. Future work will be directed towards examining the canopy understorey and the possible ecological and performance reasons why wing length, tarsus length and bill size are larger in mature forest. Selection studies using mark–recapture to estimate survival as a function of wing length and flight performance with respect to habitat (and density, see below) would be particularly useful.

Abundance was found to be fivefold higher in secondary than in mature forest, as noted previously (Louette *et al*. 1995). Density-dependent selection has been shown in both laboratory (Mueller & Ayala 1981; Shakarad *et al*. 2005) and field studies (Svensson & Sinervo 2000; Calsbeek & Smith

Table 2 Effects of environmental factors on genetic differentiation (using AFLP results) of the little greenbul using distance-based redundancy analysis. On the left are marginal tests of individual sets of predictor variables and on the right are partial (conditional) tests, where distance (the variables of latitude and longitude) have been included as covariables in each analysis. *P* indicates probability values and 'percentage var' the percentage of genetic variation explained by the particular variable. *P* values less than 0.1 are highlighted in bold

	Marginal tests		Conditional tests	
Variable set	*P*	percentage var	*P*	percentage var
(a) Tests for genetic distance (AFLP Nei's genetic distance (D_s) response matrix)				
Habitat	**0.000**	12.2	**0.098**	2.7
LAI annual mean	**0.015**	2.9	0.304	1.2
Percent tree cover	**0.000**	9.1	0.216	1.6
NDVI annual maximum	**0.000**	7.3	**0.011**	6.3
Combined Env. Variables	**0.000**	12.3	**0.017**	9.7
Distance	**0.000**	13.5	—	—
(b) Tests for genetic distance (AFLP PCO axes 1 and 2 distance response matrix)				
Habitat	**0.000**	26.9	**0.034**	3.7
LAI annual mean	**0.011**	5.6	**0.018**	4.4
Percent tree cover	**0.000**	20.4	0.183	1.6
NDVI annual maximum	**0.000**	14.6	**0.000**	14.9
Combined Env. Variables	**0.000**	25.0	**0.000**	17.8
Distance	**0.000**	27.8	—	—

2007). Given the differences in relative abundance, density-dependent selection may be acting here; however, further investigations contrasting selection under differences densities will be required.

Plumage colour and luminance

The habitat differences in plumage colour that we detected are small in magnitude and would almost certainly go unnoticed by ultraviolet-insensitive human eyes, but they should be visible to the birds. As such, with respect to plumage colour, *Andropadus virens* populations in mature and secondary forest could be classified as cryptic ecotypes. Feathers from secondary forest birds have relatively low ultraviolet reflectance compared to feathers from mature forest birds (Fig. 2). This difference goes in the direction of counteracting the change in light environment that occurs when mature forests are logged (i.e. when upper canopy trees are removed, more light reaches the understorey without being filtered through leaves, and thus the light spectrum becomes richer in short wavelengths; Endler 1993). We therefore carried out a simulation to determine whether the relatively low ultraviolet reflectance of secondary forest birds could be explained as an evolutionary adaptation to the change in the light environment between the two habitats. The simulation revealed that the observed difference in colour between birds from the two habitats was much larger than the expected colour shift caused by the change in light environment (see Supplementary materials). The absence of a strong effect of light environment on perceived colour was not surprising; relative colour constancy is a property of visual systems generally and of the specific colour vision model that we used (Endler & Meilke 2005). Nevertheless, our simulation served to show that the habitat difference in plumage colour is outside the range of perceived colour shifts expected from the change in ambient light. Thus, other factors besides ambient light ought to be considered. For example, selection for crypsis combined with a difference between mature and secondary forest in the colour distribution of understorey vegetation might be responsible for the observed colour shift. This hypothesis could be tested with data on background radiance spectra from the two habitats. The greater variability in luminance of secondary forest birds compared to mature forest birds is intriguing. This might be a product of relaxed selection on luminance in secondary forest owing to higher variability in light levels in secondary forest compared to mature forest. However, we cannot rule out the possibility that the habitat differences in plumage colour and luminance are environmentally induced rather than genetic.

Song divergence

The main difference between forest types is that the number of notes is compressed into a shorter song duration in secondary forest populations. This leads to a higher delivery rate in the songs of the birds singing in degraded

forest. High delivery rates, rapid repetition of short notes, or production of trill-like songs are better-suited to more open canopy habitats, in which air turbidity may degrade long, slow songs (Wiley & Richards 1982; Brown & Handford 2000; Slabbekoorn & Smith 2002b). For example, intraspecific geographical variation in temporal parameters related to the relative openness of the habitat has been found in the rufous-crowned sparrow *Zonotrichia capensis* across a variety of habitats (Nottebohm 1975; Handford 1981; Handford 1988), and in great tits (*Parus major*), between forest and more open urban habitat (Slabbekoorn & den Boer-Visser 2006). Degraded forest had more open canopy than mature forest based on remote-sensing data, however, one has to consider the relative openness of the vegetation layer typically used as the pathway for signal transmission. This pathway is determined by average perching height of singing little greenbuls and the average perching, foraging, or flying height of potential receivers at the time of singing (Slabbekoorn 2004). Previous sound transmission experiments through a replicated set of rainforest and ecotone forest sites, at the vegetation level used by little greenbuls, did not indicate significant habitat-dependent variation (Slabbekoorn & Smith 2002b). Little greenbuls mainly use the lower shrub layers of the forest, not well quantified by available remote-sensing instrumentation.

We should be cautious in drawing any firm conclusions, because of the small number of replicate populations and the nature of post hoc analyses. Additional song recordings in both mature and secondary forest will be needed as well as transmission experiments designed to assess song attenuation in each habitat. Nevertheless, the results suggest divergent selection pressure on acoustic signals related to forest degradation. In a previous study, songs in the ecotone, which lies north of the forest zone, were sung slower than in the primary rainforest (Slabbekoorn & Smith 2002b). If latitude was the main factor in determining delivery rate, one would have expected birds from the secondary forest, which lie closer to the ecotone, to have sung slower. In fact, the opposite was found, secondary forest songs are sung faster than mature forest songs. This suggests that the current results are not due to a simple latitudinal gradient in the delivery rate of song notes, but that habitat-dependent selection pressures are a more likely explanation.

Genetic structure and adaptive differentiation

Analysis of the AFLP genome scan were consistent with a scenario of relatively high gene flow between undisturbed and secondary forests, with all microsatellite loci and most AFLP loci showing low F_{ST} values for pairwise comparisons across the two habitats. However, the AFLP survey also detected two loci (3.2%) that showed high F_{ST} values relative to neutral expectations, suggesting that natural selection played a role in their divergence. This proportion of outlier loci is similar to that found in other studies, such as between ecotypes of lake whitefish (3.2%; Campbell & Bernatchez 2004), between parapatric populations of intertidal snails (5%; Wilding *et al*. 2001), and among-frog populations along an altitudinal gradient (2%; Bonin *et al*. 2006). Like the above studies, our results indicate that selection acts on a small number of loci, although pleiotropic effects and epistatic interactions can play a role in extending the effect of a few genes to a larger number of phenotypic traits. Our results appear to be consistent with expectations of a divergence-with-gene-flow model and other non-allopatric models of differentiation, where initial divergence takes place at relatively small number of loci under strong divergent or disruptive selection, while divergence at other loci is prevented by gene flow (Wilding *et al*. 2001; Beaumont & Balding 2004; Campbell & Bernatchez 2004).

Despite the detection of considerable genetic differentiation across habitats in two loci, the association between outlier loci and forest type was not complete. For example, individuals from the undisturbed forest site Bobo Camp shared locus #42 with secondary forest populations and thus tended to cluster more closely with populations of the latter habitat. This could possibly be due to a recent exchange of individuals. Alternatively, because peaks identified in the electropherograms are scored strictly according to fragment size, these could correspond to non-homologous fragments (Bensch & Akesson 2005), which could confound genotype–habitat associations. Finally, if the marker is indeed homologous between populations, the relationship between its relative frequency and habitat type could be spurious. A larger number of loci would be necessary to properly test these alternative hypotheses.

Nevertheless, distance-based redundancy analysis detected highly significant effects of habitat on genetic differentiation, even when controlling for spatial variation. These results implicate the effects of anthropogenic alternations on habitat structure in altering the genetic structure of populations.

Conservation implications

Rapid evolutionary change in response to introductions and environmental change have been documented in a wide variety of organisms (Reznick *et al*. 2004). Results presented here suggest that in order to preserve species in a 'pristine' state habitat will require sustained management. It cannot be assumed that the evolutionary processes affecting traits important in natural and sexual selection will remain unchanged as habitats are altered. Ultimately, conservation managers must determine what their goal is. If the goal is to maximize a given species abundance, regardless of the evolutionary consequences, then greenbuls

have clearly benefited from the clearing of mature rainforest. Abundances are as much as five times higher in human-altered secondary forest. However, if the goal is to maintain populations in a pristine state, in which evolutionary processes act in accordance, management actions will need to be designed to limit the expansion of secondary forest. Micro-evolutionary patterns in greenbuls have clearly been affected by anthropogenic changes. The proportion of greenbuls populations living under pristine evolutionary conditions today represents only a small fraction of those that did historically.

Faced with climate change, however, managers may not have the luxury of managing species under historically pristine evolutionary environmental conditions. Among all the continents, Africa is predicted to be most vulnerable to the effects of climate change (IPCC 2007). While the changes in adaptive traits caused by habitat degradation may have altered the evolutionary trajectories for the greenbuls, it may also have the effect of maximizing adaptive variation as a whole. This may help buffer the deleterious effects of climate change, ensuring a wider range of population variation and the chance that some populations will be better adapted to changing climate than others.

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Thomas B. Smith is interested in evolutionary genetics, speciation and the conservation of tropical vertebrates. Borja Milá is interested in using phylogeographic and genomic approaches to study speciation and related evolutionary processes. Greg Grether conducts research at the interface of ethology, ecology and evolutionary biology, especially how sexual selection and other forms of social selection interact with the environment to shape the evolution of behavioural strategies and signalling system. Hans Slabbekoorn is interested in the role of phenotypically plastic signals, such as learned birdsong and the role they play in population divergence. Irem Sepil will be beginning graduate studies in Fall 07 and is interested in the evolutionary relationships between coloration and disease. Wolfgang Buermann and Sassan Saatchi use satellite remote sensing to understand biotic patterns and John Pollinger applies conservation genetics to studies of carnivores and birds.

Supplementary material

The following supplementary material is available for this article:

Fig. SA1.1 Results from the ambient light simulation. Points represent mean coordinates in the YZ plane of tetrahedral colour space for the feather measurement classes that differed most in colour between mature and secondary forest sites (i.e., outer retrix outer side in parallel orientation). Solid black circles represent the position in colour space under a forest shade spectrum, open circles represent the position in colour space under a woodland shade light spectrum, and half-black circles represent a 50:50 mixture of the two ambient light spectra. All other mixtures of these two light spectra fall between the plotted points (not shown).

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