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RNDr. Darina Koubínová

Phylogeny of selected African bat species based on the cytogenetic and molecular approaches

Fylogeneze vybraných druhů letounů Afriky na základě cytogenetického a molekulárního přístupu

Ph.D. Thesis

Supervisor: prof. RNDr. Jan Zima, DrSc.

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Supervisor: prof. RNDr. Jan Zima, DrSc. (Institute of Vertebrate Biology, Academy of Sciences, Czech Republic)

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Author's declaration

I hereby certify that I am the sole author of this dissertation thesis and that all the literature and other information sources used have been cited properly. Neither whole work nor its part has been used to obtain another or same academic degree.

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ABSTRACT

Phylogenetic relationships of a sample comprising 248 bats belonging to 19 species and four families (Hipposideridae, Rhinolophidae, Molossidae and Vespertilionidae) from Senegal (Western Africa) were investigated with the use of multi-locus sequence data and non-differentially stained chromosomes.

The karyotypes of *Hipposideros ruber*, *H. tephrus*, *H. jonesi* and *H. cyclops* were described for the first time. The standard *Hipposideros* formula was recorded in *H. tephrus*, *H. jonesi* and *H. ruber* (2n = 32, FNa = 60, FN = 64). The karyotypes of *H. cyclops* (2n = 36, FN = 66) and *H. gigas* (2n = 52, FN = 64) substantially diverged from this typical chromosomal complement.

Rhinolophus landeri and *R. fumigatus* shared the same diploid number (2n = 58), but differed in the chromosome morphology (*R. fumigatus* – FNa = 60, FN = 64; *R. landeri* – FNa = 64, FN = 68). *Rhinolophus landeri* was found karyotypically distinct to other African populations, thus signalling a possible presence of cryptic forms within this species.

The karyotypes of *Chaerephon pumilus* and *Mops condylurus* had a 2n = 48, FN = 54 and were similar to other previously studied species of this chromosomally conservative family.

Chromosomal, Bayesian, maximum likelihood and genetic distance analyses revealed an indication for the existence of cryptic forms among five out of ten examined species of the West African vespertilionid bats – *Pipistrellus hesperidus*, *Neoromicia nana, N. somalica, Scotoecus hirundo* and *Nycticeinops schlieffenii*. Additionally, based on the analyses of eight mitochondrial and nuclear genes and combination of the Senegalese bats and GenBank data, *Glischropus tylopus* was found basal to the clade of the East Asian pipistrelles and *Pipistrellus rueppellii* was basal to the whole *Pipistrellus*/*Nyctalus* clade, possibly deserving its own genus. *Eptesicus* was confirmed to be polyphyletic, with *E. nasutus* and *E. dimissus* being phylogenetically distinct to other representatives of *Eptesicus*. *Neoromicia* was confirmed to be diphyletic and Scotophilini appeared as the second most basal branch of all vespertilionids. The tribes Pipistrellini and Vespertilionini were defined differently than in recent discussions.

The detection of cryptic taxa, description of new karyotypes and proposals for new systematic arrangements demonstrate that our knowledge of (West-African) Chiroptera is still incomplete and that an investigation conducted on a small area can reveal new important findings, which can considerably contribute to our understanding of both biogeography and phylogeny.

ABSTRAKT

Fylogenetické vztahy byly zkoumány ve vzorku obsahujícím 248 netopýrů patřících do 19 druhů a čtyř čeledí (Hipposideridae, Rhinolophidae, Molossidae a Vespertilionidae) ze Senegalu (západní Afriky) s použitím multilokusových sekvenčních dat a nediferenciálně obarvených chromosomů.

Karyotypy *Hipposideros ruber*, *H. tephrus*, *H. jonesi* a *H. cyclops* byly popsány poprvé. Standardní formule rodu *Hipposideros* byla zaznamenána u *H. tephrus*, *H. jonesi* a *H. ruber* (2n = 32, FNa = 60, FN = 64). Karyotypy *H. cyclops* (2n = 36, FN = 66) a *H. gigas* (2n = 52, FN = 64) se nápadně lišily od této typické chromosomální sady.

Rhinolophus landeri a *R. fumigatus* měli shodný diploidní počet chromosomů (2n = 58), ale lišili se jejich morfologií (*R. fumigatus* – FNa = 60, FN = 64; *R. landeri* – FNa = 64, FN = 68). *Rhinolophus landeri* se karyotypově odlišoval od ostatních afrických populací, což může signalizovat přítomnost kryptických forem v rámci tohoto druhu.

Karyotypy *Chaerephon pumilus* a *Mops condylurus* obsahovaly 48 chromosomů (FN = 54), což odpovídá standardní sadě nalézané u příslušníků této čeledi.

Chromosomy, Bayesiánské metody, maximální pravděpodobnost a analýza genetických distancí naznačily existenci kryptických forem u pěti z deseti vyšetřených druhů západoafrických vespertilionidních netopýrů – *Pipistrellus hesperidus*, *Neoromicia nana*, *N. somalica*, *Scotoecus hirundo* a *Nycticeinops schlieffenii*. Na základě analýz osmi mitochondriálních a jaderných genů a kombinace sekvencí senegalských netopýrů s daty z genové banky (GenBank) bylo dále zjištěno, že *Glischropus tylopus* je bazální v kladu východoasijských pipistrelů a *Pipistrellus rueppellii* je bazální v celém kladu rodů *Pipistrellus*/*Nyctalus* a pravděpodobně zasluhuje oddělení do vlastního rodu. Byla potvzena polyfilie rodu *Eptesicus*, přičemž *E. nasutus* a *E. dimissus* byli fylogeneticky odlišní od dalších zástupců rodu *Eptesicus*. Dále byla potvrzena difylie rodu *Neoromicia* a tribus Scotophilini byl druhou nejbazálnější skupinou všech vespertilionidů. Triby Pipistrellini a Vespertilionini byly definovány odlišně od nedávných zjištění.

Detekce kryptických taxonů, popis nových karyotypů a návrhy na nové systematické uspořádání ukazují, že naše znalosti (západoafrických) letounů jsou stále neúplné a že šetření provedené na malém území je schopno odhalit nové důležité poznatky, které mohou výrazně přispět k našemu chápání biogeografie a fylogeneze.

LIST OF ABBREVIATIONS AND SYMBOLS USED:

♂ – male

♀ – female

Γ – gamma distribution

2n – diploid number of chromosomes

A – acrocentric

BA – Bayesian analysis

bp – base pair(s)

BS – bootstrap support

cytb – cytochrome *b*

DNA – deoxyribonucleic acid

FISH – fluorescence *in situ* hybridisation

FN – chromosomal arms number

FNa – autosomal arms number

GTR – general time reversible

I – proportion of invariable sites

M – metacentric

MCMC – Markov chain Monte Carlo

ML – maximum likelihood

nd1 – nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 1

PCR – polymerase chain reaction

PP – (Bayesian) posterior probability

RAG1 – recombination activating gene 1

RAG2 – recombination activating gene 2

RAxML – Randomized Axelerated Maximum Likelihood

RNA – ribonucleic acid

rRNA – ribosomal RNA

SM – submetacentric

ST – subtelocentric

Thr – threonine

tRNA – transfer RNA

Val – valine

1. INTRODUCTION

1.1. GENERAL INTRODUCTION

The evolutionary relationships among organisms have been studied by various approaches. The traditionally prevailing methods involve comparison of morphological characters and the first attempts to use cytogenetics for this purpose date back to the early 20th century, with the boom of comparative cytogenetics in the second half of the century. Despite of the fact that since the 1980s the molecular methods are widely applied in this field, the species delimitations, systematics and reconstruction of their evolutionary history are still largely based on morphology. However, current molecular systematics derived from the analyses of various markers (nucleic acids, proteins) shows that many of the conventionally recognised groups among all kinds of organisms are artificial, defined on the bases of convergent similarity of their representatives.

Morphological markers have been recognised as frequently biased, as they tend to be influenced by adaptations of organisms to habitat conditions, and the assessment of some characters depends on subjective judgments of observers. The information from non-differentially stained karyotypes is often insufficient for species determination and estimation of relationships, as the number of obtained characters is limited (number and morphology of chromosomes) and the fact that some taxa share (or do not share) the same values of these characters could be just a coincidence, leading to misinterpretations. The banding techniques are more convenient and accurate for phylogenetic studies; however, their usefulness for distant, extremely chromosomally conservative, or rapidly evolving taxa with highly rearranged karyotypes is restricted (Robinson, 2001; Volobouev *et al.*, 2002). These problems can be overcome by modern molecular cytogenetics and genomic methods (FISH, gene mapping, chromosome painting, flow-cytometry) (e.g. Murphy *et al.*, 2001; Robinson *et al.*, 2002); however, their appropriateness for phylogenetics is also limited, especially when not sensibly used (Dobigny *et al.*, 2004).

Nuclear acids appeared to represent the desired neutral marker; however, some later works showed that the unequivocalness of phylogenies based on molecular characters may be disputable. Therefore, cautious handling is recommendable and combination with several independent approaches (morphology, cytogenetics, ecology, combination of nuclear and mitochondrial data) should be used to obtain unbiased correct results (Pisani *et al.*, 2007; Nabhan and Sarkar, 2012). Contrastingly, genetic species concept, delimiting the

species according to their genetic distances (similarity of sequences) usually mainly of cytochrome *b* only, is currently being applied to describe cryptic species (Baker and Bradley, 2006). However, its constraints resulting from the observed incongruences between maternally and paternally inherited genes have been identified, and the combination with other approaches has also been suggested for correct crypsis evaluation (Clare, 2011).

Chiroptera belongs to one of the most complicated orders among mammals from the phylogenetic and systematic point of view, mainly because of the plethora of extant species (over 1000) and frequent occurrence of morphologically undistinguishable forms (Simmons, 2005). However, closely related, phenotypically similar species, can differ with respect to their genetics or ecology, and, reciprocally, morphologically distinct species can be genetically very similar (Bickham, 1979; Barratt *et al.*, 1997; Jones and Barlow, 2004; Thabah *et al.*, 2006).

In the Palaearctic region or Australia, the chiropterologists' effort was always relatively intensive; however, new detections of cryptic taxa and revisions of traditional phylogenetic hypotheses are an increasingly common phenomenon, especially due to the application of molecular methods (e.g. Ibáñez *et al.*, 2006; Reardon *et al.*, 2008; Kruskop *et al.*, 2012). In comparison to this, Africa, where large geographic areas remain undersampled, the knowledge of the phylogeny and systematics reveals as highly incomplete. The number of ecological niches available in the tropics and subtropics of the African continent allows evolution and existence of greater species diversity than in higher latitudes, thus indicating potentially a high number of cryptic species still hidden to the science.

The sub-Saharan region of Western Africa belongs to one of the most underexplored areas of the continent. It is currently being threatened by deforestation, fragmentation of wooded savannah and desertification. Systematic (re)classification and investigation of the phylogenetic relationships of the local fauna are thus important not only for ordinary updating of species check-lists, but moreover for our understanding of general principles of ecology, evolution, biogeography and even (or maybe above all) for practical reasons, i.e. conservation of (rare) species endangered by a habitat loss. From this point of view, the examination of the phylogeny of the (West) African bats can be considered a challenging, cutting-edge field of the zoological research.

The main aims of this dissertation thesis were to assess the phylogenetic relationships of selected Senegalese (West-African) bat species belonging to four families

– Hipposideridae, Rhinolophidae, Vespertilionidae and Molossidae, and assess the diversity of the West-African bat fauna, specifically in the Niokolo-Koba National Park. For this purpose, cytogenetic and molecular approaches were chosen and the results were evaluated in a broader context of other (African) chiropteran populations.

The dissertation represents a part of the project "Species diversity and ecology of selected West African vertebrates" focused on the fauna of Niokolo-Koba and funded by the Grant Agency of the Academy of Sciences of the Czech Republic.

1.2. PHYLOGENY OF CHIROPTERA

1.2.1. Brief overview of current trends in systematics, cytogenetics and phylogenetics of Chiroptera

Chiroptera is the second most numerous order among mammals and currently is considered a monophyletic group, belonging together with Eulipotyphla, Cetartiodactyla, Perissodactyla, Carnivora and Pholidota to Laurasiatheria (Nery *et al.*, 2012). It comprises two suborders, and one of the most recent suggestions for their denomination is as Pteropodiformi and Vespertilioniformi (Van Cakenberghe and Seamark, 2012). Pteropodiformi should comprise family Pteropodidae (fruit bats, formerly Megachiroptera) and their closely related families of echolocating bats Hipposideridae, Rhinolophidae, Megadermatidae, Rhinopomatidae and Craseonycteridae. Vespertilioniformi should include all the remaining chiropteran families (previously referred to mainly as Microchiroptera) (see reviews and discussions about alternative classifications, i.e. Yinpterochiroptera and Yangochiroptera, or Pteropodiformes and Vespertilioniformes in Simmons, 2005; Teeling *et al.*, 2005; Hutcheon and Kirsch, 2006).

Information about chromosomes of selected groups of bats was summarised in several publications (Zima and Horáček, 1985; Zima *et al.*, 1992; O'Brien *et al.*, 2006); however, comprehensive overviews of certain geographic regions (e.g. subtropics and tropics of Africa, southeastern Asia) are still missing. Chromosomal data have been used surprisingly rarely for phylogenetic studies (e.g. Volleth and Heller, 1994; Volleth *et al.*, 2002; Volleth *et al.*, 2006; Ao *et al.* 2007), maybe because of the previous assumption, that karyotypic variability in bats occurs quite scarcely, compared to e.g. rodents or shrews (Franchini *et al.*, 2008; White *et al.*, 2010) and that the examination of non-differentially stained karyotypes therefore does not facilitate species assignment, phylogenetic

reconstructions and detection of cryptic species (Baker, 1970). However, despite of the known limitations, in several cases, especially, when using banding, FISH (fluorescence *in situ* hybridisation) or molecular cytogenetics and genomic techniques, the chromosomes were found as useful and easy tools, even enabling to estimate the evolution of whole genera and families, many times achieving the same results as molecular approaches (Volleth *et al.*, 2002, 2011).

Initially, the molecular studies of Chiroptera were targeted more at interordinal and interfamilial relationships or supertrees (Jones *et al.*, 2002; Teeling *et al.*, 2005), at the present time, the focus is concentrated rather on intrageneric and intrafamilial problematics, revisions of morphology-based systematic hypotheses, phylogeography, comprehensive reviews of bat fauna in selected geographic regions or comparative genomics (e.g. Ammerman *et al.*, 2012; Furman *et al.*, 2013; Meganathan *et al.*, 2012).

1.2.2. Cytogenetic and phylogenetic studies in African bats

In comparison to the Holarctic region (Baker and Patton, 1967; Zima and Horáček, 1985; Zima *et al.*, 1992), attempts to compile comprehensive lists of African chiropteran karyotypes are rare (Peterson and Nagorsen, 1975; Haiduk *et al.*, 1980, 1981), and the same is valid for phylogeny. While in other regions extensive phylogenetic studies including many species were performed (Hoofer *et al.*, 2006), in Africa the investigation is focused mainly on phylogeography or descriptions of new species (e.g. Lamb *et al.*, 2008; Vallo *et al.*, 2013). Information about new findings in African bats is being gathered in the African Chiroptera Reports (yearly) and African Bat Conservation News (quarterly) (both at www.africanbats.org), and an up-to-date comprehensive systematic overview has been published recently (Happold and Happold, 2013).

In contrast with rodents (Granjon *et al.*, 1992; Brouat *et al.*, 2007), the studies of bats in Senegal were focused mainly on species inventory and description of their morphological characters or biology (Aellen, 1956; Dorst, 1960; Adam and Hubert, 1972; Verschuren, 1982; Lelant and Chenaval, 2011). The first cytogenetic and molecular studies have been performed only recently (Koubínová *et al.*, 2007; Vallo *et al.*, 2008, 2011, 2013).

1.3. STUDY ORGANISMS

Bat species belonging to four families have been studied: Hipposideridae, Rhinolophidae, Vespertilionidae and Molossidae. They are presented in a different order (Vespertilionidae as the last one) than is the systematic consensus for the needs of the logical continuity of the thesis.

1.3.1. Hipposideridae

The family Hipposideridae includes more than 80 tropical and subtropical Old World species belonging to ten genera (Simmons, 2005; Van Cakenberghe and Seamark, 2012). With about 70 species, *Hipposideros* represents the largest genus within the family (Bates *et al.*, 2007). Until now there have been relatively many taxonomic reviews including the hipposiderids (e.g. Tate, 1941a; Hill, 1963; Rosevear, 1965; Koopman, 1994; Simmons, 2005), but only few phylogenetic studies (morphological characters – Bogdanowicz and Owen, 1998; molecular data – e.g. Wang *et al.*, 2003; Vallo *et al.*, 2008; Thong *et al.*, 2012).

In Africa, the hipposiderid bats are represented with 25 species belonging to five genera, and 16 species from this total number are assigned to *Hipposideros* (Van Cakenberghe and Seamark, 2012). Recently, new African genus and tribe have been defined within Hipposideridae (Benda and Vallo, 2009) and two new species have been described from the Seychelles and Madagascar (Goodman and Ranivo, 2008, 2009).

Up to date, karyotypes have been analysed in about 25 species of the hipposiderid bats (partial summary in Sreepada *et al.*, 1993), most of which had a diploid number of chromosomes 2n = 32 (all biarmed) and the fundamental number of autosomal arms FNa = 60. So far, karyotypes were reported for only five species occurring in Africa (Baker *et al.*, 1974; Đulić and Mutere, 1974; Peterson and Nagorsen, 1975; Rautenbach *et al.*, 1993; Porter *et al.*, 2010) and the DNA sequence information is available for about 15 species of this continent (e.g. Hoofer and Van Den Bussche, 2003; Eick *et al.*, 2005; Matthee *et al.*, 2006; Lim, 2007; Vallo *et al.*, 2008, 2011).

1.3.2. Rhinolophidae

The rhinolophid bats consist of approximately 80 species belonging to the single

genus *Rhinolophus* and are widely distributed in the Old World (Simmons, 2005). In Africa, 27 extant species have been recorded until now (Van Cakenberghe and Seamark, 2012).

Karyotypic investigations of approximately 40 species have shown that most have a 2n of 58 or 62, with the whole range being from 32 to 66 (Zima *et al.*, 1992). Fourteen species out of the total analysed number occur in Africa (review in Van Cakenberghe and Seamark, 2012). For about 22 African species DNA sequence information is available (e.g. Hoofer and Van Den Bussche, 2003; Lim, 2007; Rossiter *et al.*, 2007; Puerma *et al.*, 2008; Taylor *et al.*, 2012), but there have been only few molecular phylogenetic studies focused exclusively on species of this continent (e.g. Taylor *et al.*, 2012). Seven new species have been described recently from the eastern, southern and western part of Africa (Kock *et al.*, 2000; Cotterill, 2002; Fahr *et al.*, 2002; Taylor *et al.*, 2012).

1.3.3. Molossidae

Systematists recognise currently approximately 110 species of molossid bats belonging to 16 genera and two subfamilies – Molossinae and Tomopeatinae (Simmons, 2005). The representatives of this fourth largest bat family inhabit tropical and subtropical regions around the world. In Africa, 8 genera and 43 species are recognised, thus representing almost a half of the entire diversity of the family (Van Cakenberghe and Seamark, 2012). Chromosomal data are available for about 50 species (e.g. Smith *et al.*, 1986; Rautenbach *et al.*, 1993), and 21 of these are African (Van Cakenberghe and Seamark, 2012). In the majority of species, the karyotype consists of 48 chromosomes (e.g. Warner *et al.*, 1974).

Comprehensive phylogenetic study of the whole group based on molecular data has been performed for the first time only very recently (Ammerman *et al.*, 2012). In Africa, DNA sequences are available for 20 species, which have been used in several, mainly phylogeographic, studies (e.g. Hoofer and Van Den Bussche, 2003; Jacobs *et al.*, 2004; Lamb *et al.*, 2006, 2008, 2011; Ratrimomanarivo *et al.*, 2007, 2009a,b; Taylor *et al.*, 2009; Goodman *et al.*, 2010). New (cryptic) species for the African region have been discovered using molecular techniques in the last decade mainly on islands in the Indian Ocean (Madagascar, Mascarene, Pemba) and southern Africa (Goodman and Cardiff, 2004; Ratrimomanarivo *et al.*, 2007, 2009a,b; Goodman *et al.*, 2008; Stanley, 2009; Taylor *et al.*, 2009; Goodman *et al.*, 2010).

1.3.4. Vespertilionidae

With approximately 48 genera and more than 400 species, the vespertilionid bats represent the largest chiropteran family (Simmons, 2005). They are widely distributed around the world; however, the highest diversity occurs in the tropics (Simmons, 2005) and approximately one fourth of the whole vespertilionid species number is found in Africa (106 species, 16 genera; Van Cakenberghe and Seamark, 2012).

There have been many attempts of systematists to perform more or less comprehensive taxonomic revisions both with traditional morphologically based (e.g. Miller, 1907; Tate, 1941b, 1942; Koopman, 1994; Simmons and Geisler, 1998) or cytogenetic approaches (review in Zima and Horáček, 1985; Volleth and Heller, 1994; Volleth *et al.*, 2001). The karyotypes are rather diverse (with the exception of *Myotis*) with the 2n being about 26-50 (Zima and Horáček, 1985). Several dozens of African species have been studied karyologically (about 40; Van Cakenberghe and Seamark, 2012); however, the number is still low compared to the total number of occurring species.

Nucleotide sequences are available for more than 60 African species (e.g. Hoofer and Van Den Bussche, 2003; Stadelmann *et al.*, 2004; Mayer *et al.*, 2007; Vallo *et al.*, 2013, Monadjem *et al.*, 2013) and many new species have been detected, mainly with the use of molecular data, in the last years (e.g. Bates *et al.*, 2006; Goodman *et al.*, 2012, Monadjem *et al.*, 2013; comprehensive review in Van Cakenberghe and Seamark, 2012).

In comparison to other families in this study, the vespertilionid bats have been investigated relatively extensively with phylogenetic methods using DNA sequencing (e.g. Ruedi and Mayer, 2001; Lack *et al.*, 2010; Roehrs *et al.*, 2010, 2011).

1.4. STUDY AREA

Senegal is the westernmost country of the continental Africa with an area of 196, 190 sq km. The landscape consists of low plains or plateaus and hills. The climate is tropical, transitional between arid in the north and humid in the south. The north part is covered with vegetation of Sahel (wooded, bushy, or grassland savannah), a transitional semiarid zone between the Sahara desert in the north and the Sudan, i.e. the tropic forests in the south. In the southern part of Senegal there are mangroves, dense gallery forests or bamboo areas (Frederiksen and Lawesson, 1992).

Niokolo-Koba is the oldest and largest national park of Senegal. It was established in

1954 (in 1926 as a Hunting Reserve), covers approximately 9,130 sq km and belongs to the most important protected areas of Western Africa. Since 1981 it is inscribed on the List of World Heritage of UNESCO and since 2007 on the List of World Heritage in Danger. Vegetation is formed mainly by bush or woodland or grass savannah (ecoregion West Sudanese and Sudano-Guinean savannah), dry or gallery forests, bamboos and savannah grass floodplains among the banks of water bodies (Guinean forest savannah mosaic) (Madsen and Sambou, 1998). The climate is tropical, with periodical changes between rainy (May – October) and dry seasons, the average annual rainfall ranging between 800 and 1300 mm, and with a mean daily temperature of about 40 °C at its maximum (Trape *et al.*, 1996). The environment of the park is threatened by bush fires, drying up of ponds, population growth and intrusions of domesticated animals.

2. AIMS OF THE STUDY

The general aims of the dissertation thesis were to examine phylogenetic relationships within four chiropteran families (Hipposideridae, Rhinolophidae, Molossidae and Vespertilionidae) based on cytogenetic and/or molecular approaches, focused on selected species obtained from West Africa, Senegal.

Particularly, following specific aims were set:

1) to investigate non-differentially stained karyotypes of five species of hipposiderid bats, *Hipposideros cyclops*, *H. jonesi*, *H. gigas*, *H. ruber* and *H. tephrus,* and to compare the results with other congeneric species and reports from other African regions

2) to analyse non-differentially stained karyotypes of two species of rhinolophid bats, *Rhinolophus fumigatus* and *R. landeri*, and to compare the results with the data available from geographically distinct African populations

3) to examine non-differentially stained karyotypic data of two species of molossid bats, *Chaerephon pumilus* and *Mops condylurus*, and to compare them with the results obtained from other parts of Africa

4) to reconstruct phylogenetic relationships within selected representatives of the family Vespertilionidae, with the use of combined cytogenetic and molecular data

5) to assess genetic divergence of ten West African (Senegalese) vespertilionid species to other geographically distant populations, and potentially uncover cryptic taxa, gaining support from both genetic approaches

The thesis is based on three scientific works (2 published papers and 1 submitted manuscript), hereafter referred to as Supplements 1–3, in which the partial aims described above are followed.

Koubínová, **D**, Sreepada, KS, Koubek, P, Zima, J, 2010. Karyotypic variation in

rhinolophid and hipposiderid bats (Chiroptera: Rhinolophidae, Hipposideridae). Acta Chiropterol, 12:393–400. (Supplement 1, solving aims no. 1 and 2)

Sreepada, KS, **Koubínová, D,** Konečný, A, Koubek, P, Ráb, P, Rábová, M, Zima, J, 2008. Karyotypes of three species of molossid bats (Molossidae, Chiroptera) from India and western Africa. Folia Zool, 57:347–357. (Supplement 2, aim no. 3)

Koubínová, **D**, Irwin, N, Hulva, P, Koubek, P, Zima, J. Hidden diversity in Senegalese bats and associated findings in the systematics of the family Vespertilionidae. Front Zool (submitted) (Supplement 3, aims no. 4 and 5)

3. MATERIAL AND METHODS

The chiropteran samples were obtained in Senegal (Western Africa) between the years 2004 and 2008 by P. Koubek, J. Červený and others. 18 collection sites were located mainly in the Niokolo-Koba National Park and adjacent areas in the southeastern part of the country, two others were in the centre of the western coast and one in the north, close to the borders with Mauritania (Fig. 1).

The preliminary species determinations based on morphological characters were done according to the keys of Rosevear (1965), Kingdon (1997) and (Van Cakenberghe and Seamark, 2012); however, the nomenclature used follows Simmons (2005), unless revised by more recent taxonomic assessments (e.g. Lack *et al.*, 2010; Roehrs *et al.*, 2010, 2011; Van Cakenberghe and Seamark, 2012). The species determination of the hipposiderid and rhinolophid bats was further assessed by cytochrome *b* analysis done by Peter Vallo.

Altogether samples of 248 specimens were analysed in this study – 16 of hipposiderid, 2 of rhinolophid, 17 of molossid and 213 of vespertilionid bats (Table 1).

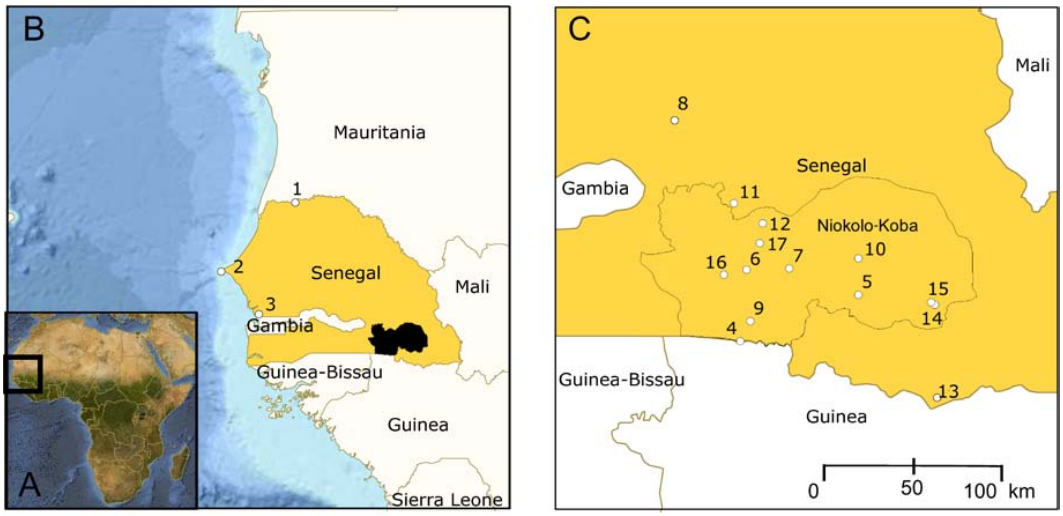


Fig. 1. Sampling localities. The vespertilionid bats were collected on localities 1-16. Sampling of members of other families is indicated after the locality name in brackets with a respective abbreviation (***H*** – Hipposideridae, ***R*** – Rhinolophidae, ***M*** – Molossidae). A – map of Africa indicating the location of Senegal; B – Senegal with the position of the Niokolo-Koba National Park (black) and the sampling localities: 1 – Mbilor, 2 – Hahn-Dakar, 3 – Bacadadgi; C – Niokolo-Koba National Park: 4 – Gué de Sambaillou (***M*****),** 5 – Mont Assirik, 6 – Simenti (***R***, ***H***, ***M***) + Camps de Lions, 7 – Lengué Kountou (***H*****),** 8 – Tambacounda + Tambacounda-Parc National de Niokolo-Koba, 9 – Dalaba, 10 – Niokolo (***M***), 11 – Niériko + Niériko-bridge, 12 – Dar Salam (***H***, ***M*****),** 13 – Dindéfélo (***R***) + Dindéfélo II (***H*** **–** Dindéfélo, Daudí Cave**),** 14 – Mako-Camp, 15 – Mako, 16 – Gué de Damantan, 17 – Badi (***H***). Numbers 6, 8, 11 and 13 show two very closely located sites, presented as one point. The map was created with www.planiglobe.com.

Family	Species	Locality	Collected samples
Hipposideridae	*Hipposideros cyclops* (Temminck, 1853)	17	1♀
	Hipposideros gigas (Wagner, 1845)	12	2♀
	Hipposideros jonesi Hayman, 1947	13	1♂
	Hipposideros ruber (Noack, 1893)	6, 7, 12, 13	7♀
	Hipposideros tephrus Cabrera, 1906	6, 13, 17	2♂, 3♀
Rhinolophidae	*Rhinolophus fumigatus* Rüppel, 1842	13	1♂
	Rhinolophus landeri Martin, 1838	6	1♀
Molossidae	*Chaerephon pumilus* (Cretzschmar, 1826)	6, 12	6♂, 7♀
	Mops condylurus (A. Smith, 1833)	4, 6, 10	3♂, 1♀
Vespertilionidae	*Glauconycteris variegata* (Tomes, 1861)	5	1♀
	Myotis bocagii (Peters, 1870)	4	1♂
	Neoromicia capensis (A. Smith, 1829)	5	1♂
	Neoromicia nana (Peters, 1852)	2, 3, 4, 5, 6, 7, 10, 11, 13, 14, 16	54♂, 44♀
	Neoromicia rendalli (Thomas, 1889)	6	2♂
	Neoromicia somalica (Thomas, 1901)	3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15	58♂, 35♀
	Nycticeinops schlieffenii (Peters, 1859)	8, 9, 10, 11, 12, 13	7♂, 1♀
	Pipistrellus hesperidus (Temminck, 1840)	5, 6, 7	4♂, 2♀
	Pipistrellus rueppellii (Fischer, 1829)	1	2♂
	Scotoecus hirundo (de Winton, 1899)	11	1♂

Table 1 List of specimens sampled in Senegal. The numbers of localities correspond to those used in Fig. 1. ♂ – male, ♀ – female.

3.1. CYTOGENETIC ANALYSES

The chromosome preparation was done by collectors in the field following the bone marrow direct methods modified after Baker (1970). Altogether 83 individuals belonging to 15 species and four families were analysed (Hipposideridae – *Hipposideros ruber*, *H. tephrus*, *H. jonesi*, *H. cyclops*, *H. gigas*; Rhinolophidae – *Rhinolophus landeri*, *R. fumigatus*, Vespertilionidae – *Scotoecus hirundo*, *Neoromicia nana*, *N. rendalli* and *N. somalica*, *Nycticeinops schlieffenii*, *Pipistrellus hesperidus*; Molossidae – *Mops condylurus*, *Chaerephon pumilus*). The numbers of specimens analysed in each family are the same as the total numbers above, with the only difference being the vespertilionid family, where only 48 (out of 213) specimens were examined. Karyotypes from selected standard non-differentially Giemsa stained chromosomal complements were assembled with the software for cytogenetic analysis Ikaros (MetaSystems GmbH, Germany). The classification of chromosomes according to the position of the centromere followed standard criteria (Hsu and Benirschke, 1967-1977). Numerous attempts for the chromosome banding failed, probably because of the unsuitable field conditions during the preparation and long storage of slides resulting in their inappropriate ageing.

3.2. MOLECULAR AND PHYLOGENETIC ANALYSES

The molecular analyses were performed from the alcohol preserved tissue samples of 213 vespertilionid bats. DNA extraction, PCRs and sequencing were performed using standard procedures, adjusted for our specific needs (see Supplement 3 for details). Altogether six mitochondrial (cytochrome *b* + $tRNA^{Thr}$ – primers newly designed – see Supplement 3; *12S* + $tRNA^{Val}$ + *16S* – primers from Springer *et al.*, 1995; NADH dehydrogenase subunit 1 – primers from Mayer *et al.*, 2007) and two nuclear (recombination activating gene 1 and 2 – primers for both from Teeling *et al.*, 2000) genes were analysed. Additional sequences of vespertilionid and other bats used for outgroups (representatives of the families Cistugonidae, Miniopteridae, Molossidae and Natalidae) were downloaded from GenBank.

Geneious Pro version 5 (Drummond *et al.*, 2010) software package was used for sequence assembling, editing, aligning and computations of genetic distances. The Bayesian phylogenetic analyses (BA) were performed using MrBayes software v. 3.1.2 and 3.2.0 (Huelsenbeck and Ronquist, 2001) and the maximum likelihood (ML) analyses were

performed with RAxML v 7.3.0 (Stamatakis, 2006) (see Supplement 3 for the settings used).

Altogether, 15 phylogenetic computations were performed. Cytochrome *b* and $tRNA^{Thr}$ sequences of all Senegalese vespertilionid specimens with the data from GenBank were analysed with ML and BA in order to reexamine the preliminary assessment based on morphological and cytogenetic characters and/or to detect possible cryptic individuals. Subsequently, 20 representative specimens (up to three per each of the ten Senegalese species) were chosen for additional phylogenetic computations. Single-gene datasets, concatenated nuclear genes and combination of the *12S* + $tRNA^{Val}$ + *16S* genes were computed with ML only (7 different analyses together). Concatenated datasets of mitochondrial genes, of published data used for testing of the phylogenetic position of *Pipistrellus rueppellii* (*nd1*, Mayer *et al.,* 2007) and of all eight genes (*cytb*, $tRNA^{Thr}$, *12S*, $tRNA^{Val}$, *16S*, *RAG1*, *RAG2*, *nd1*) were analysed with both BA and ML (for the comprehensive description of sequences and datasets see Supplement 3).

4. RESULTS

4.1. CYTOGENETIC ANALYSES

The karyotypic data were obtained in 15 species (overview in Table 2). Only the standard description of the chromosomal number (diploid number of chromosomes – 2n) and morphology (number of autosomal arms – FNa, number of chromosomal arms – FN) was provided, because differential staining was not possible to perform (see Material and Methods).

4.1.1. Hipposideridae

A typical hipposiderid karyotype was found in *H. ruber*, *H. tephrus* and *H. jonesi*: 2n = 32, FNa = 60, FN = 64, including 30 biarmed autosomes – four metacentric, eight submetacentric and three subtelocentric pairs. The X sex chromosome was a large metacentric element, the Y chromosome a medium submetacentric. A secondary constriction was found near the centromere in one pair of the medium sized submetacentric autosomes (no. 9).

Deviations from the standard hipposiderid karyotype were recorded in *H. cyclops*

(2n = 36, FNa = 62) and *H. gigas* (2n = 52, FNa = 60). The karyotype of *H. cyclops* comprised 15 meta- or submetacentric, and three small acrocentric pairs. The X was estimated as a biarmed element, because only the female karyotype was examined (Fig. 2A). The chromosomal complement of *H. gigas* included a pair of large metacentrics, three pairs of medium-sized submetacentrics, one pair of small submetacentrics, one pair of small metacentrics, and 20 pairs of acrocentrics. The two smallest acrocentric pairs were dot-like. No male specimen was available, but the X chromosome was tentatively identified as a medium sized biarmed element.

4.1.2. Rhinolophidae

A difference between the karyotypes of *Rhinolophus landeri* and *R. fumigatus* (both 2n = 58) was found in the morphology of chromosomes. *R. landeri* had four pairs of biarmed autosomes, three pairs of which were medium to small sized meta- or submetacentrics, and one was a medium sized subtelocentric. The other 24 pairs were a gradated series of acrocentrics. The X chromosome was determined only based on the comparison with other rhinolophid karyotypes, because only one female was analysed, and it is probably a large subtelocentric element (FNa = 64). The karyotype of *R. fumigatus* consisted of two pairs of small meta- and submetacentric and 26 acrocentric pairs of autosomes. The X was a medium sized subtelocentric, and the Y was dot-like (FNa = 60). There was an achromatic gap near the centromere of the medium sized acrocentric pair no. 15 (Fig. 2B).

4.1.3. Molossidae

The diploid number of chromosomes of all 13 specimens of *Chaerephon pumilus* and 4 specimens of *Mops condylurus* examined in Senegal was 2n = 48 and the fundamental number of autosomal arms (FNa) was approximately 54. One pair of autosomes was large metacentric, three pairs medium sized meta- to submetacentric and 19 pairs medium to small acrocentric. In some acrocentric pairs, short chromosomal arms were visible, but these were not included into the FNa number. The X chromosome was medium sized metacentric and the Y small acrocentric.

4.1.4. Vespertilionidae

In the chromosomal complement of *Neoromicia somalica* (2n = 28, FN = 54, FNa = 50), there were nine large metacentric and submetacentric, one medium-sized submetacentric, two small subtelocentric and one small acrocentric pairs of chromosomes. The X chromosome was a medium-sized metacentric, and the Y was a small submetacentric. A secondary constriction was detected on one pair of large submetacentric autosomes (Fig. 2C).

The karyotype of *Neoromicia rendalli* (2n = 38, FN = 54, FNa = 50) consisted of six large metacentric and submetacentric, one small subtelocentric and eleven acrocentric pairs of chromosomes. The X chromosome was medium-sized submetacentric, and the Y was dot-like (probably acrocentric). A secondary constriction was found on a pair of acrocentric chromosomes.

Neoromicia nana (2n = 34, FN = 54, FNa = 50) had eight large pairs of biarmed elements (one large pair of submetacentric chromosomes with a conspicuous secondary constriction), one small submetacentric pair and seven small acrocentric pairs. The X chromosome was a medium-sized subtelocentric, the Y was dot-like, probably acrocentric (Fig. 2D).

The karyotype of *Scotoecus hirundo* (2n = 30, FN = 50, FNa = 46) comprised six large metacentric and submetacentric, three medium-sized biarmed (submetacentric and subtelocentric), and five acrocentric autosomal pairs of chromosomes. The X chromosome was a medium-sized metacentric, and the Y dot-like, probably acrocentric (Fig. 2E).

In *Nycticeinops schlieffenii* (2n = 34, FN = 56, FNa = 52), seven metacentric and submetacentric, one medium large metacentric, two small metacentric and six acrocentric pairs of chromosomes were found. A distinct secondary constriction was recorded on the largest acrocentric pair. The X chromosome was a medium metacentric, the Y chromosome was dot-like.

The complement of *Pipistrellus hesperidus* (2n = 46, FN = 62, FNa = 58) included three pairs of large metacentrics, two smaller submetacentrics, two small submetacentrics and 15 acrocentrics (one pair with a secondary constriction). The X chromosome was medium-sized metacentric, the Y chromosome was dot-like (Fig. 2F).

Family	Species	2n	FNa	FN	X	Y	Specimens analysed
Hipposideridae	*Hipposideros cyclops*	36	(62)	66	(M/SM)	-	1♀
	Hipposideros gigas	52	(60)	64	(M/SM)	-	2♀
	Hipposideros jonesi	32	60	64	M	SM	1♂
	Hipposideros ruber	32	(60)	64	(M)	-	7♀
	Hipposideros tephrus	32	60	64	M	SM	2♂, 3♀
Rhinolophidae	*Rhinolophus fumigatus*	58	60	64	ST	D	1♂
	Rhinolophus landeri	58	(64)	68	(ST)	-	1♀
Molossidae	*Chaerephon pumilus*	48	54	58	M	A	6♂, 7♀
	Mops condylurus	48	54	58	M	A	3♂, 1♀
Vespertilionidae	*Neoromicia nana*	34	50	54	ST	D (A)	10♂, 6♀
	Neoromicia rendalli	38	50	54	SM	D (A)	2♂
	Neoromicia somalica	28	50	54	M	SM	13♂, 7♀
	Nycticeinops schlieffenii	34	52	56	M	D	4♂, 1♀
	Pipistrellus hesperidus	46	58	62	M	D	3♂, 1♀
	Scotoecus hirundo	30	46	50	M	D (A)	1♂

Table 2 Synoptic list of karyotypic characteristics of the 15 species from Senegal and numbers of specimens examined. 2n – diploid number, FNa – number of autosomal arms, M – metacentric, SM – submetacentric, ST – subtelocentric, A – acrocentric, D – dot-like chromosome, X, Y – morphology of the sex chromosomes, ♂ – male, ♀ – female. The brackets indicate the cases when only female karyotypes where analysed and therefore the identification of the X chromosomes and the FNa values could be only assumed.

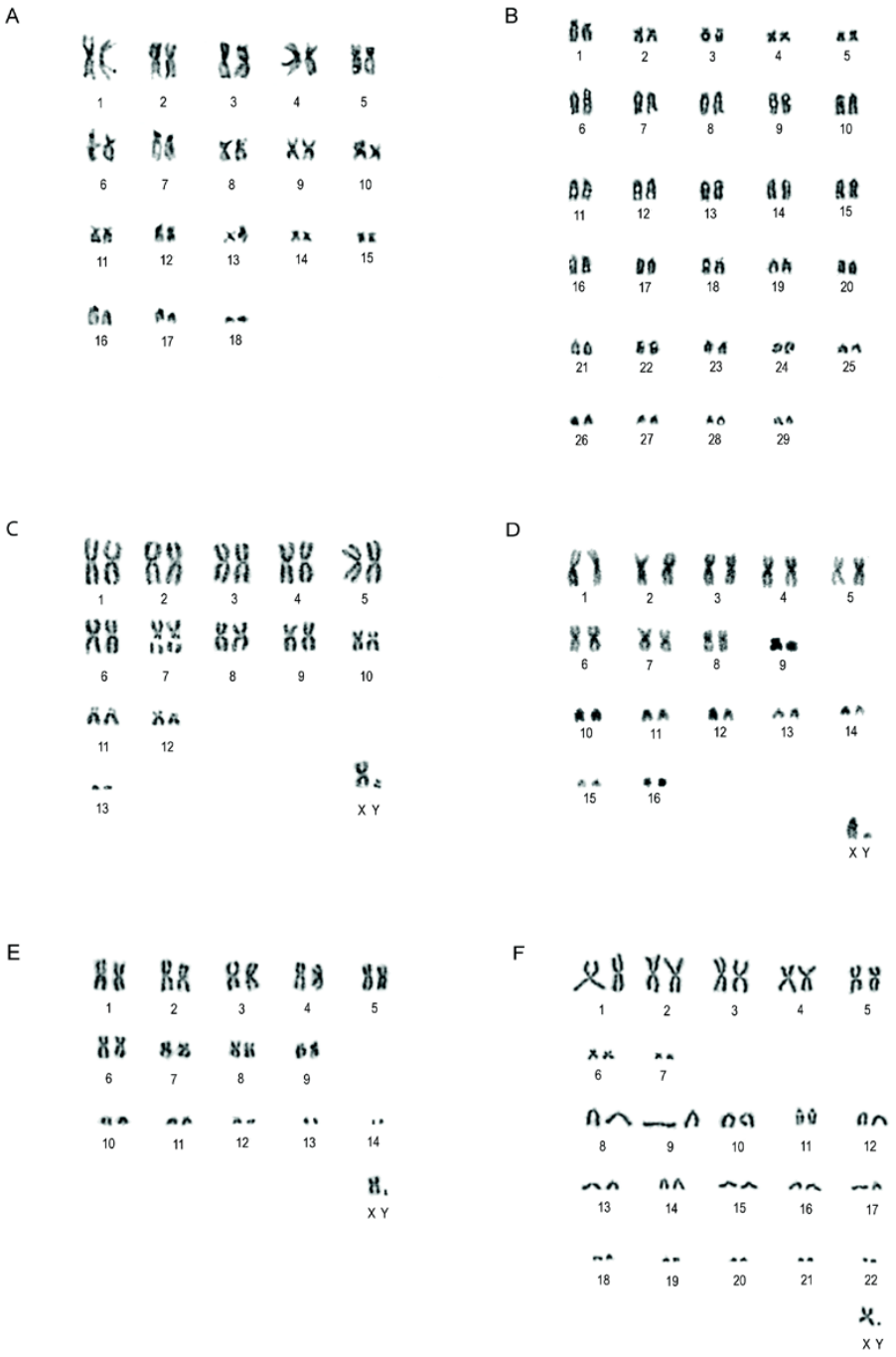


Fig. 2. Karyotypes of one hipposiderid, one rhinolophid and four vespertilionid species studied in Senegal. Only the karyotypes substantially differing from conservative complements or previous publications (indication for cryptic species) and the newly described karyotypes are presented. A – *Hipposideros cyclops*, IVB S747, female; B – *Rhinolophus landeri* IVB S110, female; C – *Neoromicia somalica*, IVB S1209, male (secondary constriction on the seventh pair of chromosomes); D – *N. nana*, IVB S1210, male (secondary constriction in the pair number eight); E – *Scotoecus hirundo*, IVB S1480, male; F – *Pipistrellus hesperidus*, IVB S592, male (secondary constriction in the eleventh pair of chromosomes).

4.2. MOLECULAR PHYLOGENY OF THE VESPERTILIONID BATS

4.2.1. Senegalese specimens

The initial analysis based on *cytb* and *tRNA^{Thr}* separated the samples from Senegal into ten different clusters (*Myotis bocagii*, *Neoromicia nana*, *N. somalica*, *N. capensis*, N. *rendalli*, *Pipistrellus hesperidus*, *P. rueppellii*, *Scotoecus hirundo*, *Nycticeinops schlieffenii*, *Glauconycteris variegata*), with the most numerous being *N. somalica* and *N. nana*. Some taxa (*Pipistrellus hesperidus*, *Nycticeinops schlieffenii*, *Scotoecus hirundo*, *Neoromicia nana* and *N. somalica* appeared notably phylogenetically distinct from their conspecifics from other populations obtained from GenBank (see the map in Fig. 3 showing the geographic origin of the specimens). The genetic distances were significantly large to consider the Senegalese forms separated (e.g. 5-13 % for *cytb*).

Subsequent ML and BA analyses of the single-gene, mitochondrial, nuclear and all eight-genes datasets showed *Pipistrellus rueppellii* as well supported on a long branch basal to the *Pipistrellus*/*Nyctalus* clade both in datasets based on our data combined with GenBank sequences and on a dataset completely obtained from Mayer *et al.* (2007) (see Fig. 3 for the tree based on all eight genes and Supplementary Material of Supplement 3 for the remaining trees). *Scotoecus hirundo* was basal to this whole group. *Pipistrellus hesperidus* was clearly distinct from *P. kuhlii*. *Neoromicia nana* was sister to *N. brunnea* and *N. rendalli*, but *N. somalica* was sister to *N. capensis* and *Laephotis*. *Glauconycteris variegata* was found basal within the *Glauconycteris* clade only in the ML analysis of all genes. In the BA, the result was influenced probably by *Arielulus cuprosus*, which fell (unsupported) to the *Glauconycteris* clade. A sister relationship of *Nycticeinops schlieffenii* to *Hypsugo eisentrauti* was recovered. *Myotis bocagii* was sister to *M. welwitschii*.

4.2.2. GenBank data

The joint phylogenetic analyses based on the Senegalese and GenBank data revealed some topologies differing from previous findings, even including the tribal position and classification.

Glischropus tylopus was basal to a clade formed by *P. coromandra*, *P. tenuis*, *P. paterculus*, *P. stenopterus*, *P. javanicus* and *P. abramus*.

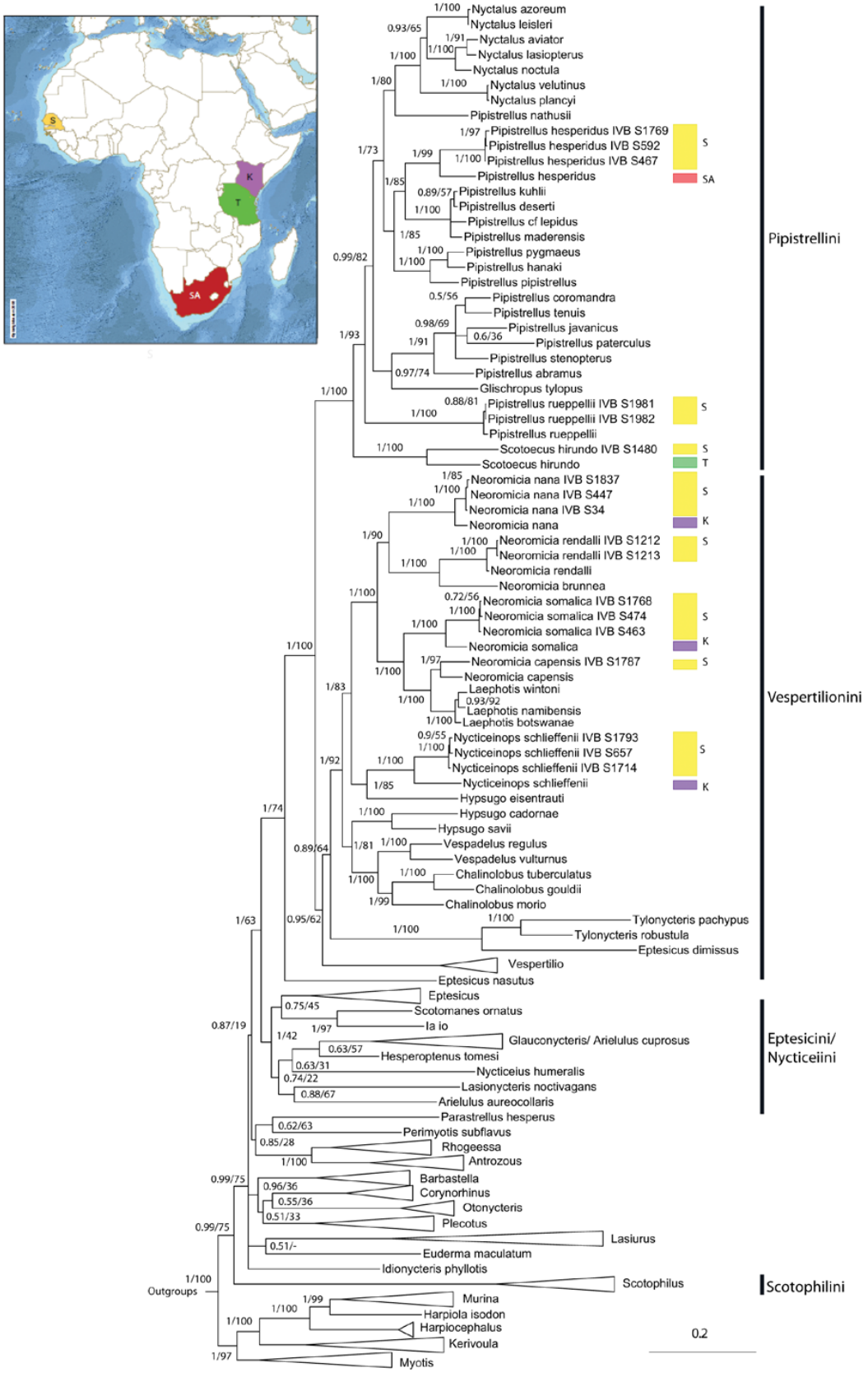
Eptesicus was found polyphyletic, with *E. dimissus* and *E. nasutus* being clearly

distinct. *E. dimissus* was sister to *Tylonycteris* within the tribe Vespertilionini, *E. nasutus* was basal to both the clades Vespertilionini and Pipistrellini. *Pipistrellus hesperidus* appeared clearly separated from *P. kuhlii.*

Nycticeius humeralis fell unsupported as one of the basal taxa of a clade that contained *Glauconycteris*, *Hesperoptenus*, *Arielulus* and *Lasionycteris*. This clade was sister to *Eptesicus*, *Scotomanes* and *Ia*.

The Pipistrellini were formed by *Nyctalus*, *Pipistrellus*, *Scotoecus*, *Glischropus* and Vespertilionini by *Neoromicia*, *Laephotis*, *Hypsugo*, *Vespadelus*, *Nycticeinops*, *Tylonycteris*, *Eptesicus dimissus*, *Chalinolobus* and *Vespertilio.* Scotophilini were the second most basal branch of all vespertilionids.

Fig. 3. Phylogram of analysed vespertilionid bat species from Senegal (IVB S) and GenBank data with selected tribes indicated. A Bayesian phylogenetic tree based on analysis of partitioned data completed in MrBayes on the concatenated dataset of 6 mitochondrial and 2 nuclear genes (*cytb* + $tRNA^{Thr}$ + *12S* + $tRNA^{Val}$ + *16S* + *nd1* + *RAG1* + *RAG2*; 5,665 bp; total number of taxa $n = 200$) is presented. ML analysis was conducted in RAxML using a GTR+Γ model on the same partitioned dataset. The nodes supports are indicated by posterior probabilities and/or bootstrap values. The nodes, which were not supported with ML and were in different position than in BA, are indicated with "-". The nodes are considered supported when bootstrap proportions are ≥0.95 for the Bayesian posterior probabilities and/or ≥75% for the ML bootstrap analysis. The bar indicates genetic distance (the number of substitutions per amino acid site). In the left top corner, see a map of Africa, showing the sampling localities of the five taxa considered to be cryptic in Senegal. The map shows the position of Senegal (S/yellow) and the countries, where the specimens from GenBank, used here for the genetic distance comparison, were sampled: SA/red – South Africa (*Pipistrellus hesperidus*), T/green – Tanzania (*Scotoecus hirundo*), K/purple – Kenya (*Neoromicia somalica*, *Nycticeinops schlieffenii*, *Neoromicia nana*). The same symbols are used to show the origin of the respective GenBank specimens in the phylogram. Some clades have been collapsed and the outgroup families have been removed for presentation. See Supplement 3 (Fig. 3) for the original tree.


S
K
T
SA
Nyctalus azoreum
Nyctalus leisleri
Nyctalus aviator
Nyctalus lasiopterus
Nyctalus noctula
Nyctalus velutinus
Nyctalus plancyi
Pipistrellus nathusii
Pipistrellus hesperidus IVB S1769
Pipistrellus hesperidus IVB S592
Pipistrellus hesperidus IVB S467
Pipistrellus hesperidus
Pipistrellus kuhlii
Pipistrellus deserti
Pipistrellus cf lepidus
Pipistrellus maderensis
Pipistrellus pygmaeus
Pipistrellus hanaki
Pipistrellus pipistrellus
Pipistrellus coromandra
Pipistrellus tenuis
Pipistrellus javanicus
Pipistrellus paterculus
Pipistrellus stenopterus
Pipistrellus abramus
Glischropus tylopus
Pipistrellus rueppellii IVB S1981
Pipistrellus rueppellii IVB S1982
Pipistrellus rueppellii
Scotoecus hirundo IVB S1480
Scotoecus hirundo
Neromicia nana IVB S1837
Neromicia nana IVB S447
Neromicia nana IVB S34
Neromicia nana
Neromicia rendalli IVB S1212
Neromicia rendalli IVB S1213
Neromicia rendalli
Neromicia brunnea
Neromicia somalica IVB S1768
Neromicia somalica IVB S474
Neromicia somalica IVB S463
Neromicia somalica
Neromicia capensis IVB S1787
Neromicia capensis
Laephotis wintoni
Laephotis namibensis
Laephotis botswanae
Nycticeinops schlieffenii IVB S1793
Nycticeinops schlieffenii IVB S657
Nycticeinops schlieffenii IVB S1714
Nycticeinops schlieffenii
Hypsugo eisentrauti
Hypsugo cadornae
Hypsugo savii
Vespadelus regulus
Vespadelus vulturnus
Chalinolobus tuberculatus
Chalinolobus gouldii
Chalinolobus morio
Tylonycteris pachypus
Tylonycteris robustula
Eptesicus dimissus
Vespertilio
Eptesicus nasutus
Eptesicus
Scotomanes ornatus
Ia io
Glauconycteris/ Arielulus cuprosus
Hesperoptenus tomesi
Nycticeius humeralis
Lasionycteris noctivagans
Arielulus aureocollaris
Parastrellus hesperus
Perimyotis subflavus
Rhogeessa
Antrozous
Barbastella
Corynorhinus
Otonycteris
Plecotus
Lasiurus
Euderma maculatum
Idionycteris phyllotis
Scotophilus
Outgroups
Murina
Harpiola isodon
Harpiocephalus
Kerivoula
Myotis
Pipistrellini
Vespertilionini
Eptesicini/
Nycticeiini
Scotophilini
0.2

5. DISCUSSION

Phylogenetic relationships on different levels of bat taxonomy have been scrutinised in many recent studies based on morphology, cytogenetics and molecular approaches (e.g. Kearney *et al.*, 2002; Lack *et al.*, 2010; Ammerman *et al.*, 2012; Taylor *et al.* 2012). The results from West Africa show that even the examination of populations on a relatively small area can reveal interesting and novel findings, including cryptic taxa, new or divergent intrageneric and intraspecific karyotypes and changes in phylogenetic relationships.

5.1. KARYOTYPIC DATA

Differences in the diploid chromosome numbers and/or in chromosome morphology between the bat populations from Senegal and from other regions of Africa were found in four vespertilionid (*Neoromicia somalica*, *N. nana*, *Scotoecus hirundo*, *Pipistrellus hesperidus*) and one rhinolophid (*Rhinolophus landeri*) species. Karyotypes of four species belonging to the family Hipposideridae were described for the first time (*Hipposideros ruber*, *H. tephrus*, *H. jonesi* and *H. cyclops*).

5.1.1. Hipposideridae

The genus *Hipposideros* is characterised by a conservative karyotype (2n = 32, FNa = 60, FN = 64; Sreepada *et al.*, 1993), with the exceptions hitherto found in *H. vittatus*, *H. gigas* (2n = 52, FNa = 60; this study; South Africa – Rautenbach *et al.*, 1993; Gabon – Porter *et al.*, 2010) and *H. obscurus* (2n = 24; FNa = 44; Philippines; Rickart *et al.*, 1999). Karyotypes of *H. cyclops* (2n = 36, FN = 66) and *H. gigas* (2n = 52, FN = 64) from Senegal represent another deviation from the standard complement. In congruence with the divergent karyotypes, *H. gigas* and *H. cyclops* were found to be sister species, separate from other (not only African) *Hipposideros* based on *cytb* (Taylor *et al.*, 2012). Thus, both karyotypic and molecular data suggest their separation to a distinct genus (this study; Vallo *et al.*, 2008; Taylor *et al.*, 2012). Furthermore, *H. gigas* from West Africa (this study; Porter *et al.*, 2010) and *H. vittatus* (Rautenbach *et al.*, 1993), both from the *commersoni* group (Simmons, 2005), may represent a single species, taking into account that they share the unusual karyotype with 2n = 52.

The karyotypes of *H. tephrus* and *H. ruber* from Senegal were identical to *H. caffer* described from eastern and southern Africa (Đulić and Mutere, 1974, 1977; Peterson and Nagorsen, 1975; Rautenbach *et al.*, 1993). Thus, the comparison of non-differentially stained karyotypes within the *H. caffer* complex, as well as in other species with the typical (2n = 32) karyotype (e.g. compare with *H. jonesi* examined in this study), does not resolve the taxonomic diversity, nor helps to identify cryptic species.

5.1.2. Rhinolophidae

The karyotypes of *R. fumigatus* and *R. landeri* from Senegal were similar to previous findings in South Africa (Rautenbach, 1986). However, the karyotype of *R. landeri* differed from the previous report by the presence of two additional pairs of biarmed autosomes, thus having one of the highest fundamental numbers (FNa = 64) among rhinolophids (review in Zima *et al.*, 1992). This difference can be explained by the variability between geographically distinct populations, or by the existence of cryptic species. The hypothesis, that the Senegalese specimen might rather belong to a sympatrically occurring, and somewhat larger *R. guineensis* (previously considered a subspecies of *R. landeri*; Van Cakenberghe and Seamark, 2012), was refused by the comparison of external morphology measurements.

5.1.3. Molossidae

Similar karyotypes for *Mops condylurus* and *Chaerephon pumilus* (2n = 48; FNa = about 54; X submetacentric/metacentric, Y small acrocentric) were reported previously from eastern, and southern Africa (Đulić and Mutere, 1973; Peterson and Nagorsen, 1975; Smith *et al.*, 1986; Rautenbach *et al.*, 1993). Both *Mops* and *Chaerephon* were removed from *Tadarida* (Freeman, 1981; Koopman, 1984; Simmons, 2005). Recent phylogenetic examination of multi-locus data (Ammerman *et al.*, 2012) supported a *Mops/Chaerephon* clade, with *Chaerephon* being paraphyletic, thus confirming previous morphology-based hypotheses (Freeman, 1981; Gregorin, 2000) and molecular analyses (Jones *et al.*, 2005; Agnarsson *et al.*, 2011). Furthermore, congruently with previous hypotheses (Rosevear, 1965; Peterson *et al.*, 1995), African *Mops condylurus* was supported as closely related to Madagascan *M. leucostigma*, and both were included into African *Chaerephon* (Ammerman *et al.*, 2012). The phylogenetic tree based on beta fibrinogen intron 7 even

showed *Mops condylurus* and *Chaerephon pumilus* as closely related (Ammerman *et al.*, 2012).

5.1.4. Vespertilionidae

All the specimens of the Senegalese *Pipistrellus hesperidus* (2n = 46, FNa = 58) had a higher diploid number of chromosomes than previously reported from South Africa and Madagascar (2n = 42, FNa = 50; Kearney *et al.*, 2002; Rautenbach *et al.*, 1993, Volleth *et al.*, 2001).

Scotoecus hirundo (2n = 30, FN = 50, FNa = 46) from Senegal had a lower number of chromosomal arms than recorded at the Ivory Coast (2n = 30, FN = 54, FNa = 50; Volleth *et al.*, 2006).

N. somalica from Senegal had one additional pair of acrocentric autosomes compared to findings from Cameroon (2n = 26, FNa = 48; McBee *et al.*, 1987). Despite sharing the same diploid number with *N. zuluensis* (previously included in *N. somalica*; Koopman, 1994; Rautenbach *et al.*, 1993), the Senegalese *N. somalica*, had a different structure of some biarmed chromosomes and the X chromosome was metacentric in *N. somalica*, while being subtelocentric in *N. zuluensis* (South Africa, 2n = 28, FNa = 50, Kearney *et al.*, 2002; southern Africa, 2n = 28, FNa = 48, Rautenbach *et al.*, 1993). From the karyotype examined in southern Africa, *N. somalica* from Senegal differed in the number of acrocentric autosomes (one pair – this study, two pairs – Rautenbach *et al.*, 1993).

Neoromicia nana lacked two pairs of acrocentric autosomes compared to previous findings, but the karyotype contained an additional metacentric pair, which could have arisen by a Robertsonian fusion of the two acrocentric pairs. The X chromosome was subtelocentric, while being metacentric elsewhere (2n = 36, FN = 50; Peterson and Nagorsen, 1975; Rautenbach *et al.*, 1993; Kearney *et al.*, 2002).

N. rendalli differed from other populations (Somalia, McBee *et al.*, 1987; Zimbabwe, Rautenbach and Fenton, 1992; Southern Africa, Kearney *et al.*, 2002) in the relative size and morphology of the sex X chromosome. It was conspicuously smaller than in the karyotypes reported from Somalia and Zimbabwe, and it was submetacentric, while being metacentric in Southern Africa.

The karyotype of *Nycticeinops schlieffenii* was similar to the findings from Somalia (Ruedas *et al.*, 1990), but it was different from the information from Southern Africa (2n =

42, FNa = 50; Rautenbach *et al.*, 1993), thus indicating the existence of two distinct species (Van Cakenberghe and Seamark, 2012).

The variability of chiropteran karyotypes is a relatively rare phenomenon (even inside genera or families such as Rhinolophidae or Molossidae; Rautenbach *et al.*, 1993), which is in contrast with e.g. rodents or shrews (White *et al.*, 2010). Vespertilionidae are quite a diverse group in this respect (with a few exceptions as *Myotis*) (Zima and Horáček, 1985; Rautenbach *et al.*, 1993; Volleth *et al.*, 2001) and many species could have a unique karyotype (Baker, 1970; Volleth *et al.*, 2001). The occurrence of intraspecific karyotype variability (as seen here in *Neoromicia somalica*, *N. nana*, *Scotoecus hirundo*, *Pipistrellus hesperidus*), therefore indicates the presence of cryptic species (Rautenbach *et al.*, 1993; Volleth *et al.*, 2001).

5.2. MOLECULAR PHYLOGENY

5.2.1. Cryptic specimens from Senegal

As seen above, four species of vespertilionid bats (*Neoromicia somalica*, *N. nana*, *Scotoecus hirundo*, *Pipistrellus hesperidus*) divergent enough to be considered cryptic species were detected on the basis of differences in karyotypes. Additionally, this conclusion was supported by the genetic species concept, which delimitates species on the basis of a genetic divergence, usually using the *cytb* distances (Baker and Bradley, 2006). Despite the fact, that the divergences of single mitochondrial DNA genes are always not sufficient to resolve the real status of supposed cryptic species, because of incongruences between maternally (mitochondrial) and paternally (Y-chromosome associated) inherited genes (Clare, 2011), the support for cryptic species recognition obtained here from the karyotypes and genetic distances was in agreement.

The Senegalese specimens of *Pipistrellus hesperidus* diverged significantly from other representative of this nominal species (Stadelmann *et al.*, 2004) as well as from *P. kuhlii* from which it was recently separated (discussion in Simmons, 2005).

Scotoecus hirundo was notably divergent in *RAG2* from a specimen from southeastern Africa (Roehrs *et al.*, 2010). Together with the differences to previous records from Western Africa found in the karyotype (Volleth *et al.*, 2006), this suggests existence of cryptic taxa within *S. hirundo* in Africa. More sampling is needed to confirm the number of cryptic taxa and their distribution ranges.

Neoromicia nana and *N. somalica* differed from the specimens examined in Kenya (Hoofer and Van Den Bussche, 2003), and this finding was also supported with the karyotypic differences (Peterson and Nagorsen, 1975; McBee *et al.*, 1987; Rautenbach *et al.*, 1993; Kearney *et al.*, 2002).

Nycticeinops schlieffenii differed from the specimens from Eastern Africa in *12S* (Hoofer and Van Den Bussche, 2003). According to the separate distribution ranges (Van Cakenberghe and Seamark, 2012) and differences in karyotypes (this study; Ruedas *et al.*, 1990; Rautenbach *et al.*, 1993), there are apparently at least two cryptic populations within this species.

5.2.2. Specimens from Senegal and vespertilionid phylogeny

The extensive dataset of mitochondrial and nuclear sequences obtained in the studied sample from Senegal, with addition of available data from GenBank, enabled to investigate various problems of vespertilionid phylogeny.

Pipistrellus rueppellii from Senegal was found basal to the *Pipistrellus*/*Nyctalus* clade, contrary to other studies where it appeared on different positions within this clade (Mayer *et al.*, 2007; Veith *et al.*, 2011). The populations occurring in the area ranging from Algeria to Senegal are presently recognised as *P. r. senegalensis*, conspicuously larger than other races (Dorst, 1960; Koopman, 1994). The external body measurements, as well as the sequence comparison confirmed that the Senegalese specimens are similar to other West African populations (compared with Adam and Hubert, 1972; Benda *et al.*, 2004; Mayer *et al.*, 2007; Happold and Happold, 2013). The discrepancy of results can be explained by influence of treating the hypervariable rRNA regions (sequences in Veith *et al.*, 2011), or of phylogenetic approaches used, as the re-analysis of published data (Mayer *et al.*, 2007) with BA and ML supported *P. rueppellii* at the same position as here. The comparison of the observed *cytb* divergence with the typical mean genetic distances in pipistrelles (Baker and Bradley, 2006) confirms that *P. rueppellii* is clearly divergent from other *Pipistrellus* species and might even belong to a distinct genus, as proposed elsewhere (Roberts, 1946; Kearney, 2005).

Scotoecus (*hirundo*) was confirmed as basal to the clade formed by *Pipistrellus* and *Nyctalus* (Roehrs *et al.*, 2010, 2011) and its pertinence to the tribe Pipistrellini (and not to Nycticeiini), was proposed as suggested before based on morphology, karyotypes or DNA analysis (Hill and Harrison, 1987; Hoofer and Van Den Bussche, 2003; Volleth *et al.*,

2006).

The examination of the Senegalese representatives of *Neoromicia* (*N. nana* and *N. somalica*) with GenBank data confirmed previous discussions based on DNA analyses, morphology and differential chromosome banding (Volleth *et al.*, 2001; Kearney *et al.*, 2002; Hoofer and Van Den Bussche, 2003; Stadelmann *et al.*, 2004; Monadjem *et al.*, 2013) that this genus is diphyletic and deserves further revision. The first lineage is formed by *N. somalica*, *N. capensis* and *Laephotis*, and the second of *N. nana*, *brunnea* and *rendalli*. The reconstruction of phylogenetic relationships and support for molecular data is not possible on the basis of diploid number and morphology of non-differentially stained chromosomes (this study; Peterson and Nagorsen, 1975; McBee *et al.*, 1987; Rautenbach *et al.*, 1993; Volleth *et al.*, 2001; Kearney *et al.*, 2002).

The sister relationship of *Nycticeinops schlieffenii* and (*H.*) *eisentrauti/H. crassulus bellieri* (see comments on species determination in Van Cakenberghe and Seamark, 2012) was supported as in other studies (Hoofer and Van Den Bussche, 2003; Roehrs *et al.*, 2011).

In the trees based on nuclear genes and ML eight-gene tree, *Glauconycteris variegata* was found basal to the clade of analysed *Glauconycteris* species, contrary to previous karyotypic (Porter *et al.*, 2010) and molecular studies (Hoofer and Van Den Bussche, 2003; Roehrs *et al.*, 2011) using the same species (or even sequences), which put it on different phylogenetic positions within this genus.

The finding of *Myotis bocagii* as sister to *M. welwitschii* concurred with previous reports (Stadelmann *et al.*, 2004).

The parts of the vespertilionid trees based on (solely) GenBank data obtained here were similar to recent studies (e.g. Hoofer and Van Den Bussche, 2003; Lack *et al.*, 2010; Roehrs *et al.*, 2010, 2011); however, some topologies concerning even tribal position were remarkably distinct, which was influenced by differences in taxon sampling, usage of different genes, gap treatment or phylogenetic methods.

The tribe Scotophilini (or subfamily Scotophilinae; Van Cakenberghe and Seamark, 2012) was fully supported as the second most basal branch of all vespertilionid bats, which was in contrast to previous multi-locus (Lack *et al.*, 2010; Roehrs *et al.*, 2010, 2011) and *cytb* analyses (Bickham *et al.*, 2004; Stadelmann *et al.*, 2004; Agnarsson *et al.*, 2011), but similar to Lack and Van Den Bussche (2010). The divergence of Scotophilini to other vespertilionids was noted previously by morphological and karyotypic data (Volleth and Heller, 1994; Horáček *et al.*, 2006).

The Malaysian *Glischropus tylopus* nested within the *Pipistrellus*/*Nyctalus* group, as basal to the (East) Asian group of pipistrelles (*P. coromandra*, *P. tenuis*, *P. paterculus*, *P. stenopterus*, *P. javanicus* and *P. abramus*), similarly as in previous study, where this position was, however, not significantly supported (Francis *et al.*, 2010). Other rare Asian species, *Eptesicus dimissus* and *nasutus* were found clearly genetically distinct from *Eptesicus*, thus confirming its polyphyly and need for revision (compare similar, however, unsupported findings of Lack *et al.*, 2010; Agnarsson *et al.*, 2011).

Contrary to recent suggestions (Roehrs *et al.*, 2010, 2011), we found the tribes Pipistrellini (*Pipistrellus*, *Nyctalus*, *Glischropus*, *Scotoecus*) and Vespertilionini (*Vespertilio*, *Neoromicia*, *Hypsugo*, *Chalinolobus*, *Laephotis*, *Nycticeinops*, *Eptesicus dimissus*, *Tylonycteris* and *Vespadelus*) supported similarly as defined previously based on morphology or in older molecular studies (Volleth *et al.*, 1994; Simmons, 2005; Volleth *et al.* 2001; Hoofer and Van Den Bussche, 2003; Csorba, 2011; Fig. 3 and Supplement 3). We also confirmed *P. nathusii* as related to *Nyctalus* (as in Hoofer and Van Den Bussche, 2003; Roehrs *et al.*, 2010), and not as basal to all pipistrelles (Veith *et al.*, 2011).

The position of *Nycticeius humeralis* was supported only in the tree based on combined mitochondrial genes. However, in the eight-gene tree it appeared in the same clade (containing members from the genera *Eptesicus*, *Ia*, *Scotomanes*, *Lasiurus*, *Arielulus*, *Glauconycteris, Hesperoptenus* and *Lasionycteris*). This topology was similar to the phylogeny based on the *mt* and *nc* genes in Roehrs *et al.* (2011), where this position, however, also lacked a significant support. If this unsupported inclusion into this clade reflects the true state, the tribe should be recognised as Nycticeiini (rather than Eptesicini) on the basis of priority (Roehrs *et al.*, 2011). More investigation, possibly including examination of other types of datasets will be necessary to clarify these relationships.

5.3. CRYPTIC SPECIES IN WESTERN AFRICA

During the geological history of Africa, there were periodic shifts from drier to more humid climatic conditions, corresponding with the glacial/moderate periods in the higher latitudes (deMenocal, 2004). Several West- and central-African plants (Maley, 1996) and rodents (Mouline, 2008; Bryja *et al.*, 2010; Nicolas *et al.*, 2011) probably survived the late Pleistocene and Holocene unfavourable conditions in refugia along the Atlantic coast of Western Africa. Additionally, the rain forests along this coast are separated into two blocks

by the Dahomey gap, a savannah belt located at the area of Benin, Togo and Ghana. This gap is thought to expand and decline repeatedly (Booth, 1958; Maley, 1996; Salzmann and Hoelzmann, 2005), thus representing a dispersal barrier between the western and the central or south/eastern parts of Africa for forest-dwelling fauna (Booth, 1954, 1958; Nicolas *et al.*, 2006). Similarly, savannah and arid-zone mammals and bird species also exhibit high genetic differences between the north/west and the south/east African populations (Muwanika *et al.*, 2003; Lorenzen *et al.*, 2010; Fuchs *et al.*, 2011). Bats are hypothesised to be able to cross the Dahomey gap (Robbins, 1978; Djossa *et al.*, 2008); however, thorough genetic samplings outside and inside of the gap are needed for an objective judgment.

Nevertheless, the detection of one rhinolophid cryptic taxon, five other cryptic taxa which belong to separate phylogenetic clades within the vespertilionids, as well as finding of cryptic species in more West African Chiroptera (Vallo *et al.*, 2008, 2011, 2013; Monadjem *et al.* 2013) and various other groups (rodents – Granjon, 2005; Nicolas *et al.*, 2006; Dobigny et al, 2008; Kouassi *et al.*, 2008; reptiles – Eaton *et al.*, 2009; Leaché and Fujita, 2010; insects – Hausberger *et al.*, 2011), indicate their long isolation from other African populations and imply high probability that more cryptic taxa could be detected in this region.

6. CONCLUSIONS

The variations both in karyotypes and DNA information found in the sample of 248 Senegalese bats belonging to 19 species and four families, showed that our knowledge of the West African bat fauna is still limited, compared to other parts of Africa or moderate climate regions and that important findings can be achieved even by investigation of a relatively small area, here of Niokolo-Koba.

Our results also demonstrate that karyotypic data can be useful, independent and simple phylogenetic markers to identify cryptic taxa (five vespertilionids and potentially one rhinolophid in this study) or divergent clades (here two *Hipposideros* species with untypical karyotypes – *H. cyclops* and *H. gigas*). Karyotypes of some species were described for the first time (*Hipposideros ruber*, *H. tephrus*, *H. jonesi* and *H. cyclops*), one was different (*Rhinolophus landeri*) and others appeared similar to other African populations (*R. fumigatus*, *Chaerephon pumilus* and *Mops condylurus*).

Combined analyses of the Senegalese bats and GenBank data gained support for some new topologies or previous findings (*Pipistrellus rueppellii* and its possible separation to distinct genus, polyphyly of *Eptesicus*, diphyly of *Neoromicia*, position of Scotophilini and classification of Pipistrellini and Vespertilionini). Furthermore, it was confirmed that in some cases the geographic distribution, rather than morphological characters, better reflects the phylogenetic relationship (Ruedi and Mayer, 2001; Hoofer and Van Den Bussche 2003; Ammerman *et al.*, 2012), e.g. the position of *Glischropus tylopus*.

New taxa and suggestions for systematic rearrangements based on the molecular and cytogenetic approaches demonstrate that in many cases morphologically similar species that occur in Africa are a result of convergent evolution, and these species belong to phylogenetically distant groups. The research is an infinite quest and there are still many details waiting to be discovered in (African) Chiroptera.

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8. CONTRIBUTION OF DARINA KOUBÍNOVÁ TO THE PAPERS AND MANUSCRIPT PRESENTED IN THE THESIS

Supplement 1

Koubínová *et al.*, 2010. Karyotypic variation in rhinolophid and hipposiderid bats (Chiroptera: Rhinolophidae, Hipposideridae). Acta Chiropterol, 12: 393–400:

co-authored the original idea of the paper, analysed the karyotypes of the Senegalese samples (approximately 70% of the samples presented) from staining of chromosomal preparation slides to karyotype assessment and figure preparation, wrote the parts of the manuscript concerning the Senegalese specimens and contributed to improvements of the final version.

Supplement 2

Sreepada *et al.*, 2008. Karyotypes of three species of molossid bats (Molossidae, Chiroptera) from India and western Africa, Folia Zool, 57:347–357:

analysed karyotypes of the Senegalese samples (approximately 95% of the samples presented) from staining of chromosomal preparation slides to karyotype assessment and figure preparation, prepared the overview table of all the molossid karyotypes, co-wrote the parts of the manuscript concerning the Senegalese specimens and contributed to improvements of the final version.

Supplement 3

Koubínová *et al.*, manuscript. Hidden diversity in Senegalese bats and associated findings in the systematics of the family Vespertilionidae:

co-authored the original idea of the paper, performed (or in some samples coordinated) analyses of all samples (from DNA extraction to assembling of DNA sequences), performed the molecular phylogenetic analyses, wrote the first version of the manuscript, contributed to its further improvements and prepared it for submission.

Prague, 19th March 2013

Prof. RNDr. Jan Zima, Dr.Sc. – supervisor