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ORIGINAL ARTICLE

Survey and DNA barcoding of flat bugs (Hemiptera: Aradidae) in the Tanzanian Forest Archipelago reveal a phylogeographically structured fauna largely unknown at the species level

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Abstract. We report results of a faunal survey of Aradidae flat bugs sampled by sifting litter in 14 wet and discrete Tanzanian primary forests (= Tanzanian Forest Archipelago, TFA) of different geological origins and ages. Images, locality data and, when available, DNA barcoding sequences of 300 Aradidae adults and nymphs forming the core of the herein analyzed data are publicly available online at dx.doi.org/10.5883/DS-ARADTZ. Three Aradidae subfamilies and seven genera were recorded: Aneurinae (*Paraneurus*), Carventinae (*Dundocoris*) and Mezirinae (*Afropictinus*, *Embuana*, *Linnavuoriessa*, *Neochelonoderus*, *Usumbaraia*); the two latter subfamilies were also represented by specimens not assignable to nominal genera. Barring the six nominal species of *Neochelonoderus* and *Afropictinus* described earlier by us from these samples and representing 11 of the herein defined Operational Taxonomic Units (OTU), only one of the remaining 52 OTUs could be assigned to a named species; the remaining 51 OTUs (81%) represent unnamed species. Average diversity of Aradidae is 4.64 species per locality; diversity on the three geologically young volcanoes (Mts Hanang, Meru, Kilimanjaro) is significantly lower (1.33) than on the nine Eastern Arc Mountains (5.67) and in two lowland forests (5). Observed phylogeographic structure of Aradidae in TFA can be attributed to vicariance, while the depauperate fauna of Aradidae on geologically young Tanzanian volcanoes was likely formed anew by colonisation from nearby and geologically older forests.

INTRODUCTION

DNA data generation and analysis targeting the cytochrome oxidase barcoding fragment (658 bp of COI-5' mitochondrial gene, Hebert et al., 2003a; b) has become established as a popular and efficient tool with various applications. Many such studies report DNA barcodes targeting members of a certain clade from a certain territory; some of the notable examples are Central European beetles (Hendrich et al., 2015) and bees (Schmidt et al., 2015), North American Noctuoidea moths (Zahiri et al., 2017), Australian hawkmoths (Rougerie et al., 2014), Neotropical Gracillariidae moths (Lees et al., 2013) and European bugs (Raupach et al., 2014; Havemann et al., 2018). Here we follow this conventional approach by reporting a DNA barcode library of Aradidae flat bugs thoroughly sampled throughout the Tanzanian Forest Archipelago (TFA) and use this newly generated dataset to test a set of hypotheses (Collins & Cruickshank, 2013).

The family Aradidae (Figs 1B–D, 2–5) is a well-supported cosmopolitan clade of about 280 recent genera and some 2,000 recent species (numbers approximated from Grebennikov & Heiss, 2014). The family is still inconsistently treated either with (Cassis & Schuh, 2010) or without (Marchal & Guilbert, 2015) the odd family Termitaphididae, a clade of 13 exclusively termitophilous, blind and wingless small-bodied pantropical species, either recent or extinct. Wet tropical forests tend to harbour the greatest diversity of Aradidae. The most recent common ancestor of Aradidae and its sister group (likely formed by the rest of the superfamily Pentatomomorpha, Schuh & Slater, 1995; Grazia et al., 2008; Yao et al., 2012; Liu et al., 2018) lived in the late Triassic (about 214 Ma, Song et al., 2016), while the Aradidae crown group dates from the late Jurassic (about 162 Ma, Song et al., 2016). The eight Aradidae subfamilies are Aneurinae, Aradinae, Calisiinae, Carventinae, Chinamyersiinae, Isoderminae, Mezirinae and Prosympiestinae. Mezirinae include more than half of the species in this family, Prosympiestinae is of doubtful monophyly (Marchal & Guilbert, 2015), while Calisiinae likely forms the sister to the remaining subfamilies (Song et al., 2016). Detailed relationships within the family have remained mostly unresolved. The easy-to-recognize appearance of Aradidae, the existence of a classical and well-illustrated global taxonomic treatment (Usinger & Matsuda, 1959)

and a catalogue (Kormilev & Froeschner, 1987) stimulated studies on the regional diversity of Aradidae, such as those by Monteith (1997) in Australia, Larivière & Larochelle (2006) in New Zealand and Baňař & Heiss (2018) in Madagascar. The degree to which aradid biodiversity has been described varies greatly among regions, and much descriptive work remains to be done in Tanzania and East Africa in general (Heiss, 2013). From an international trade perspective, Aradidae are frequently intercepted on sea-transported commodities (Chérot et al., 2011) such as wood/bark products, fruits and cut flowers, however their species level identification is difficult because of inadequate taxonomy. Smith-Pardo & Beucke (2015) documented that due to the lack of morphological and DNA diagnostic tools, only 65 among the total of 128 Aradidae interceptions made between 1992 and 2013 at the United States ports of entry were identified beyond the family level. All in all, flat bugs are just another widely distributed medium-sized monophyletic insect family suffering from taxonomic and phylogenetic neglect.

The Tanzanian Forest Archipelago (TFA), the geographical focus of this paper, is a remarkable biological phenomenon. It is formed by a dispersed cluster of wet and cool closed-canopy rainforests of variable size and altitude, separated by much greater areas of highly contrasting dry and hot savannah. Based on their age and genesis TFA forests fall into two distinct groups. One consists of ancient forests of either the Eastern Arc Mountains (EAM, Lovett & Wasser, 1993; Newmark, 2002) or those of the lowland coastal plain, both tracing their uninterrupted existence since at least the Miocene pan-African forest (>30 Myr, Hamilton & Taylor, 1991; deMenocal, 2004). The second group is formed by forests that came into existence within the last 2 Myr on geologically young volcanoes, such as Mts Kilimanjaro and Meru associated with the East African Rift (Nonnotte et al., 2008). These contrasting spatial settings facilitate the testing of the classic phylogeographic riddle as to what degree dispersal versus vicariance was the main driving force behind the distribution of organisms critically dependant on such a discrete and widely dispersed habitat (438 hits in a Google Scholar search using "Eastern Arc Mountains" and "phylogeography" on July 12, 2018).

This study of Aradidae in TFA hinges on the advantageous capacity of DNA barcoding to rapidly generate sufficient data to test hypotheses and present the results as a phylogenetic tree. Reliability of such trees at deeper levels is compromised by the well-documented shortcomings of a single quickly saturating and maternally inherited DNA marker such as COI (Funk & Omland, 2003). A significant and herein utilized practical advantage of the DNA barcoding approach is its independence from the pre-existing taxonomic framework, which makes this tool particularly applicable for studying poorly known and taxonomically neglected faunas, such as Aradidae from TFA. Instant online availability of all DNA barcoding data, including sequences, specimen images and geographical data, renders this tool particularly attractive and useful.

The goal of this study is, therefore, to perform a faunal survey of TFA Aradidae by generating their DNA barcode data. The first step in this work is to document the currently almost unknown diversity of Aradidae in TFA using DNA barcoding and then to use the data to test five hypotheses (H1 to H5):

H1: Aradidae in Tanzania are well known taxonomically at the generic and species levels.

H2: Shallow clades of Aradidae in TFA are geographically structured.

H3: All the phylogeographic structure of Aradidae in TFA can be attributed to vicariance.

H4: Aradidae fauna on geologically young Tanzanian volcanoes was formed anew and after volcanic highlands and their forests came into existence about two million years ago.

H5: If H4 is supported, then this process was driven by colonization from nearby and geologically older TFA forests.

MATERIAL AND METHODS

Specimen sampling, storing and coding

Adults and nymphs of Tanzanian Aradidae were obtained from samples of litter (Fig. 1F) collected at 14 TFA localities (Fig. 1A). Forest litter (Fig. 1E) was sifted through a hand-held sifter (Fig. 1G) and live specimens were subsequently extracted by suspending the fine litter fraction ($< 7 \times 7$ mm) in Winkler funnels. All 300 specimens were imaged (Fig. S1 in supplementary files) and uniquely linked to one of 130 samples (Fig. 1F) using two-letter and two-digit codes (explained in Table 1 in Grebennikov, 2017). Non-Tanzanian specimens included for comparative purposes (see below) were assembled from different sources. Identification to the lowest possible taxonomic rank (mainly to genus, occasionally to species or to subfamily) were done by EH based on closest match with authoritatively identified voucher specimens stored in his collection (CEHI, Ernst Heiss' Collection, Tiroler Landesmuseum, Innsbruck, Austria). Specimens are stored in either CEHI (98 non-Tanzanian specimens coded in the format BIOUG0209X-XXX and abbreviated in Figs 2–4 to EHX-XXX; previously reported in Grebennikov & Heiss, 2014) or in the Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Canada (CNC, codes are in the format CNCCOLVG0000XXXX abbreviated to the last four digits as in Figs 2–4; either reported in Heiss & Grebennikov, 2015, 2016, or new).

Hypotheses testing

To bring a DNA barcode library report into the realm of science (Popper, 1959), and as recommended by Collins & Cruickshank (2013), the herein released data, including the faunal survey counts and DNA barcode sequences, are used to test five hypotheses mentioned in the Introduction. The methodology is as follows:

H1: calculate the proportion of species of Aradidae successfully assigned to an existing Linnaean nominal genus and/or species of the total OTUs detected in the Tanzanian samples;

H2: assess whether terminal clades recovered in the Maximum Likelihood (ML) analysis are formed by specimens from the same forest of the 14 sampled;

H3: document reciprocal monophyly of geographically defined clades; the latter is considered the standard signature of vicari-

Fig. 1. A – map showing the localities in the Tanzanian Forest Archipelago sampled; B – Mezirinae specimen 5710 (not DNA barcoded) from Pugu Hills; C – *Usumbaraia* sp. from West Usambara, U; D – *Afropictinus idas* Heiss & Grebennikov, 2016 from East Usambara; E – sample WU01 in West Usambara; F – locality of sample WU07 in West Usambara; G – locality of sample RB05 in Rubeho.

ance of a widespread ancestor (Heads, 2014: 6), while deviations from this pattern might suggest long-range dispersal;

H4: compare the diversity of Aradidae on young volcanoes to that of all other sampled TFA localities (= old forests);

H5: detect whether Aradidae from young volcanoes are more closely related to those from the nearby EAM forests, than to those from other forests.

DNA sequencing, data availability and three public datasets

From a total of about five hundred specimens of freshly sampled TFA Aradidae, 300 were selected under a dissecting microscope for DNA barcoding. Sequencing of the DNA barcode fragment of these specimens (and of 234 non-Tanzanian specimens) was performed in a commercial laboratory "Canadian Center for DNA Barcode" (CCDB, http://www.ccdb.ca/) at the University of Guelph, Ontario, Canada, following the standard laboratory protocol (Ivanova et al., 2014, 2006). Two primer pairs were used to amplify the DNA barcoding fragment (Table 2 in Grebennikov, 2017). From a total of 534 specimens of Aradidae subjected to DNA barcoding, 387 resulted in DNA sequences > 200 bp (of them, 209 were those from Tanzania and they formed a dataset for a Neighbour Joining analysis, see below) and 295 resulted in sequences > 500 bp. These 295 sequences were selected for the Maximum Likelihood (ML) analysis (described below); of them, 167 sequences represented Tanzanian specimens and 128 were of non-Tanzanian origin. Among the 295 > 500 bp sequences, 96 non-Tanzanian sequences were reported by Grebennikov & Heiss (2014), one Tanzanian *Neochelonoderus* Hoberlandt, 1967 sequence was reported by Heiss & Grebennikov (2015), while 22 Tanzanian and one Ethiopian sequence of *Afropictinus* Heiss, 1986 were reported by Heiss & Grebennikov (2016); the remaining 175 sequences are newly generated (140 from Tanzania, 14 from Vietnam, nine from Ethiopia, four from Cameroon and three from China). Representatives of Mezirinae dominated the ML matrix (223 among a total of 295, 149 among a total of 167 from Tanzania). All herein utilized data consisting of images of specimens, information on their localities and, when available, DNA barcode sequences and trace files, are digitally deposited in the Barcode of Life Data System (BOLD, Ratnasingham & Hebert, 2007) and publicly available through three partly overlapping datasets, each designed to serve a unique purpose:

dataset ARADTZ available online at dx.doi.org/10.5883/DS-ARADTZ contains data from all 300 freshly sampled specimens

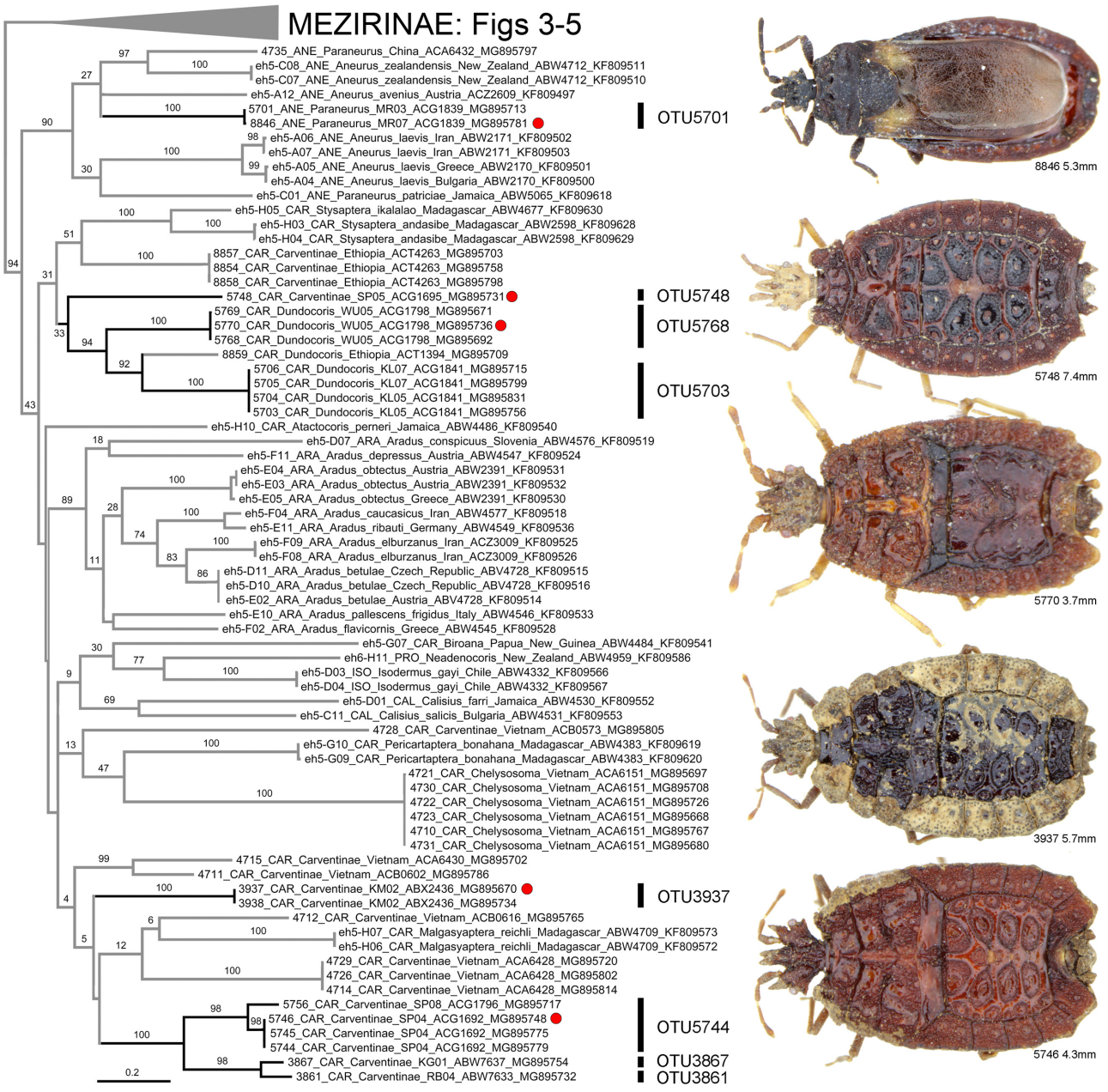

Fig. 2. Maximum Likelihood inference phylogram of non-Mezirinae flat bugs (Aradidae) using the DNA barcoding fragment. Tanzanian specimens are in black. Terminals are arranged in Operational Taxonomic Units (OTUs). Terminal labels consist of a specimen number followed by taxonomic information (a three letter subfamily abbreviation with, when available, genus and species), followed by geographic information (country of origin or, for Tanzanian specimens only, a two-letter two-digit sample code), BIN number and GenBank accession number. Red dots denote illustrated terminals.

of Aradidae from Tanzania submitted for DNA barcoding (of them, 91 specimens are without DNA barcode data) and are used to test hypothesis H1;

dataset ARADBARC available online at dx.doi.org/10.5883/DS-ARADBARC contains data from all 209 freshly sampled specimens of Aradidae from Tanzania with DNA barcode sequences > 200 bp, which were used in the BOLD Neighbour Joining (NJ) analysis (see below) to partly test hypotheses H2–H5;

dataset ARADIDAE available online at dx.doi.org/10.5883/DS-ARADIDAE contains data from all 295 specimens of Aradidae from Tanzania (167) and from the rest of the World (128) with DNA barcode sequences > 500 bp, which were used in the Maximum Likelihood (ML) analysis (see below) to partly test hypotheses H2–H5.

Neighbour Joining analysis of the 209 terminals > 200 bp Tanzanian dataset

Phenetic clustering was done using the BOLD online engine using the NJ method, without rooting and utilizing the Kimura 2-parameter. Its main purpose was to generate and make available a Tanzania-only DNA barcode dataset (see above) and to assign 42 Tanzanian specimens with short sequences (200–500 bp) to those having longer sequences and thus elucidate their species-level identity for a subsequent faunal count.

Maximum Likelihood analysis of the 295 terminals > 500 bp World dataset

Sequence alignment was trivial and introduced no insertions or deletions. Phylogenetic analysis was done using the CIPRES

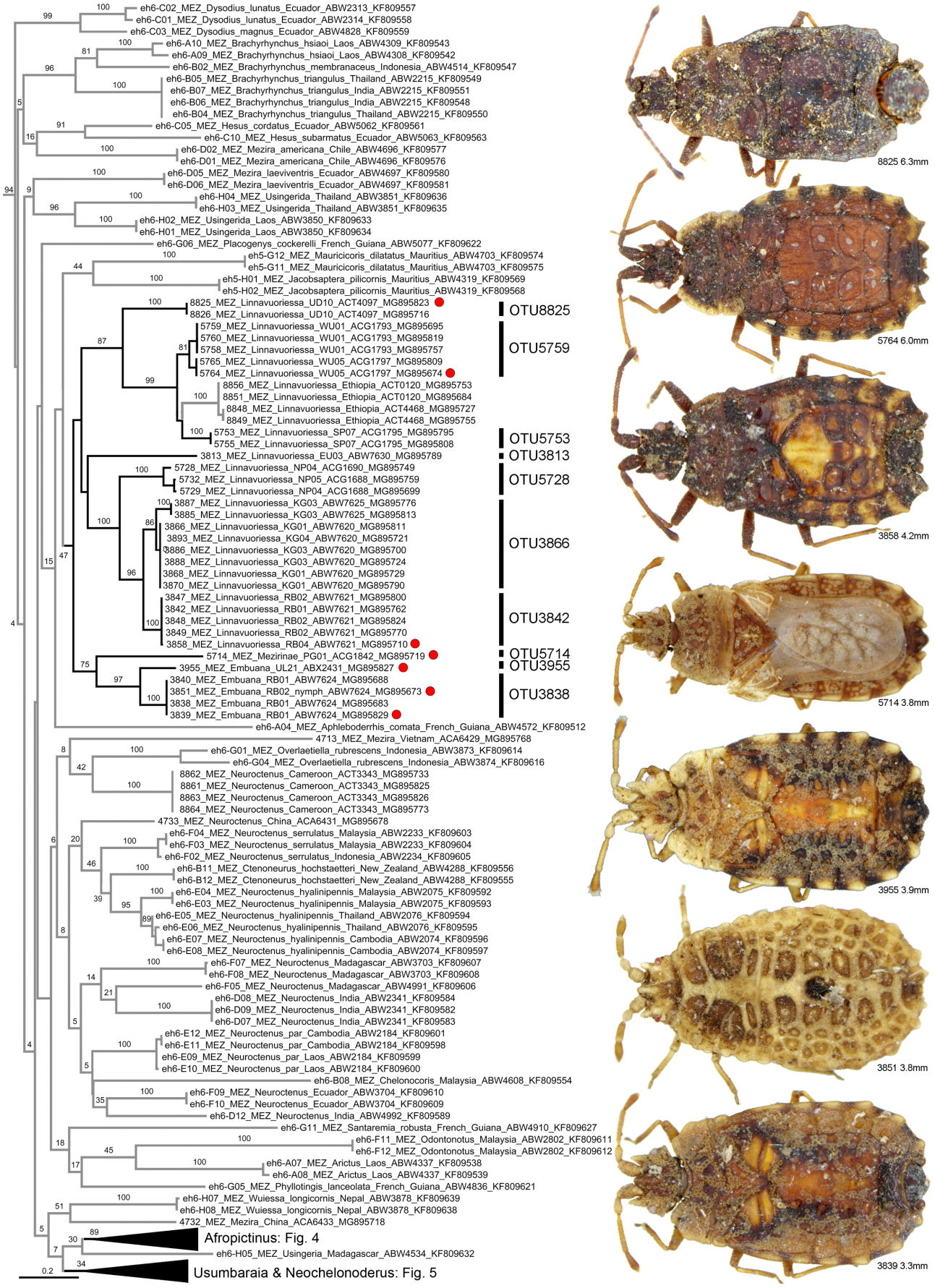

Fig. 3. Maximum Likelihood inference phylogram of Mezirinae flat bugs (Aradidae) using the DNA barcoding fragment (continued from Fig. 2).

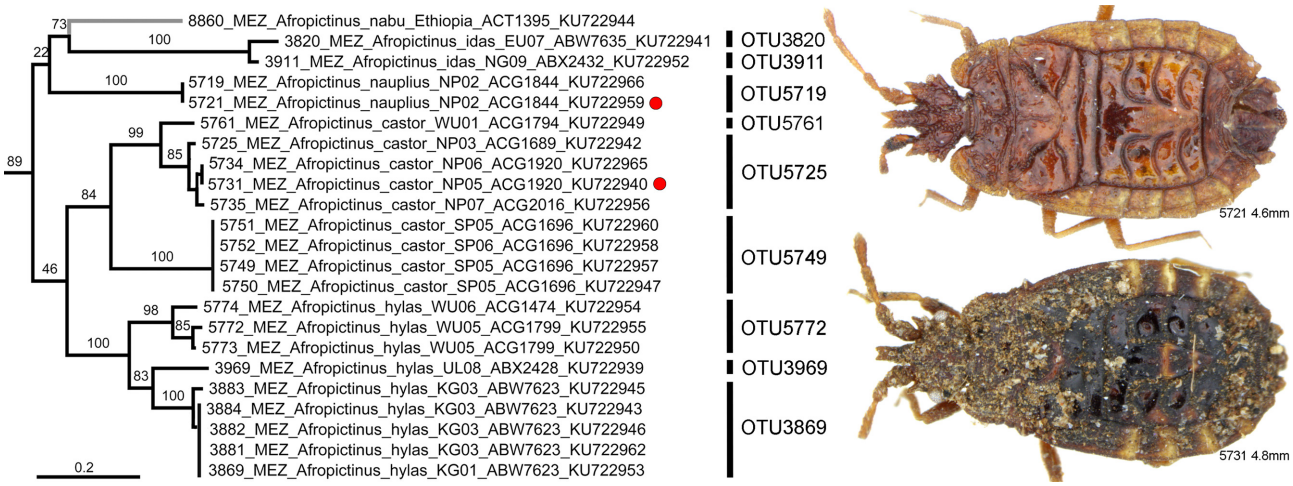

Fig. 4. Maximum Likelihood inference phylogram of *Afropictinus* flat bugs (Aradidae: Mezirinae) using the DNA barcoding fragment (continued from Figs 2–3).

Science Gateway (Miller et al., 2010) and the ML method. Topologies were generated using RAxML 7.2.7 (Stamatakis et al., 2008), with the default parameters and the GTR + G nucleotide substitution model. Clade support values were obtained with 1000 parametric bootstrap replicates (Felsenstein, 1985). Since monophyly of Aradidae is not herein doubted and the subfamily interrelationships are not tested, the resulting consensus topology was visualized and rooted arbitrarily between the largest and monophyletic Mezirinae and the rest of the tree using FigTree1.4 (Rambaut, 2014).

Operational Taxonomic Units (OTU) as candidate species, a provisional tool to document and compare the diversity of TFA Aradidae

A Linnaean species, a universally accepted unit for counting biological diversity, cannot be consistently used for TFA Aradidae due to the lack of a pre-existing taxonomic framework. Except for two genera taxonomically revised by us using some of the herein reported specimens (*Neochelonoderus* by Heiss & Grebennikov, 2015 and *Afropictinus* by Heiss & Grebennikov, 2016), only one of the TFA Aradidae could be assigned to a named species. A temporary alternative to a Linnaean species has, therefore, to be herein introduced and employed. Ideally it should meet all three primary taxon-naming criteria (Vences et al., 2013): monophyly of the taxon in an inferred species tree, clade stability and be phenotypically diagnosable. Our main practical challenge was the acute data incompatibility. Indeed, among 300 TFA specimens analyzed, only 209 have DNA barcode sequences > 200 bp and only 167 of them have DNA barcode sequence > 500 bp. To consistently assign all 300 TFA specimens to a unit comparable to a Linnaean species, we defined Operational Taxonomic Units (OTUs), which are considered to be unnamed candidate species. Like a Linnaean species, they are formed by one or more specimens meeting, depending on data availability, any of the three following combinations of criteria:

167 TFA specimens with DNA barcode sequences > 500 bp and thus meeting the minimal length criterion for the BOLD algorithm to group them into clusters with Barcode Index Numbers (BIN, Ratnasingham & Hebert, 2013) were grouped in OTUs consisting of all sympatric specimens forming the most inclusive terminal clade recovered in the ML analysis (normally represented by a single BIN, but in a few cases two or three sympatric BINs of morphologically similar specimens formed the same monophyletic OTU); in infrequent cases when allopatric specimens (= those from more than one TFA locality) share the same BIN, they are assigned to the same OTU;

42 TFA specimens with short DNA barcodes (200–500 bp), which do not meet the minimal length BIN criterion, were assigned to existing OTUs formed by sympatric specimens with a BIN (see above) using NJ clustering;

91 TFA specimens with no DNA data (= for whom our attempts to generate DNA barcode data failed) were assigned to existing OTUs, which were formed by sympatric and morphologically most similar specimens. In situations when no such OTU pre-existed, new OTUs were formed.

Generated OTUs were named using the three letters 'OTU' and four digit codes, the latter the same as one of the included specimens (Figs 2–5, Fig. S1).

Analysis limitations

Except for two taxonomically revised small genera (*Neochelonoderus* and *Afropictinus*, see Heiss & Grebennikov, 2015, 2016, respectively), no attempt was made to utilize our data for specimen identification and for species discovery (Collins & Cruickshank, 2013). The former task is currently hardly possible due to the lack of an adequately large DNA library derived from reliably identified (= name-bearing) specimens, while the latter was outside the scope of the present study due to time and resource limitations.

RESULTS

Phylogenetic analysis of Aradidae

The ML tree (Figs 2–5) represents the phylogenetic signal detected from the matrix of 295 Aradidae terminals all longer than 500 bp. Except for the subfamily Carventinae, three other Aradidae subfamilies represented in the matrix are recovered as clades: Aneurinae and Aradidnae (represented by 11 and 14 terminals, respectively; Fig. 2), as well as the most numerous subfamily Mezirinae represented by 223 terminals (Figs 2–5). Most genera represented in the analysis by more than a single species are recovered as clades, except the clade formed by reciprocally paraphyletic *Aneurus* Curtis, 1825 and *Paraneurus* Jacobs, 1986 (Fig. 2), polyphyletic *Mezira* Amyot & Serville, 1843 and *Neuroctenus* Hoberlandt, 1967 (Fig. 3), as well as *Usum-*

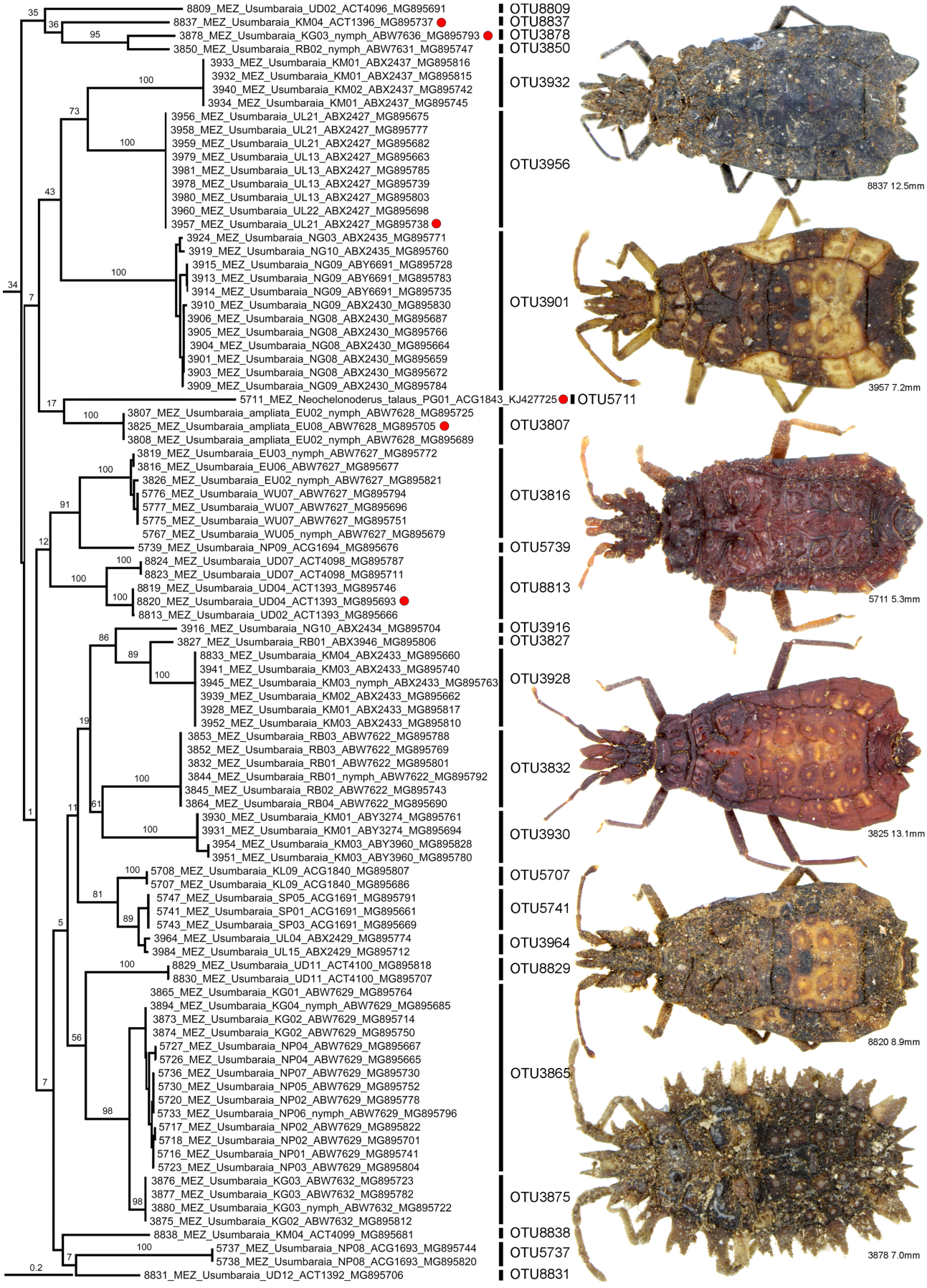

Fig. 5. Maximum Likelihood inference phylogram of *Usumbaraia* and *Neochelonoderus* flat bugs (Aradidae: Mezirinae) using the DNA barcoding fragment (continued from Figs 2–4).

Table 1. Distribution of 63 Aradidae operational taxonomic unites (OTUs) among the14 Tanzanian localities sampled and among higher taxonomic categories (subfamilies and, when possible, genera and species). The 53 OTUs delimited using the 294 terminal > 500 bp topology (Figs 2–5) are in bold; 10 delimited using morphological characters are italicized. Two OTUs (both in *Usumbaraia*) each detected at two localities are indicated with an asterisk, followed by a code of another locality.

Locality	Code	Aneurinae	Carventinae		Mezirinae						Σ
		Paraneurus	*Dundocoris*	not assigned	*Afropictinus*	*Embuana*	*Linnavuo-riessa*	*Neochelo-noderus*	*Usumbaraia*	not assigned	
Mt. Hanang	HN	none	none	none	none	none	none	none	none	none	0
Mt. Meru	MR	**OTU5701**	none	*OTU8844*	none	none	none	none	none	none	2
Mt. Kilimanjaro	KL	none	**OTU5703**	none	none	none	none	none	**OTU5707**	none	2
North Pare	NP	none	none	none	**OTU5719** (nauplius), **OTU5725** (castor)	none	**OTU5728**	none	**OTU3865***(KG), **OTU5737**, **OTU5739**	none	6
South Pare	SP	none	none	**OTU5744**, **OTU5748**	**OTU5749** (castor)	none	**OTU5753**	none	**OTU5741**	none	5
West Usambara	WU	none	**OTU5768**	none	**OTU5761** (castor), **OTU5772** (hylas)	none	**OTU5759**	none	**OTU3816***(EU)	none	5
East Usambara	EU	none	none	none	**OTU3820** (idas)	none	**OTU3813**	none	*OTU3801*, **OTU3807** (ampliata), **OTU3816***(WU)	none	5
Uluguru	UL	none	none	*OTU3982*	**OTU3969** (hylas)	**OTU3955**	none	none	**OTU3956**, **OTU3964**	none	5
Nguru	NG	none	none	none	**OTU3911** (idas)	none	none	none	**OTU3901**, *OTU3907*, **OTU3916**	none	4
Kaguru	KG	none	*OTU3871*	**OTU3867**, *OTU3879*	**OTU3869** (hylas)	none	**OTU3866**	none	**OTU3865***(NP), **OTU3875**, **OTU3878**	none	8
Rubeho	RB	none	*OTU3835*	**OTU3861**	none	**OTU3838**	**OTU3842**	none	**OTU3827**, **OTU3832**, **OTU3850**	none	7
Udzungwa	UD	none	*OTU8818*	none	none	none	**OTU8825**	none	**OTU8809**, **OTU8813**, **OTU8829**, **OTU8831**	none	6
Kimboza	KM	none	none	**OTU3937**	none	none	none	*OTU3935* (areius)	**OTU3928**, **OTU3930**, **OTU3932**, **OTU8837**, **OTU8838**	none	7
Pugu	PG	none	none	none	none	none	none	**OTU5711** (talus)	none	*OTU5710*, **OTU5714**	3
Σ		1	5	8	9	2	7	2	27	2	

baraia Kormliev, 1956, which is paraphyletic with respect to a representative of *Neochelonoderus* (Fig. 5).

Neighbour Joining analysis of Aradidae

The NJ topology of 209 Tanzania-only Aradidae terminals longer than 200 bp (Fig. S2) was mainly consistent with that obtained in the ML analysis (Figs 2–5) in recovering allopatric clusters of terminals.

Formation of Tanzanian Aradidae OTUs

Of the total of 63 OTUs representing the entire diversity of Tanzanian Aradidae sampled (Table 1), 53 are represented in the ML topology obtained from the 295 terminal matrix (Figs 2–5). Ten more OTUs are formed by sequenced specimens not represented in Figs 2–5: OTU3801 from East Usambara (specimen CNCCOLVG00003801), OTU3835 from Rubeho (specimens CNCCOLVG00003835, 3837 and likely specimens 3834, 3855, 3856), OTU3871 from Kaguru (specimens CNCCOLVG00003871, 3872), OTU3879 from Kaguru (specimens CNCCOLVG00003879, 3892, 3889, 3891), OTU3907 from Nguru (specimens CNCCOLVG00003907, 3908, 3912, 3925, 3926, 3927), OTU3935 from Kimboza (specimens CNCCOLVG00003935, 3936, 3942, 3943, 4757, 4758, 4759), OTU3982 from Uluguru (specimen CNCCOLVG00003982), OTU5710 from Pugu Hills (specimen CNCCOLVG00005710), OTU8818 from Uluguru (specimens CNCCOLVG00008818, 8815, 8817, 8821, 8822) and OTU8844 from Mt. Meru (specimens CNCCOLVG00008844, 8845, 8847).

Distribution of Tanzanian Aradidae OTUs among sampled localities and taxa

Distribution of 63 OTUs among the14 localities sampled in Tanzania (Table 1) varies between nil (Mt. Hanang, i.e. no Aradidae collected) and seven (on each of the Rubeho and Pugu Hills). The highest diversity of OTUs occurred in the genus *Usumbaraia* (27), followed by *Afropictinus* (9) and *Linnavuoriessa* Heiss & Baňař, 2016 (7). Seven Carventinae and two Mezirinae OTUs could not be assigned to a named genus. Except for the 11 OTUs belonging to the recently revised *Neochelonoderus* (2) and *Afropictinus* (9), all but one (OTU3807, *Usumbaraia ampliata* Kormliev, 1956) Tanzanian OTUs could not be assigned to a named species.

Winglessness in Tanzanian Aradidae

Except for three OTUs formed by winged specimens [OTU5701 from Mt. Meru; OTU5710 and OTU5714 (Fig. 1B) from Pugu Hills], all the Tanzanian Aradidae analyzed are apterous.

DISCUSSION

Hypothesis 1: Aradidae of Tanzania are well known taxonomically at the generic and species level

Since 52 (83%) and 12 (19%) among the total of 63 Tanzanian Aradidae OTUs were assigned to a named genus

and species (Table 1), respectively, this hypothesis could not be rejected with regard to genera, and is strongly rejected with regard to species.

Hypothesis 2: shallow clades of Aradidae in TFA are geographically structured

Since only two among 53 DNA-based OTUs were detected at more than a single TFA location (OTU3865 found at both Kaguru and North Pare, and OTU3816 found at both East Usambara and West Usambara, Table 1), our results do not reject this hypothesis.

Hypothesis 3: all the phylogeographic structure of Aradidae in TFA can be attributed to simple vicariance

Since reciprocal monophyly of all but two DNA-based OTUs is best interpreted as the standard signature of simple vicariance of a widespread ancestor (Heads 2014: 6), and since at least one non-endemic OTU (OTU3816) was detected at two nearby TFA localities (West and East Usambara), this hypothesis cannot be rejected.

Hypothesis 4: Aradidae faunas on geologically young Tanzanian volcanoes were formed anew after the volcanic highlands and their forests came into existence about two million years ago

Data in Table 1 indicate that the mean diversity of Aradidae OTUs on all three volcanoes (Mts Hanang, Meru and Kilimanjaro) is only 1.33 species (n = 3, standard deviation s = 1.15), which is far less than the same value for nine EAM (5.67, s = 1.22) and two lowland forests (5, s = 2.83). Such results indicate a depauperate Aradidae fauna on Tanzanian volcanoes, as compared to other samples from TFA localities and, therefore, do not reject this hypothesis.

Hypothesis 5: If H4 is supported, then this process was driven by colonization from nearby and geologically older TFA forests

Since the distance between a volcano and the nearest EAM (greatest for Mt. Hanang, Fig. 1A) potentially serving as a source of Aradidae is inversely proportional to the diversity of Aradidae on the three volcanoes sampled (lowest for Mt. Hanang, Table 1), this hypothesis cannot be rejected.

Diversity of Aradidae in the Tanzanian Forest Archipelago

Adequate background taxonomic information is commonly lacking for insects sampled outside a few centers of taxonomic expertise, such as Europe, Japan or North America (see, for example, a report of over 4,000 mainly unnamed species of Diptera from a single four hectare spot in Costa Rica, Borkent et al., 2018). Tanzanian Aradidae are not an exception, and, therefore, being unable to count nominal species, we had to generate, count and compare ad hoc created OTUs (Table 1). It is likely that the latter adequately represents species diversity in individual units of the TFA, which varies between nil (Mt. Hanang) and eight (Kaguru; n = 14, mean 4.64, standard deviation 2.24). The same approach, however, will likely inflate the number of species when OTUs are counted within individual genera (with the maximum of 27 OTUs recorded for *Usumbaraia*), since at least in the genus *Afropictinus* nine OTUs correspond to four nominal species (Table 1). It should be noted, however, that a biological species is not an objective category (Ward, 2011) and, therefore, is perhaps more familiar, but not intrinsically better, than OTUs for diversity assessments.

Of the total of five genera and nine species reported from the country prior to this study [*Paraneurus* (1 sp.), *Brachyrhynchus* Laporte, 1833 (1 sp.), *Mezira* (2 spp.), *Neuroctenus* (3 spp.) and *Usumbaraia* (2 spp.); Heiss, 2013], we re-sampled two: *Paraneurus* and *Usumbaraia*. It should be also noted that two additional genera with a total of six new species were added by us to the Tanzanian faunal list: *Neochelonoderus* (2 spp., Heiss & Grebennikov, 2015) and *Afropictinus* (4 spp., Heiss & Grebennikov, 2016) and they are not included as a part of the pre-existing knowledge used here for comparison. The Aradidae fauna of broadly defined East Africa (from Ethiopia to Zimbabwe) was reported by Heiss (2013) to consist of 16 genera and 37 species. Since then we added three new species of *Neochelonoderus* and six of *Afropictinus* (Heiss & Grebennikov, 2015 and 2016, respectively) and Heiss & Baňař (2016) established two new monotypic genera endemic to Kenya, *Embuana* Heiss & Baňař, 2016 and *Linnavuoriessa*. Of these 18 East African genera we re-sampled only seven (39%), possibly because the rest inhabit places other than leaf litter: Aneurinae: *Aneurillus* Kormilev, 1968 and *Breviscutaneurus* Jacobs, 1986; Aradinae: *Aradus* Fabricius, 1803; Calisiinae: *Calisius* Stål, 1860 & *Paracalisiopsis* Kormilev, 1963; Mezirinae: *Rwandaptera* Heiss, 2001, *Brachyrhynchus* Laporte, 1833, *Ctenoneurus* Bergroth, 1887, *Mezira*, *Neuroctenus* & *Strigocoris* Usinger, 1954). Excluding the new species of *Neochelonoderus* and *Afropictinus* described by us from the herein reported Tanzanian samples, our samples contain only a single nominal species: *U. ampliata* Kormliev, 1956. Such results strongly suggest that the Aradidae fauna of Tanzania is far from being completely documented.

The subfamily Aneurinae is represented in TFA by a single OTU of the genus *Paraneurus*, found only on Mt. Meru (Fig. 2, Table 10). Described originally as the "most heterogeneous" subgenus of African Aneurinae (Jacobs, 1986) and later arbitrarily elevated to the generic level (Kormilev & Froeschner, 1987), this genus-group taxon renders *Aneurus* non-monophyletic (Fig. 2) and sheds significant doubt on its phylogenetic validity.

The subfamily Carventinae is represented in TFA by 13 OTUs, five of which are assigned to the genus *Dundocoris*, while the remaining eight form at least three clades (Fig. 2) and perhaps represent undescribed genera.

The subfamily Mezirinae is by far the most diverse in TFA and accounts for 49 of the 63 recorded OTUs (Table 1). Two among five recorded nominal Mezirinae genera were recently revised by us (*Neochelonoderus* and *Afropictinus*, Heiss & Grebennikov, 2015 and 2016, respectively) and no new data on them are herein provided. The

genera *Embuana* and *Linnavuoriessa* are newly recorded from Tanzania and are likely represented by species other than their type, the only known species recently described from Kenya (Heiss & Baňař, 2016). The remaining nominal genus, *Usumbaraia*, with its 27 OTUs (Table 1) is by a wide margin the most diversified genus of Aradidae in TFA accounting for more than half of all Mezirinae (49) and nearly half of all Aradidae (63) OTUs. The genus is likely a clade, even though a single analyzed *Neochelonoderus* is nested within it (Fig. 5), and includes the largest Tanzanian Aradidae, which reach 13 mm in body length. It should be noted that the genus *Vilhenaptera* Hoberlandt, 1967 formed by a single species *V. angolensis* Hoberlandt, 1967 from Angola, seems either most closely related to, or perhaps nested within, *Usumbaraia*.

Two Mezirinae OTUs, both represented by a single macropterous specimen, could not be assigned to a named genus (Table 1). Among them, OTU5710 is represented by a specimen that is not DNA-barcoded (Fig. 1B), whose large size and the shape of its pronotum suggest *Linnavuoriessa*, rather than those of the much smaller *Embuana*. The specimen displays, however, two characters not recorded in either genus, namely: fully developed wings and the absence of bicoloured laterosternites. As for the single and much smaller male specimen representing OTU5714, it resembles the notably larger *Linnavuoriessa*, has distinct ventral stridulatory structures, and a peculiar location of the abdominal spiracles (II–V ventral, VI ventrolateral, VII lateral), which either suggests a new genus-group taxon or, considering its placement in the *Embuana* + *Linnavuoriessa* clade (Fig. 3), re-definition of these taxa.

It should be noted that the Aradidae phylogenetic tree (Figs 2–5) is similar to a tree reported earlier by us (Grebennikov & Heiss, 2014). Specifically, all represented nominal taxa are monophyletic, except the subfamily Carventinae and the genera *Neuroctenus*, *Mezira* and *Usumbaraia*. These topological similarities are partly expected, since the herein used matrix was formed by extending the 2014 matrix to include Tanzanian and a few other specimens. Still, the monophyly of Mezirinae, forming 75% of the new matrix, is newly and vigorously re-tested (since the addition of Tanzanian samples greatly diversified this subfamily' representation) and was found to be strongly supported. Tanzanian nominal genera *Dundocoris* Hoberlandt, 1952, *Linnavuoriessa*, *Embuana* and *Afropictinus* each represented by more than a single terminal are all recovered as monophyletic, while the paraphyly of *Usumbaraia* with respect to a single included *Neochelonoderus* (Fig. 5) is likely an analytical artifact, possibly attributable to long branch attraction. These results suggest that notwithstanding its well-known limitations (i.e. Funk & Omland, 2003), the DNA barcode fragment might be of phylogenetic value, particularly in the not infrequent situation when it is the only readily available source of information (see also Wilson et al., 2011 or Grebennikov et al., 2017).

Finally, it is tempting to compare this updated faunal knowledge of East African Aradidae (see above) with that of nearby Madagascar, with 35 genera and 90 species of Aradidae (Heiss, 2012; Baňař & Heiss, 2018). Both faunas share at least seven nominal genera (*Breviscutaneurus* Jacobs, 1986, *Aradus* Fabricius, 1803, *Brachyrhynchus*, *Ctenoneurus* Bergroth, 1884, *Mezira*, *Neuroctenus* and *Stigocoris* Usinger, 1954) and two nominal species (*Aradus flavicornis* Dalman, 1823 and *Neuroctenus caffer* Stål, 1860); all of these taxa contain macropterous and likely actively flying bugs. These facts, however, can hardly shed adequate light on the classic question as to whether vicariance or dispersal was the predominant biogeographic force in Madagascar (Yoder & Nowak, 2006), since the phylogenetic relationships within the family remain inadequately known and at least three among seven shared genera (*Aradus*, *Mezira*, *Neuroctenus*) are found in more than a single zoogeographical region.

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Fig. S1. Images of 300 Tanzanian Aradidae submitted for DNA barcoding.

Fig. S2. Neighbour joining clustering of 209 Tanzanian Aradidae, for which DNA barcodes of > 200 bp were obtained using the online BOLD tree-building engine and Kimura 2 parameter.