

TESTING ALTERNATIVE HYPOTHESES FOR EVOLUTIONARY DIVERSIFICATION IN AN AFRICAN SONGBIRD: RAINFOREST REFUGIA VERSUS ECOLOGICAL GRADIENTS

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Geographic isolation in rainforest refugia and local adaptation to ecological gradients may both be important drivers of evolutionary diversification. However, their relative importance and the underlying mechanisms of these processes remain poorly understood because few empirical studies address both putative processes in a single system. A key question is to what extent is divergence in signals that are important in mate and species recognition driven by isolation in rainforest refugia or by divergent selection across ecological gradients? We studied the little greenbul, *Andropadus virens*, an African songbird, in Cameroon and Uganda, to determine whether refugial isolation or ecological gradients better explain existing song variation. We then tested whether song variation attributable to refugial or ecological divergence was biologically meaningful using reciprocal playback experiments to territorial males. We found that much of the existing song variation can be explained by both geographic isolation and ecological gradients, but that divergence across the gradient, and not geographic isolation, affects male response levels. These data suggest that ecologically divergent traits, independent of historical isolation during glacial cycles, can promote reproductive isolation. Our study provides further support for the importance of ecology in explaining patterns of evolutionary diversification in ecologically diverse regions of the planet.

KEY WORDS: Acoustic adaptation, *Andropadus virens*, bird song, ecological speciation, geographic isolation.

Tropical rainforests contain the highest levels of biodiversity of any terrestrial habitat and there has been much debate on the evolutionary processes that have caused rainforest diversification. Many studies have focused on specific traits and show patterns of variation that fit a particular hypothesis invoking the role of drift or selection, but few have tested the relative effects of alternative hypotheses in the same study. Two such competing hypotheses for an explanation of evolutionary diversification through rainforest speciation are drift in isolated refugia (Haffer 1969; Diamond and Hamilton 1980; Mayr and O'Hara 1986) and selection across ecological gradients (Endler 1977; Smith et al. 1997; Schilthuizen 2000; Smith and Grether 2008).

Under the Forest Refuge Hypothesis (Haffer 1969, 1997), populations are hypothesized to have diverged, and ultimately to have evolved, into distinct species, due to genetic drift in isolation during glacial maxima. Drift is hypothesized to play an important role in divergence in these studies, because the habitats in refugial fragments are similar, minimizing potential divergent selective pressures. If secondary contact occurs after isolation, individuals may mate assortatively because of pre- or postbreeding reproductive barriers acquired while geographically separated. The Forest Refuge Hypothesis was thought to play a major role in species diversification in the Afrotropics, supported by studies showing that particular restricted-range species were confined to postulated refugia (Diamond and Hamilton 1980) and that contact zones of related species occurred somewhere between these refuges (Mayr and O'Hara 1986).

However, a series of studies on rainforest diversification in different taxa question the relative importance of rainforest refugia in species diversification (Endler 1977, 1982; Fjelds  and Lovett 1997; Moritz et al. 2000; Colinvaux and Oliveira 2001; Hill and Hill 2001; Smith et al. 1997, 2001, 2005a,b). Some phylogeographic studies have also proposed an alternative mode of speciation in montane habitats, followed by range expansion into lowland forests (Fjelds  and Lovett 1997; Fjelds  et al. 2007). Moreover, ecology may also play an important role in diversification and a number of recent studies have highlighted ecological speciation as a prominent evolutionary process in explaining patterns of biodiversity (Nosil et al. 2005; Rundle and Nosil 2005; Schluter 2009; Hoskin and Higgie 2010; Freedman et al. 2010a).

The importance of ecology in explaining patterns of biodiversity is exemplified in the ecological gradient hypothesis that posits that ecological speciation occurs as a result of divergent natural selection between populations in different environments that lead to reproductive isolation (Endler 1977; Schluter 2000, 2001, 2009). Several studies on patterns of phenotypic variation have shown greater divergence between traits across an environmental gradient than between isolated sites in the same habitat (e.g., Smith et al. 1997; Slabbekoorn and Smith 2002a; Nicholls et al. 2006; Smith and Grether 2008). Such evidence supports the

“divergence-with-gene-flow” hypothesis (Rice and Hostert 1993) as it shows that patterns of phenotypic divergence occur despite gene flow between populations in diverse habitats. In line with this hypothesis, recent molecular studies have found evidence of genomic divergence consistent with divergence in phenotypic traits under selection (Mullen and Hoekstra 2008; Freedman et al. 2010a; Michel et al. 2010).

One of the major factors leading to reproductive isolation is the evolution of premating isolation mechanisms that lead to assortative mating (Coyne and Orr 2004). It is thus essential to understand to what extent divergence in traits between populations is perceptually important in mate choice or species recognition. Acoustic signals are widely used for sexual advertisement and often serve a dual role, in competition among males over territories and mates, but also in the attraction of mates (Bradbury and Vehrencamp 1998). In birds, song typically plays this dual role, and acoustic variation among and within species is often critical for species recognition and mate choice (Collins 2004; Catchpole and Slater 2008). Many acoustic parameters can vary geographically, and any song feature, such as repertoire size or specific syllable variants, but also aspects of spectral and temporal structure, may affect relative levels of male response and female preference (e.g., ten Cate et al. 2002; Gil and Gahr 2002; Riebel 2009). Especially learned songs of songbirds are expected to diverge readily between populations, after which they can affect gene flow through variable success rates in territory establishment and mate attraction depending on postdispersal matching to local variation of male songs or female preferences (Ellers and Slabbekoorn 2003; Beecher and Brenowitz 2005).

Habitat differences can lead to the divergence of traits that function in mate choice, such as bird song (Endler 1992; Price 1998; Slabbekoorn and Smith 2002b). After the original hypothesis was postulated (Chappuis 1971; Morton 1975), many studies have demonstrated that song characteristics diverge predictably as a function of habitat (e.g., Wiley 1991; Kirschel et al. 2009a; Tobias et al. 2010). For example, songs of birds in tropical forests are typically more tonal and characterized by lower frequencies than those of birds in more open habitats (Wiley and Richards 1982; Slabbekoorn et al. 2002). The acoustic adaptation hypothesis predicts that vocalizations are adapted to the physical structure of their habitat to maximize propagation of the signal (Rothstein and Fleischer 1987; Brown and Handford 1998; Daniel and Blumstein 1998; Seddon 2005), and species may occupy those habitats where their songs are better transmitted or their songs may evolve toward better transmission through sensory drive (Luther 2009; Tobias et al. 2010). Ryan and Brenowitz (1985) offered an alternative explanation for the optimality of certain low-frequency vocalizations in dense forests. They suggested the existence of a restricted frequency window of beneficial transmission properties and low competition from ambient noise created by insects

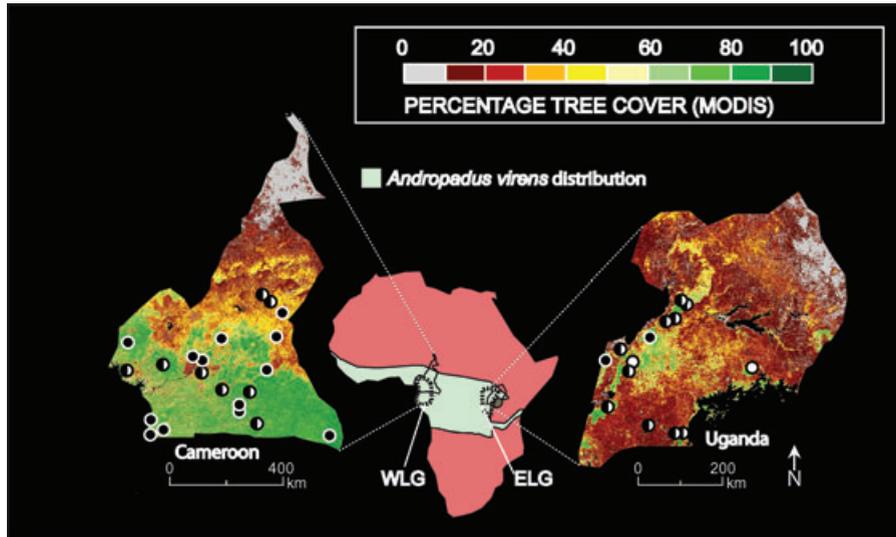


Figure 1. Map illustrating the distribution of little greenbul, *Andropadus virens* in sub-Saharan Africa, the locations of the postulated refugia of Western and Eastern Lower Guinea (WLG and ELG), and in the enlarged areas showing percent canopy cover of Cameroon and Uganda, locations of study sites where songs were recorded (filled circles), playback experiments performed (open circles) or both (half-filled circles).

and other noisy forest residents. Several subsequent studies have found that birds can adjust the frequency of their songs in response to fluctuating noise conditions (Tumer and Brainard 2007; Halfwerk and Slabbekoorn 2009; Gross et al. 2010) providing an explanation for habitat-related variation in song frequencies (e.g., Patricelli and Blickley 2006; Slabbekoorn and den Boer-Visser 2006; Kirschel et al. 2009a; Potvin et al. 2011).

Slabbekoorn and Smith (2002a) previously showed that little greenbul (*Andropadus virens*) song differed more in spectral and temporal parameters across an environmental gradient between different habitats than over a large distance between isolated populations within the same habitat. At the same time, they also reported geographic variation in the order of song type sequences, an ecologically neutral feature, which reflected more a pattern of isolation-by-distance than an influence of habitat structure (cf. Benedict and Bowie 2009). These findings were highly consistent with the habitat-dependent patterns of morphological divergence in this species, which were apparent despite ongoing gene flow, as confirmed by genetic analyses (Smith et al. 1997). Slabbekoorn and Smith (2002a) suggested that frequency variation among populations was likely driven by habitat-dependent variation in ambient noise profiles, which were distinct between sets of sites in Cameroonian rainforest and ecotone forest (Slabbekoorn 2004). However, these data only included recordings from an area associated with a single postulated rainforest refugium and we still lack all insight into whether any of the geographic song variation is meaningful to the birds.

In the current study, we examined song variation in the little greenbul in two geographically isolated regions of Central

Africa—Cameroon and Uganda; two postulated rainforest refugia (Mayr and O’Hara 1986). We compared song variation across an environmental gradient to variation between refugia but in the same habitat (Fig. 1). We used bioclimatic and remote sensing data and linear mixed models to quantify the ecological gradient between rainforest and ecotone forest and to examine the relative effects of environmental factors and refugial isolation on song characters. In their study on little greenbul song variation, Slabbekoorn and Smith (2002a) focused on two of the four song types as defined specifically for the species. Here, we examined variation in all four song types (see below). Subsequently, we tested whether refugial isolation and ecological gradients are important in driving perceptually significant song variation using a reciprocal playback experiment targeted toward territorial males. Finally, we related the results of playbacks to the song differences to evaluate which hypothesis provides the most likely explanation for driving phenotypic evolution in this sexually selected trait with potential impact on assortative mating.

Materials and Methods

STUDY SPECIES

The little greenbul is a common passerine of the Afrotropics (Slabbekoorn and Smith 2002a; Kirschel 2008). It is a member of the family Pycnonotidae, which is widespread in Africa and southern Asia (Fishpool and Tobias 2005). It inhabits both primary and secondary forest but also ecotone forest, part of the forest-savanna mosaic (Smith et al. 1997, 2005a; Slabbekoorn and Smith 2002a; Fishpool and Tobias 2005; Kirschel 2008). Little greenbul

populations in ecotones are distinct vocally and morphologically from populations in nearby rainforest (Slabbekoorn and Smith 2002a; Smith et al. 2005a,b). As described in Slabbekoorn and Smith (2002a), little greenbul sing a total of four distinct song type categories, which are produced in a consecutive sequence and repeated many times in a bout (Fig. 2). The four stereotypic song types—I, II, III and IV—are somewhat individually variable. Song types of the first three categories are most simple and stereotypic and most individuals have a single or sometimes two variants of each. Song type IV is more complex and variable and each individual might have two or three versions of it. The variation in relative stereotypy suggests that song type IV may be more important in mate attraction, and the others may be more potent signals in competition among males (Collins 2004), but no explicit tests have been performed, and, because they are always sung in continuous alternation, all song type categories could serve a role in both functions (cf Leitao and Riebel 2003).

FIELD RECORDINGS

We recorded little greenbul singing along forest roads and trails at 21 sites in Cameroon (between June 1998 and August 2007) and 12 sites in Uganda (between July 2004 and September 2007). These sites correspond with the Western and Eastern Lower Guinea Refugia (Endler 1982; Mayr and O’Hara 1986) (Table S1 and Fig. 1). Recordings were collected using either a Sony TCM-5000EV or Sony TC-D5M (Sony Corporation, Tokyo, Japan) tape recorder with TDK SA90 (TDK Corporation, Tokyo, Japan) tapes and a Sennheiser ME67 or ME88 (Sennheiser Electronic Corporation, Old Lyme, CA) directional microphone, or with a Marantz PMD 670 (Marantz Corporation, Kanagawa, Japan) solid-state digital recorder with a Sennheiser ME67 microphone recording at a 44.1 kHz sampling rate. Recordings collected with a tape recorder were subsequently digitized into WAV files at a sampling rate of 44.1 kHz in Raven 1.2 software for sound analysis (Charif et al. 2004). We do not expect any impact on our results from using a variety of recorders because recorded samples were neither associated with habitat type nor with refugium. We aimed to record at least five exemplars of each song type from at least five individuals per site to obtain sufficient high-quality recordings that characterized within population variation.

SOUND ANALYSIS

Individual songs were clipped from larger WAV files using Syrinx software (www.syrinxps.com, John Burt). High-quality songs for analysis were selected based on their signal-to-noise ratio. Background noise in each song was filtered out from each recording using Avisoft-SASlab Pro version 4.5. (www.avisoft.com, R. Specht). Song measurements were taken using the Automatic Parameter Measurement command in Avisoft-SASlab. Little greenbul song elements were detected using an amplitude

threshold of -20 dB SPL relative to the maximum SPL in the sound file, which ensures that measurements are standardized across all sound files used in the current study. Within each detected sound syllable, the following parameters were measured: Start time, End time, Entropy as the mean of all spectra, Highest Frequency as the highest frequency exceeding threshold, Lowest Frequency as the lowest frequency exceeding threshold, and the Peak Frequency as the frequency at the sampling point with the highest amplitude. Song rate was calculated by dividing the song duration (calculated from the start and end times) by the number of syllables in the song. We aimed to include five individuals per site, and up to five of each of the four song types per individual to calculate mean values. Overall, we included values for 137 individuals of song type I, 135 of type II, 139 of type III, and 143 of type IV (see Table S2 for a breakdown of number of individuals’ songs and song types measured from each site).

PLAYBACK EXPERIMENTS

We conducted playback experiments of little greenbul song on 146 individual birds at 15 sites in Uganda and Cameroon between July 2005 and February 2009 (Table S3). A total of 86 stimuli were used for the playbacks, each representing one individual recorded at one of 11 sites in Uganda and Cameroon. Stimuli were prepared from sections of recordings with a good apparent signal-to-noise ratio, with each stimulus consisting of all song types repeated in sequence multiple times. We performed playbacks to vocalizing birds encountered along roads and trails using a replicated dataset for the four playback site categories: Cameroon rainforest and ecotone and Uganda rainforest and ecotone. For each category, we tested the response strength to relatively close (51–508 km) different-habitat song recordings and distant in the other refugium (1866–2221 km) same-habitat song recordings. We used an Apple iPod with a PAL Tivoli Audio loudspeaker positioned approximately 20–30 m from the subject to project WAV file stimuli using the following protocol: 2-min playback of the selected stimulus (systematically chosen to reflect different populations from different habitats, as shown in Table S1), followed by 1-min silence and observation, a further playback of the same 2-min stimulus, and a further 1-min post playback observation. There was much individual variation in response to playback; birds often flew over the speaker, and some sang or called at some stage during the experiment. A strong response typically involved the bird stopping singing and approaching silently and searching in the immediate area of the speaker before returning to sing later during or after the experiment. The strongest responses sometimes involved much movement in the vicinity of the speaker, but always involved a very close approach, often within 2 m. After some pilot experiments, we thus elected to focus on the approach distance (closest distance to the speaker) as the measure that we believed consistently reflected strength of response. On four

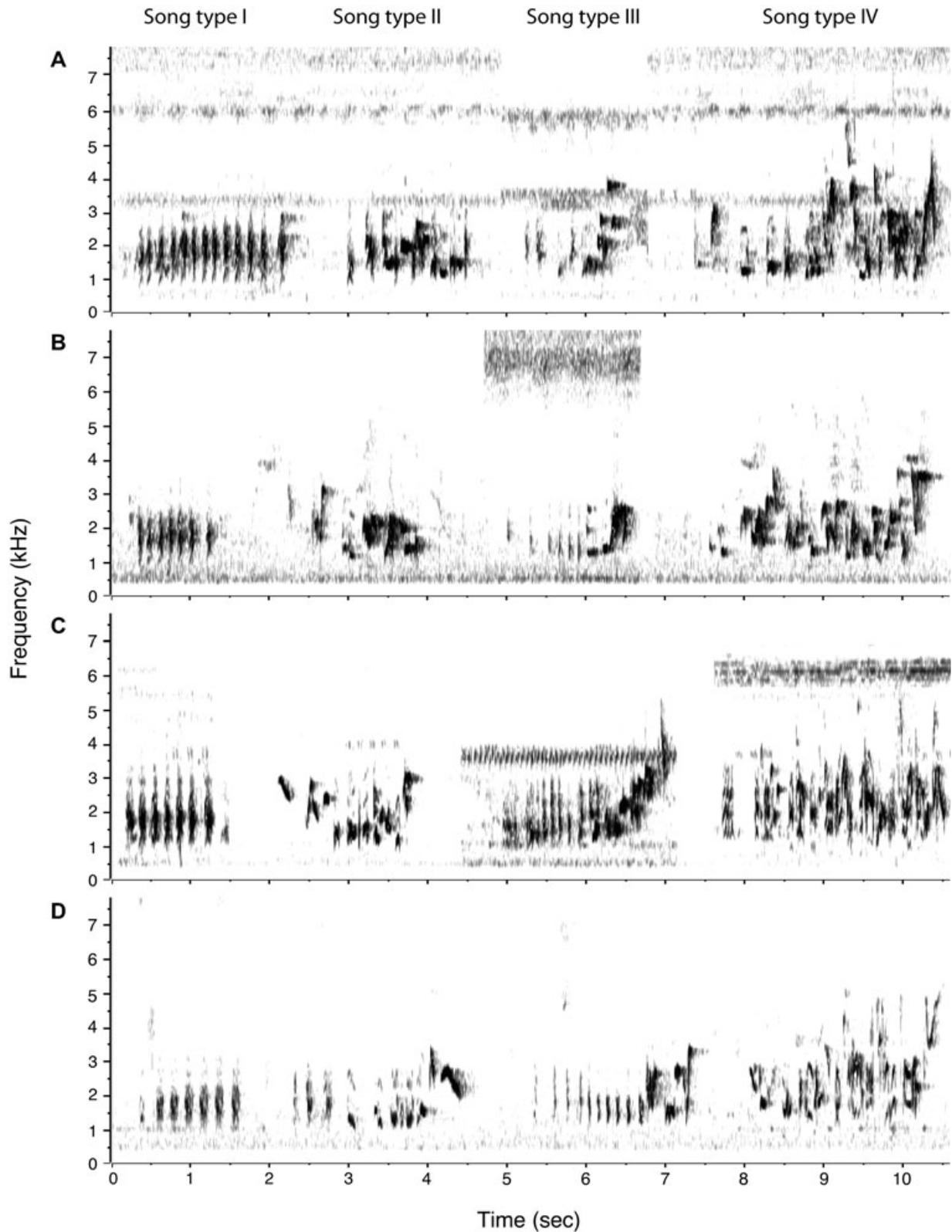


Figure 2. Spectrograms of little greenbul song, illustrating examples of the four song types from (A) Zoebefam in the Cameroon rainforest, (B) Wakwa in the Cameroon ecotone (C) Kibale National Park in the Uganda rainforest, and (D) Queen Elizabeth National Park in the Uganda ecotone. Note that the rainforest recordings, despite special recording equipment and field efforts to optimize signal-to-noise ratio, exhibit some traces of a band of ambient noise in the rainforest between 3.5 and 4 kHz. The rainforest examples for song type III illustrate how the maximum frequency of the song climax is projected above the frequency of this noise band.

occasions no visible approach or response of any kind was detected, and for those experiments we assigned a distance of 20 m (a value equal to the greatest approach distance recorded for any detectable response from the remaining experiments).

ENVIRONMENTAL VARIATION

We obtained information on habitat structure from remotely sensed estimates of percent tree cover based on the vegetation continuous field product (Hansen et al. 2002), derived from passive optical measurements of the Moderate Resolution Imaging Spectroradiometer (MODIS) sensor. We obtained space-borne estimates of elevation from the Shuttle Radar Topography Mission (SRTM) aggregated at a resolution of 1 km². We had to drop one site, Etome, from the analyses because of severe cloud contamination in the MODIS tree cover data. Because we were specifically interested in the role of natural environmental gradients as potential drivers of perceptual phenotypic variation, and not of disturbed forest, we also analyzed data by dichotomizing sites into primary forest or ecotone sites based on their percent tree cover values. We defined primary forest as that with ≥ 60% tree cover; ecotone was defined as having < 50% cover, which we determined from a tri-modal histogram distribution of canopy cover, wherein secondary sites covered the middle section of the distribution. The Afrotropical forest landscape has been disturbed by anthropogenic activities, resulting in much secondary forest (Kirschel et al. 2009b; Freedman et al. 2010b). Because we were interested in understanding which historical factors have driven divergence, we were not specifically testing for the effects of anthropogenic disturbance on song. Therefore, we did not conduct further analyses of the playback results from those disturbed forest sites with 50–60% of forest canopy cover.

DATA ANALYSIS

We analyzed variation in song characteristics using linear-mixed models with maximum likelihood estimation (Cnaan et al. 1997) to test for patterns of song variation explained by the factors: percent tree cover, elevation, and geographic isolation. We nested our data by including site as a random effect in the model. We examined: minimum, maximum, and peak frequency, song rate, and entropy for all four of the song types defined for little greenbuls (Slabbekoorn and Smith 2002a). We calculated false discovery rates (FDR) to control for multiple comparisons (Benjamini and Hochberg 1995; 2000; Verhoeven et al. 2005), which in the case of song analyses accounted for 20 tests (five song features from four song types). To analyze response to playback, we performed linear regression analyses with the standard error adjusted for within-stimulus location correlation (Williams 2000), to control for the effects of pseudo-replication in those cases where several song playback stimuli were recorded at the same sites (McGregor et al. 1992). This approach is conservative as it reduces the degrees of freedom based on the number of sites from which stimuli

were prepared, rather than the number of individuals represented. Response strength to playback may vary with the motivational state of the singer or the ambient noise present on recordings from sites where stimuli were recorded and this method controls for these effects. These regressions tested for the effect of ecological gradient versus refugial isolation (while controlling for elevation). This allowed us to assess whether, for instance Cameroon rainforest birds discriminate between songs from rainforest and ecotone populations in Cameroon, or between songs of rainforest birds from Cameroon and Uganda. As we specifically wanted to test the effect of isolation versus gradient, we excluded the results of all superfluous experiments, including any that represented both different habitat and refugium. We used FDR again here to control for the four tests (one per habitat within each region). All statistical analyses were performed using STATA 11 (StataCorp 2009).

Results

BOTH ECOLOGICAL GRADIENTS AND REFUGIAL ISOLATION INFLUENCE SONG

Geographic variation in little greenbul song was related to both the ecological gradient between rainforest and ecotone forest, as quantified by percent tree cover, as well as to geographic isolation between two rainforest refugia (Table 1). Specifically,

Table 1. Song variation by region and by habitat. P-values in bold are significant after controlling for false discovery rates.

Song type	Song measure	Geographic isolation		% Tree cover	
		<i>z</i>	<i>P</i>	<i>z</i>	<i>P</i>
Song I <i>N</i> =132	Min. Freq.	1.21	0.225	1.35	0.179
	Max. Freq.	0.6	0.546	-2.65	0.008
	Peak Freq.	0.07	0.946	-1.44	0.149
	Rate	2.34	0.019	2.21	0.027
	Entropy	-0.32	0.748	-3.59	<0.001
song II <i>N</i> =130	Min. Freq.	3.52	<0.001	0.66	0.507
	Max. Freq.	4.33	<0.001	-2.73	0.006
	Peak Freq.	3.27	0.001	-3.63	<0.001
	Rate	-0.37	0.711	-1.13	0.257
	Entropy	-3.18	0.001	-1.43	0.153
Song III <i>N</i> =134	Min. Freq.	1.14	0.256	-0.97	0.334
	Max. Freq.	-0.16	0.874	5.32	<0.001
	Peak Freq.	-0.58	0.565	0.32	0.746
	Rate	-4.95	<0.001	-1.73	0.083
	Entropy	-1.66	0.096	-1.17	0.243
Song IV <i>N</i> =138	Min. Freq.	2.92	0.004	0.56	0.577
	Max. Freq.	3.33	0.001	0.79	0.431
	Peak Freq.	-2.29	0.022	-0.78	0.435
	Rate	-3.95	<0.001	-0.75	0.455
	Entropy	-2.28	0.023	-1.39	0.164

geographic isolation better explained variation in song types II and IV, whereas percent canopy cover better explained variation in maximum frequency (Fig. 3).

Results of playbacks: ecological gradients alone drive perceptual differences in song

Cameroon rainforest birds discriminated between rainforest and ecotone forest birds from Cameroon, but not between rainforest birds from Cameroon and Uganda. The same discriminatory pattern was found for Uganda rainforest birds, whereas ecotone birds of both Cameroon and Uganda, responded equally strongly irrespective of the ecological or geographic origin of the recording. Specifically, birds from the Cameroon forest responded significantly less to Cameroon ecotone song than to Cameroon forest song (mean approach distance 8.32 m vs. 3.94 m respectively; $n = 47$, $t = 5.57$, $P = 0.001$), but there was no difference in response to Uganda forest and Cameroon forest song (6 m vs. 3.94 m; $t = 1.69$, $P = 0.141$; Fig. 4A). No differences were found, though, in responses of birds in the Cameroon ecotone to Cameroon ecotone or Cameroon forest song (3.65 m vs. 3.17 m; $n = 29$, $t = 0.10$, $P = 0.927$), or to Cameroon ecotone and Uganda ecotone song (3.65 m vs. 4.2 m; $t = 0.70$, $P = 0.510$; Fig. 4B). Birds from the Uganda forest again responded significantly less to Uganda ecotone song (2.35 m vs. 7.33 m; $n = 47$, $t = 15.06$, $P < 0.001$), but not to Cameroon forest song (2.35 m vs. 5.81 m; $t = -1.34$, $P = 0.221$; Fig. 4C) compared to Uganda forest song. Uganda ecotone birds did not respond differently to Uganda forest songs (4.18 m vs. 5.72 m; $n = 23$, $t = -0.63$, $P = 0.548$) or Cameroon ecotone songs (4.18 m vs. 7.17 m; $t = -1.84$, $P = 0.109$; Fig. 4D) than to Uganda ecotone song.

Discussion

Little greenbul songs varied significantly both across an ecological gradient and between sites from different postulated rainforest refugia. However, only ecological differentiation led to an impact on playback response and only in rainforest and not in ecotone forest. Rainforest males in Cameroon as well as Uganda responded more strongly to songs from their own than from the other habitat type independent of the refugial origin of the recorded stimulus.

SONG VARIATION INFLUENCED BY ECOLOGY AND ISOLATION

We found that song varies with both geographic isolation and ecological gradients. Refugial isolation primarily affected two song types. We suspect that these geographic differences reflect cultural evolution (Ellers and Slabbekoorn 2003), similar to patterns of variation found in song type sequences (Slabbekoorn and Smith 2002a), as spectral and temporal structure of songs may differ between populations in concert with differences in

learned song components (Slabbekoorn and Smith 2002b). Little greenbuls have very elaborate songs, sometimes coinciding with a complex courtship ritual (Kirschel 2008), and the various song components and their spectral and temporal features provide plenty of opportunity for multidimensional diversification. Several processes may drive such diversification. Distinct song elements between geographically distant regions may develop because of nonadaptive mate preferences (e.g., Prum 2010), or alternatively through parallel evolution with different adaptive mutations in similar environments (Schluter 2009), with sexual selection amplifying divergence in mate preferences (Schluter 2001). Additionally, species assemblages vary between rainforest refugia, and diverse interactions therein may also cause divergent selection despite environmental similarity in allopatry (Hoskin and Higgie 2010). Such divergent selection would thus be driven by acoustic niche differentiation (Duellman and Pyles 1983) or character displacement between interacting species (Brown and Wilson 1956; Grant 1972; Dayan and Simberloff 2005; Pfennig and Pfennig 2010), as found in the songs of African tinkerbirds (Kirschel et al. 2009b), and the direction of displacement may vary between geographic isolates in the same habitat (Duellman and Pyles 1983; Moriarty Lemmon 2009; Hoskin and Higgie 2010, A. N. G. Kirschel unpubl. data).

Ecology appeared to influence several acoustic parameters of little greenbul song. One possible explanation is that divergent ecology leads to divergence in body size and beak shape, and thus song as a byproduct (Podos 2001; Seddon 2005, cf. Kirschel et al. 2009b). With frequencies varying in different directions as a function of tree cover, as suggested by Slabbekoorn and Smith (2002a), we do not believe divergent morphology is a plausible explanation for the patterns of song variation found. Ecological gradients (as measured here by percent tree cover) primarily affected the maximum frequency of stereotypic songs. Lower maximum frequencies in the forest for two song types are consistent with expectations of lower frequencies in closed forests than in open habitats to improve transmission through dense vegetation (Morton 1975; Wiley and Richards 1982), although Slabbekoorn and Smith (2002a) found that transmission properties did not differ between rainforest and ecotone forest sites in Cameroon in the forest layer used by little greenbuls. In addition, in apparent contrast to the expected pattern of divergence in closed forests but congruent with that reported in Slabbekoorn and Smith (2002a), we found that song type III was significantly higher in maximum frequency in the rainforest than in the ecotone forest. This specific spectral adaptation could be related to a dominant noise band around 3.5 kHz in frequency, which is consistently present in the rainforest, across sites, seasons and throughout the day, but totally absent in ecotone forest (Slabbekoorn and Smith 2002a; Slabbekoorn 2004; Kirschel et al. 2009a). We suggest that little greenbuls in the rainforest may make the typical climactic notes

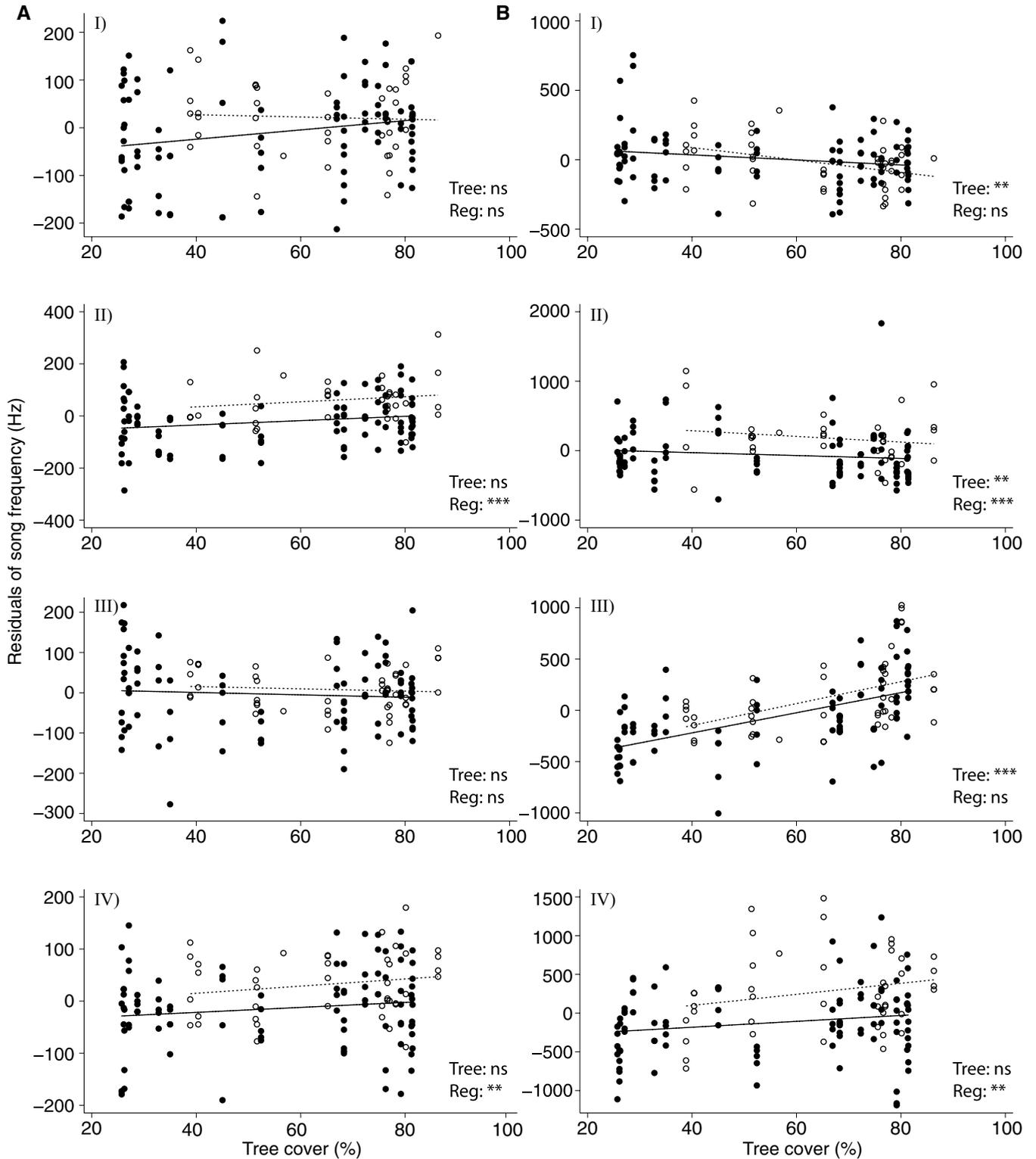


Figure 3. Little greenbul song varies in (A) minimum frequency and (B) maximum frequency for song types I, II, III, and IV (frequency residuals shown after controlling for elevation), based on percent tree cover and refugial isolation (represented by filled circles for Cameroon and open circles for Uganda). Steeper slopes of lines-of-best-fit (solid line for Cameroon, dotted for Uganda) indicate greater effects of ecological gradients on song variation, larger distances between lines-of-best-fit indicate greater song variation between regions. Geographic isolation had a significant effect on minimum and maximum frequency of song types II and IV. Ecological gradients better explained variation in maximum frequency of song types I and III, and also influenced max frequency of song type II. Significance levels for variation attributed to percent tree cover (Tree) and geographic region (Reg) *** $P < 0.001$, ** $P < 0.01$.

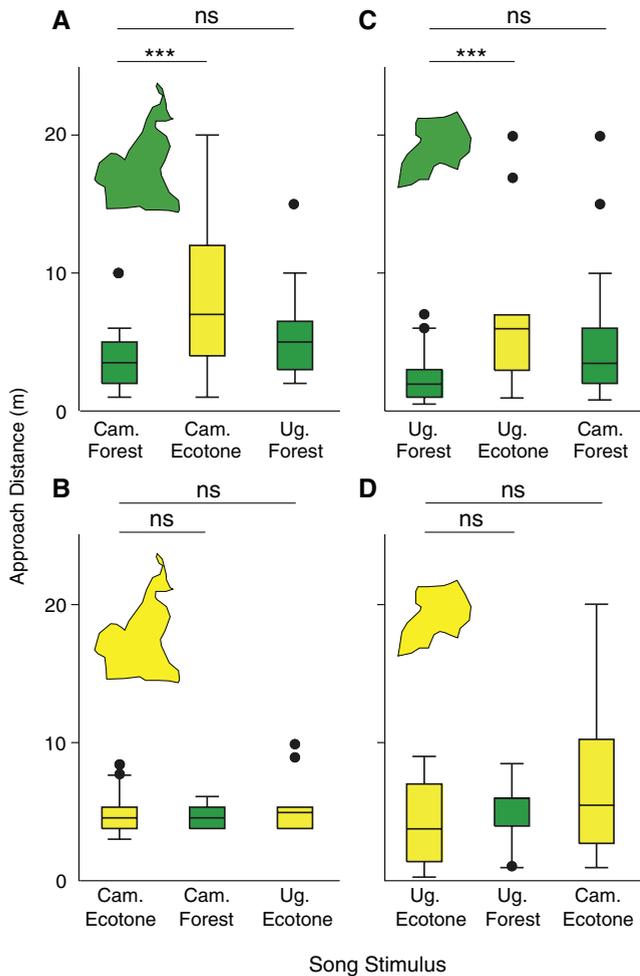


Figure 4. Responses to playback experiments from within each region; maps within each graph illustrate playback experiment location and habitat (green = forest; yellow = ecotone): (A) Birds in the Cameroon forest responded significantly less to Cameroon ecotone song than to Cameroon forest song, but not differently to Uganda forest song than Cameroon forest song, (B) Cameroon ecotone birds showed no differences in responses to Cameroon ecotone, Cameroon forest and Uganda ecotone song, (C) Uganda forest birds responded significantly less to Uganda ecotone song than to Uganda forest songs, but not differently between Cameroon and Uganda forest songs; (D) Uganda ecotone birds did not appear to differentiate between song from their own population and Uganda forest song, nor Cameroon ecotone song. Error bars represent 95% confidence intervals, *** $P < 0.001$; ns = not significant.

of the crescendo of their song type III audible to intended receivers by singing above this frequency. Little greenbuls in the ecotone forest, without consistent noise bands within the little greenbul frequency range, obviously lack this requirement for clear advertisement of this distinct song component. Although such correlative observations are still speculative, rainforest noise profiles are loud, habitat-dependent, and spectrally differentiated, and they may well explain the part of avian song variation associ-

ated with ecological gradients that is most critical to the process of ecological speciation.

PLAYBACK RESPONSE AFFECTED BY ECOLOGICAL DIVERGENCE BUT NOT REFUGIAL ISOLATION

Rainforest birds discriminated between own-habitat and different-habitat songs, independent of refugial isolation. They even showed no decline in response strength to rainforest songs from 1600 km away, while ecotone forest songs recorded at a much closer distance led to significantly lower response levels. Although some previous studies showed similar playback results with discrimination against songs across an ecological gradient (e.g., Patten et al. 2004; Seddon and Tobias 2007; Mockford and Marshall 2009; Ripmeester et al. 2010), the current study is unprecedented in both the scale of the geographical distance tested between sites and in the nature of the populations tested, which presumably have diverged historically in rainforest refugia. Although we have not specifically identified the song parameters critical for recognition, we have shown that differences in song across an ecological gradient are perceptually more important than differences between populations over vast geographic distances in similar habitat.

The asymmetry in discrimination of divergent songs among individuals from within rainforest and ecotone populations could be explained by the contrast in noise profiles mentioned above. The presence of a loud and highly persistent noise band may both favor the use of higher frequency components (in our case specifically in song type III) in rainforest songs, and might also render ecotone songs less efficient in the rainforest due to selective masking (cf., Dingle et al. 2008, 2010). The historical biogeography of little greenbul expansion may add an alternative or complementary explanation for the asymmetry found. Although we do not have data specifically for little greenbul, paleobiological analyses of other African forest birds and lizards (Freedman et al. 2010a; Smith et al. in press) show expansion out of refugial areas into ecotone following glacial maxima. A similar process in little greenbul could result in song differentiation following expansion. However, if ecotone populations were more derived, they could retain recognition of ancestral rainforest song, thus explaining their strong responses to it. Such song divergence following expansion could be driven by character release as they moved into acoustically less-complex environments, lacking the aforementioned loud ambient noise, and other competing acoustic signals. If ecotone birds produced songs that forest birds consider substandard (e.g., they do not reach the high notes of forest song type III) as a result of such character release, then forest birds may respond less to ecotone song because they considered it a weaker threat than forest song when defending territories and competing for mates. Asymmetry in responses between geographically or ecologically separated populations is not an unusual phenomenon, and has been reported in a number of recent studies (Colbeck

et al. 2010; Dingle et al. 2010; Jankowski et al. 2010), and could have a significant effect on the level and direction of gene flow.

Our field playback experiments were targeted toward singing males. Even though males typically respond aggressively to the possible presence of intruders, the reduced responses by rainforest birds toward ecotone song demonstrate the possible effect of song divergence driven by ecological differences on species recognition. Discrimination of habitat-dependent song variation by males in a territorial context can directly affect levels of gene flow (Ellers and Slabbekoorn 2003) and can reflect female response patterns in a mate choice context. Indeed, females have been shown to be even more selective in recognition of male songs (Searcy and Brenowitz 1988), and less responsive to heterospecific song than males (Seddon and Tobias 2010). Such differences may be important in mate recognition, and could thus lead to assortative mating and reproductive isolation.

In contrast to the impact of ecology, we found little support for the Forest Refuge Hypothesis in driving perceptual song divergence. Little greenbuls in rainforest populations did not respond differently to rainforest songs from different refugia. Studies that have focused on genetic structure have found evidence for reduced gene flow in neutral markers between populations from forest refuges (e.g., Marks 2010), but are patterns of differentiation in neutral molecular markers sufficient evidence for species diversification? We suggest that evidence of divergence in traits that can lead to reproductive isolation is a better indicator of the processes driving evolutionary diversification.

Conclusions

Our study shows that while both ecological gradients and rainforest refugia explain a considerable amount of geographic song variation, ecology plays a more important role in perceptual song divergence. The finding of perceptually relevant song divergence along an ecological gradient is concordant with the pattern of ecologically divergent morphology in little greenbuls (Smith et al. 2001, 2005a). This confirms the potential strength of ecology in driving evolutionary divergence, despite on-going gene flow. Recent studies have also demonstrated that phenotypic evolution along an ecological gradient despite high gene flow may reflect divergence in molecular markers under selection or those linked to them (e.g., Mullen and Hoekstra 2008; Freedman et al. 2010a). Such linkage relationships through strong selection pressures can affect the extent to which genetic divergence is isolated at loci under selection or widespread in the genome (Rice and Hostert 1993; Michel et al. 2010). Importantly, our results also suggest a potentially critical role for the interplay between ecology and behavior in yielding divergent traits, which could promote assortative mating and induce prezygotic isolation (Coyne and Orr 2004). Therefore, we believe more integrative studies, which include en-

vironmental, molecular, morphological, and behavioral analyses, are key for our understanding of the mechanisms underlying the role of ecological speciation in evolutionary diversification.

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Supporting Information

The following supporting information is available for this article:

Table S1. Recording and playback experiment sites, GPS coordinates and the regions, percent tree cover, elevation and number of playback stimuli prepared from songs recorded at those sites.

Table S2. Recording sites with no. of individuals per site whose songs were included in the analyses, and number of song types measured per site.

Table S3. Sites where playback experiments were performed, with numbers of each stimulus used.

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

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