Biomes, geology and past climate drive speciation of laminate-toothed rats on South African mountains (Murinae: Otomys)

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ABSTRACT

Mitochondrial DNA sequences (1137 bp) of the cytochrome b gene and craniodental and craniometric data were used to investigate the evolutionary relationships of six putative rodent taxa of *Otomys* (family Muridae: subfamily Murinae: tribe Otomyini) co-occurring in the Western Cape and Eastern Cape provinces of South Africa. Phylogenetic analysis of 20 new sequences together with craniodental and craniometric characters of 94 adult skulls reveal the existence of a unique lineage of *Otomys* cf. *karoensis* (named herein *Otomys willani* sp. nov.) from the Sneeuberg Centre of Floristic Endemism in the southern Drakensberg Mountain Range. Craniometric analysis distinguished *O. karoensis* from *O. willani* and identified a further four localities in the range of the latter species. We document southern range extensions of both Sloggett's ice rat, *Otomys sloggetti*, and the vlei rat *Otomys auratus* to the Sneeuberg Mountain Range, in addition to appreciable genetic divergence between Sneeuberg and southern and central Drakensberg populations of O. *sloggetti*. Our results demonstrate parallel patterns of cryptic speciation in two co-occurring species complexes (*Otomys irroratus s.l.* and *O. karoensis s.l.*) associated closely with the boundaries of biomes (fynbos vs. grassland biomes) and geological formations (Cape Fold Belt vs. Great Escarpment).

Keywords: Africa; mitochondrial DNA; phylogeny; phylogeography; taxonomy

INTRODUCTION

The genus *Otomys* F. Cuvier, 1824 (laminate-toothed rats), in the Tribe Otomyini (Family: Muridae; Subfamily: Murinae) is endemic to sub-Saharan Africa, where it is patchily associated with afro-montane regions from the Cameroon Volcanic Line in west Africa to Ethiopia in east Africa and south to Cape Town (Monadjem *et al*., 2015). The number of species in the genus is probably underestimated due to the likely existence of undescribed cryptic species occurring throughout the mountainous regions of Africa (Carleton and Byrne, 2006; Engelbrecht *et al.*, 2011; Taylor *et al.,* 2009a, b; 2011; 2014). Currently, 31 species are recognized within the genus *Otomys* (Monadjem *et al.,* 2015; Denys *et al*., 2017), compared to 15 recognized by Happold (2013). Taylor *et al.* (2009b) defined the genus *Otomys* as a group of murid rodents with unique laminate molars that distinguish them from other rodent species. Certain characters such as molar lamination have been used in *Otomys* taxonomy (Taylor and Kumirai, 2001; Carleton and Byrne, 2006). Based on these characteristics many species have been described according to progressive increases in the number of laminae in the upper molar (M3).

Laminate-toothed rats originated and diversified in South Africa from 5.0-3.5 MYA, later dispersing to east-central Africa along the African Rift mountains around 2.5-1.6 MYA, and then radiating throughout east, central and northeast Africa and the Cameroon Volcanic Line (Denys, 2003; Taylor, Denys and Mukerjee, 2004a; Taylor *et al*., 2009a; 2014). There are two genera, *Otomys* and *Parotomys*. It has been suggested that *Otomys* is polyphyletic with respect to arid-adapted *Parotomys* Thomas, 1918 (whistling rats) because some *Otomys* species such as Sloggett's ice rat, *O. sloggetti* Thomas, 1902 and the bush Karoo rat, *O. unisulcatus* F. Cuvier, 1829 are more closely related to *Parotomys* than to *Otomys* (Taylor *et al.,* 1989; 2004a; 2009a; 2011; 2014). Some have recognised *Myotomys* Thomas, 1918 as a distinct genus within Otomyini comprising *O. sloggetti* and *O. unisulcatus* (e.g. Pocock, 1976), although the above-mentioned work does not support this notion. The recent study of Phukuntsi *et al*. (2016) advocated the existence of *Myotomys*, but this was based on a narrow subset of South African taxa and thus cannot be construed as a reliable test of the validity of the genus.

There are two species of *Parotomys* (*P. brantsii* (A. Smith, 1834) and *P. littledalei* Thomas, 1918) and seven species of *Otomys* (*O. angoniensis* Wroughton, 1906, *O. auratus* Wroughton, 1906, *O. irroratus* (Brants, 1827), *O. karoensis* Roberts, 1931, *O. laminatus* Thomas &

Schwann, 1905, *O. sloggetti* and *O. unisulcatus*) currently recognized in South Africa (Monadjem *et al*., 2015). Until recently, considerable confusion surrounded the taxonomic status of populations from South Africa formerly assigned to *O. irroratus* s.l. (*O. auratus* and *O. irroratus*) and *O. karoensis* s.l. (which was formerly included in *O. saundersiae* Roberts, 1929). The present study focuses on the phylogeography, taxonomy and phylogeny of these two species groups, together with a third species, *O. sloggetti*, whose distributional limits and subspecies taxonomy have also been subject to debate and for which new molecular and morphological data are presented.

Meester *et al*. (1986) recognized disjunct western (Fynbos Biome) and eastern (Grassland Biome) subspecies of *O. saundersiae*: *O. s. karoensis* and *O. s. saundersiae* respectively. The Fynbos Biome of South Africa comprises Mediterranean-type shrub vegetation restricted to the winter rainfall climate region of the Western and Eastern Cape Provinces; the Grassland Biome comprises grasslands dominating the higher central plateau and most of the Great Escarpment of South Africa (Mucina and Rutherford, 2006). However, the taxonomic status of topotypical populations of *O. saundersiae* from Grahamstown in the Eastern Cape has been called into question (Taylor, Meester and Kearney, 1993; Taylor, Kumirai and Contrafatto, 2005). Taylor *et al*. (1993) showed that two distinct large and small-sized species (*O. irroratus* and *O. cf. saundersiae* respectively) could be distinguished morphologically in the Western Cape, and in the Eastern Cape north of 33 degrees latitude but not in the Eastern Cape south of 33 degrees, which encompassed the Grahamstown type locality of *O. saundersiae*. Based on combined molecular, karyotypic and morphometric evidence Taylor *et al*. (2009a) finally synonymized *saundersiae* under *O. irroratus* and elevated the name *O. karoensis* (with type locality in Tulbagh, Western Cape). The latter species denoted the smaller-sized, pallid-colored forms from two isolated populations from (1) the Fynbos Biome of the Western Cape and (2) the Grassland Biome of the Eastern Cape north of 33 degrees latitude. Without karyotypic and molecular data, however, the relationship between the two isolated populations has remained unknown, but will be addressed by the current study.

Otomys irroratus s.l. was formerly believed to occur throughout both the Fynbos and Grassland Biomes of South Africa extending to the Eastern Highlands of Zimbabwe. However, based on chromosomal and mtDNA evidence, Taylor *et al*. (2009a) and Engelbrecht *et al*. (2011) demonstrated the existence of two parapatric species-clades, *O. irroratus s.s.* from the Fynbos

Biome and *O. auratus* Wroughton, 1906 from the Grassland Biome of South Africa and the Eastern Highlands of Zimbabwe. Both clades occur sympatrically at Alice in the Eastern Cape. The two species cannot be distinguished morphologically; identification relies on chromosomal or molecular data. Considerable chromosomal polytypy has been revealed in *O. irroratus s.l.*, with up to five distinct cytotypes recognised among populations (Contrafatto *et al.*, 1992, Rambau, Elder and Robinson, 2001; Taylor, Kumirai and Contrafatto, 2004b; Taylor *et al.*, 2005; 2009a). Cytotypes cannot be distinguished morphologically (Taylor *et al.*, 2004b). *O. irroratus s.s.* is associated with the "B" cytotype from the Eastern Cape, that contains completely biarmed (metacentric) chromosomes, and the "C" cytotype from the Western Cape that contains an intermediate number of biarmed chromosomes. The variable number of metacentric chromosomes is due to the presence or absence of heterochromatic short arms (Contrafatto *et al.*, 1992). *O. auratus* is characterized by three cytotypes occurring in the Eastern Cape and KwaZulu-Natal provinces that have mostly acrocentric (single-armed) chromosomes ("A", "A1" and "A2). Populations of *O. auratus* in the Highveld of South Africa (Free State, Gauteng, Limpopo and Northern Cape provinces) carry the "C" cytotype but this mutation represents a convergent mutation in *O. irroratus* and *O. auratus*. Similarly, the Zimbabwe population of *O. auratus* carries a karyotype similar to the "B" cytotype found also in *O. irroratus*. Taken together with distribution, the karyotype is a highly diagnostic species character.

Given that speciation has occurred between Fynbos and Grassland populations of *O. irroratus s.l.* (comprising *O. auratus* and *O. irroratus*; Engelbrecht *et al.*, 2011), this raised the possible existence of similar disjunction and speciation within co-occurring *O. karoensis s.l.* (including isolated western Fynbos and eastern Grassland populations) from these same biomes. The availability of mtDNA and associated craniometric and craniodental evidence from new collections of *O. cf. karoensis* (small and dark-reddish colored) and *O. cf. irroratus* (large and dark-reddish-colored) from the Sneeuberg Range of the southern Drakensberg allowed us firstly to critically test this hypothesis, and secondly to determine which cryptic species within *irroratus s.l.* (*irroratus s.s.* or *auratus*) was present in the region.

A number of mtDNA sequences were also available from the current study and that of Phukuntsi *et al.* (2016) of *O. sloggetti*, which afforded the opportunity to examine the phylogeography of *O. sloggetti* from various sites in the Drakensburg from just over 2000 m to over 3000 m. Ice rats are herbivorous, burrow-dwelling rodents, currently endemic to the

southern African Drakensberg and Maluti mountains at altitudes above 2000 m. The species is adapted to living in alpine and sub-alpine habitats and generally lives in flat, grassy areas, which receive a maximum amount of sunlight, allowing the animals to sunbathe (Richter, Webb and Skinner, 1997). The range of *O. sloggetti* was once thought to include much of the Karoo and the Free State province (De Graaff, 1981, Smithers, 1983), but Lynch and Watson (1992) showed that it is mostly restricted to the Drakensberg Mountains north of 32˚S and east of 26˚E, albeit with a few relic records from isolated mountainous regions of the Karoo in the Beaufort West, De Aar, Hope Town, Britstown and Hanover districts (Lynch and Watson, 1992). These old Karoo records include the type locality of Deelfontein in the Karoo where the species has not been described since its discovery in 1902, making it likely that the range of the species has contracted in historical times. Of these isolated Karoo records, only those from Hanover and Hope Town districts could be validated by specimens (Lynch and Watson, 1992). As *O. sloggetti* is restricted to high altitude alpine and sub-alpine habitats along the Drakensberg Mountain Range and (as shown in this study) certain other parts of the Southern Great Escarpment, genetic fragmentation is likely to occur across the species' distribution range. Richter *et al.* (1997) indicated that the distribution ranges of *O. sloggetti* and *O. irroratus* meet but do not overlap because of different habitat, altitudinal and temperature preferences. However, both species were captured at one site (Asante Sana Private Game Reserve in the Sneeuberg Mountains), which shows the species can coexist (A.Kok, pers. comm.).

In summary, our study aims: (1) to reconstruct the mitochondrial phylogeny of South African *Otomys*, with special emphasis on the *O. karoensis*, *O. irroratus* and *O. sloggetti* groups; (2) to analyse morphological variation of different mitochondrial clades; (3) to synthesize the taxonomic implications of the combined information, leading to the formal description of a new species from the Sneeuberg Centre of Floral Endemism. As followed in previous revisions (Taylor *et al*., 2009; 2011; 2014), we apply the Evolutionary Species Concept and consilience principles ("integrative taxonomy") in describing and delimiting species (see Taylor *et al*., 2019).

MATERIALS AND METHODS

STUDY AREA AND SAMPLING SITES

The molecular study used samples collected from three sites in the Sneeuberg Mountains, encompassing the Sneeuberg Centre of Floristic Endemism (Clark *et al*., 2009): Asante Sana Private Game Reserve (AS), Mountain Zebra National Park (MZNP), and Sneeuberg Private Nature Reserve (SPNR) (Table 1, Figure 1). Additional samples were obtained from material collected from Sani Pass in the Drakensberg mountain range (collector: S. Maree). Intact skulls for craniodental and morphometric analysis were available for six individuals used in the molecular study above (five *cf. karoensis* and one *cf. irroratus*). In addition, a further 88 skulls were analysed from the following Museum collections: Durban Natural Science Museum (DM), Ditsong National Museum of Natural History (TM), National Museum, Bloemfontein (NMB), Amathole Museum, King William's Town (KM), and the Royal Museum for Central Africa, Tervuren, Belgium (MRAC). We selected series of skulls from localities where the species identity of cryptic species was known based on previous chromosomal or molecular studies (Table 1; Contrafatto *et al.,* 1992; Rambau *et al.,* 2001; Taylor *et al.*, 2005; 2009a; Engelbrecht *et al.*, 2011). Craniodental characters were also scored for an additional 14 voucher specimens (seven *O. cf. karoensis* and five *O. sloggetti*) from the study of Phukuntsi *et al.* (2016), currently housed in the TM.

Samples from the Sneeuberg Range were trapped between altitudes of 1740 m and 2150 m, in relatively undisturbed habitats (Table 1), and the habitat at each site was characterised according to its vegetation and physical characteristics. Animals were trapped alive using Sherman traps and standard methods were used to measure and sex animals and collect tissues from toe clipping for DNA (Kok *et al.*, 2012; 2013). Six animals were sacrificed to obtain skulls for morphological analysis, and skulls were deposited in the mammal collection of the Durban Natural Science Museum (catalogue numbers DM13657, DM13658, DM13659, DM13660, DM13661, DM13662).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

DNA was extracted from toe clips using the Invisorb® Spin Tissue MiniKit (Invitek©) following the manufacturer's protocol. Prior to addition of a lysis buffer (400 µl) and Proteinase K (40 µl),

the tissues were mechanical ground to increase lysis efficiency. Non-lysed material was removed by centrifuging.

For PCR amplification of the *cyt b* region, 5 µl of template DNA was used for PCR reactions in a total volume of 50µl (5 µl 10x PCR reaction buffer, 4 µl $MgCl_2$, 2 µl deoxynucleotide triphosphate mix (dNTP), 1 µl of each primer (L147124 and H15915) (Taylor *et al.,* 2009b), 0.1 µl Taq DNA polymerase, and 32 µl double-distilled water). In some cases, the above primers failed to produce PCR product and samples were subsequently amplified using two newly designed internal primers, namely Oto-intF′: 5′- CAG GAA ACA GCT ATG ACC CAT CRG ACA CAA CAA CAG C-3′ and Oto-intR′: 5′-TGT AAA ACG ACG GCC AGT GGA GAA GTA GCT RAT GGA RGC-3′).

PCR amplifications were performed using the following thermal conditions: initial denaturation at 94°C for 2 min and then 35 cycles of (denaturing at 94°C for 1 min, primer annealing at 52°C for 1 min and product extension at 72°C for 1 min), followed by a final extension phase at 72°C for 5 min (Taylor *et al.,* 2009b; Colangelo *et al.,* 2007). To view PCR products, 5 µl of the amplification product was mixed with 5 µl loading dye containing SYBR® Green, loaded onto a 1% agarose gel, subjected to electrophoresis at 100V for 10 min, and visualized by means of a UV transmitter. The PCR products were purified using the Invisorb PCRapace® Quick Purification Kit (Invitek) according to the manufacturer's protocol. Purified PCR products were then sequenced directly in both forward and reverse directions using the dye-terminator cycle sequencing method implemented with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Washing and precipitation of sequencing PCR products was achieved in several steps involving addition of 3M NaOAc, 99% ethanol, 70% ethanol, and centrifugation. PCR products were subsequently sequenced on an ABI 3100 genetic analyser at the Rhodes University DNA Sequencing facility.

SEQUENCE EDITING AND ALIGNMENT

DNA sequences were checked and edited using the program Sequencher ver. 4.5 (GeneCodes Corporation). Maximum sequence length was 1137 bp, though sequences for some samples were shorter than this (970 bp). Novel *cyt b* sequences for 20 samples were generated in this study, and added to 45 ingroup and 4 outgroup sequences (see below) obtained from GenBank. These

GenBank sequences have been previously published in Ducroz *et al.* (2001) and Taylor *et al.* (2009a; 2009b; 2011; 2014). We furthermore obtained 28 *cyt b* sequences of 470 bp from the study of Phukuntsi *et al.* (2016). Alignment of these sequences for phylogenetic analysis was achieved using MEGA ver. 7 (Kumar *et al.,* 2016). We chose five species of Avicanthini as outgroups since this tribe is a sister tribe of Otomyini (Lecompte *et al*., 2008).

PHYLOGENETIC ANALYSIS

Our analysis comprised 93 ingroup taxa (90 *Otomys* and 3 *Parotomys*) and five outgroup *Aethomys namaquensis*, *Dasymys incomtus* s.l., *Desmomys harringtoni*, *Hybomys univittatus*, *Rhabdomys pumilio* s.l. The majority of *cyt-b* sequences comprised 1137 bp, although we included 28 sequences from Phukuntsi et al (2016) comprising 407 bp. A substitution model of sequence evolution that best fitted the data was estimated in jModelTest ver. 2.1.10 (Posada, 2009). The model selected was Tamura-Nei plus Gamma (*G*) and Invariable sites (*I*), and this was used for Maximum Likelihood (ML) (Felsenstein, 1981) and Bayesian (Ronquist and Huelsenbeck, 2003) analyses. Phylogenetic relationships were evaluated using the Maximum Likelihood (ML) method implemented in Mega 7 (Kumar *et al.*, 2016). To estimate support for internal nodes, 1000 bootstrap replications were run using the same program (Felsenstein, 1985; Kumar *et al*., 2016). Phylogenetic relationships were also reconstructed using Bayesian Inference (BI) implemented in MrBayes ver. 3.2.7 (Ronquist and Huelsenbeck, 2003). The same program was used to obtain node posterior probabilities as support for relationships reflected in the Bayesian topology. Markov Chain Monte Carlo (MCMC) chains were run for 400 000 generations and sampled every 100th generation with 20% burn-in. The run was terminated after the standard deviation of split frequencies (SD=0.016) was below 0.05. Congruence among tree topologies emanating from analysis of *cyt b* sequences using these three different phylogenetic reconstruction methods was assessed. The final Bayesian tree was chosen as the best estimate of relationships, and support values for BI and ML were plotted onto this best tree (Figure 2).

CRANIODENTAL AND CRANIOMETRIC ANALYSES

We analysed variation in a sample of 94 intact skulls using eight craniometric variables taken to the nearest 0.01 mm using Mitutoyo digital callipers with accuracy of 0.01 mm: 1) greatest length of skull (GLS), measured dorsally, equivalent to occipito-nasal length; 2) mandible length (MDL), greatest length of the mandible excluding teeth; 3) maxillary tooth row length (MXTRL), distance from anterior edge of first maxillary tooth to posterior edge of last maxillary tooth at crown; 4) nasal width (NAW), greatest width across nasals at right angle to skull axis; 5) interorbital constriction (IOC), least distance dorsally between the orbits; 6) zygomatic width (ZYW), greatest distance between the outer margins of the zygomatic arches; 7) palatal length (PAL), from anterior edge of premaxillae to anterior-most point on posterior edge of palate; 8) greatest length of bulla (BUL), along the longitudinal axis and excluding the eustachian tube. For each skull we determined the relative age class (1-5) based on tooth eruption and the degree of tooth wear (Taylor *et al.*, 1993). Following Taylor *et al.* (1993), we took Class 4 and 5 individuals as fully adult; younger Class 3 individuals (probably young adults) were only included in a few instances where they were necessary for complete geographic or taxonomic representation. Class 1 and 2 individuals were excluded. As Taylor *et al.* (1993) did not find sexual dimorphism in *O. irroratus* or *O. saundersiae s.l.*, we pooled the sexes in our analysis. To visualise intraspecific and interspecific variation in multidimensional space of linear measurements, principal component analysis (PCA) was carried out on log-transformed cranial variables using the programme PAST (Hammer, Harper & Ryan, 2001). Univariate analyses including Analysis of Variance (ANOVA) and pair-wise Tukey tests were also carried out in PAST.

RESULTS

PHYLOGENETIC ANALYSIS

The best tree produced by Bayesian analysis of the *cyt b* alignments is shown in Figure 2. As revealed by earlier studies (Taylor *et al*., 2009; 2014), basal relationships within the Otomyini tribe are poorly resolved by *cyt b* although terminal clades are better supported. Our molecular data showed the presence of two major lineages (cryptic species) within *O. irroratus s.l.* (*O. auratus* and *O. irroratus* respectively) which supports the findings by Taylor *et al.* (2009a) and

Engelbrecht *et al.* (2011). Four specimens from Asante Sani (AS) in the Sneeuberg Range grouped clearly within the *O. auratus* clade, providing a southern range extension for this species (Fig. 1). The sister group to *O. irroratus* and *O. auratus* (with 76% bootstrap support) was a well-supported (98% bootstrap support) clade including *O. laminatus* and *O. karoensis.* Two specimens from MZNP (MZP 7 and 13) formed a well-supported and relatively deeply divergent lineage that had no supported relationship with any other Otomyini species (Figure 2); K2P genetic distances varied from 8.5% (*unisulcatus*) and 15.1% (*Parotomys*) (Table 2). Since no voucher specimens were available for this clade, it was not possible to assign this to any existing or new *Otomys* species). Three specimens from AS, one from MZNP, one from Giant's Castle in the Eastern Drakensberg and 11 from Tiffindell (classified as "Lower *sloggetti*" by Phukuntsi *et al.* (2016)) formed a new lineage (*O. cf karoensis* sp. 1), with no strongly supported link to any other clade of Otomyini.

Three distinct groups of *O. sloggetti* coincided with groups identified by Phukuntsi *et al.* (2016) as "GS1", "GS2" and "GS3". The GS1 clade includes individuals from Tiffendell Ski Resort. The GS2 clade corresponds to the Sneeuberg mountain range (AS, MZNP and SPNR) but includes two individuals from Tiffenfdell. GS3 corresponds to a northern Drakensberg clade from Sani Pass in Lesotho. The discovery of *O. sloggetti* specimens from the three Sneeuberg localities (AS, MZNP and SPNR) substantially extends the known southern (32.5˚S) and currently documented western (26.6˚E) limits of the distribution range of this species.

In order to determine lineage-level genetic divergences, average pairwise K2P distances were calculated (Table 2) between the putative species clades labelled in Figure 2. The largest divergences were observed between *Parotomys brantsii* and the other species groups (11.5 – 15.1%). The lowest divergences were detected between *O. laminatus* and *O. karoensis* (5.5%), *O. auratus* and *O. irroratus* (7.1%), *O. cf. karoensis* sp.1 and *O. auratus* (7.1%) and *O. cf. karoensis* sp.1 and *O. unisulcatus* (7.1%). The sequence divergence within groups varied from 0.0% in *O. laminatus* and *O. unisulcatus* to 2.4% in *O. auratus* and 2.6% in *O. sloggetti*.

CRANIODENTAL AND CRANIOMETRIC ANALYSES

Assessment of diagnostic craniodental characters allowed us unequivocally to distinguish *O. sloggetti* from all other *Otomys* species based on the absence of a deep groove in the lower incisors (*cf.* at least one deep groove present in *irroratus*, *auratus*, *karoensis* and *karoensis s.l.*, and/or the presence of a slit-shaped petrotympanic foramen (*cf.* round-shaped hole in other species). In seven voucher skulls in the TM identified by Phukuntsi *et al.* as "Greater *sloggetti*", the incisors possessed ungrooved or very faintly grooved incisors, confirming their identity as *O. sloggetti*: (BAR1003/TM49089, BAR1005/TM49076, BAR1007/TM49081, BAR1008/TM49075, BAR1009/TM49086, BAR1012/TM49077, BAR1023/TM49089). In one of these skulls where the bulla was intact (B1003), the petrotympanic foramen was slit-shaped, confirming the identification as *O. sloggetti*. In nine skulls identified by Phukuntsi *et al.* (2016)as "Lower *sloggetti*", a distinct deep groove was present. This character, in conjunction with maxillary tooth-row length (see below), identifies these specimens as *O. cf. karoensis*: BAR1001/TM49092, BAR1002/TM49094, BAR1006/TM49096, BAR1010/TM49093, BAR1016/TM49100, BAR1019/TM49097, BAR1020/TM49101, BAR1021/TM49091, BAR1026/TM49095. In two of these skulls (B1001/TM49092, B1020/TM49101) it was possible to confirm the presence of a round-shaped petrotympanic foramen.

PCA (Figure 3, Table 3) identified four groups consistent with the four putative species-clades identified by mtDNA sequences: *O. karoensis* from Western Cape, *O. cf. karoensis* Sp. 1 from the Eastern Cape, *O. irroratus* from the Western Cape and south-eastern Cape, and *O. auratus* from north-eastern Cape, albeit with considerable morphometric overlap between *irroratus* and *auratus*. These groups were distinguished mostly on cranial size (PC 1: Table 3) but a fifth group of three specimens from the Free State, referred to as *O*. cf. *karoensis* Sp. 2 (that were not sequenced), could be distinguished from other groups in having a proportionately large bulla (high PC2 loadings; Table 3). Even in univariate tests, BUL of these Free State animals was significantly larger than in the other four species (Table 4). The ratio BUL/GLS was 22-24% in the Free State sample compared to 15-22% in all other species samples pooled. Although *O. karoensis* and *O. cf. karoensis* Sp. 1 overlapped somewhat on PC axes 1 and 2 (Figure 3), complete separation was obtained between them on plots of PC1 and PC3. PC3 is a shape vector that contrasts interorbital constriction and palatal foramina length (high positive loadings) with nasal width (high negative loading) (Table 3). *O. cf. karoensis* Sp. 1 has a disproportionately

narrow interorbital bone, short palatal foramina and wide nasal bone compared with *O. karoensis* (Tables 3, 4, Figures 3, 4).

According to a key to *Otomys* species (Monadjem *et al.*, 2015), *O. karoensis s.l.* can be distinguished from both *O. irroratus* and *O. auratus* based on its smaller tooth-row length (< 9 mm in *karoensis s.l. cf.* > 9 mm in *O. irroratus* and *O. auratus*). This character holds true for the specimens in our study; maxillary tooth-row length (MXTRL) is always < 9 mm in specimens of *cf. karoensis* from AS, SPNR and MZNP identified by mtDNA as Clade D, as well as in all nine skulls of "Lower *sloggetti*" from Tiffendell in Phukuntsi *et al*. (2016) (Table 4, additional observations by TK), while it is always > 9 mm in *O. irroratus* and *O. auratus* (Table 4). Tukey pair-wise tests for MXTRL revealed significant differences between *O. karoensis* / *O. cf. karoensis* and *O. irroratus* / *O. auratus*, as well as significant differences between *O. karoensis* (larger tooth-row) and *O. cf. karoensis* Sp. 1 and Sp. 2 (smaller tooth-row) (Table 4). Apart from MXTRL, *O. karoensis* / *O. cf. karoensis* were also significantly smaller than *O. irroratus* / *O. auratus* in five additional variables, GLS, GLM, NAW, ZYW and PAL, while *O. cf. karoensis* Sp. 1 and Sp. 2 and *O. karoensis* differed from each other significantly in five variables, GLS, GLS, MXTRL, IOC and PL (Table 4).

DISCUSSION

INSIGHTS INTO THE BIOGEOGRAPHY, SPECIATION AND DIVERSITY OF *OTOMYS* IN THE CAPE REGION OF SOUTH AFRICA

Prior to 2009, five species of *Otomys* were known to occur in the Western and Eastern Cape provinces of South Africa: *O. sloggetti*, *O. unisulcatus O. laminatus*, *O. irroratus* and *O. karoensis* (formerly *O. saundersiae*). More recently, the work of Taylor *et al*. (2009a) and Engelbrecht *et al*. (2011), and this study, increased the diversity of *Otomys* species in the region to seven species: *O. sloggetti*. *O. unisulcatus*, *O. laminatus*, *O. irroratus*, *O. auratus*, *O. karoensis* and *O. cf. karoensis* Sp. 1 (described formally below as *O. willani* sp. nov.), and identified up to three species co-occurring at a single locality in the Sneeuberg Range. Further undescribed cryptic species await discovery in this region. These include the divergent

unidentified clade from Mt Zebra National Park in this study, which could not be corroborated due to the absence of voucher specimens. Also, specimens of *O. cf. karoensis* Sp. 2 from the Free State which are morphologically distinct due to their small skull size and proportionately expanded auditory bullae lacked corroborating molecular and/or karyotypic data. It is not clear whether the three distinct mtDNA subclades of *O. sloggetti* correspond with two or more cryptic species within this species, and further morphometric and karyotypic analyses are required.

Clark *et al.* (2009; 2011; 2015) and Nordenstam *et al.* (2009) identified the Sneeuberg Centre of Floristic Endemism and described new plant species endemic to this centre. The distribution of the newly described *O. willani* sp. nov coincides very closely with the geographical limits of the Sneeuberg Centre described by Clark et al. (2009), but extending into the main Drakensberg (Tiffendell and Giant's Castle) at its eastern limit.

Otomys cf. karoensis Sp. 1 *clade*. Vlei rat species show extreme morphological conservatism which makes them very difficult to identify in the field. Small, pallid-coloured specimens from the Sneeuberg Range (this study) and Tiffendell (from Phukuntsi *et al.* 2016) were genetically distinct from *O. auratus*, *O. irroratus* and *O. sloggetti* as well as from *O. karoensis s.s*. Morphometric and craniodental analysis (Figure 3 and Results above) confirmed their specific separation from these four species, highlighting the critical importance of retaining museum voucher specimens in molecular studies, especially in the case of cryptic species. We describe this new *cf. karoensis* lineage below as *O. willani* sp. nov. This result confirms our hypothesis, based on the cryptic speciation observed in *O. irroratus s.l.* (Engebrecht *et al.*, 2011), that co-occurring *O. karoensis s.l.* would also show evidence of speciation between Grassland and Fynbos Biomes from the Southern Escarpment and Cape Fold Belt mountain ranges respectively. The drivers of this speciation were therefore probably the same for both species complexes and are described below for the *O. irroratus – auratus* clade.

O. irroratus – auratus *clade*. Phylogenetic analysis of *cyt b* gene sequences revealed the existence of two major evolutionary lineages with no shared haplotypes, consistent with the results of Taylor *et al.* (2009a) and Engelbrecht *et al.* (2011). These two lineages that diverged 1.1 MYA (Taylor et al. 2009; Engelbrecht *et al.*, 2011) coincide with different biomes and mountain ranges (*irroratus* with the Cape Fold Belt and the Fynbos Biome and *auratus* with the Great Escarpment

and the Grassland Biome). A contact zone between the two lineages was identified at Alice in the Eastern Cape by Engelbrecht *et al.* (2011). The pattern we observed was mirrored exactly in the case of another montane rodent, the four-striped mouse *Rhabdomys pumilio s.l.* whose range corresponds closely with that of *O. irroratus s.l.*, where disjunct genetic lineages coincided with Grassland and Fynbos Biomes and also showed a contact zone in the region of Alice (Du Toit *et al.*, 2012). Similarly, Willows-Munro & Matthee (2009) identified distinct lineages of montane forest shrews from the Fynbos and Grassland Biomes of South Africa. Together with the fact that the *O. karoensis s.l.* complex shows exactly the same pattern of vicariance (see above), this striking congruence across several distinct montane-adapted rodent and shrew taxa points to a common evolutionary cause as detailed below.

Our new data from AS allowed us to extend the western limit of *O. auratus* in the Eastern Cape from around 27˚E (Hogsback) to 25˚E (AS). The origin of the two major clades within *O. irroratus s.l.* (and *O. karoensis s.l.*) may be due to vicariance processes caused by aridification. Historically, Africa has experienced periods of high-latitude glaciation cycles, which influenced African climates from the late Pliocene causing periods of aridification (Lawes *et al.,* 2007; Taylor *et al.,* 2009a). In southern Africa this climate change is thought to have caused the onset of drier and/or warmer conditions that led to shrinking of grasslands and fragmentation of biomes due to increased temperature and decrease in rainfall (De Menocal 2004; Taylor *et al.,* 2009a, 2015). Aridification of the biomes may have caused the preferred habitat of the species to shrink in size away from other suitable habitats, thereby separating the species distribution into two or more isolated ranges and preventing gene flow between them. A genetic study of snails occurring in the Southern Escarpment demonstrated the importance of aridification in the vicariance of lineages (Barker *et al.*, 2013). Temperate-adapted species such as *O. irroratus* and *O auratus* would have been displaced to higher elevations in both the Drakensberg and the Cape Fold Mountain Ranges, effectively isolating populations to these two distinct mountain ranges, whose higher elevations retained temperate climates. Therefore, climatic change linked to elevational heterogeneity is the most likely cause for the distinct difference in the two clades of *O. irroratus s.l.* Specifically the dated split between *O. irroratus* and *O. auratus* of 1.1 MYA corresponds closely with a period of aridification around 1 MYA (De Menocal, 2004; Taylor *et al.,* 2009a). The fact that the range of *O. irroratus* corresponds very closely with both the Cape Fold Belt geology and the Fynbos Biome while *O. auratus* corresponds with both the Grassland

Biome and the Great Escarpment mountain system respectively, suggest that while geology combined with climate may have been the cause for the initial divergence of lineages, subsequent speciation and evolutionary divergence may have been aided by ecological co-adaptation to the different vegetational Biomes.

From mismatch coefficients from mtDNA sequences, Engelbrecht et al. (2011) showed that *O. irroratus* had a unimodal distribution indicative of a recent population expansion, possibly explaining the eastern expansion of *O. irroratus* populations away from the Cape Fold Mountains in the E Cape Province to form a secondary contact zone with *O. auratus* at Alice on the Great Escarpment. On the other hand, *O. auratus* had a multi-modal mismatch indicative of a stable (and older) population. As also found by Taylor et al. (2009) and Engelbrecht et al. (2011), our data revealed two distinct sub-clades within *O. auratus* which are distributed west (central Plateau of South Africa and the Eastern Highlands of Zimbabwe) and east (eastern coastal escarpment populations extending from Alice and Hogsback in the W Cape to populations from the Drakensberg foothills and midlands of KwaZulu-Natal) of the Great Escarpment. This event was dated at 0.66 MYA by Engelbrecht et al. (2011) and could be explained by a period of warming and / or aridification that resulted in populations becoming fragmented and shrinking downslope on the foothills and escarpments west and east of the high Drakensberg Mountains. Significantly, populations from the Sneeuberg Range are affiliated with the western clade from the Central Plateau rather than with the eastern coastal clade even though populations from Mt Zebra (western clade) and Hogsback (eastern clade) are separated by only 100 km.

O. sloggetti *clades.* Our study revealed new distribution records for *O. sloggetti* that extend the range beyond the Drakensberg Range to the adjacent Sneeuberg region of the southern Great Escarpment. *O. sloggetti* was collected at the three localities that were sampled in the Sneeuberg Range (AS, MZNP, and SPNR). However, other mountain regions between the Sneeuberg and Drakensberg such as the Winterberg and Stormberg have not been sampled. The existence of three distinct clades of *sloggetti* from the Sneeuberg, Drakensberg and Maluti Ranges suggests that some vicariance and/or speciation may have occurred between these lineages.

IMPLICATIONS FOR CONSERVATION

The IUCN reports by Taylor and Monadjem (2008) and Taylor, Maree and Monadjem (2008) suggested that *O. irroratus s.l.* and *O. sloggetti* respectively were of least conservation concern, presumably due to perceived wide distribution including several protected areas, a presumed large population, and because populations are not believed to be in decline at present. Results from the present study and those of Taylor *et al.* (2009a) and Engelbrecht *et al.* (2001), however, refute these conclusions because lineages were identified that are separate taxonomic entities, which have restricted geographic ranges. Given the numerous threats to South African mountains including the southern Great Escarpment, the need for effective conservation measures for some of the newly identified cryptic species and lineages is imperative. Threats include a combination of habitat loss, soil erosion, land degradation and fragmentation, with consequent population isolation due to unsustainable land use practices such as overgrazing (Shroyer and Blignaut, 2003).

Based on projected declines in fynbos and grassland habitats due to climate change, Taylor *et al.* (2015) projected 12-24% and 47-60% declines by 2050 in the ranges of *O. irroratus* and *O. auratus* respectively. Partly based on these results the IUCN Red List category for *O. auratus* has been elevated to Near Threatened (Baxter, Taylor and Child, 2017), while *O. irroratus* has remained as Least Concern (Taylor and Baxter, 2017). Red List assessments of the newly described *O. willani* sp. nov. as well as *O. karoensis s.s.* have not yet been performed, but could lead to threatened categories for these species. *O. karoensis s.l.* was assessed as Least Concern (Baxter, Child and Taylor, 2017), but this assumed a wider distribution of the species across both Grassland and Fynbos Biomes, and it was stated by the above authors: "However, the effects of climate change on this species should be monitored and, should molecular research reveal a species complex, it will necessitate a reassessment". The present study indicates that such a reassessment is indeed necessary to review the Red List categories of *O. willani sp. nov.* and *O. karoensis s.s.* Further work also needs to be undertaken to resolve the identification of the different *O. sloggetti* mtDNA subclades, the divergent, unidentified clade from MZNP, and *O.* cf. *karoensis* Sp. 2., as without formal identification the threats cannot be assessed.

Based on this work, it is clear that conservation programs should be focused first on conserving the mountains (Shroyer and Blignaut, 2003) as well as preserving the populations as distinct entities. Identifying the genetic diversity of these species is a productive step towards addressing evolutionary and conservation questions, and establishing management protocols to

promote the long-term persistence of populations, species and ecosystem functions (Frankham *et al.,* 2002). Our results have shown the southern Great Escarpment is a reservoir of genetic diversity for *Otomys* and these species need to be managed as separate entities.

Ecologically, rodents play a significant role in maintaining the ecosystem functions of the region through facilitation of seed dispersal and seed germination for a number of plant species as well as improving soil fertility and aeration (Chapman and Chapman, 1999). *O. irroratus* is also an important prey species for carnivores, raptors and snakes (Bowland and Perrin, 1993). This is of great significance in terms of conservation management of the mountain ranges in which they occur. It can be argued that rodents are key ecosystem drivers, and conservation of other species may be strongly dependent on the preservation of the rodent communities that sustain them (Chapman and Chapman, 1999).

TAXONOMIC CONCLUSIONS

DESCRIPTION OF SPECIES

FAMILY MURIDAE ILLEGER, 1811

GENUS OTOMYS F. CUVIER, 1824

***OTOMYS WILLANI* SP. NOV.**

WILLAN'S VLEI RAT

Holotype: Ditsong National Museum of Natural History (TM) No. 49101 (field number BAR1020), is a male, age class 3, probably young adult (*sensu* Taylor *et al.*, 1993), with intact cranium (skin rotten and discarded), part of a series of specimens collected at Tiffendell, Eastern Cape Province in December 2013 by G. Goldner. The specimen has been included in both morphometric and molecular analyses. The following external measurements were recorded: total length 198 mm, tail length 74 mm, hind length 25 / 22 mm (*cu* / *su*), ear 16.5 mm.

Type locality: Tiffendell, Eastern Cape Province (30.653°S, 27.928°E).

Paratypes: Ten specimens collected from Tiffendell Ski Resort in December 2013. Some have formaldehyde-preserved skins and damaged skulls (TM49094 (BAR1002), TM49100 (BAR1016), TM49097 (BAR1019), TM49091 (BAR1021), TM49095 (BAR1026), TM49098 (BAR1018)), one has an intact skull, but the skin was rotten and discarded (TM49092 (BAR1001)), and three have damaged skulls and rotten skins that were discarded (TM49096 (BAR1006), TM49093 (BAR1010), TM49099 (BAR1017)). The specimens were decapitated and the brains removed from some individuals, as part of an earlier study by G. Goldner.

Referred specimens having molecular identification: (Includes the paratypes mentioned above). From Tiffendell Ski Resort, all collected by G. Goldner in December 2013: TM49099 (BAR1017), male, unknown age [Class 1 (for explanation of relative age/tooth-wear classes see Taylor et al. 1993)]; TM49098 (BAR1018), female of unknown age [Class 2]; TM49092 (BAR1001), female, unknown age [Class 1]; TM49094 (BAR1002), male, unknown age [Class 2]; TM49096 (BAR1006), male, adult [Class 3]; TM49093 (BAR1010), male, adult [Class 3]; TM49100 (BAR1016), unknown age [Class 1] and sex; TM49097 (BAR1019), male of unknown age [Class 1]; TM49091 (BAR1021), male of unknown age [Class 1]; TM49095 (BAR1026), male of unknown age [Class 1]. From Asante Sana (-32.24925S 24.936E), 2090 m: DM13662 (AS23), skull only of unknown sex, collected 7 June 2009 by Armand Kok. From Mountain Zebra National Park (-32.521S, 25.686E), 1740-1762 m: DM13657 (MZ5), skull only, unknown sex, collected 26 July 2009 by Armand Kok.
Incertae sedis: DM13661 (MZP7), female collected at Mountain Zebra National Park (-32.521S, 25.686E) on 12 February 2010 by Armand Kok. Classified in a different molecular clade to *O. willani* sp. nov. (Figure 2). DM13658 (MZ2), unknown sex, collected at Mountain Zebra National Park (-32.521S, 25.686E) on 26 July 2009 by Armand Kok. Classified in a different molecular clade to *O. willani* sp. nov. (see Figure 2).

Referred specimens having only morphological identification: TM22642, 32km from Sterkstroom on Queenstown Road, Eastern Cape Province (-31.7696S, 26.7312E), adult female, collected by A.J. Prinsloo and J.G. Greeff on 26 July 1957; TM22651, Quqodalo Location, between Glen Grey and Tarkastad, Eastern Cape Province (-31.7497S, 26.8823E), adult female,

collected by J.G. Greeff on 18 October 1955; TM22655, 32 km on road from Sterkstroom to Queenstown, Eastern Cape Province (-31.7696S, 26.7312E), adult male, collected by A.J. Prinsloo and J.G. Greeff on 26 July 1957; TM22652, Matyhantya No 11 Location, between Tarkastad and Glen Grey, Eastern Cape Province (-31.7825S, 27.0705E), adult male, collected by J.G. Greeff on 22 October 1957; TM29521, Karoo National Park, 9km NW Beaufort West, Western Cape Province (-32.2745S, 22.6266E), adult female, collected by I.L. Rautenbach, J.G. De Jager and G. De Graaff on 22 January 1979.
Incertae sedis: NMB9606 and NMB9607, unknown sex, Glen Agricultural College, Free State Province (-28.875S, 26.375E); NMB11420, unknown sex, Sandymount Park, Fauresmith, Free State Province (-29.625S, 25.125E).

Etymology: The species is named after Dr Ken Willan, a South African rodent biologist who conducted pioneering research into the social ecology and reproductive biology of *Otomys* and other African rodents. He designed the "Willan trap", which significantly improved the trap success of catching notoriously trap-shy *Otomys*.

Diagnosis: O. willani can be distinguished in the field from co-occurring *O. sloggetti* and *O. auratus* within its range in the Southern Escarpment of South Africa. It is pallid-buffy coloured, similar to *O. sloggetti* in the cheek region, but the rest of the body is dark reddish coloured, and this species has smaller body measurements than *O. sloggetti*. Although sample sizes were variable due to the poor state of preservation of some skins, the series from Tiffendell is instructive, with *O. willani* being distinctly smaller than *O. sloggetti* in body size without overlap (e.g., mean mass was 78g (75-83 g, n=4) in *O. willani* and 127g (116-158 g, n=5) in *O. sloggetti*; total body length was 170-198 mm in two individuals of *O. willani* and 224 mm in one *O. sloggetti* individual; mean hind foot (*cu*) length was 23.5 mm (21.5-25.5 mm, n=9) in *O. willani* and 27.9 mm (26.5-30.5 mm, n=7) in *O. sloggetti*). The tail of *O. willani* is relatively longer (mean 64.5 mm, n=4, 35% of total length) than in *O. sloggetti* (mean 67.5, n=2, 30% of total length). In the case of co-occurring *O. willani* and *O. auratus*, the latter species is much darker buffy-brown in colouration and also distinctly larger in body and cranial size. In terms of craniodental characters, *O. willani* is easily distinguished from *O. sloggetti* by having at least one conspicuous groove on the lower incisor (none in *O. sloggetti*) and a round-shaped

petrotympanic foramen (slit-shaped in *O. sloggetti*). On craniometric grounds, *O. willani* is easily separated from both *O. auratus* and *O. irroratus* on its smaller size, there being minimal or no overlap in most cranial variables, see Table 3 (e.g., *O. willani* has GLS < 37 mm, MXTL < 9 mm and PAL < 20 mm, *cf.* minimum values exceeding the values of these variables in the case of *O. auratus* and *O. irroratus*). While *O. willani* is generally smaller in all cranial measurements than *O. karoensis*, values usually overlap and do not differ significantly (Table 3). Only in the case of IOC is *O. willani* significantly smaller than *O. karoensis*. As shown by PCA (Figure 4) *O. willani* and *O. karoensis* are completely separated on PC3, which is defined as a "shape vector" contrasting IOC and PAL (positive loadings) and NAW (negative loadings). *O. karoensis* generally has a disproportionately large inter-orbital, longer palatal length (and palatal foramina: Figure 4) and disproportionately narrow nasal bone relative to *O. willani*, reflected in the ratio of IOC/NAW (0.69 in *O. karoensis* and 0.66 in *O. willani*). In addition to these subtle morphological differences the two species are phylogenetically distinct from each other based on mtDNA data, and they are not even sister species; instead *O. karoensis* is sister to *O. laminatus* (Figures 2 and 3). The two species are also geographically and ecologically separated with *O. karoensis* occurring mainly in Fynbos Biome habitats in the Cape Fold Belt Mountains, and *O. willani* occurring in Grassland Biome habitats in the Southern Escarpment.

Description: Like all members of the genus, it is a relatively large, robust, vole-like rodent with a large blunt head, short tail and shaggy pelage. *O. willani* is the smallest member of the genus found in Southern Africa, having a mass of 75-83 g (mean 78.2 g, n=4), total length of 170-198 mm (mean 184 mm, n=2), tail of 59-74 mm (mean 64.5, n=4), hind foot (*cu*) of 21.5-25.5 (mean 23.5, n=9), ear 15-20 mm (mean 17.3 mm, n=4) (data obtained from Phukuntsi *et al.* (2016), supplementary material). The pelage colour is overall darkish-brown with reddish to orange tints, but the facial vibrissae are a pallid creamy-brown colour (Phukuntsi *et al.*, 2016). The upper and lower incisors each have single deep grooves and an additional shallow groove in the lower incisors. The lower M1 has 4 laminae, upper M3 has 7 (in one specimen examined) or 6 laminae (in 10 specimens examined).

Distribution and biology: The species occurs along the Southern Great Escarpment at elevations higher than 1000 m, from the Karoo National Park near Beaufort West in the west to Giant's

Castle in KwaZulu-Natal Province in the east (Figure 1). Very small-sized specimens from two localities on the central plateau of South Africa north of the Drakensberg referred to as *O. cf. karoensis* Sp.2 (dark blue circles in Figure 1) are provisionally referred here to *O. willani*.

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FIGURE CAPTIONS

Figure 1. Map of South Africa showing provinces and collection localities of specimens representing the six putative species of *Otomys* investigated by mtDNA and craniometric analysis in this study. Symbols indicate three major species groups: *O. irroratus s.l.* (triangles: *O. irroratus* and *O. auratus*), *O. karoensis s.l.* (circles: *O. karoensis* and *O. cf. karoensis* Sp. 1 and Sp. 2) and *O. sloggetti* (squares). Details of numbered localities (shown in bold face) are explained in Table 1. The grey-shaded grey region indicates the Great Escarpment of South Africa. The Sneeuberg and Drakensberg Ranges of the Great Escarpment and the Cape Fold Belt that are referred to in the text are labelled using small caps. Inset (from www.en.wikipedia.org; by Oggmus - own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=40624957) shows simplified arrangement of the Great Escarpment and Cape Fold Belt of South Africa (the section marked in red indicates the extent of the Drakensberg Range).

Figure 2. Bayesian Inference consensus tree generated using the GTR + G model. Values are shown as Bayesian Inference/ Maximum Likelihood. * indicates sequences generated in the study presented here. Where available, museum catalogue numbers are provided (DM = Durban Natural Science Museum; TM= Ditsong National Museum of Natural History, Pretoria, South Africa; FMNH = Field Museum of Natural History, Chicago; MRAC = Royal Museum of Central Africa, Tervuren, Belgium). "BAR" represent field numbers from Phukuntsi et al. (2016). Labels in bold face represent new sequences added to this study. South African (SA) province names are abbreviated in the sequence labels as follows: EC = Eastern Cape; GT = Gauteng; NC = Northern Cape; WC = Western Cape; KZN = KwaZulu-Natal. Other codes represent Genbank sequence numbers.

Figure 3. PCA of eight log-transformed craniodental variables showing PC1 versus PC2 (top) and PC1 versus PC3 (bottom). Open circles = *O. cf. karoensis* Sp.1; filled triangles = *O. karoensis*; dots = *O. irroratus*; pluses = *O. auratus*, open triangles = *O. cf. karoensis* Sp. 2. PC1, PC2 and PC3 explain 80.5%, 7.4% and 4.1% respectively of total variance.

Figure 4. Different views of the skull and mandible of *Otomys willani* sp. nov. (TM49101) (A-D), *O. auratus* (TM46130) (E-H), and *O. karoensis* (TM5901 - holotype) (I-L). Images include dorsal (A,E,I), ventral (B, F, J),and right lateral (C, G, K) views of the skull, and the left lateral views of the mandible (D, H, L). The scale is the same in all the images.

Table 1. Details of localities of species and specimens included in the study (locality no. corresponds with numbers in Figure 1). Sex is indicated as male (M) or female (F) in the skulls analysed column. Where relevant museum catalogue numbers are provided for the following museums: DM = Durban Natural Science Museum, KM = Amathole Museum, King William's Town; NMB = National Museum, Bloemfontein; TM = Ditsong National Museum of Natural History, Pretoria. Other codes represent field numbers as explained in the text.

Locality no.	**Species**	**Locality and Province (South Africa)**	**Lat.**	**Long.**	**Skulls analysed (Museum No.)**	**Molecular samples (Field or Genbank No.)**
1	*O. auratus*	Asante Sana, E Cape Province	-32.25	24.94	AS24 (DM13660)	AS3, AS4, AS24 (DM13660), ASF2
2	*O. auratus*	Bloemfontein, Free State	-29.12	26.23	DM: 3573(F), 3574(M), 3070(M), 3072(M)	-
3	*O. auratus*	Chingamwe, Inyanga Mts, E Zimbabwe	-18.45	32.75	DM: 4319(M), 4323(M), 4324(F), 4326(F), 4837(F), 4838(M)	Genbank: FJ619562 (DM4319)
4	*O. auratus*	Dargle, KwaZulu-Natal Province	-29.50	30.03	DM: 8491(M), 8492(F)	Genbank: FJ619550 (DM8493); 619551 (DM8494)
5	*O. auratus*	Fort Nottingham NR, KwaZulu-Natal Province	-29.42	29.92	DM: 3487(F), 3496(M), 3497(M), 3575(F), 3576(F)	-
6	*O. auratus*	Hogsback, E Cape Province	-32.60	27.02	DM: 2933(M), 2935(M), 2936(M), 2937(F), 2938(F)	Genbank: 619553 (TM46130)
7	*O. auratus*	Kamberg NR, E Cape Province	-29.40	29.67	DM: 3309(M), 3127(F), 3128(F), 3143(F),3144(M)	Genbank: 619552 (DM3628)
8	*O. auratus*	Karkloof, KwaZulu-Natal Province	-29.32	30.20	DM: 3125(F), 3116(M), 3117(F), 3118(M), 3120(M), 3136(M), 3304(M), 3321(M)	Genbank: 619557 (DM1838)
9	*O. auratus*	Kuruman, N Cape Province	-27.45	23.43	DM: 4006(F), 4009(F), 4010(M), 4501(F)	-
10	*O. auratus*	Mgeni Vlei NR, KwaZulu-Natal Province	-29.48	29.80	DM: 3489(M), 3490(M), 3492(F)	-

11	*O. auratus*	Rietvlei, Pretoria, Gauteng	-25.82	28.53	DM: 3058(F), 3061(F), 3566(M), 3057(F),3567(F)	BAR: 1028, 1029
12	*O. auratus*	Stutterheim, E Cape Province	-32.56	27.42	DM: 2911(F), 2922(F), 2923(F), 2990(M), 2991(M),2992(M), 2993(M), 2994(M)	-
43	*O. auratus*	Tygerskloof, Kwa Zulu-Natal Province	-27.86	31.34	-	Genbank: FJ619554 (TM46512), FJ619555 (TM46513)
44	*O. auratus*	Springs Municipal Bird Sanctuary, Gauteng	-26.22	28.45	-	Genbank: FJ619556 (TM42444)
1	*O. cf. karoensis Sp1*	Asante Sana, E Cape Province	-32.25	24.94	AS23 (DM13662)	AS23 (DM13662), AS2, ASF6
13	*O. cf. karoensis Sp1*	E Cape Province: 32km from Sterkstroom on Queenstown Road	-31.77	26.73	TM: 22642, 22655	-
14	*O. cf. karoensis Sp1*	E Cape Province: Glen Grey-Tarkastad: Quqodalo Location	-31.75	26.88	TM22651	-
15	*O. cf. karoensis Sp1*	E Cape Province: Tarkastad-Glen Grey: Matyhantya No 11 Location	-31.78	27.07	TM22652	-
16	*O. cf. karoensis Sp1*	Karoo National Park, 9km NW Beaufort West	-32.27	22.63	TM29521	-
17	*O. cf. karoensis Sp1*	Mt Zebra NP, E Cape Province	-32.52	25.69	MZ3? (DM13659), MZ5 (DM13657)	MZ5
18	*O. cf. karoensis Sp1*	Tiffendell Ski Resort, E Cape Province	-30.65	27.93	BAR (TM): 1001, 1020	BAR(TM): 1001, 1002, 1006, 1010, 1016-1021, 1026
19	*O. cf. karoensis Sp1*	Giant's Castle, Drakensberg, KwaZulu-Natal Province	-29.33	29.48	-	GC1
17	Unidentified clade	Mt Zebra NP, E Cape Province	-32.52	25.69	MZ2 (DM13658), MZP7	MZ2, MZP7
20	*O. irroratus*	Algeria, Cederberg, W Cape Province	-32.38	19.06	DM: 4196(F), 4309(F), 8390(M)	Genbank: FJ619546 (DM4317), 619547 (DM8390)
21	*O. irroratus*	Alice, E Cape Province	-32.78	26.83	DM: 2008(F), 2941(F), 3090(M), 2943(M), 2944(M), 2945(M),	-

					2946(F), 2947(F), *2951(M)	
22	*O. irroratus*	Baines Kloof, W Cape Province	-33.57	19.15	DM: 4327(F), 4329(F), 4333(M),	Genbank: FJ619548 (DM4305), FJ619549 (TM46277)
23	*O. irroratus*	Cape Point NR, W Cape Province	-34.02	18.39	DM: 7134(F),7148(M)	-
24	*O. irroratus*	Constantia, Cape Town, W Cape Province	-33.97	18.48	DM: 3077(M)	-
25	*O. irroratus*	Grahamstown, E Cape Province	-33.32	26.52	DM: 2010(M), 2023(F), 8346(M), 8349(M), 8389(F), 8389(F), 8392(M), 8395(M)	Genbank: FJ619542 (DM8395), FJ619545 (DM8349), FJ619541 (DM8346), FJ619540 (DM8624), FJ619538 (DM4870), FJ61944 (DM8392)
26	*O. irroratus*	Groendal NR, E Cape Province	-33.72	25.32	DM: 4322(F) 8391(F), 8400(M)	-
27	*O. irroratus*	Paarl, W Cape Province	-33.80	19.17	DM: 4307(F), 4325(M)	-
28	*O. irroratus*	Sam Knott NR, E Cape Province	-33.11	26.68	DM: 8388(F),8397(F), 8398(M)	Genbank: FJ619543 (DM8397)
29	*O. irroratus*	Swartberg Mts, W Cape Province	-33.52	22.05	DM: 4210(M), 4328(F), 4330(M)	FJ619539 (DM4321)
30	*O. irroratus*	DeWet, Worcester, W Cape Province	-33.52	19.52	TM: 9047, 9048, 9049	-
31	*O. irroratus*	Knysna, W Cape Province	-34.05	23.05	TM: 81, 22801	-
32	*O. irroratus*	Drostdy, Tulbagh District, W Cape Province	-33.35	19.20	TM 22805	-
22	*O. karoensis*	Baines Kloof, W Cape Province	-33.57	19.15	DM: 4883(M)	-
23	*O. karoensis*	Cape Point NR, W Cape Province	-34.02	18.39	DM: 7141(F), 7147(F)	-
24	*O. karoensis*	Constantia, Cape Town, W Cape Province	-33.97	18.48	DM: 3074(M), 3075(M), 3076(M)	-
29	*O. karoensis*	Swartberg Mts, W Cape Province	-33.52	22.05	DM: 4074(M), 4076(M), 4237(M), 4256(M)	-
33	*O. karoensis*	Uitkyk Pass, Cedarberg Mts, W Cape Province	-32.41	19.10	DM: 4244(F), 4300(F), 4312(F),4401(M), 4402(F), 8396(F), 8399(F)	Genbank: FJ619559 (DM8399), 619558 (DM8396), HM363654

34	*O. karoensis*	De Hoop, Bredasdorp, W Cape Province	-34.38	20.53	KM: 31082	-
35	*O. karoensis*	Clanwilliam, Citrusdal District, W Cape Province	-32.60	19.02	KM: 04231, 4233, 4236, 4600, MRAC 34233, TM 22637	-
36	*O. karoensis*	Malmesbury, W Cape Province	-33.38	18.35	KM: 30337	-
37	*O. karoensis*	Piketburg District, W Cape Province	-32.90	18.77	KM: 4241, 30341	-
38	*O. karoensis*	Wolsley, Tulbach, W Cape Province	-33.35	19.20	TM: 5901 (TYPE)	-
1	*O. sloggetti*	Asante Sana, E Cape Province	-32.25	24.94	-	AS1, ASF15
17	*O. sloggetti*	Mt Zebra NP, E Cape Province	-32.52	25.69	MZ3 (DM 13659: skull only, unknown sex collected 26 July 2009 by Armand Kok)	MZ3
39	*O. sloggetti*	Sani Pass, Drakensberg Mts, Lesotho	-29.60	29.30	-	Genbank: FJ619563 (DM5027), Oslo3, OS3, OS4
40	*O. sloggetti*	Sneeuberg Private Nature Reserve, E Cape Province	-31.69	24.63	-	SBR11
18	*O. sloggetti*	Tiffindell Ski Resort, E Cape Province	-30.65	27.93	-	BAR1004, 1027 (“GS2”); 1003, 1005, 1007-1009, 1011, 1012, 1014, 1015, 1022-1025 (“GS1”)
41	*O. cf. karoensis Sp2*	Glen Agricultural College, Free State Province	-28.88	26.38	NMB: 9606, 9607	-
42	*O. cf. karoensis Sp2*	Sandymount Park, Fauresmith, Free State Province	-29.63	25.13	NMB: 11420	-

Table 2. Average sequence divergence between defined species-groups of *Otomys* and *Parotomys*. as determined in MEGA 7 (estimated using the Kimura 2-parameter model). UnID indicates a clade of two sequences that did not have intact voucher specimens so was unidentified.

Distance within groups	**distance between groups (K2P)**												
		Outgroups	*O. angoniensis*	*O. sloggetti*	*P. brantsii*	*O. denti*	*O. lacustris*	*O. cf. karoensis* Sp.1	*O. auratus*	*O. irroratus*	*O. karoensis*	*O. laminatus*	UnID
0.192	Outgroups	-											
0.008	*O. angoniensis*	0.188	-										
0.026	*O. sloggetti*	0.188	0.105	-									
0.017	*P. brantsii*	0.197	0.126	0.125	-								
0.027	*O. denti*	0.186	0.121	0.116	0.123	-							
0.006	*O. lacustris*	0.180	0.132	0.126	0.134	0.113	-						
0.004	*O. cf.karoensis* Sp.1	0.171	0.079	0.089	0.115	0.101	0.114	-					
0.024	*O. auratus*	0.181	0.100	0.102	0.121	0.113	0.122	0.071	-				
0.011	*O. irroratus*	0.184	0.107	0.111	0.130	0.127	0.113	0.076	0.071	-			
0.012	*O. karoensis*	0.186	0.111	0.109	0.131	0.121	0.112	0.078	0.085	0.085	-		
0.000	*O. laminatus*	0.174	0.094	0.105	0.129	0.108	0.111	0.073	0.081	0.085	0.055	-	
0.007	UnID	0.193	0.121	0.103	0.151	0.139	0.131	0.086	0.107	0.098	0.105	0.108	-
0.000	*O. unisulcatus*	0.155	0.074	0.079	0.097	0.080	0.083	0.071	0.083	0.079	0.098	0.115	0.085

Table 3. Variable loadings from PCA of 8 log-transformed variables

	PC 1	PC 2	PC 3
GLS	0.32517	0.10067	0.079919
GLM	0.44036	-0.17094	-0.0020537
MXTRL	0.31381	0.081892	0.16399
NAW	0.47298	-0.02353	-0.66254
IOC	0.19202	0.13407	0.56176
ZYW	0.30846	0.0030094	-0.21212
PL	0.48155	-0.23023	0.40843
BL	0.11941	0.93936	-0.019226

Table 4. Summary statistics for 10 cranial variables in taxa defined by this study. F-values are indicated for ANOVA tests; all tests were significant at $p < 0.01$ (***). Superscript letters indicate non-significant subsets of means based on pair-wise Tukey tests.

	O. auratus	*O. cf. karoensis* Sp.1	*O. irroratus*	*O. karoensis*	*O. cf. karoensis* Sp.2
N	**19**	**10**	**38**	**22**	**3**
GLS ($F_{4.78} = 63.58$***)					
Mean	41.35^{A}	32.61^{C}	41.01^{A}	37.16^{B}	35.00^{B}
Min	38.06	27.70	37.62	34.70	34.00
Max	44.30	36.14	45.26	39.61	36.00
Stand. dev	1.54	2.69	1.82	1.35	1.00
GLM ($F_{4.78} = 81.38$***)					
Mean	27.65^{A}	20.34^{B}	26.11^{A}	22.82	18.92^{B}
Min	24.08	17.50	22.63	21.20	18.26
Max	29.89	22.71	29.00	24.56	19.39
Stand. dev	1.22	1.75	1.54	0.96	0.59
MXTRL ($F_{4.78} = 69.63$***)					
Mean	9.76^{A}	7.89^{B}	9.68^{A}	8.62^{C}	7.88^{B}
Min	8.77	6.33	9.02	7.82	7.63
Max	10.49	8.76	10.29	9.26	8.03
Stand. dev	0.42	0.70	0.32	0.35	0.22
NAW ($F_{4.78} = 94.12$***)					
Mean	8.14^{A}	5.93^{B}	7.59^{A}	6.15^{B}	6.05^{B}
Min	7.48	5.00	6.50	5.71	6.01
Max	9.03	6.55	8.55	6.63	6.10
Stand. dev	0.40	0.44	0.50	0.26	0.05
IOC ($F_{4.78} = 25.47$***)					
Mean	4.46^{A}	3.90^{B}	4.61^{A}	4.30^{A}	3.95^{B}
Min	4.16	3.32	4.07	3.88	3.75
Max	4.90	4.34	5.03	4.63	4.24
Stand. dev	0.17	0.30	0.23	0.21	0.26
ZYW ($F_{4.78} = 70.22$***)					
Mean	20.81^{A}	16.91^{B}	20.15^{A}	17.88^{B}	17.14^{B}
Min	19.26	14.80	18.30	16.98	16.77
Max	22.18	18.52	21.91	18.94	17.56
Stand. dev	0.77	1.29	0.86	0.47	0.40
PL ($F_{4.78} = 84.74$***)					
Mean	23.28^{A}	17.11^{C}	22.53^{A}	19.74^{B}	14.10^{D}
Min	20.92	14.07	20.28	18.17	13.47
Max	26.27	19.70	25.61	21.18	14.55
Stand. dev	1.40	1.82	1.20	0.78	0.56
BL ($F_{4.78} = 11.82$***)					

Mean	6.95 [A]	6.48 [A]	7.32 [A]	6.85 [A]	8.15
Min	6.37	5.30	6.49	6.11	7.82
Max	8.66	7.33	8.51	7.94	8.48
Stand. dev	0.49	0.72	0.42	0.42	0.33

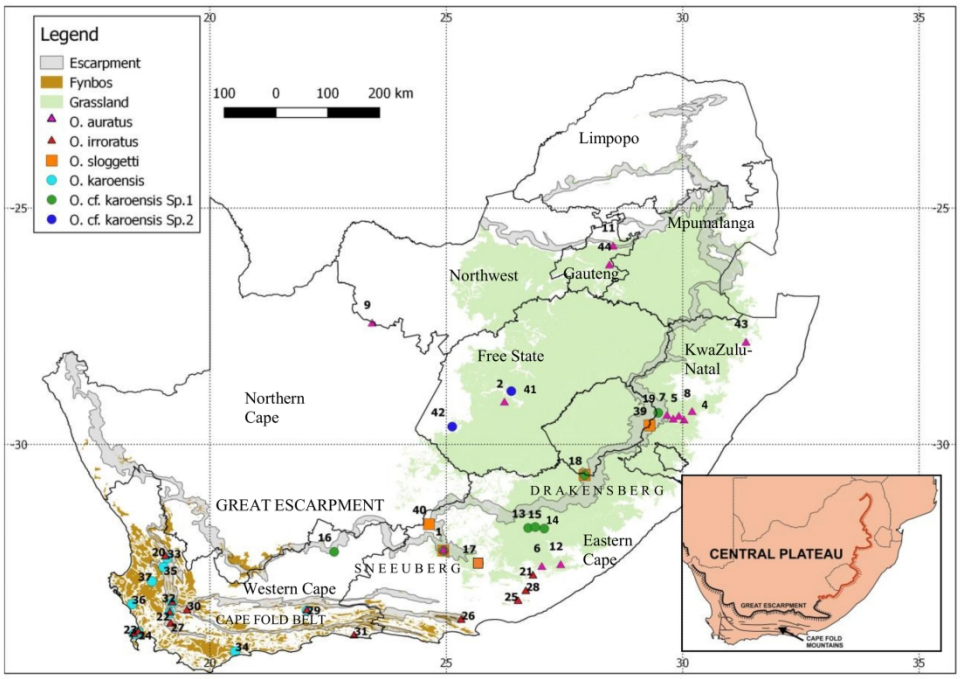


Figure 1. Map of South Africa showing provinces and collection localities of specimens representing the six putative species of Otomys investigated by mtDNA and craniometric analysis in this study. Symbols indicate three major species groups: O. irroratus s.l. (triangles: O. irroratus and O. auratus), O. karoensis s.l. (circles: O. karoensis and O. cf. karoensis Sp. 1 and Sp. 2) and O. sloggetti (squares). Details of numbered localities (shown in bold face) are explained in Table 1. The grey-shaded grey region indicates the Great Escarpment of South Africa. The Sneeuberg and Drakensberg Ranges of the Great Escarpment and the Cape Fold Belt that are referred to in the text are labelled using small caps. Inset (from www.en.wikipedia.org; by Oggmus - own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=40624957) shows simplified arrangement of the Great Escarpment and Cape Fold Belt of South Africa (the section marked in red indicates the extent of the Drakensberg Range).

170x120mm (300 x 300 DPI)

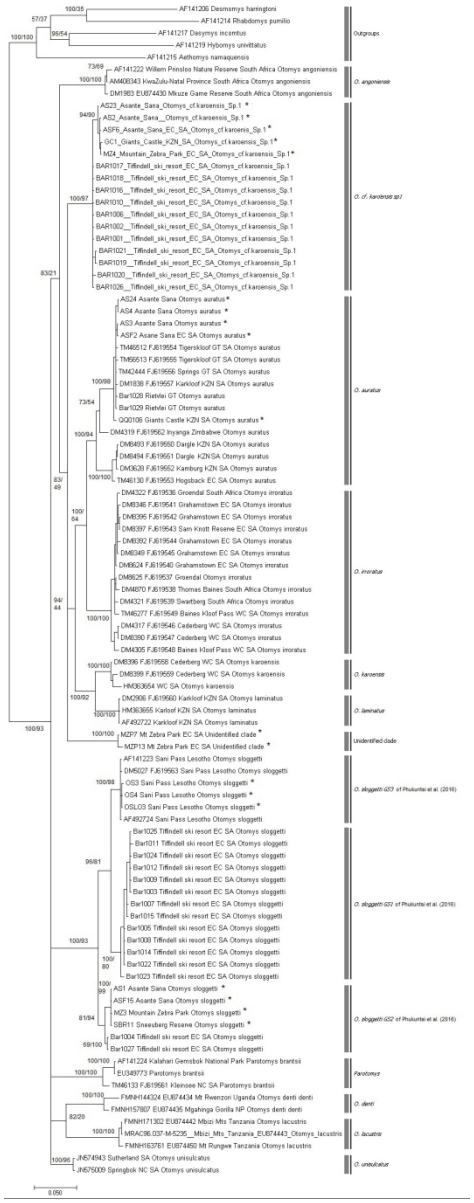
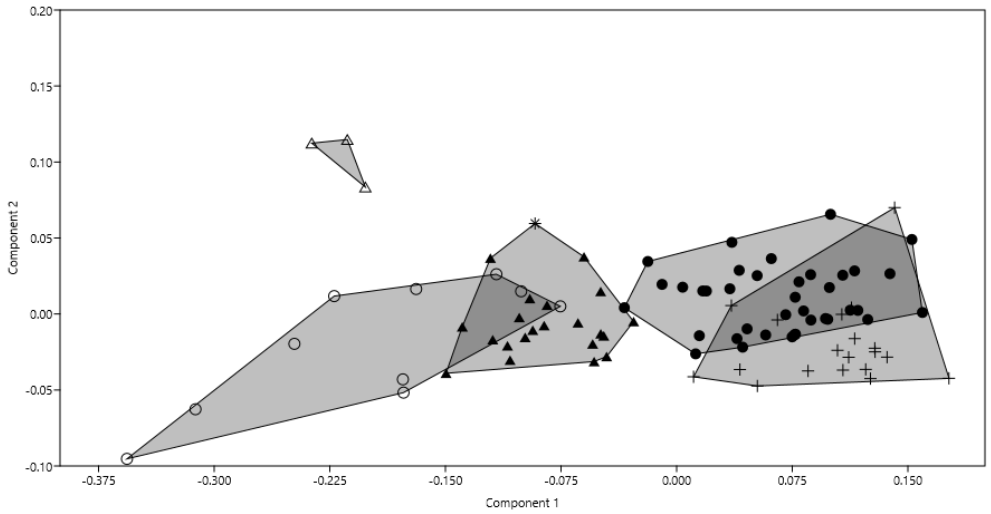
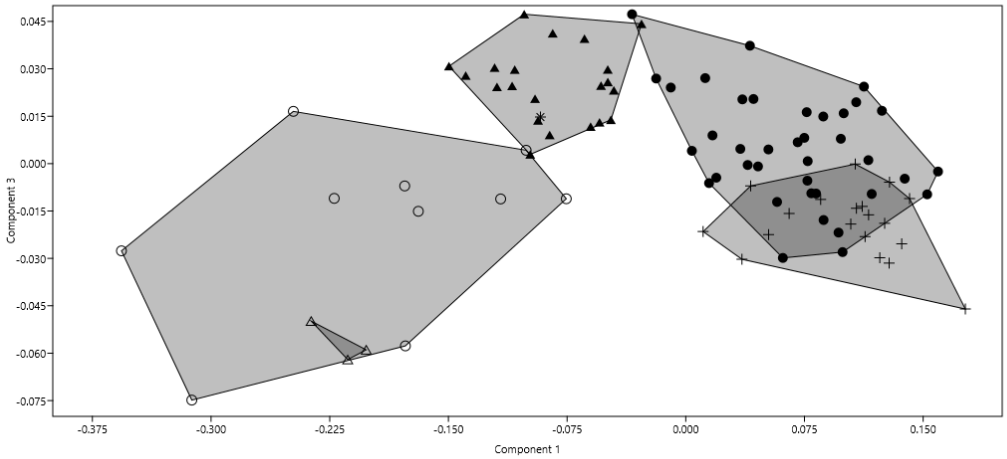


Figure 2. Bayesian Inference consensus tree generated using the GTR + G model. Values are shown as Bayesian Inference/ Maximum Likelihood. * indicates sequences generated in the study presented here. Where available, museum catalogue numbers are provided (DM = Durban Natural Science Museum; TM= Ditsong National Museum of Natural History, Pretoria, South Africa; FMNH = Field Museum of Natural History, Chicago; MRAC = Royal Museum of Central Africa, Tervuren, Belgium). "BAR" represent field numbers from Phukuntsi et al. (2016). Labels in bold face represent new sequences added to this study. South African (SA) province names are abbreviated in the sequence labels as follows: EC = Eastern Cape; GT = Gauteng; NC = Northern Cape; WC = Western Cape; KZN = KwaZulu-Natal. Other codes represent Genbank sequence numbers.


0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
Component 2
-0.375
-0.300
-0.225
-0.150
-0.075
0.000
0.075
0.150
Component 1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0.030
0.015
0.000
-0.015
-0.030
-0.045
-0.060
-0.075
Component 3
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-0.300
-0.225
-0.150
-0.075
0.000
0.075
0.150
Component 1

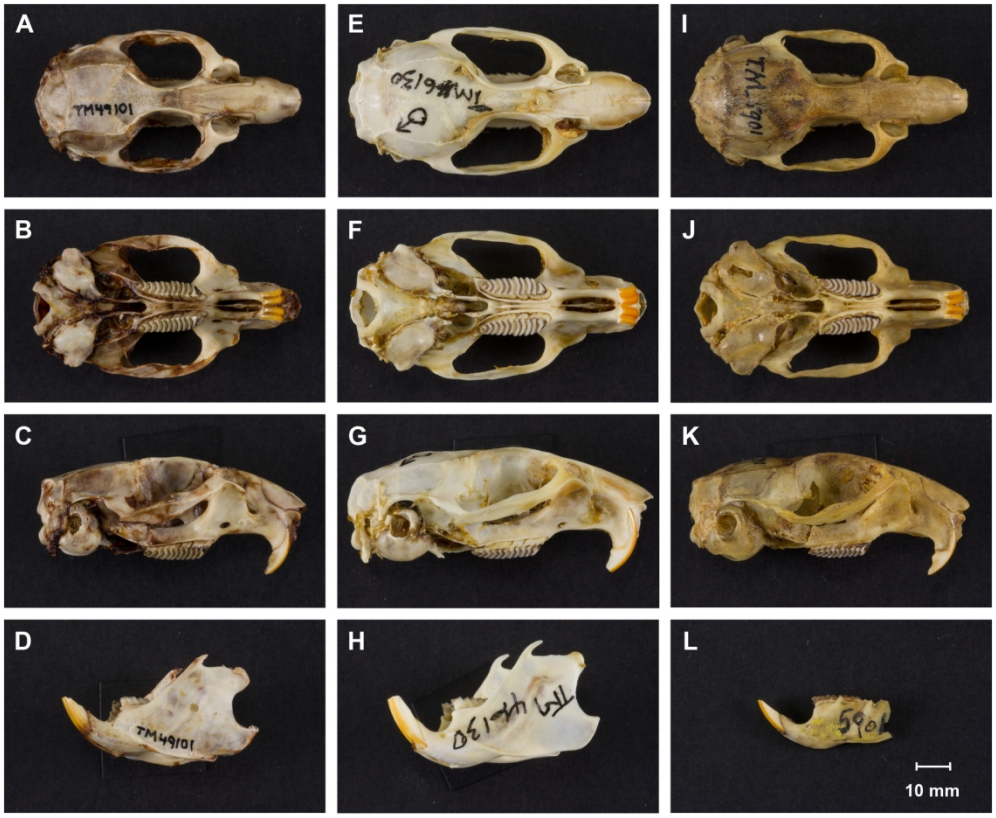

Figure 4. Different views of the skull and mandible of Otomys willani sp. nov. (TM49101) (A-D), O. auratus (TM46130) (E-H), and O. karoensis (TM5901 - holotype) (I-L). Images include dorsal (A,E,I), ventral (B, F, J),and right lateral (C, G, K) views of the skull, and the left lateral views of the mandible (D, H, L). The scale is the same in all the images.

167x138mm (300 x 300 DPI)