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

PETER JOHN TAYLOR[1,2*], TERESA KEARNEY[3,4], DESIRE LEE DALTON[1,5], GAMUCHIRAI CHAKONA[6†], CHRISTOPHER M. R. KELLY[6‡] and NIGEL P. BARKER[6§]

[1]*School of Mathematical & Natural Sciences, University of Venda, Private Bag X5050, Thohoyandou 0950, South Africa*
[2]*School of Life Sciences, University of KwaZulu-Natal, Biological Sciences Building, South Ring Road, Westville Campus, Durban, KwaZulu-Natal 3630, South Africa*
[3]*Ditsong National Museum of Natural History, PO Box 413, Pretoria 0001, South Africa*
[4]*School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Private Bag 3, Wits 2050, South Africa*
[5]*National Zoological Garden, South African National Biodiversity Institute, PO Box 754, Pretoria 0001, South Africa*
[6]*Department of Botany, Rhodes University, PO Box 94, Grahamstown 6140, South Africa*

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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).

ADDITIONAL KEYWORDS: Africa – mitochondrial DNA – phylogeny – phylogeography – taxonomy.

*Corresponding author. E-mail: peter.taylor@univen.ac.za
†Current address: Department of Environmental Science, Rhodes University, PO Box 94, Grahamstown 6140, South Africa
‡Current address: Physical Sciences Department, Graeme College, PO Box 281, Grahamstown, 6140, South Africa
§Current address: Department of Plant and Soil Sciences, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa
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INTRODUCTION

Laminate-toothed rats originated and diversified in South Africa 5.0–3.5 Mya, later dispersing to east-central Africa along the African Rift mountains ~2.5–1.6 Mya and then radiating throughout east, central and north-east Africa and the Cameroon Volcanic Line (Denys, 2003; Taylor *et al.*, 2004a, 2009a, 2014). There are two genera, *Otomys* F. Cuvier, 1824 and *Parotomys* Thomas, 1918. It has been suggested that *Otomys* is polyphyletic with respect to arid-adapted *Parotomys* (whistling rats), because some *Otomys* species, such as

Sloggett's ice rat (*Otomys sloggetti* Thomas, 1902) and the bush Karoo rat (*Otomys unisulcatus* F. Cuvier, 1829), are more closely related to *Parotomys* than to *Otomys* (Taylor *et al.*, 1989, 2004a, 2009a, 2011, 2014; Phukuntsi *et al.*, 2016).

The genus *Otomys* (laminate-toothed rats), in the tribe Otomyini (family Muridae; subfamily Murinae), is endemic to sub-Saharan Africa, where it is patchily associated with Afromontane regions from the Cameroon Volcanic Line in West Africa to Ethiopia in East Africa and south to Cape Town in South Africa (Monadjem *et al.*, 2015). The number of species in the genus is probably underestimated owing to the likely existence of undescribed cryptic species occurring throughout the mountainous regions of Africa (Carleton & Byrne, 2006; Taylor *et al.*, 2009a, b, 2011, 2014; Engelbrecht *et al.*, 2011). Currently, 31 species are recognized in the genus *Otomys* (Monadjem *et al.*, 2015; Denys *et al.*, 2017), compared with 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.

There are two species of *Parotomys* [*Parotomys brantsii* (A. Smith, 1834) and *Parotomys littledalei* Thomas, 1918] and seven species of *Otomys* [*Otomys angoniensis* Wroughton, 1906, *Otomys auratus* Wroughton, 1906, *Otomys irroratus* (Brants, 1827), *Otomys karoensis* Roberts, 1931, *Otomys 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* (ice rats), whose distributional limits and subspecies taxonomy have also been subject to debate and for which new molecular and morphological data are presented from various grassland sites in the high Drakensberg escarpment from slightly > 2000 to > 3000 m a.s.l. (present study; Phukuntsi *et al.*, 2016). Ice rats are herbivorous, burrow-dwelling rodents, currently endemic to the southern African Drakensberg and Maluti mountains at elevations > 2000 m a.s.l. The species is adapted to living in alpine and subalpine habitats and generally lives in flat, grassy areas, which receive a maximal amount of sunlight, allowing the animals to bask (Richter *et al.*, 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 & 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 relictual records from isolated mountainous regions of the Karoo in the Beaufort West, De Aar, Hope Town, Britstown and Hanover districts (Lynch & Watson, 1992). These old Karoo records include the type locality of Deelfontein, where the species has not been found since its discovery in 1902, making it likely that the range of the species has contracted since then. Of these isolated Karoo records, only those from Hanover and Hope Town districts could be validated by specimens (Lynch & Watson, 1992). Given that *O. sloggetti* is restricted to high-elevation alpine and subalpine habitats along the Drakensberg Mountain Range and (as shown in the present study) certain other parts of the Southern Great Escarpment, genetic fragmentation is likely to occur across the range of the species. Richter *et al.* (1997) indicated that the distributions of *O. sloggetti* and *O. irroratus s.l.* meet but do not overlap, because of different habitat, elevational and temperature preferences. However, both *O. sloggetti* and *O. auratus* were captured at one site (Asante Sana Private Game Reserve in the Sneeuberg Mountains), which shows that the species can coexist (A. Kok, personal communication; present study). Furthermore, Phukuntsi *et al.* (2016) recorded *O. sloggetti* and *O. karoensis s.l.* (which they termed 'lower *sloggetti*') cohabiting in the same burrows at Tiffendell at 2700 m a.s.l.

Meester *et al.* (1986) recognized disjunct western (fynbos biome) and eastern (grassland biome) subspecies of *O. saundersiae s.l.*: *Otomys saundersiae karoensis* and *Otomys saundersiae saundersiae*, respectively. The fynbos biome of South Africa comprises Mediterranean-type shrub vegetation dominated by Restionaceae, 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 & Rutherford, 2006). However, the taxonomic status of topotypical populations of *O. saundersiae* from Grahamstown in the Eastern Cape has been called into question (Taylor *et al.*, 1993, 2005). Taylor *et al.* (1993) showed that two distinct large- and small-sized species (*O. irroratus s.l.* and *O.* cf. *saundersiae*, respectively) could be distinguished morphologically in the Western Cape, and in the Eastern Cape north of 33° latitude but not in the Eastern Cape south of 33°, which encompassed the Grahamstown type locality of *O. saundersiae*. Based on combined molecular, karyotypic and morphometric evidence, Taylor *et al.* (2009a) finally synonymized *O. saundersiae* under *O. irroratus s.l.* and elevated the name *O. karoensis*

(with its type locality in Tulbagh, Western Cape). The latter species denoted the smaller-sized, pallid-coloured 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° latitude. The status of these two forms is addressed in the present study.

Otomys irroratus s.l. was formerly believed to occur throughout both the fynbos and the grassland biomes of South Africa, extending to the eastern highlands of Zimbabwe. However, based on chromosomal and mitochondrial DNA (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 recognized among populations (Contrafatto *et al.*, 1992; Rambau *et al.*, 2001; Taylor *et al.*, 2004b, 2005, 2009a).

Cytotypes cannot be distinguished morphologically (Taylor *et al.*, 2004b). *Otomys irroratus s.s.* is associated with the 'B' cytotype from the Eastern Cape, which contains completely biarmed (metacentric) chromosomes, and the 'C' cytotype from the Western Cape, which contains an intermediate number of biarmed chromosomes. The variable number of metacentric chromosomes is attributable to the presence or absence of heterochromatic short arms (Contrafatto *et al.*, 1992). *Otomys 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 s.s* and *O. auratus*. The Zimbabwean population of *O. auratus* also carries a karyotype similar to the 'B' cytotype found also in *O. irroratus s.s.*, presumably convergent. Taken together with distribution, the karyotype is a highly diagnostic species character. Based on projected declines in fynbos and grassland habitats owing to climate change, Taylor *et al.* (2016) projected 12–24 and 47–60% declines by 2050 in the ranges of *O. irroratus* and *O. auratus*, respectively. Based in part on these results, the IUCN Red List category for *O. auratus* has been adjusted to Near Threatened (Baxter *et al.*, 2017), whereas *O. irroratus* has remained as Least Concern (Taylor & Baxter, 2017).

Given that speciation occurred between fynbos and grassland populations of *O. irroratus s.l.* (comprising *O. auratus* and *O. irroratus s.s.*; Engelbrecht *et al.*, 2011), this raised the possible existence of similar disjunction and speciation in 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. karoensis s.l.* (small and pale-reddish in coloured) and *O. irroratus s.l.* (large and dark-reddish in colour) from the Sneeuberg Range of the southern Drakensberg allowed us to test this hypothesis critically and to determine which cryptic species in *O. irroratus s.l.* (*O. irroratus s.s.* or *O. auratus*) is present in the region.

In summary, the aims of our study were as follows: (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; and (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.*, 2009a, 2009b, 2011, 2014), we apply the evolutionary species concept and consilience principles ('integrative taxonomy') in describing and delimiting species (see Taylor *et al.*, 2019).

MATERIAL AND METHODS

Study area and sampling sites

Our molecular study uses 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; Fig. 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 *O.* cf. *karoensis* and one *O.* 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

Table 1. Details of localities of species and specimens included in the study

Locality no.	Species	Locality and province (South Africa)	Latitude	Longitude	Skulls analysed (museum number)	Molecular samples (field number or GenBank number)
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* sp. 1	Asante Sana, E Cape Province	–32.25	24.94	AS23 (DM13662)	AS23 (DM13662), AS2, ASF6
13	*O.* cf. *karoensis* sp. 1	E Cape Province: 32km from Sterkstroom on Queenstown Road	–31.77	26.73	TM: 22642, 22655	–
14	*O.* cf. *karoensis* sp. 1	E Cape Province: Glen Grey-Tarkastad: Quqodalo Location	–31.75	26.88	TM22651	–

Table 1. Continued

Locality no.	Species	Locality and province (South Africa)	Latitude	Longitude	Skulls analysed (museum number)	Molecular samples (field number or GenBank number)
15	*O.* cf. *karoensis* sp. 1	E Cape Province: Tarkastad-Glen Grey: Matyhantya No 11 Location	–31.78	27.07	TM22652	–
16	*O.* cf. *karoensis* sp. 1	Karoo National Park, 9km NW Beaufort West	–32.27	22.63	TM29521	–
17	*O.* cf. *karoensis* sp. 1	Mt Zebra NP, E Cape Province	–32.52	25.69	MZ3? (DM13659), MZ5 (DM13657)	MZ5
18	*O.* cf. *karoensis* sp. 1	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* sp. 1	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)	–

Table 1. Continued

Locality no.	Species	Locality and province (South Africa)	Latitude	Longitude	Skulls analysed (museum number)	Molecular samples (field number or GenBank number)
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* sp. 2	Glen Agricultural College, Free State Province	–28.88	26.38	NMB: 9606, 9607	–
42	*O.* cf. *karoensis* sp. 2	Sandymount Park, Fauresmith, Free State Province	–29.63	25.13	NMB: 11420	–

Locality numbers correspond to 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.

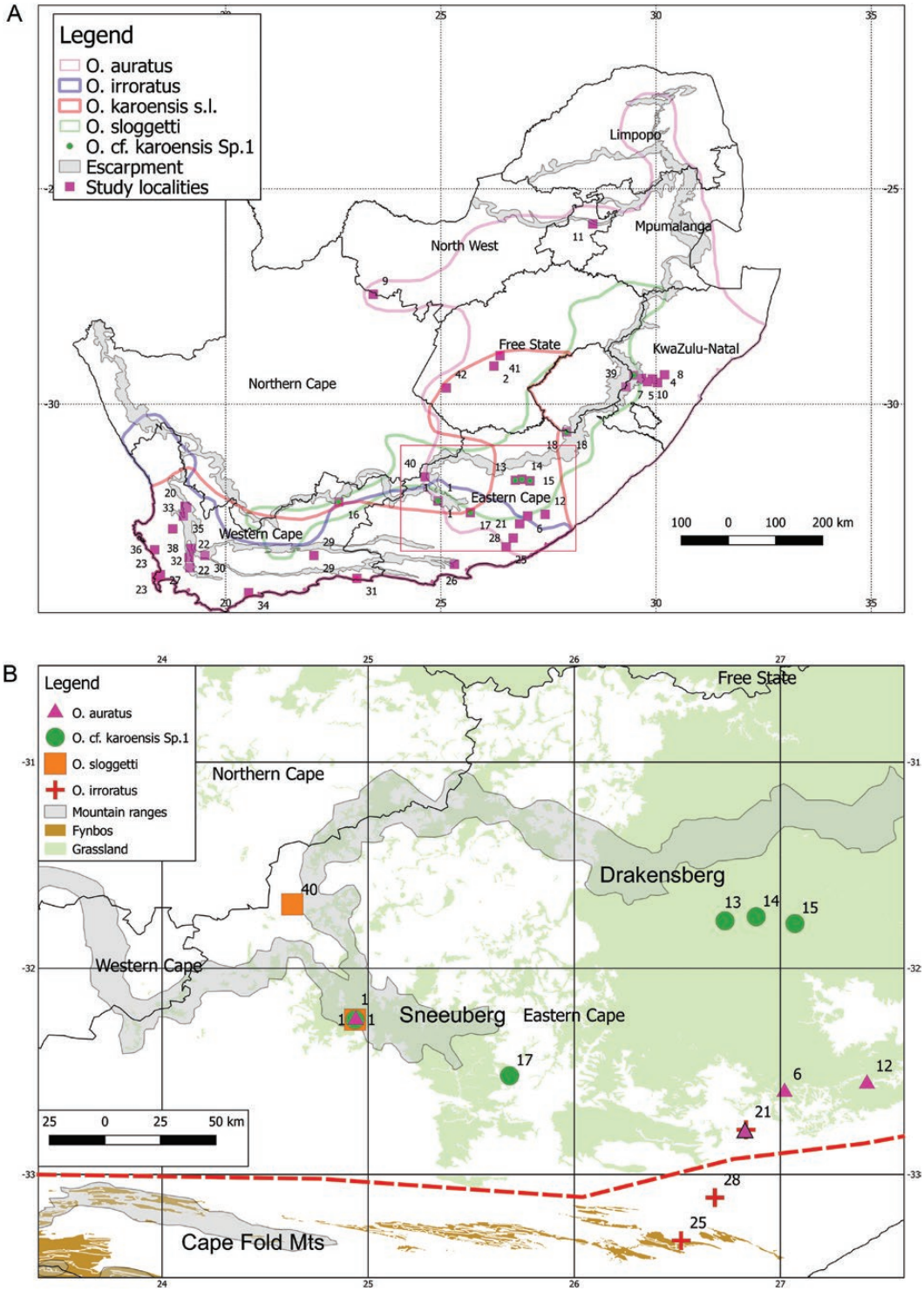

Figure 1. A, map of South Africa, showing provinces and collection localities of specimens representing five of the six putative species of *Otomys* investigated by mitochondrial DNA and craniometric analysis in the present study (*Otomys laminatus* is omitted for clarity). Details of numbered localities are explained in Table 1, and the red box indicates the extent of the inset shown in B, which is a close-up of the study region of the Sneeuberg Range. The grey-shaded region indicates the Great Escarpment and Cape Fold Belt Mountains 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. The red dashed line indicates the

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 12 voucher specimens (seven *O.* cf. *karoensis* and five *O. sloggetti*) from the study of Phukuntsi *et al.* (2016), currently housed at TM.

Samples from the Sneeuberg Range were trapped between elevations of 1740 and 2150 m a.s.l., in relatively undisturbed habitats (Table 1), and the habitat at each site was characterized 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 to collect tissues from toe clippings for DNA (Kok *et al.*, 2012, 2013). The Rhodes University Ethical Standards Committee granted ethical clearance for this work (clearance no. 2009Q-6). 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 and DM13662).

DNA extraction, amplification and sequencing

DNA was extracted from toe clippings using the Invisorb Spin Tissue MiniKit (Invitek) following the manufacturer's protocol. Before addition of a lysis buffer (400 µL) and Proteinase K (40 µL), the tissues were mechanically ground to increase lysis efficiency. Non-lysed material was removed by centrifuging.

For polymerase chain reaction (PCR) amplification of the *Cytb* region, 5 µL of template DNA was used for PCRs in a total volume of 50 µL (5 µL 10× PCR 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′-CAGGAAACAG CTATGACCCATCRGACACAACAACAGC-3′ and Oto-intR′: 5′-TGTAAAACGACGGCCAGTGGAGAAGTAG CTRATGGARGC-3′).

The 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 (Colangelo *et al.*, 2007; Taylor *et al.*, 2009b). To view PCR products, 5 µL of the amplification product was mixed with 5 µL of loading dye containing SYBR Green, loaded onto a 1% agarose gel, subjected to electrophoresis at 100 V for 10 min, and visualized by means of a ultraviolet 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 v.3.1 Cycle Sequencing Kit (Applied Biosystems). Washing and precipitation of sequencing PCR products was achieved in several steps involving addition of 3 M NaOAc, 99% ethanol, 70% ethanol and centrifugation. The 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 v.4.5 (GeneCodes Corporation). The maximal sequence length was 1137 bp, although sequences for some samples were shorter than this (970 bp). New *Cytb* sequences for 20 samples were generated in this study, and added to 45 ingroup and four outgroup sequences obtained from GenBank. These GenBank sequences have been published previously by Ducroz *et al.* (2001) and Taylor *et al.* (2009a, b, 2011, 2014). Furthermore, we obtained 28 *Cytb* sequences of 470 bp from the study by Phukuntsi *et al.* (2016). Alignment of these sequences for phylogenetic analysis was achieved using MEGA v.7 (Kumar *et al.*, 2016). We chose five species of Avicanthini as outgroups, because this tribe is a sister tribe of Otomyini (Lecompte *et al.*, 2008).

Phylogenetic analysis

Our analysis comprizes 93 ingroup (90 *Otomys* and three *Parotomys*) and five outgroup taxa (*Aethomys namaquensis*, *Dasymys incomtus s.l.*, *Desmomys harringtoni*, *Hybomys univittatus* and *Rhabdomys pumilio s.l.*). The majority of *Cytb* sequences were 1137 bp in length, 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 v.2.1.10 (Posada, 2009). The model selected was Tamura–Nei plus Gamma (G) and Invariable sites (I), and this

approximate position of the 'Bedford Gap' between the Great Escarpment and Grassland Biome in the north and the Cape Fold Belt Mountains and the Fynbos Biome in the south. Close to this line, Alice (locality 21) is a contact zone where *Otomys auratus* and *Otomys irroratus* are found to co-occur sympatrically (Engelbrecht *et al.*, 2011).

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

was used for maximum likelihood (ML) (Felsenstein, 1981) and Bayesian (Ronquist & Huelsenbeck, 2003) analyses. Phylogenetic relationships were evaluated using the ML method implemented in MEGA v.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 v.3.2.7 (Ronquist & 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 < 0.05. Congruence among tree topologies emanating from analysis of *Cytb* 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 (Fig. 2).

Craniodental and craniometric analyses

We analysed variation in a sample of 94 intact skulls using eight craniometric variables measured to the nearest 0.01 mm using Mitutoyo digital callipers with an accuracy of 0.01 mm: (1) greatest length of skull (GLS), measured dorsally, equivalent to occipitonasal 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 anteriormost point on posterior edge of palate; and (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 included in only three instances, in which they were necessary for complete geographical or taxonomic representation. Class 1 and 2 individuals were excluded. Given that 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 visualize intraspecific and interspecific variation in multidimensional space of linear measurements, principal components analysis (PCA) was carried out on $\log_{10}$-transformed cranial variables using the programme PAST (Hammer *et al.*, 2001). Univariate analyses including summary statistics of all cranial variables, ANOVA and pairwise Tukey's tests were also conducted using PAST. Univariate analysis and summary statistics provide corroboration of multivariate analysis and also allow broader comparison of morphometric data between studies and by other researchers. In some cases, non-overlap of measurements, indices or ratios based on them are useful for keys and diagnosis of museum specimens.

RESULTS

Phylogenetic analysis

The best tree produced by Bayesian analysis of the *Cytb* alignments is shown in Figure 2. As revealed by earlier studies (Taylor *et al.*, 2009a, 2009b, 2014), basal relationships within the Otomyini tribe are poorly resolved by *Cytb*, although terminal clades are better supported. Our molecular data show the presence of two major lineages (cryptic species) within *O. irroratus s.l.* (*O. auratus* and *O. irroratus*, respectively), which supports the findings of Taylor *et al.* (2009a) and Engelbrecht *et al.* (2011). Four specimens from Asante Sani (AS) in the Sneeuberg Range group clearly with 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) is a well-supported (98% bootstrap support) clade including *O. laminatus* and *O. karoensis.* Two specimens from MZNP (MZP 7 and 13) form a well-supported and relatively deeply divergent lineage that has no supported relationship with any other Otomyini species (Fig. 2); Kimura two-parameter (K2P) genetic distances vary between 8.5% (*O. unisulcatus*) and 15.1% (*Parotomys*) (Table 2). Given that no voucher specimens are 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) form 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* coincide 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. The GS3 clade 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, mean pairwise K2P distances were calculated (Table 2) between the putative species clades labelled in Figure 2. The largest divergences are observed between *P. brantsii* and the other species groups (11.5–15.1%). The lowest divergences are 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 varies 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 allows 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 *O. auratus*, *O. irroratus*, *O. karoensis* and *O. 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.* (2016) as 'greater *sloggetti*', the incisors possess ungrooved or faintly grooved incisors, confirming their identity as *O. sloggetti* (BAR1003/TM49089, BAR1005/TM49076, BAR1007/TM49081, BAR1008/TM49075, BAR1009/TM49086, BAR1012/TM49077 and BAR1023/TM49089). In one of these skulls where the bulla is intact (B1003), the petrotympanic foramen is slit shaped, confirming the identification as *O. sloggetti*. In nine skulls identified by Phukuntsi *et al.* (2016) as 'lower *sloggetti*', a distinct deep groove is 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 and BAR1026/TM49095. In two of

Table 2. Mean sequence divergence between defined species groups of *Otomys* and *Parotomys* as determined in MEGA v.7 (estimated using the Kimura two-parameter model)

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

Abbreviations: K2P, Kimura two-parameter; UnID, a clade of two sequences that did not have intact voucher specimens and was therefore unidentified.

these skulls (B1001/TM49092 and B1020/TM49101) it is possible to confirm the presence of a round-shaped petrotympanic foramen.

The PCA (Fig. 3; Table 3) identifies 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 the southern portion of the Eastern Cape, and *O. auratus* from the northern portion of the Eastern Cape, albeit with considerable morphometric overlap between *O. auratus* and *O. irroratus*. These groups are distinguished mostly on cranial size [principal component (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

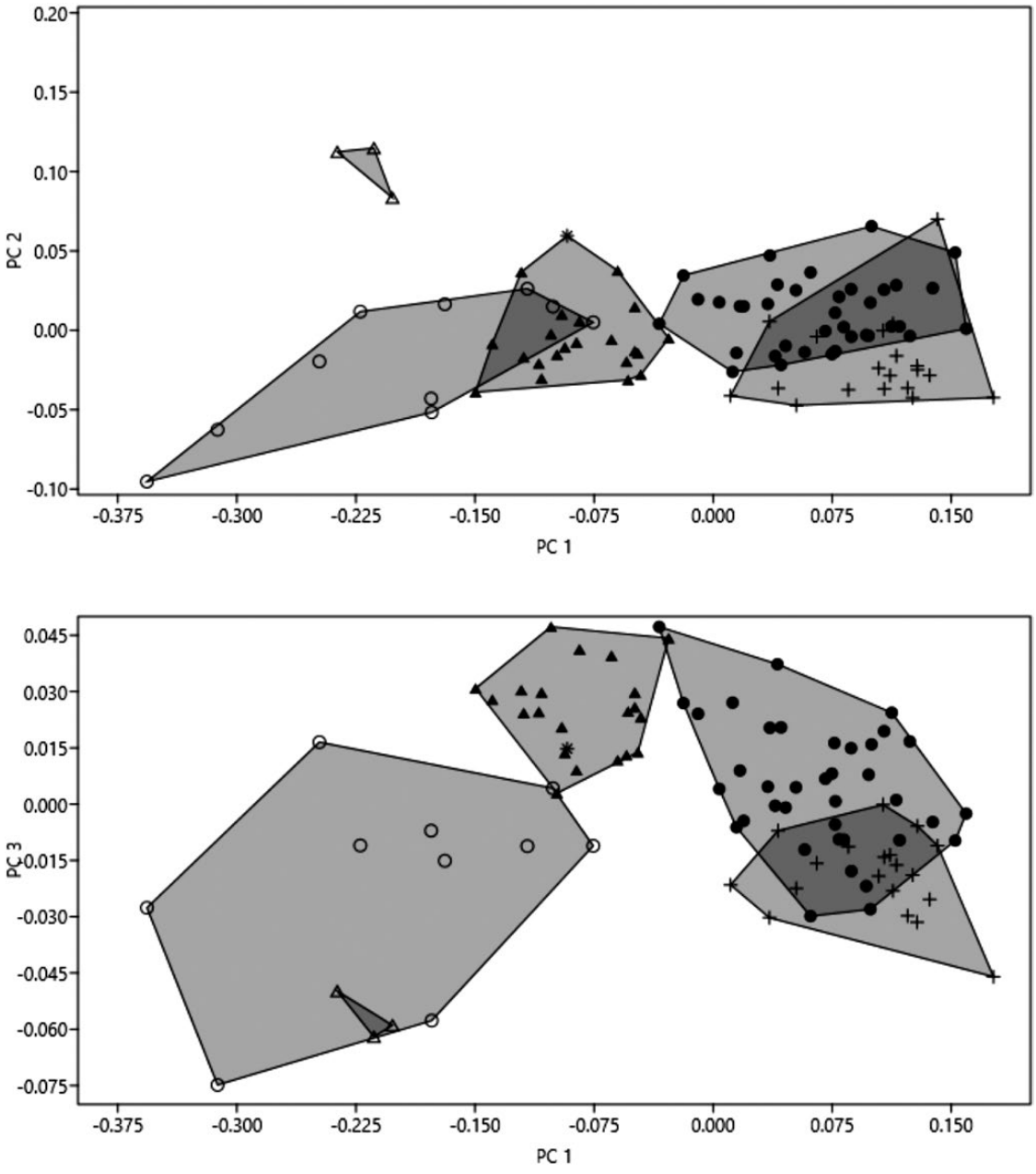

Figure 3. Principal components analysis of eight $\log_{10}$-transformed craniodental variables, showing principal component (PC)1 vs. PC2 (top) and PC1 vs. PC3 (bottom). Open circles, *Otomys* cf. *karoensis* sp. 1; filled triangles, *Otomys karoensis*; dots, *Otomys irroratus*; plus signs, *Otomys auratus*; open triangles, *O.* cf. *karoensis* sp. 2; *holotype specimen (*O. karoensis*). PC1, PC2 and PC3 explain 80.5, 7.4 and 4.1% of total variance, respectively.

Table 3. Variable loadings from principal components analysis of eight $\log_{10}$-transformed variables

	PC1	PC2	PC3
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

Abbreviations: PC, principal component; for variable abbreviations, see under text for Material and Methods.

sequenced), can 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 is significantly larger than in the other four species (Table 4). The ratio BUL/GLS was 22–24% in the Free State sample compared with 15–22% in all other species samples pooled. Although *O. karoensis* and *O.* cf. *karoensis* sp. 1 overlap somewhat on PC axes 1 and 2 (Fig. 3), complete separation is obtained between them on plots of PC1 and PC3. Principal component 3 is a shape vector that contrasts interorbital constriction and palatal foramina length (high positive loadings) with nasal width (high negative loading) (Table 3). *Otomys* cf. *karoensis* sp. 1 has a disproportionately narrow interorbital bone, short palatal foramina and wide nasal bone compared with *O. karoensis* (Tables 3 and 4; Figs 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 *O. karoensis s.l.* cf. > 9 mm in *O. auratus* and *O. irroratus*). This character holds true for the specimens in our study; maxillary tooth-row length (MXTRL) is always < 9 mm in specimens of *O.* cf. *karoensis* from AS, SPNR and MZNP identified by mtDNA as *O.* cf. *karoensis* sp. 1, and in all nine skulls of 'lower *sloggetti*' from Tiffendell described by Phukuntsi *et al.* (2016) (Table 4; additional observations by T.K.), whereas it is always > 9 mm in *O. auratus* and *O. irroratus* (Table 4). Tukey's pairwise tests for MXTRL reveal significant differences between *O. karoensis*/*O.* cf. *karoensis* and *O. irroratus*/*O. auratus*, in addition to 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* are also significantly smaller than *O. irroratus*/*O. auratus* in five additional variables (GLS, GLM, NAW, ZYW and PAL), and *O.* cf. *karoensis* sp. 1 and sp. 2 and *O. karoensis* differ from each other significantly in five variables (GLS, GLS, MXTRL, IOC and PL) (Table 4).

TAXONOMY

Family Muridae Illeger, 1811

Genus *Otomys* F. Cuvier, 1824

***Otomys willani* sp. nov.**, Willan's vlei rat

LSID:urn:lsid:zoobank.org:pub:
C891465A-E05B-4DEA-9AC4-CBF6ACE5A67E

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* (cum unguis: with claw) / *su* (sine unguis: without claw)], ear 16.5 mm.

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

Paratypes: Ten specimens collected from Tiffendell Ski Resort in December 2013. Some have formaldehyde-preserved skins and damaged skulls [TM49094 (BAR1002), male; TM49100 (BAR1016), unknown sex; TM49097 (BAR1019), male; TM49091 (BAR1021), male; TM49095 (BAR1026), male; and TM49098 (BAR1018), unknown sex], one has an intact skull, but the skin was rotten and discarded [TM49092 (BAR1001), female], and three have damaged skulls and rotten skins that were discarded [TM49096 (BAR1006), male; TM49093 (BAR1010), male; and TM49099 (BAR1017), unknown sex]. 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),

Table 4. Summary statistics for ten cranial variables in taxa defined by this study

	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}
Minimum	38.06	27.70	37.62	34.70	34.00
Maximum	44.30	36.14	45.26	39.61	36.00
SD	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}
Minimum	24.08	17.50	22.63	21.20	18.26
Maximum	29.89	22.71	29.00	24.56	19.39
SD	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}
Minimum	8.77	6.33	9.02	7.82	7.63
Maximum	10.49	8.76	10.29	9.26	8.03
SD	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}
Minimum	7.48	5.00	6.50	5.71	6.01
Maximum	9.03	6.55	8.55	6.63	6.10
SD	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}
Minimum	4.16	3.32	4.07	3.88	3.75
Maximum	4.90	4.34	5.03	4.63	4.24
SD	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}
Minimum	19.26	14.80	18.30	16.98	16.77
Maximum	22.18	18.52	21.91	18.94	17.56
SD	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}
Minimum	20.92	14.07	20.28	18.17	13.47
Maximum	26.27	19.70	25.61	21.18	14.55
SD	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
Minimum	6.37	5.30	6.49	6.11	7.82
Maximum	8.66	7.33	8.51	7.94	8.48
SD	0.49	0.72	0.42	0.42	0.33

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 pairwise Tukey's tests. For abbreviations of variables, see under text for Material and Methods.

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); and TM49095 (BAR1026), male of unknown age (class 1). From Asante Sana (–32.24925S, 24.936E), 2090 m a.s.l.: 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 ma.s.l.: DM13657 (MZ5), skull only, unknown sex, collected 26 July 2009 by Armand Kok.

Figure 4. Different views of the skull and mandible of *Otomys willani* (TM49101; A–D), *Otomys auratus* (TM46130; E–H) and *Otomys 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 left lateral views of the mandible (D, H, L). The scale is the same in all the images.

Referred specimens having only morphological identification: TM22642, 32 km 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; and TM29521, Karoo National Park, 9 km 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: There were two categories of undetermined specimens that were provisionally referred to *O. willani*: (1) those specimens without molecular data that were morphologically most like *O. willani* (e.g. small-sized in cranial dimensions), albeit slightly distinct (see Fig. 3, marked as '*O.* cf. *karoensis* sp. 2'): 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); and (2), those specimens that most closely resembled *O. willani* in morphology

but that belonged to a different mtDNA molecular clade (Fig. 2): DM13661 (MZP7), female collected at Mountain Zebra National Park (–32.521S, 25.686E) on 12 February 2010 by Armand Kok.; and DM13658 (MZ2), unknown sex, collected at Mountain Zebra National Park (–32.521S, 25.686E) on 26 July 2009 by Armand Kok.

Etymology: The species is named for 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* (Willan, 1979).

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. *Otomys 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 mm, N = 4), hindfoot (*cu*) of 21.5–25.5 mm (mean 23.5 mm, N = 9) and 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 four laminae; upper M3 has seven (in one specimen examined) or six laminae (in ten specimens examined).

Diagnosis: *Otomys willani* can be distinguished in the field from co-occurring *O. auratus* and *O. sloggetti* within its range in the southern escarpment of South Africa. It is pallid-russet in colour dorsally. Compared with *O. sloggetti*, the body is darker reddish in colour, and this species has smaller body measurements than *O. sloggetti*. In *O. willani*, the pelage is grizzled owing to interspersed black- and russet-coloured hairs (based on the colour of subterminal bands), whereas in *O. sloggetti* it is much less grizzled owing to many fewer black hairs intermixed with pale-buff-coloured hairs (Fig. 5). Compared with *O. willani*, *O. karoensis* has a more pallid-buff rather than pale-russet wash owing to the subterminal section of the guard hairs being buff to pale brown as opposed to russet. Compared with specimens of *O. auratus*, *O. irroratus* and *O. laminatus*, in addition to its distinctly smaller size, the dorsal colour of *O. willani* is conspicuously paler that the last-mentioned three species in spite of having a similar hue (Fig. 5).

Although sample sizes are variable owing 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 78 g (75–83 g, N = 4) in *O. willani* and 127 g (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 hindfoot (*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*, as mentioned above, the latter species is much darker buff-brown in colour 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. minimal values exceeding the values of these variables in the case of *O. auratus* and *O. irroratus*). Only in the case of IOC is *O. willani* significantly smaller than *O. karoensis* (Table 3). As shown by PCA (Fig. 3), *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). *Otomys karoensis* generally has a disproportionately large interorbital, longer palatal length (and palatal foramina; Fig. 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* (Figs 2, 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.

Distribution and biology: The species occurs along the Southern Great Escarpment at elevations > 1000 m a.s.l., from the Karoo National Park near Beaufort West in the west to Giant's Castle in KwaZulu-Natal Province in the east (Fig. 1). Small-sized specimens from two localities on the central plateau of South

Figure 5. Dorsal view of dry study skins showing the variation in colour between (from top to bottom) *Otomys willani* (TM22655), *Otomys sloggetti* (TM22664), *Otomys auratus* (TM46512), *Otomys irroratus* (TM22801), *Otomys laminatus pondoensis* (*TM79*, holotype) and *Otomys karoensis* (*TM5401*, holotype). Locality details are given in Table 1, with the exception of TM22664 from 13 km Warden–Vrede, Free State Province (27.625°S 28.875°E) and TM79 from Ngqeleni, E. Cape Province (31.66°S, 29.03°E).

Africa north of the Drakensberg, referred to as *O.* cf. *karoensis* sp. 2 (dark blue circles in Fig. 1), are provisionally referred here to *O. willani*.

DISCUSSION

Insights into the biogeography, speciation and diversity of *Otomys* in the Cape region of South Africa

Before 2009, five species of *Otomys* were known to occur in the Western and Eastern Cape provinces of South Africa: *O. irroratus*, *O. karoensis* (formerly *O. saundersiae*), *O. laminatus*, *O. sloggetti* and *O. unisulcatus*. More recently, the work of Taylor *et al.* (2009a), Engelbrecht *et al.* (2011) and the present study has increased the diversity of *Otomys* in the region to seven species (*O. auratus*, *O. irroratus*, *O. karoensis*, *O. laminatus*, *O. sloggetti*, *O. unisulcatus* and *O. willani*) 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 the present study, which could not be corroborated owing to the absence of voucher specimens. Also, specimens of *O.* cf. *karoensis* sp. 2 from the Free State, which are morphologically distinct owing to their small skull size and proportionately expanded auditory bullae, lack corroborating molecular and/or karyotypic data. It is

not clear whether the three distinct mtDNA subclades of *O. sloggetti* correspond to two or more cryptic species, and further morphometric and karyotypic analyses are required. Given that the above conclusions rest on only a single mitochondrial genetic marker, in addition to morphological data, there is an urgent need to add molecular data from nuclear sequences and microsatellite loci to provide further support and resolution to the taxonomic conclusions of the present study.

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* coincides 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 difficult to identify in the field. Small, pallid-coloured specimens from the Sneeuberg Range (preset study) and Tiffendell (from the study by Phukuntsi *et al.* 2016) were genetically distinct from *O. auratus*, *O. irroratus* and *O. sloggetti* and from *O. karoensis s.s*. Morphometric and craniodental analysis (Fig. 3 and Results above) confirm their specific separation from these four species, highlighting the crucial importance of retaining museum voucher specimens in molecular studies, especially in the case of cryptic species. We describe this new *O.* cf. *karoensis* sp. 1 lineage as *O. willani* above. This result confirms our hypothesis, based on the cryptic speciation observed in *O. irroratus s.l.* (Engelbert *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–O. auratus* clade.

Otomys irroratus–O. auratus clade

Phylogenetic analysis of *Cytb* gene sequences reveal 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, which diverged 1.1 Mya (Taylor *et al.*, 2009a; Engelbrecht *et al.*, 2011), coincide with different biomes and mountain ranges (*O. irroratus* with the Cape Fold Belt and the fynbos biome and *O. 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 is mirrored exactly in the case of another montane rodent, the four-striped mouse *Rhabdomys pumilio* (Sparrman, 1784) *s.l.*, whose range corresponds closely to 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). Likewise, Willows-Munro & Matthee (2011) 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 allow us to extend the western limit of *O. auratus* in the Eastern Cape from ~27°E (Hogsback) to 25°E (AS). The origin of the two major clades within *O. irroratus s.l.* (and *O. karoensis s.l.*) might be attributable 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 owing to increased temperature and decreased rainfall (deMenocal, 2004; Taylor *et al.*, 2009a, 2015). Aridification of the biomes might 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 to a period of aridification ~1 Mya (deMenocal, 2004; Taylor *et al.*, 2009a). The fact that the range of *O. irroratus* corresponds closely to both the Cape Fold Belt geology and the fynbos biome, whereas the range of *O. auratus* corresponds to both the grassland biome and the Great Escarpment mountain system, suggests that although

geology combined with climate might have been the cause of the initial divergence of lineages, subsequent speciation and evolutionary divergence might have been aided by ecological coadaptation to the different vegetation 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 Eastern Cape Province to form a secondary contact zone with *O. auratus* at Alice on the Great Escarpment. In contrast, *O. auratus* has a multimodal mismatch indicative of a stable (and older) population. As also found by Taylor *et al.* (2009a) and Engelbrecht *et al.* (2011), our data reveal two distinct subclades in *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 Western 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, although populations from Mt Zebra (western clade) and Hogsback (eastern clade) are separated by only 100 km.

Otomys sloggetti clades

Our study reveals new distribution records for *O. sloggetti* that extend the range beyond the Drakensberg Range to the adjacent Sneeuberg region of the southern Great Escarpment. *Otomys 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 *O. sloggetti* from the Sneeuberg, Drakensberg and Maluti Ranges suggests that some vicariance and/or speciation might have occurred between these lineages.

Conclusion

Our study brings to six the number of *Otomys* endemic or near-endemic species occurring in South African mountains (across the Great Escarpment and Cape Fold Belt). The Sneeuberg Centre of Floristic Endemism is clearly also a hotspot of rodent diversity and endemism, with up to three of these endemic *Otomys* species co-occurring syntopically. Given the accelerating threats of anthropogenic and climatic change in temperate mountain grassland ecosytems, the conservation status of these montane endemic rodents needs to be reappraised urgently, particularly in the case of the newly described *O. willani*, which is restricted to the southern Great Escarpment. Given expected declines in grasslands owing to climate change and the consequent adjustment of *O. auratus* from Least Concern to Near Threatened (Taylor *et al.*, 2015; Child *et al.*, 2016), we propose that *O. willani* should be classified nationally and globally as Near Threatened under the A4C IUCN criterion.

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