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Description of a new haemadipsid species of genus *Chtonobdella* Grube, 1866 from Ranomafana National Park using micro-computed tomography

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ABSTRACT

The terrestrial blood feeding leeches of Madagascar represent an understudied group with recent evidence for cryptic diversity. By combining genetic and morphological data including *cox1* molecular barcoding and micro-computed tomography (µCT) imaging, we present evidence for the presence of a new species in the genus *Chtonobdella* Grube, 1866. This description includes, for the first time, µCT rendering of known leech species from Madagascar, which were previously described solely by dissection.

RÉSUMÉ

Les sangsues terrestres hématophages de Madagascar constituent un groupe qui reste encore peu étudié en même temps que des indices récents pointent vers une diversité cryptique. En combinant des données génétiques et morphologiques, incluant le codage moléculaire par le gène *cox1* et l'imagerie par micro-tomodensitométrie (µCT), nous apportons des éléments attestant de la présence d'une nouvelle espèce au sein du genre *Chtonobdella* Grube, 1866. Cette description inclut, pour la première fois, une visualisation µCT d'espèces de sangsues connues de Madagascar, auparavant décrites uniquement par dissection.

INTRODUCTION

The terrestrial leeches of Madagascar (family Haemadipsidae) represent an understudied group, with Blanchard (1917), Harding and Moore (1927), and Richardson (1975) providing the earliest records of their diversity. Early classifications relied on morphological assessment through dissection as well as documented feeding behavior. Historical records are inconclusive in determining dietary diversity of Malagasy leeches, assigned to the genus *Malagabdella* by Richardson in 1975, save for evidence presented by Rocha et al. (2012) of the first record of Malagasy leeches feeding on frogs. This genus has since been synonymized with *Chtonobdella* (Tessler et al. 2016) following subsequent revisions (Borda et al. 2007, Tessler et al. 2016). That only four species are known to science (Borda 2006) is not indicative of their limited diversity, but rather of previously limited collection efforts.

Duognathous (two-jawed) leeches are believed to have colonized Madagascar approximately 66 million years ago during the early Cenozoic (Borda and Siddall 2011). Biogeographic and evolutionary evidence suggest this dispersal from the central Indo-Pacific to have happened after a post-Gondwanan split, allowing for the arrival of potential vertebrate hosts and the northern migration of Madagascar to its current latitude in the tropics (between 12°–25°S), enabling formation of suitable moist habitat for these terrestrial blood-sucking annelids.

Unlike their trignathous (three-jawed) relatives of the Indian subcontinent and Southeast Asia, duognathous terrestrial leeches represent endemic groups throughout the Indo-Pacific, populating such islands as Australia, Indonesia, Papua New Guinea, and Madagascar, and even with species endemic to islands as remote as Palmyra and the Juan Fernandez Archipelago (Borda and Siddall 2011). This island clade represents yet another taxonomic group endemic to Madagascar and contributes to its valued biodiversity.

Terrestrial leeches have emerged as a viable surveying tool for vertebrate biodiversity across their distribution in the tropics (Schnell et al. 2012, Schnell et al. 2018, Tessler et al. 2018, Drinkwater et al. 2021). Targeting host DNA, or invertebrate-derived DNA (iDNA) by metabarcoding leech bloodmeals has enabled researchers to document terrestrial fauna typically overlooked with other inventorying methods. Elucidating the diversity and behavior of terrestrial leeches will help improve survey design and collection methodology of leeches used for burgeoning iDNA analyses.
Here, alongside molecular data, we use morphological evidence generated by micro-computed tomography (µCT) to describe a new species from Madagascar belonging to the genus *Chtonobdella*. The field of taxonomy often benefits from advancements in imaging technology. The advent of finer scale µCT has expanded

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possibilities for morphological description and species delimitation. Micro-computed tomography captures a series of x-ray images from all angles around a specimen and reconstructs these images to produce a digital three-dimensional model. With this technology, the internal morphology of small, soft bodied taxa which are often too delicate to recover via dissection, can be observed and documented while maintaining structural integrity of the specimens. Micro-computed tomography scanning noninvasively characterizes structural morphology at micrometer-level resolution, expanding our understanding of the intricacies of leech anatomy beyond that of traditional dissections (Tessler et al. 2016).

In addition, we use iDNA to document the leeches' feeding behavior, thereby supplying genetic, morphological, and behavioral support for their classification as a species nova. For the first time, we scan leeches from Madagascar previously described exclusively by dissections for an equal comparison of anatomy across species.

METHODOLOGY

SAMPLE COLLECTION. We collected 540 terrestrial leeches from Ranomafana National Park during June 2017 which were dissected and analyzed for biodiversity surveys via iDNA analysis in prior studies (Fahmy et al. 2019, 2020). The collections and exportation of the specimens were made possible with permit number 141/17/MEEF/SG/DGF/DSAP/ SCB.Rc issued by Madagascar's Ministry of Environment and Sustainable Development. Specimens had a portion of the posterior crop region removed, making morphological assessment of diagnostic features such as number and position of testisacs and external papillation not feasible.

DNA SEQUENCING. Each individual leech was sequenced for cytochrome c oxidase subunit 1 (*cox1*), a rapidly evolving mitochondrial gene that has been used to delimit species boundaries in leeches (Apakupakul et al. 1999, Borda et al. 2007, Kutschera and Weisblat 2015, Tessler et al. 2016, Schnell et al. 2018, Tessler et al. 2018, Iyer et al. 2019) and other taxa more generally (Shen et al. 2013). We amplified a 640 bp region of *cox1* by adding the following to GE illustra PuReTaq Ready-To-Go PCR beads: 500 µM of forward primer (LCO1490 5'-GGTCAACAAATCAT AAAGATATTGG-3') 500 µM of reverse primer (500 µM of HHCO1 5'-GCTG CAAAAATRGCAAATACTGC-3') (Folmer et al. 1994), 23µl water, and 2µl DNA template. The thermocycler profile for amplification was 94°C for 1 min, 35 cycles of 94°C for 45 s, 46°C for 30 s, 68°C for 1 min, and a final cycle of 72°C for 7 min (Borda et al. 2008). Amplicons were purified using AMPure in a 0.6:1 ratio to preserve longer fragments (up to 1.8kb). They were then cycle sequenced, ethanol precipitated, and visualized on an ABI 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Resulting sequences had primers trimmed, were reconciled, and edited for quality using CodonCode Aligner (CodonCode Corporation).

PHYLOGENETIC ANALYSIS. We test leech species diversity with *cox1* following published protocols (Tessler et al. 2018). Sequences for leech *cox1* were evaluated phylogenetically in the context of congeners endemic to Madagascar as well as other species of the genus *Chtonobdella* (Table 1). We selected three-jawed terrestrial leeches *Haemadipsa picta* Moore, 1929, *Haemadipsa rjukjuana* (Oka, 1910) Lai, Nakano & Chen, 2011, comb. n., and *Haemadipsa japonica* (Whitman, 1886) as outgroup taxa following the results of Tessler et al. 2016. Sequences were aligned with MUSCLE (Edgar 2004) and the most appropriate model fit to the data with JMODELTEST2 (Darriba et al. 2012). For maximum likelihood analysis we used the selected GTR+I+G model of nucleotide evolution to reconstruct the most likely phylogeny. Maximum likelihood analysis with bootstrapping (1000 replicates) was conducted with RaxML-HPC v.8 (Stamatakis 2014) on XSEDE using the CIPRES Scientific Gateway portal (Miller et al. 2011). Phylogenetic reconstruction was edited with FigTree v.1.4.4 (Rambaut 2010).

Table 1. GenBank accession numbers for *cox1* of specimens used to reconstruct phylogeny. (Cytochrome oxidase 1 (CO1) sequences are available on the National Center for Biotechnology Information's (NCBI) Sequence Read Archive with Accession numbers PV126250 – PV126259)

	Taxon	CO1	Citation
Outgroup	*Haemadipsa picta* Moore, 1929	HQ322472	Lai et al. 2011
	Haemadipsa rjukjuana (Oka, 1910)	HQ322461	Lai et al. 2011
	Haemadipsa japonica (Whitman, 1886)	LC427763	Morishima and Aizawa 2019
Ingroup	*Chtonobdella meyeri* (Blanchard, 1894)	KT968372	Tessler et al. 2016
	Chtonobdella fallax (Blanchard, 1917)	EU100096	Borda et al. 2008
	Chtonobdella niarchosorum (Borda, 2006)	HQ203185	Borda and Siddall 2010
	Chtonobdella seychellensis (Harding, 1913)	EU100094	Borda et al. 2008
	Chtonobdella vagans (Blanchard, 1917)	HQ203186	Borda and Siddall 2010
	Chtonobdella bilineata (Richardson, 1975)	KT968384	Tessler et al. 2016
	Chtonobdella tanae Tessler et al. 2016	KT968382	Tessler et al. 2016

MICRO-COMPUTED TOMOGRAPHY SCANNING AND IMAGE RECONSTRUCTION. In preparation for µCT, six specimens grouping to the new clade of *Chtonobdella* (Figure 1) were fixed in AFA (90 mL of 70% ethanol, 5 mL glacial acetic acid and 5 ml formalin) for 48 h, rinsed in 0.2M phosphate buffer, fixed in 1% osmium tetroxide (OsO_4) for 24 h to improve contrast between soft tissues, and returned to the same buffer solution for 24 h at 4°C (Tessler et al. 2016). Specimens were then returned to ethanol after fixation. We also prepared representative specimens of known species of *Chtonobdella* from Madagascar: *C. fallax* (Blanchard, 1917), *C. vagans* (Blanchard, 1917), and *C. niarchosorum* (Borda, 2006) following the same procedure prior to scanning. Missing from this comparison is *C. morsitans* (Blanchard, 1917) with type specimens deposited at the Muséum national d'Histoire naturelle in Paris. Structural integrity of these specimens has since degraded, and their internal anatomy has not been assessed. The *cox1* region for this taxon has been sequenced and we represent it in the phylogeny, but a morphological comparison is not currently possible.

We subjected all specimens, previously stored in ethanol, to critical point drying (CPD), a procedure which reduces tension on the surface of the specimen while drying in preparation for µCT scanning. All samples were imaged with µCT at the American Museum of Natural History with resolution of 1.5 microns. Scanning was conducted in a GE v|tome|x s240 (General Electric, Fairfield,

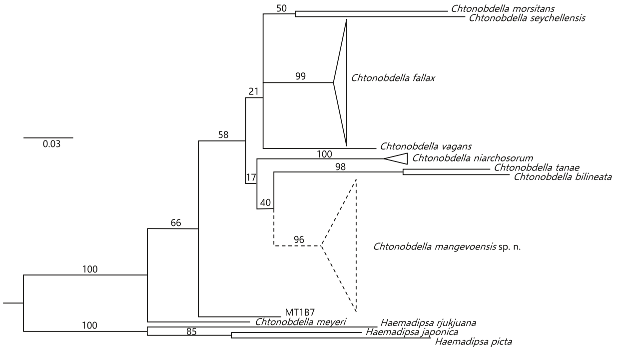

Figure 1. Molecular phylogenetic reconstruction of select members of genus *Chtonobdella* based on *cox1* with maximum likelihood bootstrap support values displayed; *Chtonobdella mangevoensis* sp. n. shown with dashed line.

CT, USA) at 100kV and 200µA with a diamond target and with exposure timing of 500ms. The holotype was scanned at a resolution of 4.82 microns/voxel. Voxel size for the remaining specimens scanned was as follows: *Chtonobdella fallax*: 6.15 microns/voxel, *C. vagans*: 5.95 microns/voxel, *C. niarchosorum*: 5.73 microns/voxel. Variability in voxel size relates to the size and thickness of each specimen (Shafiq-ul-Hassan et al. 2017). Three dimensional reconstructions of scanned images were edited with VGStudio Max 2.2 (Volume Graphics, Heidelberg, Germany) and 3DSlicer, an open-source visualization and segmentation software (Kikinis et al. 2014) and rendered in Blender (https://www.blender.org/).

RESULTS

Ninety-one percent of leeches (484/530) returned sequences for *cox1*, from which 52 were selected for phylogenetic analysis. Additional taxa analyzed are listed in Table 1. Maximum likelihood analysis reconstructed a phylogeny with strong evidence for all specimens pertaining to the new species forming a monophyletic group (Figure 1). The phylogeny also shows branch lengths that are conducive with those of other well-defined species (Tessler et al. 2016; Wang et al. 2022). Micro-computed tomography imagining of all known haemadipsids from Madagascar reflects internal anatomy uncovered previously with dissections (Borda 2006). Diagnostic male and female reproductive apparatus of the new species were easily located and successfully reconstructed and rendered with µCT. External imaging showed location of male and female gonopores (Figure 2) whereas in two-dimensional grayscale of reconstructed µCT images, location of the male gonopore was confirmed at the furrow of the fifth annulus of somite X and first annulus of XI, and female gonopore at the furrow of the fifth annulus of XI and first annulus of XII (Figure 3B). Three-dimensional reconstruction of diagnostic structures of the holotype and of previously described species visually capture morphological differences in shape, size, and location of internal anatomy (Figure 4).

SPECIES DESCRIPTION

Chtonobdella mangevoensis sp. n. (Figures 1–4)

urn:lsid:zoobank.org:pub:6C12E878-636C-41ED-94A7-7863A5F56894

<https://zoobank.org/References/6C12E878-636C-41ED-94A7-7863A5F56894>

Taxonomy: Order: Hirudinida Siddall et al. 2001; Family: Haemadipsidae Blanchard, 1893 Genus: *Chtonobdella* Grube, 1866

Type material: Madagascar, southeastern rainforests of Ranomafana National Park. Specimen collected from pristine primary forest in Mangevo field site (E047°26'50.7", S21°22'33.1"), June 2017. Specimen dissected for iDNA analysis (Fahmy et al. 2019, Fahmy et al. 2020).

Holotype: Dissected, fixed in ethanol, then stained with AFA and osmium tetroxide for µCT scanning. Subjected to Critical Point Drying and stored as a dried specimen at the American Museum of Natural History (Catalog number: AMNH_IZC 00361540).

Paratypes: 28 specimens, 18 fixed in 96% ethanol, 10 fixed in RNAlater. Dissected for iDNA analysis. Collected in Ranomafana National Park, June 2017. All specimens collected by Mai Fahmy, Aimé Tombotiana Victor, and Ny Anjara Fifi Ravelomanantsoa.

Etymology: The species is named for a field site, Mangevo, wi-

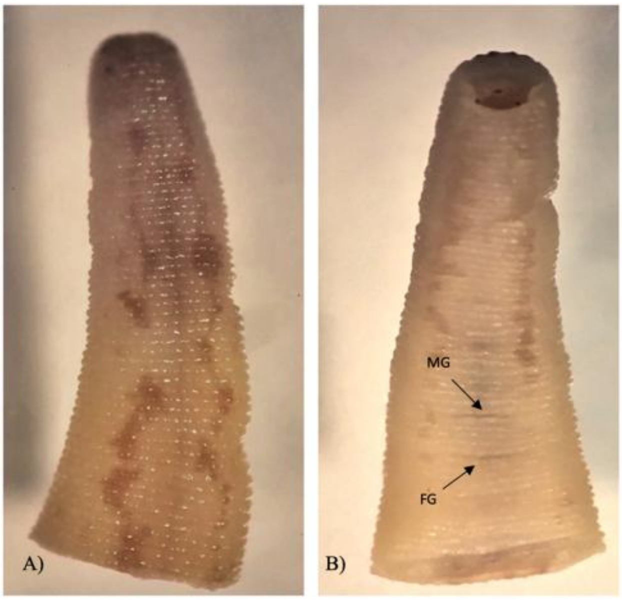

Figure 2. External morphology of holotype. (A) Dorsal view. B) Ventral view with male (MG) and female (FG) gonopores labeled)

Figure 3. Micro-computed tomography sections of the holotype of *Chtonobdella mangevoensis* sp. n. with body somites labeled (IV-XIV). (A) Coronal view showing, ejaculatory bulbs (EB), epididymal masses (EM), vaginal sac (VS), and right ovary (RO). B) Lateral view, showing full length of vaginal sac and positioning of male and female gonopores (MG, FG). Scale bars = 0.5mm)

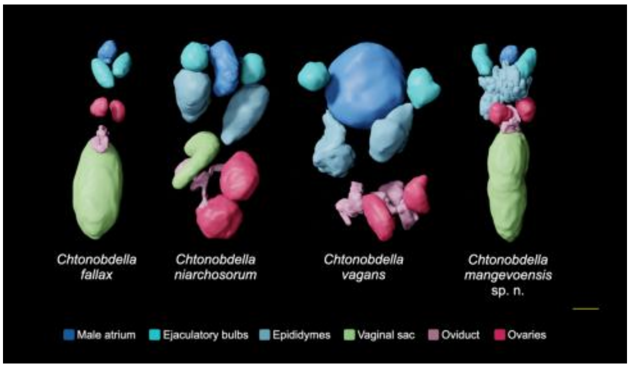

Figure 4. Three-dimensional morphometric rendering of µCT scans of diagnostic reproductive anatomy of Malagasy leeches. (Scale bar = 0.5mm. Image rendered in Blender; https://www.blender.org/)

thin Ranomafana National Park. Based on our collection efforts, it appears the species is locally endemic to this forest.

Description: *External morphology.* Dorsum light beige post fixation with darker longitudinal wavy paramedial stripes (Figure 2A). Venter light beige with darker longitudinal straight paramedial stripes, fading posteriorly (Figure 2B). Somites I-IV one-annulate; V two-annulate, VI and VII three-annulate; VIII to XXIII five- annulate. Gonopores separated by five complete annuli (Figures 2B, 3B).

Internal morphology: Ejaculatory bulbs within X and XI (Figure 3). Vaginal sac at XII, extends posteriorly to between fourth and fifth annuli of XIV (Figure 3). Male gonopore at furrow of the fifth annulus of somite X and first annulus of XI, female gonopore at the furrow of fifth annulus of XI and first annulus of XII (Figure 3B). Specimens had been dissected prior for gut content analysis, therefore posterior morphology including total number of paired testisacs, paired gastric caeca, and number of annuli on the caudal sucker could not be determined. New species is distinguished from other five-annulate leeches by the large vaginal sac extending the length of three somites (Figure 3) and curvature of full, pronounced ejaculatory bulbs (Figures 4, 5), together, not observed in other species in this genus. Large, singular epidydimal mass and micromorphic male atrium distinct in their size from other species in the genus *Chtonobdella*. Dorsal patterns resemble *C. vagans* while ventral patterns resemble *C. fallax* (Borda 2006), yet molecular data and internal structures are distinct from either (Figures 1, 4).

Distribution: Lowland eastern rainforest, Ranomafana National Park, Madagascar. Found at an elevation of 714m.

Natural history: Terrestrial, sanguivorous. Known to feed on *Fossa fossana*, *Atelornis pittoides*, *Gephyromantis redimitus*, *Homo sapiens*, felids, and mongooses as determined by iDNA analysis in prior studies (Fahmy et al. 2020).

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