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Sample design in biodiversity studies matters: a fine-scale study of Lawrence's velvet worm, *Peripatopsis lawrencei* (Onychophora: Peripatopsidae), reveals hidden diversity

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ABSTRACT

A fine-scale phylogenetic and phylogeographic analysis of *Peripatopsis lawrencei* s.l. was conducted with both mitochondrial and nuclear DNA sequence data, using both external morphology and scanning electron microscopy of taxonomically important characters. A total of 119 sequences were used for the mitochondrial cytochrome *c* oxidase subunit I (*COI*) whereas a single representative specimen from each locality was sequenced for the nuclear *18S* rRNA locus. Phylogenetic analyses were conducted on the total *COI* data set and the combined *COI* + *18S* rRNA data set using a Bayesian analysis and maximum likelihood analyses. For the combined DNA sequence data set, a divergence time estimation was further undertaken in BEAST and specimens placed in a phylogenetic framework including all the described *Peripatopsis* species from South Africa. In addition, a phylogeographic study was conducted exclusively on *P. lawrencei* s.s. (clade A) using an analysis of molecular variance and haplotype network. Phylogenetic results indicated that, at the Oubos sample locality, two highly distinct genetic lineages were present (clades A and B), whereas a divergence time estimation suggests a Miocene cladogenesis of the novel Oubos lineage. Marked phylogeographic structure was observed for *P. lawrencei* s.s. (restricted to clade A) across the distribution range with limited maternal dispersal. Morphologically, the two sympatric lineages at Oubos A and B differed in leg pair number, ventral colour and dorsal scale rank counts, as evident from scanning electron microscopy. Our results support the recognition of a distinct species that occurs in sympatry with *P. lawrencei* s.s. The new species, *P. aereus* sp. nov. (clade B) is described and the implication for fine-scale taxonomic studies on saproxylic taxa is discussed.

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Introduction

Velvet worms are soft-bodied invertebrates, typically occurring in saproxylic environments within forested regions, living in leaf litter, inside bark or under stones or moss (Hamer *et al.* 1997). The vulnerability to desiccation encourages an affinity to these sheltered terrestrial habitats from warm tropical to cold temperate climatic regimes (Ruhberg 1985; Daniels pers. obs; Giribet pers. obs). The phylum Onychophora, sister group to Arthropoda (Edgecombe 2009), consists of two families: Peripatopsidae, Bouvier, 1905 and Peripatidae, Aoudouin & Milne-Edwards, 1832. The latter is circumtropically distributed and present in South-east Asia, India, the Neotropics into Mesoamerica and Gabon (Ruhberg 1985). Members of Peripatopsidae have a Gondwanan circumpolar distribution and occur in Australasia, Chile, New Zealand, South Africa and Eswatini (formerly Swaziland) (Ruhberg 1985; Hamer *et al.* 1997). Ancient climatic oscillations, coupled with tectonic uplift followed by habitat fragmentation are considered to be the main cladogenetic drivers (McDonald and Daniels 2012; Murienne *et al.* 2014; Giribet *et al.* 2018; Sato *et al.* 2018; Myburgh and Daniels 2022).

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Two genera within Peripatopsidae, *Opisthopatus* Purcell, 1899 and *Peripatopsis* Pocock, 1894 occur in South Africa (Hamer *et al.* 1997). Towards the end of the 20th century, *Opisthopatus* and *Peripatopsis* contained eight and three species respectively, delineated by poorly defined morphological characters (Hamer *et al.* 1997; Sherbon and Walker 2004; Ruhberg and Hamer 2005). However, the recent application of DNA sequence-based systematic research and scanning electron microscopy on *Opisthopatus* and *Peripatopsis* resulted in the description of 7 and 15 species respectively (McDonald *et al.* 2012; Daniels *et al.* 2013, 2016; Ruhberg and Daniels 2013; Barnes *et al.* 2020; Barnes and Daniels 2022; Grobler *et al.* 2023). Although we note the publication by Oliveira (2023), we critique observations regarding velvet worm taxonomic diversity in South Africa. Oliveira (2023) makes several spurious, scientifically incorrect observations, for example, restricting all species to type localities. Our DNA based evidence derived from widespread sampling demonstrates the monophyly of species, suggesting that, although some species are regionally widespread, other taxa are narrowly endemic, negating the perception that all species names should be restricted exclusively to type localities (McDonald *et al.* 2012; Daniels *et al.* 2013, 2016; Ruhberg and Daniels 2013; Barnes *et al.* 2020; Barnes and Daniels 2022; Grobler *et al.* 2023). The delineation of South African species is further corroborated by SEM and to a varying degree, gross morphological data. Furthermore, Oliveira's (2023) global list lacks understanding of biogeographic context and potential barriers or the absence of barriers to gene flow, and no formal species definition is applied. Consequently, we do not adopt the alpha-taxonomic diversity proposed by Oliveira (2023). Clearly additional research into the alpha taxonomy of the South African velvet worm is required.

The greatest diversity of *Peripatopsis* occurs in the Afrotemperate forests and fynbos regions of the Western Cape province along the south-eastern Cape coast and adjacent interior, extending into the Eastern Cape and KwaZulu–Natal provinces (Daniels *et al.* 2009). Velvet worms are habitat specialists (Barnes and Daniels 2019), requiring very specific microclimatic regimes for survival, i.e. saproxylic environments and leaf litter. The Afrotemperate forests are highly fragmented and distributed along the Cape Fold Mountain belt, confined to areas of high altitude with high rainfall, often nested in deep gorges and valleys. Large areas of Afrotemperate forest remain unsampled for velvet worms. Considering the limited dispersal capability of velvet worms, several undescribed species are likely to be present in poorly sampled forested areas and fynbos regions. In South Africa, few phylogeographic studies have been conducted on velvet worms. One study assessed the *P. capensis* species complex in the western and south-western Cape regions of the Western Cape Province (McDonald and Daniels 2012), resulting in the description of two novel species, *P. overbergiensis* McDonald, Ruhberg & Daniels, 2012 and *P. lawrencei* McDonald, Ruhberg & Daniels, 2012. Daniels *et al.* (2013) examined species boundaries in the *P. balfouri* (Sedgwick, 1855) species complex and described three novel lineages within the species complex. Similarly, a fine-scale study of the *P. moseleyi* (Wood-Mason, 1979) species complex resulted in the description of five novel species (Ruhberg and Daniels 2013). Furthermore a recent study of the *P. birgeri* Ruhberg & Daniels, 2013 species complex from KwaZulu–Natal resulted in the description of a novel lineage, *P. polychroma* Grobler, Myburgh, Barnes & Daniels, 2023 (Ruhberg and Daniels 2013; Grobler *et al.* 2023). A phylogeographic study of *Opisthopatus amaxhosa* Daniels, Dambire, Klaus & Sharma, 2016, a narrowly endemic velvet worm in the Eastern Cape by Barnes and Daniels (2019) also resulted in the discovery of a novel species, *O. baziya*, Barnes & Daniels, 2022 (Barnes and Daniels 2022). These results reiterate the need for fine-scale sampling to document the alpha-taxonomic diversity of velvet worms and other saproxylic taxa including, for example, earthworms, snails, spring tails, centipedes, harvestmen and millipedes.

The first DNA sequence-based phylogeny of *Peripatopsis* by Daniels *et al.* (2009) demonstrated that at Riviersonderend, two highly divergent sympatric lineages (A & B) were present in the *P. capensis* (Grube 1866) species complex. The study by Daniels *et al.* (2009) revealed three geographically discrete, morphologically diagnosable clades in the latter species complex that were subsequently described as *P. capensis sensu stricto* (s.s.), sister group to *P. lawrencei* and *P. overbergiensis* (McDonald *et al.* 2012). The main lineage (A), present along the Riviersonderend Mountains, now known as *P. lawrencei*, also occurs in Caledon, Fernkloof, Gaansbaai, Greyton and Oubos. However, the identity of the second Riviersonderend lineage (B) is unknown, likely signifying a narrowly distributed endemic species awaiting formal description. This novel species forms the basis for the present study.

Underestimated or cryptic diversity often arises from the application of outdated taxonomic methods such as using gross morphology as the only diagnostic tool. This has been frequently demonstrated for velvet worms (Daniels *et al.* 2009; Sato *et al.* 2018; Grobler *et al.* 2023). Even when crypticity has been identified, effective species diagnosis can become problematic because of incongruence between taxonomic methods (Daniels *et al.* 2016). The most effective means of revising taxonomic lineages and cryptic species complexes involves utilising a combination of gross morphology, scanning electron microscopy (SEM) and genetic characteristics (Dayrat 2005). The fragmented nature of saproxylic environments has led to similar microclimatic pressures, promoting cryptic speciation and convergent evolution (Daniels *et al.* 2016; Barnes and Daniels 2019). Saproxylic groups show high cryptic differentiation but the alpha taxonomy is poor, further highlighting the importance of molecular systematic research to document diversity (Barnes and Daniels 2019).

During our study, we undertook fine-scale sampling along the Riviersonderend Mountains to identify the novel

species and better understand the evolutionary placement in the wider *Peripatopsis* phylogeny. The cryptic nature of these velvet worms indicates that fine-scale sampling is necessary to understand population genetic structure. The first objective was to identify and describe the novel lineage. We hypothesised that the novel lineage (B) would be morphologically very similar to *P. lawrencei* s.s. (A) but could be distinguished based on gross morphological characteristics. Additionally, SEM characteristics of the dorsal and ventral papillae would also be diagnostic. For identification, the specimens of the novel lineage were subjected to DNA sequencing, and placed into a dated phylogeny to understand the evolutionary placement and divergence relative to the wider *Peripatopsis*. In the second part of this study, we focused exclusively on *Peripatopsis lawrencei* s.s., with the objective being to examine the population genetic structure and colonisation history. Using a fine-scale phylogeographic study, we hypothesised that we would observe marked phylogeographic differentiation across the distribution range of the species that is characterised by limited dispersal based on DNA sequence data.

Materials and methods

Sample collection

A total of 69 new *Peripatopsis lawrencei sensu lato* (s.l.). specimens was collected from the known distribution range of the species in the Western Cape province, South Africa (Fig. 1). These specimens were combined with data collected from two previous studies (Daniels *et al.* 2009; McDonald and Daniels 2012), yielding a total of 119 specimens (Table 1). *Peripatopsis lawrencei* s.s. comprised 116 of the samples, whereas 3 samples were suspected to be the unknown sympatric lineage at Oubos (Riviersonderend Mountains). A handheld GPS was used to record the locality information and a permit (CN44-87-22079) was obtained from CapeNature for velvet worm sampling. Specimens were collected in Afromontane forest patches under decaying logs, leaf litter and stones (McDonald *et al.* 2012). These specimens were preserved directly in absolute ethanol and stored in a refrigerator at 4°C once returned to the laboratory.

DNA sequencing

A tissue biopsy was taken from each specimen (2–3 mm) and a Macherey–Nagel DNA extraction kit was used, following the manufacturer's protocol. DNA extractions were kept at 4°C until required for use in polymerase chain reaction (PCR). Two loci were targeted for amplification, namely mitochondrial (mtDNA) cytochrome *c* oxidase subunit I (*COI*) and nuclear *18S* rRNA subunit (*18S*). These two loci have been extensively used in velvet worm systematics studies of *Peripatopsis*, allowing us to combine our data with DNA sequences generated during earlier studies (Daniels *et al.* 2009, 2013, 2016; McDonald and Daniels 2012; Barnes *et al.* 2020; Grobler *et al.* 2023). The *COI* and *18S* rRNA primer pairs (specifically 5F and 7R for the latter locus) used in the amplification of the two fragments were obtained from Folmer *et al.* (1994) and Giribet *et al.* (1996) respectively. All newly collected specimens were sequenced for *COI*. Only a single representative of each locality was sequenced for the *18S* locus, except for the

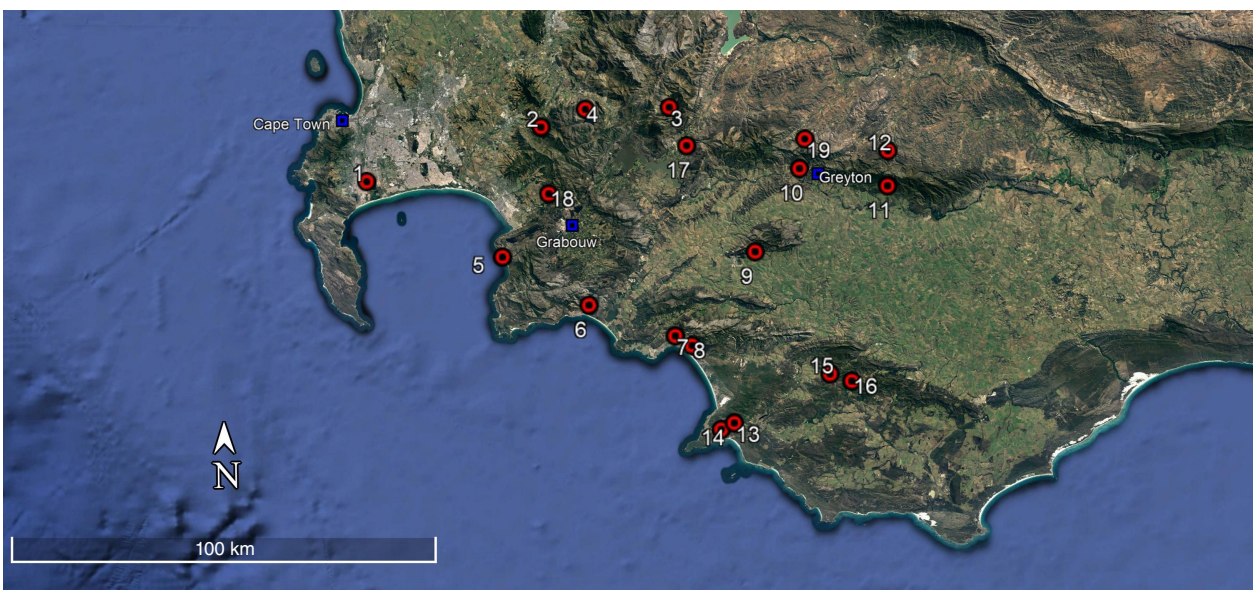

Fig. 1. Map showing all sampling localities for *Peripatopsis lawrencei* s.l., Western Cape Province, South Africa. The red markers indicate sampled localities with numbers corresponding to Table 1.

Table 1. List of the 19 sample localities at which *Peripatopsis lawrencei* s.l. was collected in the Western Cape, South Africa.

Number	Locality	Latitude (S)	Longitude (E)	Daniels *et al.* (2009)	McDonald and Daniels (2012)	Present study	Total
1	Rondevlei Nature Reserve (NR)	34°03.375′S	18°29.597′E		1		1
2	Jonkershoek NR	33°58.957′S	18°56.246′E		4		4
3	Franschhoek	33°54.292′S	19°16.560′E	4			4
4	High Noon	33°54.251′S	19°03.044′E	4			4
5	Dappat se Gat	34°13.258′S	18°50.241′E		5		5
6	Kogelberg Biosphere NR	34°05.764′S	18°50.493′E	1	1	3	5
7	Fernkloof NR	34°23.613′S	19°16.564′E	1	4	6	11
8	Vogelgat NR	34°24.021′S	19°19.330′E			10	10
9	Caledon	34°13.442′S	19°25.739′E		1	11	12
10	Greyton	34°02.015′S	19°36.573′E	3			3
11	Oubos A and B	34°04.194′S	19°49.628′E		4	15/2	19/2
12	Riviersonderend A and B	34°00.480′S	19°49.480′E	1/1			1/1
13	Grootbos NR	34°34.270′S	19°25.369′E	5	4	2	11
14	Linden Hill	34°35.109′S	19°23.871′E			5	5
15	Sandies Glen	34°28.233′S	19°40.111′E			1	1
16	Napier	34°29.237′S	19°43.202′E		6	4	10
17	Villiersdorp	33°59.007′S	19°17.180′E			1	1
18	Grabouw	34°05.470′S	18°57.550′E			8	8
19	Jonaskop	33°58.170′S	19°30.230′E			1	1

The numbers (1–19) correspond to those in Fig. 1. A forward slash (/) implies the presence of the novel lineage (clade B). Data generated during the present study were combined with *COI* sequence data from two previous studies (Daniels *et al.* 2009; McDonald and Daniels 2012).

Oubos locality at which the two lineages (A and B) occurred in sympatry. A total of 14 new *18S* rRNA sequences was generated during our study and combined with three *18S* rRNA sequences generated by McDonald and Daniels (2012).

PCRs were conducted for a 25-μL reaction and the samples held at 15°C afterwards. The reactions both contained 14.9 μL of deionised water, 2.5 μL of 10× Mg^{2+} free buffer, 0.5 μL of a 10-mM dNTP solution and 0.5 μL of the primer sets at 10 mM, 0.1 units of JMR SuperTherm Taq polymerase and 1 μL of extracted DNA. The PCR was conducted on a geneAmp PCR System Thermocycler. PCR conditions for the *COI* locus were as follows: 94°C for 4 min, 94°C for 30 s, 42°C for 35 s and 72°C for 40 s for 36 cycles, with an extension of 72°C for 10 min. For the *18S* locus, PCR conditions were as follows: 94°C for 4 min, 94°C for 30 s, 48°C for 40 s and 72°C for 40 s for 34 cycles, with an extension of 72°C for 10 min. Following successful PCR amplification, PCR products were electrophoresed in a 1% agarose gel for 180 min, at 100 V and gel-purified using a Bio flux gel purification kit, following the manufacturer's instruction. An ABI 3730 machine was used to sequence the products at the DNA sequence facility of the University of Stellenbosch.

Phylogenetic analyses

Data collected from the sequences were utilised with the DNA sequences for *P. lawrencei* s.l. from Daniels *et al.* (2009) and McDonald and Daniels (2012). Forward and reverse strands were used to compute a consensus sequence and check for base pair ambiguity, whereas sequence alignment for both the *COI* and *18S* rRNA data was computed using ClustalX (ver. 2.0, see https://www.genome.jp/tools-bin/clustalw;Thompson *et al.* 1997). For *18S* rRNA, highly variable indel regions that could not be aligned with accuracy were excluded from the phylogenetic analyses using Gblocks (ver. 0.91b, see http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks; Talavera and Castresana 2007). Using jModelTest2 (ver. 2.2.10, see https://github.com/ddarriba/jmodeltest2; Posada 2008), the Akaike information criterion (AIC) (Akaike 1973) was utilised to determine the best-fit DNA substitution model on XSEDE (ver. 2.0, see https://www.xsede.org/) in CIPRES (see http://www.phylo.org/; Miller *et al.* 2010). Bayesian inference (BI) and maximum likelihood (ML) approaches were used to construct phylogenies. Analyses were conducted on CIPRES Science Gateway (Miller *et al.* 2010), with the Bayesian

analysis taking place in MrBayes (ver. 3.2.6, see https://github.com/NBISweden/MrBayes/; Ronquist *et al.* 2012) and the phylogeny for ML in RAxML (ver. 7.2.8 alpha, see https://github.com/stamatak/standard-RAxML; Stamatakis *et al.* 2008). For the part of the study focusing on *P. lawrencei* s.s., the ML analyses were conducted on the IQ-TREE web server (ver. 2.2.2.6, see http://iqtree.cibiv.univie.ac.at/; Trifinopoulos *et al.* 2016) and BI analyses through CIPRES, to create the tree topology for the *COI* data set. For the phylogenetic placement of the new lineage, a combined tree topology for *COI* and *18S* rRNA was reconstructed using the aforementioned procedures, and DNA sequences for both loci for all described *Peripatopsis* species were included to determine the phylogenetic placement of the Riviersonderend specimens (Daniels *et al.* 2009, 2013; Ruhberg and Daniels 2013; Barnes *et al.* 2020; Grobler *et al.* 2023). Uncorrected *COI* and *18S* rRNA *p*-distances were calculated in PAUP* (ver. 4.10, see http://phylosolutions.com/paup-test/; Swofford 2002) and compared to interspecific sequence values recorded among velvet worms.

Branch support values were calculated through an ultrafast Bootstrap analysis with 10 000 pseudo replicates and bootstrap (BS) values >75% were taken as support for the nodes using IQ-TREE. For Bayesian inference, burn-in was set to 20% and a posterior probability (PP) >0.95 was considered statistically well supported, estimated through percentage of time spent on node recovery (Barnes and Daniels 2022). With a Bayesian framework, a divergence time estimation was conducted for the *COI* and *18S* rRNA loci, using a probability model to describe molecular sequences of divergent lineages. The ages of the clades were subsequently estimated using the Markov Chain Monte Carlo that was run for 50 million generations, sampling the chain every 1000th generation. In total, 20% of generations were discarded as burn-in. Convergence was assessed visually by examining the performance of the chain, as in Zhang *et al.* (2013). A relaxed molecular clock was implemented, running the analysis through BEAST (ver. 2.7.6, see https://www.beast2.org/; Drummond and Rambaut 2007) and a multiple coalescent model was used (Heled and Drummond 2010). Mutation rates of 1.5–2.3% per million years were used for the *COI* locus and the mutation rate for the *18S* rRNA locus was estimated through the use of a non-informative ($1/x$) prior. The aforementioned use of mutation rates has been applied in several *Peripatopsis* divergence time estimations (Barnes *et al.* 2020; Myburgh and Daniels 2022; Grobler *et al.* 2023). Finally, for further support we conducted a Shimodaira–Hasegawa test (Shimodaira and Hasegawa 1999) to evaluate the likelihood of a constrained tree topology, where clades A and B are combined into a single clade, rather than being represented as two separate entities.

Phylogeographic analysis of *P. lawrencei* s.s.

A haplotype network was constructed through the use of TCS (ver. 1.21, see https://bioresearch.byu.edu/tcs/), with a 95% confidence interval (Clement *et al.* 2000) and population genetic structure was computed in ARLEQUIN (ver. 3.5.2.2, see http://cmpg.unibe.ch/software/arlequin35; Excoffier and Lischer 2010) using the *COI* data for *P. lawrencei* s.s. The sample localities were subjected to a hierarchical Analysis of Molecular Variance (AMOVA), and F_{ST} values were calculated, along with standard diversity indices including nucleotide diversity, number of haplotypes and haplotype diversity at each locality.

Morphology and scanning electron microscopy (SEM)

A Canon EOS camera was used to photograph the specimens, and gross morphological traits were examined using a Leica MZ 7.5 stereomicroscope, noting dorsal and ventral integument colour, number of leg pairs and the presence of a claw on the posterior leg pair. A digital calliper was used to measure total length of the ethanol-preserved specimen from the anterior-most point to the posterior side, and additional comparisons between *P. lawrencei* s.s. and the new lineage were undertaken using a Leica S9i digital stereo microscope at the South African Museum of Cape Town. The rarity of the novel lineage (clade B) poses limitations on the methods of examination. Only two individuals of the new lineage were available for examination and due to the destructive nature of the process, traditional scanning electron microscopy (SEM) was not possible. However, the South African Museum of Cape Town has an environmental SEM, Hitachi TM4000Plus that allows for the analysis of specimens without requiring critical point drying or sputter coating. This allowed us to preserve our limited specimens for later use as holotype and paratype. Through the use of this SEM technique, dorsal and ventral scale ranks for representatives of each of the two main clades were examined. Specimens were accessioned into the entomology collection of the South African Museum of Cape Town (SAM-ENW-C013473–SAM-ENW-C013487). The new species name was deposited in ZooBank (urn:lsid:zoobank.org:act:71276915-79C6-4759-9244-20BEF6303F58).

Results

Phylogenetic analysis of *COI* for *P. lawrencei* s.l.

We amplified and sequenced a 644 base-pair (bp) fragment of the *COI* locus from 69 *P. lawrencei* s.l. specimens, two of which were the suspected novel lineage. Novel sequences were deposited in GenBank (Accession numbers OR423143–OR423211). We combined *COI* data generated during our study with 50 *P. lawrencei* s.l. sequences, of which 1 is known to be the novel species, from two previous studies to yield a total of 119 sequences, 116 of *P. lawrencei* s.s. and 3 of the novel lineage (clade B). Using corrected AIC, we selected TIM1 + G as our DNA substitution model ($-\ln L = 2224.64$).

The base frequencies for the *COI* locus were as follows: A = 25.00%, C = 12.56%, G = 16.29% and T = 46.14%, whereas the rate matrix included *R*(*a*) [A–C] = 1.00, *R*(*b*) [A–G] = 19.17, *R*(*c*) [A–T] = 2.72, *R*(*d*) [C–G] = 2.72, *R*(*e*) [C–T] = 8.76 and *R*(*f*) [G–T] = 1.00. Gamma (G) shape distribution was 0.15. Both Bayesian inference and maximum likelihood analyses yielded near-identical tree topologies, hence only the Bayesian inference topology is shown (Fig. 2). The tree topology shows two highly divergent statistically well-supported monophyletic clades (A & B) (>75% bootstrap support, BS/>0.95 posterior probability, PP). Within *P. lawrencei* s.s. (clade A) (84% BS/<0.95 PP), the most basal lineage occurs at Rondevlei NR and Jonkershoek NR (77% BS/0.95 PP). A clade comprising Villiersdorp, Jonaskop, Greyton, Riviersonderend and Oubos A (haplocluster A2; Fig. 2, 3) is sister group to a clade found exclusively at Caledon (92% BS/<0.95 PP), likely reflecting a recent colonisation history between the Riviersonderend Mountains and Klein Swartberg at Caledon (Fig. 2). A coastal clade exists at Vogelgat and Fernkloof NRs (haplocluster A1; Fig. 2, 3) that is sister group to the groups found further south-east at Grootbos, Linden Hill, Sandies Glen and Napier (haplocluster A3; Fig. 2, 3). These groups are found to be sister group to a clade comprising Grabouw, Dappat se Gat, Franschhoek, High Noon and Kogelberg Biosphere NR (haplocluster A3; Fig. 2, 3). Clade B, the novel lineage, was exclusive to the Riviersonderend Mountains and sympatric with *P. lawrencei* s.s. at Oubos B.

The uncorrected *p*-distance for the *COI* locus between *P. lawrencei* s.s. (clade A) and clade B ranged from 9.13 to 10.62%. The uncorrected *p*-distance between *P. capensis* and *P. overbergiensis* was 6.58% (our two outgroups), and between *P. overbergiensis* and *P. lawrencei* s.s., this ranged from 5.93 to 7.49%. The uncorrected *p*-distance ranged from 10.53 to 10.73% between clade B and *P. overbergiensis*. The marked *p*-distance between the two clades (A and B) of *P. lawrencei* s.l. further corroborates the genetic distinction of clade B.

Phylogeographic analysis of *P. lawrencei* s.s. (clade A)

The TCS analyses of 116 *COI* sequences revealed 57 haplotypes, comprising 3 large unconnected haploclusters that were genetically and geographically discrete (Fig. 3). Haplocluster A1 was exclusive to a narrow coastal strip at Hermanus (Fernkloof and Vogelgat Nature Reserves), whereas haplocluster A2 was restricted to the Riviersonderend Mountains (Oubos A, Greyton, Villiersdorp, Jonaskop and Riviersonderend). Finally, haplocluster A3 was geographically more widespread from Rondevlei to Napier. Within the aforementioned three haploclusters, we observed evidence for maternal dispersal exclusively between Fernkloof NR and Vogelgat, separated by 18 km because these two localities shared one haplotype (Appendix A1). Overall no evidence of shared haplotypes existed among the remaining sample localities, indicating a lack of maternal dispersal based on the *COI* sequence data (Appendix A1). The lack of maternal dispersal is validated by the AMOVA results. The AMOVA demonstrated that 83.06% of the genetic variation was present among populations (Va = 6.32, d.f. = 18, s.s. = 693.91, $P < 0.01$), whereas 16.94% of the genetic variation was present within populations (Vb = 1.29, d.f. = 97, s.s. = 125.05, $P < 0.01$). Furthermore, pairwise and statistically significant F_{ST} values showed marked to moderate levels of genetic differentiation among conspecific populations (Table 2, Fig. 4). Non-significant results in Sandies Glen, Riviersonderend, Jonaskop, Villiersdorp and Rondevlei can likely be attributed to small sample sizes ($n = 1$) at these localities.

The number of samples, haplotypes, polymorphic sites and amounts of haplotype (h) and nucleotide diversity (π) are reported in Table 3 for all 19 localities. The number of haplotypes ranged from one to seven, with most occurring between one and three. The highest haplotype and nucleotide diversities were found in Greyton ($h = 1.0000 \pm 0.2722$; $\pi = 0.009081 \pm 0.007451$) despite the comparatively low sample size ($n = 3$) and the second highest was found in Linden Hill ($h = 0.9000 \pm 0.1610$; $\pi = 0.008696 \pm 0.005872$). The lowest haplotype diversity (with $n_h > 1$) was found in Grabouw ($h = 0.4643 \pm 0.2000$) and the lowest nucleotide diversity (with $n_h > 1$) in Franschhoek ($\pi = 0.000776 \pm 0.000963$). Rondevlei NR, Riviersonderend, Sandies Glen, Villiersdorp and Jonaskop all had only one haplotype. The highest diversity was present in Greyton and Linden Hill.

Combined DNA sequence (*COI* and *18S* rRNA) phylogeny and divergence time estimation

We amplified a 519-bp fragment of the *18S* rRNA locus for 14 *P. lawrencei* s.l. specimens, one of which was the suspected novel lineage (clade B). The novel *18S* rRNA data were deposited in GenBank (Accession numbers: OR421492–OR421505). The uncorrected *p*-distance between the two sympatric Oubos lineages (A and B) was 5.58%. To determine the phylogenetic placement of the novel lineage (clade B), we combined all *18S* rRNA sequences with *COI* sequence data from published sequence data for *Peripatopsis*.

The DNA substitution model for *18S* rRNA, selected using AIC, was TIM1 + I + G (–ln*L* = 1242.71). The base frequencies for the *18S* rRNA locus were as follows: A = 19.37%, C = 27.27%, G = 30.28% and T = 23.09%, whereas the rate matrix included *R*(*a*) [A–C] = 1.00, *R*(*b*) [A–G] = 0.94, *R*(*c*) [A–T] = 2.08, *R*(*d*) [C–G] = 2.08, *R*(*e*) [C–T] = 4.02 and *R*(*f*) [G–T] = 1.00. The gamma (G) shape distribution was 1.00. For *COI* of *Peripatopsis*, the selected DNA substitution model was GTR + I + G (–ln*L* = 6422.14) and base frequencies were A = 34.69%, C = 3.71%, G = 9.53% and T = 52.07%, with the rate matrix being *R*(*a*) [A–C] = 8.47, *R*(*b*) [A–G] = 52.93, *R*(*c*) [A–T] = 2.01,

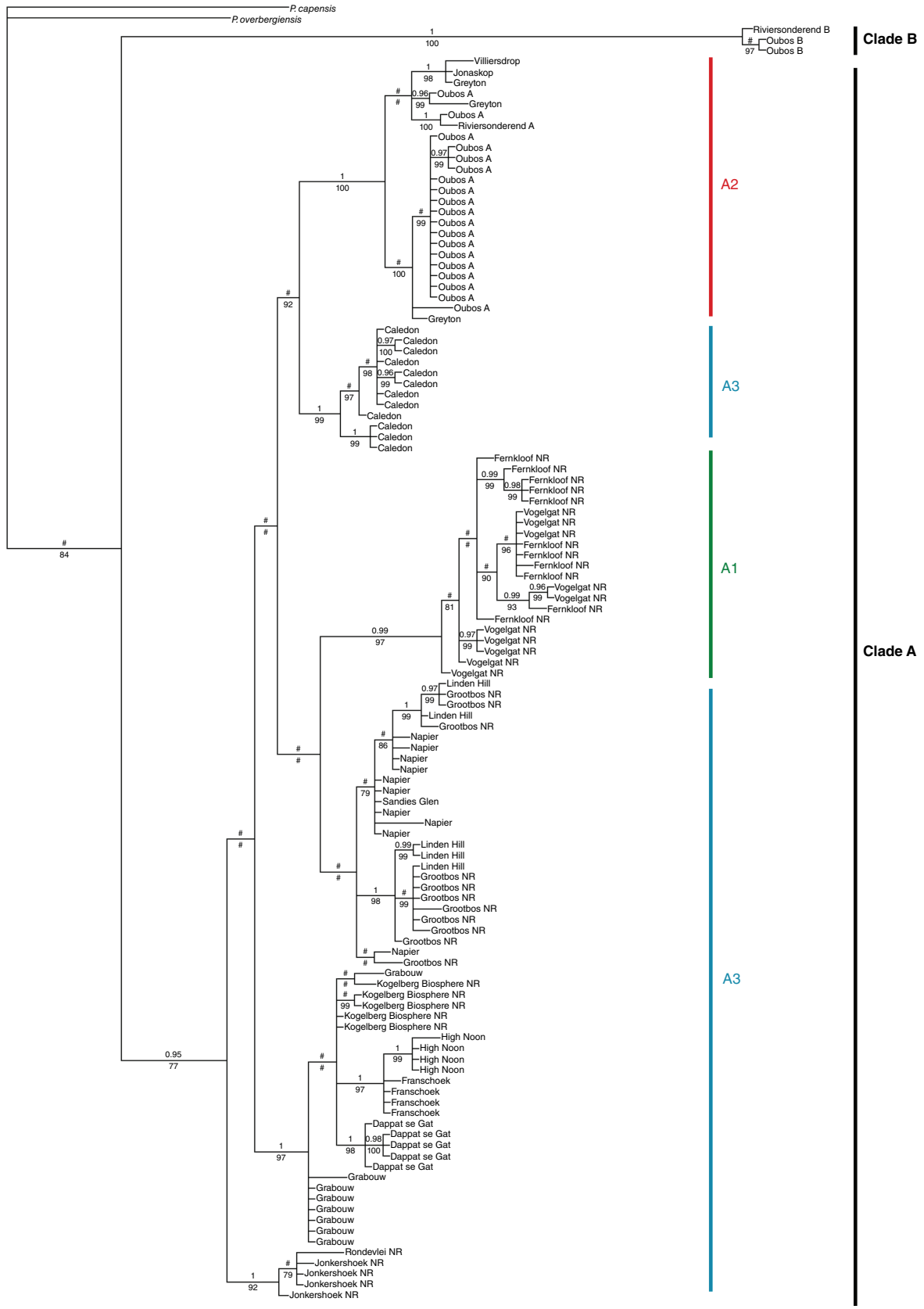

Fig. 2. (*Caption on next page*)

Fig. 2. Bayesian phylogenetic tree topology derived from the *COI* locus, showing the evolutionary relationship across the *P. lawrencei* s.s. population and separation from the sympatric novel lineage. Statistical support for the nodes is shown as posterior probability (>0.95 PP) above the nodes and bootstrap values (>75% BS) below the node. Posterior probability and bootstrap values <0.95 PP and <75% BS respectively are indicated with a hash ('#'). Clades A and B have been indicated, showing *Peripatopsis lawrencei* s.s. and the novel lineage respectively. Clade A (*P. lawrencei* s.s.) is divided into three haploclusters (A1–A3) and corresponds to the haplotype network in Fig. 3.

$R(d)$ [C–G] = 24.23, $R(e)$ [C–T] = 75.49 and $R(f)$ [G–T] = 1.00. The gamma (G) shape distribution was 0.35. The combined *COI* and *18S* rRNA data yielded a 1163-bp fragment, and the topologies produced from maximum likelihood and Bayesian inference were nearly identical, therefore only the maximum likelihood tree topology is shown (Fig. 5).

Peripatopsis was retrieved as monophyletic (>75% BS/>0.95 PP) (Fig. 5). The most basal lineage was *P. alba,* found in the Wynberg Caves on Table Mountain. The latter species was sister group to a clade of *P. balfouri* exclusively from the Kogelberg and Cape Peninsula. *Peripatopsis balfouri* was in turn sister group to a clade where *P. bolandi* Daniels, McDonald & Picker, 2013 was sister to *P. purpureus* Daniels, McDonald & Picker, 2013. The next clade comprised *P. ferrox* Barnes, Reiss & Daniels, 2020 sister to *P. edenensis* Barnes, Reiss & Daniels, 2020 and these two species were sister group to a clade comprising *P. tulbaghensis* Barnes, Reiss & Daniels, 2020, sister to *P. clavigera* Purcell, 1899 and the latter species was sister to *P. cederbergiensis* Daniels, McDonald & Picker, 2013. The novel lineage, *P. aereus* sp. nov. (clade B) was basal to the remaining *Peripatopsis* species, affirming the evolutionary distinction from the sympatric *P. lawrencei* s.s. specimens at Oubos A. In the next clade, we observed a monophyletic *P. lawrencei* s.s. (clade A) as sister group to *P. capensis.* The latter species is in turn sister group to *P. overbergiensis*. The latter clade, containing the three aforementioned species *P. lawrencei* s.s., *P. capensis* and *P. overbergiensis* was sister group to a larger clade of species distributed from the southern Cape into the Eastern Cape and KwaZulu–Natal. Within the last clade, *P. sedgwicki* Purcell, 1899 was basal to two other clades. Within these, the first clade comprised *P. janni* Ruhberg & Daniels, 2013 as sister group to three species *P. birgeri* Ruhberg & Daniels, 2013, sister group to *P. polychroma* with these two species being sister group to *P. hamerae* Ruhberg & Daniels, 2013. The second sister clade comprised *P. storchi* Ruhberg & Daniels, 2013 as sister group to *P. moseleyi*. Results of the Shimodaira-Hasegawa test indicated that the difference between the unconstrained topologies and constrained topology enforcing the monophyly of the Oubos clades (A sister to B) is highly significant (Δ $-\ln L = 185.11$, $P < 0.01$), further validating the distinction of the two clades.

Divergence time estimates (Fig. 5) based on the combined *COI* and *18S* data set suggested that *Peripatopsis* originated 28.83 Ma (95% highest posterior density, HPD 21.27–37.49 Ma) during the Oligocene–Eocene. *Peripatopsis alba* originated 13.62 Ma (95% HPD: 10.37–17.42 Ma), whereas *P. balfouri* separated shortly afterwards, 13.38 Ma (95% HPD: 9.53–15.73 Ma). Sister clades *P. purpureus* and *P. bolandi* diverged 11.21 Ma (95% HPD: 8.62–14.44 Ma), whereas *P. ferox* and *P. edenensis* diverged 10.23 Ma (95% HPD: 6.62–14.35 Ma). *Peripatopsis tulbaghensis* originated 11.07 Ma (95% HPD: 7.90–14.70 Ma) and *P. clavigera* diverged from *P. cederbergiensis* 8.72 Ma (95% HPD: 5.94–11.88 Ma). Furthermore, the novel lineage (clade B) diverged from the remaining *Peripatopsis* lineages 13.68 Ma (95% HPD: 10.46–17.73 Ma) during the Miocene. Within *P. lawrencei* s.s., sister clades *P. overbergiensis* and *P. capensis* diverged 6.02 Ma (95% HPD: 4.34–8.11 Ma), whereas *P. lawrencei* s.s. originated 5.23 Ma (95% HPD: 3.75–7.02 Ma) during the late Miocene to the early Pliocene. In a similar time period, within the more derived members of *Peripatopsis*, *P. sedgwicki* diverged 8.69 Ma (95% HPD: 6.59–11.11 Ma), whereas *P. moseleyi* diverged 5.17 Ma (95% HPD: 3.79–6.82 Ma). During the Pliocene, we observed the origin of *P. janni* 5.26 Ma (95% HPD: 3.97–6.74 Ma), *P. storchi* 3.77 Ma (95% HPD: 2.59–5.18 Ma), *P. hamerae* 4.60 Ma (95% HPD: 3.35–6.96 Ma) and the split between *P. polychroma* and *P. birgeri* 3.41 Ma (95% HPD: 2.32–4.81 Ma).

Morphology of *P. lawrencei* s.l.

Diagnostic gross morphological differences were observed between clades A and B, including the number of leg pairs and ventral colour (Table 4). In both clades, dorsal colour ranged from navy blue to slate black, to black with tints of orange and various shades of orange. The ventral colour for *P. lawrencei* s.s. was predominantly cream white and sometimes pale orange or yellow (Fig. 6*a*, *c*). For the new lineage (clade B) the ventral colour was a bronze-like golden brown (Fig. 6*b*, *d*). The presence of two claws was noted on the back legs of all individuals across both clades. The number of leg pairs differed between the two clades, as *P. lawrencei* s.s. had 17 leg pairs and clade B had 18, with the last leg pair being reduced in size. The SEM micrographs revealed marked differences in the number of scale rank counts on the primary papillae between the two lineages, with clade B having more than double the number of scale rank counts for both the dorsal and ventral papillae. *P. lawrencei* s.s. had seven scale rank counts on the dorsal papilla and four on the ventral papilla (Fig. 7*a*, *c*), whereas clade B had fifteen scale

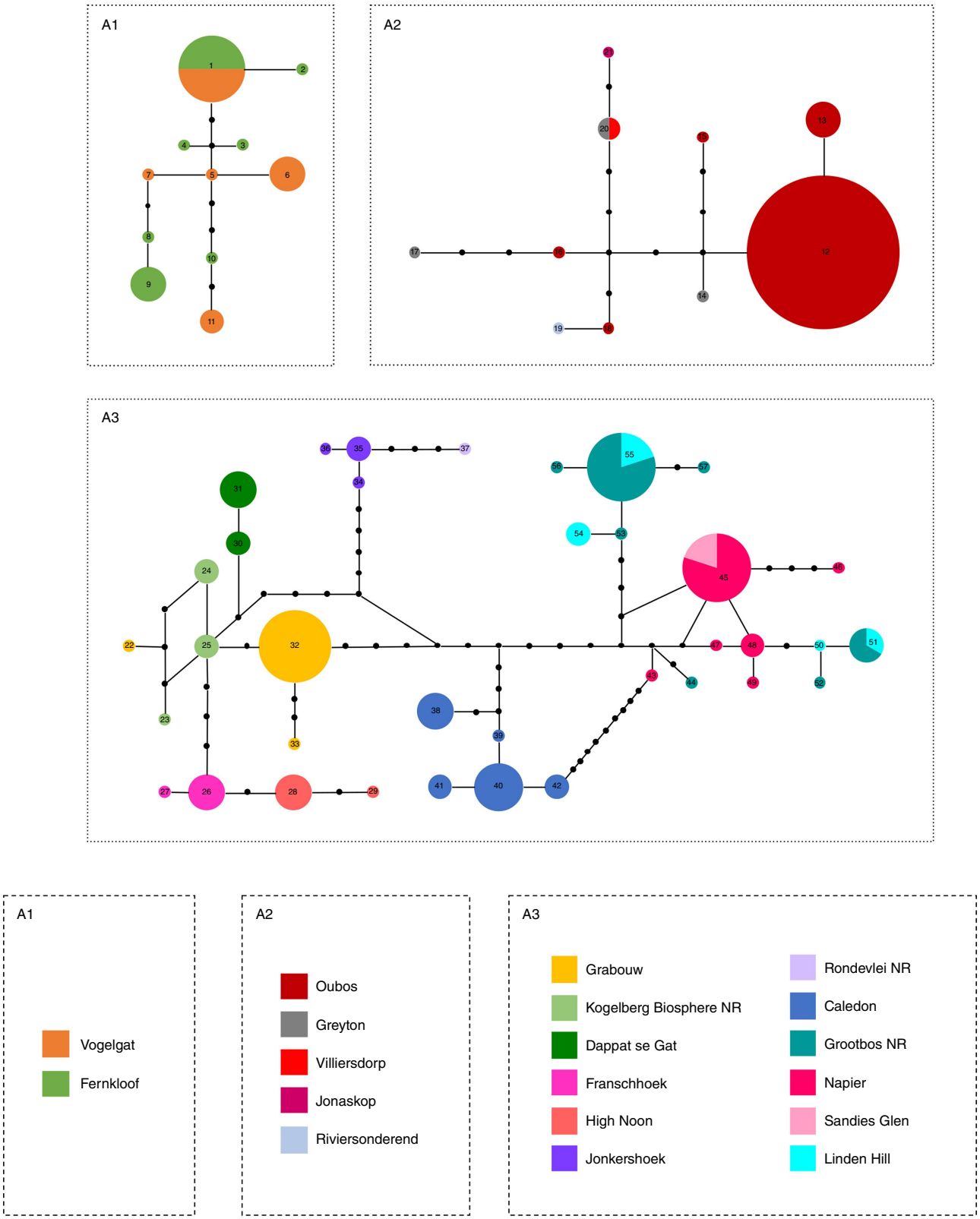

Fig. 3. The haplotype network based on the *COI* sequence data showing the genetic distribution of *Peripatopsis lawrencei* s.s. across 19 localities in the Western Cape, South Africa. A total of 57 haplotypes was identified, with circle size corresponding to the frequency of the haplotypes. The codes A1–A3 represent the clades (haploclusters) present on Fig. 2. Missing or unsampled haplotypes are indicated with a solid black circle. Haplotype numbers correspond to those in Appendix A1.

Table 2. Pairwise F_{ST} values for the *COI* locus of *Peripatopsis lawrencei* s.s. across the 19 sample localities.

Locality	Vogelgat	Fernkloof	Villiersdorp	Jonaskop	Greyton	Riviersonderend A	Oubos A	Grabouw	Kogelberg	Franschhoek	High Noon	Dappat se Gat	Jonkershoek	Rondevlei	Caledon	Napier	Grootbos	Sandies Glen	Linden Hill
Vogelgat	0.00000																		
Fernkloof	0.07913	0.00000																	
Villiersdorp	0.86029	0.85113	0.00000																
Jonaskop	0.84004	0.83004	1.00000	0.00000															
Greyton	**0.82717**	**0.82186**	0.00000	−0.63636	0.00000														
Riviersonderend A	0.86029	0.85113	1.00000	1.00000	0.05263	0.00000													
Oubos A	**0.90202**	**0.89771**	0.80117	0.73946	**0.50408**	0.73016	0.00000												
Grabouw	**0.86493**	**0.85880**	0.89260	0.86832	**0.80653**	0.89106	**0.89454**	0.00000											
Kogelberg	**0.86886**	**0.86066**	0.92929	0.91463	0.82668	0.92135	**0.91433**	0.47225	0.00000										
Franschhoek	**0.88103**	**0.86861**	0.9759	0.97183	0.85117	0.97333	**0.92541**	0.76382	0.80067	0.00000									
High Noon	**0.87900**	**0.86734**	0.95349	0.94595	0.84141	0.94872	**0.92416**	0.81674	0.83210	0.72727	0.00000								
Dappat se Gat	**0.88020**	**0.87092**	0.96939	0.96386	0.85703	0.96591	**0.92231**	0.72762	**0.70588**	0.91872	0.89109	0.00000							
Jonkershoek	**0.85682**	**0.84740**	0.95000	0.94118	0.82775	0.94444	**0.91024**	0.86012	**0.89603**	0.94118	0.92593	0.93340	0.00000						
Rondevlei	0.84004	0.83004	1.00000	1.00000	0.69492	1.00000	0.91463	0.85979	0.89855	0.96610	0.93548	0.95588	0.77778	0.00000					
Caledon	**0.85168**	**0.84670**	0.84284	0.82504	**0.77275**	0.85734	**0.87055**	**0.82117**	**0.85406**	**0.87442**	**0.87284**	**0.86850**	**0.85969**	0.85734	0.00000				
Napier	**0.83916**	**0.83248**	0.87266	0.84685	**0.80036**	0.87266	**0.87332**	**0.84500**	**0.87277**	**0.89168**	**0.88866**	**0.88780**	**0.86085**	0.85654	**0.80905**	0.00000			
Grootbos	**0.76375**	**0.76381**	0.73274	0.68632	0.69054	0.71483	**0.82002**	**0.75380**	**0.75496**	**0.77037**	0.77222	**0.76707**	**0.72019**	0.67609	**0.73679**	**0.31294**	0.00000		
Sandies Glen	0.80173	0.78936	1.00000	1.00000	0.59091	1.00000	0.88331	0.85154	0.90541	0.96825	0.93939	0.95890	0.92308	1.00000	0.79394	−0.74359	−0.12453	0.00000	
Linden Hill	**0.78157**	**0.77787**	0.72277	0.67442	**0.67846**	0.70526	**0.85379**	**0.79018**	0.79167	0.80999	**0.80593**	0.81325	0.76123	0.67059	**0.77007**	**0.30519**	−0.07664	−0.27273	0.00000

Significant values are shown in bold.

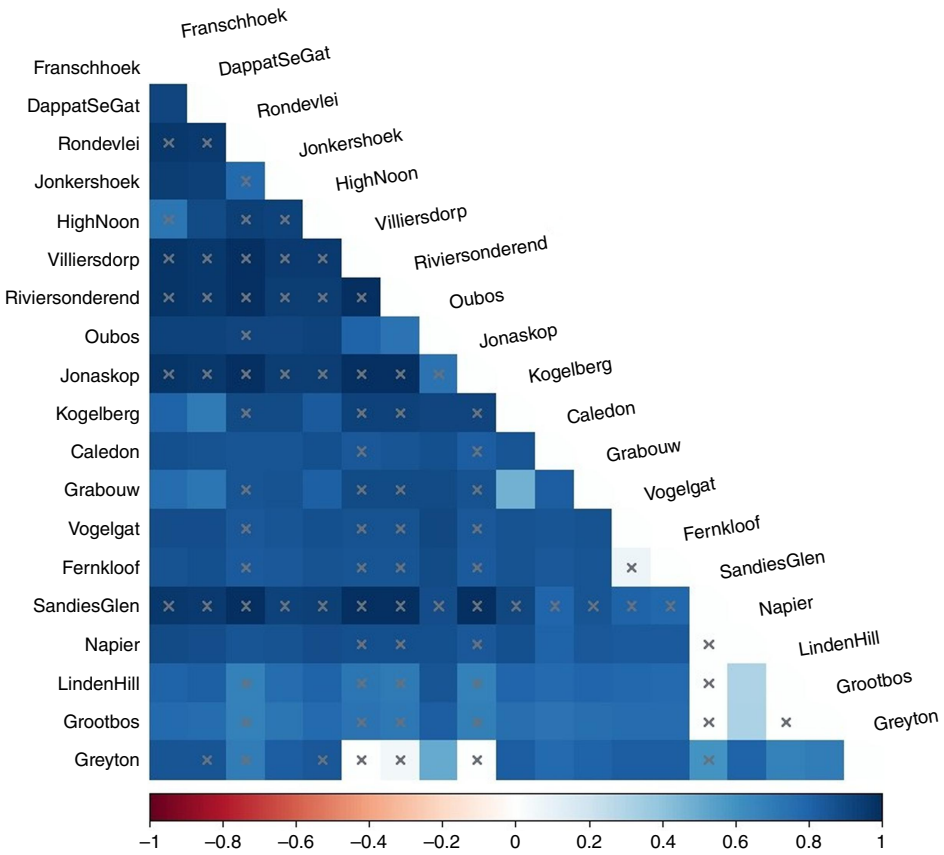

Fig. 4. Heatmap diagram with the colour showing the strength of the F_{ST} value for each respective pairwise F_{ST} combination. Darker colours show higher levels of F_{ST} between different localities and are therefore more distinct. The stronger the signal, the more easily the different populations can be distinguished from one another by looking at the individual's DNA. The lighter the colour, the less distinct. Blocks marked by a superimposed X indicate non-significance.

ranks for the dorsal papilla and nine on the ventral papilla (Fig. 7*b*, *d*).

Discussion

We observed marked genetic differentiation and detected the presence of two genetically highly divergent clades (clades A and B; Fig. 2 and 5) within *Peripatopsis lawrencei* s.l. Clade A corresponds to *P. lawrencei* s.s. whereas clade B represents a novel species that is described in the current manuscript. Marked phylogeographic structure was observed within *P. lawrencei* s.s. that was characterised by the absence of maternal dispersal evident from the *COI* haplotype network and F_{ST} heatmap (Fig. 3, 4, Table 2). Similar levels of marked genetic differentiation have been observed in several velvet worm species (Myburgh and Daniels 2015; Barnes *et al.* 2020). Clade B, the narrowly endemic lineage present exclusively in the Riviersonderend Mountains at Oubos, was not monophyletic with *P. lawrencei* s.s. (clade A) in our phylogeny (Fig. 5). Enforcing the monophyly of clades A and B retrieved a statistically less satisfactory tree topology, corroborating the evolutionary divergence. Furthermore, the two clades were characterised by marked differentiation based on the *COI* *p*-distances that fall within the interspecific range and a marked sequence *p*-distance value for the conserved *18S* rRNA locus. The two clades can be differentiated based on leg pair number, ventral colour patterns and fixed scale rank differences. Collectively these results provide supportive evidence for the recognition of a novel species for specimens in clade B. We note that the deeper nodal relationship in our *COI* + *18S* rRNA phylogeny was poorly supported and recommend the use of additional loci that represents a broader suite of evolutionary tempos to potentially resolve this problem.

Novel species delineation

Using the 'unified species concept' advocated by de Queiroz (2007), we find *P. lawrencei* s.s. (clade A) and the sympatric lineage at the Riviersonderend Mountains (clade B) to be separately evolving metapopulations, with multiple lines of evidence substantiating the classification of clade B as a divergent species. Following conventional taxonomic criteria, this can be identified as a unique species based on discernible morphological differences (Fig. 6*a–d* and 7*a–d*, Table 4). Using both gross morphology and scanning electron microscopy the two clades within *P. lawrencei* s.l. could be delineated. In clade A (*P. lawrencei* s.s.), the ventral colour was highly variable (Table 4) however in clade B, based on two samples, the ventral surface colour was bronze. In velvet worm species, the dorsal colour pattern can notably be highly variable. For example, in *Peripatopsis*

Table 3. Population genetic parameters measured with the *COI* locus in *Peripatopsis lawrencei* s.s.

Locality	*n*	n_h	n_p	Haplotype diversity (*H*)	Nucleotide diversity (π)
Rondevlei NR	1	1	0	1.0000 ± 0.0000	0.000000 ± 0.000000
Jonkershoek NR	4	3	2	0.8333 ± 0.2224	0.001553 ± 0.001538
Franschhoek	4	2	1	0.5000 ± 0.2652	0.000776 ± 0.000963
High Noon	4	2	2	0.5000 ± 0.2652	0.001553 ± 0.001538
Dappat se Gat	5	2	1	0.6000 ± 0.1753	0.000932 ± 0.001021
Kogelberg Biosphere NR	5	3	3	0.8000 ± 0.1640	0.002174 ± 0.001849
Fernkloof NR	11	7	11	0.8909 ± 0.0740	0.005590 ± 0.003463
Vogelgat NR	10	5	8	0.8444 ± 0.0796	0.005141 ± 0.003260
Caledon	12	5	6	0.8333 ± 0.0691	0.003600 ± 0.002381
Greyton	3	3	8	1.0000 ± 0.2722	0.009081 ± 0.007451
Oubos A	19	5	10	0.5263 ± 0.1266	0.002470 ± 0.001714
Riviersonderend	1	1	0	1.0000 ± 0.0000	0.000000 ± 0.000000
Grootbos NR	11	7	16	0.8727 ± 0.0891	0.008669 ± 0.005103
Linden Hill	5	4	10	0.9000 ± 0.1610	0.008696 ± 0.005872
Sandies Glen	1	1	0	1.0000 ± 0.0000	0.000000 ± 0.000000
Napier	10	6	8	0.8444 ± 0.1029	0.003520 ± 0.002384
Villiersdorp	1	1	0	1.0000 ± 0.0000	0.000000 ± 0.000000
Grabouw	8	3	5	0.4643 ± 0.2000	0.002006 ± 0.001606
Jonaskop	1	1	0	1.0000 ± 0.0000	0.000000 ± 0.000000

n represents the number of samples per locality, n_h is the number of haplotypes and n_p the number of polymorphic sites.

polychroma the dorsal colour surface ranged from olive green, through slate black to rusty brown (Grobler *et al.* 2023). Similarly, in *P. overbergiensis*, the dorsal surface colour can range from rusty brown to bright red (Daniels pers. obs). Although leg pair count is a variable indicator of species boundaries (Daniels *et al.* 2009, 2013, 2016; Barnes *et al.* 2020), the number of leg pairs differed between the species in our study, as 17 were present in *P. lawrencei* s.s. and 18 in clade B. McDonald *et al.* (2012) reported that in *P. capensis* and *P. overbergiensis* the number of leg pairs ranged from 17 to 18 respectively, with limited overlap in this character. By contrast, Grobler *et al.* (2023) reported that between sister species *P. birgeri* and *P. polychroma* the number of leg pairs always overlapped and ranged from 20 to 23. Leg pair numbers, together with the colour of the dorsal surface should be interpreted cautiously and preferably within a phylogenetic context to limit artificial splitting or lumping of species. The arrangement of dermal papillae has more often been used as a diagnostic feature in earlier velvet worm studies (Oliveira *et al.* 2011; McDonald and Daniels 2012; Daniels *et al.* 2013, 2016; Sato *et al.* 2018; Grobler *et al.* 2023). The novel lineage (Oubos B, clade B, *P. aereus* sp. nov.) was shown to have a reduced number of scale ranks on the papillae for both the dorsal and ventral surfaces.

Incorporating DNA sequence data into our argument for the recognition of a new species, we observed several lines of evidence that aid in the delineation. Marked uncorrected sequence *p*-distance values, using the *COI* locus between the two clades falls into a similar range as reported among velvet worm species. For example, the uncorrected *p*-distances between *P. capensis, P. overbergiensis* and *P. lawrencei* s.s. ranged between 5.93 and 7.49%, however, in our study the uncorrected *p*-distance *COI* distance value between *P. lawrencei* s.s. and the novel lineage (clade B) was between 9.13 and 10.62%. Similarly, between clade B and *P. overbergiensis,* the uncorrected *p*-distance was between 10.57 and 10.73%. The values found between clade B and *P. lawrencrei* s.s. are higher than what has been reported for sister species of velvet worms in prior studies (McDonald and Daniels 2012), suggesting a more distant relationship; a result corroborated by our phylogeny for *Peripatopsis* (Fig. 5). Barnes *et al.* (2020) used similar *p*-distance values ranging from 8.46 to 10.64% to delineate velvet worm species. Sequence divergence values as low as 3% have been used to delineate novel velvet worm species (Rockman *et al.* 2001). In addition, the 5.58% uncorrected *p*-distance difference between the two sympatric phylogenetically distinct Oubos lineages (A and B) observed for the highly conserved *18S* rRNA locus further corroborates the genetic distinction of

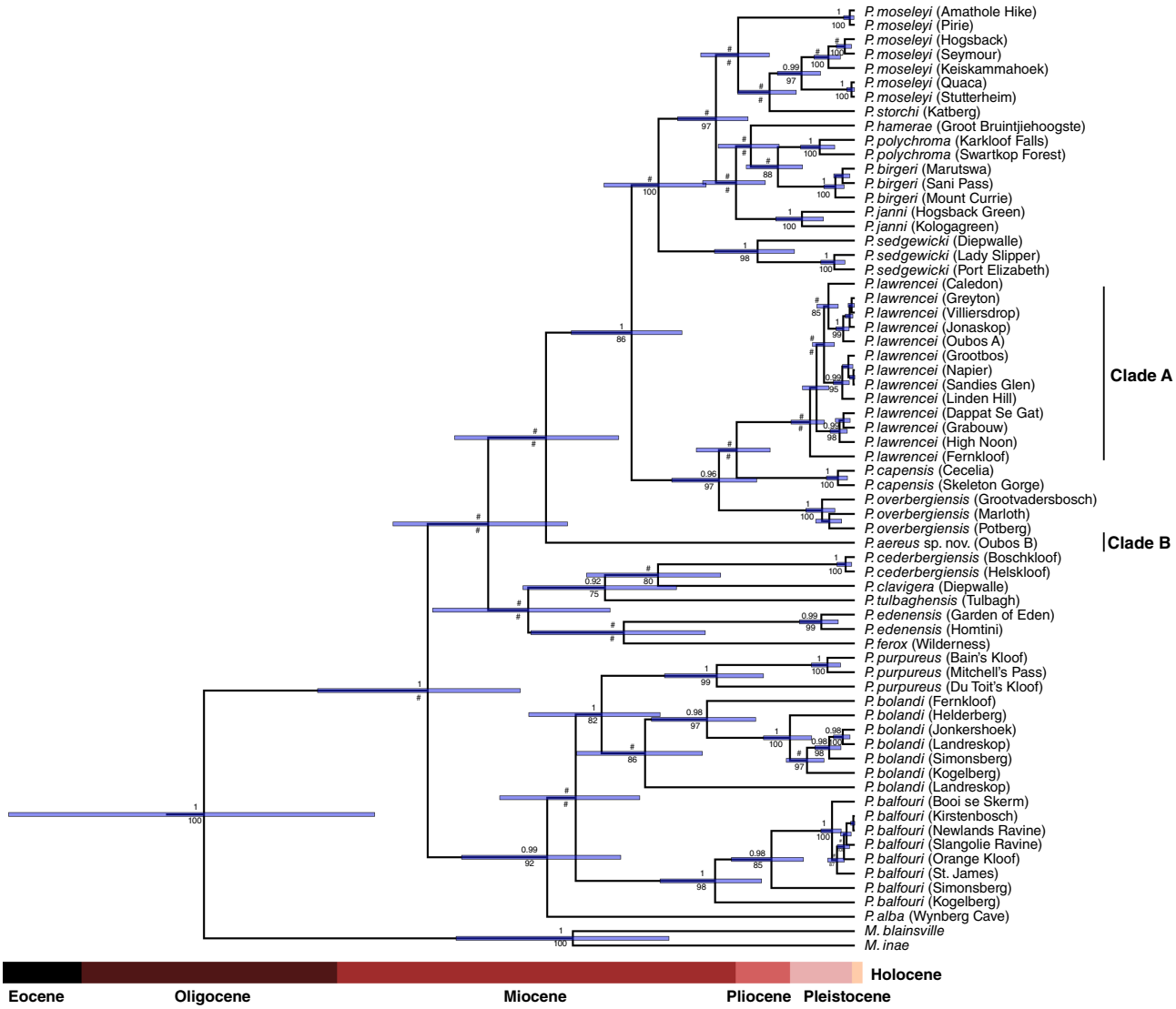

Fig. 5. Divergence time estimation based on *COI* and *18S* rRNA sequence data for *Peripatopsis*. The 95% HPD interval bars are shown at each node, with a timescale bar representing the relevant epochs. Clade A shows the monophyletic *P. lawrencei* s.s. whereas clade B indicates the ancestral novel lineage, denoted as *P. aereus* sp. nov. Blue bars indicate HPD ranges. Posterior probability values >0.95 PP are shown above each node and bootstrap values >75% BS are shown below the node. Posterior probability and bootstrap values <0.95 PP and <75% BS respectively are indicated with a hash ('#').

the two velvet worm species. Barnes *et al.* (2020) also used uncorrected *p*-distance differences in the *18S* rRNA locus to validate the recognition of species in the *P. clavigera* species complex. For the *18S* locus the latter authors noted *p*-distance values ranging from 0.68 to 3.40% to aid the recognition of novel species. The *18S* rRNA *p*-distance value we report is higher than those from earlier velvet worm studies, providing corroborative nuclear differences for the recognition of a novel lineage. To further strengthen our argument for distinct taxonomic status, a multi-locus tree topology (incorporating both mt and nuDNA sequence data) of the South African *Peripatopsis* suggests a Miocene divergence between the two Oubos clades (A and B) (Fig. 5). Given the multifaceted evidence for the distinction of this novel lineage in clade B, we are confident in the delineation and recognition of the new species.

The divergence time estimation indicates an Oligocene origin of *Peripatopsis,* 28.83 Ma (95% HPD: 21.27–37.49 Ma), followed by rapid diversification in the mid-Miocene to the Pleistocene. The Miocene divergence of the stem lineage, 13.68 Ma (95% HPD: 10.46–17.73 Ma) suggests a more ancient origin of this velvet worm and a wider distribution than previously considered (Daniels *et al.* 2009). This can be attributed to the different analytical approaches employed by the two studies. The Miocene epoch was characterised by humid subtropical forests in the Cape region of southern

Table 4. Morphological variation across 19 localities for *P. lawrencei* s.s. and the novel lineage.

Locality	Sample size (*n*)	Clade	Dorsal colour	Ventral colour	Leg pairs	Length (mm)	Width
Rondevlei NR	1	*P. lawrencei* s.s.	?	Cream white	17	?	?
Jonkershoek NR	4	*P. lawrencei* s.s.	Dark brown–orange	Cream white	17	?	?
Franschhoek	4	*P. lawrencei* s.s.	Slate black	Cream white	17	?	?
High Noon	4	*P. lawrencei* s.s.	Dark orange–orange	Cream white	17	?	?
Dappat se Gat	5	*P. lawrencei* s.s.	Slate black–dark orange	Cream white	17	?	?
Kogelberg Biosphere NR	5	*P. lawrencei* s.s.	Dark orange–orange	Cream white	17	?	?
Fernkloof NR	11	*P. lawrencei* s.s.	Navy blue–orange	Cream white	17	18–24.5	5.5
Vogelgat NR	10	*P. lawrencei* s.s.	Dark orange–orange	Cream white	17	13–20.5	1.5–4
Caledon	12	*P. lawrencei* s.s.	Slate black–orange	Cream white–pale orange	17	15–29	2–5.5
Greyton	3	*P. lawrencei* s.s.	Dark orange	Cream white	17	?	?
Oubos A	19	*P. lawrencei* s.s.	Dark orange–orange	Cream white	17	18–40.5	2–4.5
Oubos B	2	*P. aereus* sp. nov.	Dark brown	Golden brown	18	34–38	4–4.5
Riviersonderend A	1	*P. lawrencei* s.s.	Orange	Cream white	17	13.5–43	1.5–6
Riviersonderend B	1	*P. aereus* sp. nov.	Dark brown	Golden brown	18	?	?
Grootbos NR	11	*P. lawrencei* s.s.	Navy blue–dark orange	Cream white–pale orange	17	18–40.5	2–6.5
Linden Hill	5	*P. lawrencei* s.s.	Dark orange	Cream white	17	?	?
Sandies Glen	1	*P. lawrencei* s.s.	Dark orange	Cream white–olive green	17	29.5	5
Napier	10	*P. lawrencei* s.s.	Navy blue–orange	Cream white	17	13–33	1.5–3.5
Villiersdorp	1	*P. lawrencei* s.s.	Dark orange	Orange	17	21	4
Grabouw	8	*P. lawrencei* s.s.	Dark orange–orange	Cream white–pale orange	17	7.5–21	1.5–4.5
Jonaskop	1	*P. lawrencei* s.s.	Dark orange	Cream white	17	?	?

Missing data are indicated by a question mark (?). Morphological data generated during our study were combined with data from Daniels *et al.* (2009) and McDonald and Daniels (2012).

Africa (Steinthorsdottir *et al.* 2021) but was succeeded by major climatic ameliorations such as the development of the proto Benguela current along the west coast. This resulted in increased aridification, possibly contributing to cladogenesis (Siesser 1980). Cladogenesis was also observed at a comparable time period, 13.63 Ma (95% HPD: 9.53–15.73 Ma), between the sister taxa *P. bolandi* and *P. balfouri* (Daniels *et al.* 2013). Notably, these have a similar distribution, encompassing the Western Cape region and would have experienced similar climatic pressures. The dry conditions of the late Miocene persisted throughout the Plio–Pleistocene (Mucina and Rutherford 2006; Sepulchre *et al.* 2006). Contemporary populations of *Peripatopsis* generally reside at high altitudes, suggesting biome contraction in lowlands. This could have driven many lineages upward to forest refugia, further separating populations. As velvet worms are extremely mesic-adapted animals, the long-distance gene flow is highly restricted. In such cases, local adaptation or genetic drift is expected to play an important role in species divergence and genetic differentiation.

Phylogeography of *P. lawrencei* s.s.

The phylogeographic analysis of *P. lawrencei* s.s. using the *COI* dataset yielded results congruent with the tree topologies observed thus far (Fig. 2 and 5). Velvet worms are known to be highly fragmented and generally restricted to high elevation forest patches (Daniels *et al.* 2009, 2016; Barnes and Daniels 2019; Barnes *et al.* 2020; Grobler *et al.* 2023). The observed distribution of *P. lawrencei* s.s. is consistent with such habitat restriction. Haplocluster A3 had a wide distribution, A2 was confined to the Riviersonderend Mountains and A1 showed exclusivity to a narrow coastal region at Hermanus (Fig. 2 and 3). The geographically isolated haploclusters underscore the presence of strong barriers to gene flow and limited maternal dispersal among populations, as observed in prior velvet worm studies (Daniels and Ruhberg 2010; Barnes *et al.* 2020; Barnes and Daniels 2019, 2022). There is a clear interplay of geographical factors, such as physical barriers or habitat fragmentation and genetic differentiation across these

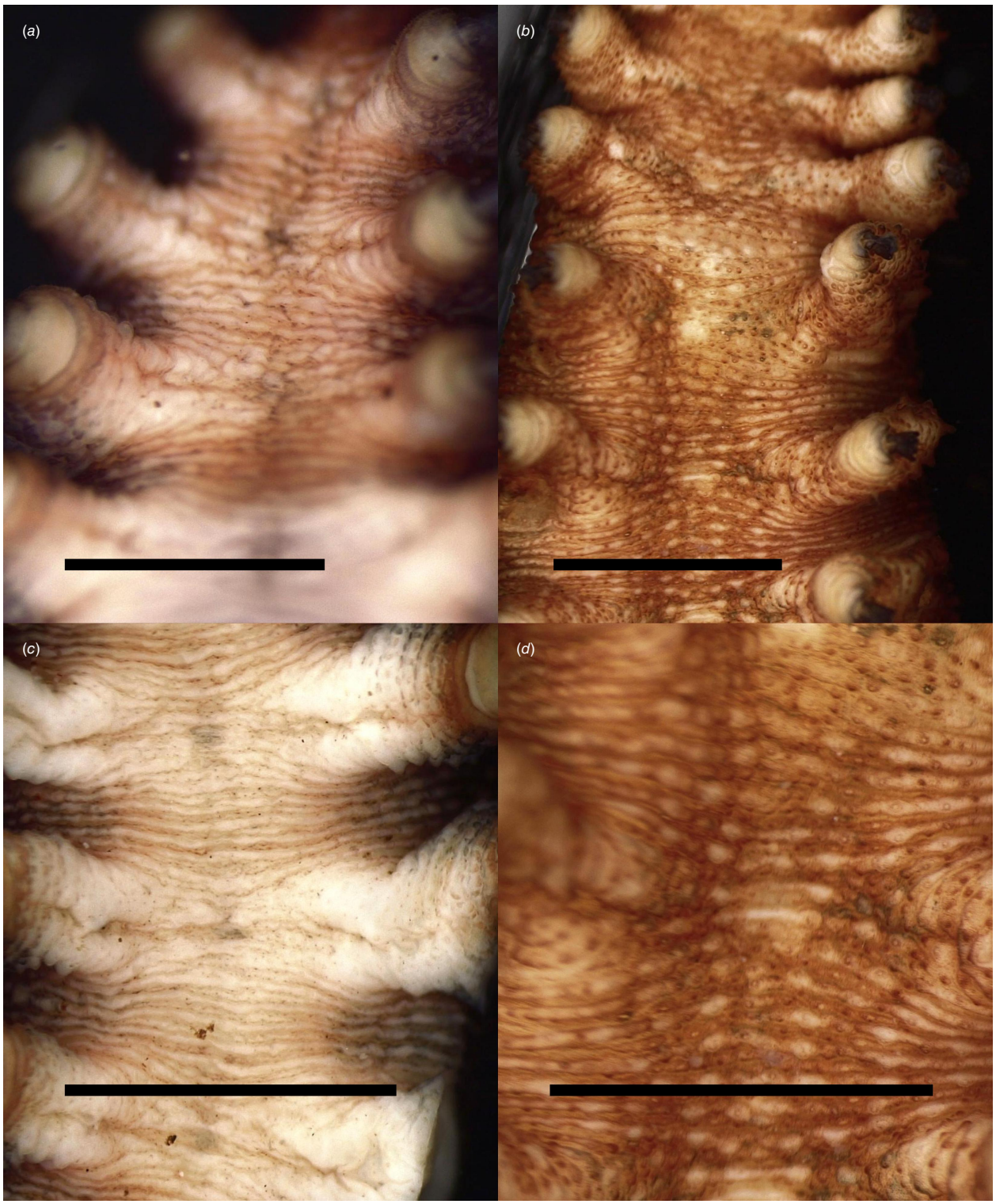

Fig. 6. Photographs of recently collected velvet worm specimens suspended in ethanol, taken with a Leica light microscope. (*a*) Ventral view of *P. lawrencei* s.s. (*b*) Ventral view of the novel lineage, *P. aereus* sp. nov. (clade B). Enhanced ventral views showing the ventral organs in (*c*) *P. lawrencei* s.s. and (*d*) the novel lineage, *P. aereus* sp. nov. Scale bars: 2 mm.

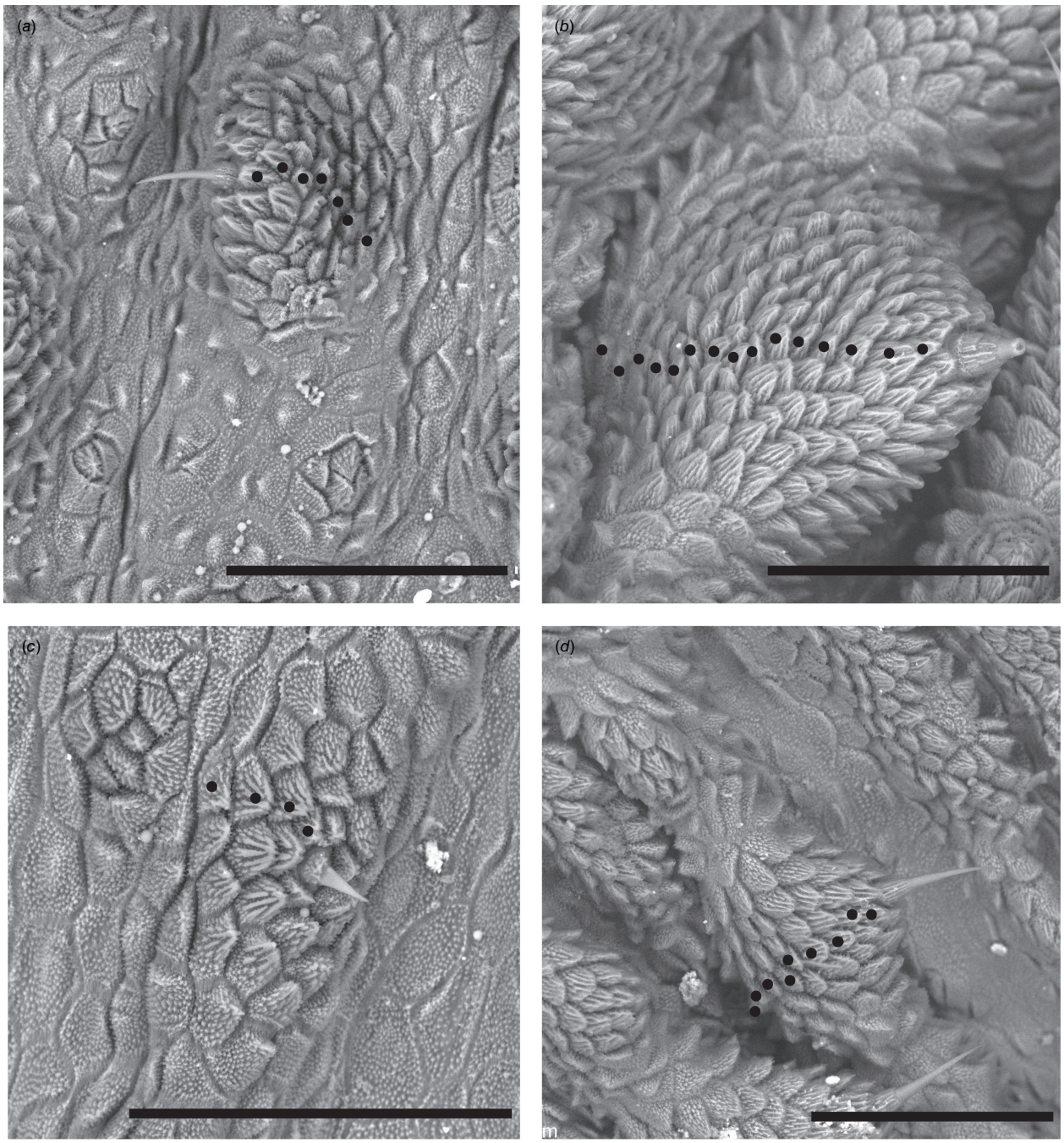

Fig. 7. Scanning electron micrographs of dermal papillae on dorsal and ventral surfaces of *P. lawrencei* s.s. and *P. aereus* sp. nov. Black dots represent a single scale rank. (*a*) Dorsal papilla for *P. lawrencei* s.s. with seven scale ranks. (*b*) Dorsal papilla for the novel lineage (clade B, *P. aereus* sp. nov.) with 15 scale ranks. (*c*) Ventral papilla for *P. lawrencei* s.s. with four scale ranks. (*d*) Ventral papilla for the novel lineage (clade B, *P. aereus* sp. nov.) with nine scale ranks. Scale bars: 100 μm.

populations. This distribution may have been influenced by localised extinctions in neighbouring or connecting populations, resulting in the observed genetic drift and allelic fixation. The F_{ST} values further support this, showing considerable genetic differentiation among the conspecific populations, indicating high genetic structuring and low rates of dispersal (Table 2). The lack of connectedness promotes genetic divergence, as supported by the AMOVA results, highlighting that a substantial portion of genetic variation occurred among populations. The higher diversity values in certain localities such as Greyton and Linden Hill could reflect the presence of refugia, allowing for accumulation of genetic diversity (Table 3). These findings suggest the need for conservation units within *Peripatopsis lawrencei*

s.s., as the Afromontane habitats are highly reduced and highly threatened. The scarcity of samples for the novel lineage further supports the need for habitat maintenance.

Fine-scale sampling and future directions

Fine-scale sampling plays a crucial role in uncovering hidden alpha-taxonomic diversity, especially in poorly studied saproxylic taxa. These organisms are often overlooked or understudied due to habitat complexity or crypticity. Saproxylic zones have been observed to harbour a wealth of taxonomic diversity, as these comprise numerous microhabitats allowing for highly specific niche divergence among taxa that may live in proximity (Daniels *et al.* 2009; Oliveira *et al.* 2011; Sato *et al.* 2018). Furthermore, weak taxonomic classifications can arise through poor sampling, leading to divergent sympatric species being lumped together into a single clade (Barnes and Daniels 2019). Many saproxylic taxa are rare and potentially endangered due to the nature of the habitats that are prone to habitat degradation and fragmentation. Fine-scale sampling allows researchers to detect these rare species and assess the conservation status precisely. A study by Barnes and Daniels (2019) demonstrated the difference between large spatial scale- and fine-scale sampling in detecting novel lineages. Fine-scale sampling, as undertaken here, enables refinement of taxonomic classifications where diversity has previously been underestimated. In our case, fine-scale sampling revealed an ancient novel lineage (clade B) living in sympatry with a well-documented species, *P. lawrencei* s.s. in the absence of these detailed studies, such species might be unnoticed, impeding conservation efforts. A fine-scale approach is highly recommended when investigating species of limited dispersal capacity and those that inhabit saproxylic zones. Future studies could intensify field surveys and assess the conservation status of this novel lineage. Population size estimates and ongoing monitoring of this lineage and the populations of *P. lawrencei* s.s. can aid in evaluating the vulnerability and informing targeted conservation strategies, including the identification of potential conservation units. Genome-wide analyses could be conducted to pinpoint specific genes responsible for morphological divergence, while also providing insights into broader evolutionary and developmental processes in velvet worms within these rapidly changing landscapes.

Conclusion

This study offers comprehensive evidence supporting the classification of a novel lineage of velvet worms found in the Riviersonderend Mountains as a distinct species from the sympatric counterpart, *P. lawrencei* s.s. Multiple lines of independent evidence are congruent and reinforce the credibility of this new species designation. Morphological differences such as ventral coloration, papillae structure and leg pair variations were observed, and ancestral tree construction demonstrated the separation of clade B from the monophyletic clade of *P. lawrencei* s.s. The genetic distinctiveness of clade B was strengthened by substantial sequence divergence, dating back to the Miocene, an epoch characterised by rapid aridification. Phylogeographic analyses revealed the role of geographical barriers and habitat fragmentation in shaping the genetic structure of *P. lawrencei* s.s. Furthermore, distinct haploclusters, characterised by limited gene flow, underscore the importance of habitat preservation and the establishment of conservation units to safeguard the unique genetic diversity. Moreover, the study's findings highlight the necessity of fine-scale sampling to uncover concealed alpha-taxonomic diversity, particularly among saproxylic organisms. The novel lineage identified in this study is described as *Peripatopsis aereus* sp. nov.

Taxonomy

Family **PERIPATOPSIDAE** Bouvier, 1907

Genus ***Peripatopsis*** Pocock, 1894

(Fig. 2, 5, 6*a*, *b*, 7*a*, *b*, Table 4.)

Peripatopsis lawrencei s.s. McDonald, Ruhberg & Daniels, 2012.

Holotype

Male (SAM-ENW-C6468a), Oubos, Riviersonderend, Western Cape Province, South Africa, collected by D. McDonald and A. Abels, 20 October 2010.

Paratypes

Four males (SAM-ENW-C6468b), Oubos, Riviersonderend, Western Cape Province, South Africa, collected by D. McDonald and A. Abels, 20 September 2010.

Additional material

One female (SAM-ENW-C6457), Fernkloof Nature Reserve, Hermanus, Western Cape province, South Africa, collected 2006 by S. R. Daniels and H. van den Worm. Three males and three females (SAM-ENW-C6458a and b), collected 2010 by D. McDonald and A. Abels from the same locality. Six males and three females (SAM-ENW-C6455), Dappat se Gat, Gordon's Bay, Western Cape province, South Africa, collected 2011 by F. Van Zyl. One male and three females (SAM-ENW-C6456a and b), Kogelberg Biosphere Nature Reserve, Kleinmond, Western Cape province, South Africa, collected 2011 by D. McDonald and A. Abels. One female and three n/a (SAMENW-C6462), Grootbos Private Reserve, Gansbaai, Western Cape province, South Africa, collected 2006 by S. R. Daniels. Five males and six females (SAM-ENW-C6463a and b), collected 2010 by D. McDonald and A. Abels from the same locality. Five females (SAM-ENW-C6464), Napier, Western Cape province, South Africa, collected 2010 by F. Van Zyl.

Two males and ten females (SAM-ENW-C6465), collected 2006 by S. R. Daniels and M. Picker from the same locality. Nine juveniles (SAM-ENW-C6454), High Noon, Villiersdorp, Western Cape province, South Africa, collected 2006 by M. Picker, R. Cowlin, and Merl. Five n/a (SAM-ENW-C6453) collected 2006 by M. Picker and R. Cowlin from the same locality. Three n/a (SAM-ENW-C6466), Greyton, Western Cape province, South Africa, (date and collector not specified). One male and three females (SAM-ENW-C6448), Jonkershoek Nature Reserve, Stellenbosch, Western Cape province, South Africa, collected 2011 by D. McDonald and A. Abels. Two males and two females (SAM-ENW-C6449) collected 2011 by D. McDonald and A. Abels from the same locality. One n/a (SAM-ENWX7290) unspecified locality, Stellenbosch, Western Cape province, South Africa, collected 1905 by Brown. One n/a (SAM-ENW-C6488), Rondevlei, Cape Flats, Western Cape province, South Africa, collected by Picker (date not specified). Five juveniles (SAM-ENW-C6467), Oubos, Riviersonderend, Western Cape province, South Africa, collected 2010 by D. McDonald and A. Abels. One female (SAMENW-C6461) Klein Swartberg, Caledon, Western Cape province, South Africa, collected 2011 by D. McDonald and A. Abels. Three males and four females (SAM-ENW-C6460), collected 2011 by G. Diedericks and C. Broeckhoven from the same locality; 15 n/a (SAM-ENW-X6390) collected by Watermeyer and Purcell (date unknown) from the same locality. Four males and two females (SAM-ENWC6450, C6451, and C6452), Berg River Dam, Franschhoek, Western Cape province, South Africa, collected 2010 by F. Van Zyl. Five n/a (SAM-ENW-X6388), Houw Hoek, Western Cape province, South Africa, collected 1900 by Purcell. One n/a (SAM-ENW-X4024), Sir Lowry's Pass, Western Cape province, South Africa, collected 1899 by Purcell; four females and two n/a (SAM-ENW-C013476), Fernkloof Nature Reserve, Hermanus, Western Cape province, South Africa, collected by S. R. Daniels and K. Gunkel, 10 June 2023. Four males, three females, and four n/a (SAM-ENW-C013486), Vogelgat Nature Reserve, Hermanus, Western Cape province, South Africa, collected by S. R. Daniels, 25 November 2022. Four males and one female (SAM-ENW-C013475), Caledon, Western Cape province, South Africa, collected by R. Basson and S.R. Daniels, 4 September 2022; three males and three females (SAM-ENW-C013480), collected by H. Barnard and R. Barnard, 24 September 2022, from the same locality. Four males, five females, and one n/a (SAM-ENW-C013487), Oubos, Riviersonderend, Western Cape province, South Africa, collected by R. Basson, S. R. Daniels and F. Gordon, 5 September 2022. Two males, two females, one n/a, and two juveniles (SAM-ENW-C013477), collected by S. R. Daniels and J. A Nieto Lawrence, 22 April 2023, from the same locality. One male, six females, and one n/a (SAM-ENW-C013481), Grootbos, Gansbaai, Western Cape province, South Africa, collected by P. Strauss, January 2022. One n/a (SAM-ENW-C013473), Sandies Glen, Western Cape province, South Africa, collected by R. Basson, 9 September 2022. One male, one female, and two n/a (SAM-ENW-C013478), Napier, Western Cape province, South Africa, collected by F. van Zyl, 8 September 2022. One male (SAM-ENW-C013483), Villiersdorp, Western Cape province, South Africa, collected by J. Durie, 29 April 2023. Four females, four n/a, and two juveniles (SAM-ENW-C013485), Grabouw, Western Cape province, South Africa, collected by J. Durie, 15 May 2023. One n/a (SAM-ENW-C013484), Jonaskop, Western Cape province, South Africa, collected by J. Durie, 2 February 2023.

Diagnosis

GenBank *COI* data

High Noon, Villiersdorp: EU855288–EU855291, Grootbos Private Nature Reserve: EU855344–EU85548, Greyton: EU855340–EU855342, Fernkloof Nature Reserve: EU855336, JN798123–JN798126, Dappat se Gat: JN798096–JN798100, Caledon: JN798101, Jonkershoek: JN798102–JN79805, Kogelberg: JN798106–JN79807, Oubos: JN798108–JN798111, Rondevlei: JN798112, Napier: JN798113–JN798118. Sequence divergence values between *P. lawrencei* and (*P. capensis* and *P. overbergiensis*) are 8.1 and 7.8% respectively (McDonald *et al.* 2012). GenBank *18S* rRNA data: High Noon, Villiersdorp: EU855531, JN798146, Greyton: EU855528, JN798153, Fernkloof Nature Reserve: EU855526, JN798145, Dappat se Gat: JN798151, Caledon: JN798142, Jonkershoek: JN798161, Kogelberg: JN798162, Oubos: JN798157, Rondevlei: JN798159, Napier: JN798160, Grootbos Private Nature Reserve: EU 855532, JN798154. In the combined DNA sequence topology, *P. lawrencei* s.l. formed a genetically distinct and statistically well supported monophyletic clade (McDonald and Daniels 2012). GenBank *COI* Data: OR423143–OR423209. GenBank *18S* rRNA Data: OR421492–OR421504 from the present study. Plus, all GenBank numbers for *COI* and *18S* included by McDonald *et al.* (2012). The uncorrected *p*-distance between *P. capensis* and *P. overbergiensis* was 6.58%; between *P. overbergiensis* and *P. lawrencei* s.s., 7.49%; and between *P. capensis* and *P. lawrencei* s.s., 8.1% (McDonald *et al.* 2012). The combined *COI* + *18S* rRNA tree topology (Fig. 5) shows *P. lawrencei* s.s. to be a statistically well supported monophyletic clade (clade A).

Morphological diagnosis and description

Large variation in dorsal colour surface, ranging from navy blues, to shades of brown and orange. Ventral surfaces are generally lighter, cream or orange, but some specimens have darker shades of red and orange. Dermal papillae are moderately spaced and there are four scale ranks on the papillae structures of the ventral surface, whereas there are seven on the dorsal papillae. There are always 17 leg pairs with two claws on each foot (Table 4).

Measurements

Holotype (male)

Length: 20 mm, Paratypes (four male adults): 12–19 mm (McDonald *et al.* 2012). Specimens from our study were between 7 and 43 mm long and 1.5–6.5 mm wide (Table 4).

Colour

Navy blue, slate black, dark brown, rust orange to lighter oranges. A dark dorsal midline is present with light lateral band above legs along the entire body. Similarly, a darker midline can be seen on the ventral surface of some individuals (Fig. 6*a*, *b*).

Integument

Moderately spaced primary dermal papillae with 2–3 intermittent accessory papillae. SEM: conical or semicircular

dermal papillae with seven scale ranks on the dorsal papillae and four scale ranks on the ventral papillae (Fig. 7*a*, *b*).

Leg pairs

17 leg pairs (Table 4). Dorsal foot surface with ridges. Three complete spinous pads with the fourth being completely to partially fragmented (McDonald *et al.* 2012). Two claws are present on each posterior leg.

Distribution

Restricted to Afromontane forest patches but widely distributed in the south-western part of the Western Cape province, from the Cape flats at Rondevlei, to Grabouw, Oubos, Greyton and Napier. This species is regionally widespread, based on genetic data (Fig. 2, 5). This result negates the observation by Oliveira (2023) that the species name be restricted to the type locality.

Ecology

All specimens were collected under or inside decaying logs or leaf litter, generally close to streams. Occasionally also found under rocks. Restricted to Afromontane forest patches.

Peripatopsis aereus Daniels & Nieto Lawrence, sp. nov.

(Fig. 2, 5, 6*c*, *d*, 7*c*, *d*, Table 4.)

ZooBank: urn:lsid:zoobank.org:act:71276915-79C6-4759-9244-20BEF6303F58

Holotype

(SAM-ENW-C013474), Oubos B, Riviersonderend, Western Cape Province, South Africa, collected by R. Basson, S. R. Daniels and F. Gordon, 5 September 2022.

Paratype

(SAM-ENW-C013479), same information as for the holotype.

Molecular diagnosis

GenBank accession numbers: *COI* Data: OR423210 and OR423211. GenBank *18S* rRNA Data: OR421505. The uncorrected *p*-distance between *P. lawrencei* s.s. and *P. aereus* sp. nov. was 10.62%, and between *P. overbergiensis* and *P. aereus* sp. nov., 10.73%. In the combined DNA sequence topology (Fig. 5), *P. aereus* sp. nov. formed a genetically distinct, statistically well-supported monophyletic clade ancestral to *P. lawrencei* s.s., *P. capensis* and *P. overbergiensis*, among other more derived members of *Peripatopsis*. The *p*-distance between *P. lawrencei* s.s. and *P. aereus* sp. nov. was 5.58% using the *18S* rRNA locus.

Morphological diagnosis and description

Dark-brown colouration with a distinct bronze-like colour on the ventral surface. Specimens with 18 leg pairs, with the posterior pair being reduced in size (Table 4). Two claws are present on each foot, including the reduced posterior legs. Deep ridges present between papillae structures and dermal papillae tightly packed. Ventrally with nine scale ranks on the primary papillae structures, whereas there are fifteen on the dorsal primary papillae (Fig. 7*c*, *d*).

Measurements

Holotype (n/a) length: 38 mm, width: 4 mm; paratype (n/a): length 34 mm, width: 4.5 mm. No additional specimens were available for measurement (Table 4).

Colour

Both specimens were dark brown, with a bronze-like ventral surface. No discernible lines could be seen on either ventral or dorsal surfaces and no other distinct colour variations were visible (Fig. 6*c*, *d*).

Integument

Densely spaced primary dermal papillae, with deeper ridges between papillae structures. Dermal papillae conical with nine scale ranks on the ventral papillae and 15 scale ranks on the dorsal papillae (Fig. 7*c*, *d*).

Leg pairs

18 leg pairs, with the posterior leg pairs being reduced in size. Two claws are present on each leg, including the posterior leg pairs (Table 4).

Distribution

The species is endemic to the Oubos site B on the Riviersonderend Mountains, Western Cape province, South Africa.

Ecology

All specimens were collected under or inside decaying logs or leaf litter, in an Afromontane forest patch.

Etymology

Named ‘aereus’ after the Latin word for bronze, referencing the consistent bronze-like ventral surface that was used to distinguish this species from the sympatric counterpart, *P. lawrencei* s.s.

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Data availability. All novel *COI* and *18S* rRNA sequences have been deposited in GenBank (accession numbers in the results).

Conflicts of interest. The authors declare that they have no conflicts of interest.

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Appendix A1. *COI* haplotype numbers and the number of sequences within each sample locality, corresponding to Fig. 2.

Locality	Haplotype number																																																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
Vogelgat NR	3				1	3	1				2																																														
Fernkloof NR	3	1	1	1				1	3	1																																															
Oubos A and B												13	3		1	1		1																																							
Greyton														1			1			1																																					
Riviersonderend A and B																			1																																						
Jonaskop																				1																																					
Villiersdorp																					1																																				
Grabouw																						1										6	1																								
Kogelberg Biosphere NR																							1	2	2																																
Franschhoek																										3	1																														
High Noon																												3	1																												
Dappat se Gat																														2	3																										
Jonkershoek NR																																		1	2	1																					
Rondevlei NR																																					1																				
Caledon																																						3	1	4	2	2															
Napier																																											1		4	1	1	2	1								
Sandies Glen																																													1												
Grootbos NR																																												1							2	1	1		4	1	1
Linden Hill																																																		1	1			2	1		