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Research article

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Integrative description of two new species of Malagasy chirping giant pill-millipedes, genus *Sphaeromimus* (Diplopoda: Sphaerotheriida: Arthrosphaeridae)

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Abstract. The species-rich giant pill-millipedes (Sphaerotheriida) often represent a microendemic component of Madagascar's mega-invertebrate fauna. Of the chirping genus *Sphaeromimus* de Saussure & Zehntner, 1902, ten species have been described. Here, we describe two new species of *Sphaeromimus* integratively, combining light microscopy, scanning electron microscopy, DNA barcoding and micro-CT technology for the first time in a taxonomic description of a giant pill-millipede. *S. kalambatritra* sp. nov. and *S. midongy* sp. nov. are the first giant pill-millipedes collected and described from the mountainous rainforests of Kalambatritra and Midongy. Both species show island gigantism compared to their congeners. Our analysis of the mitochondrial COI gene shows that the two species are related to one another with a moderate genetic distance (9.4%), while they are more closely related to an undetermined specimen from the forest of Vevembe (6.3% and 8.4%). They stand in a basal position with *S. ivohibe* Wesener, 2014 and *S. musicus* (de Saussure & Zehntner, 1897). The four aforementioned species share a high number of stridulation ribs on the male harp. Our micro-CT analysis provides a look into the head of *S. kalambatritra* sp. nov. and shows that non-destructive CT methods are a useful tool for studying the inner morphology of giant pill-millipedes.

Keywords. Biodiversity, COI, island gigantism, Kalambatritra, Midongy.

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Introduction

Madagascar, the world's 4th largest island, lies in the Indian Ocean east of Africa and has a tropical climate (Moat & Smith 2007). While its center and west is mainly covered by dry savanna and dry forest, its east is covered by dense humid rainforests (Moat & Smith 2007). Madagascar is considered one of the world's hottest biodiversity hotspots (Myers *et al.* 2000; Ganzhorn *et al.* 2001) and provides shelter to a high

diversity of endemic species. This high degree of endemism is probably due to its early isolation from Africa more than 150 Ma years ago and from India more than 80 Ma years ago (Wells 2003). Thus 99% of the amphibian species (Glaw & Verdes 2003) and 90% of all species of reptiles (Raxworthy 2003) and mammals (Goodman *et al.* 2003) in Madagascar are endemic. Since the arrival of humans ca 2000 years ago (Dewar & Wright 1993), the landscape of Madagascar has undergone tremendous changes resulting in only 9.9% of the primary vegetation remaining (Myers *et al.* 2000). A key threat to Madagascar's biodiversity is the fast rate of deforestation of habitat (Harper *et al.* 2007). Although several projects and action plans were established in the last decades to protect Madagascar's rich nature, deforestation and fragmentation still takes place at an increasing rate (Horning 2012; Waeber *et al.* 2016). Thus, from 1950 to 2000 the area covered by forests decreased by 40% (Green & Sussman 1990).

One of the highly endemic faunal groups of Madagascar are the millipedes, class Diplopoda (Enghoff 2003). Millipedes play an important role in terrestrial ecosystems as detritivores (Cárcamo *et al.* 2000) and were among the first terrestrial animals, with fossils dating back to the middle Silurian (Shear & Edgecombe 2010). Sixteen extant orders of Diplopoda are known (Blanke & Wesener 2014), of which eight occur naturally on Madagascar (Shelley & Golovatch 2011), with two of them, Polyzoniida (Wesener 2014a) and Siphonophorida (Wesener 2014b), being only recently recorded from the island. Madagascar is host to some of the most spectacular millipedes: different genera of large-bodied pitchblack/bloodred so called “Fire-Millipedes” of the order Spirobolida (Wesener *et al.* 2009a, 2009b, 2011), as well as the world's largest giant pill-millipedes, order Sphaerotheriida (see Wesener & Wägele 2008; Wesener 2009), showing island gigantism. All giant pill-millipedes of Madagascar belong to the family Arthrosphaeridae, otherwise only found in India (Wesener & VandenSpiegel 2009; Wesener *et al.* 2010) and are an example of the interesting biogeographical history of the Malagasy fauna. All species of the family are characterized by unique stridulation organs present in both sexes (see also Wesener 2014c), the male harp and the female washboard. On Madagascar, three genera with altogether 77 species of Arthrosphaeridae, all endemic, can be found (Wesener 2016): the species-rich genus *Zoosphaerium* Pocock, 1895, the dwarfed species of the genus *Microsphaerotherium* Wesener & VandenSpiegel, 2007 and the genus *Sphaeromimus*, whose members have especially well-developed stridulation organs (Wesener & Sierwald 2005). Until 2005 only known from a single specimen, *Sphaeromimus* was thought to be based on a mislabeled Indian specimen (Jeekel 1999), but then two new species and many specimens were described from SE Madagascar (Wesener & Sierwald 2005). Morphological (Wesener & VandenSpiegel 2009) and molecular (Wesener *et al.* 2010) studies suggest that *Sphaeromimus* is more closely related to the Indian genus *Arthrosphaera* Pocock, 1895 than to the other genera in Madagascar. Seven additional species were described integratively in 2014, including the first gigantic species of the genus, reaching the size of a ping-pong ball (Wesener *et al.* 2014). All specimens found so far are from south and south-east Madagascar (Wesener 2016), and inhabit humid lowland, littoral, or montane rainforests, apart from a single species (*S. musicus* (Saussure & Zehntner, 1897)) which is widespread in the spiny forest ecosystem.

Here, we describe two new species of the genus *Sphaeromimus*, both of them “giants”. The species come from previously unsampled regions, the quite remote (only approachable via a hike of >30 km) uniquely transitional forest of Kalambatritra (Irwin *et al.* 2001) and its nearest neighbour, the eastern montane rainforest of Midongy-Sud (Bora *et al.* 2007). We chose to describe both species integratively (Padiál *et al.* 2010), using drawings and scanning-electron microscopy (Oatley *et al.* 1966) combined with genetic barcoding (Hebert *et al.* 2003; Hebert & Gregory 2005). For only the second time in Diplopoda taxonomy (Akkari *et al.* 2015), we also use micro-CT technology (Ritman 2004) to study and visualize internal structures non-invasively.

Material and methods

Abbreviations:

CAS = California Academy of Sciences, San Francisco, USA

FMNH = Field Museum of Natural History, Chicago, USA

ZFMK = Zoologisches Forschungsmuseum Alexander Koenig, Leibniz Institute of Terrestrial Biodiversity, Bonn, Germany

Specimen collection and conservation

The described specimens of *Sphaeromimus* were all obtained from natural history collections and collected by hand during general inventory programs by American museums. The single specimen of the species collected in Midongy (*S. midongy* sp. nov.) by V. Soramalaia in February 2008, was provided by the FMNH. The four specimens from Kalambatritra (*S. kalambatritra* sp. nov.; Fig. 2A), collected by B.L. Fisher and colleagues in February 2009, are from the collection of the CAS.

Dissection and illustrations

The left or the right 1st, 2nd and 9th legs, the anterior and the posterior telopods of the male holotype, and the left or right 2nd leg and the subanal plate of female paratypes (if present) were dissected for drawings. Pencil-drawings were made using a camera lucida mounted on a Zeiss Discovery V8 (Carl Zeiss AG). For both species the antennae and a part of the endotergum of a mid-body segment were dissected for SEM-imaging. From the species found in Kalambatritra, where more specimens were available, the mandible and the gnathochilarium were dissected as well. All objects were dehydrated in an alcohol series (96%, 96%, 100%) and dried overnight. The parts were mounted on stubs and sputter-coated with gold. Objects were removed from the stubs and returned to alcohol after study. SEM-images were obtained with a Supra VR 300VP scanning electron microscope (Carl Zeiss AG) using the SmartSEM V05.00 software (Carl Zeiss AG).

Micro-CT imaging

For a non-invasive visualization of internal structures a micro-CT-scan was performed. The head of the female paratype of *S. kalambatritra* sp. nov. was dehydrated via an ascending ethanol series (96%, 100%) and critical point dried. A micro-CT-scan was conducted using the SKYSCAN 1272 micro-CT-scanner (Brucker microCT, Kontich, Belgium) and the accompanying Control Software v. 1.1.7 (Brucker microCT) with the following settings: source voltage = 60 kV, source current = 166 μ A, exposure = 915 ms, rotation of 180° in rotation steps of 0.2°, frame averaging = 6, random movement = 15, flat field correction ON, geometrical correction ON, filter = Al 0.25 mm. Reconstruction and thermal drift correction was performed in NRecon v. 1.7.0.4 (Brucker microCT) and the images were converted to 8bit grayscale images via Fiji ImageJ v. 1.49k (Schindelin *et al.* 2012). Volume rendering of the dataset for three-dimensional exploration and visualization was done in Drishti v. 2.6.3 (Limaye 2012). The tentorium and nebententorium were segmented automatically and corrected manually in ITK-SNAP v. 3.4.0 (Yushkevich *et al.* 2006).

DNA-extraction and sequencing

Muscle tissue was taken from the male holotypes stored in 80% ethanol and transferred to 95% ethanol. For DNA extraction the DNeasy Blood & Tissue Kit (Qiagen Sample & Assay Technologies) was used. A fragment of the cytochrome *c* oxidase subunit I (*COI*) mitochondrial gene was amplified with the primers HCO2198-JJ and LCO1490-JJ (Astrin & Stüben 2008) using the Multiplex PCR Kit (Qiagen Sample & Assay Technologies) and the Thermocycler Biometra TRIO (Biometra GmbH). The PCR protocol and other methods are the same as those used in earlier studies (Wesener 2015). The PCR-product was enzymatically purified with the Illustra ExoProStar PCR and Sequence Reaction Clean-

Table 1. Samples of species of *Sphaeromimus* de Saussure & Zehntner, 1902, depository, locality, Genbank code, and reliability of the taxonomic identification of the sampled specimen (GenSeq). Genbank numbers marked by asterisk (*) were published in previous studies (Wesener *et al.* 2010, 2014).

Species	Specimen Catalog #	Locality	Genbank #	GenSeq
<i>Procyliosoma leae</i> *	QVMAG 23:45801	Tasmania	FJ409910*	genseq-4
<i>Zoosphaerium neptunus</i> *	FMNH-INS 56005	Madagascar, Andasibe	FJ409929*	genseq-4
<i>Z. alluaudi</i> *	FMNH-INS 56000	Madagascar, Petriky	FJ409926*	genseq-3
<i>Arthrosphaera brandtii</i> *	FMNH-INS 8650	Tanzania, Usambara Hills	FJ409915*	genseq-4
<i>S. musicus</i> 01*	FMNH-INS 56016	Madagascar, Andrahomana	FJ409919*	genseq-4
<i>S. musicus</i> 02*	FMNH-INS 56016	Madagascar, Andrahomana	FJ409920*	genseq-4
<i>S. musicus</i> 03*	FMNH-INS 56008	Madagascar, Mangatsiaka	FJ409921*	genseq-4
<i>S. musicus</i> 04*	FMNH-INS 56212	Madagascar, Tsimelahy	FJ409922*	genseq-4
<i>S. musicus</i> 05 (red)*	ZFMK MYR 2273	Madagascar, Tsimelahy	KJ13244*	genseq-4
<i>S. musicus</i> 06 (red)*	ZFMK MYR 2276	Madagascar, Tsimelahy	KJ13245*	genseq-4
<i>S. splendidus</i> A*	FMNH-INS 6702	Madagascar, Sainte Luce S9	FJ409918*	genseq-3
<i>S. splendidus</i> B*	FMNH-INS 56031	Madagascar, Sainte Luce S9	FJ409917*	genseq-3
<i>S. inexpectatus</i> A*	FMNH-INS 56033	Madagascar, Enato	FJ409916*	genseq-4
<i>S. inexpectatus</i> B*	FMNH-INS 61090	Madagascar, Enato	KJ13246*	genseq-4
<i>S. titanus</i> *	CASENT 9032789	Madagascar, Manombo	KJ13247*	genseq-1
<i>S. ivohibe</i> *	FMNH-INS 8184	Madagascar, Ivohibe	KJ13248*	genseq-1
<i>S. lavasoa</i> B*	FMNH-INS 61143	Madagascar, Gr. Lavasoa	KJ13249*	genseq-2
<i>S. lavasoa</i> C*	FMNH-INS 61142	Madagascar, Gr. Lavasoa	KJ13250*	genseq-2
<i>S. andohahela</i> 01*	FMNH-INS 61135	Madagascar, Isaka-Ivondro	KJ13251*	genseq-2
<i>S. andohahela</i> 02*	FMNH-INS 61137	Madagascar, Isaka-Ivondro	KJ13252*	genseq-2
<i>S. andohahela</i> 03*	ZFMK MYR 2322	Madagascar, Isaka-Ivondro	KJ13253*	genseq-1
<i>S. andohahela</i> 04*	FMNH-INS 61136	Madagascar, Isaka-Ivondro	KJ13254*	genseq-2
<i>S. andohahela</i> 05*	FMNH-INS 61132	Madagascar, Manantantely	KJ13255*	genseq-4
<i>S. andohahela</i> 06*	FMNH-INS 61138	Madagascar, Manantantely	KJ13256*	genseq-4
<i>S. andohahela</i> 07*	FMNH-INS 56210	Madagascar, Malio	FJ409923*	genseq-4
<i>S. andohahela</i> 08*	FMNH-INS 56210	Madagascar, Malio	KJ13257*	genseq-4
<i>S. andohahela</i> 09*	ZFMK MYR 2323	Madagascar, Malio	KJ13258*	genseq-4
<i>S. saintelucei</i> *	ZFMK MYR 889	Madagascar, Sainte Luce S8	KJ13259*	genseq-1
<i>S. andrahomana</i> Cave*	FMNH-INS 56211	Madagascar, Andrahomana	FJ409924*	genseq-4
<i>S. andrahomana</i> *	FMNH-INS 56214	Madagascar, Ankapaky Plateau	KJ13260*	genseq-1
<i>S. sp. ,Vevembe</i> *	CASENT 9032816	Madagascar, Vevembe	KJ13261*	genseq-4
<i>S. kalambatritra</i> sp. nov.	CASENT 9068297-A	Madagascar, Kalambatritra	KY417112	genseq-1
<i>S. midongy</i> sp. nov.	FMNH-INS 3119888	Madagascar, Midongy-Befotaka	KY417113	genseq-1

up Kit (GE Healthcare Life Sciences). Double-strand Sanger sequencing (Sanger *et al.* 1977) of the cleaned PCR product was performed by Macrogen, Netherlands. Sequences were concatenated using Seqman (DNASTAR Inc.). BLAST searches (Altschul *et al.* 1990) were used to check the sequences for contaminations. Sequences were translated into amino acids to rule out the accidental amplification of pseudogenes. New sequences were uploaded to Genbank (see Table 1).

Sequence processing and DNA-analysis

Sequences were aligned by hand in Bioedit (Hall 1999). The final dataset consisted of 33 sequences for 15 species; two of the sequences were newly sequenced and 31 obtained from GenBank (see Table 1), which had been part of two earlier studies (Wesener *et al.* 2010, 2014). The final dataset included 33 sequences with 674 positions. Distance analysis was performed in Mega 6 (Tamura *et al.* 2013) using the uncorrected p-distance model. Variation among sites was modeled with gamma distribution with shape parameter = 1. Included were codon positions 1st+2nd+3rd. Genetic distances are displayed

Table 2. Genetic p-distances between species of *Sphaeromimus* de Saussure & Zehntner, 1902. Variation among sites was modeled with gamma distribution with shape parameter = 1. Included were codon positions 1st+2nd+3rd.

<i>Procyliosoma leae</i> (QVMAG23:45801)	
<i>Zoosphaerium neptunus</i> FMNH-INS56005)	0.199
<i>Zoosphaerium alluaudi</i> (FMNH_INS86000)	0.185 0.148
<i>Arthrosphaera brandtii</i> (FMNH-INS8650)	0.231 0.190 0.178
<i>S. musicus</i> (FMNH-INS56016)	0.244 0.182 0.169 0.193
<i>S. andohahela</i> (FMNH-INS61135)	0.220 0.215 0.210 0.218 0.199
<i>S. lavasoa</i> (FMNH-INS61143)	0.222 0.221 0.201 0.209 0.199 0.083
<i>S. lavasoa</i> (FMNH-INS61142)	0.223 0.221 0.201 0.211 0.200 0.085 0.001
<i>S. andrahamana</i> (FMNH-INS56211)	0.230 0.228 0.211 0.215 0.208 0.101 0.108 0.110
<i>S. inexpectatus</i> (FMNH-INS56033)	0.242 0.205 0.196 0.217 0.186 0.188 0.188 0.189 0.175
<i>S. saintelucei</i> (ZFMK-MYR889)	0.227 0.185 0.181 0.202 0.166 0.181 0.172 0.173 0.170 0.054
<i>S. splendidus</i> (FMNH-INS56031)	0.223 0.228 0.213 0.223 0.212 0.077 0.074 0.076 0.110 0.200 0.181
<i>S. sp.</i> Vevembe (CAS-ENT9032816)	0.230 0.203 0.207 0.199 0.149 0.181 0.178 0.179 0.190 0.192 0.180 0.179
<i>S. ivohibe</i> (FMNH-INS8184)	0.223 0.178 0.189 0.191 0.153 0.191 0.191 0.191 0.202 0.183 0.164 0.190 0.151
<i>S. titanus</i> (CAS-ENT9032789)	0.243 0.200 0.187 0.197 0.163 0.203 0.190 0.191 0.185 0.184 0.163 0.203 0.166 0.171
<i>S. midongy</i> (FMNH-INS3119888)	0.238 0.202 0.217 0.211 0.163 0.211 0.191 0.193 0.197 0.189 0.175 0.196 0.063 0.157 0.165
<i>S. kalambatritra</i> (CAS-ENT9068297-A)	0.235 0.202 0.198 0.207 0.154 0.190 0.181 0.183 0.181 0.190 0.178 0.183 0.084 0.158 0.180 0.094

in matrix format (Table 2). The best fitting substitution-model for maximum likelihood analysis was calculated with Modeltest (Tamura & Nei 1993) as implemented in Mega 6. Codon positions included were 1st+2nd+3rd. The best fitting model was the General Time Reversal (GTR)-Model (Tavaré 1986) with gamma distribution and Invariant sites (GTR+G+I) (lnL = -4010.400, Invariant = 0.5302, Gamma = 1.4196, R = 3.75520307816161; Freq A: 0.2640, T: 0.3345, C: 0.2287, G: 0.1726). Phylogenetic analysis was performed in Mega 6 based on the GTR+G+I model. A species tree was reconstructed using the maximum likelihood method with gamma-distribution of 5 categories. Initial trees were made automatically using NJ/BioNJ. The bootstrap consensus tree was calculated from 1000 replicates (Felsenstein 1985). The obtained tree was edited in Adobe Illustrator CS2.

Distribution map

The localities of specimens were mapped in the free software DIVA-GIS v. 7.5.0.0 (Hijmans *et al.* 2001) on spatial information made freely available by the GADM database v. 1.0 and the Madagascar Vegetation mapping project (Moat & Smith 2007).

Results

Order Sphaerotheriida Brandt, 1833
Family Arthrosphaeridae Jeekel, 1974

Genus *Sphaeromimus* de Saussure & Zehntner, 1902

For a recent revision, see Wesener *et al.* (2014).

Key to the species of *Sphaeromimus*:

1. Male harp on the anterior telopod with 3 stridulation ribs2
- Male harp on the anterior telopod with 4–6 stridulation ribs7

2. Body length <20 mm. Mid-body legs without a coxal lobe. Endotergum with single row of marginal bristles. Immovable finger of posterior telopod slender, apically strongly curved like a hook. Colour pink or brown3
 - Body length >20 mm. Mid-body legs at least with a weak coxal lobe. Endotergum usually with at least two, rarely only one (*S. titanus* Wesener, 2014) row of marginal bristles. Immovable finger of posterior telopod at least basally wide. Colour black or brown4
3. Colour pink, surface shiny. Process of telopoditomere 2 of anterior telopods in anterior view visible laterally. Littoral forest of Mandena and rainforest of Enato*S. inexpectatus* Wesener & Sierwald, 2005
 - Colour light brown, surface dull. Process of telopoditomere 2 of anterior telopods in anterior view not visible. Littoral forest of Sainte Luce, fragment S8*S. saintelucei* Wesener, 2014
4. Body length >30 mm, light brown. Endotergum with single row of marginal bristles. Movable finger of posterior telopod straight (Fig. 2B). Lowland forest of Manombo*S. titanus* Wesener, 2014
 - Body length 21–28 mm, black or dark brown. Endotergum with two rows of marginal bristles. Movable finger of posterior telopod curved5
5. Mid-body legs with strongly developed coxal process. Tergite surface shiny. Littoral forest of Sainte Luce, fragment S9*S. splendidus* Wesener & Sierwald, 2005
 - Mid-body legs with barely developed coxal process. Tergite surface dull6
6. Endotergum with strongly developed cuticular patterns. Movable finger of posterior telopod without small pits, carrying 20–22 crenulated teeth. Andohahela mountain chain, Manantantely, Malio and Isaka-Ivondro*S. andohahela* Wesener, 2014
 - Endotergum with weakly developed cuticular patterns. Movable finger of posterior telopod covered with small pits, carrying 23 or 24 crenulated teeth. Inside Grotte d’Andrahomana and in deep ravines N of Ankapaky*S. andrahomana* Wesener, 2014
7. Unique black pattern on orange-reddish basic colour. Harp with 5 ribs. Endotergum with three rows of marginal bristles. Widespread in the SW spiny forest*S. musicus* (Saussure & Zehntner, 1897)
 - Colour different, either uniformly black or brown. Rainforest species8
8. Harp with 4 stridulation ribs. Endotergum with two rows of marginal bristles, not reaching tergite margin. Operculum well-rounded. Dark brown to black species. Lavasoa Mountain*S. lavasoa* Wesener, 2014
 - Harp with 5 or 6 stridulation ribs. Endotergum with one to three rows of marginal bristles ...9
9. Harp with 5 stridulation ribs (Fig. 5D). Movable finger of posterior telopod with one or two membranous lobes. Marginal bristles of endotergum protruding beyond tergite margin (Fig. 4B) ...10
 - Harp with 6 stridulation ribs (Fig. 9D). Movable finger of posterior telopod with two membranous lobes (Fig. 9G)11
10. Operculum apically recessed. Medium-sized species. Movable finger of posterior telopod with single membranous lobe and 26 crenulated teeth. Eyes with >60 ocelli. Rudimentary lateral palpi consisting of 3 sensilla. Vevembe-Vatovavy area ...*S. vatovavy* Wesener, 2014
 - Operculum well-rounded, typical for genus (Fig. 6A). Gigantic species (>50 mm). Movable finger of posterior telopod with two membranous lobes and 30 crenulated teeth (Fig. 5H). Eyes with >80 ocelli. Rudimentary lateral palpi consisting of 4 sensilla (Fig. 3C). Kalambatritra Rainforest*S. kalambatritra* sp. nov.

11. Small, black species, red legs. Endotergum with single sparse row of very short marginal bristles. Posterior telopod with four spines and 36 crenulated teeth. Ivohibe Mountain
.....*S. ivohibe* Wesener, 2014
– Gigantic (>50 mm), brown species. Endotergum with two dense rows of long marginal bristles (Fig. 9B). Posterior telopod with two spines and 32 crenulated teeth (Fig. 9H). Midongy Rainforest ...
.....*S. midongy* sp. nov.

Sphaeromimus kalambatritra sp. nov.

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Figs 1–6, 11

Diagnosis

Large, massive, brown *Sphaeromimus*, >50 mm long. Differing from all other species of *Sphaeromimus* in having five stridulation ribs on the male harp (as in *S. musicus* (Saussure & Zehntner, 1897) and *S. vatovavy* Wesener, 2014) in the following characters: tarsus of leg 3 without an apical spine, coxal process almost absent, posterior telopod with two membranous lobes. >80 ocelli.

Etymology

The specific epithet ‘kalambatritra’, noun in apposition, refers to the type-locality, the Réserve Spéciale de Kalambatritra (Fig. 11).

Material examined (3 ♂♂, 1 ♀)

Holotype

MADAGASCAR: ♂, Toliaria, Réserve Spéciale Kalambatritra, Befarara, BLF21330, 23°25'04" S, 46°26'52" E, alt. 1390 m, montane rainforest, 7 Feb. 2009, B.L. Fisher *et al.* leg., general collecting (CASENT 9068297-A).

Paratypes

MADAGASCAR: 1 ♂, same collection data as for holotype (ZFMK MYR06125); 1 ♂, same collection data as for holotype (CASENT 9068297-C); 1 ♀, Toliaria, Réserve Spéciale Kalambatritra, Ampanihy, BLF21565, 23°27'49" S, 46°27'47" E, alt. 1270 m, montane rainforest, 9–10 Feb. 2009, B.L. Fisher *et al.* leg., general collecting (CASENT 9058301).

Description

MEASUREMENTS. Male holotype ca 51.5 mm long, 24.4 mm (2nd segment) up to 28.2 mm (10th segment = widest) wide, 14 mm (2nd segment) high. Female ca 52 mm long, 24.7 mm (2nd segment) wide, 15.1 mm (2nd segment) high (Fig. 1A).

COLORATION OF PRESERVED SPECIMEN (Fig. 1A). Tergites in anterior half dark brown, posterior half light brown, posterior margin with thin dark brown band. Paratergites light brown with dark brown to blackish tips. Paratergite impressions and groove of thoracic shield light brown. Antennae brown, and legs and pleurites light brown to grey. Head laterally around eyes and at posterior margin dark brown, frontally light brown. Collum dark brown. Eyes green.

HEAD. Eyes with >80 ocelli, median ocelli small and increasing in size towards lateral and posterior part of eyes (Fig. 1A), several larger ocelli on lateral margin clearly separated from eye. Organ of Tömösváry positioned in antennal-groove (Fig. 1B). Antennae short, protruding to coxa of third leg, not reaching margin of thoracic shield. Antennomere 1 as long as 2+3; antennomeres 2–5 of similar length; antennomere 6 as long as 4+5, but shorter than 1. Antennomeres 1–6 and antennal groove densely

pubescent (Fig. 2A). Antennomere 6 towards disc with single row of sensilla basiconica (Fig. 2B–C). Disc in female with 33/31, in male with 53/55 apical cones, as well as several setae shorter, or as long as, apical cones (Fig. 2B). Margin of labrum with setae.

GNATHOCHILARIUM. Gnathochilarium typical for members of the order Sphaerotheriida, stipes and lamellamentum densely pubescent (Figs 1B, 3A, E). Lateral palpi rudimentary, not distinctly projecting over level of surrounding cuticle, consisting of four sensillae (Fig. 3C). Inner palpi well developed, with field of sensory-cones and scale-like structures (Fig. 3D). Central pads (protuberance of endochilarium) with field of sensory-cones and smaller scale-like structures (Fig. 3E). Endochilarium with deep triangular incision between central pads, central pads projecting lamellamentum nearly to base of inner palpi.

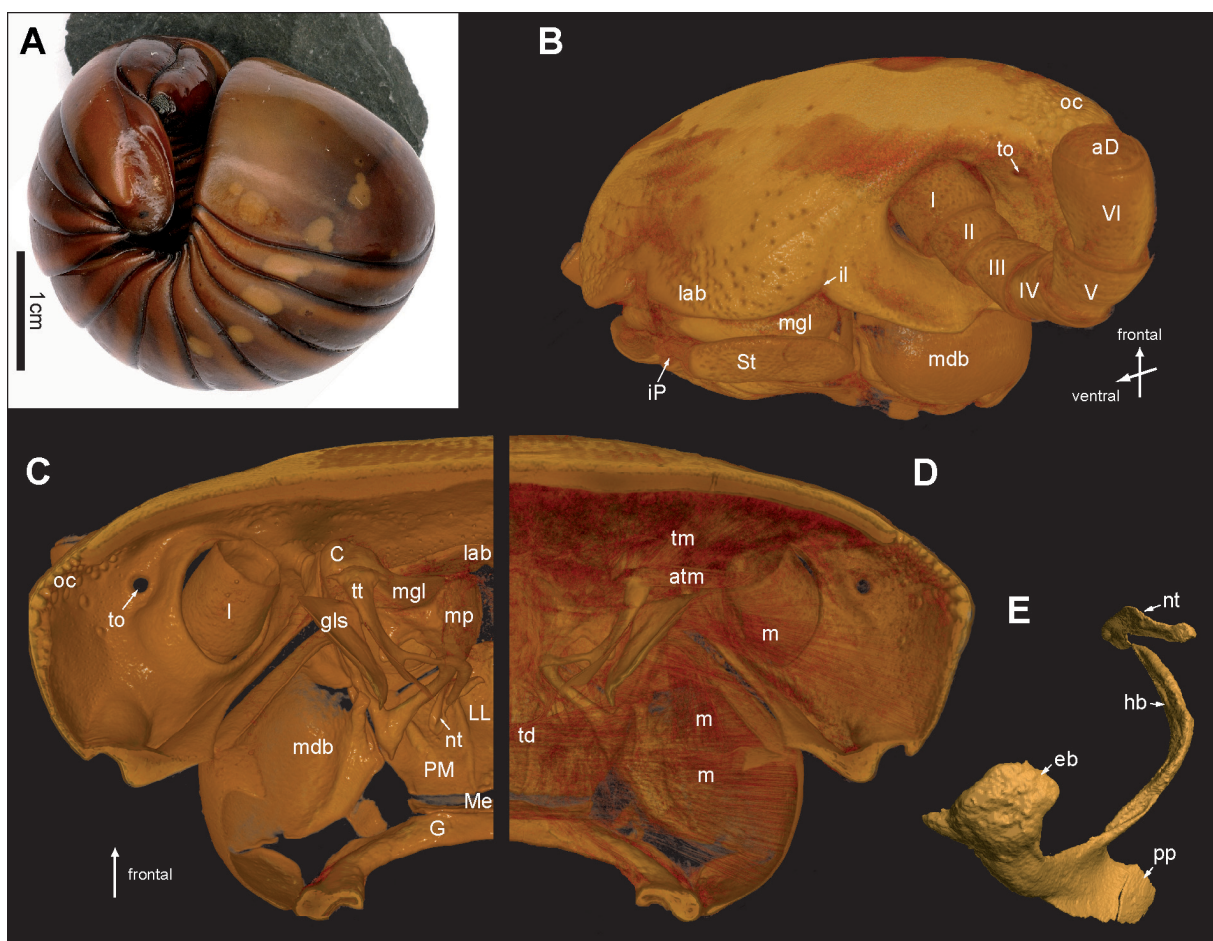


Fig. 1. *Sphaeromimus kalambatritra* sp. nov. **A.** Lateral view. **B–D.** Volume rendering of head based on μ CT-images, female paratype (CASENT 9058301). **B.** Head, outer fronto lateral view. **C.** Head, inner posterior view of sclerotized structures only. **D.** Head, inner posterior view of sclerotized structures and soft structures. **E.** Segmentation of the left tentorium and nebententorium, frontal view. Abbreviations: aD = antennal disc; atm = antennal muscle; C = condylus of mandible gnathal lobe; eb = epipharyngeal bar; G = gula; gls = gnathal lobe sclerite; hb = hypopharyngeal bar; il = incisura lateralis; iP = inner palpi; lab = labium; LL = lamella lingualis; m = mandibular muscle; mdb = mandibular base; Me = mentum; mgl = mandible gnathal lobe; mp = molar plate; nt = nebententorium; oc = ocelli; PM = paramentum; pp = posterior process; St = stipes of gnathochilarium; td = tendon; tm = tentorial muscle; to = Tömösváry organ; tt = tentorium; I–VI = antennomeres.

Lateral endochilarium with densely packed median-pointing setae. Hypopharynx with single row of teeth lateral on both sides, anterior distinctly separated row of 8 teeth (Fig. 3B).

MANDIBLE. Mandible with typical shape of Sphaerotheriida (Fig. 3F), inner tooth 3-combed, with 6 long pectinate lamellae (Fig. 3G), condylus with a single, lower step at its apex (Fig. 3F). Condylus of mandible mounted against cuticular thickening on lateral margin of labrum, anteriorly of antennae (Fig. 1C). Tentorium lacking connection to head capsule via transverse bar. Posterior process triangular and plate-like, laying parallel to plate-like gnathal lobe sclerite. Epipharyngeal bar broad and laminar, running within epipharyngeal wall in direction of mandible condylus, broadening distally. Epipharyngeal bar with short lateral offshoot. Hypopharyngeal bar rod-like, curved within hypopharyngeal wall. Distal tip of hypopharyngeal bar reaches plate-like nebententorium. Nebententorium oriented at right angle to hypopharyngeal bar (Fig. 1E).

COLLUM. Collum glabrous except for few setae at margins.

THORACIC SHIELD. Thoracic shield glabrous, with a chagrinated (leather-like) surface, few setae in grooves. Grooves deep (Fig. 1A).

BODY RINGS. Tergites 3–12 with a chagrinated surface, small hair only present at posterior margin and in grooves, paratergite tips of mid-body tergites strongly projecting posteriorly (Fig. 1A). Tergites with single black locking carina.

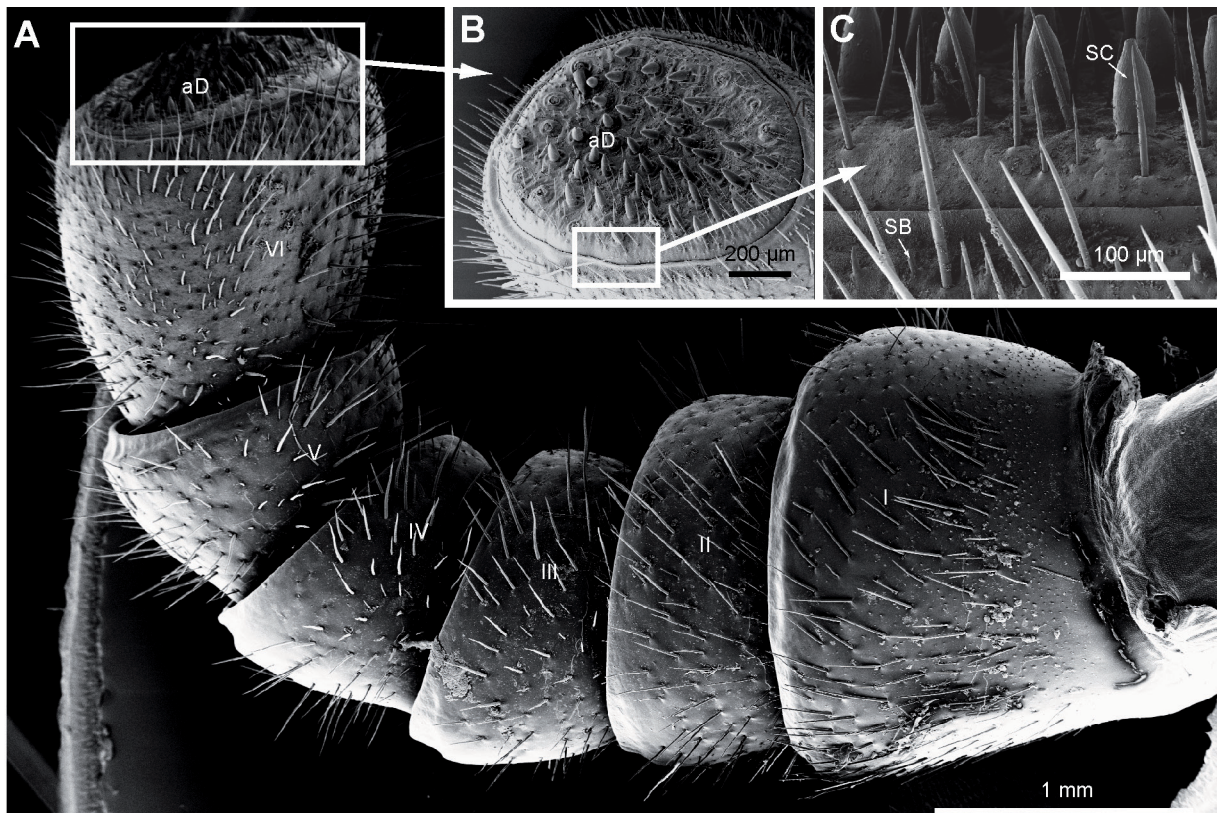


Fig. 2. *Sphaeromimus kalambatritra* sp. nov., SEM of left antenna, ♂, holotype (CASENT 9068297- A). A. Overview. B. Antennal disc, detail. C. Margin of antennal disc, detail. Abbreviations: aD = antennal disc; SB = sensilla basiconica; SC = sensory cone; I–VI = antennomeres.

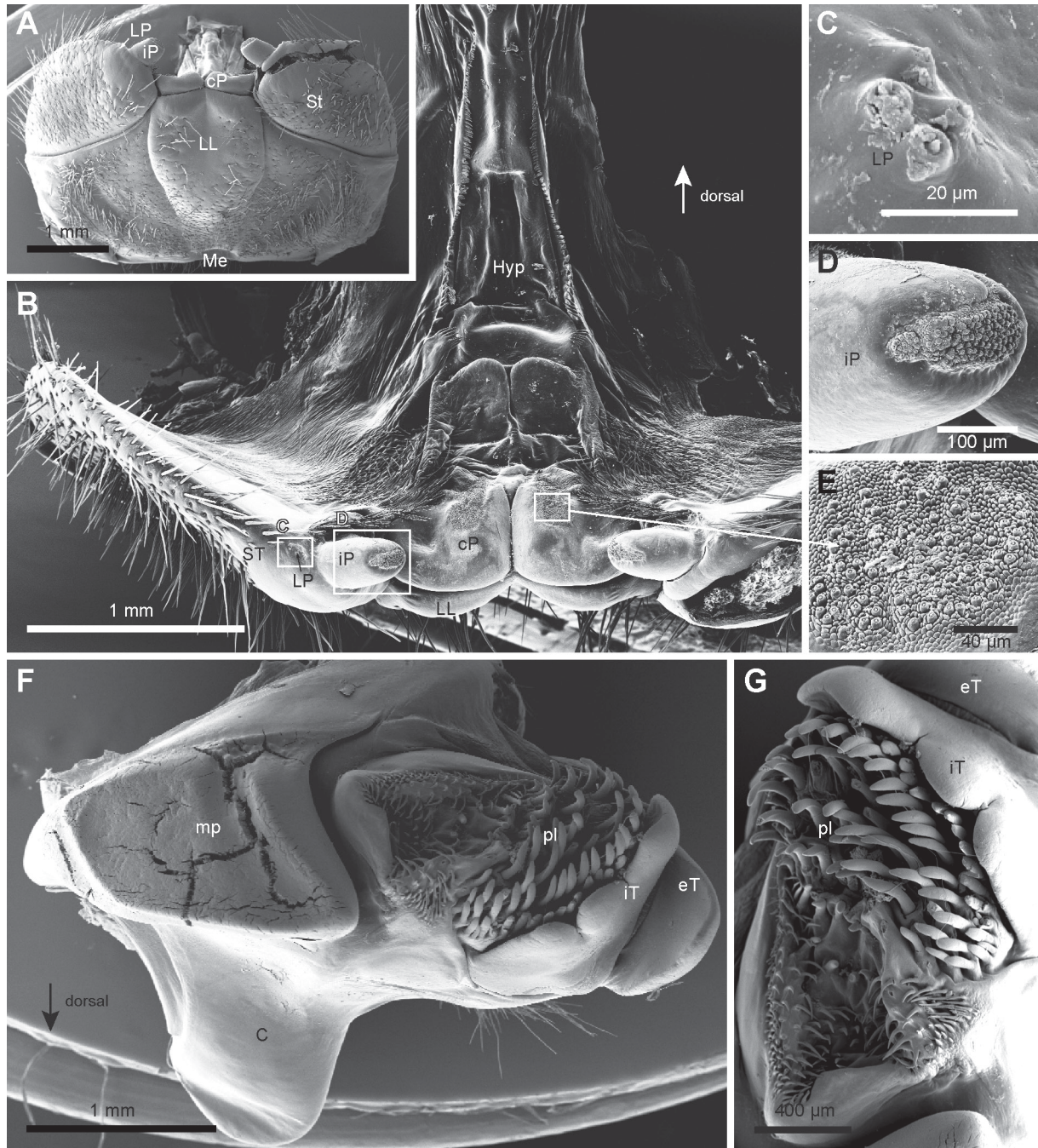


Fig. 3. *Sphaeromimus kalambatritra* sp. nov., SEM of mouthparts, ♂, holotype (CASENT 9068297- A). **A.** Gnathochilarium overview, ventral. **B.** Gnathochilarium overview, frontodorsal. **C.** Lateral palpus, detail. **D.** Inner palpus, detail. **E.** Central pad, detail. **F.** Right mandible, overview. **G.** Pectinate lamellae, detail. Abbreviations: C = condyles; cP = central pad; eT = exterior teeth; Hyp = hypopharynx; iP = inner palpus; iT = inner teeth; LL = lamella lingualis; LP = lateral palpus; Me = mentum; mp = molar plate; pl = pectinate lamellae; ST = stipes of gnathochilarium.

ANAL SHIELD. Anal shield large, with a steep edge, entirely glabrous, with lighter and darker patches (Fig. 1A). Underside with single black locking carina, located closer to tergite margins than to pleurite.

ENDOTERGUM. Endotergum inner section with loose field of short, cone-shaped spines and long setae (Fig. 4A). Externally 2–3 dense rows of long marginal bristles, slightly protruding above margin of tergite. Bristles covered with small, triangular spines, apically increasing in density (Fig. 4B).

STIGMATIC PLATES. First stigma-carrying plate with a well-rounded projecting apex, apex covered with tiny spines and setae (Fig. 5A). Second plate (Fig. 5B) without apex and spines.

FIRST PLEURITE. First pleurite laterally well-rounded, without extensions.

LEGS. Leg 1 with 2, 2 with 4, 3 with 10 ventral spines and no apical spine. Leg pairs 4–21 with 12–16 ventral spines and an apical spine. Small coxa process developed, covered with field of spines (Fig. 5C). Femur 2.0, tarsus 3.3 times as long as wide. All podomeres with few setae (Fig. 5C). Toothed ridge (cleaning comb?) of femur with >40 teeth, reaching ca. 0.8 times length of femur. Coxa in anterior aspect basally with a row of teeth, similar to “cleaning comb” on femur. Pronounced coxal process absent.

MALE GONOPORE. Male gonopore typical for genus, plate glabrous, surrounded by relative large membranous area, few small spines basally of gonopore (Fig. 5B).

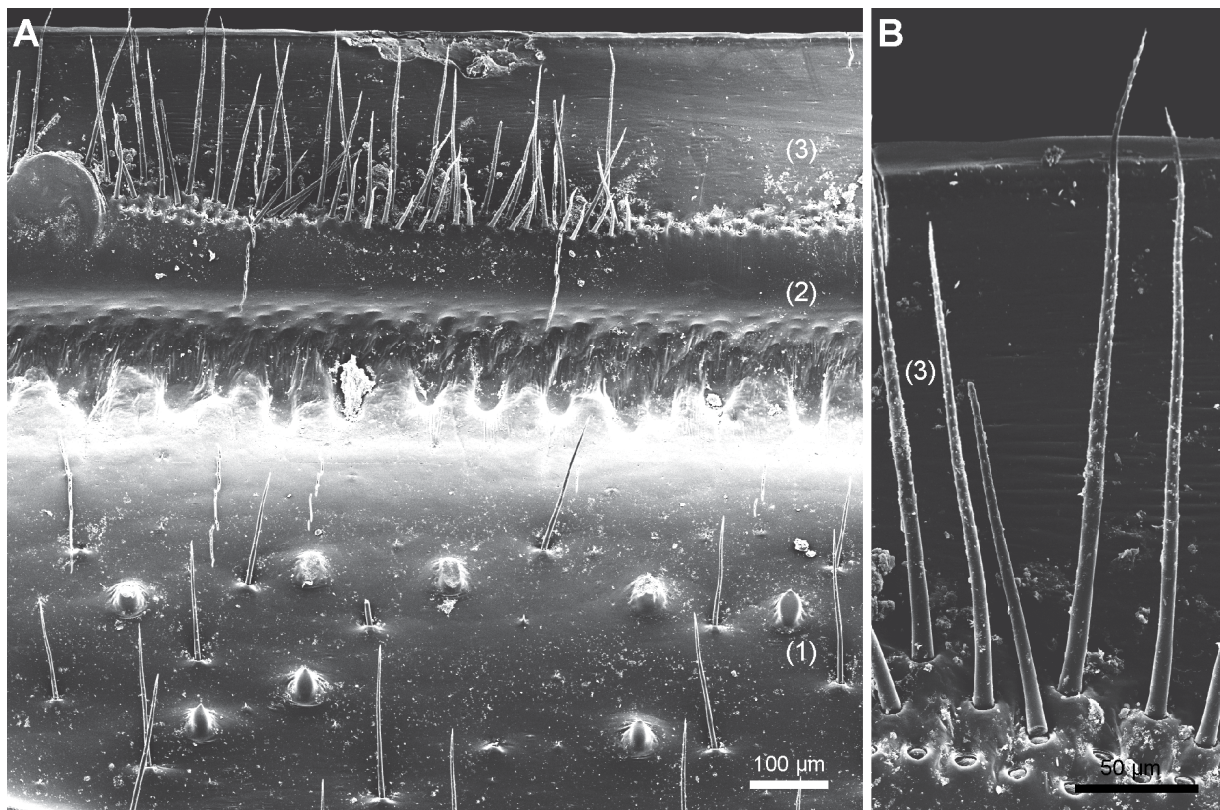


Fig. 4. *Sphaeromimus kalambatritra* sp. nov., SEM of endotergum, ♂, holotype (CASENT 9068297- A). **A.** Overview, endotergum. **B.** Margin of endotergum, detail. Abbreviations: (1) = inner part; (2) = middle part; (3) = outer part.

ANTERIOR TELOPOD. Harp carrying five well-developed stridulation ribs (Fig. 5D). Shape usual for genus, with setae and tiny teeth at apical margin. Telopoditomere 4 with one large, triangular, apically weakly sclerotized spine and 2 smaller ones (Fig. 5E–F); basally on its anterior side with several tiny teeth. Telopoditomere 3 with triangular hump laterally, located close to border to telopoditomere 4 and juxtaposed to process of telopoditomere 2 (Fig. 5E).

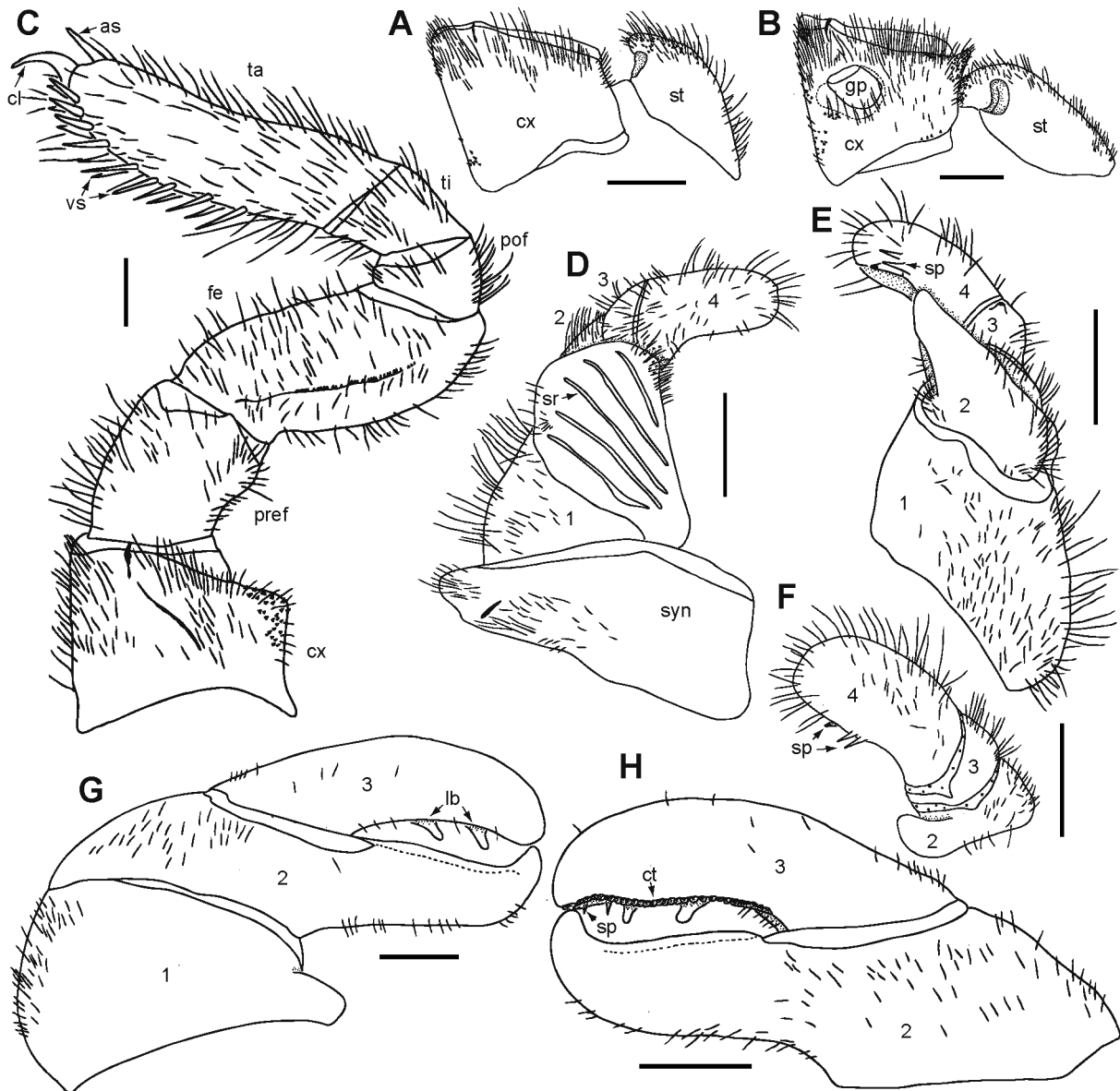


Fig. 5. *Sphaeromimus kalambatritra* sp. nov., schematic drawing, ♂, holotype (CASENT 9068297-A). **A.** 1st left coxae and stigma carrying plate. **B.** 2nd left coxa and stigma carrying plate. **C.** 9th left leg. **D.** Left anterior telopod, anterior view. **E.** Left anterior telopod, posterior view. **F.** Left anterior telopod, lateral view. **G.** Left posterior telopod, anterior view. **H.** Left posterior telopod, posterior view. Abbreviations: as = anterior spine; cl = claw; ct = crenulated teeth; cx = coxa; fe = femur; gp = gonopore; lb = lobe; pof = postfemur; pref = prefemur; sp = spine; sr = stridulation rib; st = stigmatic plate; syn = syncoxite; ta = tarsus; ti = tibia; vs = ventral spine; 1–4 = podomeres. Scale bars = 1 mm.

Etymology

The specific epithet ‘midongy’, noun in apposition, refers to the type-locality, the Parc National de Midongy.

Material examined (1 ♂)

Holotype

MADAGASCAR: 1 ♂, Province de Fianarantsoa, Parc National de Midongy-Befotaka, 11.5 km SW of Befotaka village, VS-1536, 23°53.3' S, 46°53.8' E, 1055 m alt., dense humid transitional lowland and montane forest, 27 Feb. 2008, V. Soarimalaia leg. (FMNH-INS 3119888).

Description

MEASUREMENTS. Male holotype ca 51.5 mm long, 24.1 mm (2nd segment) and 26.1 mm (9th segment) wide, 14.2 mm (2nd segment) high.

COLORATION OF PRESERVED SPECIMEN. Tergites dark brown with black tips, posterior margin with a light brown band which is bordered anteriorly and posteriorly by thinner black bands. Paratergite impressions and groove of thoracic shield lighter brown. Legs, antennae, pleurites, head and collum brown, eyes green.

HEAD. Eyes with >80 ocelli. Antennae short, protruding to third leg pair. Antennomere 1 as long as 2+3; 2 shorter than 3; 3–5 of similar length; 6 slightly shorter than 4+5. Antennomeres 1–6 densely pubescent

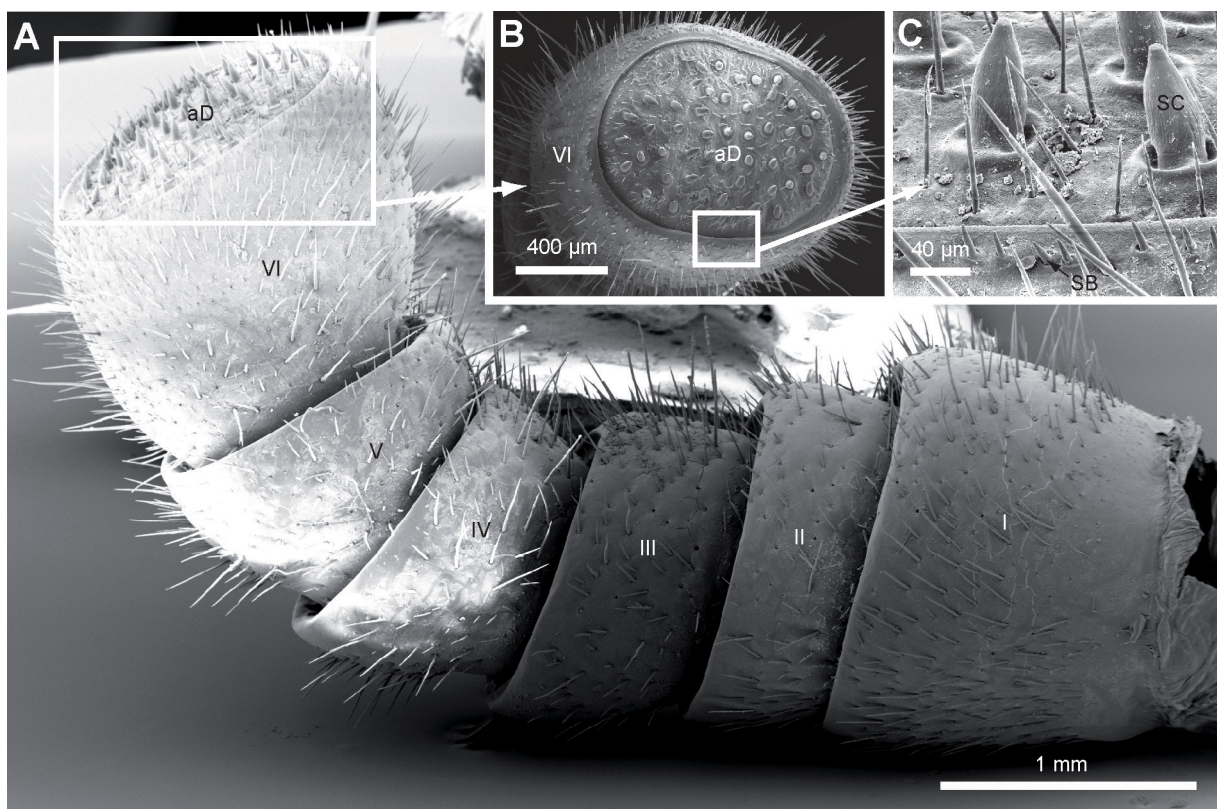


Fig. 7. *Sphaeromimus midongy* sp. nov., SEM of left antenna, ♂, holotype (FMNH-INS 3119888). **A.** Overview. **B.** Detail of antennal disc. **C.** Detail of margin of antennal disc. Abbreviations: aD = antennal disc; SB = sensilla basiconica; SC = sensory cone; I–VI = antennomeres.

(Fig. 7A). Antennomere 6 towards disc with single row of sensilla basiconica (Figs 7B, C). Disc of male with 50/49 apical cones as well as several sensillae shorter or as long as sensory cones (Fig. 7B). Organ of Tömösváry in antennal groove close to eye. Margin of labrum with setae.

MOUHPARTS. Not dissected.

COLLUM. Collum glabrous except for few setae at margins.

THORACIC SHIELD. Thoracic shield chagrinated (leather-like). Grooves deep, covered with setae.

BODY RINGS. Paratergites 3–12 with posterior margin smooth, rest chagrinated, with hairs on posterior and anterior margin, paratergite tips of mid-body tergites strongly projecting posteriorly.

ANAL SHIELD. Anal shield massive, well-rounded, lacking pubescent area. Underside with single black locking carina, located closer to tergite margins than to pleurite.

ENDOTERGUM. Endotergum inner section with setae and triangular spines (Fig. 8A). Between margin and inner area with single row of large, elliptical cuticular patterns. Externally two row of marginal bristles. Bristles protruding weakly above tergite margin. Bristles with small triangular spines, apically increasing in density (Fig. 8B).

STIGMATIC PLATES. First stigma-carrying plate with a well-rounded, projecting apex covered by hairs (Fig. 9A). Second plate without apex, but with field of teeth opposite of coxa (Fig. 9B).

FIRST PLEURITE. First pleurite laterally well-rounded, without extensions.

LEGS. Leg 1 with 4, 2 with 5 ventral spines and no apical spine; leg 3 with 10 ventral spines and a short apical spine. Leg pairs 4–21 with 14 or 15 ventral spines. Coxa process weakly developed, with small, black, triangular spines (Fig. 9C). Femur 1.5, tarsus 3.5 times as long as wide. All podomeres

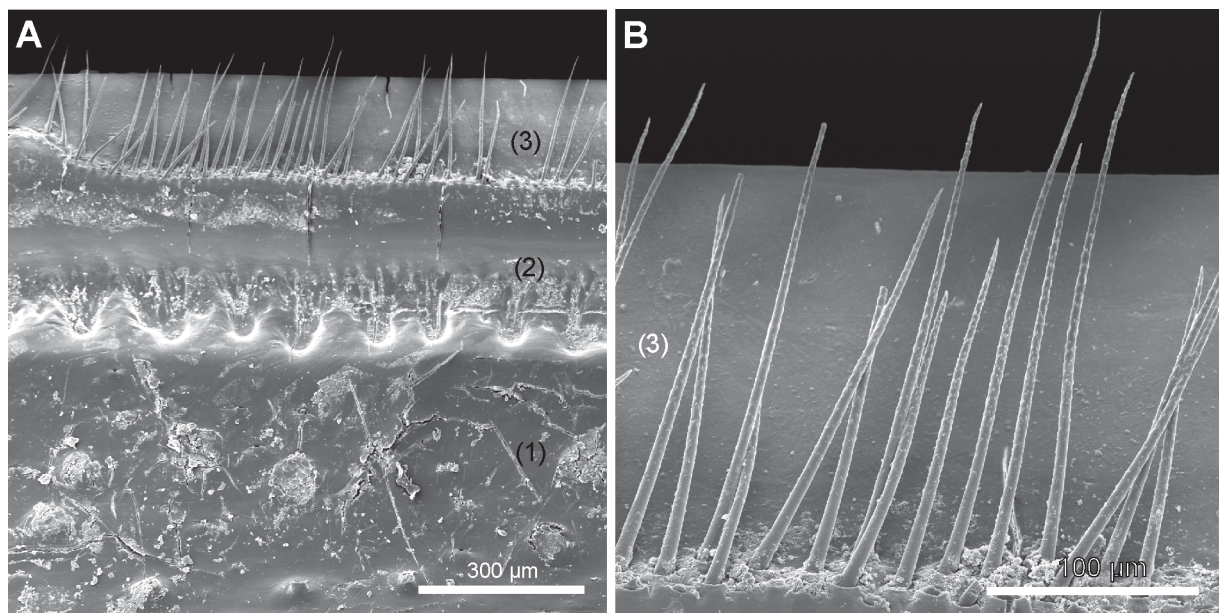


Fig. 8. *Sphaeromimus midongy* sp. nov., SEM of endotergum, ♂, holotype (FMNH-INS 3119888). **A.** Overview, endotergum. **B.** Margin of endotergum, detail. Abbreviations: (1) = inner part; (2) = middle part; (3) = outer part.

densely pubescent (Fig. 9C). Toothed ridge of femur ca $\frac{2}{3}$ length of femur, with 40 small teeth, distally increasing in size. Coxa in anterior aspect basally with a row of teeth, similar to toothed ridge on femur.

MALE GONOPORE. Male gonopore densely pubescent, shape typical for genus (Fig. 9B).

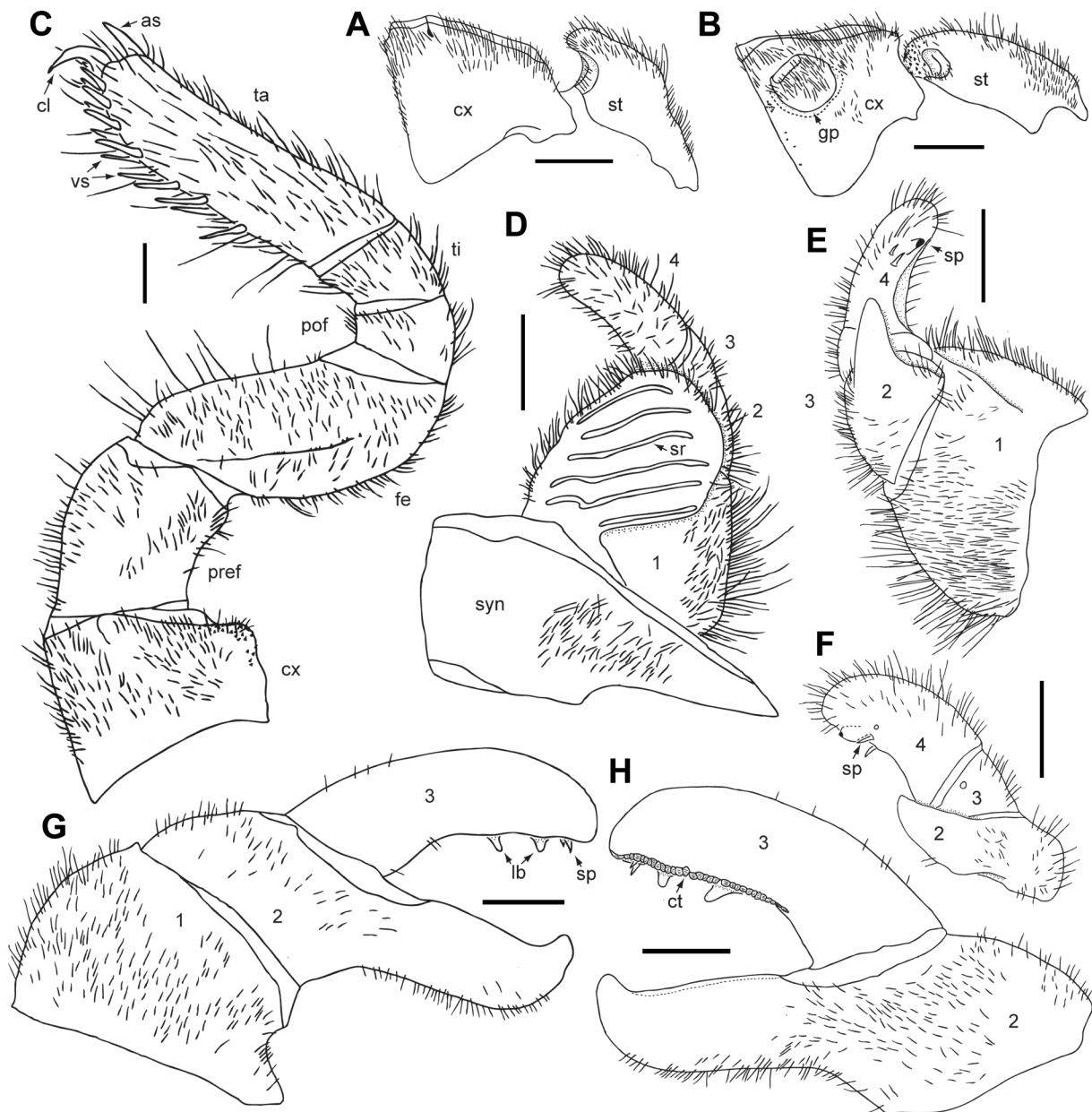


Fig. 9. *Sphaeromimus midongy* sp. nov., schematic drawing, ♂, holotype (FMNH-INS 3119888). **A.** 1st left coxae and stigma carrying plate. **B.** 2nd left coxae and stigma carrying plate. **C.** 9th left leg. **D.** Anterior view of right anterior telopod. **E.** Posterior view of right anterior telopod. **F.** Lateral view of right anterior telopod. **G.** Left posterior telopod, anterior view. **H.** Posterior view of left posterior telopod. Abbreviations: as = anterior spine; cl = claw; ct = crenulated teeth; cx = coxa; fe = femur; gp = gonopore; lb = lobe; pof = postfemur; pref = prefemur; sp = spine; sr = stridulation rib; st = stigmatic plate; syn = syncoxite; ta = tarsus; ti = tibia; vs = ventral spine; 1–4 = podomeres. Scale bars = 1 mm.

ANTERIOR TELOPOD. Harp with six stridulation ribs (Fig. 9D), all ribs of similar length. Shape usual for genus, telopoditomere 4 with one large triangular spine with sclerotized tip and 2 smaller spines (Figs 9E–F). Telopoditomeres 3 and 4 with sclerotized spots.

POSTERIOR TELOPOD. Podomere 3 slightly curved, 3 times longer than wide, slightly longer than immovable finger (Fig. 9G). Hollowed-out inner margin with two lobes and two spines, posterior aspect with ca 32 small crenulated teeth (Fig. 9H). Immobile finger constant in width, only apically tapering, tip weakly curved towards podomere 3. Podomere 1 with setae, podomere 2 with few setae on anterior and posterior side. Podomere 3 and immovable finger with only few marginal setae (Fig. 9G–H).

Female sexual characters

Unknown.

Distribution

Only known from the type-locality, the Parc National de Midongy (Fig. 11). Mountainous rainforest.

Barcoding results

S. kalambatritra sp. nov. shows the lowest genetic distance to the undetermined specimen from Vevembe (Fig. 11), with a 8.4% divergence. The distance of *S. kalambatritra* sp. nov. to *S. midongy* sp. nov. from the only rainforest located close-by is 9.4%. The highest distance of *S. kalambatritra* sp. nov. within *Sphaeromimus* is 19% to both *S. inexpectatus* and *S. andohahela* (Table 2). *S. midongy* sp. nov. also has the lowest pairwise distance to the undetermined Vevembe specimen, with 6.3%, and the highest to *S. andohahela* with 21.1% (Table 1).

The maximum likelihood tree receives high support for nodes at the species level (e.g., 100% for *S. musicus*) and shallower nodes (e.g., 100% for *S. saintelucei* + *S. inexpectatus*), while deeper nodes receive no support (e.g., 39% for *S. titanus* + (*S. kalambatritra* sp. nov. + (*S. midongy* sp. nov. + Specimen from Vevembe)) + (*S. ivohibe* + *S. musicus*)). Branches separating taxa are relatively long (Fig. 10). *Sphaeromimus* is recovered as a monophyletic group with 74% bootstrap support. A sister-group relationship of *S. midongy* with the undetermined specimen from Vevembe receives high support (99%) (Fig. 10). *S. kalambatritra* sp. nov. as the sister-group to *S. midongy* sp. nov. + the undetermined specimen from Vevembe receives 100% bootstrap support. This clade is separated by a long branch from a group comprising *S. ivohibe* and *S. musicus*. Monophyly of those four taxa is supported with 63% bootstrap support (Fig. 10).

Discussion

Updated distribution of *Sphaeromimus*

Including the two newly described species, the genus *Sphaeromimus* is still restricted to southeastern Madagascar. With *S. kalambatritra* sp. nov., we record the western-most distribution of a rainforest species (Fig. 11). *S. kalambatritra* sp. nov. was, as all other species of *Sphaeromimus*, found to live in direct sympatry with at least one species of *Zoosphaerium*.

Micro-CT in taxonomy and phylogeny

Already Akkari *et al.* (2015) showed the great potential of micro-CT in millipede taxonomy. It can be used to visualize and infer internal morphological characters, which can be exploited not only for the species description itself, but also for subsequent morphological phylogenetic reconstruction as demonstrated by Blanke & Wesener (2014). Here, we show that micro-CT is not only a useful tool to examine hard sclerotized structures like the head capsule, the gnathal lobe sclerite and the tentorium (Fig. 1C), but also to visualize soft parts like the collagenous tendon and single muscles (Fig. 1D).

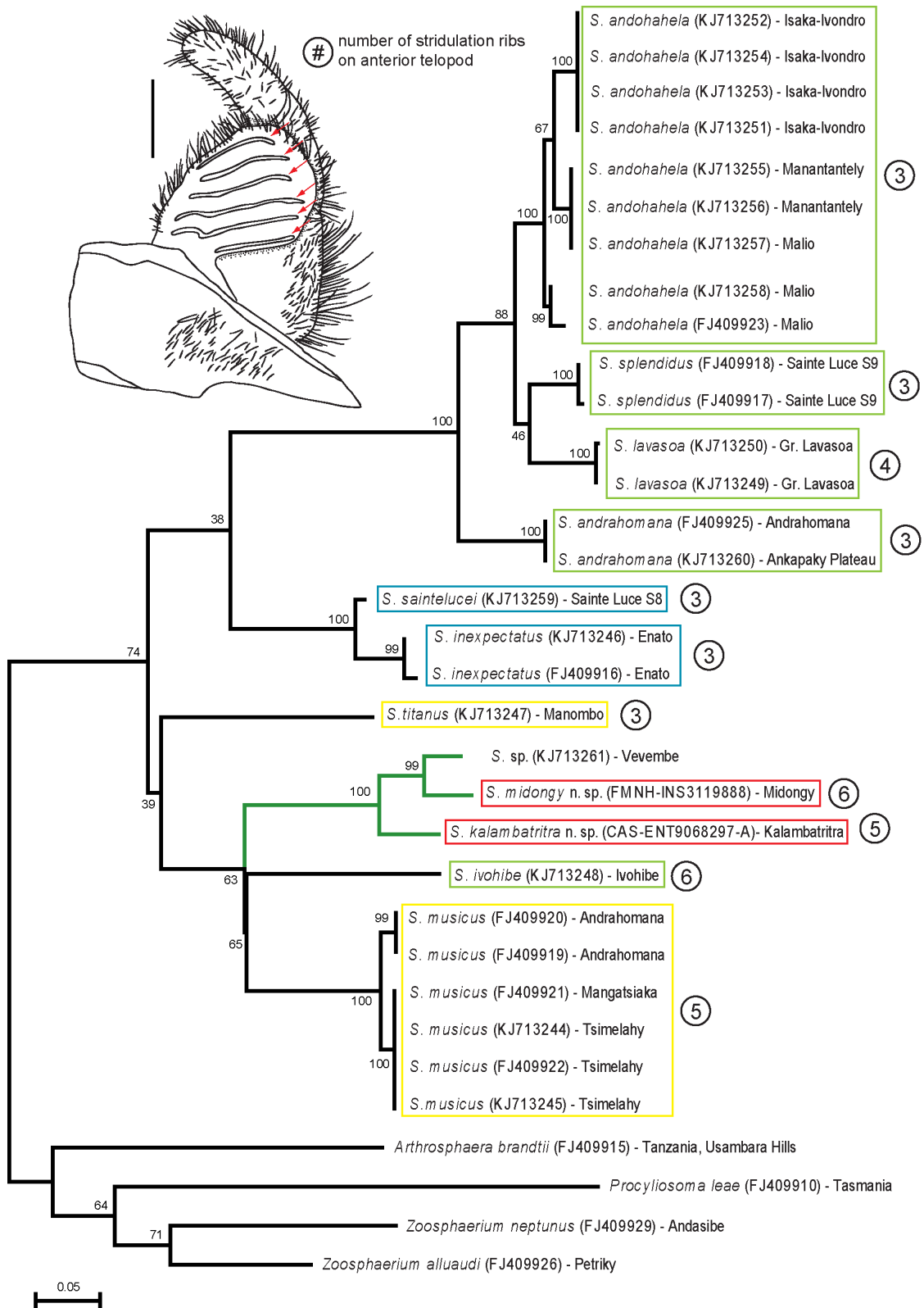


Fig. 10. Maximum likelihood tree based on the COI sequence after 1000 bootstrap replicates under the GTR+G+I model. Branch length indicates genetic distance. Numbers on branches indicate bootstrap support. Schematic drawing shows right anterior telopod of *Sphaeromimus midongy* sp. nov. Numbers in circles indicate number of stridulation ribs on the male harp.

The advantage of micro-CT compared to conventional invasive methods is that it does not damage the specimen. This is especially important, because many new species are found in museum collections and are only represented by a few or a single specimen, like the species described in this study. These specimens cannot be examined without permission and should not be damaged. This makes it nearly impossible to study internal features by dissection or histological sectioning. Furthermore, it makes it possible to share morphological information via public databases, which otherwise opens up a variety of new possibilities, as outlined by Akkari *et al.* (2015). Therefore, we suggest considering the use of micro-CT in taxonomic studies if it seems advantageous to include internal characters. For the first time we add internal characters to the taxonomic description of a member of the order Sphaerotheriida. We describe the structure of the tentorium and the articulation of the mandible condylus. At this point, it is not possible to state which of these characters show inter- or intraspecific variations, because there is no data available yet for other species, except the descriptions of the tentorium of *Arthrosphaera dentigera* (Verhoeff 1930) by Verhoeff (1932) and *Sphaeropoeus modiglianii* Silvestri, 1895, a Zephroniidae, by Silvestri (1903). Future studies will elaborate whether any useful taxonomic characters can be collected from these inner morphological features. The structure of the tentorium of *S. kalambatritra* sp. nov. corresponds to the state described for *A. dentigera*. The hypopharyngeal bar of the tentorium of *Sphaeromimus* is elongated, while the nebotentorium is more stout compared to the state depicted by Silvestri (1903) for the Zephroniidae. Furthermore, the posterior process of *Sphaeromimus* seems larger than in *Sphaeropoeus*, although this could be due to the perspective Silvestri chose to depict the tentorium. The shape of the epipharyngeal bar shows a high similarity in both.

Genetic analysis

The high genetic distances between the species of *Sphaeromimus* suggest an early speciation event, as previously suggested by Wesener *et al.* (2010, 2014). The COI gene probably lost its resolution due to the accumulation of reverse substitutions over time. Klopstein *et al.* (2010) and Townsend & Leuenberger (2011) showed that the ‘informativeness’ of the COI gene declines in older splits.

As shown in several studies on giant pill-millipedes (e.g., Wesener *et al.* 2010 for *Sphaeromimus*; Wongthamwanich *et al.* 2012 and Golovatch *et al.* 2012 for Zephroniidae), the COI gene remains nevertheless a powerful tool for the taxonomy and identification of giant pill-millipede species. It makes it possible to assign specimens of different stages, gender or with high morphological intraspecific variation to a species. Otherwise, it is not necessarily possible to assign such specimens to a species based solely on morphological evidence (see Wesener *et al.* 2014). The COI barcode shows a close and well-supported relationship between *S. midongy* sp. nov. and the undetermined (female) specimen from Vevembe (Fig. 11). In previous studies, the specimen from Vevembe was separated from *S. ivohibe* + *S. musicus* by a long branch and with weak support (see Wesener *et al.* 2014). With a genetic distance of 6.3% between *S. midongy* sp. nov. and the specimen from Vevembe, they cannot be assigned to the same species based on COI data alone until morphological characters of the male of the Vevembe population become available. Thus, *S. inexpectatus* and *S. saintelucei* show a genetic distance of only 4.1% in our analysis and 4.0% in the study conducted by Wesener *et al.* (2014), and are regarded as separate species based on morphological characters. Nevertheless, the genetic distance of the COI sequence fragment in *Sphaeromimus* is usually 8–20% between two species (Wesener *et al.* 2014). Therefore, it cannot be ruled out that the specimen from Vevembe belongs to *S. midongy* sp. nov. To draw sufficiently supported conclusions, further studies and sampling of these areas are needed. A comparison of the genetic distance and the geographical distance reveal an interesting biogeographic pattern. *S. ivohibe* and the specimen from Vevembe, which are separated by ca 43 km (Fig. 11), show a genetic distance of 15.1% while *S. midongy* sp. nov. and the specimen from Vevembe are separated by 125 km (Fig. 11), and have a genetic distance of 6.3%. The genetic distance between *S. midongy* sp. nov. and *S. kalambatritra* sp. nov.

is 9.4%, although the geographical distance is only 65 km (Fig. 11). This demonstrates that species endemic to geographically close habitats are not necessarily closely related (see also Wesener *et al.* 2014).

Number of stridulation ribs of the male harp

Comparing the number of stridulation ribs on the male harp to the species-tree, there seems to be one clade with an increased number of stridulation ribs (Fig. 10). While most known *Sphaeromimus* have three stridulation ribs (4 in *S. lavasoa*) the clade (*S. kalambatritra* sp. nov. + (*S. midongy* sp. nov. + specimen from Vevembe)) + (*S. musicus* + *S. ivohibe*) has five or six stridulation ribs. However, it has

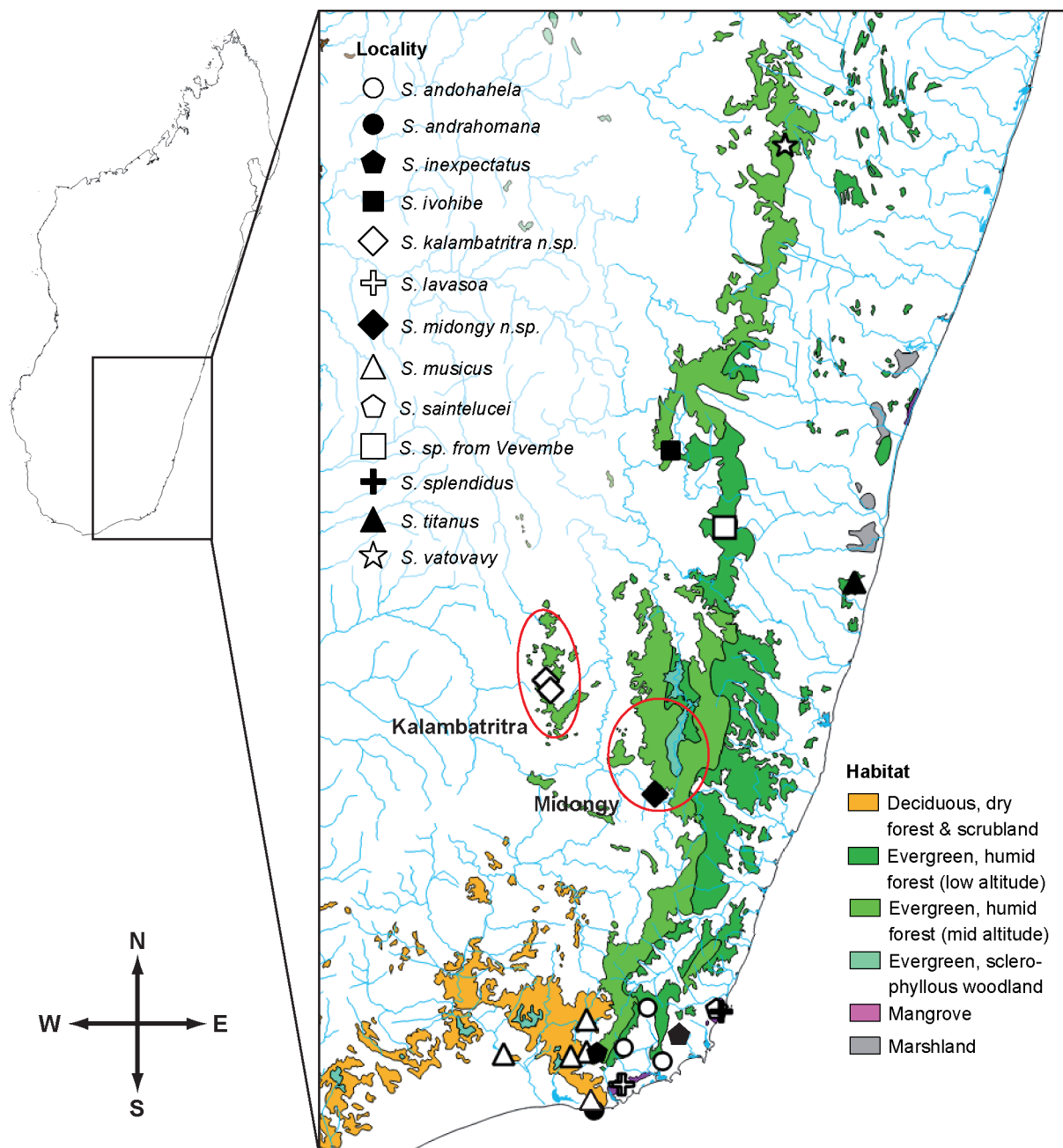


Fig. 11. Distribution of the genus *Sphaeromimus* de Saussure & Zehntner, 1902 in south-eastern Madagascar. Map modified after Moat & Smith 2007.

to be noted that there is no information on the number in the specimen from Vevembe, since we know a single female only. We can assume that the ancestral state is a low number of stridulation ribs on the male harp, as seen in other representatives of the family Arthrosphaeridae. In *Arthrosphaera* the number of stridulation ribs can vary between none and 2 ribs, *Microsphaerotherium* has one rib and *Zoosphaerium* has one or two (Wesener & VandenSpiegel 2009). The advantage of a high number of stridulation ribs remains unclear. Thus, the habitats of species with high or low numbers of stridulation ribs overlap. Species of *Sphaeromimus* and *Zoosphaerium* usually occur in sympatry (Wesener *et al.* 2014). The number of ribs might have an impact on the sound production, but only for South African species information about the produced sounds is available (Wesener *et al.* 2011).

Acknowledgements

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