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Nutlet is a little nut: disclosure of the phylogenetic position of Robbins' house bat *Scotophilus nucella* (Vespertilionidae)

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Abstract. Evolutionary parallelism complicates taxonomy of the bat genus *Scotophilus*. This implies the necessity for a careful examination of morphologically similar species. Robbins' – or "nutlet" – house bat *Scotophilus nucella* is an insufficiently known taxon of the African rainforest zone based on just a handful of recorded specimens previously included in the nut-coloured house bat *S. nux*. Because its phylogenetic relationship to *S. nux* and other congeneric species is unknown, it was assessed using analysis of DNA sequences of single mitochondrial and nuclear genes. Based on the mitochondrial cytochrome *b* sequences, *S. nucella* was placed in sister position to *S. nux*. A genetic divergence of 7.8-9.4 % between them supports the recognition of *S. nucella* as a distinct species. Analysis of partial sequences of the nuclear zinc finger protein gene on the Y-chromosome corroborated the sister relationship of *S. nucella* and *S. nux*, while showing sufficient differences to consider them as two species. Mitochondrial genetic diversity in *S. nucella* was low, whereas *S. nux* showed a rather complex genetic structure over a large geographic area, despite limited sampling. The origin of the forest group of *Scotophilus* could be dated to the Miocene-Pliocene transition and the split leading to the contemporary species *S. nucella* and *S. nux* to the Pliocene-Pleistocene transition. Both time periods are characterized by an arid climate that led to the retreat of forest environment, which likely promoted speciation in forest refugia. Mid-Pleistocene diversification in *S. nux* led to a separate lineage from Guinea, West Africa, for which a subspecific status may be considered, as it differs 3.4-4.5 % from other African populations.

Key words: cytochrome *b*, zinc finger protein Y, *zfy*, phylogeny, African rainforest, Ghana

Introduction

Evolutionary parallelism in bats is rather common due to constraints related to flight and echolocation (Norberg & Rayner 1987, Arita & Fenton 1997, Mayer & von Helversen 2001, Baker & Bradley 2006). True phylogenetic relationships between morphologically similar taxa may then be difficult to assess, which in turn can complicate the taxonomic arrangement of particular bat groups. Well-known examples of such a discrepancy are the Palaearctic vespertilionid bats of the *Myotis mystacinus* morphogroup (Ruedi & Mayer 2001, von Helversen et al. 2001, Stadelmann et al. 2004), which includes species that do not form a monophyletic assemblage and actually represent independent evolutionary units. The existence of morphological similarity without close evolutionary kinship was also revealed in the bat genus *Scotophilus* Leach, 1821 (Vespertilionidae), also known as house bats. This common bat group of tropical regions of Africa and Asia comprises taxa of similar morphological appearance, which makes their taxonomy a complicated affair (Robbins et al. 1985, Simmons 2005, Goodman et al. 2005, Horáček et al. 2006, Van Cakenberghe & Happold 2013a), despite recent advances achieved with the use of molecular genetic (Trujillo et al. 2009, Vallo et al. 2011, 2013,

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2015). Two African species, *S. dinganii* (Smith, 1833) and *S. viridis* (Peters, 1852), originally defined just morphologically, were each shown to consist of three mitochondrial lineages in a polyphyletic arrangement (Trujillo et al. 2009), which implies species status for each of them (see Brooks & Bickham 2014).

The Robbins' – or "nutlet" – house bat *Scotophilus nucella* Robbins, 1983, is a rare and insufficiently known species of the African rainforest zone, separated three decades ago from the nut-coloured house bat *S. nux* Thomas, 1904. This separation was based on diminutive size of otherwise morphologically similar specimens of brownish colouration occurring in sympatry with the larger, traditionally distinguished species. While *S. nux* occurs in Guinean rainforest from Sierra Leone to Ghana and Congolean rainforest from Nigeria to Kenya, *S. nucella* is known from just a handful of records limited to Upper Guinean rainforest of southeastern Côte d'Ivoire and southern Ghana (Robbins et al. 1985, Simmons 2005, Van Cakenberghe & Happold 2013b, c; Fig. 1). Records from western Uganda (Robbins 1983; Fig. 1) and Tanzania (Cunneyworth 1996, Doggart et al. 1999a, b; Fig. 1) were challenged by Thorn & Kerbis Peterhans (2009) and Stanley & Goodman (2011), respectively. There exists only scarce information on natural history and taxonomic relationships of *S. nucella*. Most recently, it was reviewed by Decher & Fahr (2007), who succeeded in capturing ten specimens of this species in southwestern Ghana. Their catch represented half of the known specimens of *S. nucella* captured until then, including those upon which the species description by Robbins (1983) was based. In 2014 and 2015, six additional specimens were caught during a faunistic survey in the same locality sampled more than a decade ago by Decher & Fahr (2007). The current number of *S. nucella* specimens known to science thus raised to 31, including 26 from southwestern Ghana alone, of which 16 originated from a single locality.

Phylogenetic relationships of *S. nucella* within the genus *Scotophilus* have not yet been assessed, nor was its taxonomic status as a separate species supported by molecular genetic analysis. Moreover, given the polyphyly within the congeneric *S. dinganii* and *S. viridis* morphospecies (Trujillo et al. 2009), a question arises as to the actual evolutionary relationship between *S. nucella* and *S. nux*, which may have independently evolved into species adapted to life in tropical rainforest. To fill the gap in knowledge on this enigmatic species, the phylogenetic position of *S. nucella* within the genus *Scotophilus* is inferred here based on sequence analysis of maternally inherited mitochondrial cytochrome *b* gene (cyt*b*) and the paternally inherited gene for the zinc finger protein on the Y-chromosome (*zfy*). In addition to the evaluation of the species status of *S. nucella* and its relationship to *S. nux*, the evolutionary history of both forest species is also briefly addressed.

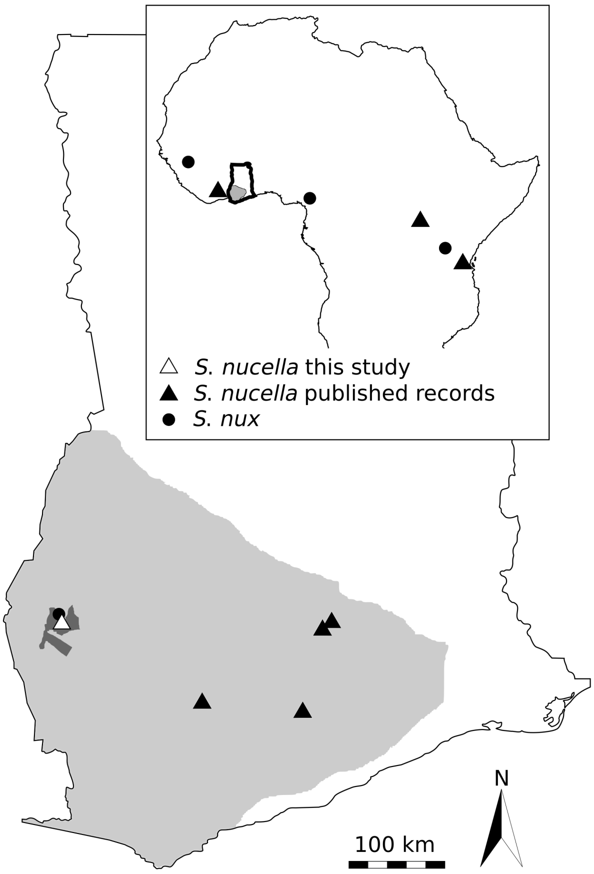

Fig. 1. Distribution of *Scotophilus nucella* shown as localities of capture of specimens analyzed in this study (white triangle) and previously published localities (black triangles) in Côte d'Ivoire, Ghana and Uganda, as summarized by Decher & Fahr (2007), and unrevised localities in Tanzania (Cunneyworth 1996, Doggart et al. 1999a, b). Localities of origin of the comparative samples of *S. nux* are also included (black circles), both new and previously published (Trujillo et al. 2009). The original extent of the Upper Guinean rainforest in Ghana as possible habitat for *S. nucella* is emphasized by shading (light grey), including the Krokosua Hills Forest Reserve (dark grey).

Material and Methods

Specimens of *Scotophilus nucella* included in this study originated from Krokosua Hills Forest Reserve (KHFR), southwestern Ghana (Table 1, Fig. 1). One was an earlier capture from a rapid assessment survey in 2003 (Decher & Fahr 2007). This specimen (ZFMK 2003.1044) was collected and preserved as ethanol voucher with skull extracted in the collection of the Zoological Research Museum Alexander Koenig

Table 1. List of *Scotophilus nucella* and *S. nux* specimens from West Africa, included in this study. Except for *S. nux* ZFMK 2008.0306 from the Simandou Range, Guinea (08°32′ N, 08°54′ W), all samples originate from the Krokosua Hills Forest Reserve, Ghana (06°37′ N, 02°51′ W). acc. nr. – accession number of haplotype in Genbank database.

species	museum or field nr.	sex	capture date	acc. nr. cyt*b*	acc. nr. *zfy*
S. nucella	ZFMK 2003.1044	♂	3.11.2003	KX010977	-
S. nucella	KHFR-T12	♂	12.9.2014	KX010980	KX010981
S. nucella	KHFR-T13	♂	12.9.2014	KX010977	KX010981
S. nucella	KHFR-B19	♂	15.5.2015	KX010978	KX010981
S. nucella	KHFR-C5	♀	16.5.2015	KX010980	-
S. nucella	KHFR-C6	♀	16.5.2015	KX010979	-
S. nucella	NMP 95434	♂	16.5.2015	KX010978	KX010981
S. nux	KHFR-B7	♂	15.5.2015	KX010976	EU751017
S. nux	KHFR-B15	♂	15.5.2015	KX010974	EU751017
S. nux	KHFR-B17	♀	15.5.2015	KX010973	-
S. nux	ZFMK 2003.1049	♀	4.11.2003	KX010973	-
S. nux	ZFMK 2003.1058	♀	11.11.2003	KX010974	-
S. nux	ZFMK 2008.0306	♂	7.3.2008	KX010975	-

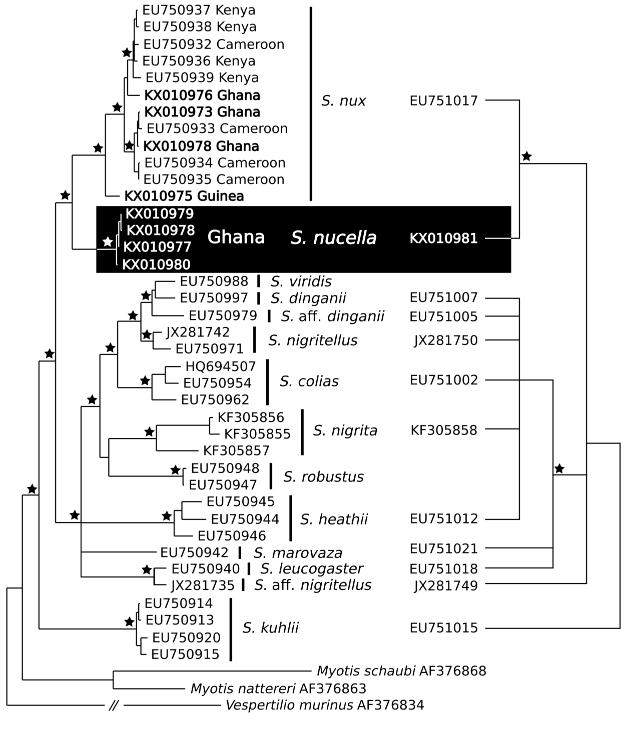

Fig. 2. Bayesian cyt*b* tree (left) and modified 50 %-majority consensus MP *zfy* tree (right) showing phylogenetic position of *Scotophilus nucella* within the genus *Scotophilus*. Newly obtained haplotypes of *S. nux* are emphasized in bold. Stars denote significant support at the respective nodes: Bayesian posterior probability of 1.00 and MP bootstrap support > 75 %.

(ZFMK) in Bonn, Germany. The other specimens were recent captures from 2014 and 2015 during a bat conservation survey, and except for one were released after measuring forearm and mass, and sampling a biopsy from the patagium. The sacrificed specimen (NMP 95434) is preserved as ethanol voucher with skull extracted in the collection of the National Museum in Prague, Czech Republic (NMP). Forearm length and cranial dimensions of this specimen were measured using a mechanical calliper as described in Vallo et al. (2011, 2013) for morphological comparison with published data on *S. nucella*. Seven specimens of the morphologically similar African forest species *S. nux* (see Robbins 1983) originating from the 2003 and 2015 surveys in Ghana, and one from a baseline study at Simandou Guinea in 2008 (Decher et al. 2015) were also included into the analysis (Table 1).

Total genomic DNA was extracted from ethanol-preserved tissue samples (muscle or patagium) using commercial kits and the complete cyt*b* and a fragment of *zfy* were PCR amplified, sequenced, and aligned as previously described (Vallo et al. 2011, 2013). Additional sequences of congeneric species published by Trujillo et al. (2009) and Vallo et al. (2011, 2013, 2015) were included into the alignment for comparison (Fig. 2). All new sequences were submitted to GenBank and can be accessed under numbers KX010973-KX010981 (Table 1). Sequences of three vespertilionids by Ruedi & Mayer (2001): *Vespertilio murinus* Linnaeus, 1758, *Myotis nattereri* (Kuhl, 1817) and *Myotis schaubi* Kormos, 1934, were used as outgroup for rooting phylogenetic trees.

Phylogenetic analysis of cyt*b* sequences was carried out using Bayesian inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) under GTR + I + Γ evolutionary model, as suggested by the program MrModeltest 2.3 (Nylander 2004). A consensus tree was computed from two independent runs of one million generations sampled each hundred generations with burn-in set to 25 %. Sequence divergences were expressed as pairwise Kimura two-parameter genetic distances (K2P; Kimura 1980) to allow comparison with other bat groups (Baker & Bradley 2006), and particularly with members of the genus *Scotophilus* (Trujillo et al. 2009, Vallo et al. 2011, 2013, 2015). Nuclear *zfy* sequences were analyzed using maximum parsimony (MP) in PAUP* 4.10b (Sinauer Associates, Sunderland, MA, U.S.A.) in two modes, with gaps coded either as missing characters or as the fifth state as described in Vallo et al. (2013, 2015).

Dating of evolutionary events leading to the origin of contemporary *S. nucella* based on cyt*b* sequences was done in BEAST 1.8.2 (Drummond et al. 2012) under a relaxed clock model with uncorrelated lognormal rate distribution and Yule model of speciation. Four independent runs of ten million generations were sampled each thousand generations and burn-in was set to 10 %. After checking for consistent results the four runs were then combined into one. Due to lack of usable fossil evidence for the genus *Scotophilus* (Horáček et al. 2006), molecular clock was calibrated by the split of *Myotis nattereri* and *M. schaubi*, which has been used in previous bat studies (Hulva et al. 2004, Stadelmann et al. 2004). These two *Myotis* species were confirmed from early Pliocene of Central Europe, more than 5 million years ago (Mya) (Horáček & Hanák 1984). Since the timing of the actual splitting event in their common ancestor remains unknown, a time period between 7.2 and 5.3 Mya is used here as an approximate estimate. Within this period of late Miocene, the origin of these *Myotis* species may be plausibly placed due to geoclimatic changes resulting from desiccation of the Mediterranean sea, the so-called Messinian salinity crisis, which was a likely trigger for speciation in bats and other mammals (Hulva et al. 2004). The prior distribution for the time of the most recent common ancestor for this calibration node was thus set as normal with the mean of 6 Mya and standard deviation of 0.5 Mya, which covers the Messinian period of late Miocene and spreads up to the already known fossils.

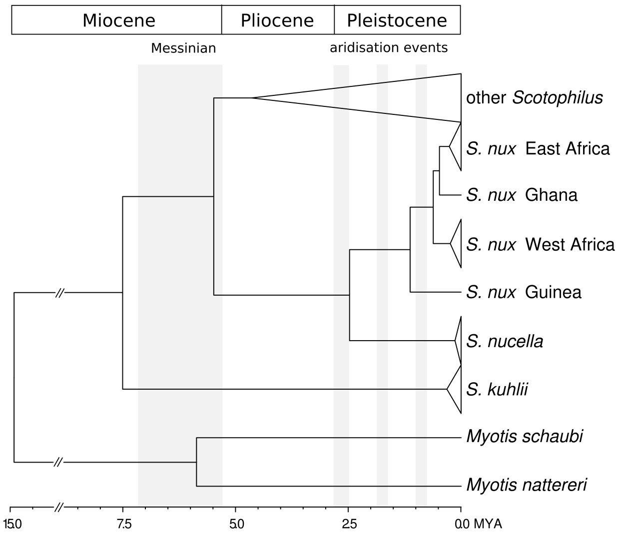

Fig. 3. Clock-like cyt*b* tree showing major splitting events within *Scotophilus* leading to origin of contemporary African high forest species *S. nucella* and *S. nux*. Grey bands denote respective geological or geoclimatical periods that may have influenced the current phylogenetic and phylogeographic structure of the target taxa.

Results

The newly collected specimen of *S. nucella* (NMP 95434) was very similar in both morphological measurements

Table 2. Selected external and cranial measurements of the *Scotophilus nucella* specimen NMP 95434 captured in 2015 in KHFR, southwestern Ghana, and comparative published data from Decher & Fahr (2007) and Robbins (1983) for the Ghana range values. *holotype, USNM – National Museum of Natural History, Washington D.C., U.S.A.

	NMP 95434	ZFMK 2003.1044	USNM 412141*	Ghana $\bar{X}$ (min-max) (N = 7)
body mass	21	25.5	22	21 (15-27)
forearm length	49.6	50.4	50.6	51.3 (49.5-52.6)
condylobasal length	16.88	17.52	17.2	17.3 (17.1-17.5)
neurocranium width	9.45	9.33	9.6	9.4 (9.2-9.6)
zygomatic width	13.28	13.61	13.7	13.6 (13.1-14.0)
rostrum width across upper canines	6.22	6.67	-	-
rostrum width across upper 3rd molars	8.18	8.46	8.5	8.3 (7.9-8.9)
length of upper toothrow	6.54	6.64	6.4	6.5 (6.5-6.6)
condylar length of mandible	13.52	14.10	-	-
length of lower toothrow	7.38	7.55	-	-

and cyt*b* sequence to the previously identified specimen from 2003 (ZFMK 2003.1044; Table 2, Fig. 2). The seven samples of *Scotophilus nucella* and the six of *S. nux* yielded each four cyt*b* haplotypes 1007 bp long. All cyt*b* haplotypes were coding for a protein of an essentially identical amino acid composition and contained no stop codons. In the cyt*b* phylogenetic tree, *S. nucella* haplotypes were placed as a monophyletic lineage in a highly supported sister position to *S. nux* (Fig. 2). The haplotypes of *S. nucella* were closely related, differing by up to 0.6 %. The southeast Asian species *S. kuhlii* was the first taxon to diverge from the basal node. The clade of sister species *S. nucella* and *S. nux* branched off next from the common ancestor with the remaining African and Asian *Scotophilus* taxa. Genetic divergence between *S. nucella* and its sister species *S. nux* reached 7.8-9.4 %, and between *S. nucella* and other *Scotophilus* species 11.6-14.1 %. Branching pattern in *S. nux* was more complex than in *S. nucella* (Fig. 2). The haplotype of *S. nux* from Simandou, Guinea, significantly diverged from its conspecifics, building a lineage of its own differing 3.4-4.5 %. Two lineages could be further delimited in *S. nux*, comprising haplotypes from Cameroon and Ghana, and Kenya and Cameroon, respectively. Variation within these two lineages reached 0.9 % and 1.2 %, respectively, and they differed by 2.0-3.0 %. The haplotype of *S. nux* from Ghana, KHFR-B7, did not cluster with any of these two lineages, and differed from them by 2.6-3.0 % and 2.0-2.4 %, respectively.

Partial *zfy* sequences obtained from four male samples of *S. nucella* were identical. Identical to each other were also newly obtained sequences of syntopic *S. nux*, which corresponded to the *S. nux* haplotype from Cameroon and Kenya available in the Genbank database under accession number EU751017. The *zfy* fragment analyzed was 454 bp long with inclusion of gaps. Its overall variation was low and genetic distance between species reached 0.9 % as computed from substitutions only. *Scotophilus nucella* and *S. nux* were represented by one haplotype each (Table 1). *Scotophilus nucella* shared one substitution with *S. nux*, through which both species differed from the other *Scotophilus*. Moreover, it showed a 3-bp stretch identical to that in *S. nux*, which they both share with *S. kuhlii* and a yet undescribed small-sized West African species of *Scotophilus* (*S.* aff. *nigritellus*) and which is missing in other *Scotophilus*. From *S. nux*, *S. nucella* differed by three unique substitutions. MP reconstruction based on *zfy* sequences supported *S. nucella* and *S. nux* as sister taxa as revealed by cyt*b* analysis (Fig. 2). In MP analysis with gaps coded as missing data, *S. nucella* and *S. nux* clustered together in all of the 13 most parsimonious trees recovered and their relationship was supported by a bootstrap value of 75 %. With the consideration of gaps coded as the fifth character, the sister relationship among the two former species was more strongly supported by a bootstrap value of 80 %. Relationships among other species were not well resolved in both analyses.

Estimates of divergence times (Fig. 3) suggest that the splitting event in *Scotophilus* leading to the modern group of African rainforest species *S. nucella* and *S. nux* occurred around 5.5 (3.6-8.0, 95 % C.I.) Mya. A subsequent split giving rise to these two species took place around 2.5 (1.5-3.8, 95 % C.I.) Mya. In *S. nux*, a rather deep split occurred around 1.2 (0.6-1.8, 95 % C.I.) Mya leading to the separation of the western Upper Guinean lineage. A subsequent split 0.6 (0.3-1.0, 95 % C.I.) Mya lead to origin of *S. nux* in eastern Upper Guinean, and Lower Guinean and Congolian rainforest regions, which was followed by radiation around 0.26 (0.12-0.4, 95 % C.I.) and 0.23 (0.1-0.4, 95 % C.I.) Mya within the East and West African lineages, respectively. In *S. nucella*, the rather shallow structure points to a recent radiation 0.13 (0.04-0.27, 95 % C.I.) Mya.

Discussion

Discordance between morphological similarity and evolutionary relationships have for a long time obscured knowledge on the bat genus *Scotophilus*, and particularly the African species (Robbins et al. 1985, Goodman et al. 2005, Trujillo et al. 2009). The previously revealed parallelism within the two African morphospecies, *S. dinganii* and *S. viridis*, implied that sister relationship between the rainforest species *S. nucella* and *S. nux* was not a straightforward assumption. Moreover, the two latter species, especially *S. nucella*, belong to the least known and rarely encountered species of the genus compared to the former common and broadly distributed savannah species. It can be considered fortunate that twelve years after the last record in Ghana (Decher & Fahr 2007), this species was confirmed to still occur in the rapidly shrinking remnants of the West African rainforest. Taxonomic affinity of the specimens of *S. nucella* from 2014 and 2015 was confirmed through close similarity of their cyt*b* sequences to the sequence of the previously identified specimen of *S. nucella* from the same locality captured in 2003. The forearm and skull measurements of the newly collected specimen NMP 95434 also corresponded to data from the type series of *S. nucella* by Robbins (1983) (Table 2).

The analysis of DNA sequences clearly confirmed that *S. nucella* deserves a position as a separate species, distinct from *S. nux*, where it was once included until size variation within West African populations prompted Robbins (1983) to separate the "nutlet" from the "nut". The two reciprocally monophyletic mitochondrial lineages occurring in sympatry, with a genetic divergence exceeding 7.8 %, are supportive of the recognition of these two size forms as separate species and of their sister relationship. This value corresponds to differences found between other *Scotophilus* species (Trujillo et al. 2009, Vallo et al. 2013, 2015), as well as other bat taxa (Baker & Bradley 2006). Besides the pattern in maternally inherited mtDNA, *S. nucella* and *S. nux* also share paternally inherited *zfy* traits unique in this pair of forest species, while attaining their own distinct characteristics. The alternative assumption to their sister relationship, namely that of possible parallel evolution of two high forest species within *Scotophilus* can thus be abandoned.

Inferred topologies from both markers corroborate the fact that the forest species of *Scotophilus* have their origin in the early evolutionary history of the genus (Horáček et al. 2006, Trujillo et al. 2009). Molecular dating using mtDNA cyt*b* sequences placed the split of the ancestor of the contemporary African taxa approximately at the transition between Miocene and Pliocene, which was characterized by gradual cooling and aridisation resulting in the retreat of forest and the spread of savannah (deMenocal 2004, Plana 2004). Very plausibly, a part of the ancestral population remained in forest habitat while the other part, ancestral to the remaining contemporary *Scotophilus* species, adapted to more arid conditions of savannah. Similar phylogenetic pattern with origin of rainforest species at this time period and later diversification of savannah species was revealed e.g. in genets of the genus *Genetta* Cuvier, 1816 (Gaubert et al. 2004). Within the forest lineage of *Scotophilus*, another split likely occurred at the transition between Pliocene and Pleistocene, which again brought severe aridisation events to Africa resulting in significant shrinking of forest areas (deMenocal 2004, Plana 2004). Most probably, this split gave origin to the contemporary sister species *S. nucella* and *S. nux* through allopatric speciation in forest refugia, as has been similarly hypothesized for African forest fruitbat species of the genera *Myonycteris* Matschie, 1899 (Nesi et al. 2013), *Casinycteris* Thomas, 1910 and *Scotonycteris* Matschie, 1894 (Hassanin et al. 2015). Creating possible phylogeographic scenarios in *S. nucella* would be rather speculative due to limited sampling of just one population. On the other hand, the basal split within *S. nux*, which delimited the Guinean haplotype, and the subsequent split leading to the West and East African lineages roughly correspond to the two other large Pleistocene aridisation waves around 1.7 Mya and 1 Mya (deMenocal 2004). The resulting pattern after the latter split is rather confusing, given that one of the Central African haplotypes from Cameroon clusters with Kenyan haplotypes and the others together with Ghanaian haplotypes, while there remains yet one more solitary divergent Ghanaian haplotype. A plausible hypothesis explaining this pattern may be an allopatric development of these lineages within known forest refugia in West, Central and East Africa (Maley 2001, Plana 2004) as summed up by Hassanin et al. (2015), followed by recent distribution range overlap. However, the determination of the possible geographic origin of *S. nux* and the direction of its distribution range expansions would be rather speculative given the current geographic sampling.

Despite limited sampling, however, the inferred sequence data indicate an allopatric speciation of the two sister taxa *S. nucella* and *S. nux*, now living in sympatry in West Africa with no evidence for hybridisation. Both species represent a remnant of the rainforest lineage of *Scotophilus*, a genus nowadays dominated by savannah forms in sub-Saharan Africa. The East African population of presumed *S. nucella* should be examined regarding its taxonomic affinity and evolutionary history. Given the current lack of morphometric data on the three known Tanzanian individuals (Cunneyworth 1996, Doggart et al. 1999a, b) and the suspect identification of the Ugandan specimen (Thorn & Kerbis Peterhans 2009), it may be quite probable that these individuals will be eventually revised as *S. nux*. The population of *S. nux* in Guinea may be considered a separate subspecies given its exclusive phylogenetic relationship to other African populations. A morphological comparison, as well as more robust genetic sampling, are definitely needed here in order to substantiate such a taxonomic amendment.

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