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

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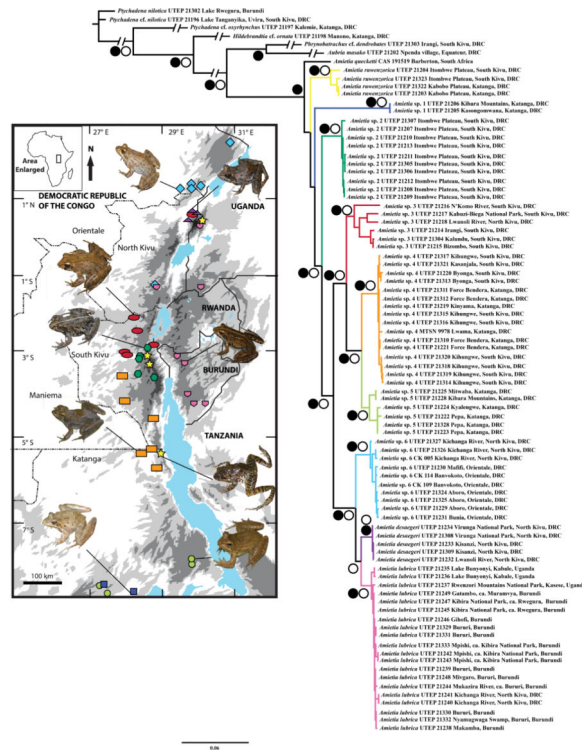
### Abstract

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.

### Graphical Abstract

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**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  $\mu$ l 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 *16S* 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 *12S* and *16S*, 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  $\Theta$  represents the ancestral population size and  $\tau_0$  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 =  $5 \times 10^{-7}$ . 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 population 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; Mosen 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

#### 3.1 Gene-tree analyses

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

Topologies for the ML and BI analyses were similar for both the *16S* and concatenated data sets (Figs. 2–3), but support for many nodes was stronger in the latter data set. The ML likelihood scores were –3497.09 for *16S* and –13957.18 for the concatenated analyses, respectively. Results for *16S* analyses included 15 haplotype clades of *Amietia*: (1) topotypic *A. angolensis* from Angola (GenBank); (2) *A. poyntoni* from South Africa (GenBank); (3) *A. hymenopus* from South Africa (GenBank); (4) *A. fuscigula* and *A. vandijki* from South Africa (GenBank); (5) *A. vertebralis* from South Africa and Lesotho (GenBank); (6) a poorly supported clade of *A. queketti* from South Africa (GenBank); (7) *A. sp. 1* from the Kibara Mountains and Kasongomwana, Katanga, DRC (1157–1428 m); (8) *A. wittei* from Mount Kilimanjaro, Tanzania above 1700 m (GenBank); (9) *A. ruwenzorica* from the Itombwe (ca. 2800 m) and Kabobo Plateaus (2440 m), DRC, and three topotypic GenBank samples from the Rwenzori Mountains, 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 *16S* 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 = 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 aberdariensis*, 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 (Zootecnica 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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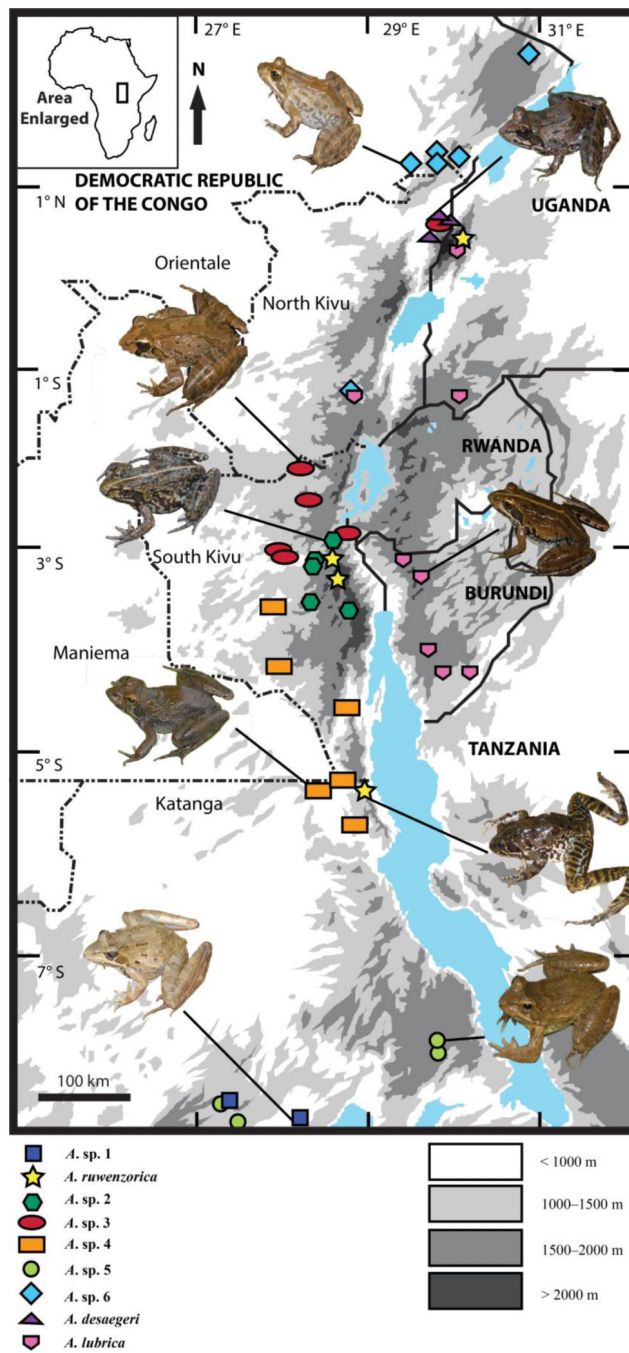
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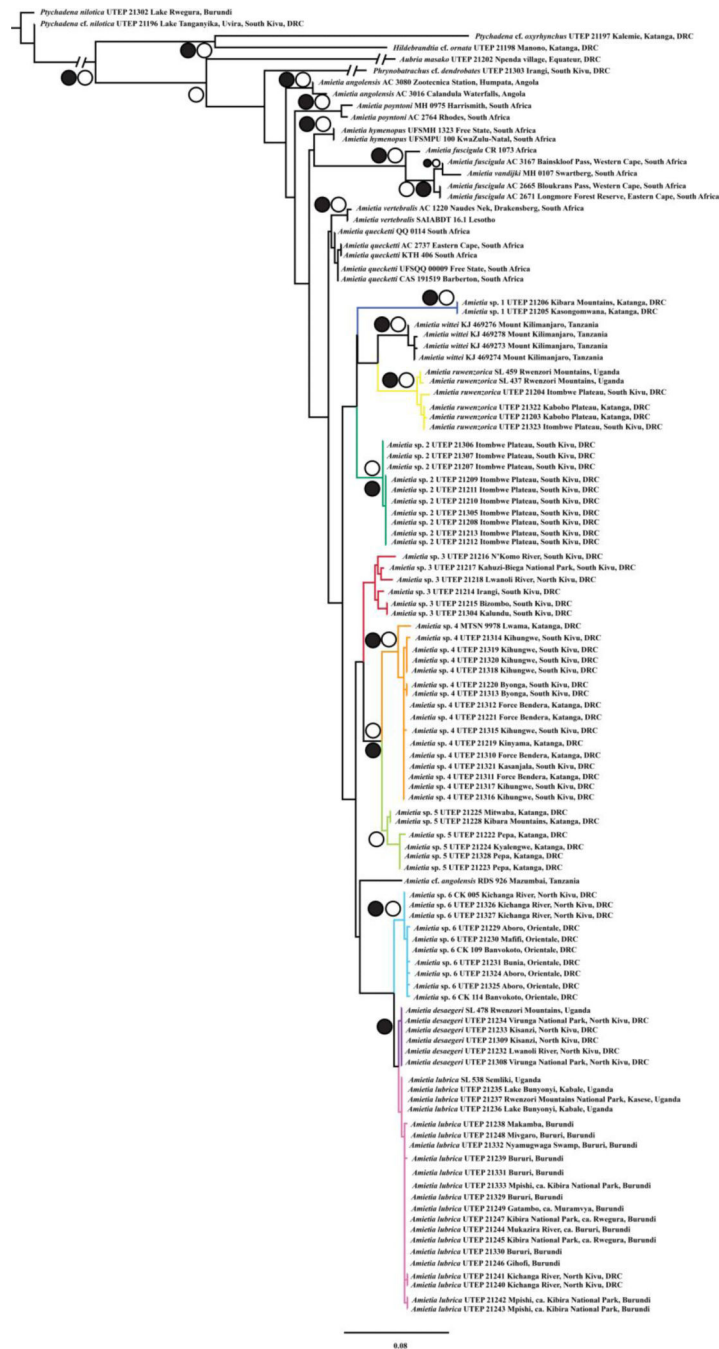
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### Highlights

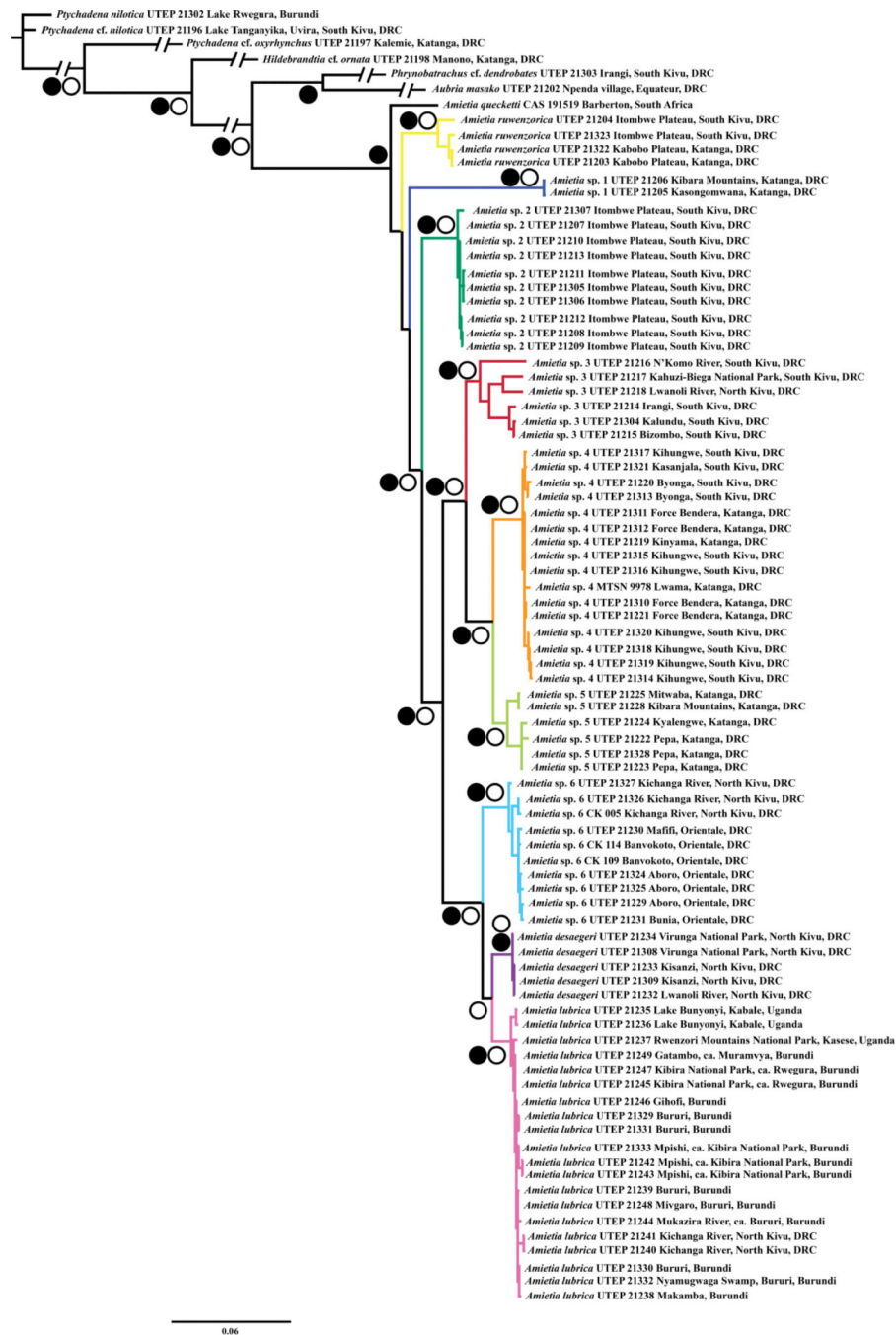
- Fifteen different lineages of *Amietia* recovered from eastern to southern Africa
- Nine distinct lineages of *Amietia* in the Albertine Rift of Central Africa
- Major radiations of *Amietia* occurred during the Miocene
- Further endemism in the Itombwe Plateau with a unique lineage of *Amietia*



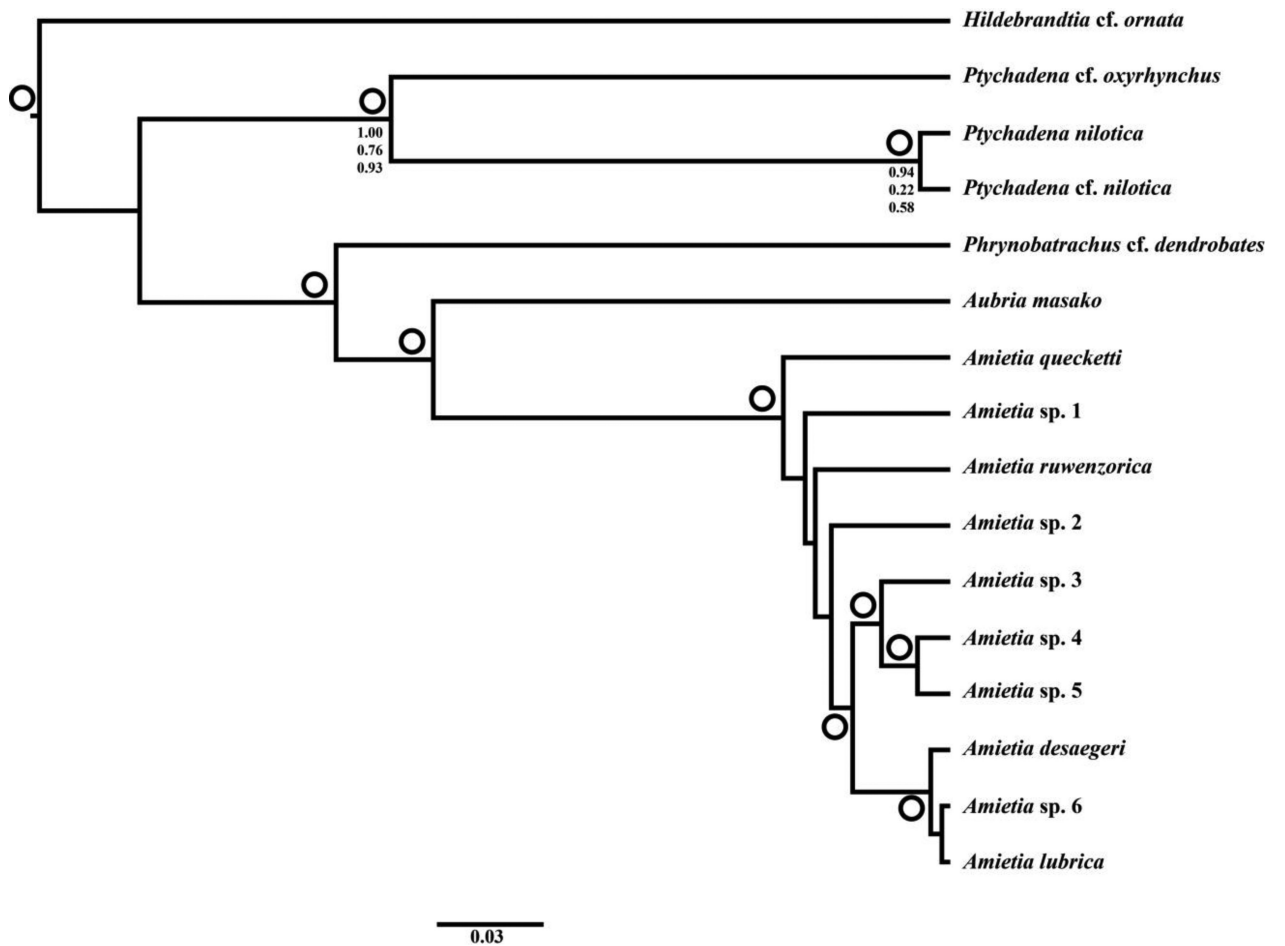
**Fig. 1.** Elevation map for the Albertine Rift showing sampling localities for *Amietia* in this study. Colored shapes correspond to localities of clades within the phylogenies of Figs. 2–3.



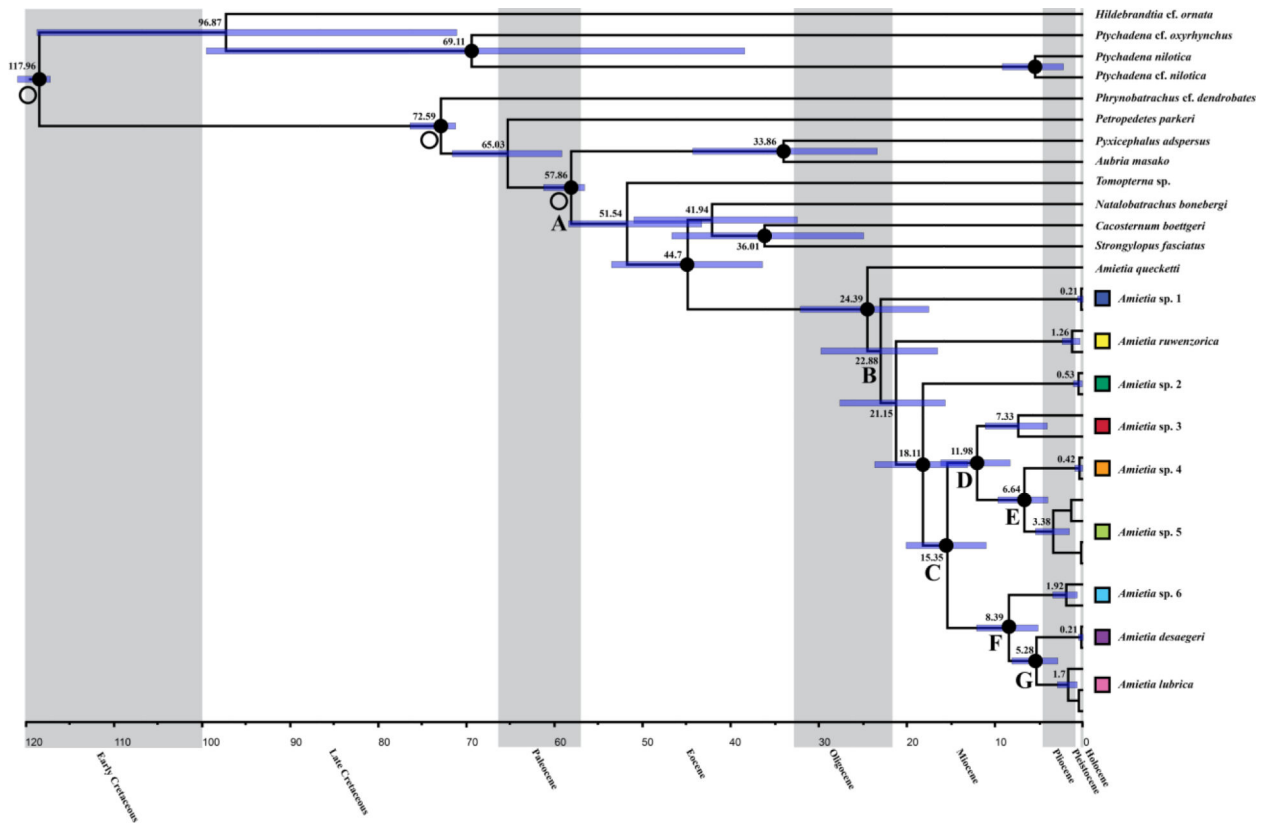
**Fig. 2.** Maximum-likelihood phylogeny with a *16S* data set of *Amietia*. Open circles denote clades with maximum likelihood bootstrap values > 70; closed circles denote clades with Bayesian posterior probability values > 0.95. Clade colors correspond to point localities in Fig. 1.



**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  $\geq 70$ ; closed circles denote clades with Bayesian posterior probability values  $\geq 0.95$ . 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  $\geq 0.95$ . 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  $\geq 0.95$ . Nodes labeled with letters correspond to Table 3. Colored boxes correspond to point localities in Fig. 1.

Table 1

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

| Species                                | Collection No. | Field No. | Locality  | 12S      | 16S      | Cyt b    | RAG1     |
|--|----------------|-----------|---|----------|----------|----------|----------|
| <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 cf. 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     | —         | Cameroun: Nguti   | AY341628 | AF124132 | —        | DQ019505 |
| <i>Phrynobatrachus cf. dendrobates</i> | UTEP 21303     | EBG 1314  | DRC: South Kivu: Irangi, ca. Kahuzi-Biega National Park | KU559937 | KU560020 | KU560105 | KU560186 |
| <i>Ptychadena cf. nilotica</i>         | UTEP 21196     | EBG 1582  | DRC: South Kivu: Lake Tanganyika, Uvira, Mutlongwe      | KU559934 | KU560017 | KU560102 | KU560183 |
| <i>Ptychadena nilotica</i>             | UTEP 21302     | ELI 1243  | Burundi: Lake Rwegura                                   | KU559933 | KU560016 | KU560101 | KU560182 |
| <i>Ptychadena cf. 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 cf. angolensis</i>          | RDS 926        | —         | Tanzania: Mazumbai, West Usambara Mountains /           | —        | 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 hynenopus</i>               | UFS MPU100     | —         | South Africa: KwaZulu-Natal                             | —        | FJ411435 | —        | —        |



| Species                  | Collection No. | Field No.         | Locality  | IZS      | 16S      | Cyt b    | RAG1     |
|--------------------------|----------------|-------------------|---|----------|----------|----------|----------|
| <i>Amietia hymenopus</i> | UFS MH1323     | —                 | South Africa: Free State  | —        | F1411434 | —        | —        |
| <i>Amietia lubrica</i>   | —              | SL 538            | Uganda: Semliki   | —        | GQ183602 | —        | —        |
| <i>Amietia lubrica</i>   | UTEF 21236     | ELI 2716          | Uganda: Kabale District: Lake Bunyonyi  | KU560012 | KU560097 | KU560178 | KU560263 |
| <i>Amietia lubrica</i>   | UTEF 21235     | ELI 2715          | Uganda: Kabale District: Lake Bunyonyi  | KU560011 | KU560096 | KU560177 | KU560262 |
| <i>Amietia lubrica</i>   | UTEF 21237     | ELI 2826          | Uganda: Kasese District: Rwenzori Mountains National Park, Ruboni Community Hotel | KU560013 | KU560098 | KU560179 | KU560264 |
| <i>Amietia lubrica</i>   | UTEF 21238     | CFS 1586          | Burundi: western Makamba  | KU560009 | KU560094 | KU560175 | KU560260 |
| <i>Amietia lubrica</i>   | UTEF 21332     | ELI 933           | Burundi: Bururi, Nyamugwaga Swamp   | KU560001 | KU560085 | KU560166 | KU560251 |
| <i>Amietia lubrica</i>   | UTEF 21239     | ELI 867           | Burundi: Bururi   | KU559998 | KU560082 | KU560163 | KU560248 |
| <i>Amietia lubrica</i>   | UTEF 21331     | ELI 877           | Burundi: Bururi   | KU560000 | KU560084 | KU560165 | KU560250 |
| <i>Amietia lubrica</i>   | UTEF 21333     | ELI 1232          | Burundi: Mpishi, ca. Kibira National Park   | —        | KU560090 | KU560171 | KU560256 |
| <i>Amietia lubrica</i>   | UTEF 21329     | ELI 865           | Burundi: Bururi   | KU559997 | KU560081 | KU560162 | KU560247 |
| <i>Amietia lubrica</i>   | UTEF 21240     | CK 051 (ELI 1562) | DRC: North Kivu: Kichanga River   | KU560014 | KU560099 | KU560180 | —        |
| <i>Amietia lubrica</i>   | UTEF 21241     | CK 006 (ELI 1607) | DRC: North Kivu: Kichanga River   | KU560010 | KU560095 | KU560176 | KU560261 |
| <i>Amietia lubrica</i>   | UTEF 21242     | ELI 1152          | Burundi: Mpishi, ca. Kibira National Park   | KU560004 | KU560088 | KU560169 | KU560254 |
| <i>Amietia lubrica</i>   | UTEF 21243     | ELI 1216          | Burundi: Mpishi, Nyanutovu River, ca. Kibira National Park                        | KU560005 | KU560089 | KU560170 | KU560255 |
| <i>Amietia lubrica</i>   | UTEF 21244     | CFS 1538          | Burundi: Mukazira River, ca. Bururi   | KU560008 | KU560093 | KU560174 | KU560259 |
| <i>Amietia lubrica</i>   | UTEF 21330     | ELI 873           | Burundi: Bururi   | KU599999 | KU560083 | KU560164 | KU560249 |
| <i>Amietia lubrica</i>   | UTEF 21245     | ELI 1266          | Burundi: Kibira National Park, ca. Rwegura  | KU560007 | KU560092 | KU560173 | KU560258 |
| <i>Amietia lubrica</i>   | UTEF 21246     | ELI 993           | Burundi: Gihofi   | KU560003 | KU560087 | KU560168 | KU560253 |
| <i>Amietia lubrica</i>   | UTEF 21247     | ELI 1258          | Burundi: Kibira National Park, ca. Rwegura  | KU560006 | KU560091 | KU560172 | KU560257 |
| <i>Amietia lubrica</i>   | UTEF 21248     | ELI 975           | Burundi: Bururi, Mivgaro  | KU560002 | KU560086 | KU560167 | KU560252 |
| <i>Amietia lubrica</i>   | UTEF 21249     | ELI 848           | Burundi: Gatambo, road ca. 12 km E of Muramvya                                    | KU559996 | KU560080 | KU560161 | KU560246 |
| <i>Amietia poyntoni</i>  | —              | MH 0975           | South Africa: Harris Smith  | —        | 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  | —        | F1411440 | —        | —        |

| Species                              | Collection No. | Field No. | Locality  | IZS      | 16S      | Cyt b    | RAG1     |
|--------------------------------------|----------------|-----------|---|----------|----------|----------|----------|
| <i>Amietia ruwenzorica</i> (tadpole) | UTEF 21322     | EBG 2226  | DRC: Katanga: Kabobo Plateau  | KU559974 | KU560058 | KU560143 | KU560224 |
| <i>Amietia ruwenzorica</i> (tadpole) | UTEF 21203     | EBG 2227  | DRC: Katanga: Kabobo Plateau  | KU559975 | KU560059 | KU560144 | KU560225 |
| <i>Amietia ruwenzorica</i>           | UTEF 21204     | EBG 2094  | DRC: South Kivu: Itombwe Plateau, Mugegema                          | KU559976 | KU560060 | KU560145 | KU560226 |
| <i>Amietia ruwenzorica</i>           | UTEF 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>           | SALAB DT16.1   | —         | Lesotho   | —        | F1411429 | —        | —        |
| <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                 | UTEF 21205     | ELI 149   | DRC: Katanga: Kasongomwana  | KU559992 | KU560076 | —        | KU560242 |
| <i>Amietia</i> sp. 1                 | UTEF 21206     | ELI 236   | DRC: Katanga: Kibara Mountains, Mayola River, ca. 3 km E of Kakunjo | KU559993 | KU560077 | KU560160 | KU560243 |
| <i>Amietia</i> sp. 2                 | UTEF 21306     | EBG 2016  | DRC: South Kivu: Itombwe Plateau, Rurambo                           | KU559949 | KU560032 | KU560117 | KU560198 |
| <i>Amietia</i> sp. 2                 | UTEF 21307     | EGB 2017  | DRC: South Kivu: Itombwe Plateau, Rurambo                           | KU559950 | KU560033 | KU560118 | KU560199 |
| <i>Amietia</i> sp. 2                 | UTEF 21207     | EBG 2018  | DRC: South Kivu: Itombwe Plateau, Rurambo                           | KU559951 | KU560034 | KU560119 | KU560200 |
| <i>Amietia</i> sp. 2                 | UTEF 21208     | ELI 824   | DRC: South Kivu: Itombwe Plateau, Mtiamba                           | KU559954 | KU560037 | KU560122 | KU560203 |
| <i>Amietia</i> sp. 2                 | UTEF 21209     | ELI 818   | DRC: South Kivu: Itombwe Plateau, Mtiamba                           | KU559953 | KU560036 | KU560121 | KU560202 |
| <i>Amietia</i> sp. 2                 | UTEF 21210     | EBG 1588  | DRC: South Kivu: Itombwe Plateau, Miki                              | KU559945 | KU560028 | KU560113 | KU560194 |
| <i>Amietia</i> sp. 2                 | UTEF 21211     | EBG 1646  | DRC: South Kivu: Itombwe Plateau, Miki                              | KU559946 | KU560029 | KU560114 | KU560195 |
| <i>Amietia</i> sp. 2                 | UTEF 21212     | EBG 2058  | DRC: South Kivu: Itombwe Plateau, Komasha                           | KU559952 | KU560035 | KU560120 | KU560201 |
| <i>Amietia</i> sp. 2                 | UTEF 21213     | EBG 2007  | DRC: South Kivu: Itombwe Plateau, Kasozo River                      | KU559947 | KU560030 | KU560115 | KU560196 |
| <i>Amietia</i> sp. 2                 | UTEF 21305     | EBG 2008  | DRC: South Kivu: Itombwe Plateau, Kasozo River                      | KU559948 | KU560031 | KU560116 | KU560197 |
| <i>Amietia</i> sp. 3                 | UTEF 21214     | EBG 1312  | DRC: South Kivu: Irangi, ca. Kahuzi-Biega National Park             | KU559942 | KU560025 | KU560110 | KU560191 |
| <i>Amietia</i> sp. 3                 | UTEF 21215     | ELI 490   | DRC: South Kivu: Bizombo  | KU559944 | KU560027 | KU560112 | KU560193 |
| <i>Amietia</i> sp. 3                 | UTEF 21304     | ELI 599   | DRC: South Kivu: Kalundu  | KU559943 | KU560026 | KU560111 | KU560192 |

| Species                          | Collection No. | Field No. | Locality  | IZS      | 16S      | Cyt b    | RAG1     |
|----------------------------------|----------------|-----------|---|----------|----------|----------|----------|
| <i>Amietia</i> sp. 3             | UTEP 21216     | EBG 1559  | DRC: South Kivu: N'Komo River near road from Uvira to Bukavu        | KU559940 | KU560023 | KU560108 | KU560189 |
| <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 |

| Species              | Collection No. | Field No.         | Locality                             | 12S      | 16S      | Cyt b    | RAG1     |
|----------------------|----------------|-------------------|--------------------------------------|----------|----------|----------|----------|
| <i>Amietia</i> sp. 6 | UTEF 21231     | EBG 2465          | DRC: Orientale: Bumia                | 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 | UTEF 21326     | CK 004 (ELI 1593) | DRC: North Kivu: Kichanga River      | KU559985 | KU560069 | KU560154 | KU560235 |
| <i>Amietia</i> sp. 6 | UTEF 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 | UTEF 21324     | EBG 2365          | DRC: Orientale: Lendu Plateau, Aboro | KU559979 | KU560063 | KU560148 | KU560229 |
| <i>Amietia</i> sp. 6 | UTEF 21325     | EBG 2368          | DRC: Orientale: Lendu Plateau, Aboro | KU559981 | KU560065 | KU560150 | KU560231 |

<sup>1</sup>This 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'—CGCCTGTTATCAAAAACAT—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'—CTACTGGTTGTCTCCGATTCATGT—3'    |  |
| <i>RAG1</i> MartF1     | 5'—AGCTGCAGYCARTAYCAYAAARATGTA—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.</i> 1 from other <i>Amietia</i>   | 22.88 (16.45–29.63) | 22.84 (17.79–28.39) |
| C: <i>A. sp.</i> 3-5 from <i>A. sp.</i> 6, <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.</i> 3 from <i>A. sp.</i> 4-5  | 11.98 (8.24–16.09)  | 9.21 (7.2–11.51)    |
| E: <i>A. sp.</i> 4 from <i>A. sp.</i> 5  | 6.64 (3.99–9.62)    | 5.0 (3.65–6.5)      |
| F: <i>A. sp.</i> 6 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)    |