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ORIGINAL RESEARCH

Environmental DNA from soil reveals the presence of a "lost" Afrotherian species

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Abstract
Environmental DNA (eDNA) sampling has attracted worldwide attention over the past few years as an emerging approach to characterising and monitoring biodiversity, and has become particularly important for species that are rare, elusive or endangered. Most animal studies to date have focused on aquatic taxa; studies on other metazoan taxa, particularly wildlife in terrestrial environments, are scarce, with only a handful utilizing soil sources. We aimed to investigate the use of DNA barcoding from soil eDNA in (1) detecting rare/elusive/threatened species and (2) as a tool to investigate and potentially monitor range distributions. Through extensive eDNA sampling along the west coast of South Africa, we aimed to refine the distributions of four golden mole species thought to occur there, and specifically to determine whether De Winton's golden mole, *Cryptochloris wintoni* (IUCN Critically Endangered; Possibly Extinct), is in fact extant or extinct. Sequences were generated for three barcode markers (mtDNA cyt *b*, 12S and nuclear GHR) using next-generation amplicon sequencing. Tissue samples from four specimens were used to generate reference sequences for species identification, along with available GenBank sequences. We were able to (1) successfully detect all four species in our data, and (2) improve records of the distributions of these species. Furthermore, we uncovered cryptic diversity in *Eremitalpa granti*. Our data conclusively reveal the presence of the elusive *Cryptochloris wintoni* and suggest that this species may in fact be widespread, but not necessarily abundant, and certainly less so in areas subjected to mining activities, which continue to pose a threat to the species.

Keywords Terrestrial eDNA · Threatened species · Small mammal · Soil sampling · Amplicon sequencing

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Extended author information available on the last page of the article

Introduction

Significant loss of biodiversity has been reported in the current era of rapid global environmental change (Díaz et al. 2019; IPBES 2019), and there is increasing concern worldwide about declines in populations of wildlife (Ripple et al. 2014; Estrada et al. 2017; Jia et al. 2018; Saha et al. 2018; Şekercioğlu et al. 2019); Beckett et al. 2023). Many national and international organizations have established biodiversity monitoring strategies to assess and mitigate the impact of this loss (Kurtz et al. 2001; UN 2003; DEAT 2005). Over the past decade, biodiversity research and monitoring has come to rely increasingly on genetic tools, with rapid and relatively affordable DNA sequencing techniques now offering the opportunity to efficiently characterize biodiversity (Corlett 2017; Alexander et al. 2020; Cowart et al. 2020; Ji et al. 2020; Leempoel et al. 2020; Yang and Zhang 2020; Sales et al. 2021). Among these tools, environmental DNA (eDNA) sampling has attracted worldwide attention (Beng and Corlett 2020) and has become particularly important for species that are rare or elusive (Piaggio et al. 2014; Franklin et al. 2019), endangered (Taberlet et al. 1997; Shelton et al. 2019; Takahara et al. 2020), dangerous (Kendall et al. 2009) or otherwise difficult to capture (Constantine et al. 2012).

Environmental DNA (eDNA) is genetic material originating from the hair, skin, urine, faeces, mucous, saliva, sperm, blood or carcasses of organisms that may be present, in a more or less degraded form, in water, soil, or sediments (Andersen et al. 2012; Taberlet et al. 2012; Taberlet et al. 2012; Thomsen and Willerslev 2015; Pedersen et al. 2016; Sigsgaard et al. 2016; Thomsen and Sigsgaard 2019). DNA can persist in the environment for varying periods of time, from hours in temperate waters, to hundreds or thousands of years in cold, dry permafrost (Thomsen and Willerslev 2015). Isolation of this DNA from the environment can facilitate detection of organisms in the absence of obvious signs of the organism's presence, and provide genetic information that can be used to identify, study and/or monitor species/individuals across time and space without having to catch, handle, or in some cases, even observe them (Waits and Paetkau 2005; Schwartz et al. 2007; Beja-Pereira et al. 2009).

The application of eDNA has the potential to revolutionize conservation science and practice in many ways (see Beng and Corlett (2020) for a review). Environmental DNA techniques are efficient and relatively cheap and simple, non-destructive and non-invasive, and can be highly effective at detecting rare, cryptic, and elusive species, even at relatively low densities (Carvalho et al. 2019; Franklin et al. 2019; Shelton et al. 2019; Takahara et al. 2020). Recent studies have employed eDNA barcoding in detecting invasive, rare, and cryptic wildlife species, map their distributions, and design management strategies (Levi et al. 2019; Qu and Stewart 2019; Reinhardt et al. 2019). A few have evaluated the efficiency of eDNA versus conventional surveys in detecting elusive species, and eDNA has typically proven comparable or more successful at accurately detecting target species (Deiner et al. 2017; Evans et al. 2017; Leempoel et al. 2020). However, most metazoan eDNA studies have focused on aquatic taxa, especially fishes and amphibians (Beauclerc et al. 2019; Deutschmann et al. 2019). Studies on other metazoan taxa, particularly wildlife in terrestrial environments, are scarce (Beng and Corlett 2020), with some making use of aquatic eDNA sources to investigate terrestrial species (Ushio et al. 2017), and only a handful utilizing soil sources (Andersen et al. 2012; Leempoel et al. 2020).

One particularly interesting group of elusive terrestrial animals that have been extremely challenging to study and/or monitor are the golden moles of sub-Saharan Africa. Golden

moles represent a family (Chrysochloridae) of highly threatened small mammals: ten of the 21 species are listed as threatened on the IUCN Red List (1 Critically Endangered, 5 Endangered and 4 Vulnerable), and a further three are listed as Data Deficient (IUCN 2022). These subterranean insectivores are dependent on soft soils for burrowing, and are severely threatened by human activities, such as mining, urbanization and agricultural development. Golden moles are notoriously obscure and understudied, mostly due to the challenges associated with finding them, trapping them and/or observing their subterranean behaviour in the wild. Effective conservation of these animals largely depends on the taxonomic delineation of species, as well as critical information about the distributional ranges and population abundance of the various taxa (Mynhardt et al. 2015; Taylor et al. 2018; Mynhardt et al. 2020; IUCN 2022). Distribution and abundance data for most species are largely lacking (IUCN 2022), and the taxonomy of the family is not well understood (Asher et al. 2010; Taylor et al. 2018).

The first character-based phylogenies of chrysochlorids were based on hyoid shape (Bronner 1991), chromosome morphology (Bronner 1995a), and craniodental anatomy (Bronner 1995b), but these were riddled with uncertainties. A more robust phylogenetic estimate for the family, based on 145 morphological characters from the cranium, dentition, and skeleton, combined with approximately 700–900 bases from exon 10 of the nuclear Growth Hormone Receptor (GHR) gene for 18 of the group's 21 recognized species, was presented in 2010 (Asher et al. 2010). While this study provided substantial insight into the evolutionary history of golden moles, some uncertainties remained, particularly pertaining to the chrysochlorid root placement, and relationships among unsampled taxa. A new phylogenetic estimate for golden moles and tenrecs is currently underway (Bronner et al. 2023), and will help to resolve some of the taxonomic uncertainties.

De Winton's golden mole, *Cryptochloris wintoni,* is a highly elusive species (Critically Endangered; Possibly Extinct; IUCN 2022) recorded only from the type locality at Port Nolloth on the west coast of South Africa (Fig. 1), where it was last seen in 1937, over 80 years ago. The coastal town of Port Nolloth lies in an area of radical habitat transformation by alluvial diamond mining (Bronner and Asher 2016b), which poses a significant threat to golden moles. *Cryptochloris wintoni* is one of four golden mole species known to occur (or have occurred) on the west coast of southern Africa, alongside the Cape golden mole, *Chrysochloris asiatica* (Least Concern; IUCN 2022), Grant's golden mole, *Eremitalpa granti* (Least Concern; IUCN 2022) and Van Zyl's golden mole *Cryptochloris zyli* (Endangered; IUCN 2022; see Fig. 1 for distributions). *Chrysoschloris asiatica* and *E. granti* are thought to be relatively abundant and widespread, although little is known about even these species, given the challenges associated with studying their subterranean life (Bronner and Asher 2016a; Maree and Bronner 2016; Taylor et al. 2018). *Cryptochloris zyli* is known only from the type locality near Lambert's Bay and Groenriviermond (Fig. 1), where it was last seen in 2003 (Bronner and Asher 2016c). Range continuity between these localities cannot be assumed as so little is known about the ecological requirements and tolerances of the species. The estimated extent of occurrence for *C. zyli* is 4999 km^2 with an area of occupancy of 32 km^2, although further field assessments are critically needed to obtain more accurate estimates (Bronner and Asher 2016c; IUCN 2022).

While *C. asiatica* is readily identifiable based on external morphology, *Cryptochloris* spp. may be easily confused with *E. granti*. Although externally similar, *Cryptochloris* is well differentiated from *E. granti* on radiographs based on malleus shape, vertebral count, and length of humeral medial epicondyle (Asher and Avery 2010). Some authors (e.g., Simonetta 1968) have treated *C. wintoni* as only subspecifically distinct from *C. zyli*, however these taxa differ consistently in pelage colour and malleus morphology, indicating that

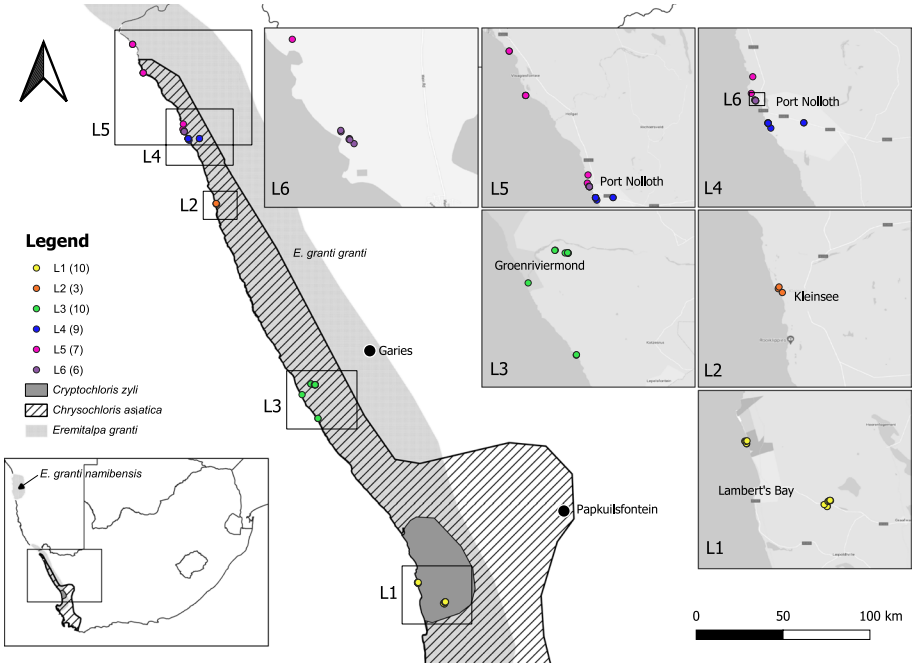


Fig. 1 Map of the west coast of southern Africa, depicting our sampling design, as well as the distributions of three of the four golden mole species found along the west coast: *Chrysochloris asiatica, Eremitalpa granti* (*E. g. granti* to the South and *E. g. namibensis* to the North—see inset), and *Cryptochloris zyli* at Lambert's Bay. *Cryptochloris wintoni* is known only from the type locality at Port Nolloth, and *C. zyli* additionally from Groenriviermond. The four tissue samples used in our study were obtained from Lambert's Bay, Port Nolloth, Papkuilsfontein and Garies. The sampling sites associated with each of the six eDNA sampling regions are colour-coded and indicated on the map, and on separate insets. Region L5 represents a vast stretch of coastline compared to the other regions, in order to encompass the mining areas, where golden mole abundance is substantially lower. Region L6 represents a comparatively small geographic area, focusing on the *C. wintoni* type locality (Port Nolloth). Since the geographic range of this sample is too small for the scale of the main map, it is indicated within the L4 inset

they are not conspecific (Meester 1974). Unfortunately *C. zyli* was one of the three species omitted from the Asher et al. (2010) study, however recent (unpublished) phylogenetic analyses based on both morphological and genetic data support the allocation of the two *Cryptochloris* taxa to separate species (Bronner and Asher 2016b).

The highly threatened, elusive and poorly understood golden moles on the west coast of South Africa may be considered flagship species for these coastal dune ecosystems, which are being severely impacted by habitat transformation due to ongoing mining and agricultural activities and associated residential developments. Furthermore, they are emblematic of the challenges associated with detecting and monitoring elusive terrestrial wildlife and the potential for eDNA applications to overcome these challenges. In this study, we aimed to evaluate the use of DNA barcoding from environmental DNA (eDNA) collected from soil (1) in detecting rare/elusive/threatened species and (2) as a tool to characterise and monitor range distributions of these species. Through extensive eDNA sampling from soil along the west coast from Lambert's Bay northwards to Port Nolloth, we aimed to refine the distributions of the four golden mole species thought to occur there, and specifically to determine whether *C. wintoni* is extant or extinct.

Materials and methods

Sample collection

Environmental DNA (eDNA) samples were collected in June 2021 from various sites along the west coast of South Africa (Fig. 1; Provincial permit: CN44-29-14184; Section-20 Permit: SDAH-Epi-21021810220). An initial scouting trip was undertaken to identify potentially active sites, through visual identification of sub-surface tunnels, and to obtain sampling permission from the relevant landowners. Soil samples were subsequently collected from these sites, wherever golden mole activity was detected, with the help of a trained scent-detection dog. GPS co-ordinates were recorded at each site. Wherever possible, soil was scraped from the inner lining of sub-surface tunnels, or alternatively as closely as possible to the furrow, in the case of very loose sand, including where possible, soil from inside the tunnel itself, and from varying depths surrounding the tunnel, up to a total of 15ml of soil per sampling site. Soil samples were collected using gloves and sterile equipment.

A total of 49 soil eDNA samples were collected, and grouped into six sequencing libraries, representing distinct geographic regions along the west coast (Fig. 1). Region L1 consisted of 14 soil samples collected from separate/distinct burrow systems from two distinct sites near Lambert's Bay (Fig. 1). Region L2 comprised 3 soil samples from distinct burrow systems near Kleinsee. Region L3 comprised 10 samples from 3 distinct sites near Groenriviermond. Region L4 comprised 9 samples from three sites on the southern outskirts of Port Nolloth, and Region L6 comprised 6 samples from the northern extent of Port Nolloth. Region L5 consisted of 7 samples from three mining sites in the vicinity of Port Nolloth, and northwards beyond Visagiesfontein towards Alexander Bay; this set of samples covers a far greater geographic range than the others, reflecting the relative dearth of golden mole activity found at the mining sites compared to the other sampling sites.

A single pitfall trap was set at Lambert's Bay (the type locality of *Cryptochloris zyli*) and at Port Nolloth beach (the type locality of *C. wintoni*). In each case, a single specimen was captured, and non-invasive DNA samples were collected via buccal and anal swabs, using a sterile cotton bud. Two additional samples were obtained opportunistically from Papkuilsfontein and Garies in the Northern Cape, through independent citizens sending in carcasses of golden moles that had allegedly been killed by pets. In these cases, a small toe clipping was taken for downstream DNA extraction. Since golden moles are morphologically conservative, we used external features (body size, pelage colour and foreclaws) only as an initial means of species identification for specimens, but relied on downstream DNA sequence analysis for more definitive identification.

This study was conducted in accordance with the regulations of the University of Pretoria's Animal Ethics Committee (ethics clearance no. EC053-18). All samples (tissue, swabs and soil) were stored dry in collection tubes at 4 °C until reaching the lab, where they were frozen at − 20 °C until further processing.

Marker selection

For animal studies, eDNA protocols typically target mitochondrial DNA (mtDNA) rather than nuclear DNA, due to the higher mtDNA copy number in cells, and consequently in the environment (Rees et al. 2014). Mitochondrial DNA barcodes commonly employed

in animal studies include cytochrome *b* (cyt *b*; Piaggio et al. 2014), 12S ribosomal RNA (Ushio et al. 2017; Leempoel et al. 2020) and cytochrome oxidase I (COI; Hebert et al. 2003). Primers are typically designed to target amplicons (amplified PCR products) of less than 150 bp (base pairs; Rees et al. 2014), based on the assumption that smaller fragments are favourable for optimizing the probability of DNA detection due to environmental degradation of DNA. However, this phenomenon is offset by the improved taxonomic resolution provided by longer fragments. Thus, eDNA barcode markers should aim to target genomic regions with adequate sequence variation, while minimising fragment length. Previous studies have shown that 210 bp of the 12S gene is suitable for successful amplification from soil eDNA, while providing sufficient resolution for mammalian species identification (Leempoel et al. 2020), therefore we targeted this marker, among others, in our study.

Three barcode markers were selected for this study: mitochondrial cytochrome *b* (cyt *b*) and 12S, and nuclear GHR intron 9 (Table 1). The markers were chosen based on availability of reference golden mole sequences (cyt *b* and GHR) and reputability for use in eDNA studies (12S). We used universal mammalian primers for amplification of a 354 bp cyt *b* fragment, and golden mole-specific 12S and GHR primers were designed using the Primer 3 web interface (Rozen and Skaletzky 2000) using the reference *Chrysochloris asiatica* genome (ChrAsi1.0; WGS: AMDV00000000.1; Genbank Assembly: GCA_000296735.1) and testing the proposed primers on alignments of all available reference golden mole sequences (12S: 16 of 21 species, including 3 of 4 target species; GHR: all 21 species).

DNA extraction and PCR amplification

All DNA extractions were performed in a standard molecular genetics laboratory at the University of Pretoria. Since we did not have access to a dedicated eDNA laboratory, special care was taken to prevent contamination of samples. Extractions and PCRs were both spatially and temporally separated.

Genomic DNA was extracted from soil samples using a NucleoSpin Soil Kit (Macherey–Nagel, Düren, Germany), and from tissue and swab samples using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quantity and quality were assessed using gel electrophoresis and QubitTM dsDNA HS and BR Assay Kits with the Qubit 2.0 Fluorometer (Invitrogen, USA).

PCR reactions were performed for each sample and consisted of 50–100 ng DNA, 1 × amplification buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP (Promega, Johannesburg, South Africa), 0.4 µM of each primer and 1 U Supertherm *Taq* polymerase (Southern

Table 1 PCR primers used in this study

Marker	Primer name	Sequence (5′–3′)	References
Cyt *b* (354 bp)	L14841-F	CCAACATCTCAGCATGATGAAA	Kocher et al. (1989)
	H15149-R	CCCCTCAGAATGATATTTGTCCTCA	Kocher et al. (1989)
12S (375 bp)	Mam12S-375-F	CCACCGCGGTCATACGATT	This study
	Mam12S-375-R	GATGGCGGTATATAGACTG	This study
GHR (275 bp)	MamGHR-275-F	GGAGACTGATTTCAATGCCA	This study
	MamGHR-275-R	AAAAGTCGATGTTTGCCAGT	This study

Cross Biotechnology, Cape Town, South Africa). A negative control, using ddH_20 instead of DNA, was run with each batch of samples, and PCR products were only retained for samples if the negative control was clear (free of contamination). The cycling parameters for the PCR involved an initial denaturation step of 4 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at the optimal annealing temperature for each marker (cyt *b*: 54 °C; 12S and GHR: 60 °C), and 20 s at 72 °C, and a final extension of 30 min at 72 °C. The quantity and quality of PCR products were assessed using gel electrophoresis and QubitTM dsDNA HS and BR Assay Kits with the Qubit 2.0 Fluorometer (Invitrogen, USA).

DNA sequencing

Cytochrome *b* PCR amplicons obtained from tissue samples were bi-directionally sequenced for species identification using a BigDye Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automated sequencer (ABI 3500xL Genetic Analyser, Applied Biosystems). PCR amplicons obtained from eDNA samples were sequenced by NGS amplicon sequencing using 600 base chemistry on the Ion Torrent S5 platform at the Central Analytical Facility (CAF) at Stellenbosch University.

For each region, or sequencing library, amplicons from all samples were pooled separately for each barcode marker. Purification of the pooled PCR products was performed using the Agencourt AMPure XP protocol (Beckman Coulter). This was followed by equimolar pooling of the three markers for each library using a QubitTM dsDNA HS Assay Kit with the Qubit 2.0 Fluorometer (Invitrogen, USA) to quantify DNA concentrations. The six pooled regional samples (libraries) were subsequently sent to CAF for library preparation and sequencing on a single sequencing chip. Ideally, negative controls would have been sequenced to provide an idea of background contamination, however the pooling strategy employed in this experiment prevented this. We decided instead to apply a stringent quality and read length filter to remove any contamination artefacts. Since we were more interested in the predominant mammalian species present (which we expected to be golden moles, as samples were taken from golden mole burrows), we were not too concerned about potentially failing to detect other rare species by over-filtering the data.

Bioinformatics

The raw sequence data were quality filtered in Geneious Prime v. 2021.2 (Biomatters Ltd.), using the BBduk plugin, with quality filter set to 30 to remove poor quality reads and size filter set to remove reads shorter than 100 bp. The de novo assembler was used to cluster reads into contigs, or operational taxonomic units (OTUs) using custom sensitivity settings, and the contigs were arranged according to depth of coverage, up to 1000 contigs per regional sample. An initial BLAST search, using the blastn function in Geneious Prime, was used to capture all golden mole OTUs (contigs) for each eDNA sample, for each of the three barcode markers. These data were exported as hit tables to .tsv files, which were subsequently filtered to remove bacterial sequences and reflect only hits with $> 90\%$ sequence identity.

Phylogenetic and phylogeographic analyses

All available golden mole reference sequences were downloaded from GenBank and aligned, first with all Sanger-sequences from sampled individuals, and then with all captured contigs from the six eDNA samples. Alignments were built separately for each barcode marker using MEGA X v.11.0.11 (Tamura et al. 2021). MEGA software was further used for model selection and subsequent phylogenetic analysis. Maximum Likelihood analysis was conducted for each marker, using the optimal BIC (Bayesian Information Criterion) model, BioNJ/NJ starting tree and 1000 bootstrap iterations. To account for missing data, we used partial deletion with a threshold of 50%, thus separate analyses were conducted for the Sanger sequenced samples and the eDNA contigs, since shorter reads among the eDNA contigs could potentially reduce the overall resolution of the tree. For the Sanger sequenced samples, all available golden mole reference sequences were included in the analyses, so as to provide context regarding each marker's taxonomic resolution within the family Chrysochloridae.

Statistical parsimony (TCS) haplotype networks were constructed using PopArt (Clement et al. 2000; Leigh and Bryant 2015). Cytochrome *b* sequence alignments were grouped into four partitions to prevent data loss resulting from missing data in a few samples/contigs. Partition A represents the full 354 bp sequence: Partition B: nt164-329 (165 bp); Partition C: nt222-331 (109 bp) and Partition D: nt217-354 (137 bp). Although there is overlap in nucleotide position between the partitions, each contig was only assigned to one of the four partitions, so that the same contig would not be reflected multiple times across the four partitions, in order to facilitate haplotype counting.

Results

Bioinformatics and preliminary species identification

The raw eDNA sequence data contained an average of 473,294 reads per regional sample. The BBduk function removed on average 279,759 reads (59%) and retained on average 193,535 reads (41%), on the basis of read length and quality (Table S1). This is a large reduction in the data, but we preferred to filter thoroughly to minimize persistence of putative sequencing errors, even at the risk of data loss (see Materials and Methods–DNA Sequencing for the rationale behind this).

De novo assembly produced contigs with coverage ranging from 60,599× to 157,600× in contig 1 (mean of 121,419× across all 6 samples) and mean coverage of 85× in contig 100 and 8× in contig 1000. We provide a comprehensive list of hits from the BLAST searches conducted in Geneious (Table S2; note that contig numbers do not correspond to those in Fig. 3 and 4, as the BLAST searches were re-run after analyses were conducted, in order to accommodate newly available GenBank reference sequences). These data should be interpreted with caution, due to the possibility of contaminants, and incorrect BLAST hits, owing to the paucity of reference sequences for some taxa.

Initial BLAST searches detected all four golden mole species thought to occur on the west coast among each of the six regions analysed. In addition, some contigs showed highest similarity to *Amblysomus* golden moles, but since these represent species from previous projects in our lab, with distribution ranges well outside our study area, it is possible that these may represent contaminant sequences. This hypothesis is supported by our finding

of Bryde's whale (*Balaenoptera brydei*; Table S2: L2 and L4), riverine rabbit (*Bunolagus monticularis*; L2, L4) mole-rats (*Cryptomys hottentotus*; L2, L3, L4, L5), and eland (*Tragelaphus oryx*; L1 and L4) sequences in our data, also species from other projects concurrently running in our lab. Additional species that were detected with high confidence in our data include human, dog, chicken, Lomi's blind legless skink (*Typhlosaurus lomii*; L4), wild turkey (*Meleagris gallopavo*; L5) and yellow-spotted rock hyrax (*Heterohyrax brucei*; L6).

BLAST searches for cyt *b* sequences generated for the four golden mole specimens from Lambert's Bay, Papkuilsfontein, Garies and Port Nolloth showed highest similarity to *Eremitalpa granti* (98, 51% sequence identity), *Chrysochloris asiatica* (97,49%), *E. granti* (90,39%) and *Cryptochloris wintoni* (98,88%), respectively.

Maximum likelihood phylogenetic analysis

Maximum likelihood analysis of cyt *b* grouped both the Lambert′s Bay sample and the Garies sample with *Eremitalpa granti* (Fig. 2a, blue shading); the Lambert's Bay sample grouped closely with *E. granti granti* (95% bootstrap support) and the Garies sample as sister to these (with weak bootstrap support), to the exclusion of the only other *E. granti* subspecies, *E. granti namibensis*. The Papkuilsfontein sample grouped with *Chrysochloris asiatica* (Fig. 2a, yellow shading), and yet appears to be somewhat divergent from reference sequences (genetic distance to nearest *C. asiatica* AJ428944.1 = 0.0302). The sample from Port Nolloth groups with *Cryptochloris wintoni*, sister to *C. zyli* (Fig. 2a, green shading).

Analysis of the 12S marker corroborated the placement of the samples from Garies with stronger support (99%) for the relationships within *E. granti* (Fig. 2b, blue shading). The placement of the Port Nolloth sample was also corroborated as sister to *C. zyli* in the

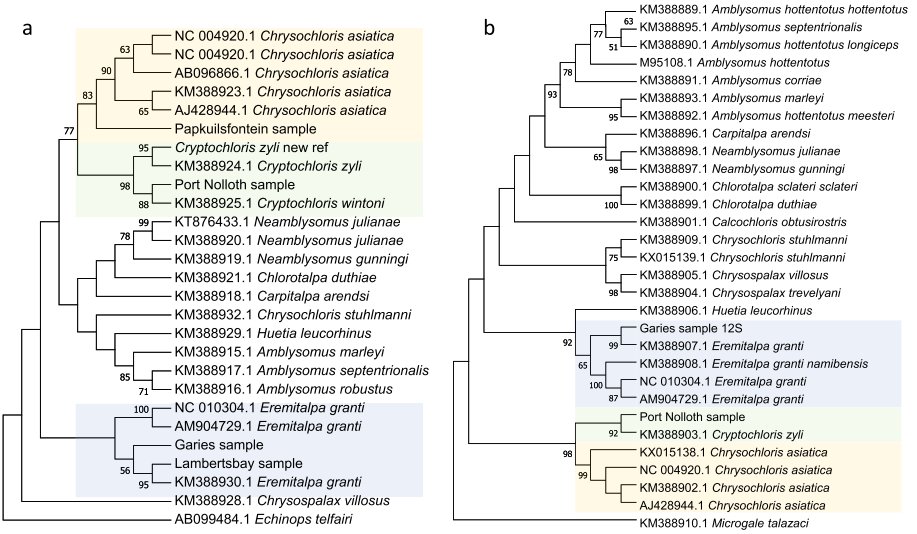


Fig. 2 Maximum Likelihood bootstrap consensus trees of sampled specimens as inferred by the Tamura-Nei model for cyt *b* (**a**) and the General Time Reversible model for 12S (**b**). Bootstrap values above 50% are shown at nodes. West coast taxa are indicated by coloured shading: *Chrysochloris asiatica*: yellow; *Eremitalpa granti*: blue; *Cryptochloris* spp. (*C. wintoni* and *C. zyli*): green

absence of a reference sequence for *C. wintoni* (Fig. 2b, blue shading). The other two samples—Lambert's Bay and Papkuilsfontein—were not sequenced for 12S, as their species identity had already been confidently established through cyt *b* analysis.

In the eDNA, *Eremitalpa granti* was detected in regions L2 and L4 (cyt *b* and 12S; Fig. S1a and b) and in L3, L5 and L6 (cyt *b* only; Fig. S1a) and *Cryptochloris wintoni* in regions L1 through L6 (cyt b; Fig. S1a) and L2 through L4 (12S; Fig. S1b). *Chrysochloris asiatica* was detected in L2 through L4 (cyt *b*; Fig. S1a), but was not detected with 12S (see *Discussion* for comparison of relative performance of markers). Resolution at the GHR marker was poor, and many samples failed to provide golden mole sequences at all, partly due to poor amplification success. Only regions L1, L4, L5 and L6 contained golden mole sequences, but species identification for these was problematic due to weak marker resolution. Thus, this marker was omitted from further analyses.

Haplotype network phylogeographic analysis

TCS network analysis revealed that the cyt *b* barcode detected one *E. granti granti* haplotype in Region L1 (Fig. 3b), three in Region L2 (Fig. 3a), three in Region L3 (Fig. 3a), two in Region L4 (Fig. 3c), four in Region L5 (Fig. 3a) and two in Region L6 (Fig. 3b). Thus *E. granti granti* was detected at each sampling site, and represents the most frequently detected taxon in our cyt *b* dataset (Fig. 5). Furthermore, the Garies specimen is grouped within *Eremitalpa* but is clearly distinct from both *E. g. granti* and *E. g. namibensis* (Fig. 3a). *Chysochloris asiatica* was not detected in Region L1, but two haplotypes were detected in Region L2 (Fig. 3a), one in Region L3 (Fig. 3a) and two in Region L4 (Fig. 3c).

The only available reference *Cryptochloris* sequences (*C. zyli* KM388924.1 and *C. wintoni* KM388925.1) were included in the analysis, but the *C. wintoni* reference sequence contains missing data, and could therefore only be included in partition B (Fig. 3b). However, since the specimen from Port Nolloth was already confidently assigned to *C. wintoni* (98.88% sequence similarity as mentioned above), we treated this as a *C. wintoni* reference across the other partitions. A single *Cryptochloris* haplotype was found in Region L1, (Fig. 3b), three haplotypes in Region L2 (Fig. 3a–c), three in Region L3 (Fig. 3a, d), three in Region L4 (Fig. 3b, c), five in Region L5 (Fig. 3a–d) and three in Region L6 (Fig. 3a, c, d). Thus, *C. wintoni* was detected at each of the six sampling sites (Fig. 5). However, partition D did not provide sufficient resolution to distinguish *C. wintoni* from *C. zyli,* therefore this particular haplotype, detected in regions L3, L5 and L6, remains unresolved and is denoted as "*Cryptochloris* sp." (Fig. 5).

The 12S barcode detected one *E. granti granti* haplotype in Region L2 and three in Region L4 (Fig. 4), corroborating cyt *b* detection of this taxon at Kleinsee and Port Nolloth (Fig. 5). The 12S marker did not detect *Chrysochloris asiatica* in any of the eDNA samples. *Cryptochloris* was the most frequently detected taxon in this dataset, with one haplotype detected in Region L2, three in Region L3, and three in Region L4, again corroborating cyt *b* findings. A further three haplotypes were detected in Regions L2 and L4, which were grouped within a *Chrysochloris-Cryptochloris* clade in ML analysis (66% bootstrap support; Fig. S1b), but which appear to be divergent from both taxonomic groups, and could not be confidently assigned to either (Fig. 4).

The GHR marker detected only a single golden mole haplotype in the eDNA samples, in Regions L1, L4, L5 and L6. This haplotype is shared with *C. asiatica* references and the Papkuilsfontein sample. However, resolution at this marker was poor, therefore, taxonomic designations could not be assigned on the basis of this marker.

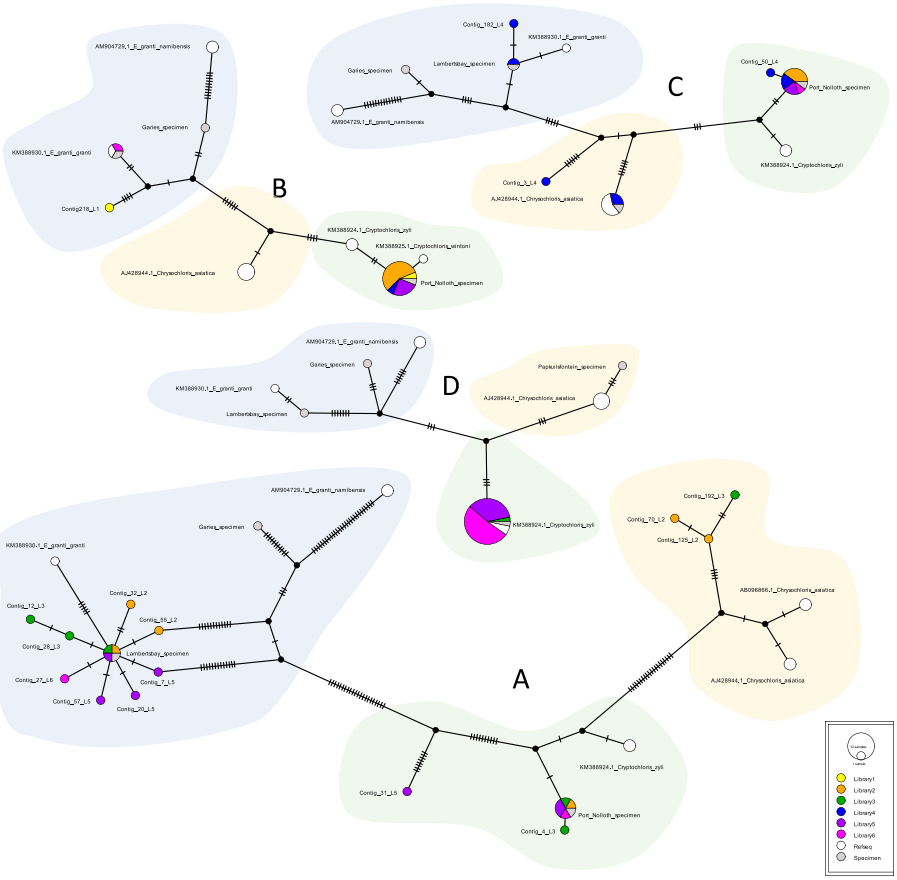


Fig. 3 TCS haplotype networks representing the evolutionary relationships among the 47 golden mole haplotypes detected across four partitions (**A**–**D**) of our cyt *b* dataset. The dataset comprises eDNA samples (contigs from six sampling regions along the west coast, tissue samples from live specimens (denoted as locality_ "sample") and GenBank reference sequences. Circle sizes reflect the number of samples sharing the same haplotype; mutational steps between haplotypes are indicated by cross-hatching, and missing haplotypes are denoted by black dots. Colours correspond to eDNA sampling regions as presented in Fig. 1, with tissue samples shown in grey and reference sequences in white. Background shading roughly denotes broader taxonomic groups: *Chrysochloris asiatica*: yellow; *Eremitalpa granti*: blue; *Cryptochloris* spp. (*C. wintoni* and *C. zyli*): green

Discussion

We investigated the use of DNA barcoding from environmental DNA (eDNA) collected from soil in detecting and investigating the distributions of rare/elusive species. We used tissue samples from trapped and opportunistically obtained specimens, along with GenBank sequences as references for taxonomic designation in our study. PCR amplicons for three barcode markers from soil eDNA samples collected at various sampling sites along the west coast of southern Africa were pooled into 6 sequencing libraries, or

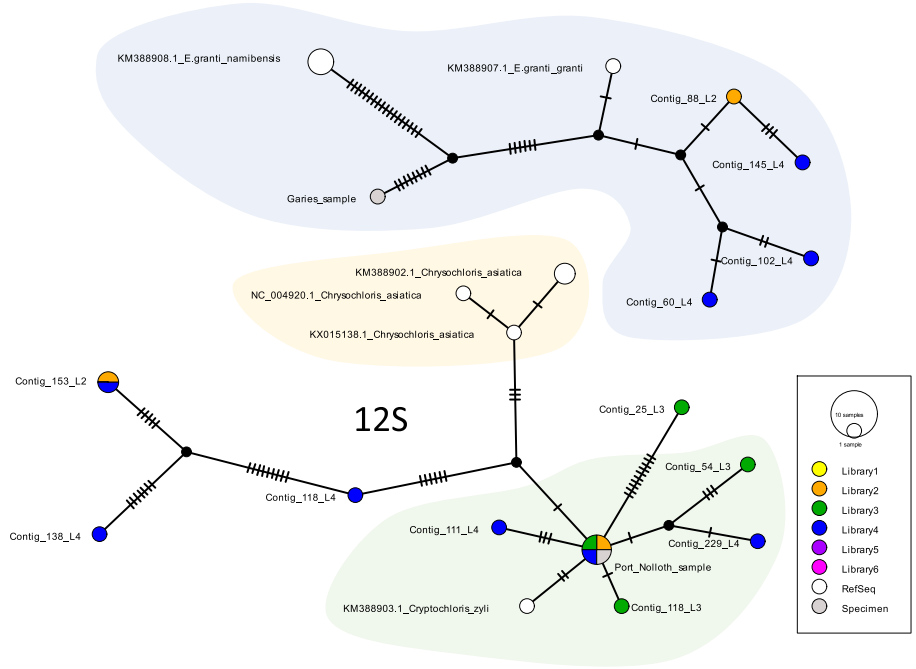


Fig. 4 TCS haplotype networks representing the evolutionary relationships among the 20 golden mole 12S haplotypes detected in our dataset. The dataset comprises eDNA samples (contigs from six sampling regions along the west coast, tissue samples from live specimens (denoted as locality_ "sample") and GenBank reference sequences. Circle sizes reflect the number of samples sharing the same haplotype; mutational steps between haplotypes are indicated by cross-hatching, and missing haplotypes are denoted by black dots. Colours correspond to eDNA sampling regions as presented in Fig. 1, with tissue samples shown in grey and reference sequences in white. Background shading roughly denotes broader taxonomic groups: *Chrysochloris asiatica*: yellow; *Eremitalpa granti*): blue; *Cryptochloris* spp. (*C. wintoni* and *C. zyli*): green

"regional samples" (see Methods – *Sample Collection* for details). Pooling of samples is common practice in eDNA studies, and generally facilitates reduced sequencing costs, by reducing the number of unique identifiers, which are required for each sample. Pooling may be achieved through direct mixing of soil samples (Taberlet et al. 2012), or pooling of PCR amplicons (Andersen et al. 2012; Leempoel et al. 2020). In our study we used the latter approach, involving separate PCR reactions for each sample and each barcode marker, which may be more labour intensive, but allows equimolar pooling of the various barcode amplicons, resulting in relatively even abundance in the sequencing reaction. It is important to note that due to the pooling of samples from various different sites into a single regional sample, the occurrence of multiple species in a single sample does not necessary indicate sympatry of these species, since a single species may have been detected at the first site, another at a second site and yet another at a third site, and when pooled into a single sample, we are not able to determine which specific sampling site each species was detected in. This sampling design was aimed at maximising detection of all golden mole species across the west coast, while testing the effectiveness of the eDNA sampling technique, and broadly refining the distributions of the various taxa.

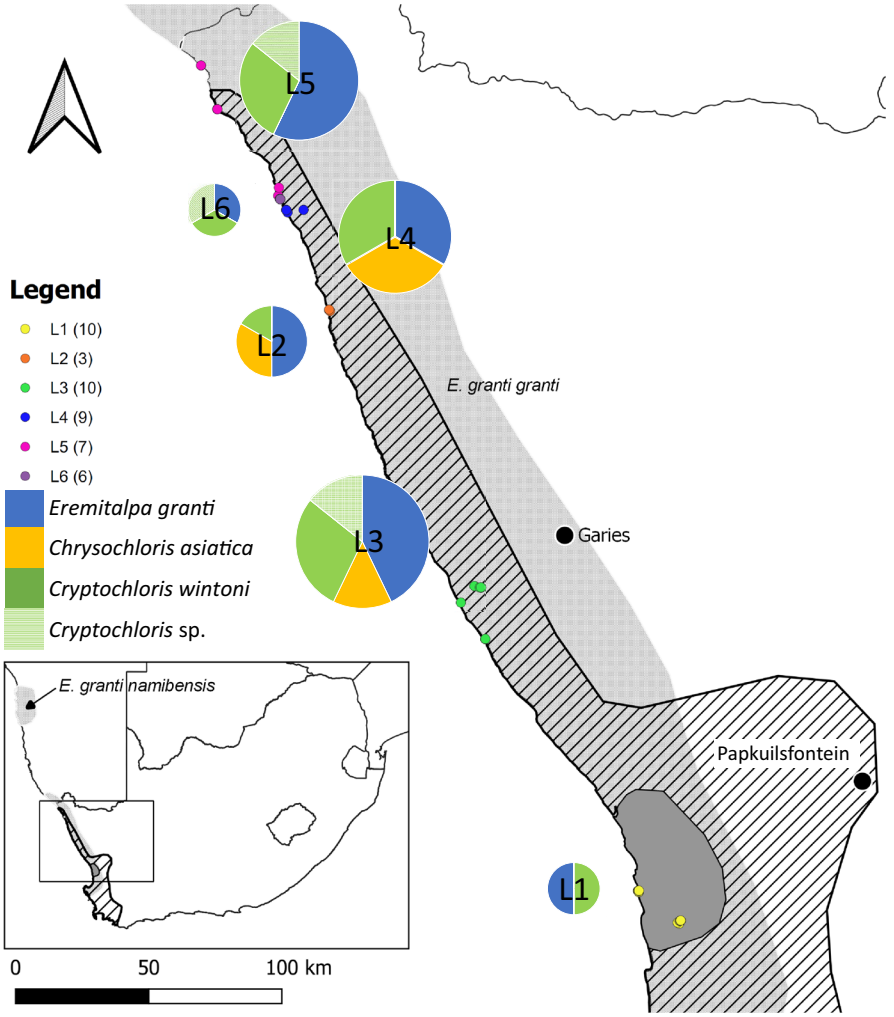


Fig. 5 Map of the west coast of southern Africa, depicting the golden mole species detected at each of six sampling regions (L1-6). Pie chart sizes reflect the number of cyt *b* haplotypes detected in each regional sample, and coloured wedges reflect the proportion of haplotypes representing each of the four species. Since some haplotypes could not be confidently assigned to either *C. zyli* or *C. wintoni*, the fourth species category is denoted as "*Cryptochloris* sp."

Given the limited availability of reference sequences for golden moles, we targeted three genes (cyt *b*, 12S and GHR) as barcode markers for our study. As expected, the two mitochondrial markers far outperformed the nuclear intron in terms of both amplification success and taxonomic resolution. This is not surprising, given the relative abundance of mtDNA in animal cells, and consequently in the environment (Rees et al. 2014), compared to nuclear DNA. We were able to detect golden mole GHR sequences in Regions L1, L4, L5 and L6, but species assignment was problematic due to weak marker resolution. Thus,

we were able to demonstrate successful amplification of mammalian nuclear eDNA from soil in our study, but the nuclear intron marker did not provide sufficient taxonomic resolution. Despite equimolar pooling of amplicons, we observed a significant bias in representation of mtDNA markers, and therefore in future studies it will be advisable to refrain from pooling nuclear and mtDNA fragments together for amplicon sequencing. Furthermore, cyt *b* outperformed 12S in the detection of golden mole species; all four species were detected in our data, whereas 12S failed to detect *Chrysochloris asiatica* in any of the samples. However, 12S provided good resolution when amplification was successful; we detected at least six *Cryptochloris* 12S haplotypes in our data, compared to only three *Cryptochloris* cyt *b* haplotypes. In contrast we detected at least nine *Eremitalpa granti* cyt *b* haplotypes, compared to only four 12S haplotypes. Taken together, both mtDNA markers performed comparatively well in terms of taxonomic resolution, and the failure of 12S to detect *C. asiatica* in our data may be explained by primer specificity and poor amplification success in this species.

Due to high mtDNA sequence similarity between *C. wintoni* and *C. zyli*, in some cases our relatively short barcode markers failed to distinguish between these sister species. Thus, we cannot confirm whether or not *C. zyli* was detected in our dataset. Unresolved *Cryptochloris* sequences were detected in Regions L3, L5 and L6, thus *C. zyli* may well be extant at these sites (Groenriviermond, Port Nolloth and Visagiesfontein). It is plausible that the two *Cryptochloris* species in fact represent a single species, however more data (additional samples and more sequence data) will be required to test this hypothesis. Limited evidence currently supports their allocation to separate species (Meester 1974; Bronner and Asher 2016b).

Our data have conclusively revealed the presence of the elusive *Cryptochloris wintoni* (IUCN Red List Critically Endangered; Possibly Extinct) on the west coast of southern Africa. Moreover, our data suggest that this species may be widespread in the area, ranging from Lambert's Bay in the south to Visagiesfontein (beyond Port Nolloth) in the north; we detected *C. wintoni* mtDNA haplotypes at each of our six sampling sites (Fig. 5). However, this cannot be taken to mean that the species is abundant across this distribution. On the contrary, we found relatively few cyt *b* haplotypes; no more than two in any given partition (Fig. 3). Generally, this was comparable to the one or two haplotypes detected for *C. asiatica*, but fewer than in *E. granti* (up to 9 haplotypes; Fig. 3). Although 12S detected six *Cryptochloris* haplotypes across the six regional samples, relative to only four for *E. granti* and none for *C. asiatica*, resolution at this marker was insufficient to distinguish *C. wintoni* from *C. zyli*, and these six haplotypes may represent both *Cryptochloris* species (Fig. 4).

The 12S barcode failed to detect *Chrysochloris asiatica*, however cyt *b* detected three to five *C. asiatica* haplotypes in Regions L2 through L4, i.e. Groenriviermond, Kleinsee and Port Nolloth (Fig. 4, Fig. 5), and additionally at Papkuilsfontein. These sites all fall within the known range of the species, however, we were surprised that *C. asiatica* was not detected at Lambert's Bay (L1), since this species is thought to be increasingly abundant further south towards Cape Town (Bronner and Asher 2016a; Taylor et al. 2018). Our relatively low detection of this species may be due to a sampling bias favouring the coastal dune habitats, which are more characteristic of *Eremitalpa* and *Cryptochloris* species. Cape golden moles are known to inhabit a wide range of habitat types, depending on soil friability and availability of invertebrate prey (Bronner and Asher 2016a; Taylor et al. 2018), and we suspect that the apparent sympatry of the four golden mole species along the west coast may be explained by subtle ecological niche preferences.

Eremitalpa granti was detected at all six sites, and additionally at Garies, all within the species' known distribution (Fig. 5). Specifically, 9–13 *E. g. granti* haplotypes were

detected in the eDNA samples across the first three cyt *b* partitions, and four from the 12S barcode. *Eremitalpa g. namibensis* was not detected in our eDNA samples, however TCS analysis for both cyt *b* and 12S placed the Garies sample within *E. granti*, but distinct from both *E. granti* subspecies. This may indicate that the sample from Garies represents a third cryptic subspecies. It is also interesting to note the high level of sequence divergence between the two *E. granti* subspecies, relative to the level of divergence between the two *Cryptochloris* species, indicating that *E. g. granti* and *E. g. namibensis* likely represent distinct species, in which case the Garies sample could represent a third cryptic species. Once again, more data (additional samples representing a broader geographic range and/or more sequence data) will be required to test this hypothesis, and to establish the taxonomic placement of the Garies sample.

Conclusion

Our study demonstrates the use of DNA barcoding from environmental DNA (eDNA) collected from soil (1) in detecting rare/elusive species and (2) as a tool to investigate and potentially monitor their distributions. Through extensive eDNA sampling along the west coast from Lambert's Bay northwards to Port Nolloth, we have not only rediscovered the "lost" De Winton's golden mole, moreover we demonstrate that this species may be widespread along the west coast, albeit in low abundance. Given that this species is listed as critically endangered (IUCN 2022) and occurs in an area under high threat of habitat transformation by alluvial diamond mining (Bronner and Asher 2016b), it will be important to gather additional data to (1) resolve the potential taxonomic uncertainty within *Cryptochloris,* (2) further refine the distribution and investigate abundance throughout the distribution, (3) determine presence vs. absence in protected areas, as well as areas under particularly high threat, and (4) assess the viability of sub-populations, and potential for connectivity between them.

The sampling design for the current study was aimed at maximising detection of all golden mole species across the west coast, while testing the effectiveness of the eDNA sampling technique, and broadly refining the distributions of the various taxa. We detected *Eremitalpa granti* and *Cryptochloris wintoni* in all six regional samples, each consisting of around 10 pooled samples from 2 to 5 proximate sampling sites. Unresolved *Cryptochloris*, potentially including *C. zyli* was detected in Regions L3, L5 and L6 (Kleinsee, Port Nolloth and Visagiesfontein), and *Chrysochloris asiatica* was detected in Regions L2 through L4 (Groenriviermond, Kleinsee and Port Nolloth). Thus, we were able to broadly investigate the distributions of the four golden mole species on the west coast, however, fine-scale sampling, targeting specific habitats or ecological niches, along with unpooled amplicon sequencing will be required to further refine the distributions of these taxa. In the meantime, regional and species-specific conservation action is both critical and urgent, in order to protect not only the threatened golden mole populations this study has revealed, but also the highly threatened west coast dune ecosystems they inhabit.

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Author contributions SM and CT designed and conceptualized the project. SM, PB and CT acquired the funding. SM and EM obtained permits and ethical clearance and JPLR acquired landowner consent. CT planned and led the field work and SM, EM, JPLR, IL and CT collected the data. SM analyzed the data and wrote the first draft of the manuscript. PB and IL commented on previous versions of the manuscript and provided advice and assistance throughout the project. All authors read and approved the final manuscript.

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Data availability The data generated and analysed in the current study are available on GenBank (accession numbers: OP279338-OP279343). Additional data generated but not analysed in the current study may be found in the supplementary information, and/or upon reasonable request from the authors.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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