

**GENETIC AND MORPHOLOGICAL EVIDENCE FOR TWO SPECIES
IN THE UZUNGWA FOREST PARTRIDGE
*XENOPERDIX UZUNGWENSIS***

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ABSTRACT

The Udzungwa forest partridge, *Xenoperdix udzungwensis* is known from only three forests within the Udzungwa and Rubeho Highlands of the Eastern Arc Mountains. Given the phenotypic differences between the Udzungwa (*X. u. udzungwensis*) and Rubeho Highland (*X. u. obscurata*) populations, it seems unlikely that there remains recurrent gene flow between these two populations. We used a combination of mitochondrial (1041 bp of NADH Dehydrogenase subunit 2) and nuclear DNA (569 bp of Fibrinogen intron 5 and 387 bp of Glyceraldehyde-3-phosphate Dehydrogenase intron 11) markers to investigate the degree to which these two taxa are separated. In mtDNA, 0.5% sequence divergence, with five-fixed mutational differences (two amino acid changes) was recovered between *X. u. udzungwensis* and *X. u. obscurata*. One fixed difference was found for Fib5 and none for Gadph11. Coalescent models suggest that no gene flow is taking place between the Udzungwa and Rubeho Highlands and that divergence between the two taxa took place about 200 000 years before present. The presence of fixed mutational differences in mtDNA and in one of the two nDNA markers analysed, the lack of gene flow, and diagnosable morphological differences (including potential display signals) between *X. u. udzungwensis* and *X. u. obscurata*, suggest that *X. u. obscurata* be accorded full species status, *Xenoperdix obscurata*, for which we put forth the common name 'Rubeho forest partridge'.

INTRODUCTION

The Udzungwa forest partridge, *Xenoperdix udzungwensis* Dinesen, Lehmberg, Svendsen, Hansen & Fjeldså, 1994, is one of Africa's rarest birds, considered threatened ("Vulnerable", see BirdLife International, 2000) because of its very small range. The species is known from only three forests within the Udzungwa and Rubeho Highlands of the Eastern

Arc Mountains in Tanzania (figure 1). Whereas population numbers may be stable as these forest patches are relatively pristine, any indication of decline should result in uplisting to "Endangered" species status (BirdLife International, 2000).

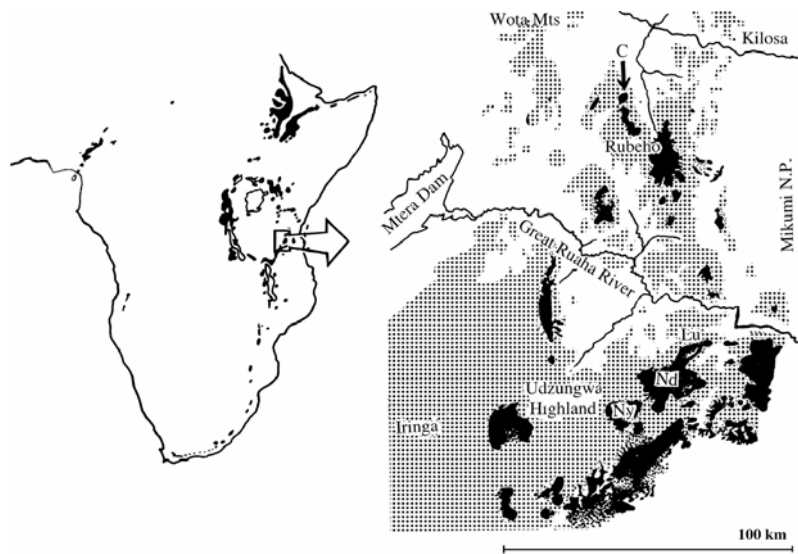


Figure 1. Distribution and collecting sites of *Xenoperdix u. udzungwensis* and *X. u. obscurata* populations sequenced in this study; black areas are remaining evergreen forest tracts. C = Chugu Hill in the Mafwemiro Forest, Lu = Luhombero Forest, Nd = Ndundulu Mts, Ny = Nyumbanitu Mts.

This species has drawn special attention because it is a relict form, whose discovery in 1991 (Dinesen *et al.*, 1994) has been characterised as one of the major surprises for the ornithological world in recent years (McGowan, 1994). Its nearest relatives are primitive forest partridges (*Arborophila*, *Rollulus* and others) of the Oriental Region (Dinesen *et al.*, 1994; Crowe *et al.*, in press). Thus, *Xenoperdix* likely diverged from its nearest living relatives in the early Miocene when the Tethys Sea closed and there was a brief opportunity for oriental forest birds to colonise Africa. Consequently, the survival of small relict populations in the Eastern Arc Mountains is remarkable. This, together with the concentration of other relict taxa in the same mountains (Burgess *et al.*, 1998; Burgess *et al.*, in press), lends support to studies that suggest that as a consequence of the highly predictable ecological conditions that these mountains experience from nearly continuous orographic rain, exceptionally low extinction rates occur (Fjeldså & Lovett, 1997; Jetz *et al.*, 2004).

Within the Udzungwa Highlands, *Xenoperdix* is known only from elevations above 1300 m in the Nyumbanitu (7°44'–50'S, 36°19'–26'E; 55 km²) and Ndundulu/ Luhombero forests (7°36'–49'S, 36°25'–42'E; 240 km²) (figure 1; Dinesen *et al.*, 1994; Butynski & Ehardt, 2003), with an estimated population of 3700 individuals (Dinesen *et al.*, 2001). Surprisingly, a small population described as a subspecies, *X. udzungwensis obscurata* Fjeldså & Kuire 2003, was recently found in the northern part (Chugu Hill) of the Mafwemiro (Mafwomero) Forest (6°49'S, 36°35'E), 100 km farther north in the Rubeho Highlands (Fjeldså & Kiure, 2003). This population most likely only numbers a few hundred individuals. In spite of extensive surveys of other forest tracts in the Udzungwa, Uvidunda, Rubeho and Wota

Highlands in central Tanzania (Fjeldså *et al.*, in press), no other populations have been found. The species may, therefore be relictual even within its present range (figure 1).

Given the phenotypic differences between the Udzungwa and Rubeho Highland populations of *Xenoperdix* (table 1; see also Fjeldså & Kuire, 2003), as well as the presence of relatively dry, low-lying, unsuitable habitat in the region between these montane highlands, it seems unlikely that there remains recurrent gene flow between these two populations. This suggests that these two populations should be managed as separate taxonomic entities. In this study, we include tissue samples from all seven specimens collected since the species was first discovered in 1991. We use a combination of mitochondrial and nuclear DNA markers to establish whether gene flow is taking place between these two isolated populations, estimate genetic diversity, and evaluate any signal in the data for recent demographic change (population expansion versus decline). Finally, we comment on the taxonomic status of *X. udzungwensis obscurata* restricted to the Rubeho Highlands.

Table 1. Description of morphological differences between Xenoperdix u. udzungwensis and X. u. obscurata. Key plumage differences are depicted in Fjeldså and Kuire (2003). Sexes are similar based on specimens examined: X. u. udzungwensis, 2 adult females, 1 adult male and 1 alcohol preserved specimen which was not sexed. X. u. obscurata: 3 specimens not sexed, 1 is almost certainly a female based on the presence of a vascularised brood-patch.

Character	<i>X. u. udzungwensis</i>	<i>X. u. obscurata</i>
Wing-length	137.5–149.0 mm	130.8–137.0 mm
Maximum tail-length	68.0–73.0 mm	57.5–61.8 mm
Face	Mostly orange-brown	Orange-brown with dense dusky speckles
Neck	Necklace of mostly white feathers with variable black spots	No necklace, but rather an arc of black spots overlaid on the olive-grey boundary between throat and breast
Under-tail coverts	Ochraceous	Only a faint trace of ochre towards feather tips
Secondaries	Prominent barring	Less distinct barring
Wing-coverts	Black and ochraceous barring	Distinctive grey to whitish distal margins giving a scaly effect
Retrices	broad 15–20 mm	Narrow, 11–14 mm, and the bars of the central feathers less distinctly black

METHODS

Sequence data were obtained from all seven specimens of *Xenoperdix* collected since the species was discovered in 1991 (table 2). This consisted of four *X. u. udzungwensis* individuals sampled from two different localities within the Udzungwa Highlands and three individuals of *X. u. obscurata* sampled from Mafwemiro Forest at Chugu Hill in the Rubeho Highlands. The hill partridge *Arborophila javanica* Gmelin 1789 was used as an outgroup. The vouchers, including the type specimens of both forms are deposited at the Zoological Museum of the University of Copenhagen, Denmark, except for one specimen, which is in the Institute of Zoology and Marine Biology, University of Dar es Salaam, Tanzania.

Table 2. List of all specimens collected since the discovery of *Xenoperdix* in 1991, as well as the outgroup taxon *Arborophila javanica*. Genbank numbers are listed for the three DNA regions sequenced in this study.

Taxon	Voucher	Locality	NADH2	Gadph11	Fib5
<i>X. u. udzungwensis</i>	ZMUC114233	Ndundulu Mts	DQ093797	DQ093805	DQ093813
	ZMUC114249	Ndundulu Mts	DQ093798	DQ093806	DQ093814
	ZMUC114257	Nyumbanitu Mts	DQ093799	DQ093807	DQ093815
	ZMUC115139	Nyumbanitu Mts	DQ093800	DQ093808	DQ093816
<i>X. u. obscurata</i>	ZMUC129213	Mafwemiro Forest at Chugu Hill	DQ093801	DQ093809	DQ093817
	ZMUC129233	Mafwemiro Forest at Chugu Hill	DQ093802	DQ093810	DQ093818
	ZMUC129234	Mafwemiro Forest at Chugu Hill	DQ093803	DQ093811	DQ093819
<i>Arborophila javanica</i>	ZMUC_JBK01-290103	Captive	DQ093804	DQ093812	DQ093820

Laboratory procedures

DNA was extracted from frozen tissues using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota) following the manufacturer's animal tissue protocols, but with an overnight proteinase K digestion at 55°C. The mitochondrial NADH Dehydrogenase subunit 2 gene was amplified using primers L5216 and H6313 (Sorenson *et al.*, 1999) under standard PCR conditions. Two nuclear DNA introns, Beta-Fibrinogen 5 (primers: Fib 5: 5'-CGCCATACAGAGTATACTGTGACAT-3' and Fib6: 5'-GCCATCCTGGCGATTCTGAA-3') and Glyceraldehyde-3-phosphate Dehydrogenase intron 11 (primers: GapdL890 and GapdH950; Friesen *et al.*, 1997) were amplified using annealing temperatures of 54°C and 64°C, respectively. PCR products were electrophoresed on 1.5% low-melting point agarose gels (FMC Bioproducts) stained with ethidium bromide and visualized under UV light. Amplicons of the appropriate length were cut out of the gel and purified using GELase™ (Epicentre Technologies, Madison, Wisconsin). The purified product was cycle-sequenced using Big Dye terminator chemistry (Applied Biosystems, Inc [ABI]). Cycle-sequencing reactions were precipitated with 3 M ammonium acetate, rinsed in ethanol, dried and re-suspended in formamide-EDTA solution and run on an ABI 3100 automated DNA sequencer. All sequences were checked using the programme Sequencher 3.0 (Gene Codes Corp.) and NADH2 aligned to the chicken *Gallus gallus* Linnaeus 1758 mtDNA sequence (Desjardins & Morais, 1990) to test for the presence of any insertions, deletions or stop codons. All sequences have been submitted to Genbank (table 2).

Coalescent-based estimates of gene flow and divergence times

The program MDIV (Nielson & Wakeley, 2001; Nielson, 2002) was used to simultaneously estimate a variety of parameters between the Udzungwa and Rubeho Highland populations of *Xenoperdix*: theta ($\theta = 2N_{ef}\mu$), migration (gene flow) rate ($M = 2N_{ef}m$), time of population divergence ($T = t/N_{ef}$), and time to most recent common ancestor ($TMRC A = t\mu$), where N_{ef} is the female-effective population size, t is the generation time, and μ is the per locus mutation rate. Six different pairwise simulations were conducted: one run each of 10 million, 25 million, and 50 million generations with priors set as $T_{max} = 15$ and $M_{max} = 10$, or $T_{max} = 50$ and $M_{max} = 10$. Multiple runs with increasing sampling of the posterior distribution were conducted to determine if convergence in the mode of the posterior distribution was being reached. A finite-sites (HKY) model was used in all analyses. Values for theta, M and T were plotted, and the mode of the posterior distribution was accepted as the best estimate. Where possible, 95% credibility intervals also were estimated for each parameter. Standardized estimates for T and $TMRC A$ are provided in which each estimate is multiplied by theta to account for different effective population sizes and allow for direct comparison among estimates. These values were converted into years before present using the widely assumed mutation rate of 2% per million years for mtDNA (see García-Moreno, 2004), or 1.915×10^{-5} substitutions per site per year, and an estimated generation time of 1 year as occurs for most galliformes (McGowan, 1994).

RESULTS

Sequence characteristics and divergence

In total 1041 bp of mtDNA (NADH2) and 956 bp of nDNA (569 bp Fib5 and 387 bp Gadph11) were amplified from all seven individuals. Fib5 was particularly difficult to sequence in both directions due to a homo-polymerous region of 22 adenines in the middle of

the intron. Five fixed nucleotide differences, two of which resulted in amino acid changes were documented in NADH2 between *X. u. udzungwensis* and *X. u. obscurata* (table 3). One fixed difference was recovered in Fib5 and none for Gadph11 (table 3). These correspond to a sequence divergence of 0.5% between *X. u. udzungwensis* and *X. u. obscurata* for NADH2, and 0.2% for Fib5. Sequence divergence between *Xenoperdix* and *Arborophila* was much larger: 20% for NADH2, 9–10% for Fib5, and 11% for Gadph11. This was also accompanied by a number of indels between *Xenoperdix* and *Arborophila* in the nDNA markers: five indels (1–8 bp in length) for Fib5, and 10 indels (1–18 bp) for Gadph11, respectively.

Table 3. Sequence characteristics, highlighting the genetic differences between *X. u. udzungwensis* and *X. u. obscurata*.

	Position	Mutation	Characteristics	Codon position	Amino acid change
NADH 2 (1041 bp)	120	T/C	Fixed	3 rd	Ile to Ile
	144	T/C	Fixed	3 rd	His to His
	464	T/C	Fixed	2 nd	Ile to Thr
	821	A/G	Fixed	2 nd	Glu to Gly
	843	C/T	Fixed	3 rd	Ile to Ile
Fib5 (569 bp)	236	A/T	Fixed	N/A	N/A
Gadph11 (387 bp)	55	C or T	Heterozygote	N/A	N/A

Almost no genetic diversity was detected within either of the populations of *Xenoperdix*. For both populations, only one haplotype for NADH2 and Fib5 was recovered (although differing by five and one fixed substitution, respectively). The only detectable within population diversity was a heterozygote at position 55 in the Gadph11 intron, and even this was found in only one of the seven individuals sampled.

Coalescent-based estimates of gene flow and divergence times

All coalescent models suggested that there is no current gene flow between populations in the Udzungwa and Rubeho Highlands (table 4, figure 2). Estimates of gene versus population divergence were more difficult to determine as the posterior distribution of T (population divergence) even after 50 million generations did not converge (table 4, figure 2). Depending on the prior set for T_{max} , gene divergence, or time to most common recent ancestor (TMRCA) of the haplotypes recovered was estimated to have occurred between 181 000 and 196 000 years before present.

DISCUSSION

The location of known *Xenoperdix* populations in mountains flanking the Great Ruaha Basin to the south and north (figure 1) could be related to long-term climatic predictability. Throughout the Pleistocene, these highlands may have been under a constant influence of warm humid winds from the Indian Ocean (Fjeldså & Lovett, 1997; Burgess *et al.*, 1998) and conditions may be particularly favourable in humid highlands bordering towards the inter-montane basin between the two highlands, which acts as a local high pressure centre with warm air rising up the mountain slopes. Several narrowly endemic bird taxa

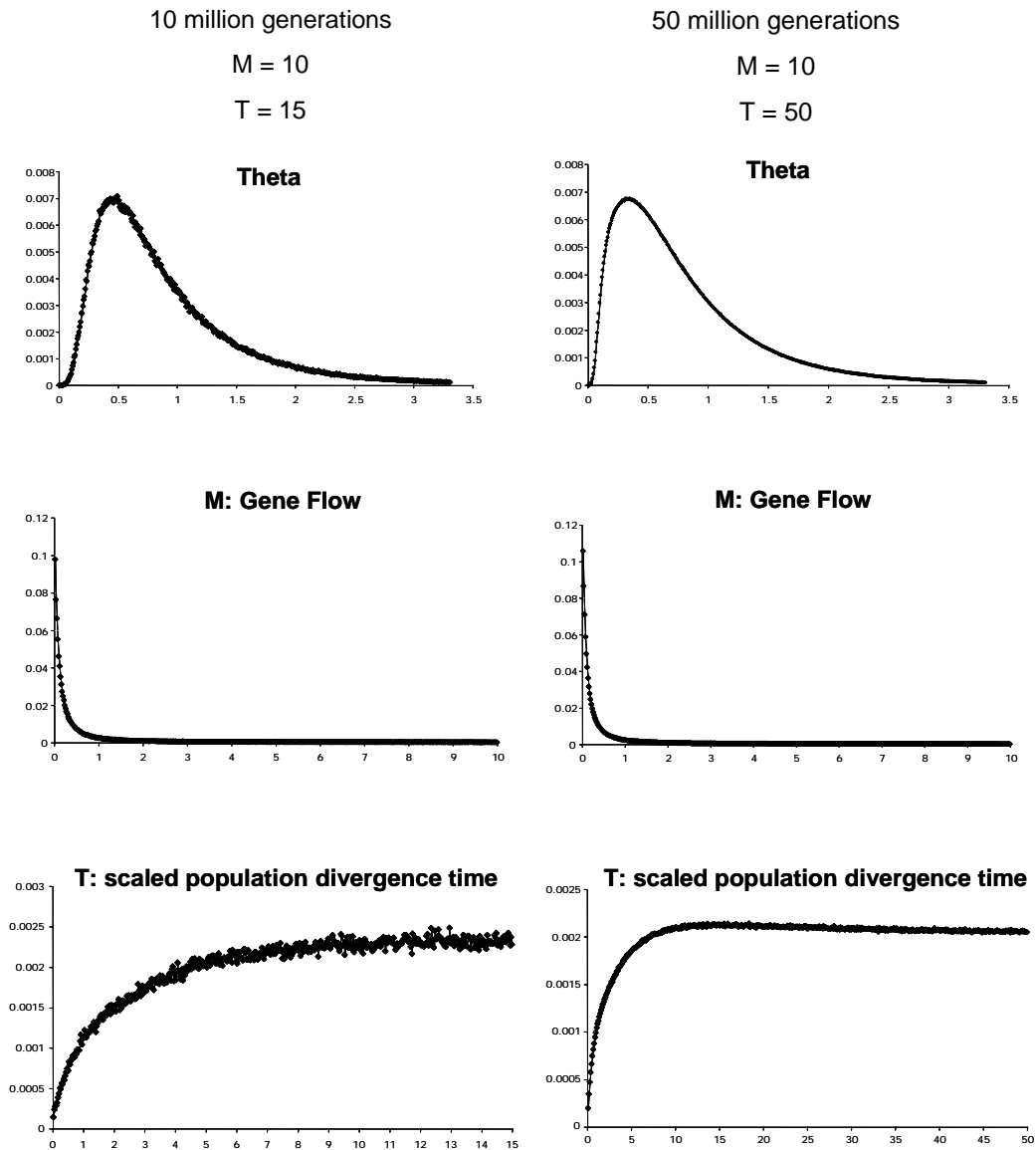


Figure 2. Pairwise estimates of theta, migration rates or gene flow ($M = N_{ef}m$) and time since population divergence (T) based on analysis of mtDNA sequence data using MDIV under two different models of T_{max} for 10, 25 and 50 million generation runs, respectively. All analyses supported a model of no gene flow between *Xenoperdix u. udzungwensis* and *X. u. obscurata*.

(*Xenoperdix*, *Bubo vosseleri* Reichenow 1908, *Batis* sp. nov. Fjelds , Bowie and Kuire unpublished, *Arcanator orostruthus* Vincent 1933, *Swynnertonia swynnertonii* Shelley 1906, *Sheppardia aurantiithorax* Beresford, Fjelds  & Kuire 2004, *S. lowei* Grant and Mackworth-Praed 1941, *Apalis chariessa* Reichenow 1879, *Scepomycter winifredae* Moreau 1938,

Nectarinia moreaui Sclater 1933, *N. rufipennis* Jensen 1983, *Ploceus nicolli* Sclater 1931, *Serinus whytii* Shelley 1897 and *S. melanochrous* Reichenow 1900) are found in this area, some of them restricted to one side of the Great Ruaha Basin, others inhabiting highlands on both sides (Bowie *et al.*, 2004; Fjeldså *et al.*, in press).

Table 4. Pairwise estimates of theta (because the mutation rate is the same for each population, differences in theta correspond to differences in N_{ef}), migration rates or gene flow ($M = N_{ef}m$), time since population divergence (T), and time to most recent common ancestor (TMRCA) between *X. u. udzungwensis* and *X. u. obscurata* based on analysis of mtDNA sequence data using MDIV. The highest posterior probability scores for theta and M are given with their 95% credibility intervals. Values for T were within undefined bounds due to likelihood estimates for the parameter not converging (see figure 2 and text for further details).

	Theta	M (Gene Flow)	TMRCA	T
10 mil generations				
Tmax = 15/Mmax = 10	0.49 (0.11-2.11)	0.0 (0.00-0.01)	7.21	Undefined
Tmax = 50/ Mmax = 10	0.35 (0.10-2.15)	0.0 (0.00-0.01)	10.08	Undefined
25 mil generations				
Tmax = 15/Mmax = 10	0.50 (0.11-2.11)	0.0 (0.00-0.01)	7.23	Undefined
Tmax = 50/ Mmax = 10	0.38 (0.09-2.12)	0.0 (0.00-0.01)	10.00	Undefined
50 mil generations				
Tmax = 15/Mmax = 10	0.49 (0.11-2.11)	0.0 (0.00-0.01)	7.24	Undefined
Tmax = 50/ Mmax = 10	0.37 (0.09-2.14)	0.0 (0.00-0.01)	9.98	Undefined

Coalescent models and fixed mutational differences in mtDNA, and one of the two nDNA markers sequenced, strongly suggest that no gene flow is currently taking place between populations of *Xenoperdix* in the Udzungwa (*X. u. udzungwensis*) and Rubeho Highlands (*X. u. obscurata*). Using the widely accepted 2% sequence divergence per million years in mtDNA (see García-Moreno, 2004), the 0.5% divergence in NADH2 suggests that the *Xenoperdix* populations on either side of the Great Ruaha Basin diverged about 250 000 years ago. Coalescent estimates of gene divergence suggest a slightly earlier divergence date of between 196–181 000 years BP. We were unable to estimate the date when gene flow ceased or population divergence occurred (T in figure 2 and table 4), but it likely followed shortly after the initial gene divergence given that both taxa are reciprocally monophyletic for two of the three markers analyzed (NADH2 and Fib5, not Gadph11).

The estimated divergence data of *ca.* 200 000 years BP is considerably more recent than the final uplifting of the Udzungwa and Rubeho Highlands around 7 million years BP (Griffiths, 1993). However, the relatively recent divergence between *X. u. udzungwensis* and *X. u. obscurata* is in agreement within ongoing studies of several forest bird species, which have demonstrated a sharing of haplotypes between birds in the Udzungwa and Rubeho Highlands, suggesting that birds do move (if rarely) across the Great Ruaha River Gorge (Bowie *et al.*, 2004, Fjeldså *et al.*, in press).

Sample sizes are small, but neither the population in the Udzungwa nor the population in the Rubeho Highlands had high levels of genetic diversity. Thus, based on the present dataset, it is not possible to identify whether the much larger Udzungwa *Xenoperdix* population acted as a source for the Rubeho population or *vice versa*. Both Fib5, a novel application to galliform systematics, and Gadph11 were variable, although Gadph11 was not as variable in *Xenoperdix* as it is in other galliform taxa (Holder *et al.*, 1999).

Almost no genetic diversity was detected within either of the populations of *Xenoperdix*. This raises the important question of whether *Xenoperdix* is inbred, or has managed to

successfully purge deleterious alleles. Due to the extremely small distribution of this species, it is critical that levels of genetic diversity and extent of local population movements within each of the highlands be accurately documented. Although we have sampled all seven specimens that have been collected for this species to date, the numbers are not sufficiently large to be statistically confident that *X. u. udzungwensis* and/or *X. u. obscurata* are genetically depauperate. However, given that two of the five fixed differences detected in NADH2 are at second codon positions, usually the most conservative because changes always result in an amino acid substitution, it is more parsimonious to assume that the *Xenoperdix* populations have been through bottlenecks, leading to low population numbers and fixation of haplotypes/alleles by genetic drift. We suggest that conservation managers would be well advised to establish a program of continued genetic assessment, obtaining samples with non-invasive methods, e.g. collecting all feathers left at dust-bathing sites in both the Udzungwa and Rubeho Highlands for further genetic analyses in order to better document genetic diversity and help meet long term objectives of conserving this unique African bird lineage.

Many biologists associate the degree of genetic diversity with extent of reproductive isolation. The rationale underlying this stems from the idea that the accumulation of genetic differences at many loci occurs over a long period of time. However, the long time periods required for sufficient genetic divergence to accumulate between two populations in order to be termed two species, are probably not often required for strong pre-mating isolation to occur (Ferguson, 2002). Indeed, the genetic basis of reproductive isolation varies so much between taxa that a predictive rate about degree of genetic divergence required for recognition of separate species is not possible (Ferguson, 2002). From a genetic perspective, a more robust statement about species status of a particular population/taxon can be made if one can demonstrate fixed genetic differences between the population/taxon in question and other populations/taxa. Such differences imply that not only has genetic differentiation taken place, but that there is a lack of gene flow as well (Ferguson, 2002). Assignment of species rank is further supported if genetic divergence is corroborated with morphological differences between populations/taxa. The two *Xenoperdix* taxa differ in facial ornaments and tail dimensions (table 1), both of which are potential display signals.

Xenoperdix u. obscurata is distinct from *X. u. udzungwensis*, with fixed mtDNA (NADH2, five fixed differences, two of which are at second codon positions resulting in amino acid substitutions) and nDNA (Fib5, one fixed) differences (table 3), and morphological characters (table 1). Thus, following Ferguson's (2002) arguments outlined above, it seems that the 0.5% mtDNA divergence between *X. u. obscurata* and *X. u. udzungwensis* is of little consequence when assigning species rank, and merely suggests that the two taxa diverged within the last 200 000 years. Further, coalescent models strongly support a model of no gene flow (figure 2, table 4). Within the framework of operational species concepts, such as the Phylogenetic Species Concept (Cracraft, 1983), and the 'Comprehensive Biological Species Concept' (Johnson *et al.*, 1999), the consistent diagnosable differences suggest that *X. u. obscurata* and *X. u. udzungwensis* can be accorded species status. Proponents of a traditional Biological Species Concept (Mayr, 1942) may argue that were the two taxa not allopatric they would interbreed. This may be the case, but we do not know, and in view of the dense human settlement, severe habitat degradation, and absence of forest cover in most parts of these highlands, it is unlikely that they will ever meet. The uncertainty of how to treat allopatric populations reflects one of the great difficulties with applying the biological species concept as an operational definition (Zink, 1997).

There is no doubt that species are the currency of conservation (Rojas, 1992; Zink, 1997).

Although an objective taxonomy should not build on non-biological criteria such as conservation policy, species rank for the Rubeho population will probably lead to higher conservation attention. Otherwise we fear that this distinctive population would be neglected as "a marginal isolate". Such taxonomic neglect has already led to the extinction of other small and isolated populations (*e.g.* Hazevoet, 1996). The genetically and morphologically distinctive Rubeho forest partridge *X. obscurata* is an endangered species in need of conservation attention.

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