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## Systematic implications of chromosome GTG-band and bacula morphology for Southern African *Eptesicus* and *Pipistrellus* and several other species of Vespertilioninae (Chiroptera: Vespertilionidae)

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Phylogenetic analyses of bacular and chromosomal GTG-band characters verify the suggestion that *Eptesicus hottentotus* (A. Smith, 1833) is the only true *Eptesicus* Rafinesque, 1820 of the six southern African species (*capensis*, cf. *melckorum*, *rendalli*, *somalicus* and *zuluensis*) formerly classified as *Eptesicus*. GTG-banded chromosomes studied in *rendalli*, *zuluensis* and *capensis* confirm the affiliation of all of them to the genus *Neoromicia*; these species were previously placed in the *Pipistrellus* Kaup, 1829, subgenus *Neoromicia* based on bacular morphology. For karyological reasons, the elevation of the subgenus *Neoromicia* to generic rank is established by the presence of three Robertsonian fusion chromosomes (7/11, 8/9, 10/12) as distinguishing characters. The move of *Hypsugo nanus* and cf. *melckorum* to the genus *Neoromicia* is indicated by chromosomal analysis and bacular morphology, respectively. The close phylogenetic relationship between *Pipistrellus* cf. *kuhlii* and *P. rusticus* is shown by a shared Robertsonian fusion element (11/12).

*Key words:* bacula, GTG-banded chromosomes, *Eptesicus*, *Pipistrellus*, *Neoromicia*

### INTRODUCTION

Differences between *Eptesicus* Rafinesque, 1820 and *Pipistrellus* Kaup, 1829, two genera of insectivorous bats of the family Vespertilionidae have long been problematic (Koopman, 1975; Horáček and Hanák, 1986). Heller and Volleth (1984) proposed that *Eptesicus* is chromosomally conservative, all species having a diploid number of 50, while *Pipistrellus* is

chromosomally variable, having diploid numbers of 44 or less. At the time of Heller's and Volleth's (1984) work the only species occurring in southern Africa that had been karyotyped were *E. hottentotus* (A. Smith, 1833) and *E. capensis* (A. Smith, 1829) (Peterson and Nagorsen, 1975). *Eptesicus capensis* with a diploid number of 32 was placed in the genus *Pipistrellus*.

On the basis of bacular morphology Heller and Volleth (1984) and Hill and

Harrison (1987) suggested the *Eptesicus* and *Pipistrellus* could be distinguished from each other by *Eptesicus* having a small, triangular baculum, and *Pipistrellus* having a medium to large, 'stick-like', elongated baculum. Applying these characters, Hill and Harrison (1987) transferred all, but one southern African species of *Eptesicus* [*capensis*, *melckorum* Roberts, 1919, *somalicus* (Thomas, 1901), *zuluensis* Roberts, 1924 and *rendalli* (Thomas, 1889)], with an exception of *E. hottentotus*, to a new subgenus, *Neoromicia*, in the genus *Pipistrellus*. A subsequent allozyme analysis by Morales *et al.* (1991), which included several southern African species of *Eptesicus* (*hottentotus*, *capensis*, *zuluensis*, cf. *melckorum*) and *Pipistrellus* (*nanus*), showed biochemical relationships between the taxa to be consistent with the suggestions of Heller and Volleth (1984) and Hill and Harrison (1987).

Several authors (Ansell and Dowsett, 1988; Cotterill, 1996; Fenton and Rautenbach, 1998; Taylor, 2000) and at least one museum (Natural History Museum of Zimbabwe, Bulawayo) have followed the suggestions of Heller and Volleth (1984) and Hill and Harrison (1987). But for the most part the caution by Meester *et al.* (1986: 56) appears to have been followed, that until all southern African species of *Eptesicus* and *Pipistrellus* have been tested against the bacula and chromosome criteria "it would be premature to depart from established generic synonymy".

Various studies have subsequently confirmed on the basis of diploid number (from non-differential staining) that *E. rendalli*, *E. somalicus* (McBee *et al.*, 1987; Rautenbach and Fenton, 1992), *E. cf. melckorum* (sensu Rautenbach *et al.*, 1993), and *E. zuluensis* (Rautenbach *et al.*, 1993) all have diploid numbers less than 50.

Chromosome banding has also proved a useful source of characters, enabling

Volleth and Heller (1994) to infer a phylogenetic relationship for Vespertilionidae. Two chromosomal characters, i.e., the banding pattern of chromosomes 11 and 23, were found to separate the tribes Vespertilionini and Pipistrellini. According to those characters, *Pipistrellus* (*Neoromicia*) *capensis* is a member of the tribe Vespertilionini, and not Pipistrellini. In order to prevent a polyphyletic classification for the genus *Pipistrellus*, Volleth *et al.* (2001) suggested the subgenus *Neoromicia* be elevated to generic rank, as had been done before for all other *Pipistrellus* subgenera sensu Hill and Harrison (1987) (*Hypsugo* — Horáček and Hanák, 1986; *Perimyotis* — Menu, 1984; *Vespadelus* — Volleth and Tidemann, 1991; *Falsistrellus* — Kitchener, 1986; *Arielulus* — Csorba and Lee, 1999). We follow the above-mentioned authors and treat all subgenera of *Pipistrellus* (sensu Hill and Harrison, 1987) as separate genera.

In this study, we present the first GTG-banded karyotypes of five southern African *Pipistrellus*-like species, and the outgroup *Myotis tricolor* (Temminck, 1832). We revisited bacular morphology to confirm the usefulness of this structure for identifying relationships. GTG-banded chromosomes and bacular morphology provided characters for cladistic analyses to assess inter- and intrageneric relationships among southern African *Pipistrellus*-like species.

## MATERIALS AND METHODS

### *Taxonomic Designations*

We followed Volleth *et al.* (2001) in calling *Pipistrellus kuhlii*-like specimens with a diploid number of 42, *P. cf. kuhlii*. Both Meester *et al.* (1986) and Koopman (1993) recognised that *N. melckorum* had not been clearly distinguished from *N. capensis*. Rautenbach *et al.* (1993) in questioning the taxonomic validity of *N. melckorum* suggested it as a synonym of *N. capensis*. This suggestion was made on the basis of

unpublished morphometric data, which showed a clinal variation within these species. We followed Rautenbach *et al.* (1993), in considering specimens (DM5630, DM5636) from Kersfontein (the type locality for *N. melckorum*) as *N. capensis*. Kersfontein specimens had the same chromosome number, GTG-banding pattern, and bacula size and shape as other *N. capensis* specimens.

Rautenbach *et al.* (1993) found specimens of *Pipistrellus* from the 'interior of South Africa' being intermediate in size between *N. capensis* and *E. hottentotus*, which matched the description of *N. melckorum*. These specimens have a different chromosome number (i.e.,  $2n = 40$ ) to *N. capensis* (Rautenbach and Schlitter, 1985), and allozyme results (Morales *et al.*, 1991) have shown them to be biochemically well differentiated, although closely allied to *N. capensis*. We accepted the suggestion by Rautenbach *et al.* (1993) that specimens found in northern South Africa and Zimbabwe be called *P. cf. melckorum*. Kearney and Taylor (1997) described a specimen of *Laephotis* Thomas, 1901 as *Laephotis cf. wintoni* since the validity of *L. wintoni* Thomas, 1901 in South Africa remains ambiguous.

## Chromosomes

GTG-banded (Seabright, 1971) karyotypes were constructed from bone-marrow metaphase spreads (for method see Green *et al.*, 1980) of *E. hottentotus*, *N. capensis*, *N. rendalli*, *N. zuluensis*, *N. nanus*, *P. cf. kuhlii*, *P. rusticus* (Tomes, 1861), and *M. tricolor*, from specimens captured at various localities in South Africa (Appendix I). Chromosomes were arranged following a standardised numbering system introduced by Bickham (1979a) for *Myotis*, where chromosome arms instead of chromosomes are numbered. This numbering system has been used subsequently in analyses of European and Asian Vespertilionidae, including *Eptesicus* and *Pipistrellus* species, by Zima (1982), Volleth (1987), Volleth and Heller (1994), and Volleth *et al.* (2001). Since complete chromosomal arms are conserved extensively in the family, it should be possible to trace the changes that have given rise to different diploid numbers, and thus infer phylogenetic relationships. Most often the chromosome changes are due to Robertsonian rearrangements, but occasionally due to inversions and tandem fusions (Baker *et al.*, 1982; Zima, 1982; Volleth and Heller, 1994).

Seven chromosome rearrangements (see Appendix II), i.e., the presence or absence of five synapomorphic Robertsonian fusion products, the state of chromosome 11 due to a small paracentric inversion

(Volleth and Tidemann, 1989; Volleth and Heller, 1994; Volleth *et al.*, 2001), and the state of the X chromosome, were used to construct a data matrix (Appendix III). Following Ando *et al.* (1977), Bickham (1979b), Zima (1982), Baker *et al.* (1985), and Volleth and Heller (1994) who all considered the *Myotis* karyotype,  $2n = 44$ , FN = 52, as closest to the hypothetical ancestral karyotype of Vespertilionidae, we used *M. tricolor* ( $2n = 44$ , FN = 52) as the outgroup.

Robertsonian fusion chromosomes are denoted as the fusion chromosome numbers linked by a forward slash. Tandem fusions are denoted as the fusion chromosome numbers linked by a hyphen.

## Bacula

Bacula were dissected, stained (Hill and Harrison 1987), cleared in glycerin (Lidicker, 1968), and drawn (Fig. 1) for *E. hottentotus*, *N. capensis*, *N. rendalli*, *N. zuluensis*, *N. cf. melckorum*, *N. nanus*, *P. rusticus*, *P. cf. kuhlii*, *P. rueppellii*, and *Hypsugo anchietae* (Seabra, 1900). Bacula from *M. tricolor*, *Laephotis cf. wintoni* (sensu Kearney and Taylor, 1997), *L. namibensis* Setzer, 1971, *L. botswanae* Setzer, 1971, *Nycticeinops schlieffenii* (Peters, 1859) and *Scotophilus dinganii* (A. Smith, 1833) were also included — these are all genera within the same subfamily Vespertilioninae, as *Pipistrellus* and *Eptesicus*. Specimen details are given in Appendix I. Since bacula of different *Laephotis* species are almost identical, their results were combined as *Laephotis* spp.

For each baculum seven qualitative characters were scored, two of which were multistate (see Appendix II), and a matrix of bacula characters was created (Appendix IV). As described for the chromosome analysis above, *Myotis tricolor* was used as the outgroup.

## Analyses

Data matrices of phylogenetically informative bacula and chromosome characters, and a matrix combining bacula and chromosome characters were analysed with Hennig86 (version 1.5; Farris, 1988). Character polarity was determined by the outgroup. Multistate characters were run as nonadditive. Characters were not weighted. The shortest possible trees were found using implicit enumeration (the 'ie\*' command in Hennig86).

In order to assess whether there was a lack of congruence between the bacula and chromosome data sets, two measures of incongruence were used, the Mickevich-Farris incongruence metric ( $i_{MF}$ ) (Kluge, 1989), and the incongruence length difference

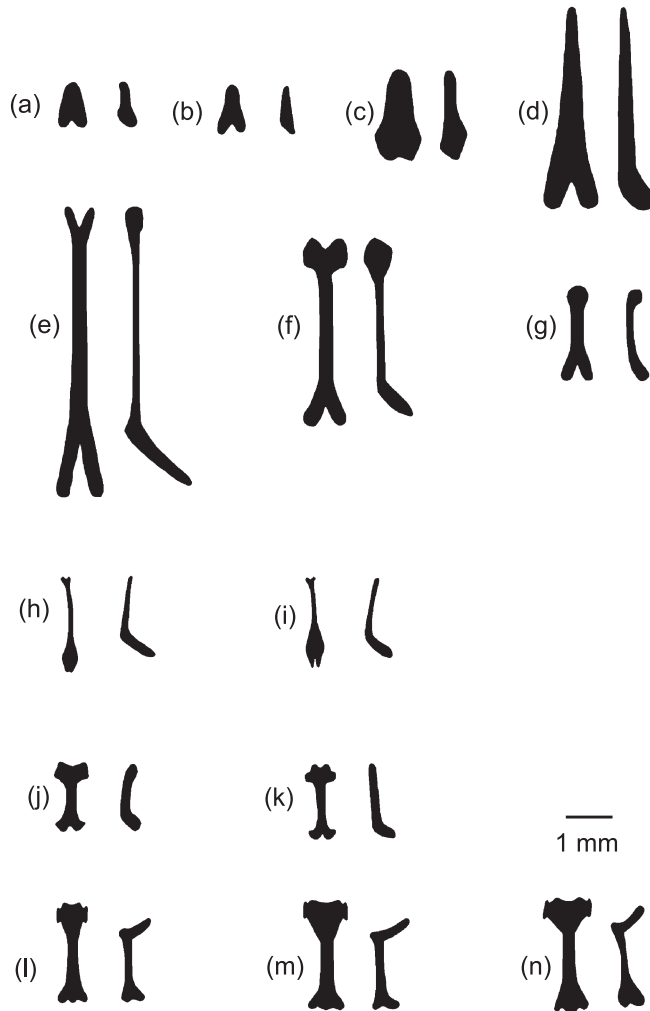


FIG. 1. Dorsal (left) and lateral (right) views of bacula from: (a) *Myotis tricolor*, (b) *Eptesicus hottentotus*, (c) *Scotophilus dinganii*, (d) *Nycticeinops schlieffenii*, (e) *Pipistrellus rueppellii*, (f) *Neoromicia rendalli*, (g) *Neoromicia nanus*, (h) *Pipistrellus rusticus*, (i) *Pipistrellus* cf. *kuhlii*, (j) *Hypsugo anchietae*, (k) *Neoromicia zuluensis*, (l) *Neoromicia capensis*, (m) *Neoromicia* cf. *melckorum*, (n) *Laephotis* cf. *wintoni*

( $D_{xy}$ ; Mickevich and Farris, 1981). The robustness of the resulting trees was assessed using the '\x;' command in Dos-equis mode of Hennig86. This identifies the additional length gained when branches are lost, by successively collapsing nodes leading to at least two taxa in the tree. This is analogous to Bremer's branch support (Bremer, 1994), which although not useful for comparison between analyses, because it is positively correlated with the number of characters in a particular analysis, it is informative within an analysis (Bremer, 1996). As a further measure of topology support, the number of unique and unreversed synapomorphies supporting each node were counted.

## RESULTS

### *Chromosome morphology*

Unfortunately bone marrow does not provide the same high GTG-band resolution that cell cultured spreads do. Thus, not all the GTG-bands obtained were of a resolution to allow detection and confirmation of possible inversions and intraspecific

variations, other than a possible polymorphism in *N. rendalli*. The banding patterns of the smallest chromosomes (including the Y chromosome) were also often difficult to detect.

*Myotis tricolor* ( $2n = 44$ , FN = 52)

The GTG-banded karyotype (Fig. 2) shows three large metacentric, one small submetacentric, and 17 acrocentric autosomal pairs. GTG-banding shows the four biarmed chromosomes are composed of chromosome arms: 1/2, 3/4, 5/6 and 16/17. The X chromosome is a medium sized submetacentric.

*Eptesicus hottentotus* ( $2n = 50$ , FN = 48)

The GTG-banded karyotype of *E. hottentotus* (Fig. 3) shows all 24 pairs of autosomes are acrocentric. Chromosome arms 16 and 17 form a single acrocentric chromosome. The X chromosome is a medium sized submetacentric.

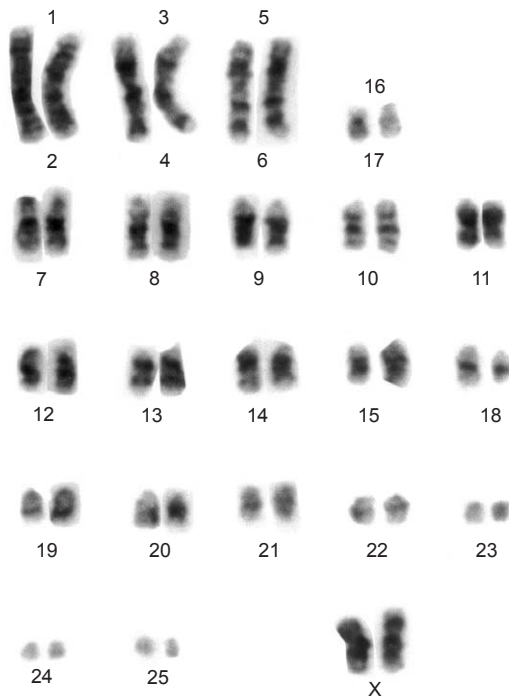


FIG. 2. GTG-banded karyotype of *M. tricolor*

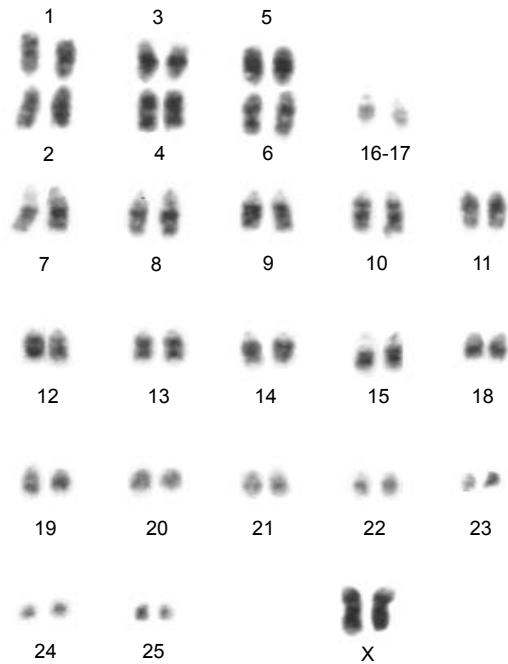


FIG. 3. GTG-banded karyotype of *E. hottentotus*

mosome. The X chromosome is a medium sized submetacentric.

*Hypsugo anchietae* ( $2n = 26$ , FN = 32)

The non-differentially stained karyogram of a male and GTG-banded karyogram of a female *H. anchietae* (Fig. 4) show one medium sized submetacentric, one small metacentric, two large and one medium sized subtelo centric, and seven acrocentric autosomes. The X chromosome is a small metacentric, and the Y a tiny acrocentric.

*Pipistrellus rusticus* ( $2n = 42$ , FN = 50)

The GTG-banded karyotype (Fig. 5) shows five biarmed, and 15 acrocentric autosomes. The X chromosome is a medium sized metacentric, and the acrocentric Y is the same size as the smallest autosome. The five metacentric chromosomes are composed of chromosome arms: 1/2, 3/4, 5/6,

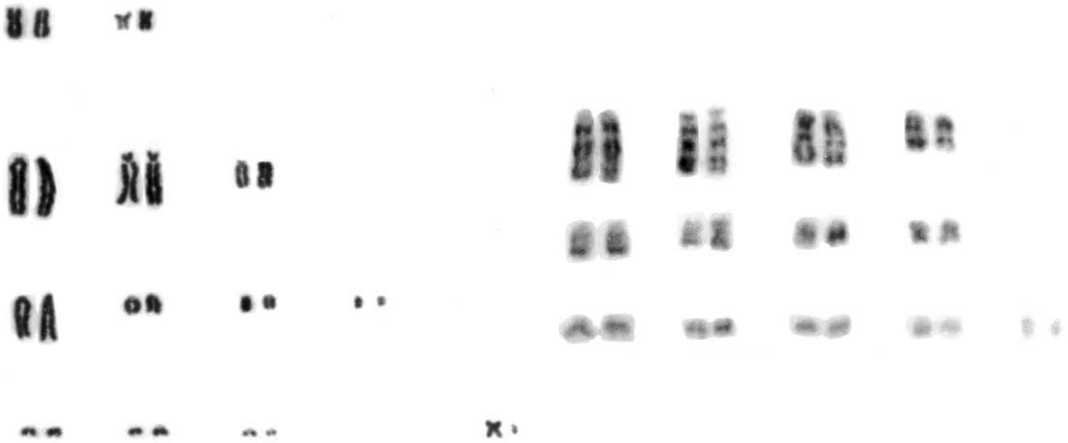


FIG. 4. Non-differentially stained and GTG-banded karyotypes of a male (left) and a female (right) of *H. anchietae*

16/17, and 11/12. GTG-banded chromosomes show that *P. rusticus* and *P. cf. kuhlii*, which have the same diploid chromosome number, share the same fusion pairs, including 11/12, which is not present in the basic karyotype (Fig. 6).

*Pipistrellus cf. kuhlii* ( $2n = 42$ , FN = 50)

The GTG-banded karyotype (Fig. 7) shows five biarmed, and 15 acrocentric autosomes. The X chromosome is a medium sized metacentric. The biarmed chromosomes are composed of arms 1/2, 3/4, 5/6, 16/17, and 11/12. The Robertsonian fusion chromosome 11/12 is the same as in *P. rusticus* (Fig. 6).

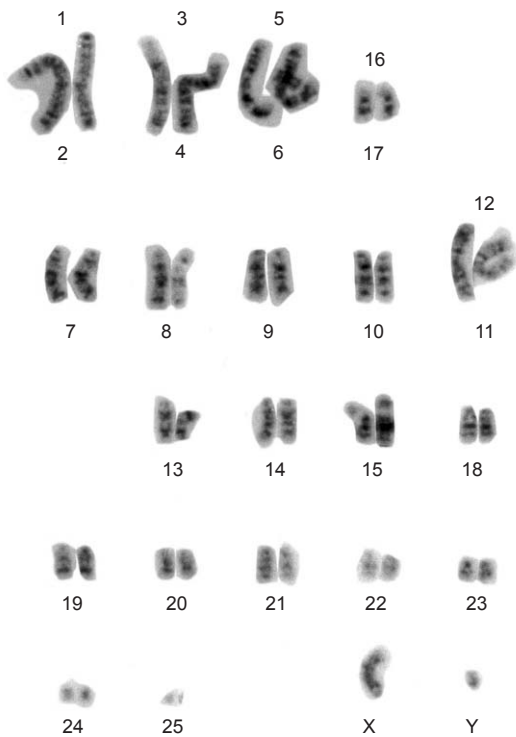


FIG. 5. GTG-banded karyotype of *P. rusticus*

*Neoromicia nanus* ( $2n = 36$ , FN = 50)

The GTG-banded karyotype (Fig. 8) shows eight biarmed, and nine acrocentric autosomes. The X chromosome is a medium sized metacentric, and the acrocentric Y chromosome is smaller than the smallest autosome. GTG-banding shows besides the metacentric chromosomes 1/2, 3/4, 5/6 and 16/17, four chromosomes which are the result of Robertsonian fusions between chromosome arms 7/11, 8/9, 10/12, and 13/14. *Neoromicia nanus* shares fusion of pairs 7/11, 8/9, and 10/12 with *N. zuluensis*, *N. rendalli*, and *N. capensis* (Fig. 6), and we therefore suggest transferring it to the genus *Neoromicia*.

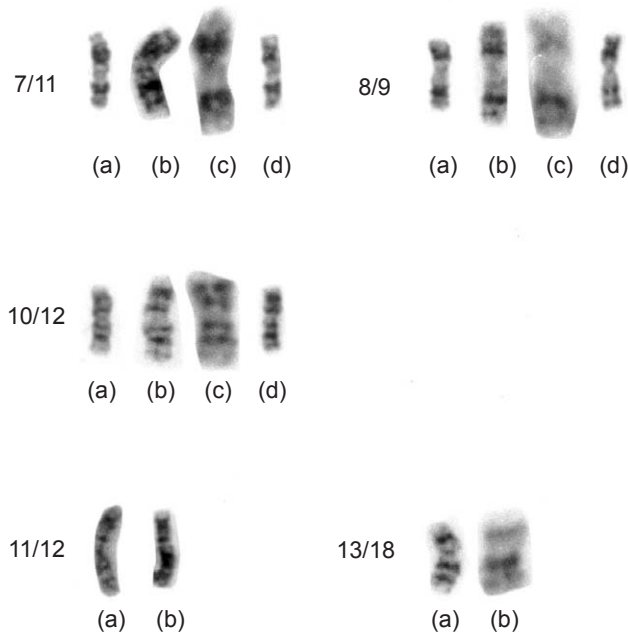


FIG. 6. Comparison of GTG-banded chromosome pairs between species: 7/11, 8/9, 10/12: (a) *Neoromicia nanus*, (b) *N. zuluensis*, (c) *N. capensis*, (d) *N. rendalli*; 11/12: (a) *Pipistrellus rusticus*, (b) *P. cf. kuhlii*; 13/18: (a) *N. zuluensis*, (b) *N. capensis*

*Neoromicia zuluensis* ( $2n = 28$ , FN = 48)

This GTG-banded karyotype (Fig. 9) shows 12 biarmed, and one acrocentric autosomes. The X chromosome is a medium sized subtelocentric. GTG-bands show the reduced chromosome number in *N. zuluensis* is due to Robertsonian fusion pairs between chromosome arms 7/11, 8/9, 10/12, 13/18, 14/21, 15/19, 20/22, and 23/24. *Neoromicia zuluensis* shares pairs 7/11, 8/9, 10/12 with *N. nanus*, *N. rendalli*, and *N. capensis*, and pair 13/18 with *N. capensis* (Fig. 6).

*Neoromicia capensis* ( $2n = 32$ , FN = 50)

The GTG-banded karyotype (Fig. 10) shows 10 biarmed and 5 acrocentric autosomes. The X chromosome is a medium sized metacentric. Robertsonian fusion pairs are between chromosome arms: 1/2, 3/4, 5/6, 16/17, 7/11, 8/9, 10/12, and 13/18.

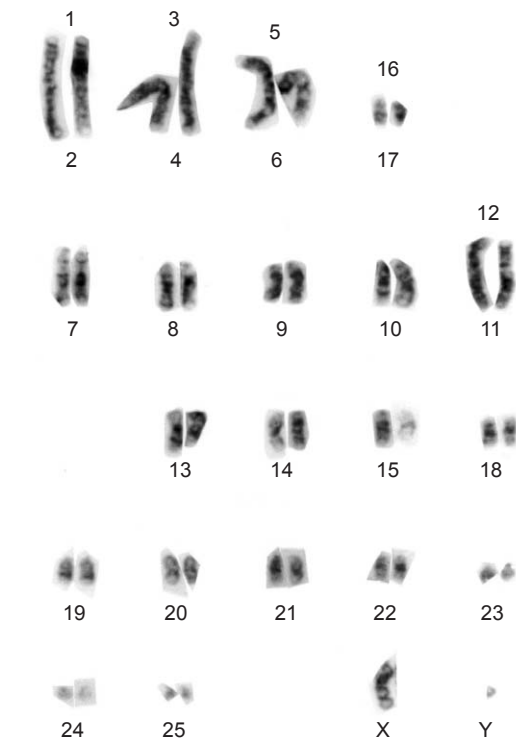


FIG. 7. GTG-banded karyotype of *P. cf. kuhlii*



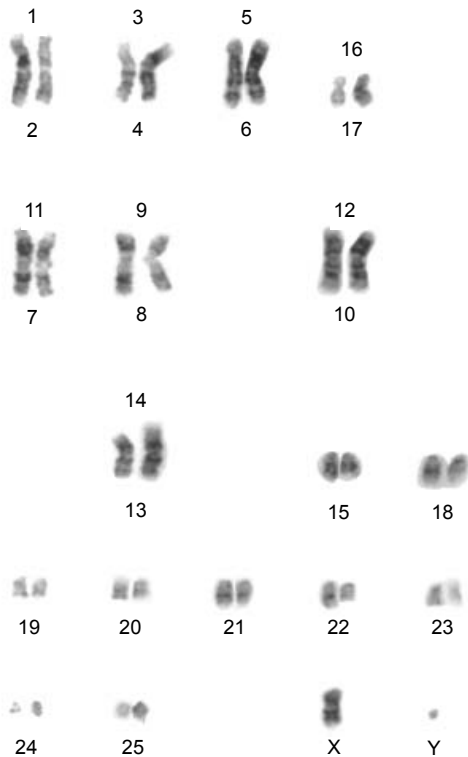


FIG. 8. GTG-banded karyotype of *N. nanus*

*Neoromicia capensis* shares pairs 7/11, 8/9, 10/12 with *N. nanus*, *N. rendalli*, and *N. zuluensis*, and pair 13/18 with *N. zuluensis* (Fig. 6).

*Neoromicia rendalli* ( $2n = 38$ , FN = 50)

The GTG-banded karyotype (Fig. 11) shows seven biarmed, and 11 acrocentric autosomes. The X chromosome is a medium sized metacentric, and the acrocentric Y is as small as the smallest autosomes. GTG-bands show the seven biarmed chromosomes are composed of 1/2, 3/4, 5/6, 16/17, 7/11, 8/9, 10/12. All pairs are shared with *N. nanus*, *N. zuluensis* and *N. capensis* (Fig. 6).

#### Cladistic Analysis of Chromosomes

Analysis of the chromosome data (Appendix III) resulted in one most

parsimonious tree (length (S) = 8; consistency index (CI) = 100; retention index (RI) = 100) (Fig. 12). The tree is not fully resolved. A trichotomy at the base is made up of the outgroup *Myotis tricolor*, forming one of the branches, *E. hottentotus* forms the second branch, while the rest of the species form the third branch.

The third branch of the trichotomy forms two clades. *Pipistrellus rusticus* and *P. cf. kuhlii* form one clade supported by a single synapomorphy (fusion of chromosomes 11 and 12), while *N. nanus*, *N. rendalli*, *N. zuluensis*, and *N. capensis* form the other clade, as a result of four synapomorphies (fusions of chromosome 7 and 11, 8 and 4, 10 and 12, and state II of chromosome 11). The relationship between these species is not fully resolved as they form a trichotomy. However, *N. zuluensis* and *N. capensis* form the terminal clade separated

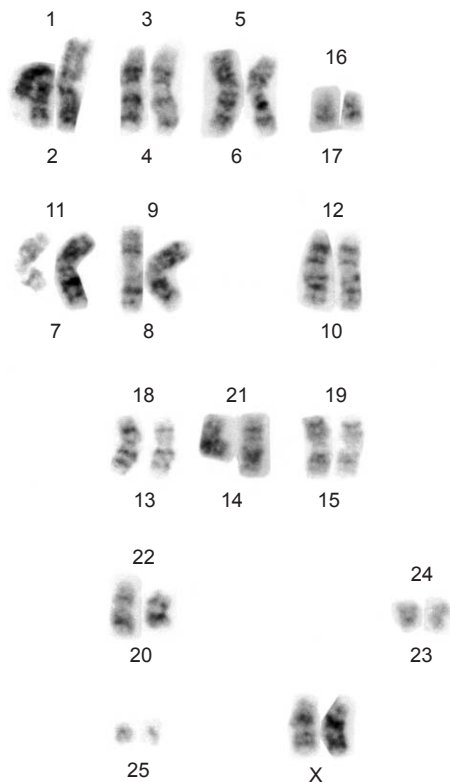
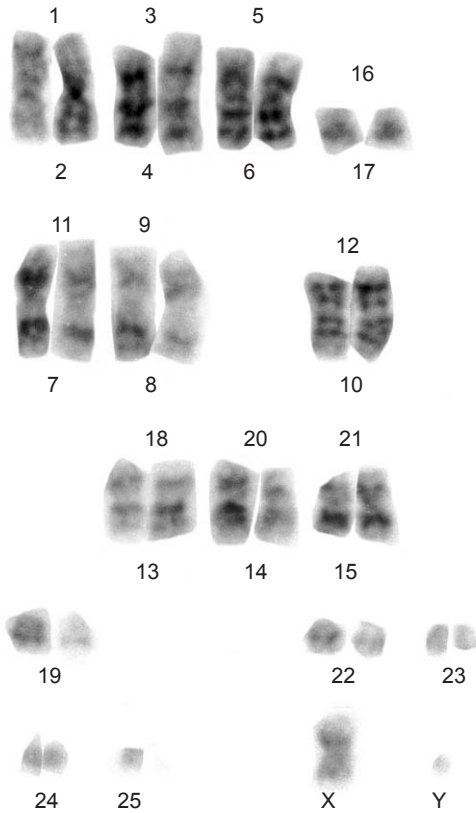


FIG. 9. GTG-banded karyotype of *N. zuluensis*

FIG. 10. GTG-banded karyotype of *N. capensis*

from *N. nanus* and *N. rendalli* due to the fusion of chromosomes 13 and 18.

As reflected by CI and RI values of 100, the steps at each node are unique and unreversed synapomorphies, and there is no homoplasy. Branch support is highest (four) for the branch linking the trichotomy, while all the other branches have the same, lower support (one).

### Bacular Morphology

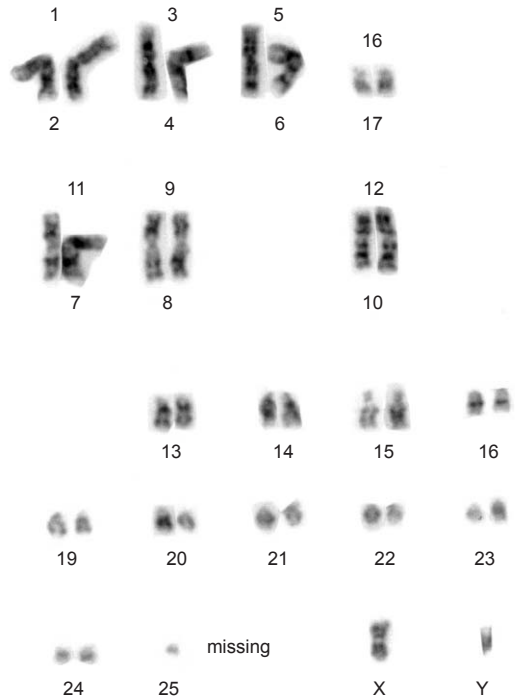
Although differences in bacular morphology are slight between certain species, there is considerable variation in the bacular morphology of all the species represented (Fig. 1). These variations in bacular morphology provided characters (see Appendices II and IV) for cladistic analysis.

### Cladistic Analysis of Bacula

Analysis of bacular characters (Appendix IV) produced nine most parsimonious trees ( $S = 12$ ;  $CI = 100$ ;  $RI = 100$ ). These cladograms give different alternatives for the resolution of the more terminal polychotomies.

The Nelsen consensus cladogram is not fully resolved (Fig. 13), with polychotomies in three places. The root is an unresolved polychotomy. The outgroup *M. tricolor* forms one branch, *E. hottentotus* another branch, *S. dinganii* yet another branch, while the rest of the species united by a single synapomorphy, bacula shape being medium to large, elongated and 'stick-like' (BS/1), form the fourth branch.

A single synapomorphy, the tip not being distinct from the rest of the bacula (TD/1), separates *N. schlieffenii* from the next unresolved polychotomy. In the polychotomy *P. rueppellii* (Fischer, 1829) forms one branch. *Pipistrellus* cf. *kuhlii* and

FIG. 11. GTG-banded karyotype of *N. rendalli*

*P. rusticus* as sister taxa separated by two synapomorphies, unique basal lobe shape (BL/4), and more than 50% of the bacula being deflected (PBD/1), form the second branch. *Neoromicia nanus* forms the third branch; while the rest of the taxa (*H. anchietae*, *N. zuluensis*, *N. rendalli*, *N. capensis*, *N. cf. melckorum*, and *Laephotis* spp.) form the fourth branch, united by a single synapomorphy, the bacula base being narrower than the tip (TB/1). *Hypsugo anchietae*, *N. zuluensis*, *N. rendalli*, *N. capensis*, *N. cf. melckorum*, and *Laephotis* spp. form the third polychotomy. *Hypsugo anchietae*, *N. zuluensis*, and *N. rendalli* each form a branch, while *N. capensis*, *N. cf. melckorum*, and *Laephotis* spp. united by three synapomorphies, a unique tip and basal shape (TS/3 and BL/2), and a ventrally deflected tip (AT), form the fourth branch. *Neoromicia capensis*, *N. cf. melckorum*, and

*Laephotis* spp. are not resolved and form a trichotomy.

Branch support of different nodes varies from one to five steps. The branch uniting *N. capensis*, *N. cf. melckorum* and *Laephotis* spp., which requires five steps, has the most support. The branch leading to the most terminal polychotomy has the next highest support, with two steps. All the other branches have least support, requiring just one step to collapse the tree at those points.

Both multistate characters (TS and BL) show homoplasy among some of the character states (TS/1, TS/2, and BL/3). *Pipistrellus rueppellii*, *P. cf. kuhlii* and *P. rusticus* all have a 'V' shaped (TS/1) bacula tip. *Neoromicia nanus*, *H. anchietae*, *N. zuluensis*, and *N. rendalli* all have a flat and broad (TS/2) bacula tip. While *N. rendalli*, *N. nanus*, *P. rueppellii*, and *N. schlieffenii* all

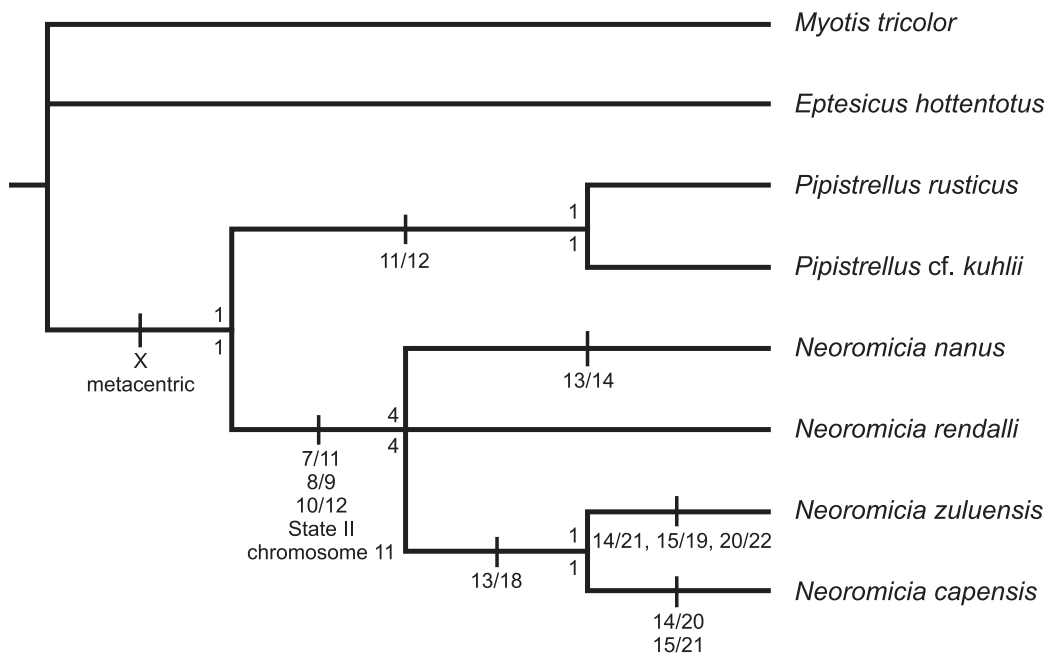


FIG. 12. Most parsimonious tree suggested by chromosome GTG-band characters, for seven taxa, of four genera of Vespertilioninae. Numbers above clade nodes are the number of unique and unreversed synapomorphies supporting each clade. Numbers below the clade nodes are branch support values (which is the number of extra steps required to collapse the particular node). Synapomorphic characters are shown below branches (abbreviations explained in Appendix II)

have evenly wide and 'V' shaped basal lobes (BL/3).

#### *Cladistic Analysis of Combined Chromosome and Bacula Datasets*

Only taxa for which there was information about both bacula and chromosomes were included. Analysis of the combined chromosome and bacula data set produced a single most parsimonious tree ( $S = 20$ ;  $CI = 100$ ;  $RI = 100$ ). The cladogram topology

(Fig. 14) is almost the same as the single most parsimonious chromosome cladogram. The combination of the two data sets however resolves the more terminal trichotomy present in the chromosome cladogram. The same characters show homoplasies as in the bacula tree. Branch support of the cladogram varies from one to four steps. The most and least supported branches are similar to those in the chromosome cladogram. Both measures of character incongruence due to disparity between

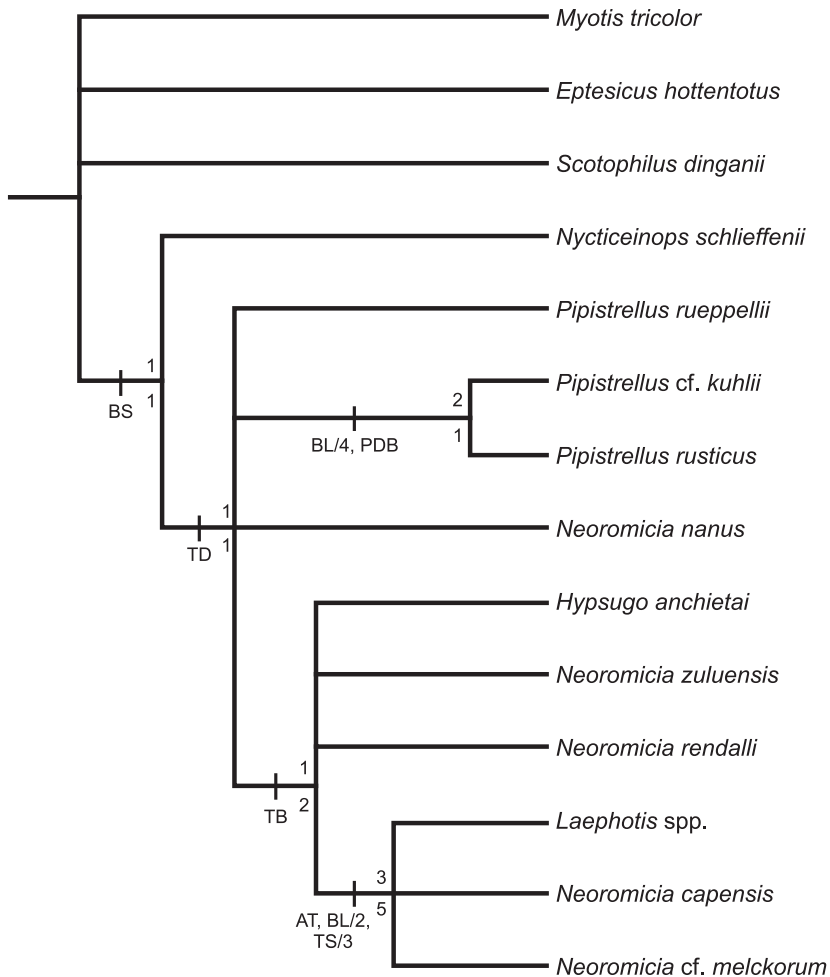


FIG. 13. A Nelsen consensus tree of nine most equally parsimonious trees suggested by bacula characters for fourteen taxa, of eight genera of Vespertilioninae. Numbers above clade nodes are the number of unique and unreversed synapomorphies supporting each clade. Numbers below the clade nodes are branch support values (which is the number of extra steps required to collapse the particular node). Synapomorphic characters are shown below branches (abbreviations explained in Appendix II)