Molecular Phylogenetics and Evolution 197 (2024) 108115

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

An ultraconserved element probe set for velvet worms (Onychophora)

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ARTICLE INFO

Keywords:
UCE
Phylogenetics
Systematics
Biogeography
Neopatida

ABSTRACT

Onychophora are cryptic, soil-dwelling invertebrates known for their biogeographic affinities, diversity of reproductive modes, close phylogenetic relationship to arthropods, and peculiar prey capture mechanism. The 216 valid species of Onychophora are grouped into two families – Peripatopsidae and Peripatidae – and apart from a few relationships among major lineages within these two families, a stable phylogenetic backbone for the phylum has yet to be resolved. This has hindered our understanding of onychophoran biogeographic patterns, evolutionary history, and systematics. Neopatida, the Neotropical clade of peripatids, has proved particularly difficult, with recalcitrant nodes and low resolution, potentially due to rapid radiation of the group during the Cretaceous. Previous studies have had to compromise between number of loci and number of taxa due to limitations of Sanger sequencing and phylotranscriptomics, respectively. Additionally, aspects of their genome size and structure have made molecular phylogenetics difficult and data matrices have been affected by missing data. To address these issues, we leveraged recent, published transcriptomes and the first high quality genome for the phylum and designed a high affinity ultraconserved element (UCE) probe set for Onychophora. This new probe set, consisting of ~ 20,000 probes that target 1,465 loci across both families, has high locus recovery and phylogenetic utility. Phylogenetic analyses recovered the monophyly of major clades of Onychophora and revealed a novel lineage from the Neotropics that challenges our current understanding of onychophoran biogeographic endemicity. This new resource could drastically increase the power of molecular datasets and potentially allow access to genomic scale data from archival museum specimens to further tackle the issues exasperating onychophoran systematics.

1. Introduction

Onychophora, commonly known as “velvet worms” or “peripatus,” are soft bodied, many-legged, elongate animals that represent the only exclusively terrestrial phylum (Fig. 1a–e). They predominantly inhabit permanently moist microhabitats to avoid desiccation (Giribet and Edgecombe, 2020; Oliveira et al., 2012). Velvet worms are particularly notable for their unique prey capture mechanism (Baer et al., 2017; Baer et al., 2019; Benkendorff et al., 1999; Haritos et al., 2010), strong biogeographic affinities (Giribet et al., 2018; Monge-Nájera, 1995; Murienne et al., 2014), and their remarkable diversity of reproductive strategies. Ranging from oviparity, through ovoviviparity, to placental viviparity, their reproductive modes have been hypothesized to be involved in their dispersal and subsequent radiation, particularly on Caribbean islands (Anderson, 1973; Baker et al., 2021; Mayer et al., 2015). Additionally, the discrepancy in diversity and disparity between Onychophora, with 216 valid species (Oliveira, 2023), and their sister group Arthropoda (Dunn et al., 2008; Laumer et al., 2019; Rota-Stabelli et al., 2010), comprising ca. 80% of living animal species, raises important questions regarding broad macroevolutionary patterns such as morphological evolution and diversification.

Onychophora are divided into two extant families with strong support from morphological (Reid, 1996) and molecular data (Baker et al., 2021; Giribet et al., 2018; Murienne et al., 2014) that have starkly disjoint distributions (Fig. 1f). Genome gigantism (Jeffery et al., 2012; Sato et al., 2023), low GC content (Mora et al., 1996), complex mitochondrial genomes (Braband et al., 2010a; Braband et al., 2010b; Podsiadlowski et al., 2008), and extremely variable regions in 18S rRNA (Giribet and Wheeler, 2001) have made molecular phylogenetics challenging and multi-locus Sanger data sets almost unattainable.

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https://doi.org/10.1016/j.ympev.2024.108115
Received 20 March 2024; Received in revised form 4 May 2024; Accepted 26 May 2024
Available online 27 May 2024
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Additionally, the cryptic nature of these animals and their low population densities (Daniels et al., 2016) has led to limited taxon sampling. However, what molecular studies have been conducted have helped increase phylogenetic resolution and revealed extensive cryptic speciation particularly within Peripatopsidae (Allwood et al., 2010; Briscoe and Tait, 1995; Oliveira and Mayer, 2017; Sato et al., 2018). Due to these limitations, onychophoran phylogenetics had only been investigated at small scales using molecular data (Allwood et al., 2010; Murienne et al., 2014; Oliveira et al., 2013) until the comprehensive phylogeny of Giribet et al. (2018) and the first phylotranscriptomic interrogation of the group by Baker et al. (2021). Despite these efforts, studies had to manage the trade-off between taxon sampling and number of loci.

Peripatopsid relationships have been relatively stable (Fig. 2b–h) with major clades corresponding to the breakup of Gondwana such as the early division between East Gondwana (Australasia, New Zealand) and West Gondwana (South America, Africa) (Allwood et al., 2010; Baker et al., 2021; Giribet et al., 2018; Murienne et al., 2014; Oliveira and Mayer, 2017; Oliveira et al., 2018; Sato et al., 2018). Furthermore, the reciprocal monophyly of mainland East and West Australian peripatopsids has been found in previous studies (Baker et al., 2021; Giribet et al., 2018; Murienne et al., 2014; Oliveira and Mayer, 2017; Oliveira et al., 2018; Sato et al., 2018). Interestingly, there are two well supported *trans*-Tasman Sea clades found in both Tasmania and New Zealand corresponding to an egg laying clade (*Ooperipatellus*) and a live bearing clade (*Peripatoides, Tasmanipatus, Diemenipatus, Leucopatus*)

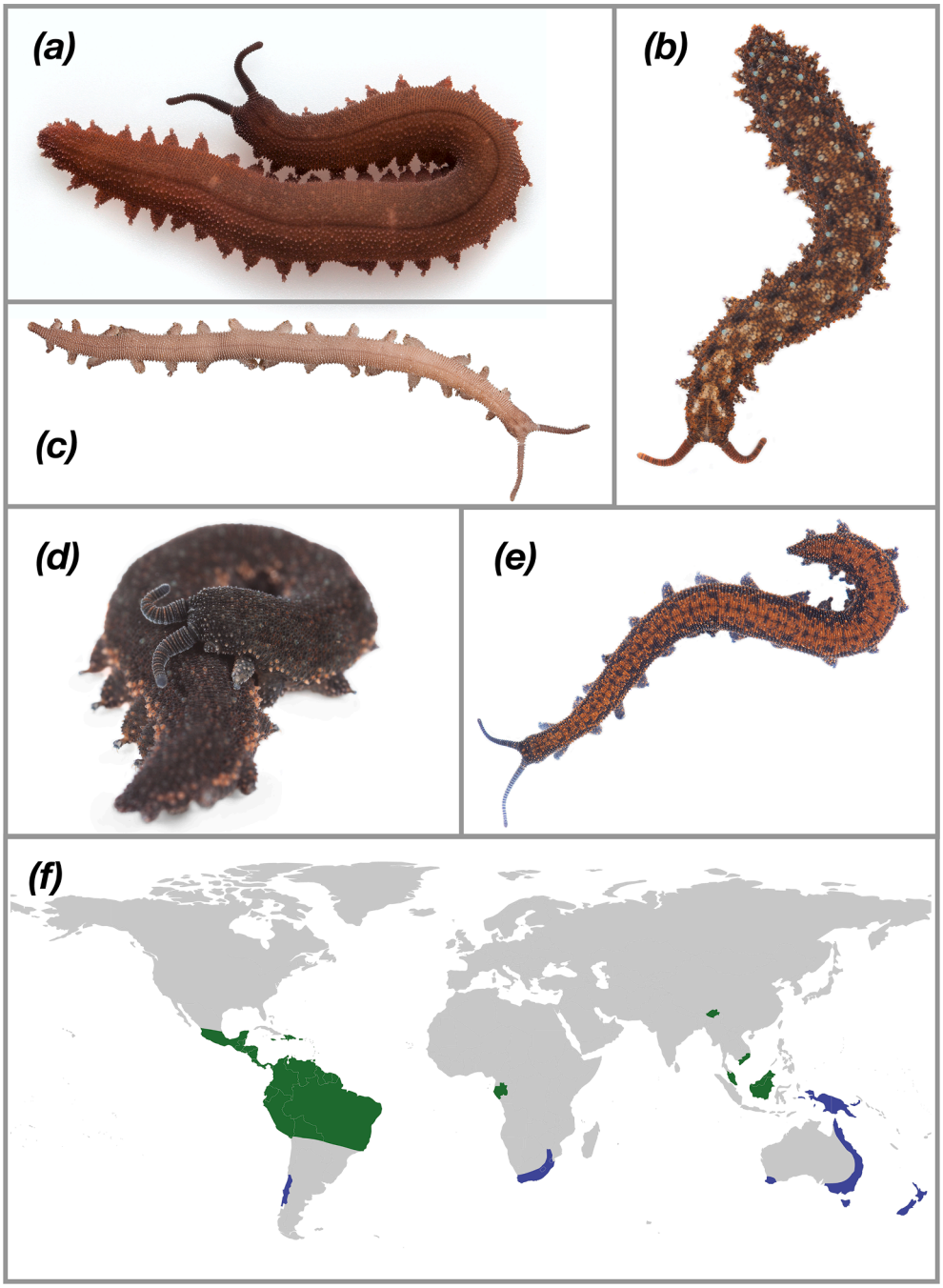

Fig. 1. The habitus of Onychophora. a) *Epiperipatus barbadensis*, Barbados; b) *Ooperipatellus* sp., TAS, Australia; c) *Tasmanipatus barretti*, TAS, Australia; d) *Austroperipatus* cf. *eridelos*, QLD, Australia; e) *Diemenipatus mesibovi*, TAS, Australia; f) map of the distribution of Peripatidae (green) and Peripatopsidae (blue).

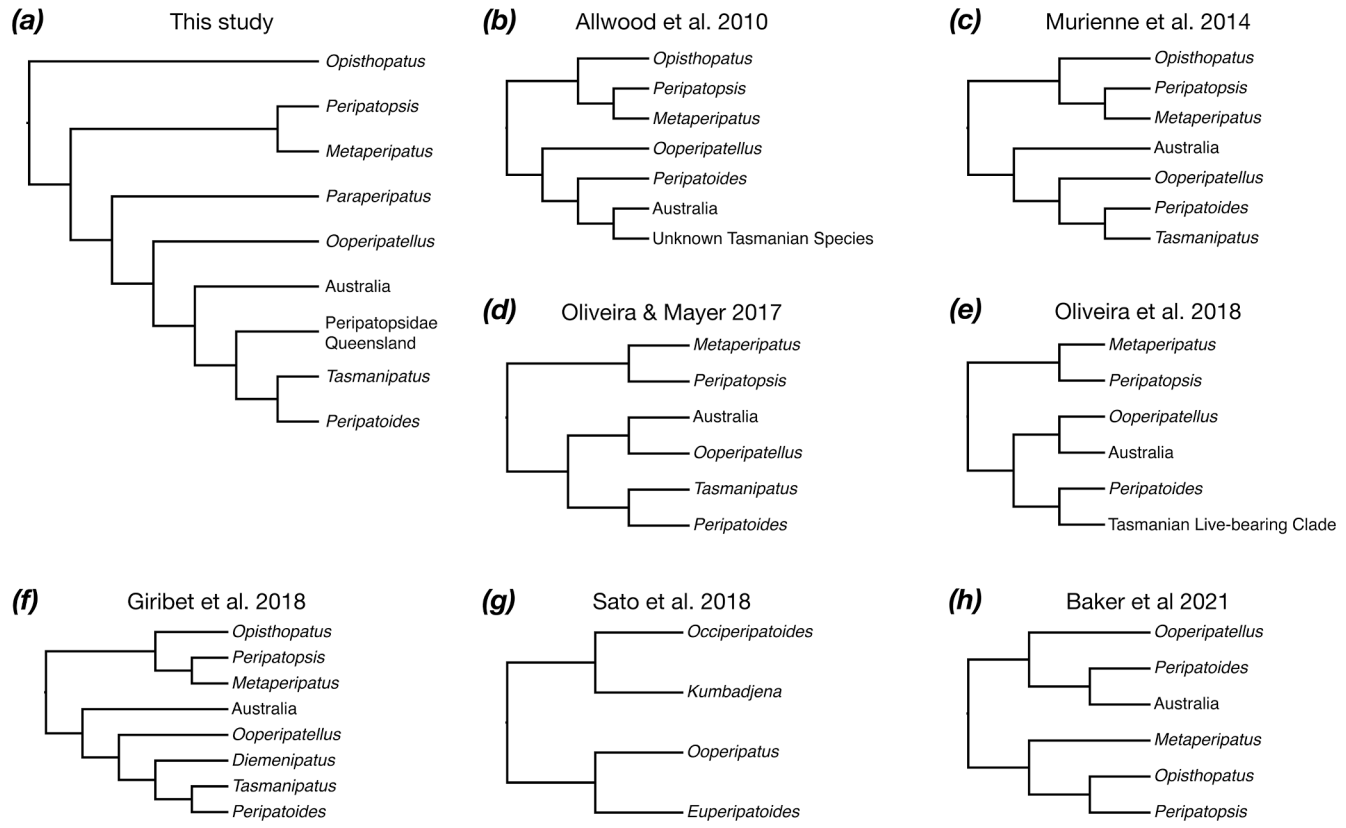

Fig. 2. Summary of the phylogenetic hypotheses within Peripatopsidae found in this study (a), compared to those from previous molecular phylogenetic analyses (b–h). Clades have been collapsed where possible for clarity.

(Baker et al., 2021; Giribet et al., 2018; Murienne et al., 2014; Oliveira and Mayer, 2017; Oliveira et al., 2018).

Peripatid relationships however (Fig. 3) have proved particularly difficult with unstable topologies and low support except for four major groups corresponding to Asia (*Eoperipatus*), Africa (*Mesoperipatus*), and two Neotropical clades (*Oroperipatus*, all other genera) (Baker et al., 2021; Costa, 2016; Giribet et al., 2018). Additionally, the most species-rich genera in Neopatida – the Neotropical clade of peripatids – have been termed "catch-all" genera with little to no support of monophyly (Fig. 3e,f) (Baker et al., 2021; Giribet et al., 2018). Several monotypic genera are also of questionable validity often nesting within these larger genera (Baker et al., 2021; Costa et al., 2021; Giribet et al., 2018). Combined with the complete lack of characters to distinguish the genera of Neopatida (Costa, 2016), this has led to a taxonomic dilemma with a proposed solution as drastic as reverting Neopatida to two historical genera: the "Péripatus andicoles" (= *Oroperipatus*) and "Péripatus

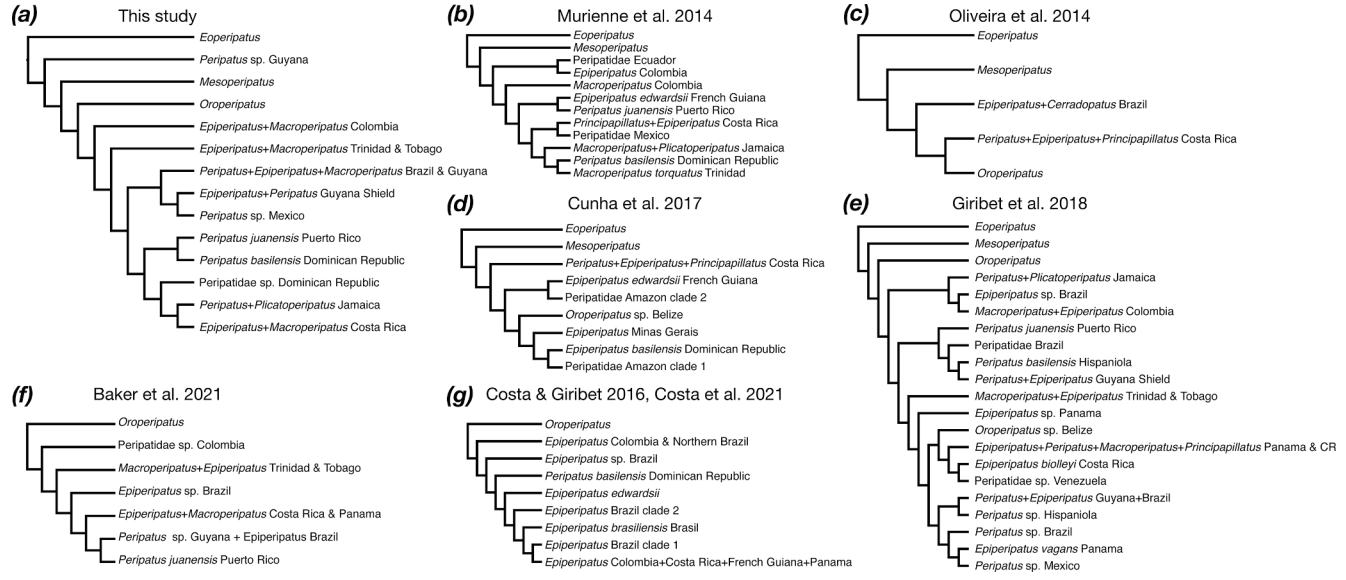

Fig. 3. Summary of the phylogenetic hypotheses within Peripatidae found in this study (a), compared to those from previous molecular phylogenetic analyses (b–g). Clades have been collapsed where possible for clarity.

caraïbes" (= *Peripatus*) of Bouvier (Bouvier, 1899a, b).

Recent advances in hybrid enrichment probe sets (Burrell et al., 2015; Faircloth et al., 2012; Suchan et al., 2016), especially with ultraconserved elements (UCEs), have generated phylogenomic data from standard, ethanol preserved specimens in natural history collections (Derkarabetian et al., 2019) and has developed into the growing field of museomics utilizing historical DNA (hDNA) (Raxworthy and Smith, 2021). Additionally, some recent analyses have found unfiltered UCE data matrices, even at modest gene occupancies (50%), may be better than standard transcriptomic amino acid data matrices at resolving rapid radiations (Chan et al., 2020; Kulkarni et al., 2021). To address these outstanding issues in onychophoran phylogenetics and taxonomy, particularly within Neopatida, we present here the first ultraconserved element probe set for the phylum Onychophora.

2. Methods

2.1. UCE probe set design

A combination of genome and transcriptome data were used to design the probe set. RNAseq data from Baker et al. (2021) were downloaded from SRA and assembled *de novo* using the pipeline from Cunha and Giribet (2019) (Supplementary Table 1). In short, reads were filtered and adaptor-trimmed with Rcorrector v1.0.4 (Song and Florea, 2015) and TrimGalore! v0.6.7 (Krueger, 2021). Ribosomal RNA and mitochondrial DNA sequences were filtered using Bowtie2 v2.3.4 (Langmead and Salzberg, 2012). Reads were assembled *de novo* with Trinity v2.13.2 (Grabherr et al., 2011; Haas et al., 2013). Assemblies were filtered again with Bowtie2 and sequence redundancy was reduced with CD-HIT-EST v4.8.1 (Fu et al., 2012). BUSCO v5.3.2 (Simão et al., 2015) was run against the Arthropoda Orthodb v10 (Kriventseva et al., 2019) to assess transcriptome quality for selection in probe set design (Supplementary Table 1). The high quality genome of *Epiperipatus broadwayi* (GenBank: GCA_028023455.1) (Sato et al., 2023) was used as the base genome for probe design. An additional genome of *Euperipatoides rowelli* was downloaded from NCBI (GenBank: GCA_003024985.2) and a newly sequenced, but fragmented and incomplete genome of *Peripatoides* sp. (unpublished) was included for probe set design.

The PHYLUCE v1.7.1 pipeline (Faircloth, 2016) was used to identify UCE loci and design the probes. Genome and transcriptome fastas were converted to 2bit format using faToTwoBit, a part of the BLAT suite v36 (Kent, 2002). 100bp reads were simulated from 11 "exemplary" taxa (Supplementary Table 1) using ART v2.5.8 (Huang et al., 2012) at 2X coverage with an insert size of 200bp (SD 150bp). Simulated reads were mapped to the base genome using stampy v1.0.31 (Lunter and Goodson, 2011). Unmapped reads were removed using SAMtools v1.16.1 (Danecek et al., 2021; Li and Durbin, 2009) and the resulting BAM files were converted to BED files using BEDtools v2.30.0 (Quinlan and Hall, 2010). Sorting, merging, and removal of repetitive intervals of the mapped reads were conducted using PHYLUCE v1.7.1 scripts.

Following the recommendation of Gustafson et al. (2019), putative UCE loci found in the base genome and only one additional taxon were used for bait design. 160bp sequences were extracted from the resulting putative UCEs and then used to design a temporary bait set with two baits per locus, 3X tiling density, and a length of 120bp. Problematic baits with >25% repeat content, GC content outside 30–70%, and >50% identity with other baits were removed. These duplicate-screened temporary baits were aligned against the base genome, the 11 exemplar transcriptomes, and an outgroup genome, *Drosophila melanogaster* (GenBank: GCA_000001215.4). Baits that were recovered from the base genome and eight other taxa were taken for final probe set design. To reduce the number of probes, those that were found in less than 50% of the taxa in the full transcriptome dataset were removed. Finally, the remaining probes were aligned to the highest quality transcriptome (*Peripatoides* sp.) using BLAST (Altschul et al., 1990) implemented in Geneious (Kearse et al., 2012). Probes that aligned to the same transcript were removed if they were less than 1kb apart. An additional run of CD-HIT-EST (Fu et al., 2012) was run with a cutoff of 97% to reduce redundancy.

2.2. In-silico testing and probe set synthesis

The final probe set was then tested *in-silico* against a full dataset of 34 transcriptomes to determine their utility and recovery of loci. Transcriptome assemblies were used as input to the PHYLUCE script phyluce_assembly_match_contigs_to_probes. Matrix construction and processing was conducted using the standard pipeline in PHYLUCE (Faircloth, 2016). An unpartitioned maximum likelihood analysis was conducted in IQ-TREE v2.2.2 including model testing, tree reconstruction, and branch support assessment with 1500 ultrafast bootstraps (Hoang et al., 2017; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015). The final probe set fasta file was subsequently sent to Arbor Biosciences for synthesis as a custom myBaits probe set.

2.3. Molecular data collection and processing

DNA from 67 specimens preserved in 95% ethanol (Supplementary Table 2) was extracted from oncopods (onychophoran legs) or trunk tissue using the DNeasy Blood and Tissue kit (Qiagen Inc.) following manufacturer protocol. Libraries were prepared using the KAPA Hyper Plus kit (Roche) following manufacturer protocol at half reaction volumes for all steps. Sequence capture followed the standard protocol in the Arbor Biosciences myBaits kit version 5. Reactions were setup with the hybridization temperature to 60 °C but were then incubated for 24h with a touchdown protocol (62 °C for 4 h, 60 °C for 16h, 55 °C for 4 h). The following bead binding and wash steps were also conducted at 60 °C. Final hybridized libraries were amplified with universal Illumina primers and sequenced at 150bp paired-end (PE) reads in an Illumina NovaSeq S4 flow cell at the Bauer Core Facility at Harvard University to at least 2 million reads per sample.

Raw reads were processed, assembled, and aligned into final matrices using PHYLUCE v.1.7.1 (Faircloth, 2016). Adaptor removal and quality filtering was done with illumiprocessor (Faircloth, 2013), a wrapper for Trimmomatic (Bolger et al., 2014). Processed reads were assembled using SPAdes v3.15.5 (Bankevich et al., 2012). Additional genomes were downloaded from NCBI for outgroups (Supplementary Table 3). Contigs matching UCE probes were aligned using MAFFT (Katoh and Standley, 2013) and trimmed with Gblocks (Castresana, 2000; Talavera and Castresana, 2007) using the following settings (−b1 0.5, −b2 0.5, −b3 6, −b4 4). Both steps were run in PHYLUCE v1.7.1. Loci were further filtered using CIAlign v1.1.0 (Tumescheit et al., 2022) to remove divergent sequences (<60% identity) and gaps. A 50% (M1) and 95% (M2) occupancy matrix, determined by the percentage of ingroup taxa only, were created from the resulting loci and concatenated. This means that for a 50% occupancy matrix, loci that are represented at least in 50% of the ingroup taxa were selected, effectively using 50% as the minimal threshold per locus for that matrix.

Locus statistics including length, GC content, and parsimony informative sites were calculated with PhyKIT (Steenwyk et al., 2021) using alignments of all 1,465 loci with outgroup samples removed. Sequences were aligned with MAFFT and trimmed with moderate Gblocks settings (−b1 0.5, −b2 0.5, −b3 6, −b4 4) prior to the calculation of statistics. A second set of statistics were calculated using more stringent Gblocks settings (−b1 0.5, −b2 0.85, −b3 4, −b4 8).

2.4. Phylogenetic methods

Maximum likelihood analyses, including model testing, tree reconstruction, and branch support assessment with 1500 ultrafast bootstraps, were conducted for all matrices in IQ-TREE v2.2.2 (Hoang et al., 2017; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015). The best

partitioning scheme was identified using PartitionFinder (Lanfear et al., 2012), implemented in IQ-TREE using the relaxed hierarchical clustering algorithm (Lanfear et al., 2014). Bayesian analyses were run in ExaBayes v1.5.1 (Aberer et al., 2014) under a GTRGAMMA model on matrices M1 and M2. The Markov Chain Monte Carlo was configured with two runs, each with one cold and three heated chains and run for 10 million generations sampling every 500 until the average standard deviation of split frequencies (ASDSF) was <0.02. Log files were combined with LogCombiner v1.10.4 (Drummond and Rambaut, 2007) and parameter convergence was checked in Tracer v1.7.2 (Rambaut et al., 2018). The first 25% of trees were discarded as burn-in. All trees were rooted between Onychophora and all outgroup taxa and thus conclusions should not be drawn from the relationships of the outgroups. Gene concordance factors (gCF) and site concordance factors (sCF) were calculated using matrix M2 (95% occupancy) in IQ-TREE (Minh et al., 2020; Mo et al., 2023). The resulting values were then plotted in R v4.3.0 (R Core Team, 2021).

2.5. Testing Peripatidae sp. MCZ:IZ:32029

Given the initial unorthodox position of a specimen of Peripatidae sp. (MCZ:IZ:32029) from Guyana (Sato, 2023) (specimen data and images available at: https://mczbase.mcz.harvard.edu/guid/MCZ:IZ:32029), we set out to test the validity of its phylogenetic position and rule out potential contamination sources. The same specimen was resequenced and reads were processed for UCEs. A new 50% occupancy matrix (M3) was created, and a maximum likelihood phylogeny was estimated with IQ-TREE using the methods previously outlined.

2.6. Microscopy methods

Color photos of Peripatidae sp. (MCZ:IZ:32029) were taken using a Canon EOS 5D mark III and MP-E 65mm f2.8 1-5x macro lens along with macro flashes. Images were focus stacked in Photoshop. Prior to critical point drying, tissue samples were transferred to fresh 100% ethanol for 30 min two times to ensure proper dehydration. The tissues were then critical point dried in a Tousimis 931 GL 2.5 and mounted on an SEM stub using biadhesive carbon tape. The samples were then sputter coated in 10nm of Pt/Pd 80/20 using a Quorum Technologies 150T S and imaged using a Zeiss FESEM Ultra Plus at the Center for Nanoscale Systems, Harvard University.

3. Results and discussion

3.1. Design and in-silico testing

A total of 730,129 putative UCEs were shared between the base genome and at least one of the 11 exemplar taxa. The temporary baits designed from this initial pool of potential UCEs recovered 2,720 loci from the base genome and at least eight other taxa. These loci were used to design a draft probe set with 1,547 loci. After duplicate removal and filtering, the final probe set resulted in 19,267 probes targeting 1,465 loci. *In-silico* testing of the probe set using transcriptomes and genomes recovered a topology identical to the 75% occupancy amino acid analysis in Baker et al. (2021) except for the placement of *Epiperipatus* sp. MCZ:IZ:136557 from Amazonas (Supplementary Fig. 1). The *in-silico* test recovered this taxon as sister group to *Epiperipatus* sp. MCZ:IZ:46445 from Guyana, a result never recovered in the original transcriptomic analyses of Baker et al. (2021). However, this taxon was known to be rogue in the original transcriptome dataset, and was recovered in several places with most analyses supporting a sister group relationship to the clade from Central America and Puerto Rico-Guyana-Brazil or to just the Puerto Rico-Guyana-Brazil clade (Baker et al., 2021).

3.2. Locus recovery

We were able to recover contigs matching to most loci (>95%) from all samples except *Oroperipatus peruvianus* MCZ:IZ:83623 which was the oldest sample in the dataset, collected in 1994, nearly 30 years before our experiments (Supplementary Table 4). Raw locus recovery was high with the 95% occupancy matrix totaling >700 loci (Supplementary Table 5). Loci trimmed with low stringency Gblocks settings showed substantial variation outside of the core UCE both within genera (Fig. 4a,b) and within species (Fig. 4c,d), across both families. This, in conjunction with the resolution within *Plicatoperipatus jamaicensis, Peripatus juanensis,* and *Oroperipatus,* suggests the utility of this probe set for species level phylogenetic analyses.

Loci trimmed with moderate Gblocks settings (−b1 0.5, −b2 0.5, −b3 6, −b4 4), averaged 747 bp in length with a GC content of 29%. The raw alignment of ingroup samples averaged 75% parsimony informative sites per locus totaling 779,239 sites. More stringent Gblocks trimming (−b1 0.5, −b2 0.85, −b3 4, −b4 8) drastically reduced these values to an average length of 231 bp, 36% GC content and 59% parsimony informative sites per locus. Due to this loss of information, we conducted phylogenetic analyses with moderate Gblocks settings as more stringent trimming of UCEs is known to have potentially negative downstream effects (Bossert et al., 2021; Portik and Wiens, 2021).

3.3. Phylogenetic reconstruction

Maximum likelihood and Bayesian analysis of matrix M1 recovered identical topologies (Supplementary Figs. 2, 3). For clarity, they will be referred to together as topology M1 (Fig. 5). All nodes were found with high support (bootstrap support [BS]≥95%, posterior probability [PP]= 1) except for the clade consisting of the Guyana Shield group, two Brazilian samples, and a *Peripatus* from Mexico (BS=44%, PP=1). An additional clade consisting of samples from Puerto Rico, Costa Rica, and Jamaica was found with low support (BS=64%, PP=1). Both maximum likelihood and Bayesian topologies of matrix M2 were nearly identical except in the placement of Peripatidae sp. MCZ:IZ:131426 (Supplementary Figs. 4, 5). All ingroup nodes had high support (BS≥90%, PP=1) except for the sister group relationship between *Peripatus* sp. MCZ:IZ:131331 (from Mexico) and *Epiperipatus edwardsii* (BS=60%, PP=1) and the sister group relationship of Peripatidae sp. MCZ: IZ:131426 (from the Dominican Republic) to a large clade consisting of *Peripatus juanensis,* Costa Rican species, and Jamaican species (BS=56%, PP=1). Maximum likelihood analysis of M3 resulted in a well-supported tree with all nodes receiving moderate support (BS>85%, PP=1) except for the sister group relationship between the Costa Rica + Jamaica clade and *Peripatus juanensis* + a South American clade comprised of MCZ: IZ:131445 (from Guyana), MCZ:IZ:136557 (from Brazil), and MCZ: IZ:131441 (from Brazil) (BS=61%, PP=1).

Gene and site concordance factors of matrix M2 ranged from 0 to 75% and 21–75% respectively (Supplementary Fig. 6,7,8). Low gCF could originate from systematic issues such as gene tree estimation error or could reflect true biological signal from gene tree incongruence (e.g. incomplete lineage sorting) (Lanfear and Hahn, 2024). Gene tree estimation error is particularly exacerbated by short alignment lengths which is known to be an issue with UCE datasets (Camargo et al., 2012; Meiklejohn et al., 2016; Van Dam et al., 2021). Relatively higher sCF values (Supplementary Fig. 8) could suggest the phylogenetic signal is spread across alignments or are masked by noise in individual loci but the lower overall values indicate the data are still affected by conflicting signal. This is not surprising given the conflicting signal found even in phylotranscriptomic datasets with extensive reticulation and radial topology of SuperQ networks (Baker et al., 2021).

3.4. Relationships within Peripatopsidae

Notably the South African genus *Opisthopatus* was recovered as sister

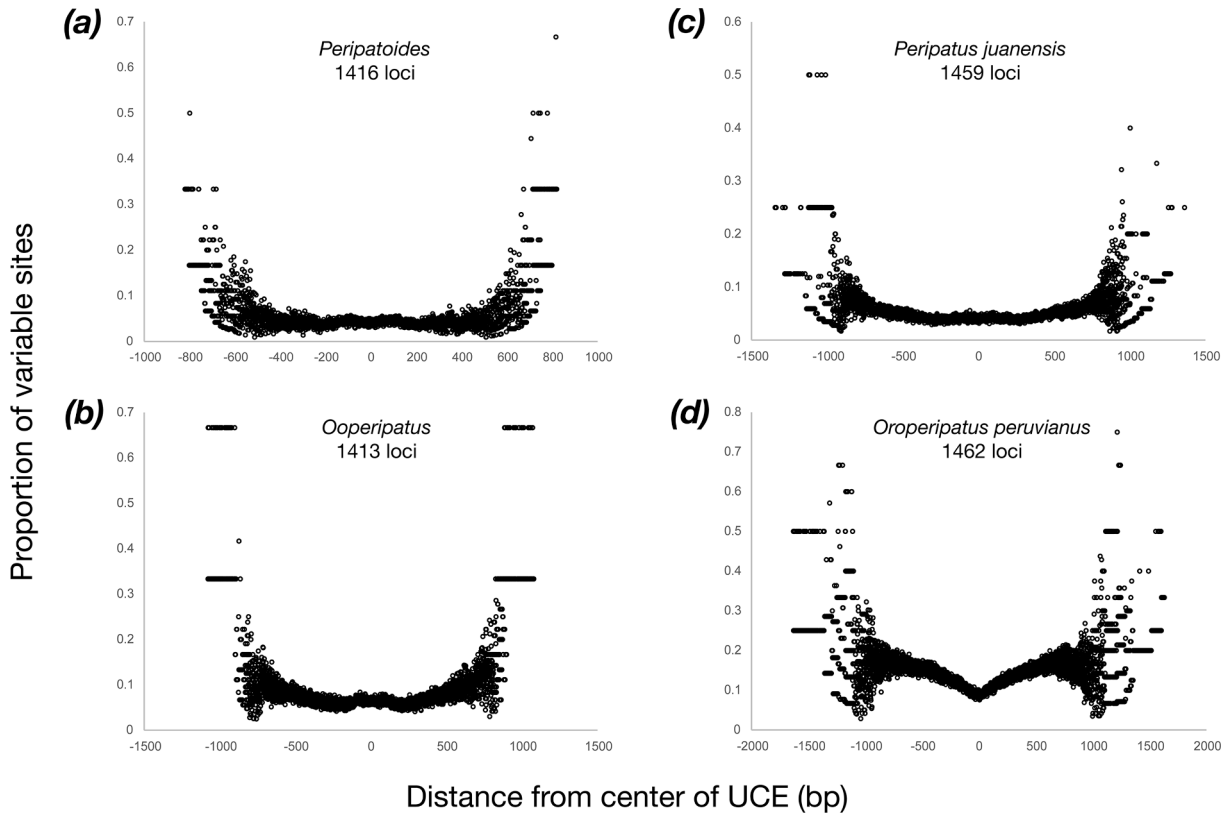

Fig. 4. Smilograms of UCE loci at species and genus level. Variation measured as proportion of variable sites is plotted against distance from the center of the UCE locus. a) all loci recovered for species in the genus *Peripatoides*, b) loci for species in the genus *Ooperipatus,* c) all loci for *Peripatus juanensis,* d) all loci for *Oroperipatus peruvianus*.

group to the rest of Peripatopsidae in all analyses (Fig. 5, Supplementary Figs. 2–5). This is a novel result as all previous molecular analyses including *Metaperipatus* (from Chile), *Opisthopatus,* and *Peripatopsis* (from South Africa) recovered a Western Gondwanan clade with these three genera (Fig. 2b,c,f,h) (Allwood et al., 2010; Baker et al., 2021; Giribet et al., 2018; Murienne et al., 2014). Additionally, the sister group relationship of the Chilean *Metaperipatus* and the South African *Peripatopsis* was found in only one ASTRAL analysis of the 75% amino acid occupancy matrix in Baker et al. (2021) and some Sanger-based studies with variable support (Allwood et al., 2010; Giribet et al., 2018; Murienne et al., 2014). All other phylogenomic analyses to date have found a monophyletic South African clade of *Opisthopatus* + *Peripatopsis*. Non-monophyly of South African taxa has been found in other coeval Gondwanan organisms (e.g. Baker et al., 2020; Derkarabetian et al., 2021; Giribet et al., 2022). In light of the relationships between South America and South Africa in these other dispersal-limited soil taxa, our results could be interpreted as ancestral cladogenesis in West Gondwana prior to the separation of Africa and South America. The deep divergence between the Eastern Gondwanan and Western Gondwanan taxa has been previously estimated to precede the opening of the Atlantic (Baker et al., 2021). However, due to the signal conflict at this node (Supplementary Fig. 6,7) further investigation into the nature of this relationship is needed.

A clade of East Gondwanan peripatopsids (Australasia, including Papua New Guinea, Australia and New Zealand) was recovered with full support in all analyses (BS=100%, PP=1) (Fig. 5, Supplementary Figs. 2–5). The egg-laying *trans*-Tasman Sea genus *Ooperipatellus* appears as monophyletic, and forms either the sister group to all remaining Australian and New Zealand taxa or to the rest of Australasia (including *Paraperipatus*). This result was also found in the phylotranscriptomic analysis of Baker et al. (2020), although that study did not include the New Guinean *Paraperipatus*. This however contrasts with the Sanger-based phylogenetic analyses of Giribet et al. (2018) and Murienne et al. (2014) (Fig. 2c,f), which supported a sister group relationship of *Ooperipatellus* to the live-bearing *trans*-Tasman Sea clade (represented here by *Tasmanipatus* from Tasmania and *Peripatoides* from New Zealand). This could be the result of limited resolving power of Sanger datasets and in fact, the relationship between *Ooperipatellus* and the *trans*-Tasman Sea clade was found with low bootstrap support in the untrimmed peripatopsid only dataset (Giribet et al., 2018). Additionally, the sister group relationship of mainland Australian peripatopsids with the live bearing *Peripatoides* to the exclusion of *Ooperipatellus* was found in transcriptomic data (Fig. 2h) (Baker et al., 2021). The genera *Tasmanipatus* and *Peripatoides* were recovered as sister groups forming a *trans*-Tasman Sea clade, a result corroborating most prior studies (Giribet et al., 2018; Murienne et al., 2014; Oliveira and Mayer, 2017; Oliveira et al., 2018). This live-bearing clade was found to be nested within a larger clade from mainland Australia containing live-bearing as well as egg-laying species (MCZ:IZ131436, the Western Australian clade of *Occiperipatoides* and *Kumbadjena*, and the larger clade containing the genera *Aethrikos*, *Euperipatoides*, *Ooperipatus*, and *Phallocephale*), a result that was suggested by the phylotranscriptomic analysis but not explicitly tested as the dataset did not include representatives from Tasmania (Baker et al., 2021). The mainland Australia clade was further split into a Western Australia clade (*Occiperipatoides* and *Kumbadjena*) and an Eastern Australia clade (*Aethrikos*, *Euperipatoides*, *Ooperipatus*, and *Phallocephale*), corroborating previous analyses (Baker et al., 2021; Giribet et al., 2018; Murienne et al., 2014; Oliveira and Mayer, 2017; Oliveira et al., 2018; Sato et al., 2018).

Topologies M1, M2, and M3 were almost identical with regard to peripatopsid relationships except for the placement of *Paraperipatus* (New Guinea). In analyses of M1 and M3, *Paraperipatus* was recovered as sister group to the rest of the East Gondwana clade whereas analyses of M2 placed *Ooperipatellus* stemwards and *Paraperipatus* as sister group to

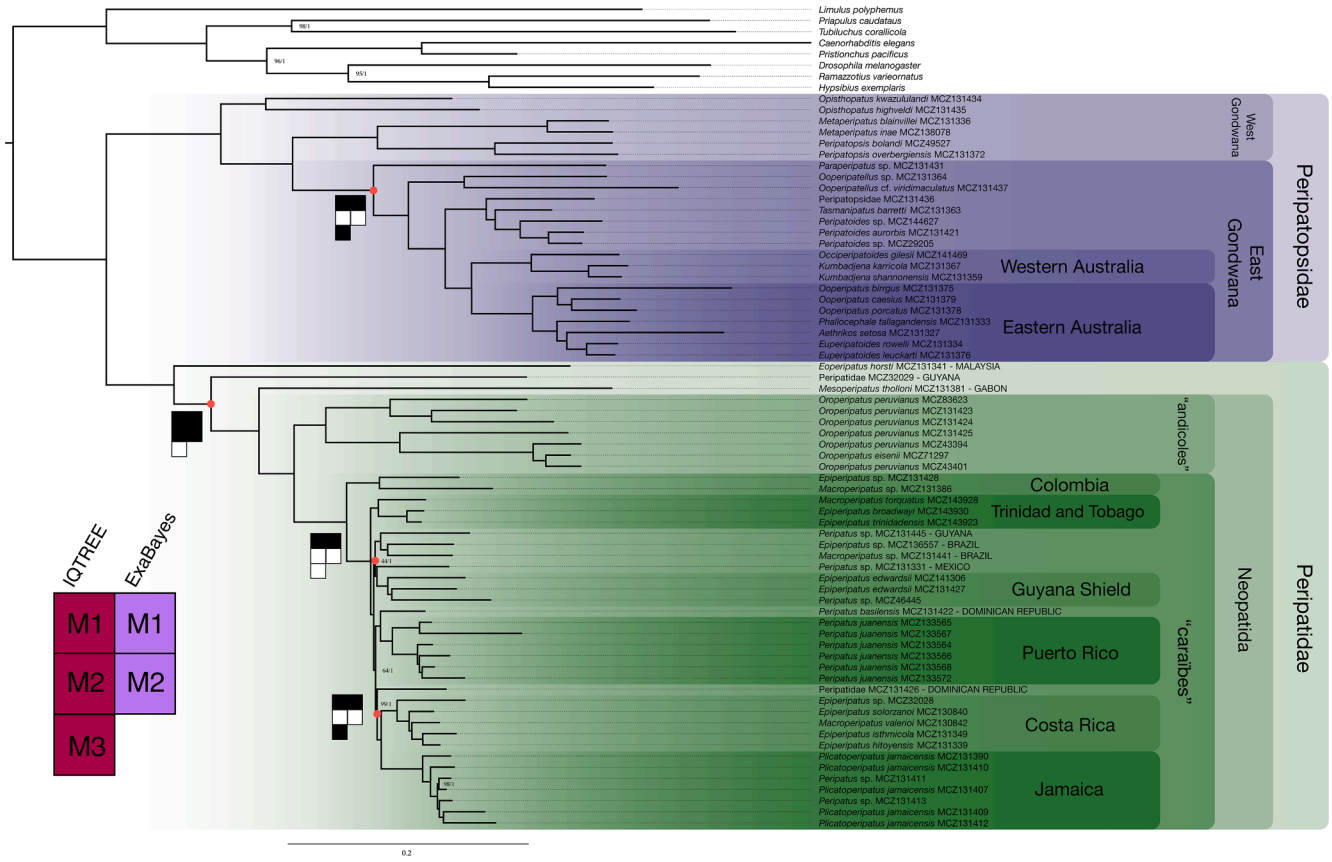

Fig. 5. Summary of phylogenetic analyses plotted on the 50 % occupancy matrix (M1) topology. All nodes received full support (BS = 100 %, PP = 1) in both the maximum likelihood and Bayesian analysis of all matrices unless otherwise noted. Heatmaps represent topological conflict across the five analyses conducted in this study. Clade colors correspond to the distribution map (Fig. 1f).

the rest of the Australia + New Zealand peripatopsids (Supplementary Figs. 2–5,9).

3.5. Relationships within Peripatidae

The evolutionary relationships within Peripatidae have been recalcitrant and poorly supported in previous analyses (Giribet et al., 2018; Murienne et al., 2014) particularly among the Central American, Amazonian, and Caribbean taxa termed the "Eastern clade" (Costa et al., 2021; Cunha et al., 2017; Oliveira et al., 2011; Oliveira et al., 2014a). However, some stable relationships among major clades have been found including an early divergence of *Eoperipatus* from Southeast Asia, followed by *Mesoperipatus* from West Africa, which is sister group to the clade of Neotropical peripatids, the Neopatida (Fig. 3b-e). In the absence of the monotypic Indian genus *Typhloperipatus*, this makes for a split between East and West Gondwana. Additionally, within Neopatida, there is strong support for the division between *Oroperipatus* (Mexico, Galapagos, Andes), and the rest of Neopatida in previous studies (Fig. 3b,d,e,f,g) (Baker et al., 2021; Costa et al., 2021; Giribet et al., 2018; Murienne et al., 2014). This relationship corresponds to the historical division of "Péripatus andicoles" and "Péripatus caraïbes", respectively (Bouvier, 1899a, b). The UCE phylogeny was able to recover these main lineages with strong support (BS≥95%, PP=1) (Fig. 5, Supplementary Figs. 2–5). However, an undescribed Peripatidae sp. from Guyana (MCZ:IZ:32029) was recovered as sister group to *Mesoperipatus* + Neopatida *sensu stricto* (topologies M1 and M2) or as sister group to *Eoperipatus horsti* (topology M3). This sample has never been included in any analysis to date but represents the first contradiction to the stable relationships among the earliest branching lineages within Peripatidae. The non-monophyly of South American or Neotropical taxa has been found in other groups of soil-dwelling, tropical invertebrates with low-vagility (e.g. Benavides et al., 2019; Derkarabetian et al., 2021) and could represent cladogenesis prior to the breakup of Gondwana. Within the problematic Eastern clade, resolution and support was higher than in previous analyses and clades corresponded to geography rather than current taxonomic groupings (Fig. 5) with multiple genera from one region being more closely related than to other members of their respective genera (e.g., in Trinidad and Tobago, Costa Rica or Jamaica).

There was more discordance among peripatid relationships between M1 and M2 with more basal positions of Peripatidae sp. MCZ:IZ:131426 albeit with low support (BS=56%, PP=1) and the clade of three specimens from Guyana and Brazil (MCZ:IZ:131445, MCZ:IZ:136557, MCZ:IZ:131441) (BS=100%, PP=1) in analyses of M2. There were additional slight differences among the relationships in *Peripatus juanensis* between M2 topologies and the other two topologies.

3.6. Peripatidae sp. MCZ:IZ:32029

Resequencing of Peripatidae sp. MCZ:IZ:32029 (Fig. 6a–c) produced poorer libraries indicated by Qubit and Tapestation resulting in fewer recovered loci (1,421 vs 815 raw loci recovery; 1,144 vs 633 loci in matrix M3) (Supplementary Table 4). Inspection of individual alignments revealed the non-zero branch lengths between the two replicates resulted from a handful of spurious alignments and variable regions outside of the core UCE region potentially due to misassembly and poor library construction. Regardless, analysis still recovered this replicate in a basal position with the original sequences (Supplementary Fig. 9). Additionally, the *Peripatus edwardsii* clade (MCZ:IZ:313331, MCZ:IZ:141306, MCZ:IZ:131427, MCZ:IZ:46445) was recovered in a more

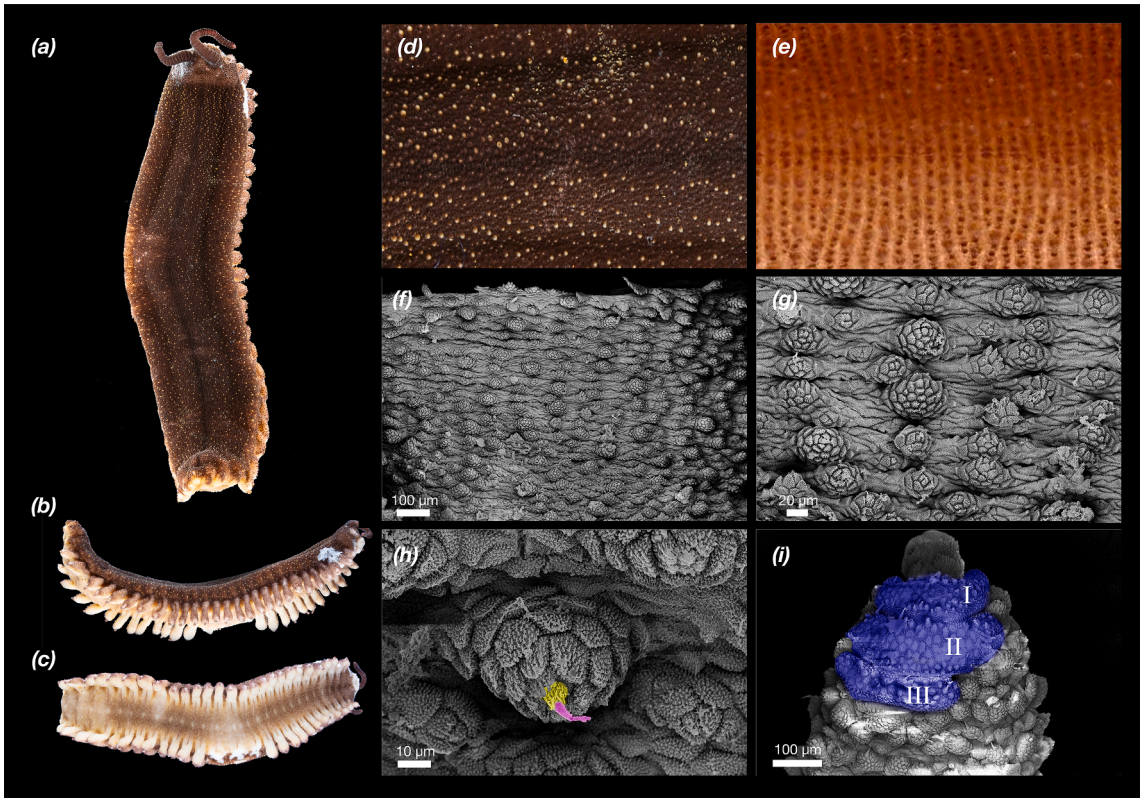

Fig. 6. Light photographs and scanning electron microscopy of Peripatidae sp. MCZ:IZ:32029 from Guyana. a) light image of the dorsal view of the specimen, b) light image of lateral view, c) light image of ventral view, d) light image of dorsal integument showing lack of plicae, e) typical view of plicae found in all Onychophora taken of a live specimen of *Epiperipatus barbadensis*, f) SEM of lack of plicae on dorsal integument, g) detail of primary and accessory papillae on dorsal integument, h) detail of primary papilla, i) detail of spinous pads on oncopod IV. Spinous pads have been numbered. Colors have been added to SEM images to highlight key characteristics: yellow = apical piece, pink = sensory bristle, blue = spinous pads.

basal position subsequently joining the *juanensis* + *basilensis* clade with a small Brazil + Guyana clade (MCZ:IZ:131445, MCZ:IZ:136557, MCZ:IZ:131441).

Examination of Peripatidae sp. MCZ:IZ:32029 with light microscopy revealed peculiar morphological characteristics (Fig. 6). The most striking aspect of the specimen is the lack of annulations or plicae on the dorsal integument (Fig. 6d). Even with scanning electron microscopy, plicae could not be easily delimited (Fig. 6f,g) because they were incomplete and anastomosed, i.e. either fusing or dividing into separate rows. Additionally, the primary papillae were nearly identical to accessory papillae with an inconspicuous apical piece (Fig. 6h). The absence of well-defined primary papillae could account for the lack of clear plicae delimitation in the specimen. All specimens studied to date in a variety of fixation methods have clearly defined rings of papillae (Fig. 6e) which have been taxonomically informative (Oliveira et al., 2014b; Reid, 1996). The specimen was collected using modern and standard preservation methods. Thus, it is unlikely that the unusual plicae morphology is due to preservation artefacts, as specimens collected by the same collectors and using the same methods clearly show plicae (e.g., MCZ:IZ:46445). Analysis of oncopod four revealed the presence of three spinous pads (Fig. 6i), a characteristic shared with *Mesoperipatus tholloni,* the only other peripatid known to have three spinous pads (Costa and Giribet, 2016). The morphological characters displayed by this sole specimen of this putative new lineage appear to be unique or intermediate with regard to Peripatidae, like the inconspicuous apical pieces and the number of spinous pads, respectively.

4. Conclusion

Limitations of previous molecular approaches have restricted our understanding of onychophoran biogeography and evolutionary history, including their radiation across Caribbean islands and the role of their reproductive modes in their diversity. Additionally, their taxonomic history spanning 200 years has led to confusion due to the heterogeneity and inconsistency of the morphological characters used to define genera and species. The advent of next generation sequencing and the emergence of the field of “museomics” have opened new avenues of research into rare and understudied groups. Leveraging these recent advances, we designed the first UCE probe set for Onychophora. This new resource is highly informative at multiple levels from deep divergences within the phylum to species level relationships. Testing of the probe set on a limited set of taxa has shown the potential to resolve the relationships within the particularly problematic Neopatida and has already revealed a new lineage that contradicts long held phylogenetic and biogeographic hypotheses for Peripatidae. UCE datasets have shown utility across a wide range of taxa and we believe this new resource will enable novel avenues of research into this understudied and charismatic group of animals.

Funding sources

This work was supported by the National Science Foundation (grant number 2154245) “Collaborative Research: PurSUiT: Understanding the Neotropical Velvet Worms (Onychophora, Peripatidae, Neopatida), a Cretaceous Radiation of Terrestrial Panarthropods”; MCZ Putnam Expedition Grants to Gabon, Puerto Rico, South Africa and Trinidad & Tobago; and National Geographic Society [9043-11] to Brazil. Published by a grant from the Wetmore Colles Fund.

CRediT authorship contribution statement

Shoyo Sato: Writing – original draft, Methodology, Investigation, Formal analysis. **Shahan Derkarabetian:** Writing – review & editing, Methodology. **Arianna Lord:** Writing – review & editing, Investigation. **Gonzalo Giribet:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Adam Baldinger provided collections support at the MCZ. Computations for this project were done on the Cannon cluster, supported by the FAS Division of Science at Harvard University. SEM was conducted at the Center for Nanoscale Systems. For field companionship, we are indebted to Gregory D. Edgecombe, Michele K. Nishiguchi & Y.-Y. Zhen (Australia, 2000); Stephanie Aktipis & Michele K. Nishiguchi (Australia, 2004); Gregory D. Edgecombe (Australia, 2006); Ligia R. Benavides & Jêrome Y. Murienne (Gabon, 2009); Savel R. Daniels and Benjamin L. de Bivort (South Africa, 2011); Gustavo Hormiga (Brazil, 2012); Gustavo Hormiga & Abel Pérez-González (Chile, 2014); Scott Bartlam & Rosa M. Fernández (New Zealand, 2014); Savel R. Daniels & Christine D. Palmer (South Africa, 2014); Miquel A. Arnedo, Caitlin M. Baker & Rosa M. Fernández (New Zealand, 2016); Ligia R. Benavides, Julia G. Cosgrove & Gustavo Hormiga (Trinidad & Tobago, 2017). Additional specimens were provided by numerous colleagues, without whose help, this work would have not been possible: Ligia R. Benavides (Colombia and Ecuador), Michael G. Branstetter (Costa Rica, Guyana), Rebecca Buckman-Young & Julia G. Cosgrove (Puerto Rico), J.E. Cadle (Peru), C. A. Car (Australia), Roberto Carrera Martínez (Puerto Rico), Gerardo Contreras & Griselda Montiel (Mexico), Savel R. Daniels (Australia and South Africa), Luis Espinasa (Ecuador), Jeremy Huff (Dominican Republic), Milan Janda (Papua New Guinea), John T. Longino from the LLAMA project (Mexico), Diego Luna (Colombia), Luke Mahler (Dominican Republic), Georg Mayer (Australia, Chile, and Costa Rica), Igor V. Muratov (Jamaica), Jêrome Y. Murienne (French Guiana), J. Ochoa Camara (Colombia), Mandy Read (Australia, E. Realpe (Colombia), Gary Rosenberg (Jamaica), Carlos J. Santos (Puerto Rico), Tedd Shultz, U. Meller & John S. Lapolla (French Guiana), Peter J. Schwendinger (Malaysia), D. Seaborg (Jamaica), Diana Tamariz (Puerto Rico), Ana L. Tourinho (Brazil), Sebastián Vélez (New Zealand), Hannah Wood (Australia and New Zealand), M. Zumbado & J.F. Corrales (Costa Rica). Editor-in-chief Guillermo Ortí and two anonymous reviewers provided comments that greatly improved the manuscript.

Collecting permits

All specimens acquired by the authors for this project were collected under valid permits (New Zealand multiple permits [38002-RES]; Australia [QLD #WITK00845202; WA Permits #OF000190, #CE000648, #SF004565]; Chile [Autorización #026/2014]; South Africa [Eastern Cape permits #CRO 108/ 11CR and CRO 109/11CR; KZN #OP 4085/2011; Western Cape Permit #AAA007-00344-0035]; Puerto Rico [#2015-IC-056]; Trinidad & Tobago [Trinidad permit A No. 1176; B No. 000573; Tobago Special Game License without number]. Additional specimens were obtained from Museum loans or from colleagues with additional permits.

Data Statement

New sequences are deposited in the Sequence Read Archive (BioProject PRJNA1076361, SRA Accession Numbers SRR28030502–SRR23080567, SRR28385900). Probe file, individual locus statistics, final matrices, and tree files can be found online at Harvard Dataverse (https://doi.org/10.7910/DVN/MTYWIX).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108115.

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