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Conflicting mitochondrial and nuclear paraphyly in small-sized West African house bats (Vespertilionidae)

Peter Vallo, Petr Benda, Jaroslav Červený & Petr Koubek

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Hybridization between species may result in introgression of mitochondrial DNA from one species to another. Phylogenetic inference, therefore, may not recover true evolutionary relationships. In bats, there are only a few reported cases of introgressive hybridization. House bats are a genus with obscure phylogeny and taxonomy, caused mainly by morphological similarity. We undertook a detailed analysis of small-sized West African house bats (*Scotophilus*), tentatively identified as *S. nigritellus*, to clarify relationships between two sympatric colour forms. These forms were recovered in paraphyletic position to each other in both mitochondrial and nuclear phylogenies, signifying that they are two distinct species. While the yellow-bellied form could be assigned beyond doubt to *S. nigritellus* s. str., the white-bellied form may be an as yet undescribed species. Moreover, the white-bellied form clustered as a sister mitochondrial lineage to another species, *Scotophilus leucogaster*. These sister lineages differed by only 2.6–2.8% sequence divergence, which lies within the intraspecific range for this genus. Two nuclear markers, however, contradicted the sister relationship, showing them instead to be distantly related. The apparent conflict between the mitochondrial and nuclear signals suggests that past hybridization may have occurred between these morphologically distinct species.

Corresponding author: *Peter Vallo, Institute of Vertebrate Biology AS CR, v.v.i., Květná 8, 603 65 Brno, Czech Republic. E-mail: vallo@ivb.cz*
Petr Benda, Department of Zoology, National Museum (Natural History), Václavské nám. 68, 115 79 Praha 1, Czech Republic & Department of Zoology, Faculty of Science, Charles University, Viničná 7, 128 44 Praha 2, Czech Republic. E-mail: petr_benda@nm.cz
Jaroslav Červený, Department of Forest Protection and Game Management, Institute of Vertebrate Biology AS CR, Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Kamýcká 129, 165 21 Praha 6, Czech Republic. E-mail: jardaryscerveny@seznam.cz
Petr Koubek, Department of Forest Protection and Game Management, Institute of Vertebrate Biology AS CR, v.v.i., Květná 8, 603 65 Brno, Czech Republic. E-mail: koubek@ivb.cz

Introduction

Over recent years, phylogenetic inference based on mitochondrial DNA (mtDNA) has become a standard approach in taxonomic evaluation of animal species (Avise 2000; Baker & Bradley 2006; Galtier *et al.* 2009). Use of mtDNA has become firmly established because of a number of favourable characteristics, including maternal inheritance, haploid status, lack of recombination, high mutation rate and widely available protocols (Moritz *et al.* 1987; Ballard & Whitlock 2004; Galtier *et al.* 2009). Although mtDNA-based phylogenies are considered robust, they may also reflect other evolutionary processes that can obscure species phylogenies, for example, introgressive hybridization (Moore 1995; Nichols 2001; Galtier *et al.* 2009). While introgressive hybridization has been shown to be a relatively common phenomenon in natural populations (Berthier *et al.* 2006; McGuire *et al.* 2007; Currat *et al.* 2008), its occurrence and direction is dependent on barriers to interspecific reproduction (Wirtz 1999; Avise 2000). Despite its confounding effect on species phylogeny, revelation of introgression within a multigene assay plays an important role in understanding the evolutionary history and delimitation of species (Funk & Omland 2003; Petit & Excoffier 2009).

Few cases of mtDNA introgression have been documented in bats. It has been observed, however, both in sister species, for example, large European mouse-eared bats *Myotis myotis* (Borkhausen, 1797) and *M. blythii* (Tomes,

1857) (Berthier *et al.* 2006), and in distantly related congeneric species, for example, European serotine bats *Eptesicus nilssonii* (Keyserling & Blasius, 1839) and *E. serotinus* (Schreber, 1774) (Mayer & von Helversen 2001; Artyushin *et al.* 2009). In both cases, mtDNA introgression was explained as a result of expansion of one of the species into range of another following a change of environmental conditions. Recently, hybridization has even been hypothesized to have occurred between different genera, that is, the Central African fruit bats *Micropteropus pusillus* (Peters, 1867) and *Epomophorus gambianus* (Ogilby, 1835) (Nesi *et al.* 2011). MtDNA introgression, as revealed in European bats, has yet to be described in bats of sub-Saharan Africa. However, some features of bats, such as powered flight, which is a unique means of crossing geographical barriers impenetrable to terrestrial animals, and roosting in mixed colonies, which enables close contact between species, allow reasonable presumption of presence of this phenomenon also in this area.

The genus *Scotophilus* Leach, 1821 (house bats; Vespertilionidae), is a common faunal element of bat communities in the Old World tropics (Robbins *et al.* 1985; Simmons 2005; Horáček *et al.* 2006). These bats represent a morphologically uniform group with variation predominantly in body size and pelage colouration, differing from other vespertilionids mainly through their derived dental and cranial character states (Menu 1987; Horáček *et al.* 2006). Because of similarity in external appearance, taxonomic and phylogenetic structure of the genus has traditionally been confused (Hayman & Hill 1971; Robbins *et al.* 1985; Koopman 1994; Simmons 2005; Jacobs *et al.* 2006). Eleven species are currently recognized as occurring in sub-Saharan Africa, including two endemic Madagascan species (Robbins *et al.* 1985; Goodman *et al.* 2005, 2006; Simmons 2005; Trujillo *et al.* 2009; Vallo *et al.* 2011). Six species are reported from West Africa. Two of these, *S. nux* (Thomas, 1904) and *S. nucella* Robbins, 1973, are limited in their distribution to equatorial forest regions, and the others are distributed throughout the savannah zone. *Scotophilus nigrita* (Schreber, 1774), the largest representative of the genus, can be unambiguously identified by its size [forearm length (LAt) over 70 mm]. There are two other large forms, the yellow-bellied *S. dinganii* (Smith, 1833) (LAt ca. 50–58 mm) and the white-bellied *Scotophilus leucogaster* (Cretzschmar, 1830) (LAt ca. 48–54 mm), and a small form (LAt ca. 42–48 mm) that has traditionally been included into *S. viridis* (Peters, 1852) based on morphology (Robbins *et al.* 1985) but has recently been confirmed as a separate species, *S. nigritellus* de Winton, 1899, based on DNA analysis (Trujillo *et al.* 2009).

Our recent (2004–2008) inventory of bat communities of the Niokolo Koba National Park (NKNP) in south-eastern Senegal resulted in numerous catches of house bats. Small-sized individuals were tentatively identified as *S. nigritellus* based on currently accepted taxonomy (Simmons 2005; Trujillo *et al.* 2009). Several individuals, however, exhibited an apparent inconsistency in pelage colouration, that is, while the majority of bats had yellowish-brown backs and yellow venters, which agrees with the description of *S. nigritellus* by de Winton (1899), aberrant bats had greyish-brown dorsal and white ventral colouration, some having a reddish-brown spot in addition that was probably attributable to gland excretion. These differences were originally considered as intraspecific variation as Afro-tropical bats are known to vary in this trait and recent studies (Goodman *et al.* 2005; Jacobs *et al.* 2006) have considered pelage colouration unreliable, particularly in house bats.

In this study, we examine genetic variation in small-sized house bats collected in Senegal and search for potentially significant differences between the aberrant white-bellied and the prevailing yellow-bellied individuals. We use sequences of the mitochondrial gene for cytochrome *b* (*cytb*), a traditional choice in molecular taxonomy (Baker & Bradley 2006), for inference of a phylogenetic relationship between the two forms within the broader spectrum of African congeneric species. To cover the possible confounding effect of mtDNA on species phylogeny, we analyse variation in fragments of two nuclear genes, the paternally inherited gene for zinc finger protein on the Y chromosome (*zfy*), and the bi-parentally inherited gene coding for the intron 7 of beta fibrinogen (*fgb7*). Amplified portions of both genes include intron regions, which are considered reasonable counterparts of mtDNA for phylogenetic inference in closely related species (Cathey *et al.* 1998; Prychitko & Moore 2000; Matthee *et al.* 2007). These nuclear genes have been successfully used in previous phylogenetic studies on bats (Trujillo *et al.* 2009; Nesi *et al.* 2011). We further assess morphometric variation using external and cranial dimensions and link it to genetic variation. Based on a combination of genetic and morphological evidence, we address the taxonomic status of the small-sized *Scotophilus* of West Africa.

Material and methods

Sampling

Most of the bats included in this study were captured between 2004 and 2008 in the NKNP in south-eastern Senegal (Fig. 1, Table 1). The NKNP is the largest natural protected area in Senegal and covers over 9130 km^2 of well-preserved Sudanese and Sudano-Guinean savannah belt, which is characterized by woodlands and a mosaic of grassland and wooded savannah (Madsen & Sambou 1998; Arbonnier 2002). Voucher specimens were fixed in ethanol

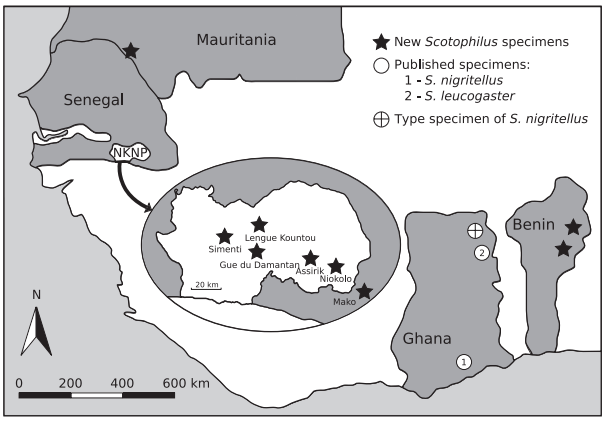

Fig. 1 Map of West Africa with localities of origin of specimens used in the study.

and deposited at the Institute of Vertebrate Biology of the Academy of Sciences of the Czech Republic, v. v. i. (IVB). The Senegalese specimens identified as *S. nigritellus* (N = 20) were compared with a set of syntopic *S. leucogaster* captured during the same fieldwork in the NKNP (N = 8) and additional specimens of both species from the collection of the National Museum in Prague, Czech Republic (NMP): *S. leucogaster* from Mauritania (N = 1) and *S. nigritellus* from Benin and Mauritania (N = 2).

For mtDNA phylogenetic comparison, we included additional GenBank sequences of African *Scotophilus* by Trujillo *et al.* (2009): *S. nux* (EU750933), *S. nigritellus* from Ghana (EU750971, EU750974), *S. leucogaster* from Ghana (EU750940), *S.* aff. *dinganii* from Ghana and westernmost Kenya (EU750977, EU750979, EU750982),

Table 1 List of newly processed West African *Scotophilus* specimens and conspecific samples published in GenBank used in the study

Sample	*Scotophilus* form	Sex	Country	Locality	*cytb*	Acc. #	*zfy*	Acc. #	*fgb7*	Acc. #
Sen0524	YB *nigritellus*	F	Senegal	Dalaba	Sen1	JX281737	–	–	–	–
Sen1236	YB *nigritellus*	F	Senegal	Simenti	Sen2	JX281738	–	–	YB1, YB2	JX281755, JX281756
Sen1264	YB *nigritellus*	F	Senegal	Niokolo	Sen3	JX281739	–	–	–	–
Sen1265	YB *nigritellus*	F	Senegal	Niokolo	Sen3	–	–	–	–	–
Sen1333	YB *nigritellus*	F	Senegal	Mako	Sen4	JX281740	–	–	–	–
Sen1685	YB *nigritellus*	M	Senegal	Niokolo	Sen5	JX281741	zfyYB	JX281750	YB1	JX281755
Sen1691	YB *nigritellus*	M	Senegal	Niokolo	Sen5	–	–	–	YB1, YB3	JX281755, JX281757
Sen1759	YB *nigritellus*	M	Senegal	Assirik	Sen6	JX281742	zfyYB	–	YB4, YB5	JX281758, JX281759
Sen1760	YB *nigritellus*	M	Senegal	Assirik	Sen1	–	zfyYB	–	–	–
Sen1763	YB *nigritellus*	F	Senegal	Assirik	Sen6	–	–	–	–	–
pb3530	YB *nigritellus*	F	Benin	11-km NW of Alafiarou	Ben1	JX281743	–	–	–	–
–	*nigritellus*	–	Ghana	Accra region	Gha1	EU751071	–	EU751009	–	–
–	*nigritellus*	–	Ghana	Accra region	Gha2	EU751074	–	–	–	–
Sen0382	WB *nigritellus*	F	Senegal	Simenti	Sen7	JX281735	–	–	–	–
Sen1148	WB *nigritellus*	M	Senegal	Simenti	Sen7	–	zfyWB	JX281749	WB1	JX281751
Sen1208	WB *nigritellus*	M	Senegal	Simenti	Sen7	–	zfyWB	–	–	–
Sen1231	WB *nigritellus*	F	Senegal	Simenti	Sen7	–	–	–	–	–
Sen1581	WB *nigritellus*	M	Senegal	Lengue Kountou	Sen7	–	zfyWB	–	–	–
Sen1582	WB *nigritellus*	M	Senegal	Lengue Kountou	Sen7	–	zfyWB	–	WB1	–
Sen1584	WB *nigritellus*	M	Senegal	Lengue Kountou	Sen7	–	zfyWB	–	WB1	–
Sen1633	WB *nigritellus*	F	Senegal	Gue du Damantan	Sen7	–	–	–	–	–
Sen1634	WB *nigritellus*	F	Senegal	Gue du Damantan	Sen7	–	–	–	WB1	–
Sen1635	WB *nigritellus*	F	Senegal	Gue du Damantan	Sen7	–	–	–	–	–
pb4787	WB *nigritellus*	F	Mauritania	Kaedi	Mau1	JX281736	–	–	WB1	–
Sen0523	*leucogaster*	F	Senegal	Dalaba	Sen8	JX281744	–	–	–	–
Sen1232	*leucogaster*	M	Senegal	Simenti	Sen8	–	zfySL	–*	SL1, SL2	JX281752, JX281753
Sen1271	*leucogaster*	M	Senegal	Niokolo	Sen9	JX281745	–	–	–	–
Sen1293	*leucogaster*	F	Senegal	Mako	Sen8	–	–	–	–	–
Sen1579	*leucogaster*	M	Senegal	Lengue Kountou	Sen10	JX281746	zfySL	–	–	–
Sen1630	*leucogaster*	M	Senegal	Gue du Damantan	Sen9	–	zfySL	–	SL2, SL3	JX281753, JX281754
Sen1632	*leucogaster*	M	Senegal	Gue du Damantan	Sen9	–	zfySL	–	SL2, SL3	–
Sen1758	*leucogaster*	F	Senegal	Assirik	Sen9	–	–	–	–	–
pb4782	*leucogaster*	M	Mauritania	Kaedi	Mau2	JX281747	zfySL	–	SL2, SL3	–
pb3526	*leucogaster*	M	Benin	10-km W of Parakou	Ben2	JX281748	–	–	–	–
–	*leucogaster*	–	Ghana	Yendi	Gha3	EU750940	–	EU751018*	–	–

**Zfy* sequence of *Scotophilus leucogaster* in this study was identical to the published sequences EU751018 and EU751019 on the 844 bp analysed. Acc. # – GenBank accession number.

S. colias (Thomas, 1904) from Ethiopia and Kenya (EU750954, EU750960, EU750960) – see Vallo *et al.* (2011) for this taxonomic assignation – and *S. dinganii* s. str. (EU750995, EU750997) from South Africa. A sequence of the Asian congeneric species *S. kuhlii* Leach, 1821 (EU750915), which has been shown to be the basal taxon of the genus (Trujillo *et al.* 2009), was used as an outgroup for rooting the phylogenetic trees.

For *zfy* comparison, we included the following additional *Scotophilus* sequences by Trujillo *et al.* (2009): *S. dinganii* morphospecies from Eastern, Western and Southern Africa (EU751002–EU751010), *S. leucogaster* (EU751018, EU751019), *S. nux* (EU751017) and the outgroup taxon *S. kuhlii* (EU751015). The only published *zfy* sequence of *S. nigritellus* was identical with sequence EU751009 of *S.* aff. *dinganii* from Ghana.

DNA processing and phylogenetic analysis

Total genomic DNA was extracted from ethanol-preserved tissue samples (spleen, muscle or patagium) using Dneasy Tissue Kit (Qiagen, Hamburg, Germany) or JetQuick Spin Tissue Kit (Genomed, Löhne, Germany) according to the manufacturers' protocols. The complete *cytb* mitochondrial gene was amplified via polymerase chain reaction (PCR) using primers F1 (modified; 5′-CCACGACC AATGACAYGAAAA-3′) and R1 (5′-CCTTTTCTGGTT TACAAGACCAG-3′) by Sakai *et al.* (2003) in 25-μL reaction volume consisting of 12.5-μL Combi PPP Master Mix (Top-Bio, Prague, Czech Republic), 200 μM of each primer, and 1.5–3 μL of extracted DNA. Alternatively, several samples were amplified using a PCR cocktail containing 0.8 mM dNTP (Fermentas) and 1U of HotMaster DNA polymerase and corresponding 10× buffer (Eppendorf, Germany). Initial denaturation of the PCR at 94 °C for 3 min was followed by 35 cycles of denaturation for 40 s at 94 °C, annealing for 40 s at 50 °C, and extension for 90 s at 65 °C, with final extension at 65 °C for 5 min. Partial sequences of *zfy* were amplified using primers 33X5YF (5′-GCAGCAGCTTATGGTAAGTGA-3′) (Trujillo *et al.* 2009) and LGL331 (5′-GCAAATCATGCAAGGATA GAC-3′) (Cathey *et al.* 1998), and a PCR protocol similar to the protocol for *cytb*, differing in an annealing temperature of 58 °C and extension time of 150 s. Partial sequences of *fgb*7 were amplified using primers Bfib1 (5′-ATTCACAACGGCATGTTCTTCAG-3′) and Bfib2 (5′-AANGKCCACCCCAGTAGTATCTG-3′) by Seddon *et al.* (2001) and a PCR protocol differing from the *cytb* protocol in an annealing temperature of 57 °C. The resulting PCR products were purified using JetQuick PCR Purification Kit (Genomed) or the QiaQuick PCR Purification Kit (Qiagen), and sequenced commercially (Macrogen, Seoul, Korea) using BigDye Terminator sequencing chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl sequencer in both directions for *cytb*, and in forward direction for *zfy* and *fgb*7, using the same primers as for PCR amplification. Sequences were assembled and edited in Sequencher 4.6 (Gene Codes, Ann Arbor, MI, USA) and the Contig Assembly Programme (CAP; Huang 1992) implemented in BioEdit 7.0 (Hall 1999). All newly obtained unique sequences were submitted to GenBank under accession numbers JX281735–JX281748 (*cytb*), JX281749–JX281750 (*zfy*) and JX281751–JX281759 (*fgb*7). Sequences were aligned in BioEdit 7.0 (Hall 1999) either by eye (mtDNA sequences) or using ClustalW (Thompson *et al.* 1994) under default settings for gap penalties in nuclear sequences.

MtDNA *cytb* phylogeny was computed in PAUP* 4.10b (Sinauer Associates, Sunderland, MA, USA) using maximum parsimony (MP) and maximum likelihood (ML). Tree space was searched heuristically with a tree bisection-reconnection swapping algorithm on 100 random sequence additions. Reliability of branching pattern was assessed by bootstrapping using 1000 and 100 pseudoreplicates in MP and ML, respectively. In ML bootstrapping, only 10 random sequence additions were run to reduce computation time. The model of evolution used in ML analysis was the transversion-weighted model with gamma distributed evolutionary rates split into four rate classes (TVM + Γ; Yang 1996), as suggested by the program Modeltest 3.7 (Posada & Crandall 1998). Phylogeny was further estimated using Bayesian inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) under the most closely related, more complex model of evolution incorporated in MrBayes, the general time-reversible model with gamma distributed evolutionary rates (GTR + Γ; Tavaré 1986, Yang 1996). We used two independent simultaneous Metropolis-coupled MCMC runs of four chains running for 10^6 generations, sampled every 100th generation and starting from random trees. The first 2500 sampled trees were discarded as burn-in. A 50% majority rule consensus tree was constructed from the remaining trees with posterior probabilities representing credibility estimates of topology. Sequence divergences were expressed as percentage pairwise Kimura two-parameter genetic distances (K2P; Kimura 1980) to allow comparison with other bat groups (Baker & Bradley 2006).

Nuclear *zfy* sequences were first described with regard to sequence polymorphism and characteristic differences between lineages. They were then compared using MP analysis with a heuristic search of 100 random sequence additions. Gaps were treated as fifth state to keep as much phylogenetic information as possible (Simmons & Ochoterena 2000). Nodal support was assessed through 1000× bootstrapping. ML and Bayesian trees were also computed

under TrN (Tamura & Nei 1993) and GTR evolutionary models, respectively, as suggested by Modeltest 3.7. ML analysis of *zfy* was carried out in an identical fashion as MP analysis. Bayesian analysis of *zfy* was run as in the *cytb* assay.

Nuclear *fgb*7 sequences were analysed for sequence polymorphism and characteristic differences noted. Double peaks in otherwise clearly readable chromatograms were considered to represent heterozygous positions of different alleles, as the marker used is diploid. For phylogenetic analysis, sequences containing such double peaks had to be split into haplotypes, each having just one of the two bases present in diploid sequence. As several individuals showed more than one heterozygous position, we used PHASE 2.1 software (Stephens *et al.* 2001) under default settings to estimate haplotypes from these sequences. As in *zfy* analysis, gaps were treated as the fifth-state character. A median-joining network was computed from all reconstructed haplotypes using the Network 4.6 program (Bandelt *et al.* 1999) to visualize phylogenetic relationships among the bats.

Morphological comparison

All specimens sequenced for inference of phylogenetic relationships were included in the morphological comparison. The type specimen of *S. nigritellus* [Natural History Museum, London, UK (BMNH 99.6.15.9), collected at Gambaga, Ghana; de Winton, 1899] was also included for taxonomic evaluation of small-sized *Scotophilus* bats. We used only one external measurement, forearm length (LAt), as this is the only traditionally examined dimension based on a bony body part and which is not biased, therefore, by tissue shrinkage caused by the fixation agent. Skulls were extracted from the voucher specimens and measured with mechanical callipers to the nearest 0.01 mm along the following 15 dimensions: greatest length of skull (LCr), condylobasal length (LCb), zygomatic width (LaZ), width of interorbital constriction (LaI), rostrum width across infraorbital foramina (LaInf), neurocranium width (LaN), mastoidal width (LaM), height of neurocranium (ANc), largest horizontal diameter of tympanic bulla (LBT), width across upper canines at crowns (CC), width across third upper molars (M^3M^3), length of upper tooth-row from front of canine to back of third molar (CM^3), condylar length of mandible (LMd), height of coronoid process (ACo) and length of lower tooth-row from front of canine to back of third molar (CM_3).

For size comparison of the two *S. nigritellus* forms, individual dimensions were compared using the analysis of variance (ANOVA) on raw data. Additionally, six indices were computed as ratios of selected skull dimensions (ANc, LaN, LMd, CM^3, LaInf, LaZ) against LCb and used to compare relative size of skull proportions. The skulls were further compared using principal component analysis (PCA) to assess overall morphological difference between specimens of *S. nigritellus* and to reveal potential presence of distinct morphotypes. The PCA used the 15 original skull dimensions only; these variables being entered as raw data. Statistical analyses were performed using Statistica 6.0 software (StatSoft, Tulsa, OK, USA).

Results

Variation of mtDNA sequences

Twenty small Senegalese house bats tentatively identified as *S. nigritellus* were sequenced for the complete *cytb* mitochondrial gene [1140 base pairs (bp)]. These *cytb* sequences corresponded to seven unique haplotypes Sen1–Sen7 (Table 1). The position of these haplotypes was inferred within a phylogeny including additional specimens of *S. nigritellus* (haplotypes Mau1, Ben1) and *S. leucogaster* (haplotypes Sen8–10, Mau2, Ben2) from Senegal, Mauritania and Benin, and previously published *Scotophilus* sequences (Table 1). MP analysis recovered six most parsimonious trees (600 steps long), which differed in minute rearrangements of terminal nodes within well-supported clades (Fig. 2). ML analysis yielded two virtually identical trees (-lnL = 4326.16287) that agreed with the arrangement of the main clades in the MP trees (Fig. 2). Topology of the Bayesian consensus tree was basically identical to that of the MP and ML trees. Support for the clades and their position within phylogeny was high under all three methods [bootstrap >90%, posterior probability (pp) > 0.95], except for Bayesian support of the *S. leucogaster* clade (pp = 0.88). Interestingly, the small-sized house bats tentatively identified as *S. nigritellus* clustered in two paraphyletic lineages. Yellow-bellied (YB) *nigritellus* specimens (haplotypes Sen1–Sen6) were placed as a crown lineage in sister relationship to the *S. dinganii* morphospecies clade from Southern and West Africa, including the westernmost part of Kenya. On the contrary, the white-bellied (WB) *nigritellus* specimens, all represented by haplotype Sen7, clustered at the base of the phylogenetic tree in sister relationship to the *S. leucogaster* clade. The small-sized bat from Benin (haplotype Ben1) and two haplotypes from Ghana previously identified as *S. nigritellus* clustered together with the Senegalese YB *nigritellus* specimens. The small-sized bat from Mauritania (haplotype Mau1) clustered with the Senegalese WB *nigritellus* specimens. Genetic divergence between the two lineages of West African small-sized bats ranged from 12.3 to 13.5%. Variation in YB *nigritellus* ranged from 0.2 to 0.5% within the Senegalese group only, and up to 3.2% when haplotypes from Ghana and Benin were included. The two WB *nigritellus* haplotypes differed from each other by 0.1%.

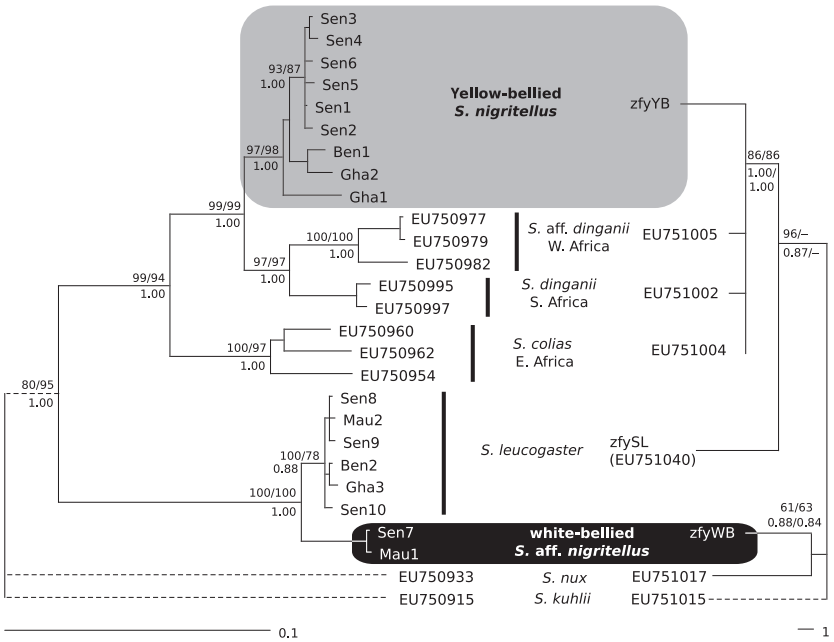

Fig. 2 Bayesian consensus tree based on *cytb* sequences (left) and MP tree based on *zfy* sequences (right) depicting phylogenetic position of small-sized *Scotophilus* of West Africa within a spectrum of congeneric species. MP and ML bootstrap values are given above, Bayesian posterior probabilities below the respective branches. In *zfy* tree, Bayesian nodal support is given for analysis with and without inclusion of indels. Branches depicted as dashed lines are for representative purposes only and their length does not reflect the amount of evolutionary change.

Most of the remaining taxa differed from each other, and from the two *nigritellus* lineages, by 5.1–13.6%. Lowest divergence values 2.6–2.8% were found between the sister lineages of WB *nigritellus* and *S. leucogaster*.

Variation of nuclear sequences

Partial *zfy* sequences were obtained from most of the males present in our set of specimens (Table 1). Sequences of the three forms, YB *nigritellus*, WB *nigritellus* and *S. leucogaster*, were each represented by one unique haplotype. These haplotypes were aligned with the comparative sequences of other *Scotophilus* to a block with a length of 844 bp. The *zfy* haplotypes of both *nigritellus* forms were different from published *Scotophilus* sequences (haplotypes zfyYB and zfyWB), while the *S. leucogaster* sequence was identical to the published sequence (sequences EU751018 and EU751019 were identical on the 844-bp fragment analysed). The three haplotypes differed by several characteristic insertions/deletions (indels): *S. leucogaster* differed from both *nigritellus* forms by a striking indel of 152 bp. Other unique indels included a 4-bp stretch in YB *nigritellus* and a 3-bp stretch in WB *nigritellus* that were not present in the other two forms. Nucleotide polymorphism was present at 12 positions of the alignment: WB *nigritellus* differed from YB *nigritellus* by 10 substitutions and from *S. leucogaster* by nine substitutions, and YB *nigritellus* differed from *S. leucogaster* by seven substitutions. The published sequences of *S. dinganii* morphospecies EU751002–EU751010 were reduced to only three unique haplotypes in the 844-bp alignment, represented by sequences EU751002 (identical with EU751003), EU751004 (identical with EU751006-EU751010) and EU751005. Sequence EU751004, which also represents *S. nigritellus* from Ghana (EU751009), differed from the recovered haplotype of *S. nigritellus* (zfyYB) by just one 4-bp indel.

In the final alignment, only four from 30 variable positions without consideration of gaps, and seven from 209 with gaps, were parsimony informative. MP analysis yielded one parsimonious tree (60 steps) with a rather shallow structure, except for the *S. leucogaster* branch, whose length was greatly inflated because of the characteristic 152-bp indel. Re-calculation without this alignment section produced two MP trees (31 steps) with the same arrangement and a visually more comprehensible structure with regard to inferred branch length. In contradiction to the mtDNA phylogeny, *S. leucogaster* clustered within a supported monophyletic clade together with the *S. dinganii* morphospecies and YB *nigritellus*, while its sister *cytb* lineage WB *nigritellus* was placed in paraphyly at the base of the tree (Fig. 2). ML and Bayesian analyses supported monophyly of *S. dinganii* morphospecies and YB *nigritellus*, but could not fully resolve the position of *S. leucogaster*. Nevertheless, the latter species and WB *nigritellus* were clearly not in sister relationship (Fig. S1).

Partial *fgb7* sequences were successfully obtained from 4 to 6 specimens from each of the two *nigritellus* forms and from *S. leucogaster*. The final alignment was 449-bp long and included a characteristic 20-bp indel in YB *nigritellus*. A further 1-bp indel was shown to differentiate *S. leucogaster*

from both *nigritellus* forms. Without indels, 11 alignment positions were polymorphic, five containing unresolved characters indicating the presence of heterozygous alleles. These alleles were reconstructed using PHASE software. Several heterozygous positions were decomposed into haploid phases with 50% uncertainty; however, this was not a serious issue with regard to the purpose of the analysis. The 26 sequences of the final alignment corresponded to nine unique haplotypes. In the reconstructed median-joining network, these haplotypes clustered into three distinct groups corresponding to the previously identified mtDNA lineages of the three respective *Scotophilus* forms (Fig. 3). The haplotype representing all five WB *nigritellus* specimens and *S. leucogaster* differed by five nucleotide substitutions and one indel, while YB *nigritellus* differed from *S. leucogaster* by one nucleotide substitution and two indels.

Morphological comparison

Comparison of dimensions showed close size similarity in all the small-sized house bats examined (Table 2; Fig. 4). Detailed examination, however, revealed morphological differences between the two groups separated by genetic analysis. YB *nigritellus* specimens had slightly larger LAt

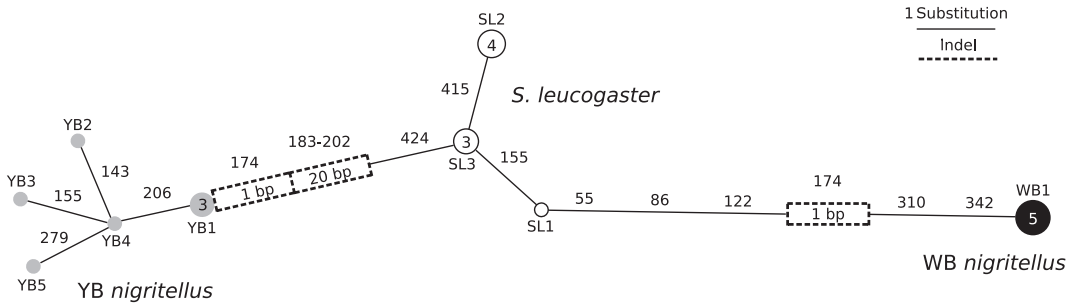

Fig. 3 Haplotype network calculated from *fgb7* sequences. Size of nodes is proportional to frequency of particular haplotypes. Except for single haplotypes, frequency is given in the nodes. Variable positions are indicated above the branches.

Table 2 External and cranial dimensions, selected indices of skull dimensions and statistical comparison (ANOVA) of the examined specimen sets of the small-sized West African *Scotophilus* bats. Data on the type specimen *Scotophilus nigritellus* de Winton, 1899; are also presented, but these were not included in ANOVA

	YB *nigritellus*					**WB *nigritellus***					**Type *nigritellus***	**ANOVA**		
	N	M	min	max	SD	*N*	M	min	max	SD	BMNH 99.6.15.9.	d.f.	*F*	P
LAt	10	44.43	42.6	46.6	1.520	10	42.07	40.6	43.2	0.817	44.4	18	18.70	***
LCr	10	16.60	15.86	17.03	0.322	10	16.21	15.92	16.54	0.234	16.62	18	9.74	*
LCb	11	15.63	15.07	16.13	0.369	10	15.38	15.12	15.68	0.213	15.63	19	3.51	
LaZ	9	12.01	11.53	12.49	0.296	8	11.31	10.98	11.44	0.151	12.21	15	36.62	***
LaI	11	4.18	3.98	4.43	0.139	10	4.46	4.36	4.55	0.062	4.38	19	32.80	***
LaInf	11	5.91	5.52	6.36	0.255	10	5.38	5.08	5.63	0.159	6.37	19	31.77	***
LaN	11	8.18	7.68	8.44	0.248	10	8.57	8.27	8.85	0.167	8.07	19	17.55	***
LaM	10	10.15	9.38	10.62	0.389	8	9.89	9.58	10.02	0.139	–	16	3.17	
ANc	10	6.83	6.31	7.14	0.299	10	6.25	5.94	6.48	0.167	6.72	18	28.79	***
LBT	11	3.49	3.29	3.76	0.154	10	3.50	3.27	3.74	0.151	–	19	20.56	***
CC	11	5.73	5.49	5.92	0.157	10	5.48	5.31	5.58	0.085	6.11	19	41.16	***
M^3M^3	11	7.62	7.44	7.88	0.149	10	7.23	7.02	7.41	0.125	8.08	19	23.03	***
CM^3	11	5.86	5.76	6.09	0.108	10	5.63	5.48	5.84	0.110	5.95	19	19.04	***
LMd	11	12.09	11.78	12.61	0.253	10	11.67	11.32	11.93	0.189	12.18	19	45.74	***
ACo	11	4.77	4.47	5.09	0.167	10	4.37	4.17	4.47	0.090	4.76	19	8.57	*
CM_3	11	6.55	6.33	6.82	0.139	10	6.38	6.21	6.56	0.128	6.71	19	0.00	
ANc/LCb	10	0.436	0.397	0.462	0.020	10	0.406	0.384	0.424	0.011	0.430	18	16.60	**
LaN/LCb	11	0.523	0.479	0.553	0.022	10	0.557	0.546	0.573	0.010	0.516	19	19.65	***
LMd/LCb	11	0.774	0.754	0.792	0.013	10	0.758	0.743	0.770	0.008	0.779	19	10.31	**
CM^3/LCb	11	0.375	0.364	0.386	0.008	10	0.366	0.355	0.372	0.005	0.381	19	9.01	*
LaInf/LCb	11	0.378	0.352	0.407	0.015	10	0.350	0.333	0.361	0.009	0.408	19	27.41	***
LaZ/LCb	9	0.767	0.747	0.782	0.012	8	0.735	0.724	0.755	0.010	0.781	15	36.13	***

P* value indicated as significant on the level of 0.05*, 0.01 or 0.001***

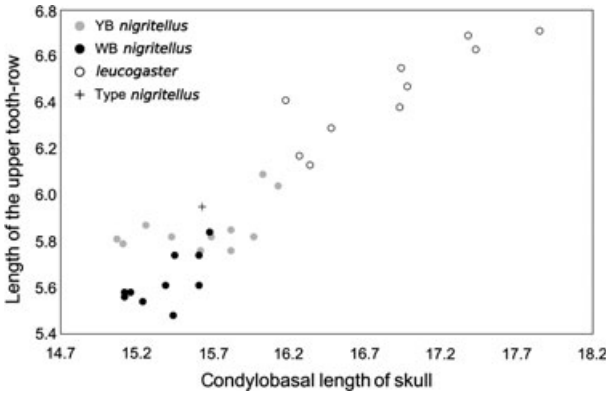

Fig. 4 Bivariate plot of two basic skull dimensions of compared *Scotophilus* specimens including the holotype specimen of *Scotophilus nigritellus* de Winton, 1899. Specimens of *Scotophilus leucogaster* are included to show size distinction from the small-sized forms of *S. nigritellus*.

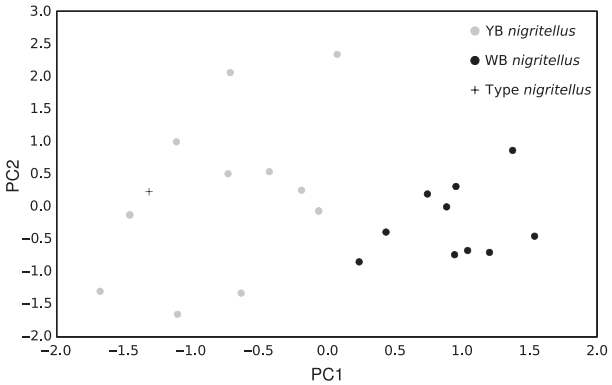

Fig. 5 Principal component analysis of skull dimensions of compared *Scotophilus nigritellus* specimens including the holotype specimen of *S. nigritellus* de Winton, 1899.

and skull length dimensions than WB *nigritellus*. Although dimension ranges for most skull measurements overlapped in these two groups, two width dimensions (LaZ, M^3M^3) and the height of the coronoid process (ACo) were clearly larger in YB *nigritellus* bats, with no overlap with WB *nigritellus*, while WB *nigritellus* specimens were larger in width of neurocranium (LaN). Thus, these groups differed in skull shape more markedly than in skull size. YB *nigritellus* specimens had an absolutely and relatively higher, but relatively much narrower, braincase; a relatively wider rostrum; and relatively longer jaws than WB *nigritellus* bats (see indices and ANOVA results in Table 2).

Specimens of the two haplotype groups, therefore, represented two distinct morphotypes of slightly different sizes but significantly different skull shapes. The clear difference between the two groups was best demonstrated by the PCA results based on all 15 skull dimensions (Fig. 5; PC1 = 56.53% of variance; PC2 = 9.99%), wherein the two groups are clearly separated; YB *nigritellus* (PC1 > 0.4) and WB *nigritellus* (PC1 < 0.0). The holotype specimen of *S. nigritellus* de Winton, 1899, which was included in all comparisons, consistently fell within variation ranges of the YB *nigritellus* specimens.

Discussion

Mitochondrial phylogeny

House bats have long been considered a group with confused intrageneric relationships and ambiguous delimitation of taxa, especially in sub-Saharan Africa (Hayman & Hill 1971; Robbins *et al.* 1985; Koopman 1994; Goodman *et al.* 2005, 2006; Simmons 2005; Jacobs *et al.* 2006; Trujillo *et al.* 2009; Vallo *et al.* 2011). To a large extent, this situation has been caused by the similar external appearance of bats from populations throughout the continent. Pelage colouration has often been used for identification, but this trait has recently been shown to be as unreliable for species identification as morphological measurements, where considerable overlap exists among most taxa (Goodman *et al.* 2005; Jacobs *et al.* 2006; Trujillo *et al.* 2009; Vallo *et al.* 2011). Captured specimens, therefore, have been identified to species rather intuitively, as was the case for the small-sized bats identified as *S. nigritellus* in this study. Molecular phylogenetic comparison of two forms with different pelage colouration, however, indicated that, surprisingly, the white-bellied form was actually not closely related to the yellow-bellied form, the latter agreeing with the original description of *S. nigritellus* by de Winton (1899). The paraphyletic relationship between the two forms, inferred from *cytb* sequence analysis, clearly indicates that they represent different species. This conclusion is further supported by a deep sequence divergence of over 12%, as such values are generally considered interspecific in bats (Baker & Bradley 2006) and are also reported among other well-defined house bat species (Jacobs *et al.* 2006; Trujillo *et al.* 2009; Vallo *et al.* 2011; this study). On the other hand, the sister relationship of WB *nigritellus* to *S. leucogaster* and the rather low genetic divergence between them (2.6–2.8%) indicate that these two lineages could belong to the same species. Similar levels of divergence are usually considered as intraspecific variation in bats, including *Scotophilus* (Baker & Bradley 2006; Jacobs *et al.* 2006; Trujillo *et al.* 2009), and even in this study, similar values ranging around 3% were obtained between YB *nigritellus* from Senegal and Ghana. The size of *S. leucogaster*, however, which alone allows unambiguous discrimination between *S. leucogaster* and YB *nigritellus* (Fig. 4), tends to contradict the potential intraspecific relationship between the two sister lineages.

Conflicting nucDNA signal

Additional data from nuclear markers provided interesting information on the mutual position of the previously defined mtDNA lineages. Comparison of male-inherited *zfy* gene sequences unambiguously identified three distinct haplotypes that significantly differed from each other by characteristic indels and substitutions. WB *nigritellus* was placed in paraphyletic position to both *S. leucogaster* and YB *nigritellus*, supporting its evolutionary independence from both species. Interestingly, *S. nigritellus* from Ghana lacks the 4-bp indel present in its conspecifics from Senegal, and its sequence is identical to *S.* aff. *dinganii* from the same region. Not only does this point to a close evolutionary relationship between these two house bat species but, based on the mtDNA data, it also implies that *S. leucogaster* and WB *nigritellus* should have very similar or even identical *zfy* sequences, given the much reduced mtDNA sequence divergence in comparison with the divergence between YB *nigritellus* and *S.* aff. *dinganii* (around 6%). The phylogenetic pattern inferred from *zfy* thus strongly contrasts with that from *cytb*, with WB *nigritellus* placed in a different position, albeit still paraphyletic with respect to YB *nigritellus*.

Data from *fgb*7 indicate a similar conflicting pattern to *cytb* as for *zfy*, with WB *nigritellus* differing substantially from both YB *nigritellus* and *S. leucogaster*. Omission of both indels from the network further increases the evolutionary relatedness between YB *nigritellus* and *S. leucogaster*, while WB *nigritellus* remains in a distant position. This pattern actually corroborates the *zfy* phylogeny, where WB *nigritellus* was placed in a paraphyletic position to YB *nigritellus* and *S. leucogaster*. The close, and basically unresolved, evolutionary relationship between YB *nigritellus* and *S. leucogaster* may be explained by the lower mutation rate and larger coalescence time of nuclear genes in comparison with mtDNA, which further stresses the distant position of WB *nigritellus*. Regarding the magnitude of divergence, the five substitutions that represent the difference between WB *nigritellus* and the other two species largely correspond to interspecific difference between the fruit bat species *E. gambianus* and *M. pusillus* (6 substitutions), and *E. gambianus* and *Epomops franqueti* (Tomes, 1860) (eight substitutions) on a proportionally longer fragment of *fgb*7 (700 bp) reported by Nesi *et al.* (2011).

Introgression of mtDNA: a likely explanation

Taxonomic evaluation of sister lineages based on the percentage value of mtDNA genetic divergence can be unreliable as it depends on recognition of true phylogenetic relationships that may be obscured by, for example, transfer of DNA between unrelated lineages via hybridization (Moore 1995; Nichols 2001; Galtier *et al.* 2009). This, in turn, strongly biases determination of threshold values of interspecific variation. A commonly used example of the lower interspecific threshold value in bats is based on the sequence divergence between the European serotine bats *Eptesicus serotinus* and *E. nilssonii* (Mayer & von Helversen 2001). These sympatric species have been shown to differ by as little as 0.7–1.4% in the NADH dehydrogenase subunit 1 gene, which strongly overlaps with their intraspecific variation. Such values roughly correspond with the *cytb* divergence of 1.2% recently reported by Artyushin *et al.* (2009). Large morphological differences between the species were originally explained as a rapid phenotype change over short evolutionary time (Mayer & von Helversen 2001). Recently, a more plausible explanation has been put forward for the mtDNA pattern observed in serotine bats. Based on the inferred paraphyletic position of Asian *E. nilssonii* to the European population and distant position of Asian *E. serotinus*, Artyushin *et al.* (2009) suggested that current mtDNA structure in European serotine bats was caused by ancient interspecific hybridization followed by fixation of local *E. nilssonii* mtDNA in the genome of alien *E. serotinus* following colonization of Europe.

Low genetic divergence between the morphologically distinct sister mtDNA lineages of *S. leucogaster* and WB *nigritellus* could be explained in a similar manner to the original serotine bat hypothesis of Mayer & von Helversen (2001). Indeed, the hypothesis is even more plausible in this case given that genetic divergence in the house bats (2.6–2.8%) is twice that of the serotine bats, signifying roughly twice as much evolutionary time for phenotypic differentiation. In our study, comparative data from a distant population were available for just one species of the sister pair, *S. leucogaster*. Despite a distance of ca. 2000 km between the localities in Senegal and Mauritania and those in Ghana and Benin, comparable to the area included in the serotine bat study of Artyushin *et al.* (2009), we found only limited mtDNA sequence variation and were unable to produce any alternative explanation. A reasonable interpretation of the mtDNA pattern can be inferred, however, from comparison with the nuclear data available in our study. The conflicting mtDNA and nucDNA signals clearly deny close evolutionary relatedness between WB *nigritellus* and *S. leucogaster* and support the hypothesis of ancient introgression of mtDNA.

As there are no known data for WB *nigritellus* from West Africa, we can only speculate about the possible evolutionary event that led to the hypothesized hybridization. Both species live in sympatry, and current morphological and genetic data suggest the existence of reproductive barriers between them. Historically, the species may have come into contact following changes in their distribution ranges owing to, for example, habitat shifts resulting from

climate change (Hewitt 2001). Prezygotic reproductive isolation, like size difference, may not have played a large role, as assumed from reports of European serotine bats or African fruit bats. Hybridization may have been further facilitated by karyotype compatibility, as both species bear the same chromosome number ($2n = 36$) and chromosome morphology (autosomal fundamental number NFa = 50) (Koubínová 2007).

Taxonomic implications

As mentioned earlier, the paraphyletic relationship between the yellow-bellied and white-bellied forms of tentatively identified *S. nigritellus*, inferred from *cytb* phylogeny and over 12% sequence divergence, indicates that these two forms actually represent separate species. Relationships inferred from both nuclear markers also support this conclusion. Morphologic comparison, which clearly indicates two distinct morphogroups of significantly different size and skull shape, provides yet further evidence for the specific distinctness of the two small-sized house bat forms. Additionally, the type specimen of *S. nigritellus* from Ghana was grouped within the YB *nigritellus* specimen morphogroup. This morphological similarity, as well as the molecular proximity of Senegalese specimens to those from Benin and Ghana, which can be regarded as almost topotypic, confirms the assignation of the yellow-bellied form to *S. nigritellus* de Winton 1899, beyond doubt. On the other hand, the taxonomic affiliation of the white-bellied form remains rather obscure.

Taxonomy of the genus *Scotophilus* has long been confused and controversial (e.g. Hayman & Hill 1971; Hill 1980; Robbins *et al.* 1985; Koopman 1994; Jacobs *et al.* 2006). Aside from the large *S. nigrita* (Schreber, 1774), African populations previously described under various names were synonymized under *S. borbonicus* (Geoffroy, 1803) in the first revision of the genus by Dobson (1878). Availability of this name for the African taxa was later advocated by Hill (1980), amongst others; however, this name is currently considered unavailable for all African house bats because of the unclear origin of the type specimen (S. Goodman, pers. comm.). Several other names have been suggested for African small-sized house bat populations at the lower range of the size spectrum (forearm 42–50 mm), which have been variously synonymized (see Robbins *et al.* 1985). In the last taxonomic revision of *Scotophilus* by Robbins *et al.* (1985), all populations of small-sized house bats were included into *S. viridis* (Peters, 1852), and the West African form *S. nigritellus* de Winton, 1899; was included as a junior synonym naming the respective West African populations as a subspecies. Only recently, a molecular phylogenetic analysis by Trujillo *et al.* (2009) revealed that the small-sized West African *S. viridis* actually represents an evolutionary lineage distinct from the Southern African S. *viridis* s. str., and should thus be recognized as a separate species, *S. nigritellus* de Winton, 1899.

Given the synonymy of *S. viridis* s.l. by Robbins *et al.* (1985), that is, including *nigritellus*, three other names are available for the white-bellied form *S.* aff. *nigritellus*: *S. altilis* Allen, 1914, described from the Blue Nile Valley in SE Sudan; and *S. murinoflavus* (von Heuglin, 1861) and *S. flavigaster* (von Heuglin, 1861) described from Eritrea. The status of the latter two species is currently obscure because of a lack of information on type specimens. The original descriptions, meanwhile, do not correspond with the white-bellied *S.* aff. *nigritellus* in pelage colouration or size. Rather, these two species conform to the East African *S. colias* (Thomas, 1904), as we discussed elsewhere (Vallo *et al.* 2011). The remaining name, *S. altilis*, applies to populations of the East African Sahel and conforms in pelage colouration to the white-bellied *S.* aff. *nigritellus* rather than to *S. nigritellus* s. str.. Unfortunately, as the type specimen of *S. altilis* was unavailable to us, we were unable to provide a definitive conclusion on taxonomic affiliation of the West African white-bellied small-sized house bat to this name. On the other hand, there appear to be some size differences between the West African (aff. *nigritellus*) and East African (*altilis*) populations, for example, forearm lengths of ca. 42 and 46 mm, respectively, which cannot be explained by phenotypic variation over large geographical distance (over 5000 km) without additional morphological and genetic information. A new name may still be needed for this form, therefore, which would raise the number of African *Scotophilus* species to twelve.

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Supporting Information

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

Fig. S1. Phylogenetic trees of *Scotophilus* based on the *zfy* sequences without indels recovered under maximum likelihood (A) and Bayesian (B) methods. Nodal support is given at the respective nodes.

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