

Miocene uplift and Pleistocene forest connectivity drove the evolution of large-bodied Afrotropical pill scarabs (Coleoptera: Hybosoridae: *Afrocloetus* and *Congomostes*)

VASILY V. GREBENNIKOV

Canadian Food Inspection Agency, 960 Carling Ave., Ottawa, ON, K1A 0Y9, Canada [vasily.grebennikov@canada.ca]

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Abstract. This study targets the post-Oligocene evolution of low-vagility beetles in wet African forests. Two nominal genera of large-bodied pill scarabs form the focus: the East African *Afrocloetus* with two nominal species and the Central African *Congomostes* with three nominal species. Both genera are rarely sampled, particularly *Afrocloetus* which is known until now from two holotypes collected in 1948 and 1957. Long series of *Afrocloetus* are newly reported from four Tanzanian localities: Kimboza forest, Kaguru, Rubeho and Udzungwa; the last three belong to the biodiverse chain of the Eastern Arc Mountains. A phylogenetic analysis utilized a matrix consisting of 52 terminals (including five *Afrocloetus* and two *Congomostes*) and an alignment of 2,940 bp from one mitochondrial and two nuclear fragments. The analysis recovered both genera as monophyletic and forming the sister clade to the Afrotropical *Philharmostes* group of genera. Molecular clock analysis of 39 DNA barcodes of both genera estimated their separation within a temporal window (13.0–6.8 Ma) that is fully concurrent with the Miocene uplift of the East African Plateau causing aridification and forest fragmentation. Three Tanzanian localities each support an endemic mitochondrial *Afrocloetus* clade evolving in allopatry, following the pre-Pleistocene range breakup of their originally widespread ancestor (= first cycle of forest expansion and contraction). Two morphologically and genetically distinct *Afrocloetus* clades co-exist in Udzungwa; this sympatry is attributed to secondary re-colonization of Udzungwa by the younger and reproductively isolated clade at 2.4–1.2 Ma (= second cycle of forest expansion and contraction). This hypothesised re-colonisation of Udzungwa by flightless beetles coincides with the Pleistocene climatic cycles and suggests temporal connectivity between Rubeho and Udzungwa forests. Adults of *Afrocloetus* are extensively illustrated, compared among themselves and with those of *Congomostes*, and their relevant evolutionary markers are discussed. The taxonomy of both nominal and likely synonymous genera is unmodified pending further research. Habitus images, DNA sequences and other supplementary information of all sequenced specimens are available online at dx.doi.org/10.5883/DS-VGDS002 and dx.doi.org/10.5883/DS-VGDS003.

Key words. DNA barcode, ITS2, 28S, phylogeny, forest litter, phylogeography.

1. Introduction

This study addresses evolution of a clade of low-dispersing and forest-dependent scarab beetles within the historical and geographical context of the Miocene fragmentation (and occasional subsequent Pleistocene reconnections) of the once widespread African wet forest. It is fitting, therefore, to provide separate introductions for both focal biological phenomena: the beetles and the forest.

1.1. Ceratocanthini beetles

Spheroid-forming Ceratocanthini (Hybosoridae: Ceratocanthinae) pill scarabs are a pantropical clade of 38 extant nominal genera comprising about 358 species (BALLERIO & GREBENNIKOV 2016). The tribe is formed by three distinct geographical clusters of genera, none of the latter shared: those in the New World, those in Afrotropics (including Madagascar and nearby islands) and those of a triangle delimited by India, southern Japan and Queensland, Australia (BALLERIO & GREBENNIKOV 2016).

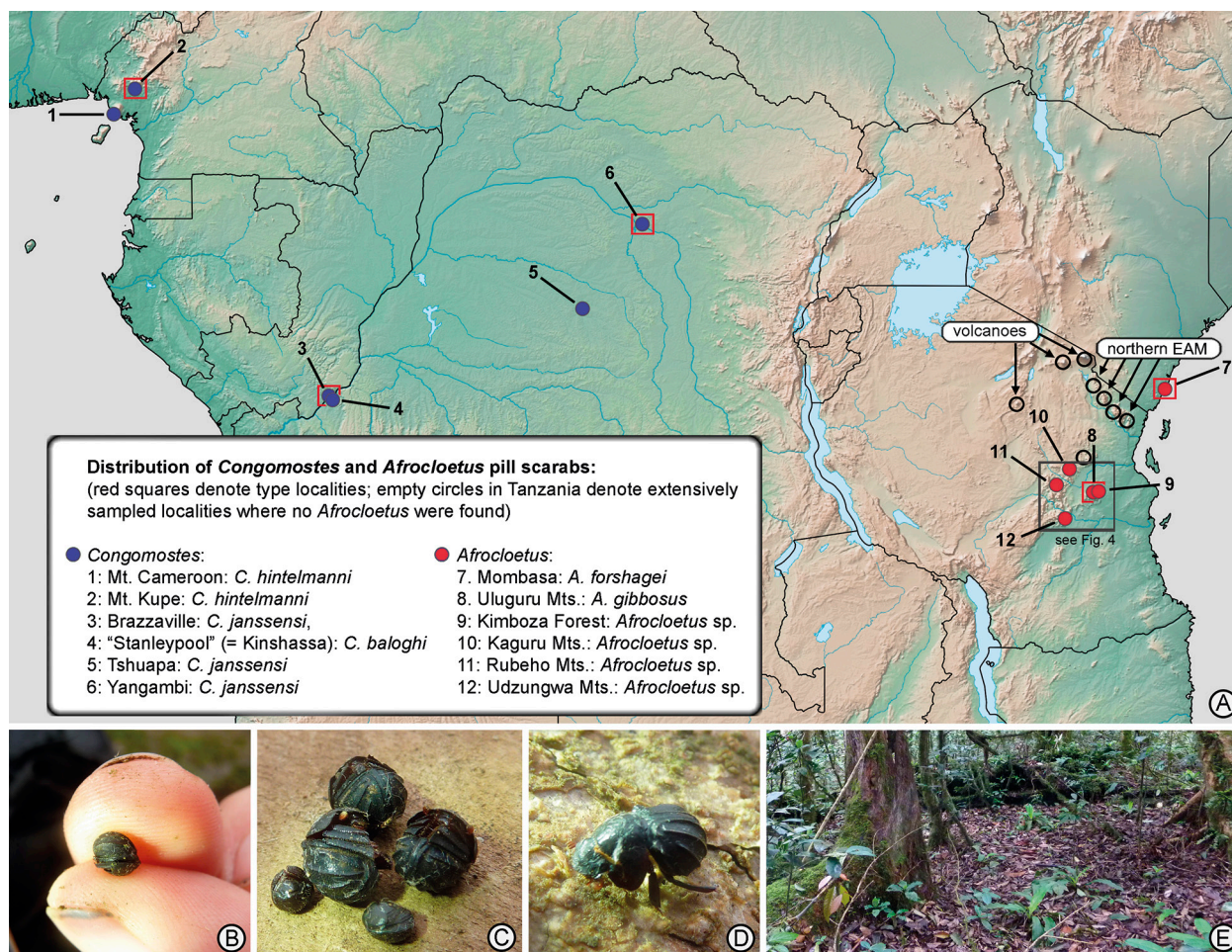


Fig. 1. A: Geographical distribution of large-bodied Afrotropical pill scarabs of the nominal genera *Afrocloetus* and *Congomastes*; B–D: live adults of *C. hintelmanni* in proportion to a human (B) or other pill scarabs in enroled position (C), or not (D); E: forest leaf litter, preferred habitat of many pill scarabs, including those of *Afrocloetus* and *Congomastes*.

This evolutionary suggestive distribution has not been adequately studied due to the absence of a sufficiently broad and robust phylogenetic hypothesis. So far only four studies attempted to resolve relationships among pill scarabs using quantitative methods applied to data from larval morphology (GREBENNIKOV et al. 2004), adult morphology (BALLERIO 2016; BALLERIO & GREBENNIKOV 2016) and DNA sequences (GREBENNIKOV 2019a). The present paper forms a sequel to the latter and attempts, among its other goals, to resolve relationships of the until now exceedingly rare large-bodied Afrotropical pill scarab genus *Afrocloetus* Petrovitz, 1968, which was re-discovered in the wild for the first time since 1957.

The mainland Afrotropical fauna of 13 pill scarab genera (excluding Madagascar) was thought (BALLERIO 2006) to contain three clades: (1) the monophyletic *Philharmostes* group of seven nominal genera (= PhG, GREBENNIKOV 2019a), (2) the reciprocally monophyletic *Melanophilharmostes* Paulian, 1968 and *Pseudopterorthochaetes* Paulian, 1977 (BALLERIO 2016), and (3) the South African genera *Acanthocerodes* Péringuey, 1901 and *Anelobolus* Hesse, 1948. Two other mainland Afrotropical genera remain not phylogenetically assigned. They are

Congomastes Paulian, 1968, with three nominal species in the Congo basin and its western periphery (Cameroon, Fig. 1; a single record from Zambia by BALLERIO & GREBENNIKOV 2016: 32 could not be verified) and *Afrocloetus* with two nominal species in Kenya and Tanzania (Fig. 1). Remarkably, both *Congomastes* and *Afrocloetus* include by far the largest Afrotropical pill scarabs (Figs. 1B,C, 2A–D, 3) notably similar among themselves (Fig. 2A–D). Their maximum body length in the enroled (= spheroid, = rolled up) position is about 4.5–5.5 mm, while those of the majority of Afrotropical Ceratocanthini are notably smaller (Fig. 1B) and vary between 2 mm and 3 mm, rarely approaching 4 mm, as in *Carinophilharmostes vadoni* (Paulian, 1937) (Fig. 3). Besides being uniquely large, specimens of both genera share three other similarities: they are dark and non-metallic, have strongly carinate elytra and have an “almost glabrous and relatively convex” (BALLERIO 2006) proximal surface of the first antennomere of the club. Both genera are parapatric in distribution (Fig. 1A) and inhabit primary forests (Fig. 1E). Remarkably, both genera were established in the same year by authors who were likely unaware of each other’s work (PAULIAN 1968; PETROVITZ 1968) and are mainly distinguished by the absence (*Afrocloetus*,



Fig. 2. Holotypes of large-bodied Afrotropical pill scarabs. Images were taken by different people at different times, therefore some lack scale bars (C, D) and labels (C).

Fig. 5B) versus presence (*Congomostes*, Fig. 7B) of a partial interocular bridge (= “genal canthus”) extending posteriorly from the anterior corner of each adult eye and separating it into dorsal and ventral parts. In view of their suggestive similarities it is, therefore, surprising that the nominal genera *Afrocleotus* and *Congomostes* have never been considered as closely related, or perhaps synonymous. Conversely, both genera were placed far apart on a phylogenetic tree based on an analysis of 97 parsimoniously informative adult morphological characters (BALLERIO & GREBENNIKOV 2016).

The difficulty of assessing relationships of *Afrocleotus* and *Congomostes* is partly due to the rarity of their records. The genus *Congomostes* contains macropterous *C. janssensi* (Basilewsky, 1955, described as *Philharmonistes*, Fig. 2B) and *C. baloghi* Paulian, 1968 (the type species, Fig. 2A), both from the Congo basin, as well as flightless *C. hintelmanni* Ballerio, Gill and Grebennikov, 2011 from Cameroon (Figs. 1B,C, 7A–I). The latter species has been recently resampled in both known localities and emerged as a sister to PhG of seven Afrotropical genera (GREBENNIKOV 2019a). Prior to 2015, however, the total number of all known *Congomostes* specimens was likely expressed by a single digit.

The East African genus *Afrocleotus* is even more obscure. It contains the type species *A. gibbosus* Petrovitz, 1968 (Fig. 2C) from around Bunduki village in the Ulu-guru Mountains in Tanzania (Fig. 1A), and *A. forshagei* Ballerio, 2006 (Fig. 2D) from the Bamburi Bay at Mombasa, in eastern Kenya (Fig. 1A). The holotypes were collected in 1957 and in 1948, respectively, and remained the only known specimens of the genus. Long series of *Afrocleotus*, however, were recently sampled in four Tanzanian localities: in Kimboza forest situated less than 20 km from the type locality of *A. gibbosus* (Fig. 1A), and in three relatively nearby (less than 150 km from Kimboza forest) forest blocks of the Eastern Arc Mountains (EAM): Kaguru, Rubeho and Udzungwa (Fig. 1A). Rediscovery of the enigmatic forest-dwelling *Afrocleotus* is, therefore the main trigger for this paper.

1.2. African forest since the Oligocene

During the Oligocene the African forest extended latitudinally across the entire continent (MALEY 1996; Fig. 9.24 in KIRK-SPRIGGS & MULLER 2017). In the Miocene its eastern part became fragmented as a result of

aridification linked with the uplift of the East African plateau (BOBE 2006). Dramatic and repeated forest expansions and shrinkages followed, coinciding with the Pleistocene glacial cycles (PLANA 2014). These events are expected to leave their traces in the patterns of distribution and phylogeny of the present day African forest and its inhabitants, particularly those with reduced capacity for dispersal. Particularly promising in this respect is the phenomenon of the continent sky islands formed by isolated forested mountains located on the periphery of the Congo basin and surrounded by drastically different lowland environments of the dry and hot savannah or grassland. At least three such African sky island formations are remarkable for their high and geographically structured biodiversity. They are: (1) the Cameroon volcanic line extending between the island of Annobón in the southwest and Lake Chad in the north-east; (2) the meridional oriented Albertine Rift delimited by Lake Albert and Lake Tanganyika (highest vertebrate diversity in the continent, references in VOELKER et al. 2010) and (3) EAM mainly in Tanzania (Fig. 1A), harbouring the newly detected *Afrocloetus* populations and, therefore, forming the geographical focus of the present study. Advantageously, the latter chain of highlands has been extensively studied to test its long-assumed climatic and biotic stability. Analyses of pollen, charcoal and carbon isotopes from deep soil probes taken in two EAM highlands (Udzungwa and Uluguru, MUMBI et al. 2008 and FINCH et al. 2009, respectively) revealed stable forest cover for the past 48,000 years, a period greatly exceeding the Last Glacial Maximum with its peak about 25,000 years ago.

1.3. Design and goals of the study

Two non-excluding hypotheses were advanced to explain the high biodiversity of EAM. The **refugia hypothesis** links it to the Miocene uplift of the East African plateau (LOVETT 1993) and subsequent forest fragmentation resulting in a long evolution of isolated allopatric populations. This hypothesis agrees well with patterns seen in most of the few East African clades studied in sufficient detail, such as *Atheris* Cope, 1862 bush vipers (MENEGON et al. 2014), *Grammomys* Thomas, 1915 arboreal rodents (BRYJA et al. 2016) or minute and blind *Antireicheia* Basilewsky, 1951 ground beetles (GREBENNIKOV et al. 2017). The **species-pump hypothesis** (PAPADOPOULOU & KNOWLES 2015), on the other hand, emphasises the role of the repeated cycles of the connectivity and isolation during the subsequent Pleistocene, occasionally resulting in sympatry of closely related EAM endemics (FJELDSÅ et al. 2010). Advantageously, the large-bodied Afrotropical pill scarabs possess three biological characteristics that could make them a potential tool to infer the post-Oligocene evolution of the African forest. Firstly, both nominal genera might form a clade distributed between the western (Cameroon) and eastern (Tanzania and Kenya) historical extremities of the Congo forest (Fig. 1A).

Secondly, the Cameroonian and East African populations (e.g. peripheral to the Congo basin) lack hind wings and are, therefore, a low-dispersing flightless proxy for old forests. Thirdly, populations of Tanzanian *Afrocloetus* are known from four highly isolated forests, three of which belong to the sky island chain of the biodiverse EAM. These considerations, together with the necessity to document distribution and morphology of these understudied beetles, determined these six goals of the present study:

1. to report *Afrocloetus* pill scarabs from four new Tanzanian localities and discuss distribution of this nominal genus;
2. to test a hypothesis (**H1**) that the evolution of the large body among Afrotropical Ceratocanthini, as exemplified in the genera *Afrocloetus* and *Congomostes*, was a unique event;
3. to test a hypothesis (**H2**) that the observed distribution and diversity of *Afrocloetus* and *Congomostes* is attributable to the Miocene East African forest fragmentation preventing gene flow among isolated populations of their broadly distributed most recent common ancestor (MRCA) (= the refugia hypothesis);
4. to test an alternative (and not mutually exclusive) hypothesis (**H3**) that the observed distribution and diversity on *Afrocloetus* in fragmented Tanzanian forests is attributable to re-colonization of already occupied EAM areas via normal ecological dispersal when the currently isolated forests might have been re-connected during the wetter periods of the Pleistocene climatic cycles (= the species-pump hypothesis);
5. to document adult morphological characters of a representative of *Afrocloetus* and *Congomostes* (particularly those of the male genitalia which are understudied in pill scarabs), and highlight potential synapomorphies;
6. to review the classification pertaining to both nominal genera and assess its compatibility with the current state of phylogenetic knowledge.

2. Material and methods

All herein reported *Afrocloetus* and *Congomostes* specimens were sampled by sifting forest litter using the methods and sample codes described earlier (GREBENNIKOV 2017, 2019b). A hand-held sifter 35 cm in diameter and with 5 × 5 mm wire mesh was used to obtain the fine fraction of forest litter containing live litter organisms. Mesh bags of non-electric Winkler funnels were then loaded (normally one handful of litter per bag, nine bags per funnel, six funnels in operation) and suspended for 2–8 hours, twice followed by re-loading and re-suspension of the same litter for the same period of time. Live extracted organisms accumulated in a container at the bottom of each funnel were transferred into a Whirl-Pak sealable plastic bag with 96% ethanol; the latter was changed at least twice with intervals of a few days to maintain its high concentration. Samples were sorted under a dissecting microscope about a month

Fragment	#	min	max	aligned	Positions
COI-5P	51	561	658	658	1 to 658
ITS2	49	206	867	1522	659 to 2180
28S	50	284	648	760	2181 to 2940

Table 1. DNA fragments used in the three-locus phylogenetic analysis; total number of sequenced terminals, followed by minimal, maximal and aligned length of each fragment, and the first and the last position of each aligned fragment in the concatenated matrix.

Table 2. GenBank accession numbers of five newly sequenced *Afrocloetus* and one *Congomostes* terminals (for the remaining 46 terminals see GREBENNIKOV 2019a).

Voucher	Taxon	Locality	COI	ITS2	28S
CNCCOLVG00003734	<i>Afrocloetus</i>	Tanzania: Rubeho	MH778032	MH981217	MH981212
CNCCOLVG00003738	<i>Afrocloetus</i>	Tanzania: Kaguru	MH778030	MH981216	MH981211
CNCCOLVG00008766	<i>Afrocloetus</i>	Tanzania: Udzungwa	MH778014	none	MH981209
CNCCOLVG00008767	<i>Afrocloetus</i>	Tanzania: Udzungwa	MH778013	MH981214	MH981208
CNCCOLVG00008778	<i>Afrocloetus</i>	Tanzania: Kimboza Forest	MH778026	MH981215	MH981210
CNCCOLVG00009720	<i>Congomostes hintelmanni</i>	Cameroon: Mt. Kupe	MH916844	MH777802	MH777846

later. All herein sequenced specimens of pill scarabs (including those of both target genera) are stored in the Canadian National Collection of Insects, Arachnids and Nematodes in Ottawa, Canada.

2.1. Hypotheses testing

The analytical part was designed to test three hypotheses H1–H3 (enumerated in Introduction) following the principle of falsifiability (POPPER 1959). Testable predictions from each hypothesis were formulated and subjected to statistical scrutiny. To be corroborated, each hypothesis required the following conditions to be met:

H1 (= single origin of gigantism among Afrotropical pill scarabs) requires one condition: monophyly of *Afrocloetus* + *Congomostes*;

H2 (= the refugia hypothesis) requires three conditions: (A) monophyly of West African *Congomostes* sister to East African *Afrocloetus*; (B) Miocene timing of their divergence and (C) *Afrocloetus* consisting of strictly allopatric clades diverging before the Pleistocene;

H3 (= the species-pump hypothesis) requires three conditions: (A) monophyly of *Afrocloetus*; (B) detection of at least one case of sympatry of at least two genetically (and perhaps morphologically) distinct *Afrocloetus* clades; (C) the younger of them having a Pleistocene time of its divergence.

2.2. DNA methods and three-loci phylogenetic analysis

To test hypothesis H1 (and, in part, H2 and H3), a DNA-based phylogenetic analysis was designed. All laboratory and analytical methods are the same, as those described in GREBENNIKOV (2019a). In brief, freshly sampled specimens of *Afrocloetus* (four localities in Tanzania, 34 specimens) and *Congomostes* (two localities in Cameroon, five specimens) were DNA barcoded (= sequenced for 658 bp of the 5-end of the cytochrome c oxidase subunit

I) in the Canadian Centre for DNA Barcoding, University of Guelph, Canada (CCDB, <http://www.ccdb.ca>). Obtained DNA barcodes 473–658 bp in length were preliminarily clustered using the Neighbour Joining engine of the Barcode of Life online database (= BOLD, RATNASINGHAM & HEBERT 2007, <http://www.boldsystems.org/>). Five *Afrocloetus* and one *Congomostes* DNA barcoded specimens representing terminal clusters (= evolutionary significant units, = candidate species) on the NJ tree were additionally sequenced for two nuclear ribosome-coding regions: internal ribosomal spacer 2 (ITS2) and 28S rDNA (Table 1). This newly generated six terminal dataset of three markers (COI, ITS2, 28S) was added to the 46 terminal matrix used in GREBENNIKOV (2019a; one of them *Congomostes* specimen 9720; two others are non-Ceratocanthinae Hybosoridae outgroup taxa). The ingroup of the phylogenetic analysis was, therefore, formed by seven terminals (five *Afrocloetus* and two *Congomostes*). Alignments of the ITS2 and 28S sequences were made using the MAFFT 7 online platform (<http://mafft.cbrc.jp/alignment/server/>) and the Q-INS-i algorithm utilising the secondary structure information. No parts of the alignments were excluded from the analysis. The resulting three-locus matrix consisted of 52 terminals (specimens are listed and illustrated in [dx.doi.org/10.5883/DS-VGDS003](https://doi.org/10.5883/DS-VGDS003) online dataset) and 2,940 aligned positions. All laboratory work other than DNA barcoding was done in CCDB using protocols and primers described in GREBENNIKOV (2019a). GenBank accession numbers of new sequences are listed in Table 2; those of newly generated DNA barcodes used in the temporal analysis (see below) are shown in Fig. 4. Phylogenetic analysis was conducted on the CIPRES Science Gateway online platform (MILLER et al. 2010; <http://www.phylo.org/>) using the Maximum Likelihood (ML) method and RAxML 7.2.7. The concatenated matrix was partitioned into three fragments corresponding to three used loci (Table 1) and an independent GTR+G model was applied to each data partition. Invariant sites were not omitted from the analysis. Support values were obtained with 1000 standard bootstrap replicates.

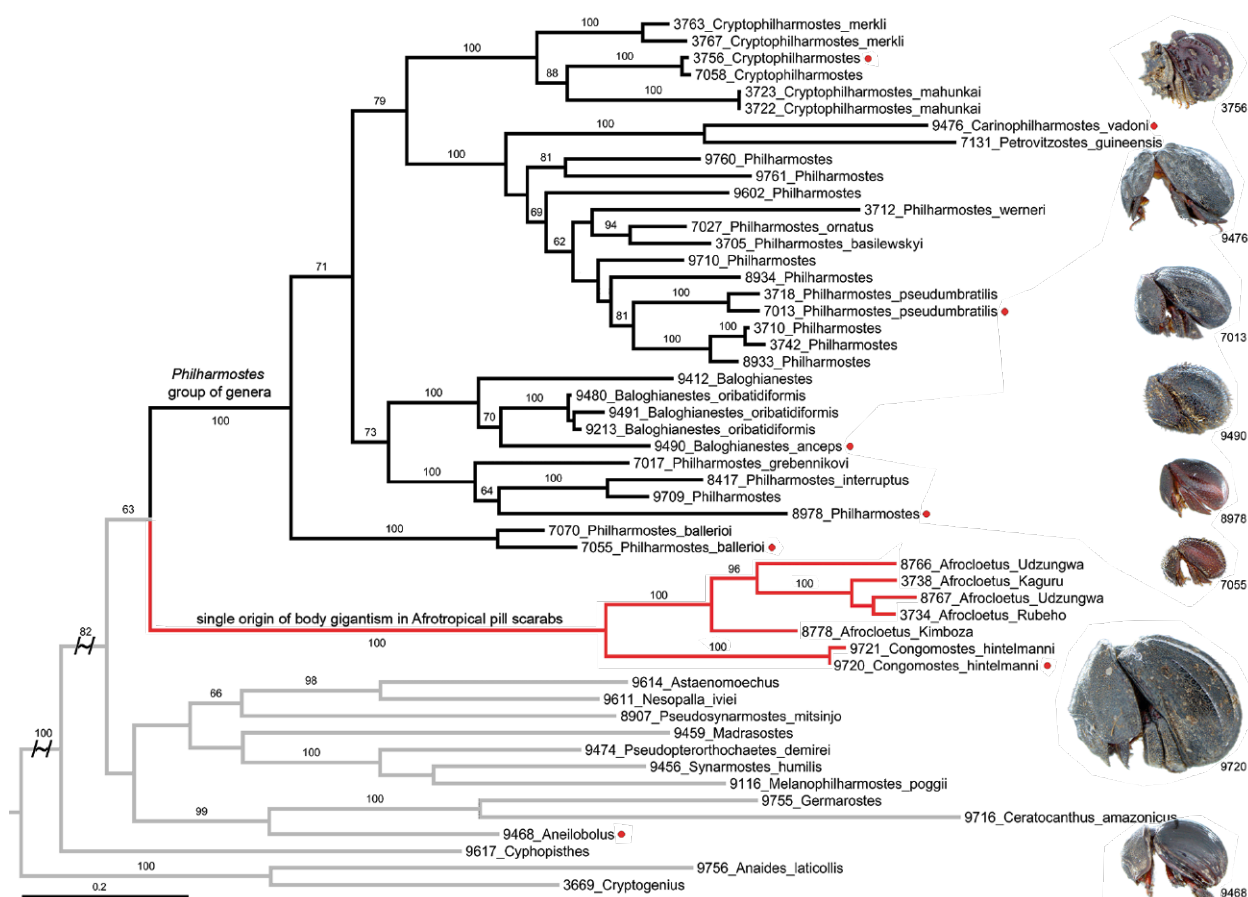


Fig. 3. Maximum Likelihood inference phylogram from a three-locus analysis revealing the clade of large-bodied Afrotropical pill scarabs of the genera *Afrocloetus* and *Congomostes* sister to the *Philharmostes* group of genera. Digits at internodes are bootstrap values > 60%. Red dots denote specimens imaged on the right (to scale).

2.3. Temporal DNA barcode analysis

Obscurity and phylogenetic neglect affecting the clade of pill scarabs severely limited methods available for testing temporal hypotheses H2 and H3. Considering the challenges, namely (1.) the monophyly of Tanzanian *Afrocloetus* and their sister group relationships with Cameroonian *Congomostes* (see Results), (2.) the lack of calibrating points within the ingroup, such as fossils or relevant geological events, and (3.) the lack of a sufficiently well-known outgroup for the *Afrocloetus* + *Congomostes* clade with reliable calibrating points, a flat-rate temporal analysis was the only option available for time estimation. For this purpose, a new matrix was formed from 39 DNA barcodes representing both genera (specimens are listed and illustrated in [dx.doi.org/10.5883/DS-VGDS002](https://doi.org/10.5883/DS-VGDS002) online dataset). To estimate divergence time, a flat molecular clock of 0.018 nucleotide substitutions per site per million years per lineage (subs/s/Myr/l) was applied. This value is consistent with those obtained in studies of various beetles (PAPADOPOULOU et al. 2010; ANDÚJAR et al. 2012), other insects (BROWER 1994) and other arthropods (CICCONARDI et al. 2009; BAUZÀ-RIBOT et al. 2012), and is frequently used for time estimates in situations when no alternative exists (i.e. GARCÍA et al. 2019). Bayesian phylogenetic ana-

lysis in BEAST 1.8 (DRUMMOND et al. 2012) was used to simultaneously estimate an ultrametric phylogenetic tree and ages of diversification. The GTR+G+I evolutionary model (estimated in MEGA 7, KUMAR et al. 2016) was applied and the MCMC chains and run for 10 million generations. Convergence of all parameters was checked in TRACER (DRUMMOND et al. 2012) and consensus trees were estimated with TreeAnnotator (DRUMMOND et al. 2012) discarding the first 25% of replications as the burn-in fraction. No *a priori* topological constraints were added in the analysis.

2.4. Morphological study, illustrations, dissections and terminology

Previous publications on pill scarabs were consulted in search for morphological characters that are diagnostic for adults of *Afrocloetus* and *Congomostes*, particularly those listed in BALLERIO (2006) and BALLERIO et al. (2011). Considering that 34 *Afrocloetus* specimens from four Tanzanian localities formed five geographically structured clusters (see Results), an effort was made to find reliable external morphological differences between them and, particularly, between two sympatric and genetically distinct Udzungwa populations. Taxonomic revision of

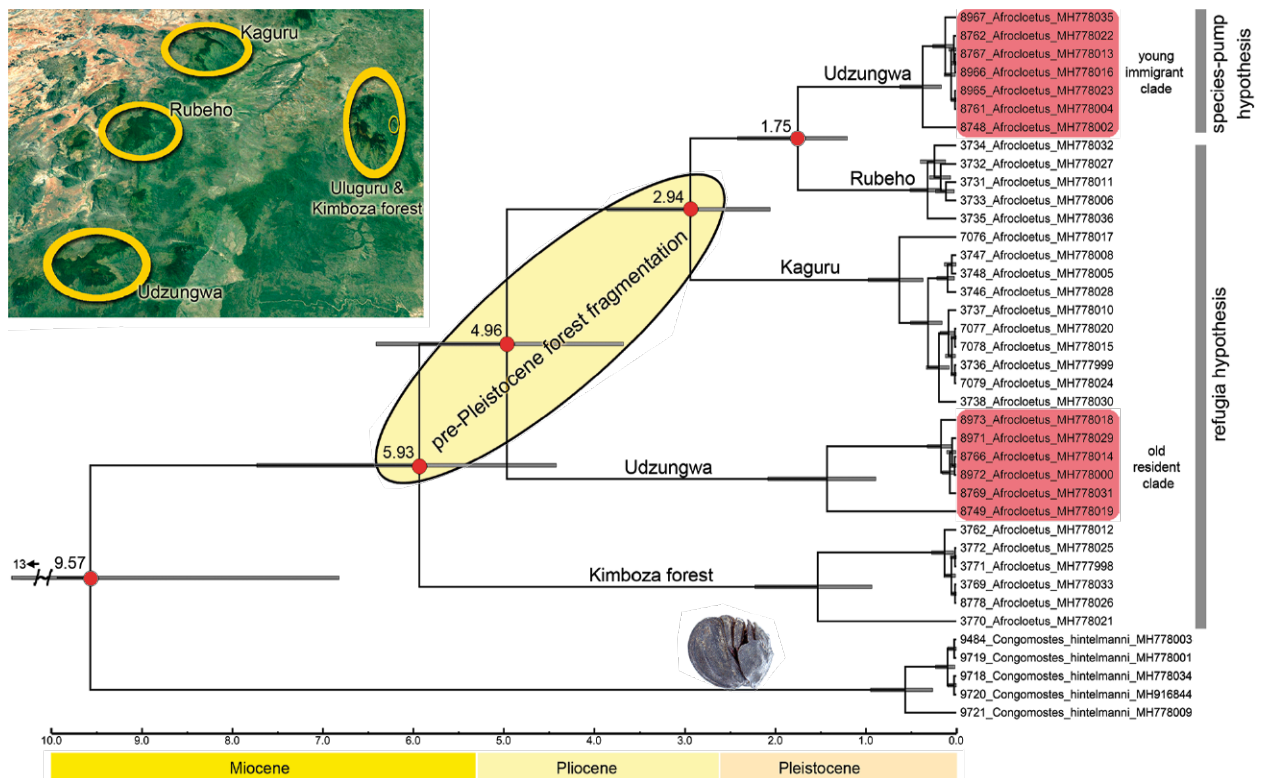


Fig. 4. Ultrametric time tree providing temporal interpretation of the post-Oligocene evolution of large-bodied Afrotropical pill scarabs applying flat substitution rate of 0.018 subs/s/Myr/l to the DNA barcoding fragment. Numbers on the time scale and at the nodes denote millions of years before present. Node bars represent 95% confidence interval of age estimate. Note that Udzungwa supports two genetically and morphologically distinct clades of *Afrocloetus*.

Afrocloetus was outside of the paper's scope, mainly due to unavailability of DNA (and partly morphological) data from the toptotypical populations represented by both holotypes (see below on destruction of forest in both type localities). Male and female genitalia are two character systems that are understudied and underutilized in pill scarabs (supplying only six phylogenetically informative characters among the 97 used in BALLERIO & GREBENNIKOV 2016), while their dissection method is labour-intensive and highly destructive to specimens. For these reasons, only a limited study of genitalia is herein undertaken with the purpose of illustrating a male specimen from both the *Afrocloetus* and *Congomostes* clades, and a female from either of them. A total of three dry specimens glued to points were imaged in an enrolled position in five standard views (Figs. 5A, 6A, 7A), relaxed in water, submerged in glycerol, disarticulated, sexed and additionally imaged (Figs. 5B–I, 6B–F, 7B–I). Genitalia of both sexes were macerated in hot 5–10% KOH water solution. Morphological terms, including those of male (from D'HOTMAN & SCHOLTZ 1990) and female genitalia, are those illustrated in GREBENNIKOV (2019a). Special effort was made to use strictly morphological nomenclature of male genital sclerites (WANAT 2007). No attempt was made to revise and re-analyse the *Afrocloetus* and *Congomostes* matrix entries of the recent morphological analysis of pill scarabs (BALLERIO & GREBENNIKOV 2016), which (likely erroneously, see Results) placed the two nominal genera widely apart.

3. Results

Afrocloetus specimens were detected in four among 14 sampled Tanzanian localities: Kimboza forest, Kaguru, Rubeho and Udzungwa (Figs. 1A, 4). Attempts to re-sample *Afrocloetus gibbosus* in its type locality (Uluguru) were not successful. Specimens of both sexes of the older resident *Afrocloetus* population of Udzungwa (Fig. 4) could be consistently separated from those of the younger immigrant Udzungwa clade (and from other newly sampled congeners) by two external characters: (1) presence of longitudinal, linear and mainly non-ocular punctures on elytral disc, Fig. 6D (versus mainly ocular; Fig. 5C) and (2) absence of transverse ridge on frons, Fig. 6B (versus its presence, Fig. 5B). These and other morphological characters of *Afrocloetus* and *Congomostes* specimens were extensively documented in Figs. 5A–I, 6A–F and 7A–I. Except for the old resident Udzungwa clade, all newly sampled *Afrocloetus* specimens appeared indistinguishable among themselves.

The ML tree of 52 terminals analysed using the concatenated 2,940 bp matrix of three loci resulted in a topology (Fig. 3) with a herein newly detected clade of large-bodied pill scarabs. All *Afrocloetus* formed a clade (bootstrap support 100%) sister (100%) to a monophyletic *Congomostes* (100%). *Afrocloetus* from Kimboza forest was sister to the rest of the genus (96%) consisting of the older population from Udzungwa (represented by

specimen 8766) sister (100%) to a clade of *Afrocloetus* from Kaguru, Rubeho and the younger Udzungwa population (represented by specimen 8767). The clade of giant Afrotropical pill scarabs was sister (63%) to a monophyletic PhG (100%).

The BEAST temporal analysis using 39 DNA barcodes resulted in a topology (Fig. 4) with five geographically structured clades of *Afrocloetus* as sister to *Congomostes*. Divergence between both nominal genera was dated in the late Miocene, ca. 9.6 million years ago (= Ma, c.i. 13.0–6.8 Ma). The divergence pattern among five terminal clades of *Afrocloetus* was the same as in the topology from the three-locus analysis (Fig. 3) and in all cases pre-dated the Pleistocene, except for the most recent divergence ca. 1.74 Ma (c.i. 2.4–1.2 Ma) between *Afrocloetus* from Rubeho and the younger among two sympatric Udzungwa clades.

4. Discussion

4.1. Goal 01: Distribution of nominal *Afrocloetus*

Numerous adults of *Afrocloetus* pill scarabs were found in four Tanzanian forests, after more than 60 years since the last congeneric specimen had been seen in nature. This re-discovery reflects neglect for these organisms, rather than their genuine “rarity”. Flightless Afrotropical pill scarabs can be only infrequently detected by methods other than forest litter sifting, followed by specimen extraction in suspended Winkler funnels. A lack of focused collecting efforts is perhaps the main factor allowing *Afrocloetus* to successfully evade humans so efficiently. Absence of *Afrocloetus* in samples taken in Nguru and in four northern blocks of EAM (empty circles in Fig. 1A: East and West Usambara, South and North Pare) is difficult to explain by factors other than random events of either specimen sampling and/or clade survival, since at least one flightless species of other pill scarabs was detected in all of them (except for North Pare). Conversely, *Afrocloetus* and other flightless pill scarabs likely are truly absent on the forested slopes of all three sampled volcanoes (Mts. Kilimanjaro, Meru and Hanang, Fig. 1A). These forests formed de novo not before respective geologically young highlands came into existence < 2 Ma (NONNOTTE et al. 2008), therefore their subsequent colonization by flightless pill scarabs was likely efficiently obstructed by the intervening dry non-forested landscapes. This hypothesis is frequently evoked to explain underrepresentation of low-vagility forest-dependent organisms in the forests of these Tanzanian volcanoes, be they flightless Aradidae flat bugs (GREBENNIKOV & HEISS 2018) or montane frogs (ZANCOLLI et al. 2014).

Both type localities of *Afrocloetus* might be genuinely devoid of these beetles due to human encroachment (BALLERIO 2006). The Bamburi Bay in Kenya, the type locality of *A. forshagei*, is now a part of the city of

Mombasa, which likely devastated the original forest and its biota. The “vallée Ululu-Ndogo, 1500 m”, the type locality of *A. gibbosus* in Uluguru, likely refers to the presently heavily cultivated valley of Mgeta River on the western slope of Uluguru above Bunduki village and below the forest lower edge at 1,700–1,900 m. Extensive sampling in Uluguru detected only two Ceratocanthinae species (*Philharmostes basilewskyi* Paulian, 1977 and *P. ornatus* Ballerio, 2004), none of them *Afrocloetus*. The congeneric population found in Kimboza forest only 18 kilometers to the east (Figs. 1A, 4) is not necessarily conspecific with *A. gibbosus*, since the phylogeographic history of similarly flightless and forest-dependent *Ty-poderus* weevils inhabiting both forests are highly distinct (GREBENNIKOV 2019b). Summing up, while thriving in the forests of Kimboza, Kaguru, Rubeho and Udzungwa, *Afrocloetus* remain unknown from anywhere else and might be extinct in both type localities.

4.2. Goal 02: Testing single origin of body gigantism in Afrotropical pill scarabs

Results of the ML phylogenetic analysis strongly suggest that the Afrotropical pill scarabs of the nominal genera *Afrocloetus* and *Congomostes* form a clade (Fig. 3). This is consistent with the hypothesis H1 that the large body size in Afrotropical pill scarabs was an evolutionary novelty possessed by the most recent common ancestor of both nominal genera. Notably less confidence might be assigned to the de novo hypothesis suggesting that PhG is sister to the large-bodied Afrotropical pill scarabs (Fig. 3). The bootstrap support of this more inclusive clade (63%) is low and, moreover, such a relationship has never been considered in the historical studies utilizing adult morphology. The only independent piece of evidence consistent with this more inclusive clade is its Afrotropical distribution. A more inclusive analysis preferably involving representatives of all 20 herein absent nominal genera of Ceratocanthini would be required to adequately test this hypothesis.

4.3. Goals 03 and 04: Testing the refugia and species-pump hypotheses

The newly generated phylogeographic data on *Afro-cloetus*, including the first temporal interpretation of their post-Oligocene evolution (Fig. 4), display signs of at least two subsequent cycles of forest expansion and contraction. All but one among five extant geographical *Afrocloetus* clades reveal originally strictly allopatric geographical distributions and pre-Pleistocene divergences. These results are consistent with the refugia hypothesis of speciation driven by the Miocene uplift of the East African Plateau causing aridification and forest fragmentation. The only exception is the younger Udzungwa clade (= sister to Rubeho clade, Fig. 4), which is herein interpreted as a secondary invasion of (= immigration to)

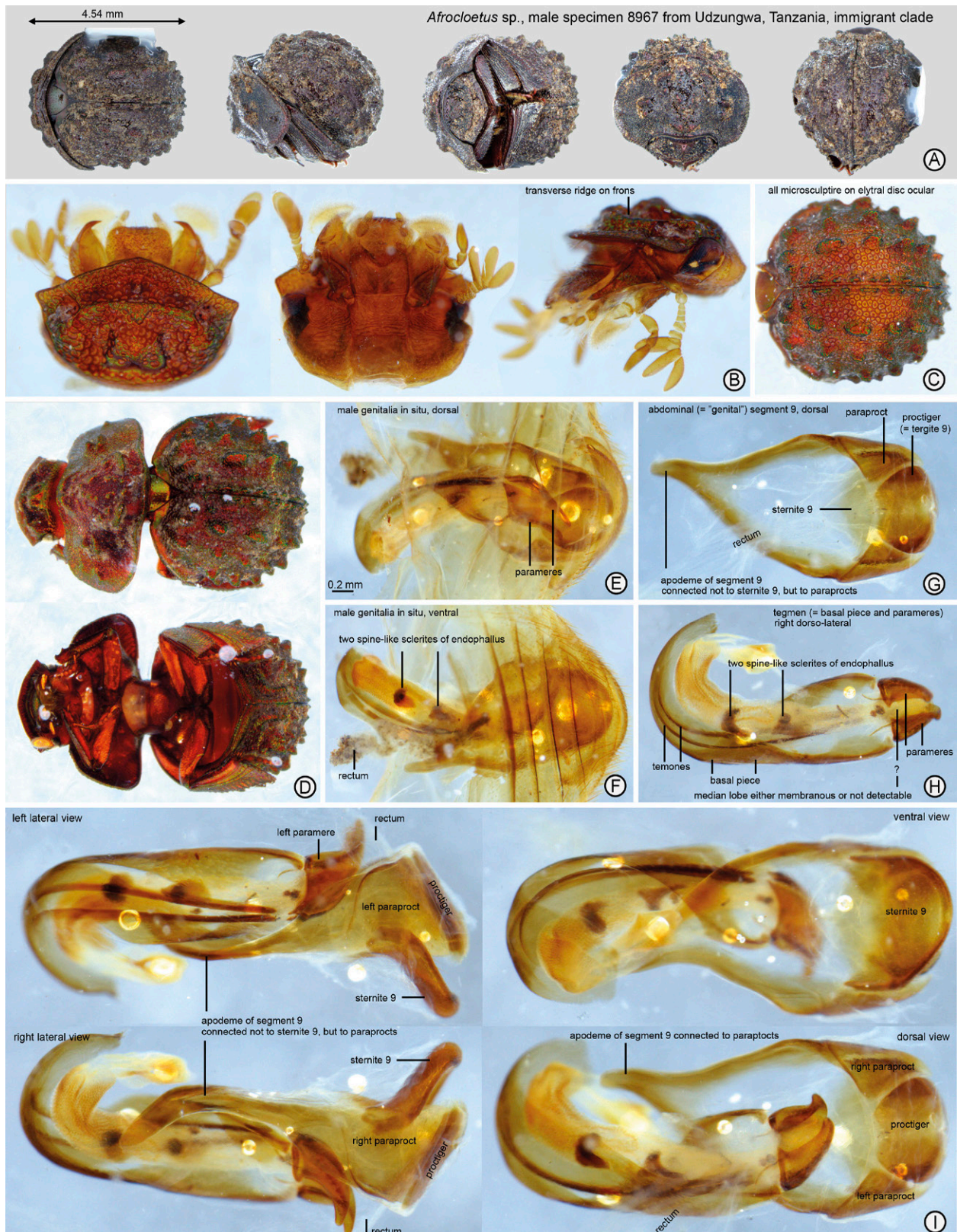


Fig. 5. External and internal morphology of *Afrocloetus* sp., male specimen 8967 from Udzungwa, Tanzania.

Udzungwa forests which were already occupied by the older resident *Afrocloetus* clade. This secondary invasion is deduced from the sympatry of two genetically and morphologically distinct *Afrocloetus* populations coexisting without signs of introgression in Udzungwa, each

of which having a different sister group and notably different divergence time (Fig. 4). The estimated date of the Udzungwa re-colonisation by the immigrant *Afrocloetus* population is well inside the Pleistocene, interglacial periods which manifested themselves in Africa by a compar-

atively wetter climate and significant forest expansions. It follows that Udzungwa forest, already supporting the resident *Afrocloetus* population, has been at least once temporary re-connected with other nearby forests, from where a younger genetically different population arrived by means of normal ecological dispersal (Figs. 1A, 4). After being initially separated by forest fragmentation about 4.96 Ma (Fig. 4), both Udzungwa populations met 1.75 (1.2–2.3) Ma. Considering their observed genetic and morphological distinctness, the interim period of allopatric evolution lasting about 3.2 Myr was sufficient for both lineages to accumulate enough mutations to become reproductively isolated (= speciate), which is consistent with the species-pump hypothesis. Although infrequently documented in the relatively understudied African context, speciation mechanics through cycles of habitat expansion and contraction has been suggested for a number of late Miocene clades, e.g., European Hae-nydra clade of *Hydraena* Kugelann, 1794 water beetles (RIBERA et al. 2011).

Mitochondrial sequences formed a significant source of evolutionary signal in the herein implemented phylogenetic analysis and the only source of information in the temporal analysis. Considering their maternally-inherited (= non-recombinant) nature, both topologies used as the basis of evolutionary interpretations (Figs. 3, 4) might be biased by any of the relatively well-known causes of mitochondrial non-monophyly, particularly by incomplete lineage sorting (FUNK & OMLAND 2003). This, however, is unlikely to have significantly affected the topologies because of three reasons. Firstly, both mitochondrial (COI) and nuclear (ITS2 and 28S) fragments provide consistent signal. Secondly, considering the random nature of incomplete lineage sorting, its traces should be detected evenly across the mitochondrial tree (Fig. 4), which is not the case (except, perhaps, for relatively early diverging specimens 3770 from Kimboza forest and 8749 from the older Udzungwa clade, which, however, cluster as expected with their geographical neighbours). Thirdly and perhaps most convincingly, morphological differences of both Udzungwa populations are fully consistent with the mitochondrial topology (Fig. 4).

Perhaps the main weakness of the herein implemented temporal analysis is its reliance on the flat substitution rate of a single fragment (COI). This is, however, an unavoidable shortcoming when analysing obscure clades from inland localities that lack reliable and preferably multiple well-dated calibrating fossils (as in Gyrinidae beetles, GUSTAFSON et al. 2017) or well-documented geological events (as in the Western Mediterranean during the last 35 Myr, FAILLE et al. 2018). Furthermore, even if deviating little from 0.018 subs/s/Myr/l (see Material and Methods), this value was thought to be about four times higher (0.0793 subs/s/Myr/l) in *Trigonopterus* Fauvel, 1862 weevils (analysis 2 in TÄNZLER et al. 2016). With all these limitations, however, the herein presented attempt to measure the time dimension of pill scarab evolution is the first ever attempted and, therefore, is unavoidably preliminary.

4.4. Goal 05: Morphological diagnostics of *Afrocloetus*, *Congomastes* and nearby clades

Comparative morphological study of the ingroup pill scarabs is outside the scope of the present paper. Recovery of the strongly supported topology (Fig. 3) is, however, an inviting opportunity to discuss adult morphology as a source of phylogenetically relevant data and to document morphological characters of an ingroup representative. The latter is herein employed to illustrate diagnostic (and potentially synapomorphic) characters of the following clades: 1. *Afrocloetus*; 2. *Congomastes*; 3. *Afrocloetus* + *Congomastes*; 4. PhG; 5. PhG + (*Afrocloetus* + *Congomastes*).

BALLERIO (2006) listed four *Afrocloetus* characters unique among all Afrotropical pill scarabs. His character of “genal canthus indistinct, with eyes not visible from above” should be treated as three independent characters (two multistate and one binary), resulting in a total of six characters:

- interocular bridge (= “genal canthus”) extending posteriorly from anterior edge of eyes: absent (as in *Afrocloetus*, Figs. 5B, 6B) versus present; when present, then either subdividing eyes only in anterior half (as in *Congomastes*, Fig. 7B) or completely (as in many members of closely related PhG) into externally visible dorsal and ventral eyes (and in a manner similar to that of whirling beetles, Gyrinidae; BEUTEL et al. 2017);
- eyes, their part visible from above when head is not covered by pronotum: barely visible as longitudinal strips (as in *Afrocloetus*, Fig. 5B), obviously visible (as in *Congomastes*, Fig. 7B), or not visible (as in *Ph. ballerioi* Grebennikov, 2019, which forms sister to the rest of entire PhG; GREBENNIKOV 2019a);
- dorsal part of eyes, when beetle is in enrolled position: more than half covered by anterior angles of pronotum (as in *Afrocloetus*, Figs. 5A, 6A) or fully visible (as in *Congomastes*, Fig. 7A).

Three other characters mentioned by BALLERIO (2006) as unique for *Afrocloetus* could not be reliably used to distinguish this nominal genus from *Congomastes* (and perhaps from other pill scarabs): “fore tibiae robust, with two apical teeth and some other smaller teeth on the outer side”, “meso- and metatibiae flattened” and “metatibiae expanded apically (“triangular”)”. Three other characters, however, seem to be diagnostic for each of both nominal genera:

- elytra with longitudinally oriented tubercles arranged in interrupted ridges (*Afrocloetus*, Figs. 2C, 5C,D, 6D) or elytra with even and uninterrupted carinae (*Congomastes*, Figs. 2A,B, 7D); *A. forshagei* from Kenya has, however, the *Congomastes* character state (Fig. 2D);
- each elytron with longitudinal sutural ridge, plus four more (*Afrocloetus*, Fig. 5C,D) or each elytron with sutural longitudinal carina, plus three more (*Congomastes*, Figs. 2A,B, 7D);
- short ridges and tubercles on pronotum and head dorsally, in addition to microsculpture: present (*Afrocloetus*,

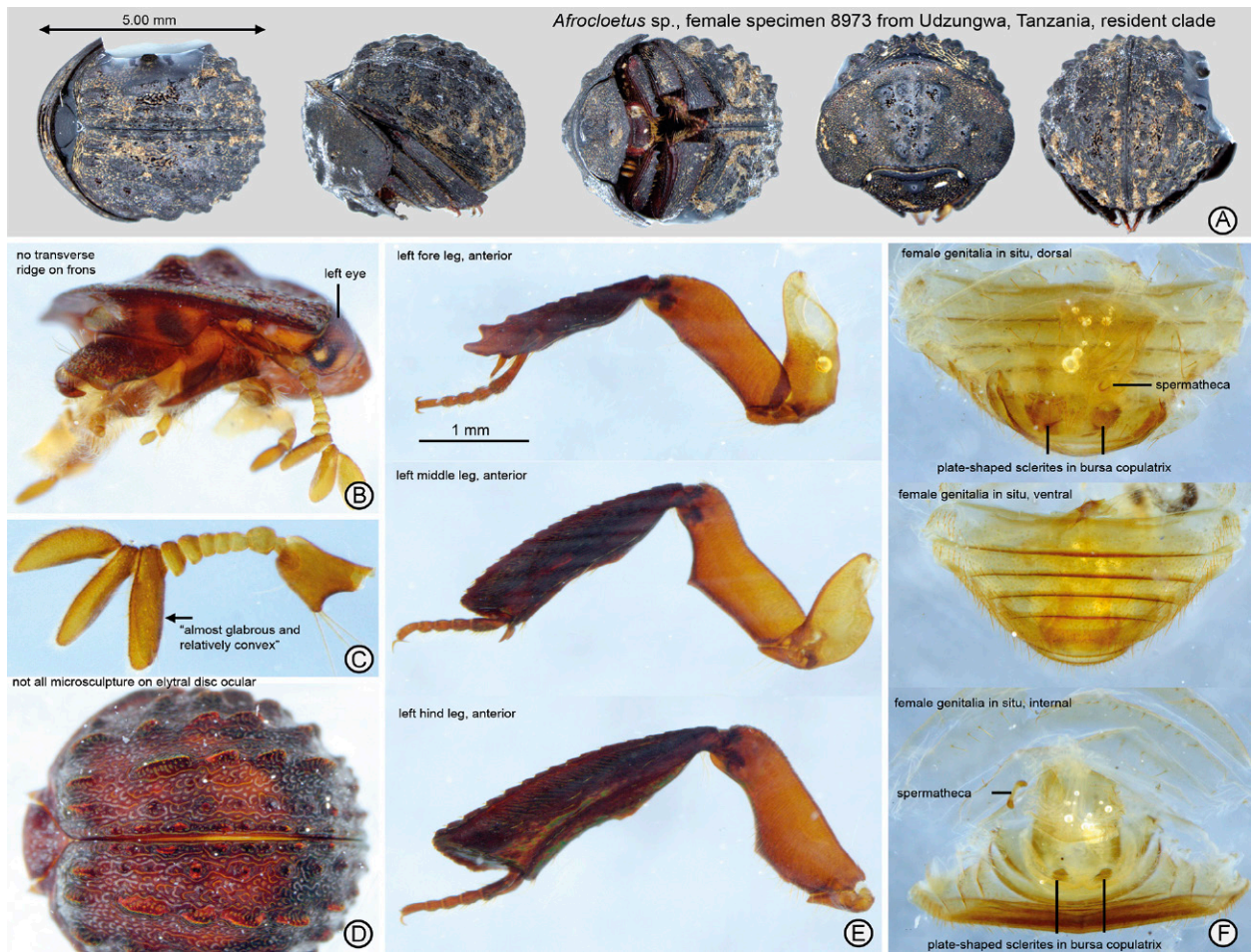


Fig. 6. External and internal morphology of *Afrocloetus* sp., female specimen 8973 from Udzungwa, Tanzania.

Figs. 2C,D, 5C,D, 6A) or absent (*Congomostes*, Figs. 2A, B, 7A).

Three characters are diagnostic (and perhaps synapomorphic) for the clade of *Afrocloetus* and *Congomostes*: (1) body large, its maximal dimension in enrolled position 4.5–5.5 mm; (2) dark and non-metallic body and (3) strongly carinate elytra. Even though occasionally found in other pill scarabs, the combination of these three characters is unique for the clade in question. BALLERIO (2006) mentioned “almost glabrous and relatively convex” proximal surface of the proximal club antennomere (Fig. 6C) as characteristic for *Afrocloetus*; this, however, could not be herein adequately assessed for the lack of comparative material.

The *Philharmostes* group of genera, sister to the *Afrocloetus* + *Congomostes* clade (Fig. 3), is diagnosable by the shape of their protibiae, which are curved (= “broadly arcuate”) and without large spines (= “teeth”) on outer margin (versus the straight and spiny protibiae of the majority of pill scarabs, including *Afrocloetus* and *Congomostes*, Figs. 6E and 7C, respectively). No morphological character is presently known to diagnose the more inclusive clade formed by PhG and its sister (if, indeed, one exists).

Sexual dimorphism could not be detected among large-bodied Afrotropical pill scarabs, which is similar

to the condition found in *Ph. ballerioi* and many other members of PhG. Assuming that all these beetles form a clade (Fig. 3), sexual dimorphism in the shape of the protibiae among some members of PhG would likely be an evolutionary novelty of the more restricted group (*Callophilharmostes fleutiauxi* (Paulian, 1943), *Car. vadoni*, *Petrovitzostes guineensis* (Petrovitz, 1968); see character 80 in BALLERIO & GREBENNIKOV 2016).

As presently known, the female genitalia of pill scarabs are weakly sclerotized (Fig. 6F) and offer only the four characters listed in BALLERIO & GREBENNIKOV (2016). The male genitalia, particularly the internal sclerotization of the endophallus, appear to be a richer source of data, which is in line with results obtained for Scarabaeinae dung beetles (MEDINA et al. 2013; TARASOV & GÉNIER 2015; TARASOV 2017). Herein documented male genitalia of a representative of *Afrocloetus* (Fig. 5E–I) and *Congomostes* (Fig. 7E–I) corroborate this supposition and invite a more detailed study. Such a study, however, will likely require significant specimen disarticulation, since sexing of many non-sexually dimorphic pill scarabs cannot be done before genitalia dissection. The later normally results in significant specimen damage, particularly when the disarticulated abdomen has to be retracted through the relatively narrow (= bottle neck) ventral opening of the subelytral cavity (Figs. 5D, 7D).

Except for the aedeagus (D'HOTMAN & SCHOLTZ 1990), all other sclerites of the male genital chamber (i.e. those posterior to abdominal segment 7, particularly those of abdominal segment 9) have never been homologized across the superfamily Scarabaeoidea in terms comparable with those used in other beetles. Some of these structures are consistently referred to by vague historical or descriptive terms, such as “genital segment” or “spiculum gastrale”. Following WANAT's (2007) studies of weevils (where he illustrated for comparative purposes two scarab genera), a morphological assessment of the sclerites on abdominal segment 9 (Figs. 5I, 7I, = “genital segment”) was conducted, the first such treatment in the superfamily Scarabaeoidea. This interpretation suggests that the proctiger (= tergite 9, Figs. 5I, 7I; not tergite 10) and paraprocts (Figs. 5I, 7I) belong to abdominal segment 9, while no sclerites of abdominal segment 10 can be detected in Coleoptera. Consistent use of these terms promotes sclerite homologization and, therefore, a phylogenetic (rather than phenetic) approach to morphological data.

Apart from BALLERIO & GREBENNIKOV (2016), the only phylogenetic hypothesis linked *Afrocloetus* with the South African genera *Aneilobolus* and *Acanthocerodes* (BALLERIO 2006: 312). It was supported by similarities in the proximal surface of the proximal antennomere of the club (Fig. 6C), which is “almost glabrous and relatively convex” (and by less definite characters of “the shape of the labrum, that of mandibles and the teeth on fore tibiae”). Remarkably, *Congomostes* was not mentioned as a possible relative of *Afrocloetus* (BALLERIO 2006). The genus *Acanthocerodes* is not represented in the present analysis, while *Aneilobolus* forms a strongly supported clade (99%) with two New World terminals (Fig. 3), thus refuting any hypothesized closest relations with either *Afrocloetus* or *Congomostes*.

4.5. Goal 06: Inadequate taxonomy of *Afrocloetus* and *Congomostes*

The relationships among five newly detected *Afrocloetus* populations in four Tanzanian forests do not offer a simple taxonomic solution. Both sympatric and morphologically distinct Udzungwa populations of *Afrocloetus* maintain their genetic identity (Figs. 3, 4) and fully deserve to be treated as separate Linnaean species, regardless of a species concept and/or definition (DE QUEIROZ 2007). If so, then the Kimboza population, sister to the clade containing them both (Figs. 3, 4), must be treated as a third Linnaean species, even though it is morphologically indistinguishable from all herein sequenced *Afrocloetus*, except for the resident Udzungwa clade. If this three-species scheme is implemented, then it is uncertain how to treat the taxonomy of the Kaguru and Rubeho populations (Figs. 3, 4), which form a clade with the immigrant Udzungwa population and are morphologically indistinguishable from it (and from that in Kimboza). The situation is compounded by the fact that both nomi-

nal *Afrocloetus* species are likely not represented in the DNA analysis and perhaps will never be (see above on forest destruction in both type localities). These uncertainties result in a taxonomic stalemate in naming new *Afrocloetus* species, at least for the time being.

Instead of fine-tuning the existing taxonomy of the ingroup in accordance with the results of the phylogenetic analysis, this study opens a Pandora's box of unsettled discrepancies between phylogeny and taxonomy. The absence in the analysis of the type species of *Congomostes* is particularly problematic. Assuming, however, that *C. hintelmanni* adequately represents a monophyletic *Congomostes* (but see BALLERIO et al. 2011), maintenance of two generic names for the morphologically, biologically and geographically coherent clade of the giant Afrotropical pill scarabs is redundant. The most notable morphological dissimilarity between at least the type species of both nominal genera (complete versus multiply interrupted elytral ridges of *Congomostes* and *Afrocloetus*, respectively) is paralleled by two other Afrotropical pill scarabs forming the genus *Cryptophilharmostes* Ballerio, 2000 (*C. mahunkai* Ballerio, 2000 and *C. merkli* Ballerio, 2005, respectively). It is, therefore, likely, that both generic names of the ingroup must be synonymized. If so, PAULIAN's (1968) paper introducing nominal *Congomostes* came out in the first issue of a journal published on an unspecified date in September 1968 (colophon on page 164), while the issue containing Petrovitz's *Afrocloetus* (1968) was printed on December 30, 1968 (recto of title page); this would establish seniority of the former name over the latter (not herein implemented). It should also be mentioned that PAULIAN (1977) expressed concerns about whether his own nominal species *C. baloghi* was different from *C. janssensii* (their holotypes are in Figs. 2A and 2B, respectively). Considering all these uncertainties and pending further research, the current taxonomy of the nominal genera *Afrocloetus* and *Congomostes* is not herein modified.

5. Concluding remarks

Rediscovery in the wild of multiple specimens and populations of previously enigmatic *Afrocloetus* pill scarabs led to a number of notable results. A DNA-based analysis suggested that the large body is a non-reversed evolutionary novelty (= autapomorphy) of the newly hypothesized clade of the two nominal (and likely synonymous) genera *Afrocloetus* and *Congomostes*. Clade evolution was intimately linked with, and is indicative of, the post-Oligocene history of wet African forest. Widely distributed East African descendants of the most recent common ancestor of *Afrocloetus* were separated from those of *Congomostes* by the Miocene uplift of the East African plateau and were multiply fragmented, together with the ancient forests that they had inhabited. Subsequently, strictly allopatric populations of *Afrocloetus* evolved in isolation until Pleistocene. Subsequent forest expan-

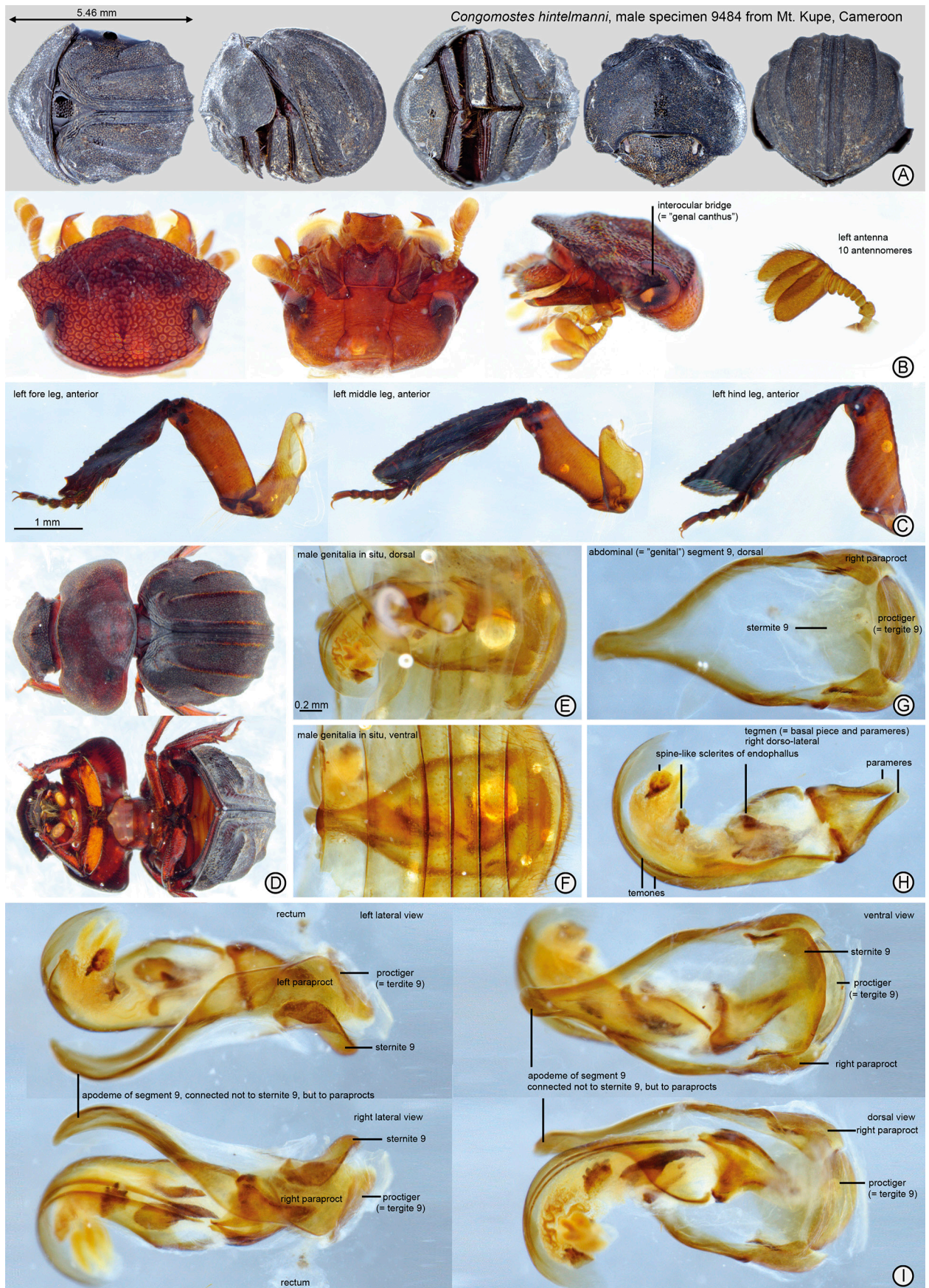


Fig. 7. External and internal morphology of *C. hintelmanni*, male specimen 9484 from Mt. Kupe, Cameroon.

sion and contraction cycles facilitated re-colonization of Udzungwa by an immigrant population through normal ecological dispersal along a hypothetical forest connection. Since then both Udzungwa populations co-existed in sympatry as different species, i.e. without introgression and while accumulating morphological distinctness. The evolution of *Afrocloetus* during the last 15 Myr is, therefore, consistent with both the refugia and the species-pump hypotheses, each of them leaving its unique and recognizable signatures in the phylogeographic pattern of these beetles.

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