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Identifying *Rhabdomys* museum specimens following taxonomic changes: use of short COI sequences

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Abstract

The African four-striped grass mouse (*Rhabdomys* sp.) is a widespread rodent genus which is currently divided into two geographically distinct species. These rodents' wide distribution range makes them perfect candidates for molecular studies related to adaptations to different habitats. Museum collections form valuable sources of samples for molecular analyses, but use of older classifications or mislabelling of specimens can be problematic. In this study, we attempt to use medium sized (~300 bp) cytochrome oxidase subunit I (COI) mitochondrial DNA fragments to correctly group *Rhabdomys* museum specimens into their now known taxonomic groups. Samples were sourced from four South African museum collections. We were able to group the majority of the specimens into their anticipated taxonomic groups, with 65 specimens identified as *R. pumilio* and 35 as *R. dilectus*. The phylogenetic grouping of the specimens largely correlated with work done by Du Toit *et al*. (2012). These results will facilitate the use of the selected specimens for a future study, which will focus on the genetic variation observed at selected adaptively linked genes, among *Rhabdomys* populations.

Key words

Rhabdomys, species identification, museum specimens, COI.

Introduction

The African four-striped grass mouse (*Rhabdomys* sp.) has for many years been regarded as a monotypic genus (*R. pumilio* Sparrman, 1784). Currently two geographically distinct species are recognized for this genus, with *R. pumilio* occurring in the arid to semi-arid western regions of southern Africa and *R. dilectus* found in the mesic to humid eastern parts (Rambau *et al*., 2003). A more recent molecular study using mitochondrial data showed that *R. dilectus* has a more diverse genetic structure than previously thought, consisting of three unique lineages (Castiglia *et al*., 2012). Du Toit *et al*. (2012) showed that there are four genetically distinct monophyletic cytochrome c oxidase I (COI) lineages within *Rhabdomys*, with three unique clades for *R. pumilio*. These authors argued that these lineages should be seen as separate species, not only on the basis of the mtDNA monophyly observed, but also based on ecological divergences such as habitat specialisation. Contact zones between *pumilio* and *dilectus* lineages were also identified; in the Sandveld region of the Free State and the Fort Beaufort area in the Eastern Cape (Du Toit *et al*., 2012). Meynard *et al*. (2012) also proposed the existance of these contact zones.

Museum specimens provide a valuable resource for molecular research, especially if adequate funding for field-work is a problem. Although the split of *Rhabdomys* into two species is widely accepted, the museum collections in South Africa still reflect the original nomenclature of *R. pumilio*. The use of older classifications or mislabelling of specimens are well-known problems within museum collections (Sonet *et al*., 2011; Phukuntsi *et al*., 2016) and it is not always possible to identify specimens according to morphology, as coat colour fade with time (Downing, 1945; Davis *et al*., 2013). Cryptic species can

Fig. 1. Sampling localities of the 159 sourced specimens. The overall *Rhabdomys* distribution range is indicated by the light grey shaded area, with possible *R. pumilio* and *R. dilectus* contact zones identified by Meynard *et al*. (2012) indicated by the dark grey shading. The distribution map is based on data from IUCN (2008).

also lead to misidentified museum specimens, as these taxa are morphologically similar and often grouped into a single species (Pfenninger & Schwenk, 2007). The existence of cryptic species in southern African rodent species is well studied (Engelbrecht *et al*. 2011; Taylor *et al*. 2013) and is therefore a valid concern when identifying museum specimens for analysis. The use of species distribution ranges can also be inadequate for species identification, as overlaps do occur between the two *Rhabdomys* species. It is therefore necessary to identify the *pumilio* and *dilectus* clades using molecular techniques before using these specimens in downstream genetic studies. In this regard, the use of short to medium sized COI sequences is a well-accepted method for the identification of ancient or degraded biological specimens (Hajibabaei *et al*., 2006; Robins *et al*., 2007; Sonet *et al*., 2011). We therefore aimed in this study to group the *Rhabdomys* specimens obtained from four South African archival collections into the now accepted species (*R. pumilio* and *R. dilectus*) using a medium length (~300 bp) COI mtDNA fragment and the COI haplotypes from Du Toit *et al*. (2012).

Materials and Methods

Compliance with Ethical Standards

The appropriate ethical approval was obtained from the Inter-Faculty Animal Ethics Committee of the University of the Free State, South Africa (Ref no: UFS-AED2016/0044).

Samples

Museum specimens were sourced from four South African archival collections (National Museum, Bloemfontein [Bm]; Ditsong National Museum of Natural History, Pretoria [Tm]; Durban Natural Sciences Museum, Durban [Dm]; and McGregor Museum, Kimberley [Mc]). In total 159 specimens sampled from 31 localities were sourced from these collections (see Supplementary Table S1), covering a large section of the *Rhabdomys* distribution range (Fig. 1). The majority of the samples were dried skins (n = 108), with 51 tissue samples stored in absolute ethanol.

DNA extraction

The Purelink Genomic DNA Kit (Life Technologies ™, Carlsbad, CA) was used for all DNA extractions. The tissue samples stored in ethanol were first washed to remove the surface ethanol, by implementing two wash steps with dH_2O. The samples were then incubated in dH_2O for 4 h to re-hydrate the samples. The manufacturer's protocol was then followed for extraction. The dried skin samples were also first washed to remove any surface contaminants. Three wash steps were performed (96% ethanol, 70% ethanol and dH_2O), with a final hy-

dration step in dH_2O for 4 h. The manufacturer's protocol was then followed. The elution step was, however, also modified, with the DNA eluted in 50 µl elution buffer. The elution buffer was added to each column and incubated at 55 °C for 2 min followed by centrifugation at 10000 × g for 1 min. The DNA quality and quantity were assessed by spectrophotometry using a NanoDrop® Spectrophotometer ND-1000. All working surfaces were decontaminated with a 1.25% hypochlorite solution before and after each set of DNA extractions.

Primer design and DNA amplification

Previously published sequences for *R. pumilio* (n = 3) and *R. dilectus* (n = 3) were downloaded from Genbank to design primers suitable for degraded DNA samples (Accession numbers: JQ003437, JQ003455, JQ003456, JQ003459, JQ003461, JQ003470). Following manually inspection of the alignment of the six downloaded *Rhabdomys* sequences, primer set Rhd_COIb

(F: 5′—ACAGACCGAAACCTTAATAC—′3;
R: 5′—TTTACTCCTGTTGGGATAGC—′3)

spanning a ~ 300 bp region of the COI gene was designed using Primer3 (Rozen & Skaletsky, 1999) as implemented in Geneious R9 (Kearse *et al*., 2012). PCR setup was performed in a DNA free laboratory, and all working surfaces were decontaminated using a 1.25% hypochlorite solution, prior to setup. The PCR reaction mixture consisted of: 3 – 4 µl DNA (10 – 150 ng/ul DNA, depending on sample quality), 6.25 µl Ampliqon TEMPase Hot Start 2 × Master Mix, 0.5 µl of each primer (10 uM stock) and dH_2O to make up a 12.5 µl mixture. A touchdown PCR cycling protocol was followed. An initial denaturation at 95 °C for 3 min was followed by a 7-cycle touchdown protocol of 95 °C for 30 s, 58 °C to 52 °C for 40 s and 72 °C for 30 s; this was followed by 30 cycles of 95 °C for 30 s, 52 °C for 40 s and 72 °C for 30 s, with a final extension step of 72 °C for 1 min. The successfully amplified PCR products were identified by gel electrophoresis, using a 1% agarose gel. PCR product purification was performed with the BioSpin PCR Purification Kit (BioFlux, Tokyo, Japan). Sequencing PCR reactions were performed using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Division, Perkin-Elmer, Foster, CA). The ZR DNA Sequencing Clean-up™ Kit (Zymo Research, Orange, CA) was used for sequencing clean-up, with sequencing analysis performed on an ABI 3500 Genetic Analyzer.

Sequence analyses

Forward and reverse sequences were assembled, edited and aligned using Geneious R9. Summary statistics, including the number of polymorphic sites (P), the number of parsimony informative sites (PI) and the nucleotide diversity (π), were estimated using DnaSP v5 (Librado & Rozas, 2009). Published *Rhabdomys* COI sequences (Accession numbers: JQ003320 – JQ003470) from Du Toit *et al*. (2012) were downloaded from Genbank to serve as a reference dataset (n = 151). The reference dataset was aligned and trimmed to the 299 bp region amplified by primer set Rhd_COIb. To assess the usefulness of the COI region in clustering individuals in the correct species groups, a Bayesian inference (BI) tree was estimated in MrBayes v3.2 (Ronquist *et al*., 2012) using the reference dataset and three outgroup taxa (*Rattus norvegicus*, AY172581; *Rattus rattus*, EU273707; *Mus musculus domesticus*, FJ374665). The most optimal nucleotide substitution model was identified using the Akaike information criterion (AIC; Akaike, 1974) as implemented in jModelTest v.2.1 (Darriba *et al*., 2012). The BI analysis consisted of 10 million generations, a sampling frequency of 1,000 and a burn-in of 0.25. The run reached convergence once the average standard deviation of split frequencies was below 0.01. The COI sequences obtained from this study was deposited in Genbank (Accession numbers: MG773379 – MG773478).

Results

DNA extractions and sequencing

Of the 159 samples used in this study, 26 samples did not provide DNA of sufficient quantity or quality for PCR amplification. Twenty-five of these 26 samples were tissue samples stored in absolute ethanol and one was a dried skin sample. One of the museum curators indicated that some of their samples might have been stored in formalin in the past, which could have led to the problems with DNA extraction. A wide range of DNA concentrations and quality was obtained from the archival samples. The average DNA concentration over all samples was 41.51 ng/ul (Average 260/280 ratio: 1.67; Average 260/230 ratio: 1.77). Good quality DNA sequences were obtained for 100 specimens. The post trimming sequence length for the datasets was 299 bp. The number of polymorphic sites and number of parsimony informative sites for the museum dataset (P = 55; PI = 54) was lower than that observed for the Du Toit *et al*. (2012) dataset (Table 1). Similar nucleotide diversity values were, however, obtained (museum: $\pi = 0.07$; Du Toit *et al*. (2012): $\pi = 0.075$).

Classification of archival specimens

The phylogenetic tree generated closely resembles the tree produced by Du Toit *et al*. (2012), with the museum specimens grouping according to the four main clades observed (*R. dilectus* clade and *R. pumilio* Coastal, Central and Northern clades). The Central *R. pumilio* clade was, however, found to be sister to all the other taxa (Fig. 2).

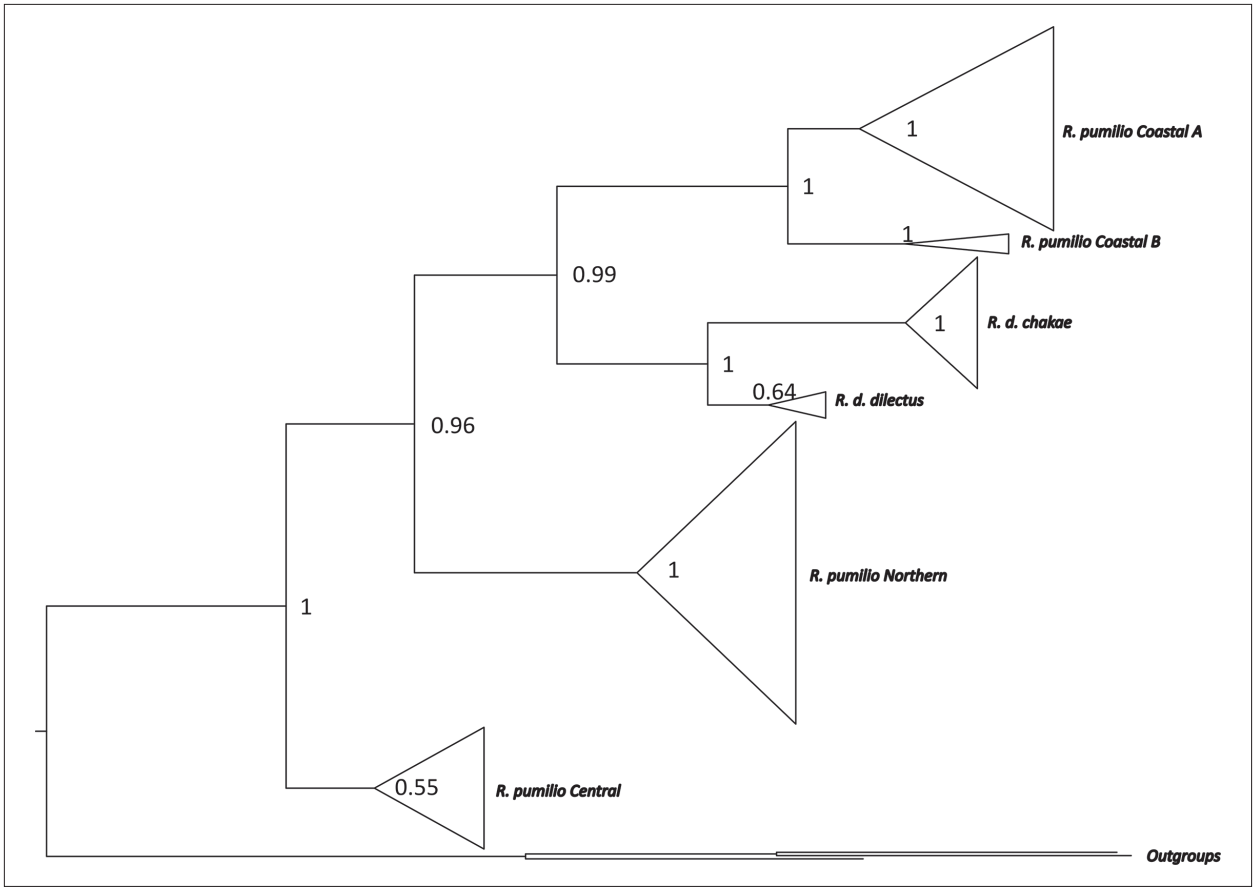

Fig. 2. The phylogenetic tree obtained through Bayesian inference (BI) for the combined archival and reference *Rhabdomys* COI dataset. The posterior probability for each major node is provided. The naming of the clades follows that of Du Toit *et al*. (2012). The sample information for the archival specimens used in the current study is provided in Table 2.

Thirty-five of our specimens were identified as *R. dilectus*. The majority of these grouped with the *R. d. chakae* subspecies (n=32) and only three specimens grouping with the *R. d. dilectus* subspecies (Castiglia *et al*., 2012; Du Toit *et al*., 2012). The *R. d. chakae* specimens mainly originated from the eastern parts of the Eastern Cape, KwaZulu-Natal, Mpumalanga and Lesotho. Two specimens identified as *R. d. dilectus* were sampled from the Soutpansberg region of Limpopo. Surprisingly, four *Rhabdomys* specimens sampled in the Western Cape grouped with the *dilectus* clades (*R. d. chakae*: Tm12, Tm13, Tm14; *R. d. dilectus*: Tm16).

The remainder of the specimens (n=65) grouped with the three identified *R. pumilio* clades, and largely correlates with the results from Du Toit *et al*. (2012). Three specimens from Western Cape grouped with the *pumilio* Coastal A clade as expected, with five Eastern Cape specimens (EC) falling in the *pumilio* Coastal B clade. A large portion of the identified *pumilio* specimens (n=37) formed part of the *pumilio* Northern clade and were sampled in the Free State, Northern Cape and Western Cape Provinces. The remaining 20 specimens were sampled from the Free State, Northern Cape, Eastern Cape, Western Cape and Limpopo Provinces and from southern Namibia, and grouped with the Central clade. Not all our archival specimens grouped according to the expected geographical clades, with the Namibian and Limpopo specimens falling within the Central clade and not the Northern as expected. It also was observed that specimens from the Kimberley, NC, and Nieuwoudtville, NC, populations mainly grouped to the Northern clade, with one specimen from each falling in the Central clade. All the reference sequences did, however, group to the same clades as seen by Du Toit *et al*. (2012).

Discussion

In this study we successfully sequenced 62.9% of our specimens using a medium length COI fragment. This level of amplification success is in line with observations by other researchers (Hajibabaei *et al*., 2006; Sonet *et al*., 2011). The same four clades were observed in our analysis as seen by Du Toit *et al*. (2012), with one difference. The Central and Northern clades were not observed as sister taxa, but the Central clade was instead found to be basal to all the other taxa. The differences observed between our phylogenetic tree and that from Du Toit *et al*. (2012) could be linked to the shorter fragment we used in our analyses, which contained slightly less polymor-

Table 1. The summary statistics for the COI fragment as estimated for the museum specimen dataset and compared to the Du Toit *et al*. (2012) dataset. The number of sequences (n), sequence size in basepairs (bp), the number of polymorphic sites (P), the number of parsimony informative sites (PI) and the nucleotide diversity (π) are provided for each dataset.

Dataset	n	bp	P	PI	π
Current study	100	299	55	54	0.07
du Toit *et al*. (2012)	151	299	78	67	0.075
R. pumilio	136	299	69	57	0.07
R. dilectus	15	299	22	15	0.028

phic sites. Except for the uncertain position of the Central and Northern clades, all reference specimens grouped in the same clades as in Du Toit *et al*. (2012), and we are therefore certain of the species identity of our archival specimens. The uncertainty of the position of specimens within the Central and Northern clades could be further investigated by using an additional or longer COI fragment. We were, however, mainly interested in identifying the specimens to species level and this was achieved.

The four Western Cape specimens which were identified as *R. dilectus* was unexpected. According to museum records, all four specimens originated from the Postberg Nature Reserve near Langebaan on the west coast of the Western Cape. The distribution range of *R. dilectus* does, however, not include the Western Cape (Rambau *et al*., 2003). Several factors could explain this observation. For one, this could be due to DNA contamination, either during DNA extraction or during specimen storage. Dried skin specimens are typically stored together in drawers or similar storage units where specimens can come into contact with each other. Another possibility could be mislabelling of specimens, and that these specimens thus originate from an alternative location. A third possibility could be that these are *dilectus* specimens. While examining supplementary table A.1 from Du Toit *et al*. (2012) to verify our results, we observed that haplotypes H110 and H111 were identified as *R. d. chakae*, but were isolated from specimens from Stellenbosch (SB), Western Cape. The authors did, however, not discuss why specimens from the Western Cape grouped with the *R. d. chakae* clade. Two possibilities come to mind. One, these individuals could have established themselves in the Western Cape through accidental translocation via ship or another form of anthropogenic action from any of the eastern coast port cities to the western coast ports of South Africa. The human-mediated spread of rodents, particularly *Rattus* sp. and *Mus* sp., are well documented (Atkinson, 1973; Copson, 1986; Robins *et al*., 2016). Another explanation, might be that these are the remnants of a relic population which could have been isolated during the Quaternary ice ages. These ice ages occurred in cycles of 40,000 to 100,000 years, causing habitat expansions and contractions on a global scale (Hewitt, 2000; Hewitt, 2004). This period roughly started around the time of

Table 2. Archival specimen grouping according to the Bayesian inference (BI) tree in Fig. 2. The clade annotation, number of samples and samples identified are provided.

Clade	No of samples	Samples
***R. pumilio* Coastal A**	3	Dm37-39_WP1
***R. pumilio* Coastal B**	5	Dm02-05_EC2
		Dm16_EC2
***R. pumilio* Northern**	37	Bm01, 02, 04-11_FS1
		Mc01-10_NC1
		Mc11-13, 15-20_NC2
		Mc26, 29_NC3
		Mc39, 43, 44_NC4
		Bm34_NC6
		Tm36_WP4
		Tm41_WP1
***R. pumilio* Central**	20	Bm32, 33, 35-37, 39_NC6
		Dm08-10_EC3
		Mc32_NC3
		Mc33_NC4
		Tm24-25_Lim2
		Tm26, 28_Nam
		Tm32, 33, 35_WP4
		Tm38_WP5
		Tm45_WP6
R. d. dilectus	*3*	Dm32-33_Lim1
		Tm16_WP3
R. d. chakae	*32*	Bm12, 15-17, 20_EC1
		Bm22, 24, 25, 28-31_Les
		Dm14_EC2
		Dm21_KZN1
		Dm22-24_KZN2
		Dm25-26_KZN3
		Dm30_KZN4
		Dm34-36_MP1
		Tm01_KZN5
		Tm06_KZN6
		Tm12-14_WP2
		Tm46_MP3
		Tm47_MP4
		Tm48-49_MP5
Outgroups		*Rattus norvegicus*
		Rattus rattus
		Mus musculus domesticus

R. dilectus speciation, with the most recent common ancestor (MRCA) for *R. dilectus* dated from about 2.17 mya (CI = 1.25 – 3.21; Du Toit *et al*. 2012) to 2.9 mya (Rambau *et al*. 2003). Currently our translocation hypothesis is more plausible, and finer scale sampling of the *Rhabdomys* populations of the Western Cape is needed

to better understand the pattern we have observed. Additional molecular techniques like population structure and migration analyses using microsatellite data is also needed.

A contact zone between *pumilio* and *dilectus* was identified, which correlates with Du Toit *et al*. (2012). This zone was observed in the Grahamstown region, which forms part of the 'Bedford-gap' where different biomes meet (Lawes, 1990), providing suitable habitats for both mesic and xeric *Rhabdomys* species. Ganem *et al*. (2012) also identified contact zones between *pumilio* and *dilectus* in the Free State province of South Africa, where different biomes converge. Another possible contact zone might occur in the Limpopo Province, seeing as specimens from the Settlers region were identified as *pumilio* and specimens in the Soutpansberg region were identified as *dilectus*. Three biomes occur in the Soutpansberg region, namely the Grassland, Forest and Savanna biomes. This combination of biomes could provide adequate habitat for the occurrence of both *Rhabdomys* species. Meynard *et al*. (2012) predicted that contact zones in northern South Africa could occur around the edge regions of the Grassland and Savanna biomes. It is also important to note that the Grassland and Forest biomes in the Soutpansberg area are in the middle of an area dominated by Savanna, therefore forming habitat islands (Macarthur & Wilson, 1967). There are a number of similar habitat islands which can be observed along the northern parts of the Drakensberg mountain range stretching through the Limpopo and Mpumalanga Provinces. These regions can thus form population refugia for *R. dilectus*. Further research in these areas are, however, needed to elucidate the population dynamics of *Rhabdomys* in the northern parts of South Africa.

The current study clearly shows that the medium sized mtDNA fragments can be used with high success to identify unknown or mislabelled archival specimens. It is also clear that the *Rhabdomys* genus is highly complex, and more in-depth studies of possible contact zones and possible refugia is needed to better understand the evolution of this widespread African rodent.

Conclusion

The use of a medium length COI gene fragment has proven to be a useful tool for the identification of unknown or mislabelled *Rhabdomys* museum specimens. Failure to obtain usable DNA from some of the samples could be addressed by alternative extraction protocols. Many specialized DNA extraction kits are now available for the extraction of DNA from difficult samples such as formalin fixed or treated samples. Resampling and re-extraction of suspect specimens can also be pursued to ensure the correct classification of said specimens. Finer scale sampling of the Western Cape and the use of additional genetic tools should help to clarify the anomaly observed in the current study. It is also clear that the *Rhabdomys* genus is highly complex, and more in-depth studies of possible contact zones and possible refugia is needed to better understand the evolution of this widespread African rodent. The reclassified specimens can now be used for further analyses of the adaptive variability among the various *Rhabdomys* groups occurring across the South African biomes.

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Electronic Supplement File

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File 1: Supplementary_TableS1.xlsx