Evolutionary history of the river frog genus *Amietia* (Anura: Pyxicephalidae) reveals extensive
diversification in Central African highlands

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ABSTRACT

The African river frog genus *Amietia* is found near rivers and other lentic water sources throughout central, eastern, and southern Africa. Because the genus includes multiple morphologically conservative species, taxonomic studies of river frogs have been relatively limited. We sampled 79 individuals of *Amietia* from multiple localities in and near the Albertine Rift (AR) of Burundi, Democratic Republic of the Congo, and Uganda. We utilized single-gene (*16S*) and concatenated (*12S*, *16S*, *cyt b* and *RAG1*) gene-tree analyses and coalescent species-tree analyses to construct phylogenetic trees. Two divergence dating approaches were used in BEAST, including secondary calibration points with *12S*, *16S*, *cyt b* and *RAG1*, and a molecular clock with the *12S*, *16S*, and *cyt b* genes. All analyses recovered *Amietia* as monophyletic with strong support, and revealed several well-supported cryptic lineages, which is consistent with other recent phylogeography studies of AR amphibians. Dating estimates were similar, and *Amietia* diversification is coincident with global cooling and aridification events in the Miocene and Pliocene, respectively. Our results suggest additional taxonomic work is needed to describe multiple new species of AR *Amietia*, some of which have limited geographic distributions that are likely to be of conservation concern.

Keywords: Biodiversity; Albertine Rift; speciation; amphibians; Montane Forest

1. Introduction

The genus *Amietia* currently contains 16 species of frogs (Frost, 2015) associated with permanent water sources such as rivers and lakes in central, eastern, and southern Africa. Although typically similar in morphology, *Amietia* vary slightly in toe webbing (Laurent, 1972; Channing, 2015), body proportions (Poynton, 1964), pattern and coloration (Channing and Baptista, 2013), and call (Visser and Channing, 1997). *Amietia* generally occur in habitats ranging from forests to open savannas, but they are rarely found far from water sources (Channing and Howell, 2006; Largen and Spawls, 2010). In the last decade, several species have been described, including *A. lubrica* (Pickersgill, 2007), *A. poyntoni* Channing and Baptista, 2013, *A. tenuoplicata* (Pickersgill, 2007), and *A. viridireticulata* (Pickersgill, 2007).

The current classification of the species in the genus *Amietia* was a result of morphological (Dubois, 2005) and genetic (van der Meijden et al., 2005) analyses that supported a monophyletic group containing both *Amietia* and *Afrana*. The species in these genera were subsequently combined into the genus *Amietia* in the Family Pyxicephalidae (Frost et al., 2006). Several recent studies have clarified the taxonomic status of some *Amietia* species (Pickersgill, 2007; Channing and Baptista, 2013; Channing, 2015), but many areas within the distribution of *Amietia* remain poorly sampled.

Five species of *Amietia* (*A. amieti*, *A. angolensis*, *A. desaegeri*, *A. ruwenzorica*, and *A. wittei*) are currently known from the Albertine Rift (AR) of Central Africa, where the vertebrate communities are considered to be the most species-rich in continental Africa (Plumptre et al., 2007; Greenbaum and Kusamba, 2012; Menegon et al., 2014; Greenbaum et al., 2015a; Portillo et al., 2015). Many threatened and endemic species reside within the AR (Plumptre et al., 2007; Jenkins et al., 2013; Portillo et al., 2015), making it one of the most irreplaceable and important

sites for conservation in Africa (Brooks et al., 2006). Many vertebrate species in the AR are morphologically cryptic and endemic to a small number of sites, including small mammals (Kerbis Peterhans et al., 2010; Demos et al., 2014), birds (Prigogine, 1971, 1977, 1978, 1984, 1985; Bowie et al., 2006), reptiles (Greenbaum et al., 2011, 2015a; Menegon et al., 2014) and amphibians (Laurent, 1964, 1972; Evans et al., 2008, 2011; Greenbaum and Kusamba, 2012; Portillo and Greenbaum, 2014a,b; Portillo et al., 2015). Viertel et al. (2012) recently described tadpole morphology and chytrid fungal infections in a Ugandan population of *A. ruwenzorica*, but no previous studies have assessed AR *Amietia* populations with molecular data.

Herein, we utilize the General Lineage Concept of species (de Queiroz, 1998, 2007), which postulates that species are separately evolving lineages. Lineages are recognized as candidate species based on unique morphological (i.e., size, color, toe webbing), ecological, or behavioral differences in congruence with genetic differentiation (Wiens and Penkrot, 2002). We utilize concatenated gene-tree and species-tree analyses to evaluate the following questions: (1) Do populations of AR *Amietia* form a monophyletic group? (2) Are cryptic lineages of *Amietia* present in the AR? (3) Can species-tree analyses help to resolve relationships of rapidly radiating *Amietia* lineages? (4) When did AR *Amietia* populations diverge from one another? (5) Do estimated divergence dates correspond to temporal and spatial biogeographic events?

2. Materials and Methods

2.1 Taxon sampling

We sequenced 79 samples from the genus *Amietia* from locations throughout the AR in Democratic Republic of the Congo (DRC), Burundi, and Uganda (Fig. 1). Additional *Amietia*

sequences were obtained from the studies of Dawood and Uqubay (2004), Scott (2005), van der Meijden et al. (2005, 2011), Bossuyt et al. (2006), Tarrant et al. (2008), Tolley et al. (2010), Viertel et al. (2012), Channing and Baptista (2013), and Zancolli et al. (2014), with collections as listed by Sabaj Pérez (2013). Outgroup samples included: *Ptychadena nilotica*, *Ptychadena* cf. *nilotica*, *Ptychadena* cf. *oxyrhynchus*, *Hildebrandtia* cf. *ornata* (Ptychadenidae), *Phrynobatrachus* cf. *dendrobates* (Phrynobatrachidae), and *Aubria masako* (Pyxicephalidae). Newly sequenced samples were deposited into GenBank (Table 1).

2.2 Laboratory protocols

The Qiagen DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA) or IBI Scientific Genomic DNA Mini Kit (IBI Scientific, Peosta, IA, USA) were used to extract genomic DNA from alcohol-preserved muscle or liver tissue samples. We used gene-specific primers (Table 2) in 25 µl PCR reactions with an initial denaturation at 95°C for 2 min, followed by denaturation at 95°C for 35 s, annealing at 50°C for 35 s, and extension at 72°C for 95 s with 4s added to the extension per cycle for 32 (mitochondrial genes) or 34 (nuclear gene) cycles. The PCR products were visualized using a 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen Corporation, Carlsbad, CA, USA). Sequencing reactions were purified with CleanSeq magnetic bead solution (Beckman Coulter, Inc., Brea, CA, USA) and then sequenced using an ABI 3130xl automated sequencer at the University of Texas at El Paso (UTEP) Border Biomedical Research Center (BBRC) Genomic Analysis Core Facility.

2.3 Sequence alignment and phylogenetic analyses

Chromatograph data were interpreted with the program SeqMan Pro v. 8.0.2 (Swindell and Plasterer, 1997). Sequences were aligned using T-COFFEE (Notredame et al., 2000) with further manual adjustments in MacClade v. 4.08 (Maddison and Maddison, 2005). Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI) criteria for both single-gene and concatenated data sets. The program RAxML v. 7.2.6 (Stamatakis, 2006) was used with the GTRGAMMA model for ML analyses, with a random starting tree and all parameters estimated. Clade support values inferred by ML analyses were estimated with the rapid bootstrap algorithm with 1000 replicates (Stamatakis et al., 2008). MrBayes v. 3.2.3 (Ronquist et al., 2012) was utilized to conduct BI analyses on the CIPRES Science Gateway v. 3.3 web portal (Miller et al., 2010). Single-gene analyses were conducted for *I6S* to determine genetic differences and phylogenetic relationships between all previously sequenced *Amietia*, because most samples from Genbank include only this gene. Concatenated analyses included GenBank samples with additional genes, but were mostly limited to newly sequenced samples for this study. Eight data partitions were used in our concatenated model: single partitions for *I2S* and *I6S*, and independent codon positions for the protein-coding genes *cyt b* and *RAG1*. The models of evolution most consistent with our data for BI analyses were determined with the Bayesian information criterion in PartitionFinder (Lanfear et al., 2012). Bayesian analyses were conducted with random starting trees, run for 20,000,000 generations, and Markov chains were sampled every 1000 generations. Convergence of multiple runs was verified through the graphical exploration using the compare tool of the MCMC program “Are We There Yet?” (AWTY) (Wilgenbusch et al., 2004; Nylander et al., 2008). A conservative estimate of 25% of trees were discarded as “burn in” once convergence was reached. Phylogenies were visualized using FigTree v. 1.4.2 (Rambaut and Drummond, 2014).

2.4 Species trees and species delimitation

Species-level phylogenetic estimates were generated using the program *BEAST v. 1.8.1 (Drummond et al., 2012) for the concatenated *12S*, *16S*, *cyt b*, and *RAG1* data set. Species assignments for *BEAST were based on gene trees, collection locality, and morphology. Sequence evolution models were determined with PartitionFinder, and analyses were run with a Yule tree prior and unlinked loci and substitution models. Each analysis was run for 50,000,000 iterations with Markov chains sampled every 1000 generations. Tracer v. 1.6 (<http://tree.bio.ed.ac.uk/software/tracer/>) was used visually to determine that convergence was reached, assess adequate sampling of all parameters, and determine “burn in.” A conservative 25% of the trees were discarded as “burn in” once convergence was reached. Trace plots were reviewed to ensure the convergence of Markov chain Monte Carlo (MCMC) runs. Gametic phases of heterozygous sites were resolved through a coalescent-based Bayesian method (PHASE v. 2.1) (Stephens et al., 2001) as executed in the software DnaSP v. 5.10.3 (Librado and Rozas, 2009). Gametic phase of alleles for polymorphic sites were inferred with probabilities ≥ 0.7 .

After generating a species guide tree in *BEAST, species delimitation was tested using Bayesian Phylogenetics and Phylogeography (BPP v. 2.0; Yang and Rannala, 2010). The MCMC analyses were run for 500,000 generations, with a sampling interval of five, and a burn-in period of 10,000. Analyses were initiated with different starting seeds. Because the prior distributions on the ancestral population size and root age can affect the posterior probabilities for models, the effect of different priors was evaluated by implementing three different combinations of priors. Prior combinations were chosen from Leaché and Fujita (2010) where Θ

represents the ancestral population size and τ_0 represents the divergence rates. The first combination of priors assumed small ancestral population sizes and relatively small divergence rates: $\Theta = G(2,2000)$ and $\tau_0 = G(2,2000)$, with a prior mean = 0.001 and variance = 5×10^{-7} . The second combination of priors assumed large ancestral population sizes and deep divergence rates: $\Theta = G(1,10)$ and $\tau_0 = G(1,10)$, with a prior mean = 0.1 and variance = 0.01. The third combination mixes priors that assume large ancestral populations sizes $\Theta = G(1,10)$ and relatively small divergence rates among species $\tau_0 = G(2,2000)$. The third combination favors models containing fewer species and is considered to be a conservative combination of priors that would favor models with the least number of species. Each of the species delimitation models was assigned equal prior probability. Classifying of species was determined when the nodes of all three prior combination runs were > 0.95 (Smith et al., 2013).

2.5 Divergence dating

Two separate divergence dating analyses were conducted—one with secondary calibration points and another with a molecular clock for the mitochondrial genes. The program BEAST 1.8.1 (Drummond et al., 2012) was used with data sets for secondary calibrations (*12S*, *16S*, *cyt b*, and *RAG1*) and molecular clocks (*12S*, *16S*, and *cyt b*) to estimate the divergence dates for the lineages of *Amietia* in this study. Additional outgroups (lacking *cyt b* only) were sampled from the van der Meijden et al. (2005) study, including *Petropedetes parkeri* (Petropedetidae) and the pyxicephalids *Pyxicephalus adspersus*, *Tomopterna* sp., *Natalobatrachus bonebergi*, *Cacosternum boettgeri*, and *Strongylopus fasciatus* (Table 1). For the secondary calibration analyses, we applied an uncorrelated log-normal relaxed clock model with a coalescent tree prior. Analyses were run for 50,000,000 generations, sampling every 1000

generations. Based on dating estimates from Roelants et al. (2007), we used the following secondary calibrations: (1) the ancestral node of Ranoidea, which included *Ptychadena*, *Hildebrandtia*, *Phrynobatrachus*, *Petropedetes*, *Pyxicephalus*, *Tomopterna*, *Natalobatrachus*, *Cacosternum*, *Strongylopus*, *Aubria*, and *Amietia*, was constrained with a zero offset of 116.7 million years ago (mya), a log-normal mean of 0.01, and a log-normal standard deviation of 1.0; (2) Africanura, comprising all the previous genera with the exception of *Ptychadena* and *Hildebrandtia*, was constrained with a zero offset of 70.9 mya, a log-normal mean of 0.01, and a log-normal standard deviation of 1.0; and (3) Pyxicephalidae, comprising *Pyxicephalus*, *Tomopterna*, *Natalobatrachus*, *Cacosternum*, *Strongylopus*, *Aubria*, and *Amietia*, was constrained with a zero offset of 56.3 mya, a log-normal mean of 0.01, and a log-normal standard deviation of 1.0.

For molecular-clock rates, we applied a strict clock model with a coalescent tree prior to our mitochondrial data sets. Analyses were run for 50,000,000 generations, sampling every 1000 generations. We assumed a range of substitution rates from 0.60% to 1.00% per million years for *cyt b* and 0.20% to 0.30% per million years for both *12S* and *16S*, based on rates previously published for amphibians (Macey et al., 1998, 2001; Monsen and Blouin, 2003; Fouquet et al., 2009; Pröhl et al., 2010; Portillo et al., 2015).

We used the program Tracer v. 1.6 (Drummond et al., 2012) to determine the MCMC analysis stationarity, adequate effective sample sizes of the posterior probabilities, and the appropriate “burn-in” percentage. Maximum clade credibility trees were summarized using the program TreeAnnotator v. 1.8.1 (Drummond et al., 2012) and visualized in FigTree v. 1.4.2.

3. Results

188

189 *3.1 Gene-tree analyses*

190 Our concatenated data set consisted of 2499 base pairs (*12S* [449 bp], *16S* [576 bp], *cyt b*
 191 [616 bp], and *RAG1* [858 bp]). Five samples of *Amietia* failed to amplify for the gene *cyt b*
 192 (Table 2). Samples with missing data were still used in concatenated analyses, because branch
 193 lengths are not biased by modest amounts of missing data (Pyron et al., 2013; Jiang et al., 2014).
 194 The program PartitionFinder selected the following models of nucleotide substitution: *12S* (GTR
 195 + I + G); *16S* (GTR + I + G); *cyt b* 1st codon position (GTR + I + G), *cyt b* 2nd codon position
 196 (HKY + G), *cyt b* 3rd codon position (TrN + G); *RAG1* 1st and 3rd codon positions (K80 + I +
 197 G), *RAG1* 2nd codon position (HKY + G). When a model was not available in MrBayes, the
 198 least restrictive model (GTR) was implemented.

199 Topologies for the ML and BI analyses were similar for both the *16S* and concatenated
 200 data sets (Figs. 2–3), but support for many nodes was stronger in the latter data set. The ML
 201 likelihood scores were –3497.09 for *16S* and –13957.18 for the concatenated analyses,
 202 respectively. Results for *16S* analyses included 15 haplotype clades of *Amietia*: (1) topotypic *A.*
 203 *angolensis* from Angola (GenBank); (2) *A. poyntoni* from South Africa (GenBank); (3) *A.*
 204 *hymenopus* from South Africa (GenBank); (4) *A. fuscigula* and *A. vandijki* from South Africa
 205 (GenBank); (5) *A. vertebralis* from South Africa and Lesotho (GenBank); (6) a poorly supported
 206 clade of *A. quecketti* from South Africa (GenBank); (7) *A. sp. 1* from the Kibara Mountains and
 207 Kasongomwana, Katanga, DRC (1157–1428 m); (8) *A. wittei* from Mount Kilimanjaro, Tanzania
 208 above 1700 m (GenBank); (9) *A. ruwenzorica* from the Itombwe (ca. 2800 m) and Kabobo
 209 Plateaus (2440 m), DRC, and three topotypic GenBank samples from the Rwenzori Mountains,
 210 Uganda (above 2400 m); (10) *A. sp. 2* from the Itombwe Plateau, DRC (1965–2848 m); (11) a

weakly supported clade of *A. sp. 3* from multiple forest localities in eastern DRC (811–2289 m); (12) *A. sp. 4* from South Kivu and Katanga provinces, DRC (721–1324 m); (13) *A. sp. 5* from the Marungu Plateau and proximate locations in Katanga Province, DRC (1428–2037 m), which was well supported as the sister taxon to *A. sp. 4*; (14) *A. sp. 6* from North Kivu and Orientale Provinces, DRC (823–2088 m); and (15) *A. desaegeri* from Virunga National Park (DRC) and Rwenzori Mountains in DRC and Uganda (742–1543 m), and *A. lubrica* from multiple localities in Burundi, Uganda, and eastern DRC (1173–2303 m). *Amietia vandijki* was nested within the *A. fuscigula* clade; the former taxon is primarily distinguished from the latter by male advertisement call (Visser and Channing, 1997). Recent taxonomic changes to *Amietia* based on morphology (recognition of *A. vertebralis* and *A. hymenopus*) by Channing (2015) are well supported within our tree.

Our concatenated phylogeny (Fig. 3) recovered a well-supported group of *Amietia* that included nine strongly supported groups of recognized or candidate species from the AR: (1) *A. ruwenzorica*; (2) *A. sp. 1*; (3) *A. sp. 2*; (4) *A. sp. 3*; (5) *A. sp. 4*; (6) *A. sp. 5*; (7) *A. sp. 6*; (8) *A. desaegeri*; and (9) *A. lubrica*. In general, the concatenated tree showed stronger support and improved resolution compared to the single-gene analyses. There was strong support for a clade with all AR *Amietia* populations except for *A. ruwenzorica* and *A. sp. 1*. Another well-supported clade included *A. sp. 3*, *A. sp. 4*, *A. sp. 5*, *A. sp. 6*, *A. desaegeri*, and *A. lubrica*. The following two clades were strongly supported as sister taxa: (1) *Amietia sp. 3*, *A. sp. 4* and *A. sp. 5*, and as in the single-gene tree, the latter two taxa were strongly supported as sister taxa; and (2) *A. sp. 6*, *A. desaegeri* and *A. lubrica*, with the latter two taxa as strongly supported sister taxa. There was weak support for the monophyly of the genus *Amietia* and the *Amietia sp. 3* clade in the *I6S*

analyses, but the genus was recovered as a well-supported monophyletic group in the concatenated analyses.

3.2 Species tree and species delimitation

Major clades in *BEAST analyses (Fig. 4) of AR populations of *Amietia* recovered the same recognized and candidate species as the concatenated gene tree. Estimates from *BEAST also recovered strong support ($pp \geq 95\%$) for the clade comprising *A. sp. 3*, *A. sp. 4*, *A. sp. 5*, *A. sp. 6*, *A. desaegeri*, and *A. lubrica*. Within this group, the same two major sister clades as the concatenated analyses were recovered, except that *A. lubrica* and *A. sp. 6* were weakly supported as sister taxa. All three BPP analyses revealed nine distinct evolutionary lineages of AR *Amietia* (identical to those in the concatenated analyses) with the maximum possible posterior probability values ($pp = 1.0$). Conservative BPP models that favored fewer species did not collapse species that had relatively shallow divergences between them (e.g., *A. lubrica* and *A. sp. 6*).

3.3 Divergence dating

Results from the secondary calibration-based dating analysis suggest a basal divergence of *Amietia* in the late-Oligocene to early-Miocene 24.39 mya (17.44–31.97 mya, 95% highest posterior densities [HPD]) (Fig. 5). The common ancestor of AR *Amietia* diverged 22.88 mya (16.45–29.63 mya, HPD) (Table 3). Most AR *Amietia* lineages continued to diverge in the mid- to late Miocene. The most recent divergence between *A. desaegeri* and *A. lubrica* was 5.28 mya

(2.86–8.05 mya, HPD) in the late Miocene to early Pliocene. Dating estimates from molecular-clock analyses were comparable (Table 3).

4. Discussion

4.1 Biogeography

Our results are mostly congruent with other phylogenetic studies of amphibians and reptiles from the AR (Evans et al., 2011; Greenbaum et al., 2012a,b, 2013; Portillo et al., 2015) with regard to several areas of endemism within the AR, including the Itombwe, Kabobo, Lendu, and Marungu Plateaus. Although our concatenated tree supported the monophyly of *Amietia* and a clade of AR *Amietia* (Fig. 3), the *16S* analyses did not (Fig. 2), and some weakly supported clades included AR populations with *A. wittei* from Mt. Kilimanjaro, Tanzania and a single sample of *A. cf. angolensis* from Mazumbai (West Usambara Mountains), Tanzania.

Diversification of AR *Amietia* coincided with a global cooling trend in the late Miocene and the aridification of Africa in the Pliocene, and not the formation of the AR in the late Oligocene/early Miocene ca. 25 mya (Roberts et al., 2012). With the spread of savannas in the mid-Miocene (ca. 16 mya), *Amietia* associated with open, non-forested habitats may have become widespread throughout these habitats, whereas forest-specialist *Amietia* (i.e., *A. sp. 3*) were likely restricted to forest refugia. Relatively vagile and habitat generalist species of *Amietia* (e.g., *A. lubrica*) likely spread throughout large areas of the AR during relatively xeric conditions from the Miocene to Pleistocene (Fig. 5). Forest species, including snakes (Menegon et al., 2014; Greenbaum et al., 2015a), chameleons (Tolley et al., 2013), and frogs (Evans et al., 2011; Portillo et al., 2015) have shown similar patterns and dates of diversification under similar biogeographical processes. In contrast, dating analyses of AR species of birds (Fjeldså and

Bowie, 2008) and mammals (Demos et al., 2014) recovered most divergence dates somewhat younger in the Pliocene, which might be explained by increased vagility of both birds and mammals, differences in molecular-clock rates, dating methods, or more recent colonization events.

The Rwenzori Mountains within the AR are unique because they likely originated relatively recently (Bauer et al., 2013) from a peneplain landscape around 2 mya (Kaufmann et al., 2015). This estimate is consistent with the relatively small genetic divergence between *A. lubrica* and *A. desaegeri*, which highlights formation of the mountain range as a potential driver of speciation for other vertebrates in the AR. However, this geological process did not influence the diversification of *A. ruwenzorica*, which is not restricted to the higher elevations of the Rwenzoris alone, but rather extends to several additional highlands within the AR as suggested by Frost (2015), including the Itombwe and Kabobo Plateaus.

The Itombwe Plateau harbors an unusually large number of endemic amphibians (Greenbaum and Kusamba, 2012), including recent species descriptions for *Xenopus itombwensis* (Evans et al., 2008) and *Leptopelis anebos* (Portillo and Greenbaum, 2014a). Haplotypes from *Amietia* sp. 2 from Itombwe formed a clade that is distinct from other AR populations, which suggests this candidate species is likely endemic to the plateau. The distinct *Amietia* sp. 6 lineage from the Lendu Plateau, a savanna-forest mosaic, and surrounding environs is geographically concordant with a recently described, endemic *Kinyongia* chameleon species (Greenbaum et al., 2012b) and a genetically distinct population of *Boaedon fuliginosus* (Greenbaum et al., 2015a). The historical geographic pattern relating *A. sp. 6* (including the Lendu Plateau), *A. desaegeri*, and *A. lubrica* is similar to *Xenopus lenduensis* from the Lendu

Plateau, which is closely related to *X. victorianus* populations from eastern DRC, Burundi, and Uganda (Furman et al., 2015).

The geological complexity of Katanga Province in DRC has led to impressive species richness, and approximately 12% of Katanga's amphibians and reptiles are endemic (Broadley and Cotterill, 2004). Our study demonstrates that the northern part of Katanga contains at least four distinct species of *Amietia*, which are genetically distinct from *A. angolensis* and all other species known from the AR. For example, *A. sp. 5* is found in the Marungu Plateau and Mitwaba regions in Katanga. The Marungu Plateau is known to harbor several endemic vertebrate species, including several bird taxa, a cordylid lizard, and at least one distinct species of *Hyperolius* (Greenbaum et al. 2012a). Although *Amietia sp. 5* seems to be the only lineage that occurs in Marungu, our study revealed that it is not endemic to the plateau.

4.2 Species limits and taxonomy

The results of our coalescent-based analyses suggest six unique genetic lineages likely warrant full species status, but additional sampling and morphological analyses are needed. *Amietia sp. 1* occupies a woodland/savanna mosaic of the Kibara Mountains and nearby region from 1157–1428 m. *Amietia sp. 2* was found in montane forest edges and marshes from 1965–2848 m on the Itombwe Plateau. *Amietia sp. 3* seems to be the only species that is restricted to forest—it was found in transitional and montane forests from multiple localities in eastern DRC from 811–2289 m. *Amietia sp. 4* occupies forest, forest edges, and woodland/savanna mosaic habitats in the regions west and south of the Itombwe Plateau in South Kivu and Katanga Provinces from 744–1324 m. *Amietia sp. 5* occupies montane grassland in the Marungu Plateau and a woodland/savanna mosaic of the Kibara Mountains at elevations from 1428–2037 m. One

sample of the latter clade (UTEP 21228) was found in sympatry with *Amietia* sp. 1 (UTEP 21206) at the Mayola River in the Kibara Mountains. Differences between *A.* sp. 1 and *A.* sp. 5 are strongly supported by both gene-tree analyses (Figs. 2–3), the species-tree analysis (Fig. 4), and by distinctive color patterns and other morphological characters (EG and TRL, unpubl. data). *Amietia* sp. 6 occurs in the montane woodland/savanna mosaic of the Lendu Plateau and surrounding region, marshes near the volcanoes of the southern sector of Virunga National Park (where it is sympatric with *A. lubrica* at the Kichanga River), and forest edges of the Ituri region from 823–2088 m. In future studies, additional morphological evidence will be combined with the genetic data presented within this study to confirm the recognition of these candidate species.

Our study revealed that *Amietia angolensis* does not occur in the AR, and it is likely much more limited in its distribution than currently recognized (Frost, 2015). Two distinct *A. angolensis* lineages were recovered in our single-gene phylogeny, and it is likely that the one from Tanzania represents a new species. Further investigation throughout the range of *A. angolensis*, including populations in Malawi (Conradie et al., 2011) and Tanzania (Zancolli et al., 2014), will likely reveal additional lineages in the *A. angolensis* complex.

Amietia lubrica is currently known only from the type locality (Lake Bunyonyi, Uganda) (Pickersgill, 2007). Our study clearly demonstrates that *A. lubrica* is the most widespread species within the AR, with a geographic distribution extending from western Burundi to the North Kivu Province of eastern DRC and western Uganda. These results underscore how poorly known the AR herpetofauna is in general. *Amietia desaegeri* is currently considered to be restricted to the Rwenzori Mountains (Frost, 2015) and southwestern Uganda (AmphibiaWeb, 2015), and although we did not detect the species in Uganda, our study shows that *A. desaegeri* also occurs near lowland rainforests of Virunga National Park, DRC. As noted above, *A.*

ruwenzorica is found above 2000 m at the Itombwe Plateau, Kabobo Plateau, and Rwenzori Mountains, and the species is likely to occur in other montane regions of the AR.

According to Loveridge (1957) and Frost (2015), *A. wittei* should occur in eastern DRC. There is no evidence of *A. wittei* in eastern DRC based on our single-gene analysis, which included GenBank data from this species (Fig. 2). It is possible that the confusion over the distribution of *A. wittei* can be attributed to its complicated taxonomic history. *Rana aberdarensis*, currently a synonym of *Amietia wittei*, was synonymized with *Rana nutti* by Barbour and Loveridge (1928), then removed from this synonymy (Loveridge, 1929), and subsequently placed into the synonymy of *R. (Amietia) wittei* (Loveridge, 1936).

The poorly known taxon *Rana chapini* is currently considered to be a synonym of *A. angolensis* (Frost, 2015), but given the enormous disjunct distribution and ecoregion differences between the lowland Congo Basin rainforest type locality for the former taxon (Batama, Orientale Province, DRC) and the presumably topotypic *A. angolensis* GenBank samples from Calandula Falls and Humpata (Zootechnica Station) in Angola (Angola-Miombo Woodlands Ecoregion, *sensu* Burgess et al., 2004), it is likely that these taxa are not conspecific. Locality data for UTEP 21230 (Table 1) (Fig. 1) at Mafifi (near Epulu, elevation 844 m) places our most western sample of *A. sp. 6* about 230 km east of Batama (528 m elevation), the only known locality for *R. chapini*. Noble (1924) noted that the single specimen of *R. chapini* was collected “in grass bordering the brook at Batama,” suggesting the habitat might have been at the edge of the rainforest and not inside it, which is consistent with *A. sp. 6* at Mafifi. However, preliminary morphological data from webbing of the fourth toe of these taxa (TRL, unpubl. data) suggest *A. sp. 6* is not consistent with the original description of *R. chapini* (Noble, 1924). *Rana (Amietia) chapini* is likely a distinct species, but additional sampling is needed to confirm this hypothesis.

The candidate species *Amietia* sp. 3 contains samples collected within 100 km of the type locality of *A. amieti* at Lubile (Maniema Province, DRC). The samples were collected in forests ranging from 811–2289 m. There is extensive morphological variation among the vouchers, and comparisons to type specimens are needed to clarify its taxonomic status.

4.3 Conservation concerns

Although five countries (DRC, Burundi, Rwanda, Tanzania, and Uganda) currently contain at least 13 national parks (Plumptre et al., 2007) associated with the AR, there is a need for improved habitat conservation for many of the amphibian species in the AR, including *Amietia*. The AR contains the most endemic vertebrate fauna in continental Africa, including four currently recognized species of *Amietia*, and many of these areas have high levels of endemism and are either poorly protected (e.g., Itombwe and Kabobo Plateaus) or completely unprotected (e.g., Lendu and Marungu Plateaus) (Greenbaum and Kusamba, 2012; Greenbaum et al., 2012a,b). Candidate species of *Amietia* that were found within protected areas of the AR include *A.* sp. 2 on the Itombwe Plateau (parts of which were recently made into a reserve), *A.* sp. 3 in the highlands of Kahuzi-Biega National Park, *A. desaegeri* in Virunga National Park, *A. ruwenzorica* in Virunga National Park and the Itombwe Plateau, and *A. lubrica* in Kibira National Park. The remaining candidate species of *Amietia*, including *A.* sp.1, 4, 5, and 6, have all been found outside of protected areas.

Political instability, corruption, and weak law enforcement have allowed militias to control significant parts of the region, often blocking scientific and conservation efforts (Greenbaum and Kusamba, 2012). Even where protected areas have been established, law enforcement officials are disregarded or even attacked. Emmanuel de Merode, director and

chief warden of Virunga National Park, was recently shot several times, and 140 park rangers were killed for protecting the park's resources and animal fauna, which include mountain gorillas (Worrall, 2015). Political instability in the region reduces the effectiveness of law enforcement and also decreases the likelihood of significant long-term conservation goals (Omari et al. 1999; Sodhi et al., 2007). Human activities involved with the destruction of natural habitats, including deforestation, cattle ranching, and mining have been harmful to amphibian populations in the AR (Behangana et al., 2009; Greenbaum and Kusamba, 2012). Trends indicate that subsistence farming will continue to increase and place more pressure on wildlife reserves and other protected areas in many parts of Africa, including the AR (de Klerk et al., 2003; Fjeldså et al. 2004). Because many of the samples in this study were collected in relatively pristine habitats, additional conservation protection in these areas is important. A notable exception is *A. lubrica*, which is common in degraded and disturbed habitats (topotypic samples were collected in a hotel swimming pool adjacent to Lake Bunyonyi, Uganda).

Another major cause for concern to AR amphibian populations is the recent increase in species known to be afflicted with *Batrachochytrium dendrobatidis*, commonly called *Bd*, or chytrid fungus (Skerratt et al., 2007). The effects of *Bd* infection on AR amphibians are relatively unknown. However, samples of *Amietia* sp. 1, 2, 4, and 5 from our study tested positive for *Bd* (Greenbaum et al., 2014, 2015b), and samples of *A. ruwenzorica* from the Rwenzori Mountains (Uganda) also tested positive (Viertel et al., 2012). These studies and additional ones from Malawi (Conradie et al., 2011) and Kenya (Kielgast et al., 2010) suggest that *Amietia* species have relatively high rates of *Bd* infection compared to other African genera of frogs (Conradie et al., 2011; Greenbaum et al., 2015b). Given the high susceptibility of *Amietia* to chytrid infection, the limited distribution of many species within the declining pristine

habitats of the AR (Fig. 1), and the suitability of the AR highlands for chytrid fungus (Seimon et al., 2015), it is likely that many additional *Amietia* populations are threatened by chytrid infections.

With the new understanding of *Amietia* diversity that is evident in this study, concomitant conservation assessments will be needed after candidate species are formally described. Given the recent discovery of other cryptic, candidate species of amphibians in the AR (e.g., Portillo et al., 2015), it is likely that many additional amphibian species await discovery in the remaining natural habitats of the region, underscoring the increasing conservation value of the AR relative to other regions of continental Africa. Because the AR has one of the highest human population densities on the continent (Barnes and Lahm, 1997), additional biodiversity studies and conservation efforts are urgently needed.

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Table 1

Voucher numbers, localities, and GenBank numbers for genetic samples. DRC = Democratic Republic of the Congo.

Species	Collection No.	Field No.	Locality	<i>12S</i>	<i>16S</i>	<i>Cyt b</i>	<i>RAG1</i>
<i>Aubria masako</i>	UTEP 21202	ELI 2156	DRC: Equateur: Npenda village	KU559938	KU560021	KU560106	KU560187
<i>Cacosternum boettgeri</i>	ZFMK 66727	—	Namibia: Hardap	AF124096	AF215414	—	AY571645
<i>Hildebrandtia</i> cf. <i>ornata</i>	UTEP 21198	ELI 359	DRC: Katanga: 7 km south of Manono	KU559936	KU560019	KU560104	KU560185
<i>Natalobatrachus bonebergi</i>	ZFMK 74837	—	South Africa: The Haven	AF215198	AF215396	—	DQ019502
<i>Petropedetes parkeri</i>	No voucher	—	Cameroon: Nguti	AY341628	AF124132	—	DQ019505
<i>Phrynobatrachus</i> cf. <i>dendrobates</i>	UTEP 21303	EBG 1314	DRC: South Kivu: Irangi, ca. Kahuzi-Biega National Park	KU559937	KU560020	KU560105	KU560186
<i>Ptychadena</i> cf. <i>nilotica</i>	UTEP 21196	EBG 1582	DRC: South Kivu: Lake Tanganyika, Uvira, Mulongwe	KU559934	KU560017	KU560102	KU560183
<i>Ptychadena nilotica</i>	UTEP 21302	ELI 1243	Burundi: Lake Rwegura	KU559933	KU560016	KU560101	KU560182
<i>Ptychadena</i> cf. <i>oxyrhynchus</i>	UTEP 21197	EBG 2873	DRC: Katanga: road south of Kalemie	KU559935	KU560018	KU560103	KU560184
<i>Pyxicephalus adspersus</i>	ZFMK 66446	—	South Africa: KwaMbonambi	AF206091	AF215505	—	DQ019508
<i>Strongylopus fasciatus</i>	ZFMK 66444	—	South Africa: Little Brak	DQ019594	AF215412	—	DQ019513
<i>Tomopterna</i> sp.	ZFMK 66403	—	Namibia: Khorixas	DQ019595	DQ019610	—	DQ019514
<i>Amietia angolensis</i>	—	AC 3080	Angola: Humpata, Zootechnica Station	—	KC756286	—	—

<i>Amietia angolensis</i>	—	AC 3016	Angola: Calandula Waterfalls	—	KC756291	—	—
<i>Amietia</i> cf. <i>angolensis</i>	RDS 926	—	Tanzania: Mazumbai, West Usambara Mountains ¹	—	DQ022350	—	—
<i>Amietia desaegeri</i>	—	SL 478	Uganda: Rwenzori Mountains	—	GQ183600	—	—
<i>Amietia desaegeri</i> (tadpole)	UTEP 21232	EBG 1879	DRC: North Kivu: Lwanoli River	KU559959	KU560042	KU560127	KU560208
<i>Amietia desaegeri</i>	UTEP 21233	EBG 1845	DRC: North Kivu: Kisanzi	KU559957	KU560040	KU560125	KU560206
<i>Amietia desaegeri</i>	UTEP 21234	EBG 1811	DRC: North Kivu: Virunga National Park, Ndjuma	KU559955	KU560038	KU560123	KU560204
<i>Amietia desaegeri</i>	UTEP 21309	EBG 1846	DRC: North Kivu: Kisanzi	KU559958	KU560041	KU560126	KU560207
<i>Amietia desaegeri</i>	UTEP 21308	EBG 1812	DRC: North Kivu: Virunga National Park, Ndjuma	KU559956	KU560039	KU560124	KU560205
<i>Amietia fuscigula</i>	—	CR 1073	Africa	—	DQ347347	—	—
<i>Amietia fuscigula</i>	—	AC 3167	South Africa: Western Cape: Bainskloof Pass	—	KC756306	—	—
<i>Amietia fuscigula</i>	—	AC 2671	South Africa: Eastern Cape: Longmore Forest Reserve	—	KC756317	—	—
<i>Amietia fuscigula</i>	—	AC 2665	South Africa: Western Cape: Bloukrans Pass	—	KC756321	—	—
<i>Amietia hymenopus</i>	UFS MPU100	—	South Africa: KwaZulu-Natal	—	FJ411435	—	—
<i>Amietia hymenopus</i>	UFS MH1323	—	South Africa: Free State	—	FJ411434	—	—
<i>Amietia lubrica</i>	—	SL 538	Uganda: Semliki	—	GQ183602	—	—

<i>Amietia lubrica</i>	UTEP 21236	ELI 2716	Uganda: Kabale District: Lake Bunyonyi	KU560012	KU560097	KU560178	KU560263
<i>Amietia lubrica</i>	UTEP 21235	ELI 2715	Uganda: Kabale District: Lake Bunyonyi	KU560011	KU560096	KU560177	KU560262
<i>Amietia lubrica</i>	UTEP 21237	ELI 2826	Uganda: Kasese District: Rwenzori Mountains National Park, Ruboni Community Hotel	KU560013	KU560098	KU560179	KU560264
<i>Amietia lubrica</i>	UTEP 21238	CFS 1586	Burundi: western Makamba	KU560009	KU560094	KU560175	KU560260
<i>Amietia lubrica</i>	UTEP 21332	ELI 933	Burundi: Bururi, Nyamugwaga Swamp	KU560001	KU560085	KU560166	KU560251
<i>Amietia lubrica</i>	UTEP 21239	ELI 867	Burundi: Bururi	KU559998	KU560082	KU560163	KU560248
<i>Amietia lubrica</i>	UTEP 21331	ELI 877	Burundi: Bururi	KU560000	KU560084	KU560165	KU560250
<i>Amietia lubrica</i>	UTEP 21333	ELI 1232	Burundi: Mpishi, ca. Kibira National Park	—	KU560090	KU560171	KU560256
<i>Amietia lubrica</i>	UTEP 21329	ELI 865	Burundi: Bururi	KU559997	KU560081	KU560162	KU560247
<i>Amietia lubrica</i>	UTEP 21240	CK 051 (ELI 1562)	DRC: North Kivu: Kichanga River	KU560014	KU560099	KU560180	—
<i>Amietia lubrica</i>	UTEP 21241	CK 006 (ELI 1607)	DRC: North Kivu: Kichanga River	KU560010	KU560095	KU560176	KU560261
<i>Amietia lubrica</i>	UTEP 21242	ELI 1152	Burundi: Mpishi, ca. Kibira National Park	KU560004	KU560088	KU560169	KU560254
<i>Amietia lubrica</i>	UTEP 21243	ELI 1216	Burundi: Mpishi, Nyanutovu River, ca. Kibira National Park	KU560005	KU560089	KU560170	KU560255

<i>Amietia lubrica</i>	UTEP 21244	CFS 1538	Burundi: Mukazira River, ca. Bururi	KU560008	KU560093	KU560174	KU560259
<i>Amietia lubrica</i>	UTEP 21330	ELI 873	Burundi: Bururi	KU599999	KU560083	KU560164	KU560249
<i>Amietia lubrica</i>	UTEP 21245	ELI 1266	Burundi: Kibira National Park, ca. Rwegura	KU560007	KU560092	KU560173	KU560258
<i>Amietia lubrica</i>	UTEP 21246	ELI 993	Burundi: Gihofi	KU560003	KU560087	KU560168	KU560253
<i>Amietia lubrica</i>	UTEP 21247	ELI 1258	Burundi: Kibira National Park, ca. Rwegura	KU560006	KU560091	KU560172	KU560257
<i>Amietia lubrica</i>	UTEP 21248	ELI 975	Burundi: Bururi, Mivgaro	KU560002	KU560086	KU560167	KU560252
<i>Amietia lubrica</i>	UTEP 21249	ELI 848	Burundi: Gatambo, road ca. 12 km E of Muramvya	KU559996	KU560080	KU560161	KU560246
<i>Amietia poyntoni</i>	—	MH 0975	South Africa: Harrismith	—	KC756304	—	—
<i>Amietia poyntoni</i>	—	AC 2764	South Africa: Rhodes	—	KC756283	—	—
<i>Amietia quecketti</i>	—	QQ 0114	South Africa	—	EF136555	—	—
<i>Amietia quecketti</i>	—	KTH 406	South Africa	—	EF136562	—	—
<i>Amietia quecketti</i>	—	AC 2737	South Africa: Eastern Cape	—	KC756354	—	—
<i>Amietia quecketti</i>	CAS 191519	—	South Africa: Barberton	DQ019576	DQ019596	—	DQ019493
<i>Amietia quecketti</i>	UFS QQ00009	—	South Africa: Free State	—	FJ411440	—	—
<i>Amietia ruwenzorica</i> (tadpole)	UTEP 21322	EBG 2226	DRC: Katanga: Kabobo Plateau	KU559974	KU560058	KU560143	KU560224
<i>Amietia ruwenzorica</i> (tadpole)	UTEP 21203	EBG 2227	DRC: Katanga: Kabobo Plateau	KU559975	KU560059	KU560144	KU560225

<i>Amietia ruwenzorica</i>	UTEP 21204	EBG 2094	DRC: South Kivu: Itombwe Plateau, Mugegema	KU559976	KU560060	KU560145	KU560226
<i>Amietia ruwenzorica</i>	UTEP 21323	EBG 1703	DRC: South Kivu: Itombwe Plateau, Magunda	KU559977	KU560061	KU560146	KU560227
<i>Amietia ruwenzorica</i>	—	SL 437	Uganda: Rwenzori Mountains, Mubuku River	—	JF809877	—	—
<i>Amietia ruwenzorica</i>	—	SL 459	Uganda: Rwenzori Mountains, Mubuku River	—	JF809880	—	—
<i>Amietia vandijki</i>	—	MH 0107	South Africa: Swartberg	—	HQ014418	—	—
<i>Amietia vertebralis</i>	SAIAB DT16.1	—	Lesotho	—	FJ411429	—	—
<i>Amietia vertebralis</i>	—	AC 1220	South Africa: Drakensberg: Naudes Nek	—	AY255097	—	—
<i>Amietia wittei</i>	—	—	Tanzania: Mount Kilimanjaro	—	KJ469278	—	—
<i>Amietia wittei</i>	—	—	Tanzania: Mount Kilimanjaro	—	KJ469273	—	—
<i>Amietia wittei</i>	—	—	Tanzania: Mount Kilimanjaro	—	KJ469274	—	—
<i>Amietia wittei</i>	—	—	Tanzania: Mount Kilimanjaro	—	KJ469276	—	—
<i>Amietia</i> sp. 1	UTEP 21205	ELI 149	DRC: Katanga: Kasongomwana	KU559992	KU560076	—	KU560242
<i>Amietia</i> sp. 1	UTEP 21206	ELI 236	DRC: Katanga: Kibara Mountains, Mayola River, ca. 3 km E of Kakunko	KU559993	KU560077	KU560160	KU560243
<i>Amietia</i> sp. 2	UTEP 21306	EBG 2016	DRC: South Kivu: Itombwe Plateau, Rurambo	KU559949	KU560032	KU560117	KU560198

<i>Amietia</i> sp. 2	UTEP 21307	EBG 2017	DRC: South Kivu: Itombwe Plateau, Rurambo	KU559950	KU560033	KU560118	KU560199
<i>Amietia</i> sp. 2	UTEP 21207	EBG 2018	DRC: South Kivu: Itombwe Plateau, Rurambo	KU559951	KU560034	KU560119	KU560200
<i>Amietia</i> sp. 2	UTEP 21208	ELI 824	DRC: South Kivu: Itombwe Plateau, Mitamba	KU559954	KU560037	KU560122	KU560203
<i>Amietia</i> sp. 2	UTEP 21209	ELI 818	DRC: South Kivu: Itombwe Plateau, Mitamba	KU559953	KU560036	KU560121	KU560202
<i>Amietia</i> sp. 2	UTEP 21210	EBG 1588	DRC: South Kivu: Itombwe Plateau, Miki	KU559945	KU560028	KU560113	KU560194
<i>Amietia</i> sp. 2	UTEP 21211	EBG 1646	DRC: South Kivu: Itombwe Plateau, Miki	KU559946	KU560029	KU560114	KU560195
<i>Amietia</i> sp. 2	UTEP 21212	EBG 2058	DRC: South Kivu: Itombwe Plateau, Komesha	KU559952	KU560035	KU560120	KU560201
<i>Amietia</i> sp. 2	UTEP 21213	EBG 2007	DRC: South Kivu: Itombwe Plateau, Kasozo River	KU559947	KU560030	KU560115	KU560196
<i>Amietia</i> sp. 2	UTEP 21305	EBG 2008	DRC: South Kivu: Itombwe Plateau, Kasozo River	KU559948	KU560031	KU560116	KU560197
<i>Amietia</i> sp. 3	UTEP 21214	EBG 1312	DRC: South Kivu: Irangi, ca. Kahuzi-Biega National Park	KU559942	KU560025	KU560110	KU560191
<i>Amietia</i> sp. 3	UTEP 21215	ELI 490	DRC: South Kivu: Bizombo	KU559944	KU560027	KU560112	KU560193
<i>Amietia</i> sp. 3	UTEP 21304	ELI 599	DRC: South Kivu: Kalundu	KU559943	KU560026	KU560111	KU560192
<i>Amietia</i> sp. 3	UTEP 21216	EBG 1559	DRC: South Kivu: N'Komo River	KU559940	KU560023	KU560108	KU560189

			near road from Uvira to Bukavu				
<i>Amietia</i> sp. 3	UTEP 21217	EBG 1467	DRC: South Kivu: Kahuzi-Biega National Park, Mugaba	KU559939	KU560022	KU560107	KU560188
<i>Amietia</i> sp. 3	UTEP 21218	EBG 1882	DRC: North Kivu: Lwanoli River	KU559941	KU560024	KU560109	KU560190
<i>Amietia</i> sp. 4	—	MTSN 9978	DRC: Katanga: Lwama	KU560015	KU560100	KU560181	—
<i>Amietia</i> sp. 4	UTEP 21219	MTSN 9837	DRC: Katanga: Kinyama	KU559963	KU560047	KU560132	KU560213
<i>Amietia</i> sp. 4	UTEP 21314	ELI 1389	DRC: South Kivu: Kihungwe	KU559970	KU560054	KU560139	KU560220
<i>Amietia</i> sp. 4	UTEP 21319	ELI 1405	DRC: South Kivu: forest stream near Kihungwe	KU559968	KU560052	KU560137	KU560218
<i>Amietia</i> sp. 4	UTEP 21320	ELI 1426	DRC: South Kivu: forest stream near Kihungwe	KU559969	KU560053	KU560138	KU560219
<i>Amietia</i> sp. 4	UTEP 21318	ELI 1404	DRC: South Kivu: forest stream near Kihungwe	KU559967	KU560051	KU560136	KU560217
<i>Amietia</i> sp. 4 (tadpole)	UTEP 21313	ELI 555	DRC: South Kivu: Byonga	KU559973	KU560057	KU560142	KU560223
<i>Amietia</i> sp. 4	UTEP 21312	EBG 2259	DRC: Katanga: Force Bendera	—	KU560046	KU560131	KU560212
<i>Amietia</i> sp. 4	UTEP 21315	ELI 1400	DRC: South Kivu: forest stream near Kihungwe	KU559964	KU560048	KU560133	KU560214
<i>Amietia</i> sp. 4	UTEP 21310	EBG 2200	DRC: Katanga: Force Bendera	KU559960	KU560043	KU560128	KU560209
<i>Amietia</i> sp. 4	UTEP 21220	ELI 529	DRC: South Kivu: Byonga	KU559972	KU560056	KU560141	KU560222
<i>Amietia</i> sp. 4	UTEP 21321	ELI 1471	DRC: South Kivu: Kasanjala	KU559971	KU560055	KU560140	KU560221

<i>Amietia</i> sp. 4	UTEP 21311	EBG 2203	DRC: Katanga: Force Bendera	KU559962	KU560045	KU560130	KU560211
<i>Amietia</i> sp. 4	UTEP 21221	EBG 2201	DRC: Katanga: Force Bendera	KU559961	KU560044	KU560129	KU560210
<i>Amietia</i> sp. 4	UTEP 21317	ELI 1402	DRC: South Kivu: forest stream near Kihungwe	KU559966	KU560050	KU560135	KU560216
<i>Amietia</i> sp. 4	UTEP 21316	ELI 1401	DRC: South Kivu: forest stream near Kihungwe	KU559965	KU560049	KU560134	KU560215
<i>Amietia</i> sp. 5	UTEP 21222	EBG 2926	DRC: Katanga: Marungu Plateau, Pepa	KU559989	KU560073	KU560157	KU560239
<i>Amietia</i> sp. 5	UTEP 21223	EBG 2978	DRC: Katanga: Marungu Plateau, Pepa	KU559991	KU560075	KU560159	KU560241
<i>Amietia</i> sp. 5	UTEP 21224	EBG 2897	DRC: Katanga: Marungu Plateau, Kyalengwe	KU559988	KU560072	KU560156	KU560238
<i>Amietia</i> sp. 5 (metamorph)	UTEP 21225	ELI 186	DRC: Katanga: Mitwaba	KU559994	KU560078	—	KU560244
<i>Amietia</i> sp. 5	UTEP 21228	ELI 235	DRC: Katanga: Kibara Mountains, Mayola River, ca. 3 km E of Kakunko	KU559995	KU560079	—	KU560245
<i>Amietia</i> sp. 5	UTEP 21328	EBG 2942	DRC: Katanga: Marungu Plateau, Pepa	KU559990	KU560074	KU560158	KU560240
<i>Amietia</i> sp. 6	UTEP 21229	EBG 2381	DRC: Orientale: Lendu Plateau, Aboro	KU559980	KU560064	KU560149	KU560230
<i>Amietia</i> sp. 6	UTEP 21230	EBG 2309	DRC: Orientale: Mafifi	KU559978	KU560062	KU560147	KU560228
<i>Amietia</i> sp. 6	No Voucher	CK 114	DRC: Orientale: Banvokoto	KU559983	KU560067	KU560152	KU560233
<i>Amietia</i> sp. 6	UTEP 21231	EBG 2465	DRC: Orientale: Bunia	KU559982	KU560066	KU560151	KU560232

<i>Amietia</i> sp. 6	No Voucher	CK 005	DRC: North Kivu: Kichanga River	KU559986	KU560070	KU560155	KU560236
<i>Amietia</i> sp. 6	UTEP 21326	CK 004 (ELI 1593)	DRC: North Kivu: Kichanga River	KU559985	KU560069	KU560154	KU560235
<i>Amietia</i> sp. 6	UTEP 21327	CK 027 (ELI 1605)	DRC: North Kivu: Kichanga River	KU559987	KU560071	—	KU560237
<i>Amietia</i> sp. 6	No Voucher	CK 109	DRC: Orientale: Banvokoto	KU559984	KU560068	KU560153	KU560234
<i>Amietia</i> sp. 6	UTEP 21324	EBG 2365	DRC: Orientale: Lendu Plateau, Aboro	KU559979	KU560063	KU560148	KU560229
<i>Amietia</i> sp. 6	UTEP 21325	EBG 2368	DRC: Orientale: Lendu Plateau, Aboro	KU559981	KU560065	KU560150	KU560231

¹This locality was erroneously listed as Muzambai, Tanzania by Scott (2005).

Table 2 Primers used for sequencing mitochondrial and nuclear genes.

Primer name	Primer sequence	Primer source
<i>12SA</i>	5'—AAACTGGGATTAGATACCCCACTAT—3'	Kocher et al. (1989)
<i>12SB</i>	5'—GAGGGTGACGGGCGGTGTGT—3'	
<i>16SA</i>	5'—CGCCTGTTTATCAAAAACAT—3'	Palumbi et al. (1991)
<i>16SB</i>	5'—CCGGTCTGAACTCAGATCACGT—3'	
<i>cyt b</i> -CBJ10933	5'—TATGTTCTACCATGAGGACAAATATC—3'	Bossuyt and Milinkovitch (2000)
<i>cyt b</i> -C	5'—CTACTGGTTGTCCTCCGATTCATGT—3'	
<i>RAG1</i> MartF1	5'—AGCTGCAGYCARTAYCAYAARATGTA—3'	Chiari et al. (2004); Pramuk et al. (2008)
<i>RAG1</i> AmpR1	5'—AACTCAGCTGCATTKCCAATRTCA—3'	

Table 3 Comparison of calibrated and molecular-clock time estimates for important nodes in the BEAST phylogeny of *Amietia*. Lettered nodes correspond to those shown in Figure 5. Data are mean highest posterior densities (in million years ago), with ranges in parentheses. Secondary calibration points are provided in the methods.

Node	Calibrated	Molecular Clock
A: Split of <i>Aubria</i> and <i>Amietia</i>	57.86 (56.33–60.98)	55.38 (43.44–68.32)
B: <i>A. sp. 1</i> from other <i>Amietia</i>	22.88 (16.45–29.63)	22.84 (17.79–28.39)
C: <i>A. sp. 3–5</i> from <i>A. sp. 6</i> , <i>A. desaegeri</i> and <i>A. lubrica</i>	15.35 (10.96–20.0)	12.02 (9.4–14.87)
D: <i>A. sp. 3</i> from <i>A. sp. 4–5</i>	11.98 (8.24–16.09)	9.21 (7.2–11.51)
E: <i>A. sp. 4</i> from <i>A. sp. 5</i>	6.64 (3.99–9.62)	5.0 (3.65–6.5)
F: <i>A. sp. 6</i> from <i>A. desaegeri</i> and <i>A. lubrica</i>	8.39 (5.1–12.04)	6.1 (4.53–7.81)
G: <i>A. desaegeri</i> from <i>A. lubrica</i>	5.28 (2.86–8.05)	3.95 (2.77–5.26)

FIGURE LEGENDS

Fig. 1. Elevation map for the Albertine Rift showing sampling localities for *Amietia* in this study. Colored shapes correspond to localities of clades within the phylogenies of Figs. 2–3.

Fig. 2. Maximum-likelihood phylogeny with a *16S* data set of *Amietia*. Open circles denote clades with maximum likelihood bootstrap values ≥ 70 ; closed circles denote clades with Bayesian posterior probability values ≥ 0.95 . Clade colors correspond to point localities in Fig. 1.

Fig. 3. Maximum-likelihood phylogeny with combined *12S*, *16S*, *cyt b*, and *RAG1* data sets of AR *Amietia*. Open circles denote clades with maximum likelihood bootstrap values ≥ 70 ; closed circles denote clades with Bayesian posterior probability values ≥ 0.95 . Clade colors correspond to point localities in Fig. 1.

Fig. 4. Species tree generated in *BEAST for AR *Amietia*. Circles denote Bayesian posterior probability values ≥ 0.95 . Posterior probabilities for three independent runs in BPP were 1.0 for all nodes except for those with *Ptychadena*, which are listed as: top run 1; middle run 2; bottom run 3 (most conservative).

Fig. 5. Phylogenetic tree from secondary calibration-based BEAST analyses. Open circles denote calibration points, and numbers at the base of nodes denote mean highest posterior densities (HPD). Bold circles at the nodes denote Bayesian posterior probability values ≥ 0.95 . Nodes labeled with letters correspond to Table 3. Colored boxes correspond to point localities in Fig. 1.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5