Supplementary file

Molecular Methodology

DNA extraction and molecular identification of cryptic Aethomys and Mastomys species

Genomic DNA extractions were performed using ear clip samples collected from cryptic *Aethomys* and *Mastomys* species. These samples were collected from live individuals, and immediately frozen and stored at -20°C, before transporting them to the University of Pretoria (UP) for processing and analysis. Following this, DNA extraction was performed at the UP molecular laboratory using the Roche® High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Germany), using the extraction protocol prescribed for mammalian tissue. Briefly, all samples underwent an overnight Proteinase K digestion at 56°C, the DNA was bound to a cartridge and was eluted in a final volume of 200 μL subsequent to a number of inhibitor removal and wash steps. Touchdown PCR reactions were performed in a final volume of 40 μl, which contained 1.5 units of DreamTaq (Thermo Fisher Scientific, USA), 2.5 mM dNTPs (Fermentas), 3 μl of template DNA and 0.4 μM of each of the murid rodent primers L14724 (TGAYATGAAAAAYCATCGTTG) and H15915 (CATTTCAGGTTTACAAGAC). The aforementioned primers, which bind in regions flanking the cytochrome *b* (cyt *b*) gene, amplify a mitochondrial genome region of ~1,2 kbp. Thermal cycling was performed on an ABI 3500 (Applied Biosystems) PCR machine using the following conditions: an initial denaturation step at 96°C for 10s, annealing for 30s, and elongation at 70°C for 1 minute, with a final elongation step at 70°C for 1 minute. Following Bastos et al. [37] the annealing temperature for the first two cycles was 50°C, 48°C for the next three cycles and 46°C for the final 35 cycles.

Nucleotide sequencing

The PCR products were separated using a 1.5% agarose gel electrophoresis and run against a 1kb DNA molecular weight marker (Thermo Scientific Fisher, United States) to estimate product sizes. Agarose gels were stained with Goldview and were visualized using UV irradiation. PCR products of the expected size were purified directly from the tube using the Roche PCR High Pure Product Purification Kit (Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions. Cycle sequencing was performed using the BigDye v.3.1 terminator cycle-sequencing kit (Perkin Elmer, USA), and the external PCR primer L14724 at an annealing temperature of 48°C. Unincorporated primers, dNTPs and fluorescently-labelled ddNTPs were removed by sodium-acetate precipitation and the purified products were then submitted to the Core Sanger sequencing facility of the University of Pretoria.

Sequence analysis

Sequence chromatographs were visualized and edited in the Chromas program embedded in MEGA 7 and used to generate contiguous sequences (contigs) [38]. Sequences were aligned using ClustalW and each sequence was then used in a nucleotide blast search against the Genbank database (https://www.ncbi.nlm.nih.gov/blast) to identify the most closely related sequences (>98% identity) linked to published peer-reviewed papers, in the database. Reference and outgroup sequences were identified for the cryptic species that were sourced from the Genbank database and added to the final alignment for phylogenetic analysis (sequence information is provided in a supplementary file).

Table S1. Gene regions, accession numbers, and publication sources obtained for reference sequences used in the construction of the maximum likelihood tree.

Species	**Gene region**	**Accession number**	**Publication**
Aethomys ineptus	*cytb*	MW537307	Krásová et al. (2021)
Aethomys ineptus	*cytb*	MW537308	Krásová et al. (2021)
Mastomys coucha	*cytb*	MT524718	McDonough & Sotero-Caio (2019)
Mastomys coucha	*cytb*	MT524719	Eiseb et al. (2021)
Micaelamys namaquensis	*cytb*	GQ471959	Russo et al. (2010)
Micaelamys namaquensis	*cytb*	GQ472095	Russo et al. (2010)

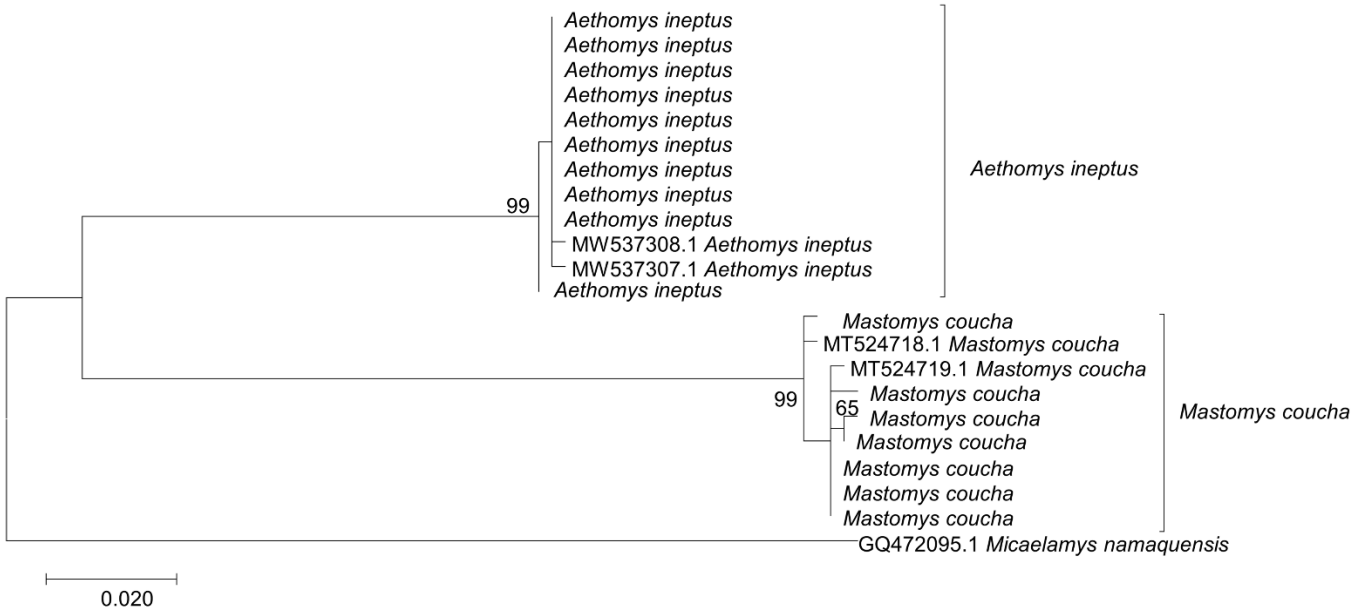

Figure S1. Maximum likelihood tree generated using cytochrome b (*cytb*) gene region for the identification of cryptic rodent species from *Aethomys* and *Mastomys* genera. Bootstrap support above 65 percent is indicated at relevant nodes. Reference and outgroup sequences were downloaded from Genbank, with accession numbers provided at the beginning of each sequence label.

References

1. Eiseb, S.J.; Taylor, P.J.; Zeller, U.; Denys, C.; Nicolas, V. Rapid peripatric speciation linked with drainage evolution in a rare African rodent, *Mastomys shortridgei* (Rodentia: Muridae). *Journal of Zoological Systematics and Evolutionary Research*. **2021**, 59(2), 522-542.
2. Krásová, J.; Mikula, O.; Bryja, J.; Baptista, N.L.; António, T.; Aghová, T.; Šumbera, R. Biogeography of Angolan rodents: The first glimpse based on phylogenetic evidence. *Diversity and Distributions*. **2021**, 27(12), 2571-2583.
3. McDonough, M.M.; Sotero-Caio, C.G. New karyotypic information for small mammals from Botswana with implications for regional biogeography. From field to laboratory: A memorial volume in honor of Robert J. Baker. **2019**, 775-804.
4. Russo I-RM; Chimimba CT; Bloomer P. Bioregion heterogeneity correlates with extensive mitochondrial DNA diversity in the Namaqua rock mouse, *Micaelamys namaquensis* (Rodentia:

Muridae) from southern Africa-evidence for a species complex. *BMC Evolutionary Biology*. **2010**, 10, 307.