

Hidden Identities: Cryptic Species in the *Otomys* Genus (Cuvier 1824) (Rodentia: Muridae: Otomyinae) Revealed by Mitochondrial and Nuclear DNA in South Africa

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Abstract

The recent taxonomy and phylogeny of Otomyinae has been in a state of flux as new systematic revisions combining molecular, karyotypic and morphometric information have identified changes at various taxonomic levels. Currently two genera of Otomyinae and eight species of *Otomys* are recognized in South Africa. However, the position of *Otomys sloggetti* on the phylogeny of Otomyinae has not been resolved, and since this species was not well represented in recent revisions it may also reveal multiple cryptic evolutionary species. In this study four mitochondrial and one nuclear gene regions and external morphological characters were analysed to elucidate relationships within *O. sloggetti*, as well as between *O. sloggetti* and other *Otomys* species occurring in South Africa. The data from this study suggested *O. sloggetti* belongs to neither the *Otomys*, nor the *Parotomys* genera. Instead, we propose returning to the classification of Otomyinae and recognize *Myotomys* as the valid genus for *O. sloggetti*. Within *O. sloggetti*, our data does not support the traditional view of the distribution and intraspecific variation of the species, and invites a new hypothesis. Specimens identified in the field as *O. sloggetti* were found to represent two different clades. One of the clades was genetically and morphologically consistent with the description for *O. sloggetti*, while the other was distinct from *O. sloggetti* and other *Otomys* species known to occur in South Africa. Our data suggests that this is a novel species within the *Otomys* genus.

Keywords: DNA barcoding; *Otomys*; *Myotomys*; Cryptic species; *O. sloggetti*; DNA taxonomy

Introduction

The recent taxonomy and phylogeny of Otomyinae has been unstable due to incongruent results from different data sets [1-3]. Recognised three genera of the subfamily in South Africa: *Myotomys* [4], *Otomys* (Cuvier 1824) and *Parotomys* (Thomas [4], Smith 1834). Musser et al. [1] retained this taxonomy. Taylor and Taylor et al. [3,5] did not recognize *Myotomys* as a genus as the monophyly of *Myotomys* was not supported by molecular and morphological analyses. Various studies reported by Musser [1] showed *Otomys* to be polyphyletic, with allusion to the close affinity of *O. sloggetti* (Thomas 1902) and/or *O. unisulcatus* (Cuvier 1829) to species in *Parotomys*.

At the species level, Musser [1] recognised four *Otomys* species, namely *Otomys angoniensis* (Wroughton 1906), *Otomys irroratus* (Brants 1827), *Otomys laminatus* (Thomas and Schwann 1905), and *Otomys saundersiae* (Roberts 1929) and two species of *Myotomys* (*M. sloggetti* and *M. unisulcatus*) occurring in South Africa. Taylor [5] recognised six species of *Otomys* in South Africa, reverting species previously in *Myotomys* to *O. sloggetti* and *O. unisulcatus*. More recently, Monadjem [3] recognized seven *Otomys* species occurring in South Africa. This included the six species indicated by Taylor [5], although *O. saundersiae* was synonymized with *O. irroratus* and *O. saundersiae karoensis* was elevated to full species level (*O. karoensis*). The seventh species they recognized was *O. auratus*, which was recently elevated, having previously been a synonym of *O. irroratus*, following analyses involving molecular, chromosomal and morphometric information [6,7]. Similar systematic revisions of *Otomys* in other parts of Africa have revealed additional cryptic species; while Taylor [5] recognized 15 *Otomys* species. More recently, Monadjem et al. [3] recognized 29 species of which two still need to be formally described.

Otomys sloggetti is the smallest *Otomys* species, and as with many other *Otomys* taxa, has a limited distribution range, being restricted to 2000 m above sea level in the Drakensberg mountain range with isolated populations on mountains in the Karoo [3] (Figure 1). Monadjem et al. [5] recognized the following five sub-species of *O. sloggetti* from different geographic regions: *O. s. sloggetti* (Northern Cape Province, South Africa), *O. s. jeppe* (Northern Cape Province, South Africa), *O. s. turneri* (eastern Free State Province, South Africa), *O. s. robertsi* (high Drakensberg Mountains in Lesotho and north Eastern Cape Province, South Africa), and *O. s. basuticus* (low lying areas in south-eastern Lesotho).

The current taxonomy of *O. sloggetti* is based on morphological, allozyme, karyological and geographic data, and the position of *O. sloggetti* in the phylogeny of Otomyinae has not been resolved [1-3]. Various authors have shown that taxa defined by these parameters may harbour cryptic species that may be uncovered by phylogenetic analyses based on DNA sequence data [6-8]. DNA barcoding is a DNA sequencing method, using a short fragment of a gene region of mitochondrial DNA, which was introduced to speed up the process

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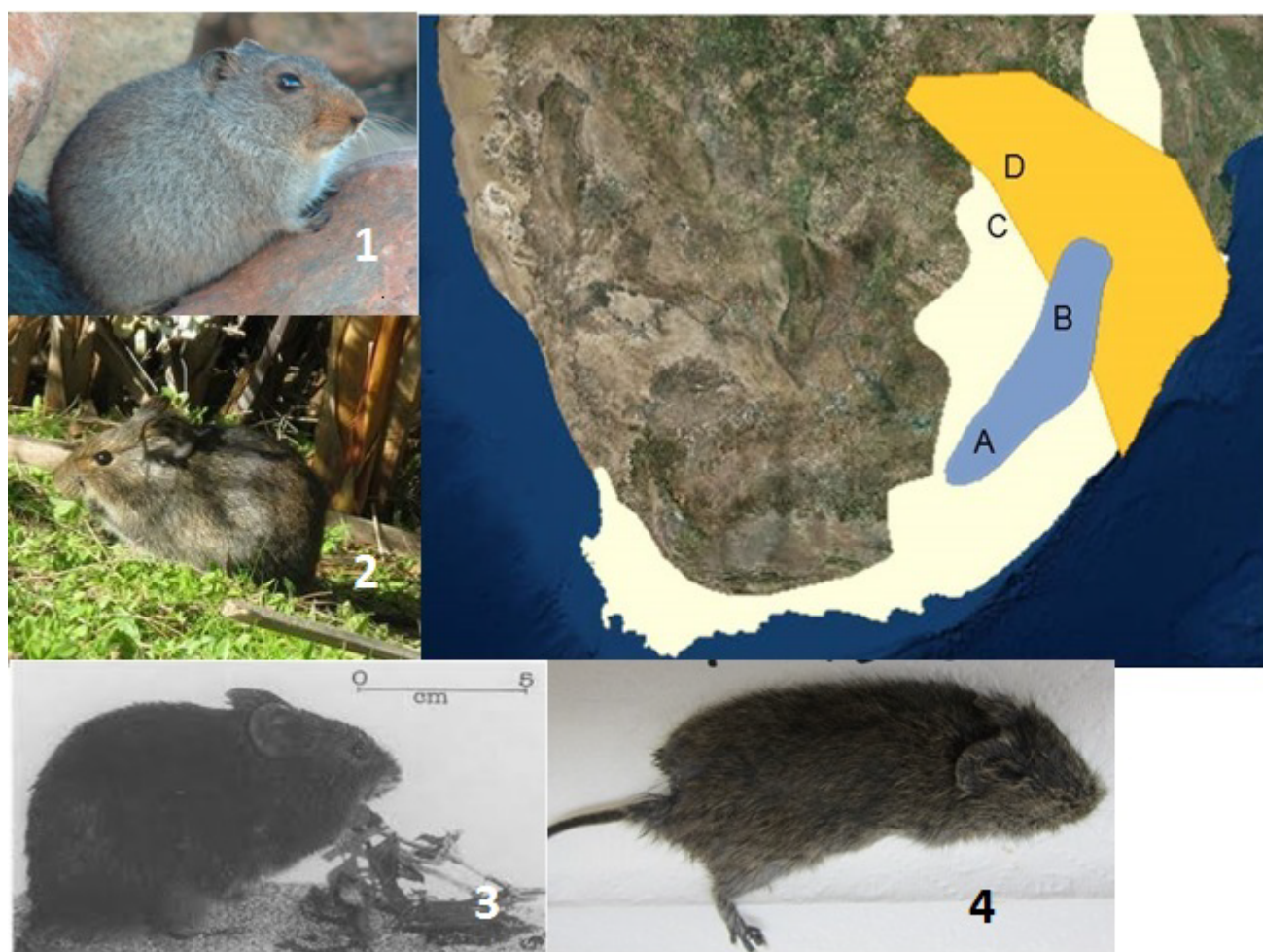


Figure 1: Distribution map of *Otomys irroratus* (indicated in white), *Otomys angoniensis* (indicated in yellow) and *Otomys sloggetti* (indicated in blue) [42,43]. Sampling localities for this study include (a) Tiffendale, (b) Wakkerstroom, (c) Rietvlei and (d) Lajuma. Photographic images of the studied species include: *Otomys sloggetti* (1), *Otomys irroratus* (2) *Otomys angoniensis* (3) and an unidentified species previously assumed to be *O. sloggetti* (4) [44-46].

of species identification [9-10]. The most widely accepted gene region for DNA barcoding of vertebrates is a 658 bp fragment of cytochrome oxidase subunit I (COI) [9]. According to a comprehensive search in Genbank (<http://www.ncbi.nlm.nih.gov/genbank/>) and the literature, cytochrome b (Cyt b), 16S rDNA subunit (16S) and displacement loop of the mitochondrial control region (D-loop) are also frequently used gene regions for vertebrate species identification. This paper investigates intraspecific variation within some *O. sloggetti* by using new and existing information from four mitochondrial gene regions, 16S, COI, Cyt b, and D-loop, and one nuclear gene region, *IRBP*, as well as morphological measurements. In addition, the genetic diversity between *O. sloggetti* and other *Otomys* and *Parotomys* species occurring in South Africa was explored [10].

Materials and Methods

Sample collection

Twenty-eight whole specimens, collected in the Eastern Cape and provisionally identified as *O. sloggetti* based on general appearance, locality and habitat (Figure 1), were obtained from another study [11]. The specimens were previously euthanized in that study, which was approved by the University of Pretoria Animal Ethics

Committee (EC003-13) and the Eastern Cape Nature Conservation (CRO157/14CR). The project was also approved at the Research Ethics and Scientific Committee of the National Zoological Gardens of South Africa (P13/12). Following analyses, these specimens were deposited in the Ditsong National Museum of Natural History (DNMNH) with accession numbers DNMNH49075-DNMNH49101 (Supplementary Table S1). A further three dried skin samples were obtained from the DNMNH (accession numbers 68, 22683 and 22684). The skin samples were collected from *O. sloggetti* originating from Kwa-Zulu Natal, Eastern Cape and Free State, respectively (Supplementary Table S2).

Sample analyses

DNA isolation and gene region amplification: DNA was isolated, amplified and sequenced as described in Phukuntsi et al. The primers used for amplification are also provided in Phukuntsi et al. [12].

Phylogenetic analyses: Sequences were manually edited and aligned in MEGA6 [13]. A BLAST search using the Genbank sequence database provided by the National Centre for Biotechnology Information (NCBI) was performed for each sequence, and the best matches for each sequence were downloaded as reference sequences (Supplementary Table S3). Sequences of other *Otomys* species from

South Africa [5,14] were obtained from a broader study [15] and GenBank and subsequently added for genetic diversity comparison. Taylor et al. [16] identified a close kinship between *O. sloggetti* and *Parotomys*; however, its position on the phylogeny of Otomyinae has remained unresolved [3]. Available *Parotomys* (*P. littledalei* and *P. brantsii* where available) sequences were obtained from Genbank and added onto the alignment. Sequences of *Rattus norvegicus* and *R. exulans*, downloaded from Genbank, were used as outgroups to root the tree. The Rattini tribe is genetically basal to Otomyinae [17-19]. The *R. exulans* sequence was used for *IRBP* only, as *R. norvegicus* sequences were unavailable for *IRBP* in Genbank.

Phylogenetic analyses were performed using MEGA6 (distance-based) [13], PHYML 3.1 (maximum likelihood) [20] and MrBayes 3.2.2 (Bayesian-based) [21,22] programs, as indicated by Phukuntsi [12]. The model of evolution was determined on MEGA6 for distance-based analyses, while jModeltest 2.1.1 [23] was used for both maximum likelihood and Bayesian methods using the Akaike Information Criterion (AIC) [24]. A concatenated dataset of all five gene regions was created manually using MEGA6 [13]. Partitioned multilocus analyses in MrBayes 3.2.2 [21,22] and Garli 2.01 [25] were used to infer phylogenetic trees based on Bayesian and maximum likelihood methods, respectively. For the Bayesian analyses, the same parameters were used as indicated in Phukuntsi et al. [12]. For the maximum likelihood analyses, a heuristic maximum likelihood search of the best tree was performed twice, for 5 million generations, using 20 bootstraps. Thereafter, PAUP 4.0a149 [26] was used to generate a 50% majority rule consensus tree from all the bootstrap trees. According to [27], phylogenetic trees based on concatenated datasets perform as well as, if not marginally better than, simulated species estimation methods. BEST [28] analyses was used to simulate a species tree based on the five gene regions for comparison.

Species delimitation: Three species delimitation methods namely best close match (BCM), nearest-neighbour (NN) and optimised thresholds (OpThr) were performed using the SPIDER package [29] in R Core Team [30], to evaluate the accuracy of assignments from the data in this study. A test for monophyly, as well as for presence of a genetic distance or barcoding gap between the clades observed, was also performed using the R package.

Morphological analyses: Traditional external body measurements [2,3] were not taken from specimens collected as part of the previous Pretoria University study. Most of the specimens were decapitated in this earlier study, and together with removal of the cranium and perfusion of some of the corpses with PFA, it was not possible to measure head body or total body length, tail and ear lengths for many of the specimens. Where possible, five external body measurements (total body length, tail length, mass, hind foot (with and without nail) and ear length) were taken from 23 specimens (Supplementary Table S1). To avoid age related bias four juveniles (represented by BAR1004, BAR1017, BAR1024 and BAR1025) were not included in the morphological analyses. Additional characters, which are considered key to *Otomys* species in the aforementioned publications, were also obtained from the specimens; these included dental, cranial and tail characteristics, and were assessed on specimens represented by BAR1003, BAR1005, BAR1009, BAR1006, BAR1010 and BAR1020. These individuals were chosen as they had intact features, and they represented the distinct groups discovered by genetic analyses in this study (see Results section).

Results

Phylogenetic trees

DNA sequence information of the five gene regions included in this study is provide in Supplementary Table S4. The topologies of the gene trees (Figures 2 and 3; Supplementary Figure S1) were essentially congruent, with the exception of the D-loop (Supplementary Figure S1) tree which consisted of only sequences included in this study. The *O. irroratus* and *O. angoniensis* sequences formed distinct monophyletic clades. The *O. sloggetti* sequences formed two distinct clades, Lower Sloggetti (LS) and Greater Sloggetti (GS) in all datasets. Lower Sloggetti, consistently grouped with either *O. angoniensis* or *O. irroratus* forming an *Otomys* clade. In the Cyt b tree, the Lower Sloggetti clade formed part of a monophyletic clade that included four other established South African *Otomys* species (*O. angoniensis*, *O. irroratus*, *O. laminatus* and *O. s. karoensis*). Sequences of Lower Sloggetti gave the highest percentage match to *O. irroratus* followed by *O. angoniensis* as shown in Supplementary Table S5. Greater Sloggetti grouped separately from the rest of the other *Otomys* sequences, while grouping closer to a *Parotomys* clade (Figures 2 and 3). The exception was in the Cyt b tree, where *O. unisulcatus* grouped with both the Greater Sloggetti clade and *Parotomys* species. However, there was a clear separation between the Greater Sloggetti clade and the *Parotomys* species, although support was low (>70% maximum likelihood bootstrap (BS) and >0.90 Bayesian posterior probability (BPP)). The Greater Sloggetti clade was divided into three subclades, namely GS1, GS2 and GS3. However, in the D-loop tree (Supplementary Figure S1), the GS2 subclade was paraphyletic to the GS1 subclade.

In the concatenated dataset tree (Figure 4), the Lower Sloggetti clade grouped separately from *O. angoniensis* and *O. irroratus*, but formed the same monophyletic group (1 BPP) and 100% (BS) with the two species. Similarly, the Greater Sloggetti clade grouped separately from the other *Otomys* clades, with a high support for the monophyly of the clade (1 BPP and 100% BS). The Greater Sloggetti was split into two subclades (GS1 and GS2), with similarly strong support (1BPP and 100% BS). The monophyly of both GS1 and GS2 was strongly supported (over 0.99 BP and over 87% BS). A similar topology was observed in the species tree simulated using BEST (Figure 5), with similarly strong support for the *Otomys* species (1 BPP), *M. sloggetti* (Greater Sloggetti) species (1 BPP), as well as GS1 (1 BPP) and GS2 (1 BPP) lineages. The monophyly of each of the three species in the *Otomys* clade were strongly supported (1 BPP each).

Genetic distance

The sequence divergence among all major *Otomys* clades was high and ranged from 4.7-14.9% for 16S, 6.8-25.3% for COI, 8.0-33.1% for Cyt b, and 2.3-3.6% for *IRBP* (Table 1). The sequence divergence between the Greater Sloggetti clade and the Lower Sloggetti clade ranged from 3.6% (*IRBP*) to 20.1% (COI). In comparison, the Lower Sloggetti clade was less divergent to *O. irroratus* in three gene regions (4.7% in 16S, 6.8% in COI and 0.086 in Cyt b) than to all the other clades, but similarly divergent to *O. irroratus* and *O. angoniensis* in *IRBP* (2.8%). Sequence divergence between the Greater Sloggetti and the other clades was observed at 10.6% and over for 16S, 19.1% and over for COI, 15.7% and over for Cyt b, 9.2% and over for D-loop and 2.8% and over in *IRBP*. The values for 16S, COI and Cyt b overlapped with inter-generic distances reported in rodents by Baker et al. and Nicolas et al. [17,31,32]. Incidentally, the sequence divergence of the Greater Sloggetti clade from the *Parotomys* sequences was close to, but less than, the sequence divergence between the Lower Sloggetti clade and the *Parotomys* sequences (Table 1). Sequence divergence within

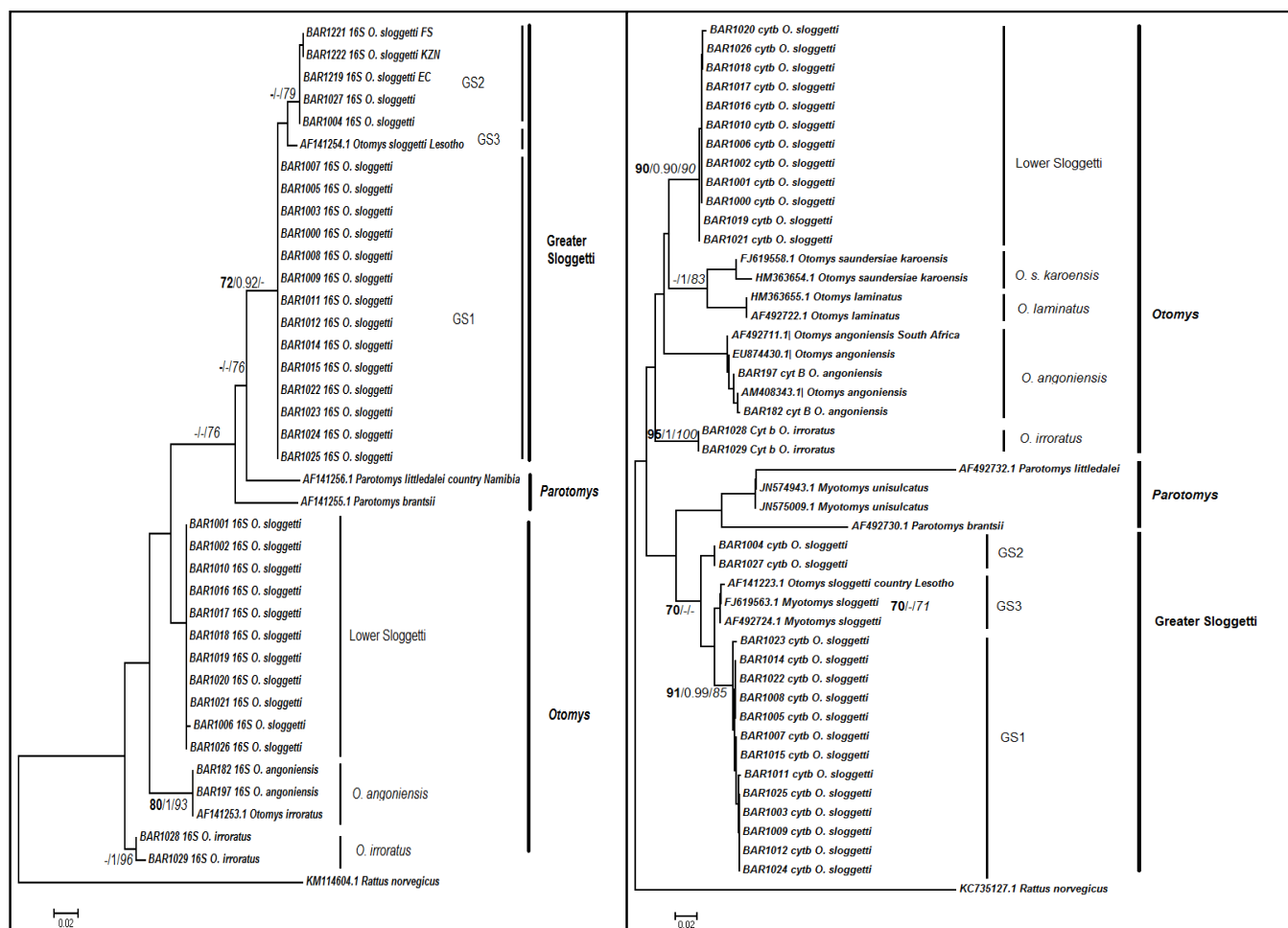


Figure 2: Neighbour-joining trees of two mitochondrial gene regions, 16s (a) and cyt b (b), showing the major clades and lineages in otomys and parotomys genera. The bootstrap values are shown in bold. Bayesian posterior probabilities and maximum likelihood bootstrap (italics) values indicate a topology that was supported in bayesian and maximum likelihood analyses. Only values equal to and above 70% and 0.7 (bayesian probabilities) are shown.

| | 16S | COI | Cyt b | D-loop | IRBP |
|-----------------------------------|---------------|---------------|---------------|---------------|---------------|
| Model of evolution | TN93+G=0.12 | TN93+G=0.21 | TN93+G=0.19 | HKY+G=0.25 | K2+G=0.36 |
| Within | - | - | - | - | - |
| Greater Sloggetti (GS) | 0.009 (0.004) | 0.008 (0.003) | 0.019 (0.005) | 0.023 (0.006) | 0.005 (0.001) |
| Lower Sloggetti (LS) | 0.000 | 0.002 (0.002) | 0.002 (0.002) | 0.005 (0.002) | 0.009 (0.002) |
| <i>O. irroratus</i> (IR) | 0.002 (0.002) | 0.002 (0.002) | 0.001 (0.001) | 0.008 (0.005) | 0.003 (0.002) |
| <i>O. angoniensis</i> (AN) | 0.000 | nc | 0.000 | nc | 0.001 (0.001) |
| <i>O. laminatus</i> (LA) | nc | nc | 0.013 (0.006) | nc | nc |
| Between | - | - | - | - | - |
| GS/LS | 0.106 (0.045) | 0.201 (0.056) | 0.161 (0.054) | 0.092 (0.020) | 0.036 (0.007) |
| GS/IR | 0.149 (0.075) | 0.253 (0.070) | 0.157 (0.050) | 0.105 (0.022) | 0.031 (0.007) |
| GS/AN | 0.142 (0.066) | 0.191 (0.047) | 0.208 (0.083) | nc | 0.028 (0.006) |
| LS/IR | 0.047 (0.022) | 0.068 (0.015) | 0.097 (0.031) | 0.111 (0.026) | 0.028 (0.006) |
| LS/AN | 0.082 (0.041) | 0.081 (0.021) | 0.112 (0.036) | nc | 0.028 (0.006) |
| IR/AN | 0.073 (0.036) | 0.095 (0.024) | 0.140 (0.052) | nc | 0.023 (0.006) |
| GS/SK | nc | nc | 0.221 (0.078) | nc | nc |
| GS/LA | nc | nc | 0.225 (0.088) | nc | nc |
| GS/UN | nc | nc | 0.194 (0.074) | nc | nc |
| LS/SK | nc | nc | 0.117 (0.036) | nc | nc |
| LS/LA | nc | nc | 0.124 (0.044) | nc | nc |

| | | | | | |
|-------|---------------|----|---------------|----|----|
| LS/UN | nc | nc | 0.157 (0.058) | nc | nc |
| IR/SK | nc | nc | 0.130 (0.041) | nc | nc |
| IR/LA | nc | nc | 0.120 (0.042) | nc | nc |
| IR/UN | nc | nc | 0.221 (0.092) | nc | nc |
| AN/SK | nc | nc | 0.186 (0.062) | nc | nc |
| AN/LA | nc | nc | 0.142 (0.049) | nc | nc |
| AN/UN | nc | nc | 0.185 (0.079) | nc | nc |
| SK/LA | nc | nc | 0.080 (0.026) | nc | nc |
| UN/SK | nc | nc | 0.218 (0.091) | nc | nc |
| LA/UN | nc | nc | 0.331 (0.200) | nc | nc |
| BR/GS | 0.090 (0.040) | nc | 0.260 (0.099) | nc | nc |
| LI/GS | 0.073 (0.030) | nc | 0.513 (0.267) | nc | nc |
| BR/LS | 0.091 (0.034) | nc | 0.285 (0.112) | nc | nc |
| LI/LS | 0.119 (0.053) | nc | 0.334 (0.135) | nc | nc |
| BR/LI | 0.105 (0.045) | nc | 0.444 (0.204) | nc | nc |

Table 1: Estimates of sequence variation within and average sequence divergence between *Otomys* groups as determined with MEGA6 [13] using *ad hoc* calculated models of evolution (using modeltest on MEGA6). Values in brackets indicate standard errors. Species with less than two representatives were not added for within sequence variation. NC=Not calculable.

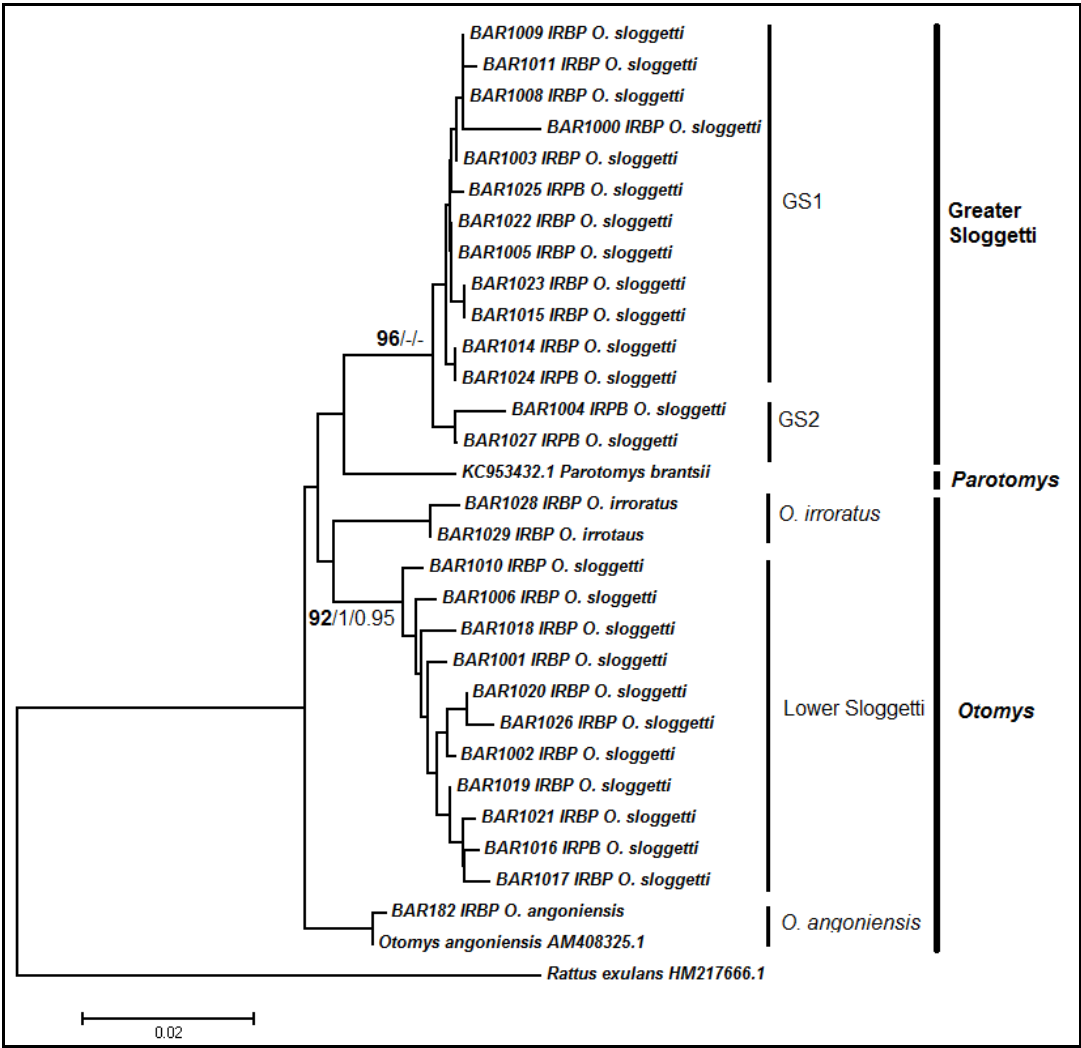


Figure 3: Neighbour-joining IRBP gene tree showing the major clades and lineages in *Otomys*, *Parotomys* and *Myotomys* genera. The bootstrap values are shown in bold. Bayesian posterior probabilities and maximum likelihood bootstrap (italics) values indicate a topology that was supported in Bayesian and maximum likelihood analyses. Only values equal to and above 70% and 0.7 (Bayesian probabilities) are shown.

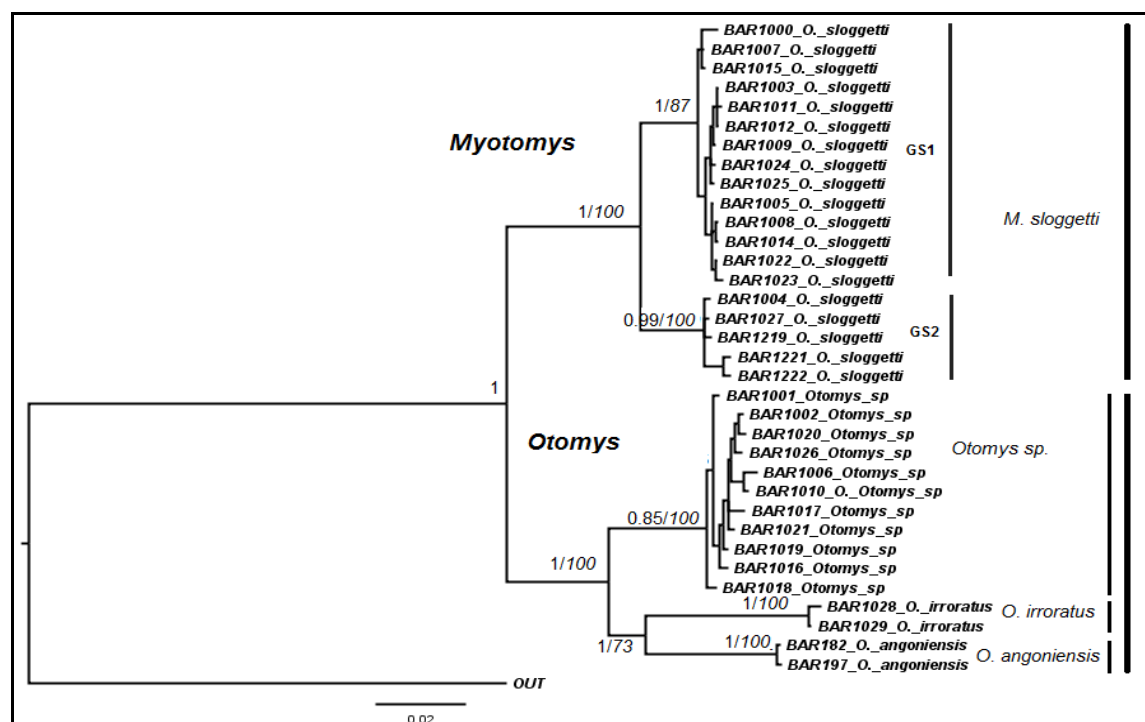


Figure 4: Concatenated dataset Bayesian tree of five gene regions (16S, COI, Cyt b, D-loop and IRBP) showing the major clades and lineages in *Otomys* and *Myotomys* genera. Bayesian posterior probabilities are shown. Maximum likelihood bootstrap values (italics) indicate a topology that was supported in maximum likelihood analyses. Only values equal to and above 0.7 are shown.

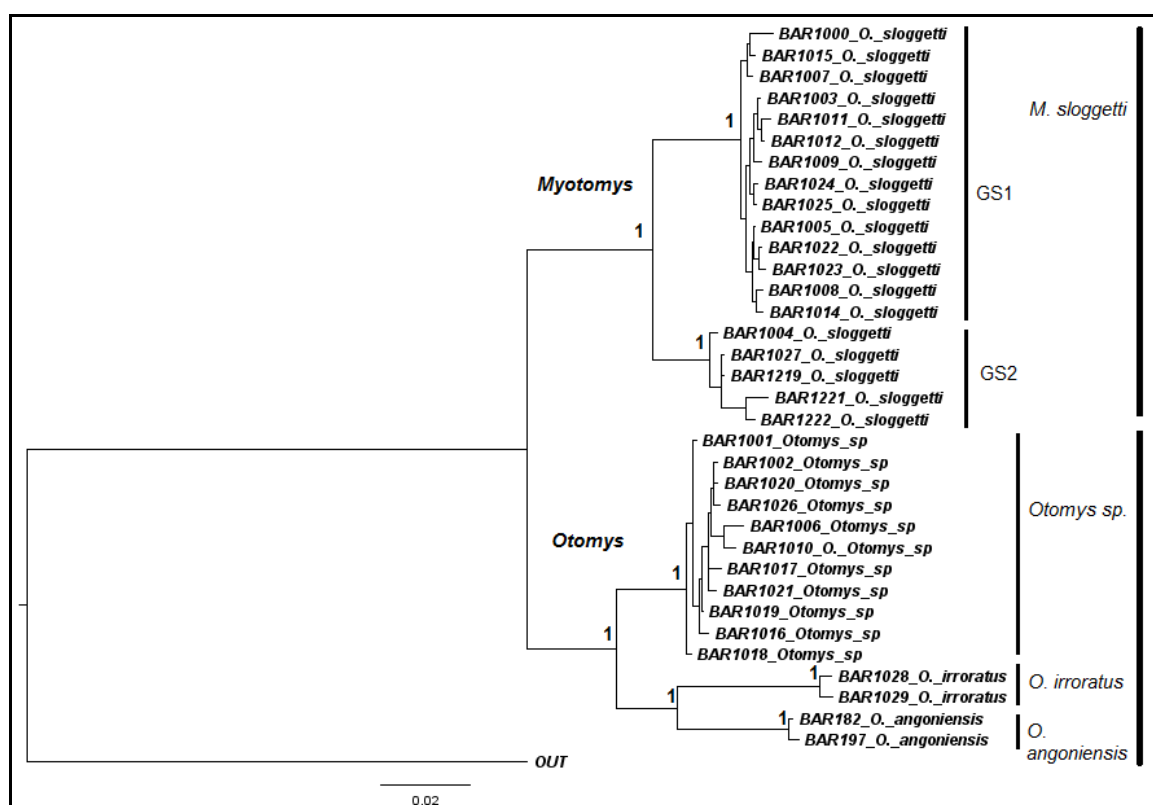


Figure 5: Species tree based on five gene regions (16S, COI, Cyt b, D-loop and IRBP), showing the species in *Otomys* and *Myotomys* genera, as well as two lineages of *M. sloggetti*. Bayesian posterior probabilities (BPP) are shown. The tree was determined using BEST [28].

the Greater Sloggetti was over four times greater (0.9-2.3%) than that within the Lower Sloggetti clade (0.0-0.5%) for all four mitochondrial gene regions. Conversely, sequence divergence within the Lower Sloggetti was almost twice greater (0.9%) than within the Greater Sloggetti clade (0.5%) in the nuclear gene region. This was similarly observed in the gene trees (Figures 1 and 2; Supplementary Figure S1), with the Greater Sloggetti being more variable in the mitochondrial genes than in the nuclear genes, suggesting different evolutionary histories between specimens of the two clades.

Species delimitation

In four gene regions, and the combined tree, with the exception of D-loop, the Greater Sloggetti clade was indicated as a single species under *Myotomys*, *M. sloggetti* and was considered as such in the SPIDER analyses. The rationale behind that was that a) the Greater Sloggetti was excluded from the *Otomys* genus based on the gene trees, b) it was clearly separated from the *Parotomys* clade and c) Musser et al. [1] recognise *Myotomys* as a genus with *O. sloggetti* and *O. unisulcatus* as species under the genus. For 16S, COI and IRBP, three species of *Otomys*, *O. angoniensis*, *O. irroratus* and the Lower Sloggetti clade were indicated. In the Cyt b gene trees, three more species were indicated, *O. laminatus*, *O. saundersiae* and *M. unisulcatus*, all represented by reference sequences from Genbank. In the D-loop gene tree, BAR1004 and BAR1027 were indicated as a paraphyletic clade from *M. sloggetti*, and as such were considered as a distinct species for the analyses in SPIDER. The *Parotomys* sequences were indicated as a separate genus, with two species in each of the 16S and Cyt b gene trees and one species in the IRBP gene tree. The results of the species assignment evaluation are summarized in Table 2.

According to the BCM method, all samples were either correctly identified, or they did not match any available reference sequences. All samples were correctly identified using the D-loop, according to the BCM method, while one did not have any match using COI and IRBP. Three and four samples did not have any match using 16S and Cyt b, respectively. Based on the nearest neighbour method, two samples each in the 16S and Cyt b trees and one each in the COI and IRBP trees did not meet the requirements for correct species assignment, while all samples in the D-loop region were correctly assigned. According to the OpThr method, two samples each in 16S and Cyt b and one in COI were identified as true negative, meaning they were correctly excluded from any of the available species groups. In the D-loop and IRBP tree, all species were correctly assigned. There was a barcoding gap between the major groups in all gene regions. The monophyly of the clades in 16S, COI and IRBP was well-supported, while two out of nine and one out of four clades were rejected in Cyt b and D-loop respectively.

Morphological analyses

Specimens corresponding to the Greater Sloggetti and Lower Sloggetti clades exhibited differences in size, dental and cranial characters, as well as tail morphology and pelage colour (Tables 3 and 4). Greater Sloggetti were larger in body size and mass (121.74% and 158.96%, respectively). Furthermore, the hindfoot length of the two specimens do not over (Lower Sloggetti max=25.5 mm vs. Greater Sloggetti min=26 mm). The hair in the vibrissae region of the face is reddish orange in the Greater Sloggetti specimens compared to the Lower Sloggetti's creamy brown, while the rest of the hair was a buffy, beige, greyish-brown colour (compared to darkish brown with reddish to orange tinge in Lower Sloggetti). The tail tip was broader with less distinct hairs in the Greater Sloggetti compared to the Lower Sloggetti specimens. The Greater Sloggetti specimens had a shallower single groove in the lower incisor than the Lower Sloggetti, with less laminae

(5) compared to the Lower Sloggetti (6) in the upper third molar.

Discussion

According to Musser et al. [16], the *Otomys* genus is largely paraphyletic, with *O. sloggetti* and *O. unisulcatus* generally grouping with *Parotomys* species rather than with other conspecific *Otomys* species. A similar observation was made in this study. Musser et al. suggested that *Parotomys* and *Otomys* be grouped within one genus of *Otomys*, despite the differences shown between *Parotomys* and *Otomys* species based on multidisciplinary data. This recommendation by the authors was made on the observation that two clearly separated groups of Otomyinae rodents could not be consistently established, an observation also made in the Cyt b tree in this study. The data in this study partly supports Musser et al. [1], following Thomas [4], WHO recognized *O. sloggetti* and *O. unisulcatus* in a third genus, *Myotomys*, in moving *O. sloggetti* to *Myotomys*. However, this study did not support the monophyly of *O. sloggetti* and *O. unisulcatus*, instead grouping *O. unisulcatus* with *Parotomys*. Three possible solutions are to a) consider *O. unisulcatus* as a *Parotomys* species, b) consider *Myotomys* as a subgenus of either *Parotomys* or *Otomys* or c) follow Thomas [16] and consider *Otomys* as a single genus under Otomyinae. *O. sloggetti* and *O. unisulcatus* clearly should not be considered species of *Otomys* without the inclusion of *Parotomys*. It is, however, clear that there is paraphyly between *O. sloggetti* and the other *Otomys* species previously termed as a 'mesic group' by other investigators [16]. The Lower Sloggetti clade grouped as a distinct species under *Otomys* consistently in all gene trees, concatenated dataset tree as well as the species tree, even when all South African Otomyinae species were taken into account. This shows that, based on the phylogenetic trees, the Lower Sloggetti specimens should be considered a previously undescribed species of *Otomys* in South Africa.

Of the sequence divergence observed in the gene regions in this study, only 16S and COI reflected the taxonomic hierarchy observed in the gene trees. These values overlap with interspecific distances in rodents reported in published studies, for example Baker et al. [31] (1.3-13.0% in Cyt b), Russo et al. [33] (1.9-16.2% in Cyt b) and Nicolas [32] (0.0-10.1% in 16S; 1.0-19.8% in COI and 1.3-31.1% in Cyt b). Stanhope et al. [34] reported genetic distance of 0.9% for IRBP between two undisclosed *Tonatia* species. It is however important to note that the interspecific ranges in Nicolas et al. [32] included non-congeneric species. The genetic distances between the major clades therefore reflect the genetic distances between species reported in published studies, suggesting that each major clade, including the Lower Sloggetti clade, is a distinct species. There is no Cyt b sequence in Genbank with similarity of above 95% to the Lower Sloggetti Cyt b sequences, with *O. irroratus* sequences being the best match (Supplementary Table S6). While the genetic distances calculated in this study between the other *Otomys* species and *O. sloggetti* overlap intergeneric sequence divergence in reported studies, it is perhaps prudent to note that the aforementioned authors calculated simple Kimura-2-parameter (K2P) distances. Although K2P genetic distances are universally used for species delineation [35], the authors suggested that when calculating genetic distance for phylogenetic analyses beyond species identification, the best-fit model should be used for each dataset, across loci and across sample sets. In this study it was observed that the genetic distances calculated using the best-fit models (Table 1) are remarkably higher than genetic distances calculated using the universal Kimura-2-parameter model in this study (Supplementary Table S6). Based on this incongruence, it is ambiguous whether or not these genetic distances provide empirical evidence of whether *O. sloggetti* is congeneric with the other *Otomys* species or not. However, there is generally a greater

| | 16S | COI | Cyt b | D-loop | IRBP |
|--|-------|------|-------|--------|-------|
| Best Close Match (1% Threshold) | | | | | |
| Correct | 35 | 26 | 40 | 29 | 29 |
| Incorrect | 0 | 0 | 0 | 0 | 0 |
| No ID | 3 | 1 | 4 | 0 | 1 |
| Ambiguous | 0 | 0 | 0 | 0 | 0 |
| Total | 38 | 27 | 44 | 29 | 30 |
| Nearest neighbor | | | | | |
| True | 36 | 26 | 42 | 29 | 29 |
| False | 2 | 1 | 2 | 0 | 1 |
| Total | 38 | 27 | 44 | 29 | 30 |
| Optimized Threshold | 0.014 | 0.02 | 0.013 | 0.008 | 0.009 |
| Cumulative error | 0 | 0 | 0 | 0 | 0 |
| True negative | 2 | 1 | 2 | 0 | 0 |
| True positive | 36 | 26 | 42 | 29 | 29 |
| False negative | 0 | 0 | 0 | 0 | 0 |
| False positive | 0 | 0 | 0 | 0 | 0 |
| Total | 38 | 27 | 44 | 29 | 29 |
| Monophyly of clades | | | | | |
| True | 6 | 4 | 7 | 3 | 5 |
| False | 0 | 0 | 2 | 1 | 0 |
| Total | 6 | 4 | 9 | 4 | 5 |
| Barcoding gap | | | | | |
| True | 38 | 27 | 44 | 29 | 30 |
| False | 0 | 0 | 0 | 0 | 0 |
| Total | 38 | 27 | 44 | 27 | 30 |

Table 2: Results of simulation of species assignment of *Otomys* groups as determined by SPIDER package in R. For the best close match, “No ID” indicates that a match was not found for the particular query, while “ambiguous” indicates that more than one match was found for the query. For the nearest neighbour method, “TRUE” and “FALSE” indicate that a query was correctly assigned and that a query was incorrectly assigned, respectively. For optimized threshold, “true negative” indicate that a query was correctly excluded from a taxon, “true positive” indicates correct assignment, “false negative” indicates that a query was incorrectly excluded from a taxon, while “false positive” indicates that a query was incorrectly assigned. The barcoding gap indicates a positive difference is obtained when subtracting the greatest genetic distance between an individual and conspecific member from the distance between the individual and the closest non-conspecific individual.

| Character | Greater Sloggetti | Lower Sloggetti |
|--|--|---|
| Hair colour in facial vibrissae area | Reddish-orange | Creamy-brown |
| Number and depth of grooves on the upper incisor | One-deep | One-deep |
| Number of laminae on the upper third molar | Five | Six |
| Number and depth of grooves on the lower incisor | One-shallow to non-existent | One-deep |
| Number of laminae on the lower first molar | Four | Four |
| Petrotympenic foramen shape | Slit-shaped (BAR1003) | Round (BAR1020) |
| Tail hair | Smooth along tail, not that noticeable | ‘Spiny’, sticks out away from the tail, so noticeable |
| Tail tip shape | Broad | Narrow |
| General body colour | Buff, beige, greyish-brown | Darkish brown with reddish to orange tinges |

Table 3: Morphological comparisons between specimens of *O. sloggetti* (Greater Sloggetti-BAR1005, 1009 and 1003) and specimens of a previously undescribed *Otomys* species (Lower Sloggetti-BAR1006, 1020 and 1010), which were collected in the same burrows at Tiffindell in the Drakensberg Mountains, Eastern Cape, South Africa.

| Feature | Lower Sloggetti (<i>Otomys</i> sp.) | | | Greater Sloggetti (<i>O. sloggetti</i>) | | |
|--------------|--------------------------------------|------------------|----|---|-------------------|----|
| | Range | Mean(SD) | n | Range | Mean(SD) | n |
| Mass | 45-83 | 71.6 (+/-15.16) | 5 | 78-158 | 113.82 (+/-21.71) | 11 |
| Total length | 170-198 | 184.0 (+/-19.80) | 2 | 224-224 | nc | 1 |
| Tail | 59-74 | 64.5 (+/-7.14) | 4 | 54-73 | 65.13 (+/-5.80) | 8 |
| Hf (cu) | 21.5-25.5 | 23.4 (+/-1.27) | 10 | 26-30.5 | 27.5 (+/-1.35) | 13 |
| Hf (su) | 20-23 | 21.1 (+/- 0.94) | 10 | 23-28 | 24.81 (+/-1.41) | 13 |
| Ear | 15-20 | 17.13 (+/-2.10) | 4 | 15-23 | 16.67 (+/-2.60) | 9 |

Table 4: Summary of external measurements showing comparison between specimens of *O. sloggetti* (Greater Sloggetti) and a previously undescribed *Otomys* species (Lower Sloggetti), which were collected in the same burrows at Tiffindell in the Drakensberg Mountain, Eastern Cape, South Africa. Standard deviation (SD) values are in the parentheses. Hf (cu)=Hindfoot with nail; Hf (su)=Hindfoot without nail; NC=Not calculable.

genetic distance between *O. sloggetti* and the other 'mesic' *Otomys* species than among the 'mesic' species themselves. The genetic analyses in this study indicate that the Lower Sloggetti specimens are a different species from *O. sloggetti*, as well as from the other *Otomys* species.

Three lineages of *O. sloggetti* were observed; however, they do not correspond with the distribution indicated by Taylor [5]. While the authors only consider one subspecies in Eastern Cape, the data in this study shows two very divergent lineages in Eastern Cape (GS1 and GS2). Furthermore, GS2 comprises individuals from Eastern Cape, Free State and Kwa-Zulu Natal. At the very least, the data in this study indicates that there is one distinct taxon within *O. sloggetti* that has not been described before, and indicates that the distribution of *O. sloggetti* requires revision. The three methods of species identification accept GS2 both as a separate species from GS1 and GS3 and as conspecific with GS1 and GS3. However, the data indicating GS2 as a separate species is based on D-loop, which has a higher evolution rate than the rest of the gene regions used in this study [12,36-37]. Although the sample size in this study is too small and lacks adequate geographic diversity to make definite conclusions, the data from the mitochondrial and nuclear gene trees, as well as genetic distances, suggest that the traditional view of the intraspecific and geographic variation in *O. sloggetti* may not be a true reflection of the taxonomy of the species. A multidisciplinary analysis of data from a more extensive data sample set may provide a clearer picture of the intraspecific taxonomy of *O. sloggetti*.

The morphological features described above for the Greater Sloggetti specimens concur with those previously indicated for *O. sloggetti* [3,5]. As for the Lower Sloggetti specimens, the only other *Otomys* species occurring in South Africa that has a slit-shaped petrotympanic foramen is *O. angoniensis*. However, this species has either six or seven laminae on the upper third molar, as well as a noticeable groove on the lower incisor [1,2]. Of the other *Otomys* species in Africa, *Otomys denti* (Thomas 1906), albeit larger, is closest in shared features to Lower Sloggetti. *Otomys denti* also possesses one deep groove on the lower and upper incisors, a round petrotympanic foramen, and six laminae in the upper third molar [3,5]. Furthermore, the specimens associated with the Lower Sloggetti clade in this study are generally smaller (see Table 4) than *O. denti*. Monadjem [3] record *O. denti* having a mean mass of 92.4 g (n=6), total body length of 240.4 mm (152.2 mm head and body+88.2 mm tail; n=11) and hindfoot length of 29.8 mm (n=11).

The specimens associated with the Lower Sloggetti clade in this study were assumed to be *O. sloggetti* as they were collected from the same alpine habitat and burrows as the Greater Sloggetti (i.e., *O. sloggetti*) individuals. However, morphological data indicate that the specimens associated with the Lower Sloggetti and Greater Sloggetti clades are different. Specimens associated with the Greater Sloggetti clade concur with the morphological features of *O. sloggetti* [3,5], and therefore support the genetic data in this study that the Greater Sloggetti clade individuals belong to the *O. sloggetti* taxon and should be identified as *O. sloggetti* species. In contrast, the Lower Sloggetti specimens do not fit the description of *O. sloggetti* [3,5], supporting the genetic data in this study that they are not conspecific with *O. sloggetti*. Specimens associated with the Lower Sloggetti clade do not concur with any southern African species of *Otomys*. The closest species that may be associated with the Lower Sloggetti specimens morphologically is *O. denti*. However, *O. denti* is a central and eastern African species, with Malawi as it's previously reported most southerly distribution [3,5]. The specimens associated with the Lower Sloggetti clade in this study are generally smaller than *O. denti*. While this could indicate that Lower Sloggetti may be a geographic variant of *O. denti* in a previously

unrecognized geographic range, the two species are genetically distinct. A Genbank BLAST search indicated that maximum similarity between the Lower Sloggetti Cyt b sequences and *O. denti* Cyt b sequences was less (92%) than that between the Lower Sloggetti Cyt b sequences and South African *Otomys* species (93-95%; Supplementary Table S6). To our knowledge, there is no species, current or in previous reviews, matching the description of the Lower Sloggetti specimens, considering geographic, genetic and morphological data. We therefore propose that the specimens associated with the Lower Sloggetti clade represent an undescribed species that is most similar to *O. denti*, i.e., *Otomys* cf. *denti*. Further studies on these two species, *O. sloggetti* and *Otomys* cf. *denti*, may assist in providing new insights into the evolution of small mammals, as they have different evolutionary histories although they occupy the same habitat and ecological niche. This study supports the proposal of various authors (e.g. Hebert and Gregory [9], Luttikhuisen et al. [38], Chaval et al. [39]) that phylogenetic analyses based on DNA sequence data are an invaluable tool in species discovery and cryptic species identification, as well as defining geographical boundaries for existing species. Studies on rodents are especially important due to them being carriers of disease that are of zoonotic risk [40,41].

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