The mitochondrial cytochrome c oxidase I gene reveals phylogeographic structure in the African Goshawk Accipiter tachiro (Accipitridae)

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We used a 298 bp fragment of the mitochondrial cytochrome c oxidase I subunit I gene (COI) to examine sequence variation in (mostly) museum specimens of the African Goshawk Accipiter tachiro. Our results showed two clades with high bootstrap support in a phylogenetic analysis and two groups in a nonmetric multidimensional scaling (NMDS) analysis. Each of the two phylogenetic clades corresponded to one of the NMDS groups. One clade comprised haplotypes of the subspecies A. t. lopezi, A. t. macrocelides, A. t. toussenelli and A. t. canescens and corresponded to the morphospecies A. toussenelli. This taxon has a more north-western distribution. The second clade comprised haplotypes of the subspecies A. t. sparsimfasciatus, A. t. pembaensis and A. t. tachiro and corresponded to the morphospecies A. tachiro, which has a more south-eastern distribution. Furthermore, one branch corresponded to the morphospecies A. t. unduliventer, which is confined to the Ethiopian highlands. The genetic divergence observed among the three A. tachiro morphospecies appeared concordant with the ecological and morphological divergence and suggests the existence of three putative species. Within A. tachiro and A. toussenelli there is substantial morphological, but very little genetic, differentiation among subspecies.

Keywords: Accipiter tachiro, African Goshawk, COI, nonmetric multidimensional scaling, phylogeny, taxonomy

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Introduction

The African Goshawk Accipiter tachiro (Daudin, 1800) is a common, widespread and medium-sized raptor that feeds on a wide spectrum of prey. Its distribution range covers the African equatorial evergreen forest belt (with some coastal outliers towards Senegal), the woodlands in southern and eastern Africa (but not those in the area from Senegal to Sudan), forest islands on the Ethiopian highlands, and the islands of Zanzibar, Pemba and Bioko (Figure 1) (Ferguson-Lees and Christie 2001). It is the only Accipiter species with endemic taxa on circum-African islands with A. t. lopezi on Bioko (Louette 2001) and A. t. pembaensis on Pemba (Benson and Elliott 1975). These birds can be found from sea level to over 3000 m, at the level of the montane forest (e.g. Louette 2007).

Accipiter tachiro is morphologically variable with respect to body size, barring of the ventral plumage in adults, the ventral colour pattern in juveniles, and the presence/absence of sexual dimorphism in general colour. Moreover, the large morphological variation is regularly accompanied by a large ecological and behavioural variation (e.g. Louette 2000, 2003a, 2007, 2010; see Table 1). Eight subspecies have been described based on morphological and life-history characteristics (Table 1). These are well-differentiated and recognised in recent works (e.g. Brown et al. 1982; Kemp 1994; Ferguson-Lees and Christie 2001). Most authors consider these eight subspecies (sometimes nine, if croizati is recognised) as a single species (e.g. Amadon 1953; Prigogine 1980; Brown et al. 1982; Dowsett and Dowsett-Lemaire 1993; Ferguson-Lees and Christie 2001), but often recognising two or three groups within the species. Other authors separate these birds into two morphospecies, viz. A. tachiro, with a more south-eastern distribution, and A. toussenelli, with a more north-western distribution (e.g. Chapin 1932; Kemp 1994; Clark and Davies 2000). Based on plumage characteristics and habitat preferences, Louette (2007) recognised two morphospecies, viz. a generally more cryptically (especially in the female) plumaged woodland taxon (tachiro), comprising A. t. tachiro, A. t. sparsimfasciatus and A. t. pembaensis, and a generally more colourful (especially in the female, which shows masculine plumage type) forest taxon (toussenelli), comprising A. t. toussenelli, A. t. lopezi, A. t. mascrocelides and A. t. canescens. The subspecies A. t. unduliventer (of which croizati is probably a synonym; Louette 2003b) is a geographically isolated, rather colourful form (Louette 2003b) from the Ethiopian highlands that is
intermediate in this respect (Louette 2007). Breman et al. (2013), as a result of a larger DNA barcoding project on African birds, studied the evolutionary relationships of the African and European representatives of Accipiter using the mitochondrial cytochrome c oxidase subunit I gene (COI). These authors suggested that the three A. tachiro morphospecies might represent different evolutionary species, based on their monophyly in the phylogenetic trees. Indeed, COI has been used to evaluate the taxonomic status of plumage variants in birds (e.g. Avise and Nelson 1989; Cooper et al. 2001) and may provide a common phylogenetic yardstick whereby divergences can be compared among taxa. In this study, we therefore examined DNA sequence divergence of the COI gene among (mostly) museum specimens of all subspecies of the African Goshawk and compared these sequence divergences with those of other Accipiter species. Unlike Breman et al. (2013), we also performed nonmetric multidimensional scaling (NMDS) because this ordination technique makes no assumptions on the nature of the data (unlike, for instance, principal component analysis) and can be used for any distance measure used (e.g. Legendre and Legendre 1998). The latter technique also allows speculation on the potential maternal lineage of a morphologically hybrid specimen (from the Democratic Republic of Congo) between A. t. canescens and A. t. sparsimfasciatus (for more details, see Louette 2003a) that was not studied by Breman et al. (2013).

**Methods**

Total genomic DNA was extracted from toe pads and feather shafts obtained from 43 (mostly) museum specimens of A. tachiro and several outgroup specimens (Supplementary Table S1) with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Most specimens were old museum vouchers (up to 150 years old) so that the DNA was often fragmented. Hence, we focused on a small fragment of 298 bp of the mitochondrial COI gene that was amplified with the primers Bird 1Fd (TCAACCAACCACAAAGAYATYGGYAC; modified after Hebert et al. 2004 and Lohman et al. 2008) and BirdH_351d_370d (CCTGCTCCWGCTTCTAYDGT; Sonet et al. 2011). This fragment corresponds to the first half

![Figure 1: Distribution map of the eight subspecies of Accipiter tachiro (modified after Louette 2007a). The dashed line shows the presumed border of the northern and southern localities of A. t. sparsimfasciatus](image)

| Subspecies          | Mainland/ insular | Habitat type | Body size | General colour pattern | Sexual dimorphism underneath | Barring underneath | Ventral side of juveniles       |
|---------------------|-------------------|--------------|-----------|------------------------|-----------------------------|-------------------|--------------------------------|
| A. t. macroscelides | Mainland          | Forest       | Small     | Advertising            | No                          | Rufous, barred grey| Clearly spotted                |
| A. t. toussenelii   | Mainland          | Forest       | Intermediate | Advertising   | No                          | Rufous, barred white| No spots or sparsely spotted   |
| A. t. canescens     | Mainland          | Forest       | Intermediate | Advertising   | No                          | Rufous, almost unbarred| No spots or sparsely spotted   |
| A. t. lopezi        | Insular           | Forest       | Small     | More deeply than in A. t. toussenelii coloured | No                          | Rufous, barred white| Strongly spotted               |
| A. t. tachiro       | Mainland          | Woodland     | Large     | Variable image avoiding in both sexes | Yes                         | White, barred grey | Strongly spotted               |
| A. t. sparsimfasciatus | Mainland          | Woodland     | Large     | Variable image avoiding in both sexes | Yes                         | White, barred grey | Strongly spotted               |
| A. t. pembaensis    | Insular           | Woodland?    | Intermediate | Rufous below, on breast, flanks and thighs especially | No                          | Rufous, barred white| Strongly spotted               |
| A. t. unduliventer  | Mainland          | Woodland?    | Intermediate | Variable image avoiding in both sexes | Intermediate | White, barred grey | Strongly spotted               |
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of the standard DNA barcode (Hebert et al. 2004). Reactions were carried out in a total volume of 30 µl, containing 2 µl genomic DNA, 1× PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 2.0 mM MgCl₂, 0.5 U Taq DNA polymerase (Platinum Taq, Invitrogen) and mQ-H₂O. The PCR profile was 4 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, with a final extension of 7 min at 72 °C. Museum samples that failed in a first PCR attempt were redone with 4 µl DNA added to the reaction mix. PCR products were purified using the NucleoFast® 96 PCR clean-up protocol (Macherey-Nagel) and sequenced using the BigDye Terminator v1.1 chemistry on an ABI 3130xl automated capillary DNA sequencer (Applied Biosystems). Sequences were checked, assembled and aligned in SeqScape 2.5 (Applied Biosystems). A neighbor-joining (NJ) tree was constructed on the basis of the Kimura-2-parameter (K2P) model in MEGA 5.05 (Tamura et al. 2011). Maximum parsimony (MP) and maximum likelihood (ML) trees were constructed in PAUP* 4.0b10 (Swofford 2002) using a heuristic search with the tree-bisection-reconnection branch-swapping algorithm and random addition of taxa. The ML phylogenetic tree was inferred with the evolution model selected using the Akaike information criterion as implemented by jMODELTEST 0.1.1 (Posada 2008), which for this data set was the TPM3uf-G model. Relative branch support was evaluated with 1000 bootstrap replicates (Felsenstein 1985) for the NJ and MP trees, and 200 for the ML tree. For ML and MP we present the 50% majority-rule consensus tree. Phylogenetic trees were rooted with Accipiter castanillus (voucher numbers: RMCA 11890 and RMCA 7622A41), an outgroup that is a close relative of A. tachiro (Wattel 1973; Breman et al. 2013). We excluded nine sequences (one of which was the single specimen of A. t. tachiro) from the phylogenetic analyses that lacked part of the beginning or end of the gene fragment to obtain sequences of equal length for analyses that lacked part of the beginning or end of the gene fragment to obtain sequences of equal length for the phylogenetic analyses. These were the individuals I5, I10, I14, I24, I25, I26, I34, I40 and I42 (Supplementary Table S1) resulting in a trimmed data set of 34 A. tachiro sequences of 298 bp length.

Sequence divergence between haplotypes and mean sequence divergence within and among phylogenetic lineages, subspecies and morphological taxa were calculated with MEGA 5.05 (Tamura et al. 2011) and were visualised via NMDS (Legendre and Legendre 1998) combined with a minimum spanning tree (MST) as implemented by NTSYS 1.8 (Rohlf 1993). For this, we used the K2P model and pairwise deletion of missing data. The pairwise deletion option allowed us to use the nine excluded sequences of the phylogenetic analysis as well (i.e. individuals I5, I10, I14, I24, I25, I26, I34, I40 and I42). More specifically, the NMDS/MST analysis allowed us to investigate the presumed morphological hybrid between A. t. canescens and A. t. sparsimfasciatus (i.e. individual I24; Louette 2003a).

Results

The 298 bp COI fragment yielded 21 variable sites (7%) and 15 sites (5%) were parsimony informative. The 34 sequences of 298 bp of A. tachiro represented 11 unique haplotypes. Phylogenetic relationships among these haplotypes are shown in Figure 2. There were two clades with high bootstrap support (≥90%) in both the NJ, ML and MP analyses. One clade (clade A) comprised three haplotypes, viz. one of A. t. lopezi, one of A. t. macroscelides and one shared by A. t. toussenelii and A. t. canescens. Within this clade there was high support, but only in the NJ analysis, for a sister-species relationship between A. t. lopezi and A. t. macroscelides. The other clade (clade B) comprised seven haplotypes of A. t. sparsimfasciatus. The haplotypes of A. t. pembaensis and A. t. tachiro were similar and were also similar to one of the sparsimfasciatus haplotypes. Within the latter clade there were two subclades that corresponded to the haplotypes of the northern and southern sampling localities, respectively, but the clade with the haplotypes of the southern localities was not supported in the MP and ML analyses. The position of the haplotype of A. t. unduliventer was not resolved.

The results of the NMDS/MST analysis are given in Figure 3. This analysis showed that the sequences of A. t.

![Figure 2: Neighbor-joining (NJ) tree with indication of major lineages of 11 mitochondrial cytochrome c oxidase subunit I (COI) gene haplotypes of Accipiter tachiro and an outgroup haplotype of Accipiter castanillus. Values for node support are: bootstrap values for NJ (Kimura 2-parameter model with 1000 replicates) / maximum likelihood (200 replicates) / maximum parsimony (1000 pseudoreplicates) trees. Bootstrap values ≥50% are shown, lower values are indicated with ‘-’. Branch lengths are according to the NJ tree. S and N denote the haplotypes of the southern and northern localities of A. t. sparsimfasciatus, respectively](image-url)
lopezi and A. t. macroscelides cluster closely together, as well as those of A. t. toussenellii and A. t. canescens. Furthermore, the group of sequences from the northern localities of A. t. sparsimfasciatus and those from the southern localities appear differentiated. The A. t. tachiro specimen (I43) had the same haplotype as 12 A. t. sparsimfasciatus individuals from the southern localities and the specimen from A. t. pembaensis (I32). The sequences of A. t. unduliventer held an intermediate position. Finally, one individual (I24) showed a somewhat distinct position but showed close affinities to the northern A. t. sparsimfasciatus.

The mean sequence divergence among the 43 A. tachiro sequences is given in Supplementary Table S2. The mean sequence divergence between the subspecies of clade A (A. t. tachiro, A. t. sparsimfasciatus and A. t. pembaensis) was very low (0–0.9%), as well as between the subspecies of clade B (A. t. toussenellii, A. t. canescens, A. t. macroscelides and A. t. lopezi) (0.1–1.6%). The mean sequence divergence between subspecies from both phylogenetic groups was higher (range: 3–4.4%) and was of the same magnitude as the mean sequence divergence between A. t. unduliventer and both clades (A. t. unduliventer – clade A: 5–6.6%; A. t. unduliventer – clade B: 5.7–6.5%) or between A. tachiro and A. castanilius (4.7–6.6%). It is also well above the 2.5% threshold for interspecific COI sequence divergences observed within 25 species of the genus Accipiter (Breman et al. 2013). Hence, the morphological and molecular data suggest three species within A. tachiro, viz. A. tachiro (Daudin, 1800), A. toussenellii (Verreaux, Verreaux & Des Murs, 1855) and A. unduliventer (Rüppell, 1836). Remarkably, the biogeographic pattern that we depicted for the African goshawk corresponds very well to the biogeographical regionalisation of sub-saharan Africa as recently observed for African birds in general (Linder et al. 2012).

Figure 3: Three-dimensional plot of 43 specimens belonging to eight subspecies of Accipiter tachiro via nonmetric multidimensional scaling combined with a minimum spanning tree based on Kimura 2-parameter distances of mitochondrial cytochrome c oxidase subunit I (COI) gene sequences. For full information of the individuals refer to Supplementary Table S1. Individual I24 is a morphological hybrid between A. t. canescens and A. t. sparsimfasciatus (Louette 2003a). For A. t. sparsimfasciatus: S = southern localities, N = northern localities.
distributions of *A. tachiro*, *A. toussenelli* and *A. unduliventer* correspond to the avian-biogeographic regions Zambesian/ Somalian, Congolian and Ethiopian, respectively (see Figure 1b in Linder et al. 2012).

Within both clades there was little, if any, differentiation among the subspecies (mean sequence divergence between subspecies <1.6%) which is well below the threshold for interspecific divergences in *Accipiter* (Breman et al. 2013). Within clade A, the subspecies *A. t. canescens*, which occurs in the eastern part of Lower Guinea, could not be distinguished with our data set from the subspecies *A. t. toussenelli*, which has an adjacent, more western distribution (Figure 1). In addition, the morphological distinction between these subspecies is very subtle and may reflect clinal differences in the adult plumage, although the subspecies differ in juvenile plumage (Louette 2003a). The insular subspecies *A. t. lopezi* from Bioko showed strong affinities in the phylogenetic trees and haplotype network with *A. t. macroscelides*, which occurs on the nearby mainland, although they are morphologically quite distinct (e.g. Louette 2001). *Accipiter t. lopezi* thus most probably originated from the central western part of the African continent.

Within clade B, the subspecies *A. t. tachiro*, *A. t. pembaensis* and several *A. t. sparsimfasciatus* individuals shared the same haplotype. The morphological differentiation between *A. t. tachiro* and *A. t. sparsimfasciatus* is weak (Brown et al. 1982; Allan 2005). Our data also suggest that the subspecies *A. t. pembaensis* has evolved from the nearby southern *A. t. sparsimfasciatus*, rather than from *A. t. tachiro*, which has a more south-eastern African distribution (Figure 1). *Accipiter t. pembaensis* is smaller in size than *A. t. sparsimfasciatus* and this hints at the ‘island rule’ of Van Valen (1973), which predicts that ‘large’ animals evolve to a smaller size on islands. For birds, Lomolino (2005) demonstrated that phenotypic evolution of island birds indeed follows the island rule and that the ‘tipping point’ for change is between 70 and 120 g (i.e. when the mean weight of a species is >70–120 g, then island species are smaller than their mainland sister species). The average weight of male and female *A. t. sparsimfasciatus* is 160–235 g and 230–510 g, respectively (Ferguson-Lees and Christie 2001) but the weight of *A. t. pembaensis* is unknown (Louette 2007). Finally, there may be a northern group within *A. t. sparsimfasciatus*. There is, however, no support for a southern group and the mean sequence divergence between haplotypes from northern and southern localities is very low (1.6%) so that this pattern appears to be intraspecific geographic substructuring rather than suggesting a taxonomic differentiation.

The NMDS/MST analysis showed that individual I24 held a distinct position. Morphologically, this specimen appears to be a hybrid between *A. t. canescens* and *A. t. sparsimfasciatus* (Louette 2003a). The sequence divergence between this individual and the other *Accipiter* specimens, and the NMDS/MST analysis, strongly suggest that the maternal line is (northern) *A. t. sparsimfasciatus*. However, mitochondrial markers alone cannot identify hybridising parapatric species pairs (e.g. Aliabadian et al. 2009) and assignment to species in those cases should be done by character-based approaches or with the use of complementary nuclear markers to account for mitochondrial introgression in hybridising species. Therefore, we examined 10 microsatellite markers that were previously developed for other *Accipiter* species (Topinka and May 2004; Takaki et al. 2009). However, because the DNA was probably highly degraded and of low amount, we obtained very few successful amplifications. With this very limited data set, we were unable to find microsatellites that would allow to differentiate among the different taxa and we therefore could not unambiguously pinpoint the two parental subspecies of this presumed hybrid.

In summary, the genetic divergence observed among the three morphological *A. tachiro* taxa appears concordant with the ecological and morphological divergence and suggests three putative species within *A. tachiro*, viz. *A. tachiro*, *A. toussenelli* and *A. unduliventer*. Within the species there is substantial morphological, but very little genetic, differentiation among subspecies. Our study also emphasises that, despite a more general distinct morphological appearance between *A. tachiro* (cryptic), *A. toussenelli* (more colourful) and *A. unduliventer* (intermediate), many of the phenotypic traits studied (see Table 1) earlier have little, if any, phylogenetic information. For instance, there is a remarkable morphological similarity between *A. t. lopezi* and *A. t. pembaensis*, but both subspecies belong to well-differentiated clades. Clearly, additional genetic markers and individuals are needed to fully understand the evolutionary history of the African goshawk.

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