

Genetic continuity of brood-parasitic indigobird species

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Abstract

Speciation in brood-parasitic indigobirds (genus *Vidua*) is a consequence of behavioural imprinting in both males and females. Mimicry of host song by males and host fidelity in female egg laying result in reproductive isolation of indigobirds associated with a given host species. Colonization of new hosts and subsequent speciation require that females occasionally lay eggs in the nests of novel hosts but the same behaviour may lead to hybridization when females parasitize hosts already associated with other indigobird species. Thus, retained ancestral polymorphism and ongoing hybridization are two alternative explanations for the limited genetic differentiation among indigobird species. We tested for genetic continuity of indigobird species using mitochondrial sequences and nuclear microsatellite data. Within West Africa and southern Africa, allopatric populations of the same species are generally more similar to each other than to sympatric populations of different species. Likewise, a larger proportion of genetic variation is explained by differences between species than by differences between locations in alternative hierarchical AMOVAS, suggesting that the rate of hybridization is not high enough to homogenize sympatric populations of different species or prevent genetic differentiation between species. Broad sharing of genetic polymorphisms among species, however, suggests that some indigobird species trace to multiple host colonization events in space and time, each contributing to the formation of a single interbreeding population bound together by songs acquired from the host species.

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Introduction

Population subdivision as a consequence of host fidelity is perhaps the most plausible mechanism for sympatric differentiation. In phytophagous insects, rearing environment influences subsequent habitat choice and in turn promotes assortative mating that isolates populations associated with different hosts (e.g. Bush 1994; Berlocher & Feder 2002; Nason *et al.* 2002; Diegisser *et al.* 2004). Similar mechanisms are responsible for speciation in brood parasitic indigobirds (genus *Vidua*, Payne 2004), in which host–parasite associations are established and maintained through behavioural imprinting. Species-specific parasites of various estrildid finches, both male and female indigobirds

learn the songs of their respective hosts. Adult males combine mimicry of host song with indigobird vocalizations and thereby attract female indigobirds reared by the same host species (Nicolai 1964; Payne 1985; Payne *et al.* 1998, 2000), whereas females preferentially parasitize nests of the species that reared them (Payne *et al.* 2000). Thus, behavioural imprinting results in prezygotic isolation of indigobirds associated with different hosts and promotes rapid speciation after a new host is colonized (Payne *et al.* 2000; Sorenson *et al.* 2003).

Both morphological and genetic data are consistent with a recent diversification of indigobird species through host colonization rather than a more ancient process of host–parasite cospeciation (Klein & Payne 1998; Sorenson & Payne 2002; Sorenson *et al.* 2004). Although morphologically similar, indigobird species are distinguished by consistent differences in adult male plumage colour and iridescence, and for certain species, by differences in body size and colours of the feet, legs and bill (Payne 2004).

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Additional morphological variation reflects adaptation of indigobirds to their hosts: young indigobirds, which are reared along with the host's young, mimic the species-specific mouth markings of host nestlings (Payne 1982; Payne *et al.* 1992; Payne & Payne 1994; Payne 2004). These markings are displayed as part of the distinctive begging behaviour shared by estrildid and parasitic finches and undoubtedly play a role in obtaining parental care (see Payne *et al.* 2001). Although mouth patterns are unknown for some indigobird species (the markings fade and disappear in adult birds), many indigobirds show remarkably precise mimicry of host mouth markings, as well as mimicking other attributes of host young, such as begging calls, skin colour, natal down, and juvenile plumage (Payne & Payne 2002; Payne 2004).

In contrast to consistent morphological differences among indigobird species, genetic data for indigobirds meet an expectation of 'profound genetic similarity' for sympatrically derived species (Via 2001). Sympatric indigobirds share a limited diversity of both mitochondrial haplotypes and nuclear microsatellite alleles and are more similar to each other than to indigobirds in other regions (e.g. southern Africa vs. West Africa) (Sorenson *et al.* 2003). Slight but statistically significant interspecific differentiation in haplotype and allele frequencies, however, is consistent with current reproductive isolation and suggests that the genetic similarity of indigobirds reflects incomplete sorting of ancestral polymorphisms following diversification from a recent common ancestor (Sorenson *et al.* 2003). Ongoing hybridization and introgression, however, might retard the lineage-sorting process and homogenize neutral genetic markers across species boundaries even as selection produces and maintains host-specific adaptations. This is an important consideration because the same behavioural mechanisms promoting speciation may facilitate hybridization between established indigobird species. If females occasionally lay in the nest of an alternate host species, the resulting offspring will either attract (if male) or choose (if female) mates from the indigobird species already associated with the alternate host, leading to hybridization between indigobird species in the second generation (see Payne & Sorenson 2004).

Further consideration suggests a continuum of hypotheses for the genetic similarity of indigobirds that reflects the spatial and/or temporal scales of host colonization events. At one end of this continuum, the minimal differentiation of indigobird species is simply a consequence of 'retained ancestral polymorphism' following recent speciation. Given speciation by host shift, however, and broad sharing of genetic polymorphisms across species, the origin of each new species must have involved multiple founding females, raising the question of when and where these females laid in nests of the novel host. Moving to the middle of this continuum of hypotheses, an indigobird

species might trace to 'multiple colonization' events in space and time, giving rise to local populations that later annealed to form a single interbreeding population bound together by shared song mimicry. If individual host shifts mediated by female egg laying are extended through time, multiple colonization grades into a model of 'ongoing hybridization' between established species.

If hybridization is rare, indigobird species should gradually differentiate in neutral genetic markers while intra-specific gene flow should help to homogenize conspecific populations in different areas, whether they originated from one or many colonization events. In contrast, if host shifts and hybridization are frequent, sympatric indigobirds associated with different hosts may be more similar to each other than to conspecific populations in other locations, particularly if allopatric populations became associated with the same host independently. The same pattern could develop over time if limited dispersal allows allopatric populations of the same species to diverge through genetic drift while ongoing hybridization homogenizes sympatric populations associated with different hosts. At the extreme, hybridization might keep indigobirds in a perpetual state of incomplete differentiation and obscure a longer history of continuous host-parasite associations than is suggested by neutral genetic variation. It is important to specify that these predictions apply to neutral genetic markers only: if hybridization is frequent and ongoing, then selection must maintain the clear and consistent morphological differences between indigobird species.

To test the previously mentioned predictions, we used mitochondrial and nuclear markers to assess the genetic continuity of indigobird species on a regional scale. Almost doubling the number of indigobirds samples used in Sorenson *et al.* (2003), we first test for significant differentiation among indigobird species while controlling for possible biases associated with sampling different species in different locations. We also compare the level of genetic differentiation among conspecific populations sampled at different localities with interspecific differentiation of populations from the same site and test whether geographical location or species membership is a better predictor of genetic similarity.

Materials and methods

In fieldwork from 1966 to 1980 and 1991 to 2000, indigobirds were collected at various locations in Cameroon and Nigeria (West Africa) and Zambia, Zimbabwe, Malawi, Botswana and South Africa (southern Africa). Seven indigobird species occur in West Africa: *Vidua chalybeata*, *Vidua camerunensis*, *Vidua larvaticola*, *Vidua maryae*, *Vidua nigeriae*, *Vidua raricola* and *Vidua wilsoni* (Payne 2004). *V. chalybeata* also occurs in southern Africa along with *Vidua codringtoni*, *Vidua funerea*, and *Vidua purpurascens*. In West Africa,

individuals recognized as *V. camerunensis* based on morphology are associated with several host species (Payne & Payne 1994), although individual males mimic only one host. Our sample includes two 'song populations' of *V. camerunensis* from Tibati, Cameroon, where individual males sang either African firefinch *Lagonosticta rubricata* or black-bellied firefinch *Lagonosticta rara* song (Payne *et al.* 2005). Given the importance of behavioural imprinting in this system (Payne *et al.* 1998, 2000), *V. camerunensis* associated with different hosts may be reproductively isolated and we therefore treated *V. camerunensis* from Tibati as two separate populations in the analyses presented here.

In general, the genetic divergence between western and southern indigobirds is greater than among the species within each region (Sorenson *et al.* 2003), suggesting independent diversification of indigobirds in the two regions. Given the relatively deep genetic divergence between regions, including a nearly complete lack of shared mtDNA haplotypes, we generally present separate analyses for the two regions in this study.

Sources of DNA included the calamus of one or two primary or secondary feathers obtained from specimens at the University of Michigan, Museum of Zoology (birds recorded in the field and collected from 1966 to 1980) and either tissue or feather samples from more recent field work. Genomic DNA was obtained using QIAamp tissue kits (QIAGEN) with the addition of 30 μ L of 100 mg/mL dithiothreitol for lysis of feather samples (Cooper 1994). When working with museum samples, all preamplification steps were carried out in a separate room with dedicated equipment.

Mitochondrial DNA sequences comprising 1100 base pairs and including most of the nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 6 (*ND6*) gene, tRNA glutamine and the 5' half of the control region were determined for 174 western and 230 southern indigobirds. Microsatellite genotypes were obtained from nine loci (*INDIGO* 7, 8, 15, 28, 30, 37, 38, 40, 41; Sefc *et al.* 2001). Laboratory methods for mtDNA and microsatellite data collection were described by Sorenson & Payne (2001) and by Sefc *et al.* (2001), respectively. Polymerase chain reaction (PCR) conditions and replication strategies for genotyping museum feather extracts were described by Sefc *et al.* (2003). The sample of microsatellite genotypes was slightly smaller (166 western and 194 southern birds) because of difficulties with some of the older museum samples (Sefc *et al.* 2003). Sequence data have been deposited in GenBank (Accession nos: AF090341, AY322613–AY322833, AY865372–AY865554).

Individuals within each region were assigned to species based on their morphology and host association (inferred from host song mimicry). One male *V. codringtoni* and one male *V. chalybeata* sang the songs of *L. rubricata* rather than their usual hosts, presumably because their mothers laid an egg in an alternate host nest (see Payne *et al.* 1992; Payne

& Sorenson 2004). These two individuals were assigned to species based on their morphology. We also divided each species into a number of 'local' subpopulations, ranging from one to five per species depending on the spatial distribution of available samples. Given the small number of available samples, all *V. larvaticola* from Nigeria were included in a single subpopulation. For other species, the average distance between sampling locations within subpopulations varied from essentially zero (all individuals from a single locality) to 110 km, whereas maximum distances were less than 220 km. Average distances between subpopulations of a given species ranged from 160 to 1460 km. A few individuals were excluded from population-level analyses because they were from locations with inadequate sample sizes (\leq nine individuals). Sample sizes and geographical locations for species and subpopulations are indicated in Table 1 and Fig. 1, respectively.

Using GENEPOP version 3.4 (Raymond & Rousset 1995), we found no significant deviations from Hardy–Weinberg expectations in species or subpopulation samples after correcting for multiple comparisons (Benjamini & Hochberg 1995). Tests for linkage disequilibrium yielded one significant result for the locus pair *IND* 28–*IND* 38 in *V. funerea*. As linkage equilibrium was not rejected in any other species, we assumed that these two loci are independent and included both in our analyses.

We used ARLEQUIN (Schneider *et al.* 2000) to obtain pairwise estimates of population differentiation based either on haplotype frequencies (F_{ST}) or uncorrected genetic distances between haplotypes (Φ_{ST}). ARLEQUIN was also used for hierarchical analyses of molecular variance and to estimate pairwise F_{ST} values based on microsatellite allele frequencies. Differentiation estimates accounting for evolutionary distances between microsatellite alleles under the assumption of stepwise mutation (R_{ST}) were obtained using RSTCALC (Goodman 1997). Type I errors in tests involving multiple comparisons were controlled using the method of Benjamini & Hochberg (1995).

Genetic similarity among all populations was summarized in a UPGMA phenogram constructed in PHYLIP (Felsenstein 1989) based on combined microsatellite allele and mitochondrial haplotype sharing between populations (calculated in *microsat* as compiled by Eric Minch, <<http://hpgl.stanford.edu/projects/microsat/>>).

Finally, we tested for correlations between genetic distance, geographical distance and species membership using individual birds as the sampling unit, an approach that allowed us to include all of the data for each region in a single analysis. Mitochondrial distance matrices were based on the observed number of nucleotide differences between pairs of sequences, whereas pairwise estimates of relatedness were used to build a microsatellite similarity matrix. Even if divergence among populations is too recent to result in obvious allele frequency differences, we still

Table 1 Sample sizes and genetic diversity of indigobird populations analysed in this study

Population	<i>n</i>	<i>H</i>	mtDNA haplotype diversity	mtDNA nucleotide diversity	Microsatellite allelic diversity
Southern Africa					
<i>Vidua chalybeata</i>					
Maun 1967	10 (0)	7	0.867	0.00461	—
Marble Hall 1967	9 (0)	7	0.944	0.00399	—
Merensky Nature Reserve 1967	15	14	0.990	0.00339	0.779
Lochinvar 1967 (6), 1997 (5)	11 (0)	7	0.873	0.00334	—
Chipinge 1967	19	12	0.942	0.00263	0.750
Species sample	106 (64)	40	0.943	0.00350	0.757
<i>Vidua codringtoni</i>					
Mutare 1967 (6), 1991 (6)	12	8	0.894	0.00219	0.775
Chipinge 1967 (7), 1991 (2)	9	4	0.583	0.00152	0.716
Species sample	29	11	0.899	0.00274	0.774
<i>Vidua funerea</i>					
Mutare 1967 (13), 1991 (3)	16	14	0.983	0.00328	0.768
Species sample	39 (30)	24	0.954	0.00265	0.766
<i>Vidua purpurascens</i>					
Merensky Nature Reserve 1967	21	13	0.919	0.00216	0.767
Chipinge 1967	13	10	0.949	0.00238	0.784
Northern Malawi 1991	9	8	0.972	0.00369	0.750
Southern Malawi 1991	9	8	0.972	0.00293	0.789
Species sample	56	27	0.932	0.00246	0.771
West Africa					
<i>Vidua chalybeata</i>					
Garoua 1980 (1), 1992 (9)	10	9	0.978	0.00335	0.754
Central Nigeria 1968 (8), 1991 (2)	10	6	0.844	0.00764	0.754
Species sample	22	13	0.947	0.00673	0.764
<i>Vidua nigeriae</i>					
Garoua 1980 (3), 1992 (6)	9	5	0.722	0.00444	0.788
Central Nigeria 1995	10	4	0.533	0.00055	0.783
Species sample	20	8	0.589	0.00243	0.790
<i>Vidua larvaticola</i>					
Central Nigeria 1968 (17), 1975 (2), 1995 (2)	21 (15)	15	0.943	0.00339	0.783
Species sample	22 (19)	15	0.926	0.00325	0.789
<i>Vidua maryae</i>					
Central Nigeria 1968 (5), 1995 (4), 1997 (2)	11	6	0.891	0.00264	0.778
<i>Vidua wilsoni</i>					
Central Nigeria 1968 (9), 1995 (7)	16	9	0.817	0.00364	0.762
Species sample	23 (22)	13	0.818	0.00392	0.774
<i>Vidua camerunensis</i> , <i>L. rubricata</i> mimics					
Tibati 1980 (7), 1992 (6), 2000 (5)	18	16	0.960	0.00352	0.802
<i>Vidua camerunensis</i> , <i>L. rara</i> mimics					
Tibati 1992 (12), 2000 (8)	20	12	0.811	0.00272	0.810
<i>Vidua raricola</i>					
Tibati 1980 (16), 1992 (10), 2000 (8)	34	13	0.904	0.00221	0.806
Species sample	38	13	0.875	0.00211	0.803

The first column lists sampling location and sampling date(s), with the number of samples obtained in different years indicated in parentheses. For each population, the number of individuals sampled (*n*) and the number of different mitochondrial haplotypes (*H*) observed in each sample are provided along with estimates of genetic diversity calculated in DNASP version 3.52 (mtDNA; Rozas *et al.* 2003) and FSTAT (microsatellites; Goudet 2001). If the number of individuals typed for microsatellite data was smaller than for mtDNA, this is indicated in parentheses. The total sample for many species includes additional birds not assigned to any of the listed populations, therefore resulting in a larger sample size than the sum of the individual population samples.

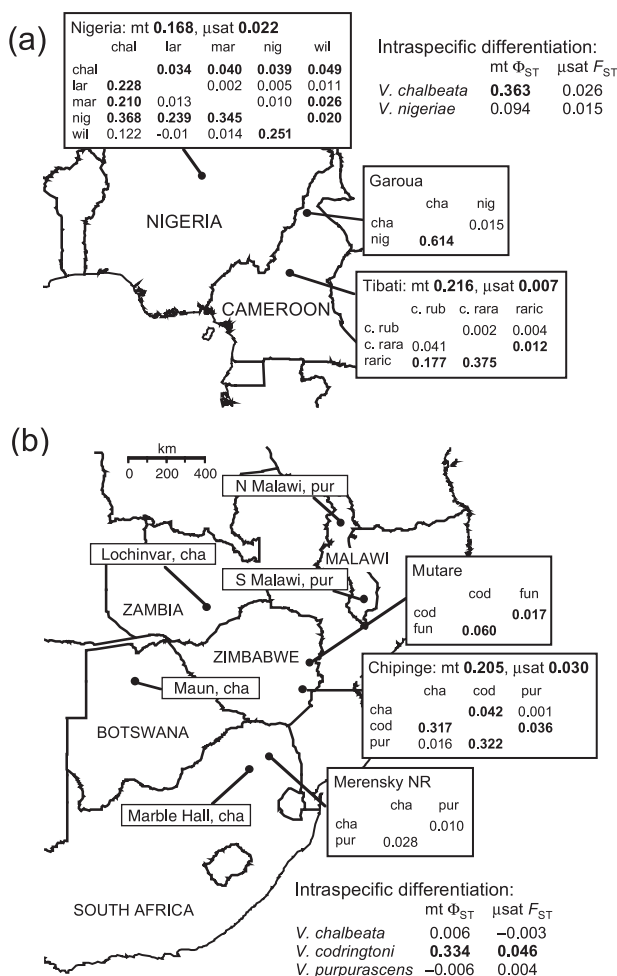


Fig. 1 Sampling locations of West African (a) and southern African (b) indigobird populations. The tables associated with each sampling location show the differentiation between sympatric heterospecific populations at each location, with mitochondrial Φ_{ST} values below the diagonal and microsatellite F_{ST} values above the diagonal. Values in bold remain significant at the 0.05 level when using the method of Benjamini & Hochberg (1995) to control the rate at which null hypotheses are falsely rejected. Species abbreviations in tables: cha, *Vidua chalybeata*; lar, *Vidua laroaticola*; mar, *Vidua maryae*; nig, *Vidua nigeriae*; wil, *Vidua wilsoni*; c.rub, *Vidua camerunensis* mimicking *Lagonosticta rubricata*; c.rara, *Vidua camerunensis* mimicking *Lagonosticta rara*; rar, *Vidua rariola*; pur, *Vidua purpurascens*; cod, *Vidua codringtoni*; fun, *Vidua funerea*.

expect individuals within species to be more closely related to each other than to individuals from different species. Coefficients of relatedness between pairs of individuals were calculated in RELATEDNESS version 4.2 (Queller & Goodnight 1989). To minimize prior assumptions, no bias correction was applied for background allele frequencies, which were based on all individuals of all species within each region. As sample sizes are large (> 150), the bias introduced by including a few relatives, and thus the

underestimation of relatedness, should be weak. Physical distances between sampling sites were calculated from geographical coordinates in the R package (Legendre & Vaudor 1991), and species membership matrices were coded as 0 for conspecific or 1 for heterospecific pairs. Partial Mantel tests (executed using the program ZR, Bonnet & Van de Peer 2002) were then used to test for correlation between genetic distance and geographical distance with species membership held constant, and correlation between genetic distance and species membership with geographical distance held constant.

Choice of differentiation statistics

Population differentiation can be quantified using traditional F -statistics or more recently developed metrics that account both for marker frequencies and coalescence times (Excoffier *et al.* 1992; Slatkin 1995). We obtained counter-intuitive results when using F_{ST} for mtDNA. For example, F_{ST} values for population pairs with divergent haplotypes but high within-population diversity were lower than for populations that shared closely related haplotypes but had limited haplotypic diversity, illustrating the effect of marker polymorphism on F_{ST} values (Hedrick 1999; Balloux *et al.* 2000). In contrast, by incorporating divergence resulting from both drift and mutation, Φ_{ST} appears to be a more robust measure of mtDNA differentiation.

As a measure of microsatellite differentiation, R_{ST} does not necessarily have the same advantages because of the homoplasy inherent in a stepwise mutation process. Because stepwise mutations at microsatellite loci will most often give rise to alleles already present in a population, divergence in allele size may take a long time to develop. Although all but one (*IND 28*) of the loci we used complied with the stepwise-mutation model (Cornuet & Luikart 1996), some population pairs that differed significantly in allele frequencies (as measured by F_{ST}) showed no significant differentiation using R_{ST} . Thus, the reduced sensitivity of R_{ST} to genetic drift (Slatkin 1995) is not compensated by incorporation of coalescence times (measured by proxy as difference in allele size), at least during the initial phases of population differentiation. In contrast, most mitochondrial mutations increase the level of divergence among haplotypes, making the consideration of genetic distances informative for both recent and more ancient splits. According to this logic, F_{ST} and Φ_{ST} were employed for microsatellite and haplotype data, respectively, in most of our analyses.

Results

Genetic differentiation among indigobird species

With approximately twice as many samples included, mitochondrial haplotype networks and estimates of genetic

	<i>V. chalybeata</i>	<i>V. funerea</i>	<i>V. codringtoni</i>	<i>V. purpurascens</i>
<i>V. chalybeata</i>	—	0.007*	-0.002	0.003
<i>V. funerea</i>	0.053***	—	0.009	0.005
<i>V. codringtoni</i>	0.102***	0.047*	—	-0.004
<i>V. purpurascens</i>	0.037**	-0.004	0.057***	—

Adjusted significance levels of 0.001, 0.01 and 0.05 are indicated as ***, ** and *, respectively.

Table 2 Pairwise population differentiation values between southern African indigobird species. Below diagonal, mitochondrial Φ_{ST} values; above diagonal, microsatellite F_{ST} values. Bold values remain significant after controlling for false rejection rate using the method of Benjamini & Hochberg (1995)

Table 3 Pairwise population differentiation values between West African indigobird species

	<i>V.cam.-1</i>	<i>V.cam.-2</i>	<i>V. chal.</i>	<i>V. lar.</i>	<i>V. mar.</i>	<i>V. nig.</i>	<i>V. rar.</i>	<i>V. wil.</i>
<i>V. camerunensis-1</i>	—	-0.001	0.017**	-0.011	0.002	0.009	0.010*	0.008
<i>V. camerunensis-2</i>	0.050	—	0.027***	-0.014	0.019*	0.007	0.011*	0.008
<i>V. chalybeata</i>	0.449***	0.528***	—	0.017*	0.030**	0.021**	0.037***	0.039***
<i>V. larvaticola</i>	0.123**	0.309***	0.433***	—	0.004	0.001	0.004	0.003
<i>V. maryae</i>	0.019	0.186*	0.404***	0.010	—	0.007	0.009	0.023*
<i>V. nigeriae</i>	0.319***	0.490***	0.443***	0.197***	0.258***	—	0.017**	0.020**
<i>V. raricola</i>	0.193**	0.404***	0.512***	0.047*	0.091*	0.174***	—	0.029***
<i>V. wilsoni</i>	0.096*	0.272***	0.365***	-0.005	-0.013	0.178***	0.036*	—

Below diagonal, mitochondrial Φ_{ST} values; above diagonal, microsatellite F_{ST} values. Bold values remain significant after controlling for false rejection rate using the method of Benjamini & Hochberg (1995). Adjusted significance levels of 0.001, 0.01 and 0.05 are indicated as ***, ** and *, respectively. *Vidua camerunensis-1* and *Vidua camerunensis-2* refer to song mimics of *Lagonosticta rubricata* and *Lagonosticta rara*, respectively.

differentiation among indigobird species within each region were similar to those reported by Sorenson *et al.* (2003). Both mitochondrial and nuclear data indicate greater genetic diversity and stronger genetic structure among species in West Africa than in southern Africa. Interspecific mitochondrial differentiation was highly significant (West Africa: $\Phi_{ST} = 0.310$, $P < 0.0001$; southern Africa: $\Phi_{ST} = 0.053$, $P < 0.0001$) and of similar magnitude to that found previously, whereas microsatellite data yielded slightly lower estimates of differentiation than those previously reported (West Africa: $R_{ST} = 0.025$, $P < 0.0001$, $F_{ST} = 0.014$, $P < 0.0001$; southern Africa: $R_{ST} = 0.010$, $P < 0.01$, $F_{ST} = 0.003$, $P = 0.052$). For comparison, microsatellite R_{ST} values reported by Sorenson *et al.* (2003) were, West Africa, $R_{ST} = 0.034$, $P = 0.0015$; southern Africa, $R_{ST} = 0.027$, $P < 0.0001$.

Pairwise estimates of differentiation among indigobird species range from essentially zero in some comparisons to limited but statistically significant differentiation among many species pairs to relatively strong differentiation, particularly for mtDNA (Table 2, Table 3). Both mitochondrial and microsatellite data suggest that *V. chalybeata* is most strongly differentiated from the other species in both West Africa and southern Africa, but results from the two sources of data are not always congruent, raising issues of stochasticity in the lineage sorting process and the comparability of differentiation statistics designed for different kinds of genetic data (see Material and methods).

A model-based clustering method without a priori assignment of individuals to species (STRUCTURE version 2.1, Pritchard *et al.* 2000) yielded little resolution and nearly identical allele frequency estimates for many populations, reflecting minimal genetic differentiation among species, and perhaps also our relatively small sample sizes for some species.

Interspecific and intraspecific genetic structure among local populations

To rule out the possibility that significant interspecific differentiation is an artefact of geographical structure combined with sampling different species in different locations, we assessed differentiation of sympatric heterospecific populations at six locations where more than one species was sampled. Mitochondrial differentiation among sympatric populations of different species was significant at all but one location (Merensky), and nuclear microsatellites indicated significant differentiation in all but two locations (Merensky and Garoua). Differentiation in pairwise comparisons of sympatric populations ranged from zero to highly significant in both marker systems (Fig. 1).

In contrast we found limited evidence of significant genetic differentiation among subpopulations of the same species, suggesting genetic continuity of indigobird species within each region. No significant intraspecific

differentiation was detected among populations of *Vidua nigeriae* in West Africa or among populations of *Vidua chalybeata* and *Vidua purpurascens*, respectively, in southern Africa (Fig. 1). Mitochondrial differentiation as measured by Φ_{ST} was relatively high and statistically significant for two western *V. chalybeata* populations but was nonetheless smaller than the level of differentiation between *V. chalybeata* and other species. This result is the consequence of a divergent clade of haplotypes found in all *V. chalybeata* from Garoua, Cameroon, but in only half of *V. chalybeata* individuals from Nigeria. Intraspecific genetic continuity was not supported for two *Vidua codringtoni* populations, which differ from each other in both allele and haplotype frequencies (Fig. 1b). This result, however, reflects the distinctiveness of the *V. codringtoni* population from Chipinge rather than similarity to sympatric populations of different species. Six of nine *V. codringtoni* from Chipinge shared a unique mtDNA haplotype that was also detected in two *V. codringtoni* from Malawi, but not in birds of other species. The Malawi *V. codringtoni* were not included in population-level analyses because only seven individuals were available, but the Chipinge population may be more similar to conspecifics from Malawi than to the population from nearby Mutare, which was used in the analysis for Fig 1.

The previous analysis included only species represented by two or more populations within each region. The phenogram in Fig. 2 summarizes the level of genetic similarity among all populations sampled in this study based on a composite measure of allele and haplotype sharing. Conspecific populations of West African *V. nigeriae* and *V. chalybeata*, respectively, cluster together to the exclusion of heterospecific populations. The southern populations of *V. chalybeata* also cluster together, whereas *V. purpurascens* from different locations cluster with populations of other species. As noted previously, however, genetic differentiation among *V. purpurascens* populations was not statistically significant. Both *V. codringtoni* populations are relatively divergent from other southern African indigobirds.

Partitioning genetic variance between geographical and species factors

As a more inclusive test of genetic continuity, we examined whether species membership or geographical location was the stronger determinant of genetic similarity within each region by comparing two different hierarchical AMOVAs: populations nested within species and populations nested within localities. When using Φ_{ST} values for the West African data, the largest proportion of variance was always allocated to the lowest nesting level, because the frequency of a highly divergent mtDNA clade found primarily in *V. chalybeata* varies both geographically and between species, thus rendering the comparison between the alternative

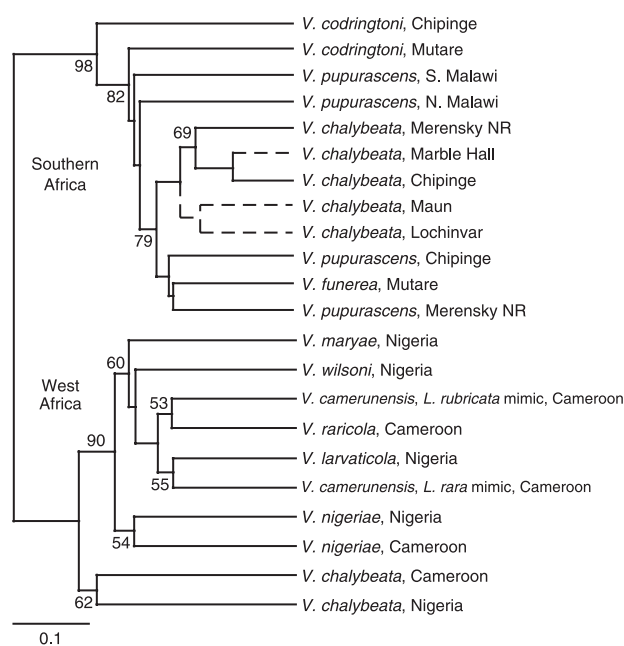


Fig. 2 Phenogram summarizing population similarity based on a composite measure of microsatellite and mtDNA differentiation. Populations on dashed branches were typed for mitochondrial haplotypes only. Branch support greater than 50% (based on 1000 bootstrap replicates) is shown.

nesting regimes uninformative. We therefore used F_{ST} for both mitochondrial and microsatellite data in this analysis.

In both regions, > 90% of the mitochondrial variation was attributable to variation within individual populations (Table 4). When populations were grouped by species, variation among groups (species) was greater than variation among populations within species. In contrast, when populations were grouped according to location, all of the variation resulting from substructure among populations was allocated to differences within locations. Thus, populations of the same species from different localities are more similar in their mtDNA than to sympatric populations of other species.

Less than 2% of the total variation in microsatellites was attributable to population structure. Most of this variation was allocated to the lowest nesting level (i.e. within groups) regardless of whether groups were defined by species or by locality. This result, however, is largely the result of the divergence of single populations (Garoua *V. chalybeata* in West Africa and Chipinge *V. codringtoni* in southern Africa). When Chipinge *V. codringtoni* were excluded from the analysis of southern populations, species groups were more homogeneous than locality groups, and among-group variation was higher between species than between localities, consistent with genetic continuity of species across localities. Excluding Garoua *V. chalybeata* leaves only one conspecific

Table 4 Hierarchical analysis of molecular variance between species and between localities

	West Africa statistical model		Southern Africa statistical model	
	Populations within species	Populations within localities	Populations within species	Populations within localities
Mitochondrial haplotype variation				
Among groups	$F_{CT} = 0.0798^*$	$F_{CT} = -0.0057$	$F_{CT} = 0.0252^*$	$F_{CT} = -0.0139$
Among populations within groups	$F_{SC} = 0.0137^{**}$	$F_{SC} = 0.0945^{***}$	$F_{SC} = 0.0191^{**}$	$F_{SC} = 0.0497^{**}$
Proportion of total variation explained by model	9.24%	8.93%	4.38%	3.65%
Nuclear microsatellite variation				
Among groups	$F_{CT} = -0.0014$	$F_{CT} = 0.0053$	$F_{CT} = -0.0006$ ($F_{CT} = 0.0025$)	$F_{CT} = -0.0083$ ($F_{CT} = -0.0061$)
Among populations within groups	$F_{SC} = 0.0191$	$F_{SC} = 0.0142^{***}$	$F_{SC} = 0.0095^*$ ($F_{SC} = 0.0018$)	$F_{SC} = 0.0159^{**}$ ($F_{SC} = 0.0089^*$)
Proportion of variation explained by model	1.77%	1.94%	0.90% (0.43%)	0.78% (0.29%)

The partitioning of mitochondrial and nuclear variance into within- and between-group components is compared between two nesting regimes, populations grouped by species and populations grouped by location. Results of analyses excluding the *Vidua codringtoni* population from Chipinge are shown in parentheses. Significance levels of 0.001, 0.01 and 0.05 are indicated as ***, ** and *, respectively.

Partial correlation	West Africa		Southern Africa	
	mtDNA	microsatellites	mtDNA	microsatellites
Genetic – geo. spec	$r = 0.057$ $P = 0.0079$	$r = -0.004$ $P = 0.326$	$r = 0.054$ $P = 0.078$	$r = -0.008$ $P = 0.221$
Genetic – spec. geo	$r = 0.132$ $P < 0.0001$	$r = -0.047$ $P < 0.0001$	$r = -0.024$ $P = 0.162$	$r = -0.026$ $P = 0.024$

Table 5 Partial correlations of genetic distance (mtDNA) or similarity (microsatellite loci) with geographical distance or species association, while holding the alternative variable constant

The partial correlation between genetic and geographical distance was calculated with species membership held constant (Genetic-geo.spec) whereas the correlation between genetic distance and species membership held geographical distance constant (Genetic-spec.geo). Note that significant correlation coefficients for microsatellite data are negative because a genetic similarity matrix (microsatellite relatedness values) was tested against a distance matrix (species or geographical distance). *P* values based on 10 000 randomizations.

population pair among West African samples and prevents a meaningful analysis.

A second approach to assessing the relative effects of geography and species membership on the distribution of genetic polymorphism was based on individual genotypes. This approach allowed us to include all of the individual birds we sampled ('species samples' in Table 1), whereas the previous analyses were limited to subpopulations with sufficient sample sizes. Partial correlation coefficients between pairwise genetic distances (mitochondrial sequences) or relatedness (microsatellite data) and geographical distance were compared to partial correlation coefficients between genetic matrices and species membership (Table 5). The possibly interacting effects of species membership and spatial distribution on the genetic composition of individuals were accounted for by keeping each of these variables

constant in the respective partial Mantel tests. In West Africa, higher correlation coefficients between genetic and species matrices than between genetic and geographical matrices indicate that genetic relatedness is more strongly determined by species membership than by geographical location. Note that significant correlation coefficients for microsatellite data are negative because a genetic similarity matrix (microsatellite relatedness values) was tested against distance matrices (species and geographical distance). A low but significant correlation of mtDNA genetic distances with geographical distances suggests a role for geographical distance in shaping intraspecific genetic structure, but this effect was weaker than the correlation between species membership and genetic distance. Relatedness based on microsatellite data was significantly correlated only to species membership. In southern Africa,

the correlation between microsatellite relatedness values and species membership was statistically significant. In contrast, mtDNA genetic distances for southern Africa showed a stronger correlation with geographical distance than with species membership, but neither correlation was significant (Table 5).

Discussion

Interspecific differentiation and intraspecific genetic continuity

Consistent morphological differences between indigobirds that mimic the songs of different hosts, field observations of assortative mating, and nestling mimicry of host-specific mouth markings all support the recognition of distinct indigobird species. Likewise, compelling experimental evidence for the importance of host imprinting in indigobird social behaviour (Payne *et al.* 1998, 2000) supports a model of rapid speciation following the colonization of new hosts and in turn provides a plausible explanation (i.e. retained ancestral polymorphism) for the limited differentiation of indigobird species in neutral genetic markers (Sorenson *et al.* 2003). The same mechanisms, however (host imprinting and imperfect host fidelity in female egg laying, a necessary condition for speciation in this system), may also lead to hybridization, suggesting an alternative hypothesis for the genetic similarity of indigobirds. Are indigobird species in the process of diverging following a recent radiation or does ongoing hybridization maintain these species in a perpetual state of incomplete genetic differentiation? If individual host shifts leading to hybridization and introgression are infrequent, we should find greater genetic differentiation between sympatric populations of different indigobird species than between allopatric populations of the same species. Our results were generally consistent with this prediction.

In species level analyses, we found significant genetic differentiation between most pairs of indigobird species in both nuclear and mitochondrial markers. In analyses restricted to sympatric populations, significant mitochondrial and nuclear differentiation was detected in more than half of pairwise comparisons (Fig. 1), suggesting that species-level differentiation is not simply an artefact of geographical differentiation combined with sampling different species in different locations. Similarly, average relatedness and haplotype sharing were always higher among the individuals of a given population than between individuals of sympatric heterospecific populations.

More inclusive analyses based on local populations (Table 4) or individual birds (Table 5) also indicate that allopatric populations of the same species are more similar to each other than to sympatric populations of different species. Although genetic differentiation among indigo-

bird species is incomplete in mtDNA and often minimal in nuclear microsatellites, and although many of the comparisons we made were not statistically significant, the general pattern that emerges from our results is one of genetic continuity of the different species within each region. More extensive sampling of indigobirds both in number of localities and individuals might allow stronger conclusions, but the available data suggest that each indigobird species represents a reproductive entity joined by a common origin and/or intraspecific gene flow and that the rate of introgression between sympatric populations of different species is not sufficient to prevent significant genetic differentiation.

An exception to the general pattern of intraspecific genetic continuity was the population of *Vidua codringtoni* from Chipinge, which was significantly differentiated from a conspecific population in Mutare. This population, however, showed no similarity to sympatric populations of *Vidua chalybeata* and *Vidua purpurascens* and therefore does not support the alternative hypothesis of frequent hybridization and introgression between sympatric populations. A relatively low level of genetic diversity suggests that a bottleneck or founding event may be responsible for the distinctiveness of the Chipinge population, which also differs in adult male plumage colour from other *V. codringtoni* populations (Payne 1973).

Host colonization hypotheses

In contrast to a classic vicariant model of speciation, the origin of new indigobird species depends on a behavioural phenomenon that may be repeated in space and time. Shared genetic polymorphisms among species imply that multiple females contributed to the origin of each indigobird species by laying eggs in the nests of a novel host. Genetic differentiation among indigobird species and populations should therefore depend not only on time since common ancestry but also on the timing and spatial distribution of host colonization events contributing to the establishment of each new species. An intriguing possibility is that indigobird populations that became associated with a new host at different times and locations might later merge as a result of their shared songs. Even in this case, however, the genetic adaptation to a particular host species in terms of mouth mimicry need not evolve independently after each host shift. A low level of gene flow from populations with appropriate mouth markings could establish the allelic variants necessary for the evolution of mimetic markings in each population (Slatkin 1996). Sexual selection might also facilitate the homogenization of adult morphology across 'conspecific' populations with independent origins. Multiple independent shifts to the same host species could be viewed as parallel speciation (Schluter & Nagel 1995; Rundle *et al.* 2000; Turner 2002), in the sense that the

isolating mechanism – songs acquired through imprinting on the new host – develops more than once.

While gene flow between populations imprinted on the same host could eventually produce intraspecific genetic continuity and obscure a history of multiple colonization, clear examples of independent origins of ‘conspecific’ populations were generally lacking in our within-region analyses. Only the *V. codringtoni* population at Chipinge was clearly distinct from conspecific populations elsewhere in southern Africa. Perhaps a more likely model is that each new indigobird species experiences a gradual accrual of genetic lineages as females at different times and places occasionally lay in nests of a novel host species, a process that grades into ongoing hybridization between established species as time goes on.

Differentiation estimates among indigobird populations covered a wide range of values, with overlapping estimates for inter- and intraspecific comparisons (Fig. 1). This variation likely reflects stochasticity in the lineage sorting process (e.g. Rosenberg & Nordborg 2002), differences in the age and demographic histories of populations, and perhaps asymmetric gene flow from more numerous to less numerous species. In addition, host shifts may be more successful and introgression more likely between some species than others, as a result of differences in the abundance of syntopic alternative hosts or variable discrimination by different host species against nonmimicking parasites (e.g. Payne & Payne 2002; Payne *et al.* 2002).

The mitochondrial data, which directly reflect the history of female lineages, are more easily interpreted with respect to patterns of host colonization. The mitochondrial data suggest examples of both a single restricted origin and multiple colonization on broader spatial scales. Almost all *V. nigeriae* individuals shared a single common mtDNA haplotype or one of several rare haplotypes just one base divergent from the common one, suggesting that a single colonization event led to the origin of this species. Interestingly, *V. nigeriae* parasitizes quailfinch, *Ortygospiza atricollis*, a distant relative to most other indigobird hosts that may be more difficult to successfully colonize. Two individual *V. nigeriae*, however, had divergent haplotypes characteristic of *V. chalybeata*, indicating subsequent shifts by female *V. chalybeata* to the host of *V. nigeriae*. These events must have occurred at least several generations in the past, as the bearers of these haplotypes were morphologically *V. nigeriae*. In contrast, *V. larvaticola* haplotypes are scattered across the mitochondrial network, suggesting that multiple host shifts at various points in space and time led to the observed mtDNA diversity in this species. Mitochondrial polyphyly of indigobird species must reflect not only their recent origin and incomplete lineage sorting, but also independent shifts of female lineages between hosts.

In contrast to the general pattern of within-species genetic continuity within each region, genetic data suggest

a lack of gene flow between regions (Sorenson *et al.* 2003). The most broadly distributed indigobird, *V. chalybeata*, has a continuous distribution from Senegal across to Ethiopia and down to southern Africa. Because western and southern *V. chalybeata* are genetically most similar to other species in their respective regions, and exhibit geographical variation in adult plumage and bill colour, they likely represent independent colonization and adaptation to the same host in different regions. *V. chalybeata* is nonetheless treated as a biological species (e.g. Payne 2004) because western and southern populations apparently interbreed in East Africa. Analysis of *V. chalybeata* population samples from East Africa is needed to test whether western and southern populations are disjunct or continuous. Two isolated samples from Ethiopia and Kenya clustered with West African and southern African indigobirds, respectively.

The African firefinch, *L. rubricata*, is parasitized by *V. funerea* in southern Africa and *V. camerunensis* in West Africa, providing another example of independent colonization of the same host in different regions. In this case, however, the parasitic birds are recognized as two different species because they have disjunct distributions and are genetically and morphologically distinct.

Hybridization between indigobird species

Indigobird social behaviour suggests two possible pathways for hybridization and introgression, either through the choice of mates or through the choice of host nests. While egg-laying mistakes (i.e. parasitism of species other than the usual host) are necessary for the colonization of new hosts and indigobird speciation, the same mechanism can lead to hybridization (see Payne & Sorenson 2004). Egg-laying mistakes should result in an equal number of male and female offspring imprinted on the alternate host and should not introduce a sex bias in genetic introgression. The alternative route to hybridization, mating between males and females of different song types combined with host fidelity in egg laying, should lead to subsequent backcrosses with the maternal species, as dictated by host imprinting, and would be tantamount to male-biased gene flow.

Asymmetric and symmetric gene flow leave different signatures in the nuclear and mitochondrial genome. In the present study, information from nuclear microsatellite and mitochondrial sequence data was largely congruent, but mitochondrial estimates of differentiation were considerably higher than corresponding nuclear values (although to a greater or lesser degree depending on the differentiation estimators – F_{ST} , Φ_{ST} or R_{ST} – applied to the two marker systems). Microsatellite differentiation, however, should develop more slowly following speciation even in completely isolated populations as a result of the homoplasy resulting from constraints on allele sizes (Nauta & Weissing

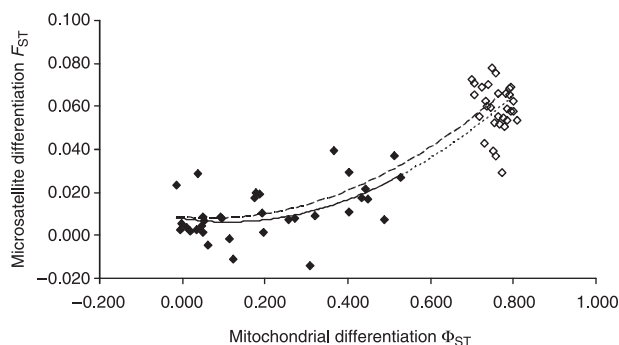


Fig. 3 Scatterplot of mitochondrial Φ_{ST} (x -axis) vs. microsatellite F_{ST} values (y -axis). Filled diamonds represent differentiation estimates between species within West Africa and within southern Africa, open diamonds represent differentiation estimates between West African and southern African species. The solid curve depicts the polynomial trendline for within-region data points, the dotted curve is the extension of the within-region polynomial according to its equation ($y = 0.1242x^2 - 0.0277x + 0.0078$) and the dashed curve represents the polynomial fitted to both within-region and between-regions data points ($y = 0.1166x^2 - 0.0156x + 0.0067$).

1996), as well as the fourfold larger effective population size as compared to mitochondrial genomes. In addition, symmetric introgressive hybridization introduces, on average, four nuclear alleles per mitochondrial haplotype. Only after these factors have been taken into account need one invoke male-biased gene flow to explain low microsatellite differentiation contrasting with higher mitochondrial estimates. In the absence of male-biased gene flow, nuclear markers should eventually reflect genetic differentiation, even if the initial phases are evident only in the divergence of mitochondrial haplotype frequencies. To separate marker-dependent lineage sorting issues from the effects of gene flow, we compared nuclear and mitochondrial differentiation estimates between potentially hybridizing species (i.e. between species within each major region), and in the absence of gene flow (i.e. between species in West Africa vs. southern Africa). If male-biased gene flow was responsible for the lower level of microsatellite differentiation among sympatric species, the discrepancy between mitochondrial and microsatellite differentiation estimates should be reduced in the absence of gene flow.

Figure 3 plots microsatellite differentiation values (F_{ST}) between indigobird species within and between each major region against the corresponding mitochondrial Φ_{ST} estimates. A polynomial curve fitted to the within-region data has a positive slope for mitochondrial Φ_{ST} values > 0.112 (Pearson $r = 0.49$; note that the correlation coefficient may be inflated by nonindependence of multiple comparisons). The extension of the polynomial curve derived from the species comparisons within each region (i.e. with potential gene flow) closely predicts the between-region differentiation estimates (i.e. differentiation in the absence of gene

flow). Similar trajectories of differentiation within and between regions suggest that the forces at work are similar in both situations. Patterns of mitochondrial and microsatellite differentiation between sympatric species therefore need not be attributed to male-biased gene flow, but may be solely the result of marker specific effects on lineage sorting.

The aim of this study was to distinguish between ongoing gene flow and ancestral polymorphism as the underlying cause of genetic similarity among indigobird species. This endeavor was complicated by mostly low and somewhat variable differentiation estimates among populations, leaving little room for pronounced quantitative differences between classes of pairwise comparisons. Nonetheless, our data provide some important qualitative insights into indigobird population dynamics and speciation. While the process of lineage sorting in reproductively isolated populations is stochastic, the genetic homogenization of panmictic populations is not; the detection of significant differentiation between sympatric heterospecific populations is therefore evidence of reproductive isolation between birds imprinted on different host species. Occasional shifts to established hosts and, consequently, ongoing hybridization likely occurs, but not so frequently as to prevent the species from evolving as distinct genetic units. The behavioural and genetic data collected on indigobirds in the past few decades perhaps represent a snapshot of the early stages of divergence between incipient species, which, over time, may develop into a much clearer picture of speciation through shifts in host association.

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- Kristina Sefc is interested in the imprint of evolutionary processes on patterns of genetic diversity in populations and is currently involved in similar research on African cichlids. Robert Payne is interested in avian behavior and systematics and has worked on indigobirds as well as other brood parasitic birds since the 1960's. His research on indigobirds in the field and more recent experiments in captivity provide the behavioral and ecological framework for understanding the results presented here. Michael Sorenson has long-standing interests in the evolutionary ecology of avian brood parasitism and in recent years has focused on the application of molecular methods to research in this area.
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