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Research Article

Light at the end of the tunnel: insights into the molecular systematics of East African puddle frogs (Anura: Phrynobatrachidae)

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To investigate intra- and interspecific variability of 'Little Brown Frogs' (LBFs) from East Africa, we analysed molecular data from 23 species of *Phrynobatrachus* using 16S rRNA, a mitochondrial gene considered a universal DNA barcoding marker in amphibians. Conspecific populations, excluding *P. natalensis*, exhibited low (0–0.5%) sequence divergences, while conspecific populations of *P. natalensis* exhibited a wide range of differentiation (0.2–6.4%) with a distinct bimodal distribution, affirming that this may be a complex of cryptic species. Sister species yielded a range of differentiation from 1.5–8.3% with median of 2.3%. Results revealed that at least three non-sister populations of miniaturized puddle frogs are currently identified as *P. mababiensis*, and a new species from Ethiopia was identified. This first step into revising East African *Phrynobatrachus* systematics illustrates the necessity of rigorous taxonomic study, especially for groups with confusing or highly incomplete taxonomy.

Key words: 16S rRNA, DNA, Ethiopia, genetic divergence, Kenya, *Phrynobatrachus*, Tanzania, Uganda

Introduction

Anuran biodiversity is largely understudied in tropical ecosystems, which is mainly due to incomplete information on taxonomy and distribution (e.g. Vieites *et al*., 2009). This is especially true for species that are small in size, cryptically coloured, and morphologically similar, often with similar pattern morphs occurring in numerous species (e.g. Zimkus & Blackburn, 2008). Some taxonomists have come to refer to these problematic amphibians as 'Little Brown Frogs' (LBFs). One such group is the Afrotropical puddle frogs, genus *Phrynobatrachus* (Günther, 1862), which has been a long-standing source of confusion to systematists due to the aforementioned reasons, as well as inadequate original descriptions (Stewart, 1974; Rödel, 2000; Largen, 2001). Puddle frogs are one of the most diverse sub-Saharan anuran lineages, with approximately 75 species currently recognized (Frost, 2009). The majority of species are extremely small with snout-vent lengths ranging from 15 to 25 mm, and at least 18 of the 27 known East African species exhibit diminutive body size (Channing & Howell, 2006; Pickersgill, 2007; Frost, 2008). A number of species show remarkably restricted ranges (e.g. *P. inexpectatus* Largen, 2001, *P. pakenhami* Loveridge, 1941), while others have comparatively wide distributions (e.g. *P. mababiensis* FitzSimons, 1932, *P. natalensis* (Smith, 1849)). Recent analyses have identified significant cryptic diversity, leading to descriptions of new taxa and resurrections of synonymized species (i.e. Largen 1997, 2001; Rödel & Ernst, 2002; Crutsinger *et al*., 2004; Uyeda *et al*., 2007; Schick *et al*., 2010).

To investigate intra- and interspecific variability of East African LBFs, we analysed molecular data from 23 *Phrynobatrachus* species, using the 16S rRNA gene. This gene region has significant species-level variability and conserved flanking sites are present, which allow universal primers to work for the majority of species. These characteristics make this marker a valuable tool for taxonomic investigation in both large-scale phylogenetic studies and population-level haplotype analyses of amphibians (Vences *et al*., 2005*a*; Fouquet, 2007). The great numbers of amphibian 16S rRNA sequences currently present in GenBank (13 393 entries as of January 2010) support the usefulness of this gene in taxonomic investigations. Most recently this molecular marker was used as part of an integrative approach to identify an incredible diversity of amphibian species in Madagascar,

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many of which are new to science (Vieites *et al*., 2009). The purpose of this study was to: (1) illustrate the applicability of both inter- and intraspecific genetic variation of the 16S rRNA gene in identifying candidate species within the genus *Phrynobatrachus* and (2) present a phylogeny of East African *Phrynobatrachus*, representing a first step into unravelling the taxonomically problematic East African LBFs.

Materials and methods

DNA extraction, PCR amplification and sequencing

Molecular data are included from 136 *Phrynobatrachus* specimens obtained from various museum collections (Fig. 1), as well as sequence data downloaded from GenBank (Appendix 1, see supplementary material which

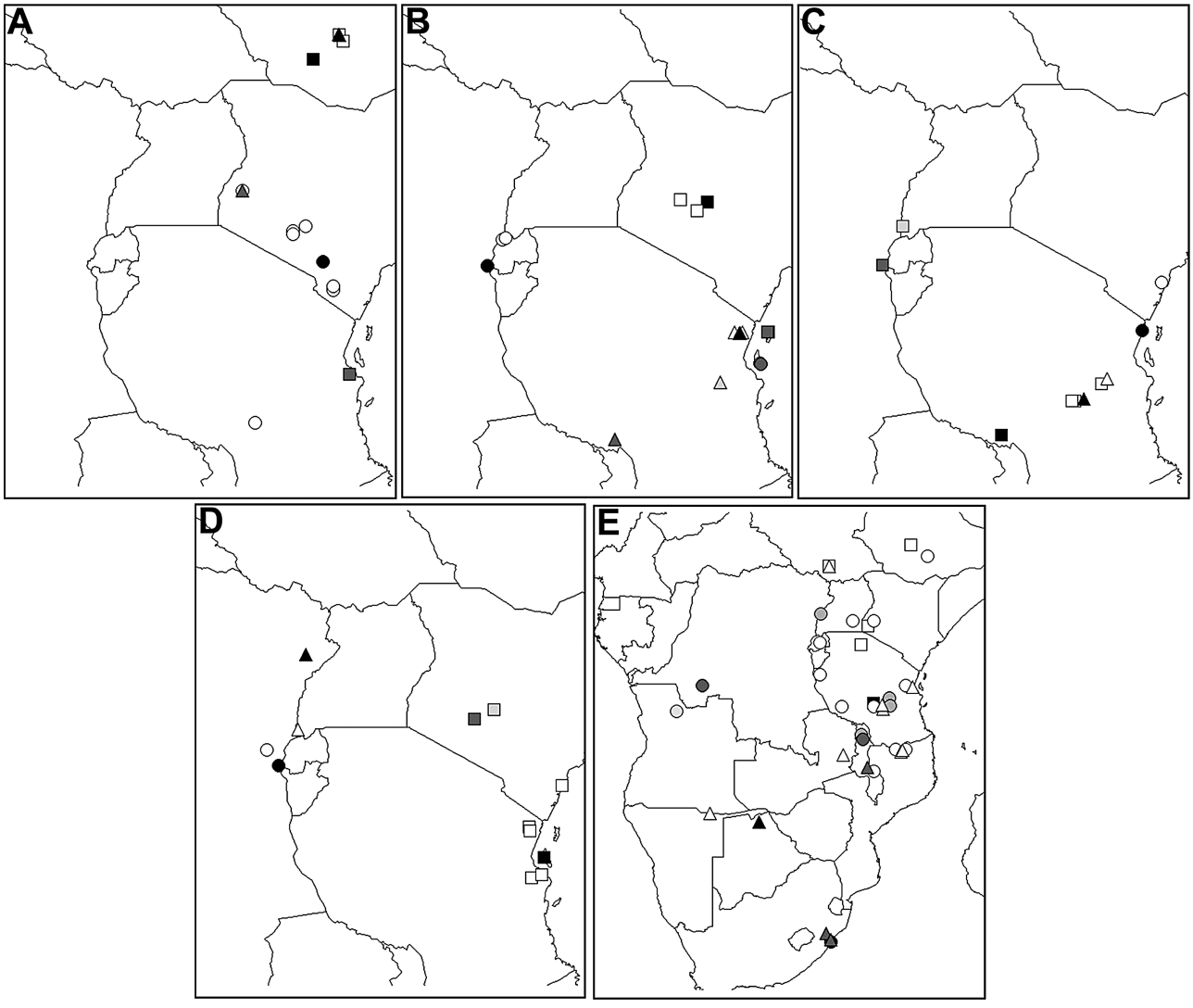

Fig. 1. Maps showing type localities and sites of East African *Phrynobatrachus* sampled in this study. A, *P.* sp. nov 'Kakamega': grey triangle (type locality; no other localities known); *P. scheffleri*: black circle (type locality), white circles; *P. inexpectatus*: black triangle (type locality and additional sites); *P. minutus:* black square (type locality), white squares including *P.* sp. nov 'Oromia', grey square (synonym = *P. albifer*); B, *P. keniensis*: black square (type locality), white squares; *P. krefftii*: black triangle (type locality), white triangles; *P. graueri*: black circle (type locality), white circles; *P. pakenhami*: grey square (type locality and additional sites); *P. ukingensis*: dark grey triangle (type locality), light grey triangle; *P. ungujae* grey circle (type locality and additional sites); C, *P. pallidus*: black circle (type locality), white circle; *P. rungwensis*: black square (type locality), white squares; *P. uzungwensis*: black triangle (type locality), white triangle; *P. versicolor* dark grey square (type locality), light grey square. D, *P. acridoides*: black square (type locality), white squares; *P. acutirostris*: black circle (type locality), white circle; *P. dendrobates*: black triangle (type locality), white triangle; *P. kinangopensis*: dark grey square (type locality), light grey square; E, *P. mababiensis*: black triangle (type locality), white triangles, grey triangles (synonyms = *P. broomi*, *P. chitialaensis*, *P. vanrooyeni*); *P. natalensis*: black circle (type locality), white circles; *P. bullans*: black square (type locality), white squares; *P. parvulus*: dark grey circle (type locality), medium grey circle, light grey circles (synonyms = *P. schoutedeni*, *P. ukingensis nyikae*).

is available on the Supplementary content tab of the article's Informaworld page at http://www.informaworld.com/mpp/uploads/zimkus-schick_appendix_1.pdf). *Petropedetes yaku-sini* (MWK 11002) was used as the outgroup for this study. Institutional abbreviations follow Leviton *et al.* (1985) with the following additions: AC (A. Channing), EBG (E. Greenbaum), MV (M. Vences), RdS (R. de Sa), and SL (S. Lötters).

Different protocols were utilized by B. Zimkus and S. Schick to collect 16S rRNA sequences. B. Zimkus extracted DNA from liver tissue fixed in 99% ethanol using Qiagen DNeasy tissue kits. The polymerase chain reaction (PCR) was used to amplify approximately 750 base pairs (bp) of the 16S rRNA genes using the following primers (Darst & Cannatella, 2004): 16Sc (5′-GTRGGCCTAAAA GCAGCCAC-3′) and 16Sd (5′-CTCCGGTCTGAACTCA GATCACGTAG-3′). Amplification followed the standard PCR conditions (Palumbi, 1996) with the following thermal cycle profile: 2 min at 94°C, followed by 35 cycles of 94°C for 30 s, 46°C for 30 s and 72°C for 60 s, and a final extension phase at 72°C for 7 min. All amplified PCR products were verified using electrophoresis on a 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen Corporation, Carlsbad, CA, USA). PCR products were purified using the Qiagen DNeasy DNA Purification System (Qiagen Inc, Valencia, CA, USA) according to manufacturer's recommendations. DNA sequences of both strands were sequenced using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems) on an automated DNA sequencer (ABI PRISM 3730). Editing and assembly of contigs was completed in Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI). S. Schick extracted DNA from muscle tissue fixed in 99% ethanol using Highpure PCR Template Preparation Kits (Roche Diagnostics). The polymerase chain reaction (PCR) was used to amplify approximately 550 bp of the 16S rRNA gene using the following primers of Palumbi *et al*. (1991): 16SA (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SB (5′-CCGGTCTGAACTCAGATCACGT-3′). Amplification followed the standard PCR conditions (Palumbi, 1996) with the following thermal cycle profile: 90 s at 94°C, followed by 33 cycles of 94°C for 45 s, 55°C for 45 s and extension 72°C for 90 s. All amplified PCR products were verified using electrophoresis on a 1.4% agarose gel stained with ethidium bromide. PCR products were purified using Highpure PCR Product Purification Kits (Roche Diagnostics). Single-stranded DNA was sequenced using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems) on an automated DNA sequencer (ABI PRISM 377). Editing was completed in MEGA4 (Tamura *et al*., 2007). All novel nucleotide sequence data was deposited in GenBank (Appendix 1, see supplementary material).

Sequence alignment and phylogenetic analyses

DNA sequences were aligned using ClustalX (Thompson *et al.*, 1997) and further improved by eye with MacClade 4.08 (Maddison & Maddison, 2000). Approximately 550 bp present in the majority of samples were included in phylogenetic analyses as two different sets of primers were used to collect DNA sequence data. Intra- and interspecific sequence divergence was calculated as per cent pairwise uncorrected molecular distance using PAUP* 4b10 (Swofford, 2002). Estimates of phylogeny were obtained using maximum likelihood (ML) and Bayesian methods. A model of molecular evolution was selected from the data using MrModeltest v2 (Nylander, 2004). ML analyses were implemented in GARLI v0.94 (Zwickl, 2006). Three independent GARLI analyses were completed to assure that the program was not trapped at a local optimum, and each analysis was terminated 3 million generations after the last topological improvement. Nodal support for ML topologies was assessed through nonparametric bootstrap analysis using GARLI; 1000 bootstrap replicates were performed. Branches present in $\geq$ 70% of replicates were considered well supported (Hillis & Bull, 1993). Bayesian inference (BI) was conducted with MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). Four Markov chains were run for 10 million generations, which were sampled every 100 generations using four chains and a temperature of 0.2. Three additional replicates were performed to confirm that MCMC searches were not trapped at local optima. Plots of likelihood versus generation number were used to determine when stationarity was reached; post burn-in trees from all four replicates were combined, and a 50% majority rule consensus tree was calculated by using PAUP* 4b10. Topologies with posterior probabilities $\geq$ 95% were considered well supported (Wilcox *et al.*, 2002). Haplotype networks were constructed using the algorithm given by Templeton *et al*. (1992) and implemented in the TCS software package version 1.21 (Clement *et al*., 2000). The statistical parsimony algorithm calculated the number of mutational steps by which pairwise haplotypes differ and computes the probability of parsimony for pairwise differences until the probability exceeds 0.95. Ambiguous connections in the haplotype network were resolved following the criteria recommended by Pfenninger & Posada (2002): (1) haplotypes are more likely to be connected to common than to rare haplotypes; (2) haplotypes are more likely to be connected to interior than to exterior haplotypes; and (3) haplotypes are more likely to be connected to haplotypes from the same population or region than to those occurring in distant populations.

Results

Phylogenetic reconstruction utilizing the 16S rRNA gene resulted in the same topology using ML and Bayesian methods (Fig. 2). Both the Hierarchical Likelihood Ratio Tests and Akaike Information Criterion implemented in MrModeltest selected a GTR+I+G model with a gamma distribution of 0.5091 and a proportion of invariable sites of 0.3086 (estimated base frequencies: A: 0.3356, C: 0.2442, G: 0.1752, T: 0.2450; rate matrix: A–C: 2.9967, A-G: 10.8980, A-T: 4.9484, C-G: 0.8863, C-T: 21.9403, G-T: 1.0000). The likelihood score of the tree computed using GARLI was –4892.862. For Bayesian analyses, plots of likelihood versus generation number suggest that stationarity was reached at fewer than 500 000 generations. However, the first one million generations were excluded from computation of posterior probabilities to ensure a sufficient burn-in value.

All but two *Phrynobatrachus* species were recovered as monophyletic and highly supported by both ML bootstrap values and posterior probabilities; specimens identified as *P. mababiensis* are recovered in three different places within the topology (*P. mababiensis* A–C; Fig. 2), and specimens of *P. ungujae* Pickersgill, 2007 form a polytomy with *P. ukingensis* (Loveridge, 1932). A potential new species sister to *P. pallidus* Pickersgill, 2007 is also identified and highly supported by ML bootstrap values and posterior probabilities (*P.* sp. nov. 'Oromia'; Fig. 2).

Sequence divergences (uncorrected molecular distances) were calculated within conspecific populations, among conspecific populations, excluding *Phrynobatrachus natalensis*, among conspecific populations of *P. natalensis*, and between sister species (Fig. 3). Sister species were defined as morphologically similar species that are phylogenetically closest to each other in results of ML and Bayesian analyses conducted as part of this study (Fig. 2). There was no sequence divergence within conspecific populations, while divergences among conspecific populations, not including *P. natalensis*, were surprisingly low (0–0.5%; Fig. 3A, B). Conspecific populations of *P. natalensis* yielded a distinct bimodal distribution with a range of differentiation of 0.2–6.4% and a median of 2.3% (Fig. 3C). Although all species that included more than one representative specimen were well supported by both ML bootstrap values and posterior probabilities, relationships among numerous *Phrynobatrachus* species are not resolved (Fig. 2), and therefore only five sister species pairs could be identified: *Phrynobatrachus acridoide*s (Cope, 1867) and *P. pakenhami* Loveridge, 1941; *P. inexpectatus* Largen, 2001 and *P. minutus* (Boulenger, 1895); *P.* sp. nov. 'Kakamega' and *P. mababiensis* C; *P. pallidus* and *P.* sp. nov. 'Oromia'; *P. rungwensis* (Loveridge, 1932) and *P. uzungwensis* Grandison & Howell, 1984. Molecular divergences among those sister species revealed a range of differentiation from 1.5–8.3% with median of 2.3% (Fig. 3D). Only the divergence between *P. pallidus* and *P.* sp nov. 'Oromia' (8.3%) was significantly different from the median value of 2.3% (Fig. 3D).

Discussion

Identification of East African puddle frogs using 16S rRNA

Numerous taxonomists have attempted to resolve relationships among *Phrynobatrachus* species using morphology alone with only minor success. Some taxonomic confusion involving East African species was recently resolved using both morphological, bioacoustical and molecular data, leading to the resurrection of *P. scheffleri* from synonymy with *P. minutus* and description of a new species, *P.* sp. nov. 'Kakamega' (Schick *et al.*, 2010). Results of molecular analyses conducted in this study confirm the validity of two species recently described using morphological and acoustic data: *P. bullans* Crutsinger, Pickersgill, Channing, & Moyer, 2004 and *P. pallidus*. In addition, our results identify a potential new species closely related to *P. pallidus* from Ethiopia (*P.* sp. nov. 'Oromia'; Fig. 2), previously identified as *P. minutus*. *Phrynobatrachus* sp. nov. 'Oromia' differed from *P. pallidus* (MVZ 234153; Kenya) by a 16S rRNA sequence divergence of 8.3%.

Clearly, difficulties in distinguishing small (snout-vent length <25 mm) phrynobatrachines have led taxonomists to confuse a number of species, and molecular analyses of 16S rRNA reveal that there are at least three different populations currently named *Phrynobatrachus mababiensis* (*P. mababiensis* A–C; Fig. 2). According to Poynton (in Frost, 1985), *P. mababiensis* more closely resembles *P. parvulus*, while Laurent (in Frost, 1985) believes *P. mababiensis* was likely a subspecies of *P. ukingensis*. Results of the 16S rRNA phylogeny demonstrate that two populations currently identified as *P. mababiensis* (A and C; Fig. 2) are more closely related to *P. parvulus* (Boulenger, 1905) than *P. ukingensis*. Results also demonstrate that one population, *P. mababiensis* B, is sister to the clade containing *P. ukingensis* and *P. ungujae* (Fig. 2). Additional study is therefore necessary to determine which population represents *P. mababiensis* and describe the remaining two populations if it is determined that they are not currently described species. In addition, molecular analyses suggest that *P. ungujae* may be synonymous with *P. ukingensis*, which is not surprising given that this species was often confused with *P. ukingensis* prior to its description (Pickersgill, 2007). However, only a single sequence of *P. ukingensis* was included in this study, and, as a result, further investigation, including additional genetic samples of both species, is needed.

Using sequence divergence thresholds alone to define or identify species is not recommended (e.g. Moritz & Cicero, 2004; Will *et al.*, 2005); however, analysis of sequence divergences within lineages may assist in the identification

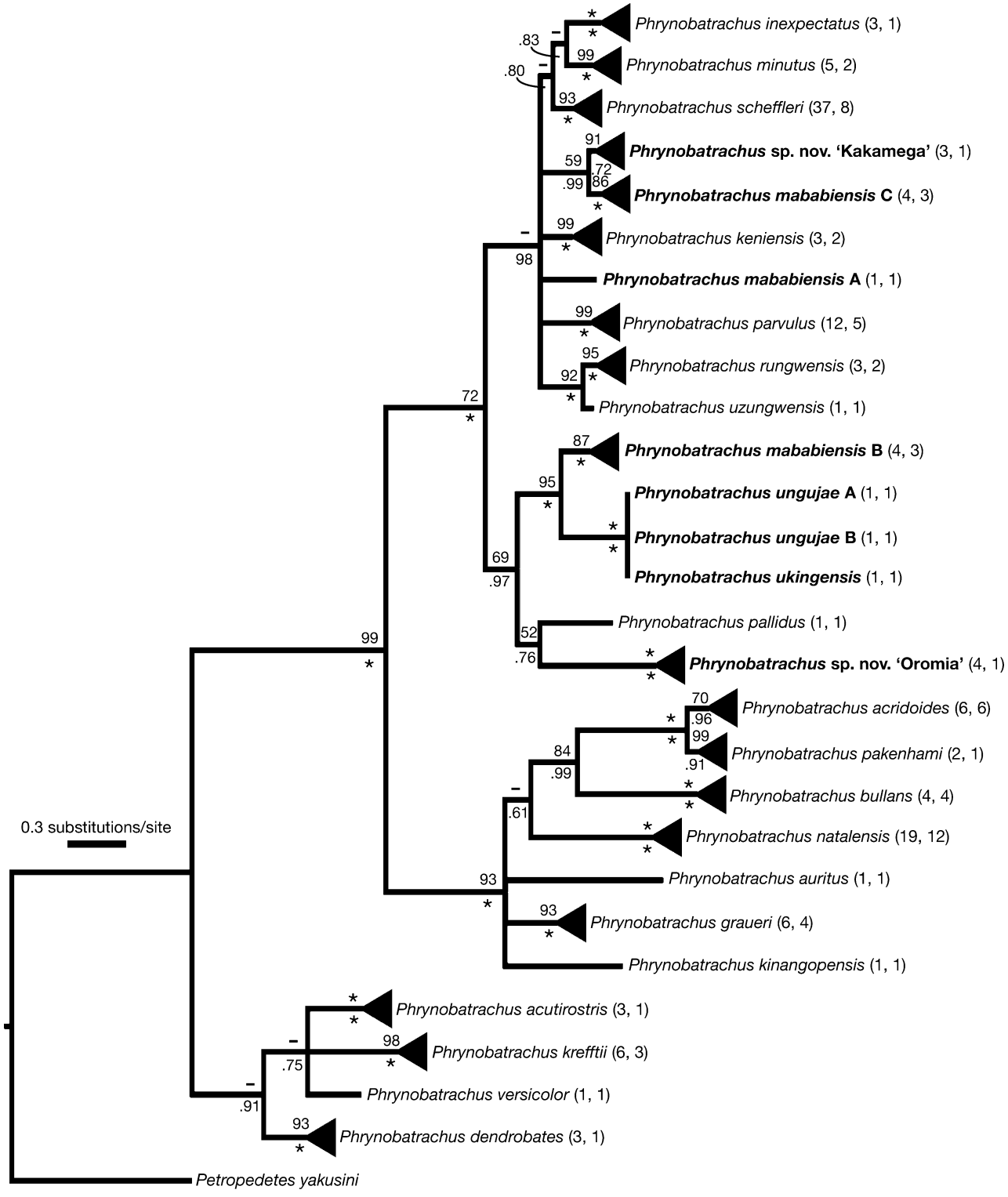

Fig. 2. Bayesian phylogram inferred from nucleotide sequence data from mitochondrial 16S rRNA. Numbers in parentheses after scientific names indicate the number of sequences included for particular species and number of populations sampled, respectively. Candidate species, cryptic species complexes and species that may require synonymy are shown in bold. Numbers above branches are nonparametric bootstrap proportions from GARLI analysis; numbers below branches are Bayesian posterior probabilities. 100% non-parametric bootstrap support and 1.00 posterior probability are indicated by asterisks (*); less than 50% bootstrap support and 0.50 posterior probability are indicated by dashes (-).

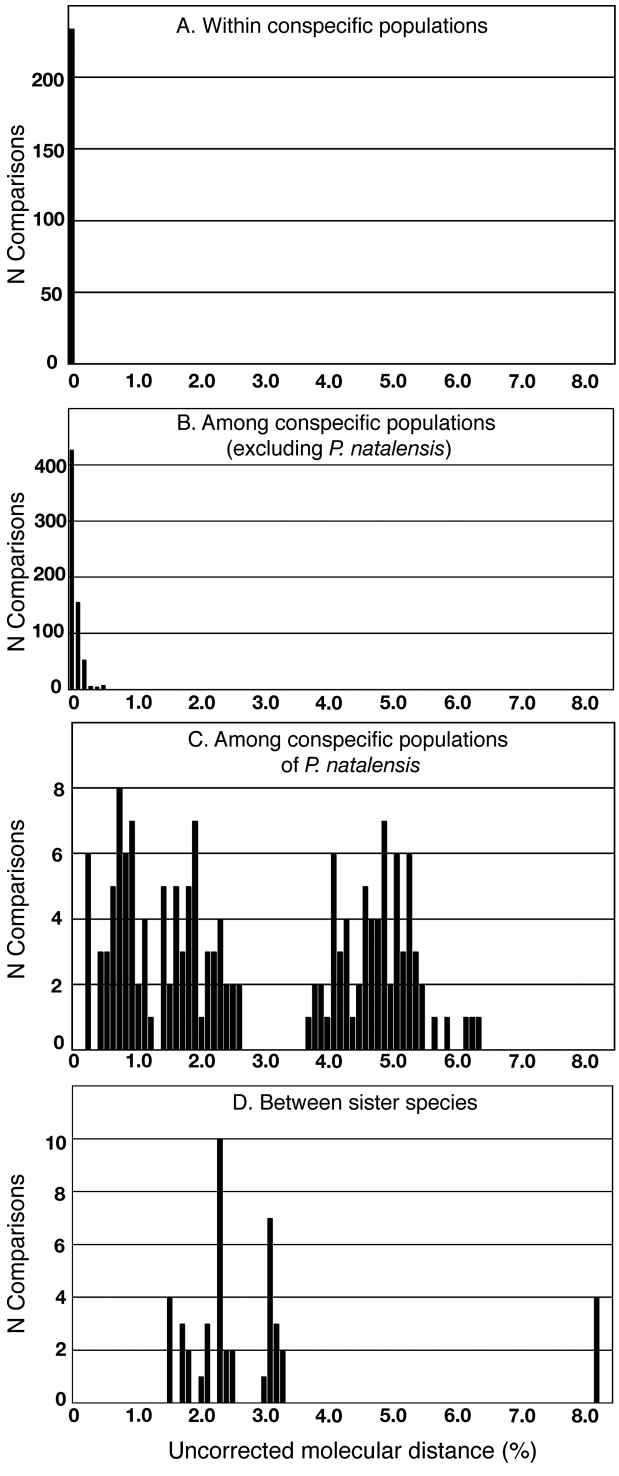

Fig. 3. Inter- and intraspecific genetic variation of the 16S rRNA gene (550 base pairs) of East African *Phrynobatrachus*. Uncorrected p-distances (%) from pairwise comparisons (*a*) within conspecific populations; (*b*) among conspecific populations, excluding *P. natalensis*; (*c*) among conspecific populations of *P. natalensis*; and (*d*) among five sets of sister species identified by phylogenetic analyses.

of species complexes or be used as part of an integrative approach to identify candidate species (Vieites *et al.*, 2009). Unlike the overlapping differentiation found among conspecific and sister species of Mantellidae by Vences *et al*. (2005*a*), sequence divergences within conspecific populations of puddle frogs, excluding *Phrynobatrachus natalensis*, did not overlap with those found between sister species: 0–0.5% and 1.5–8.3%, respectively. Sequence divergences among conspecific populations of *P. natalensis* (0.2–6.4%) did overlap with those found between sister species, both exhibiting medians of 2.3% (Fig. 3C, D). Conspecific *P. natalensis* also exhibited a bimodal distribution of sequence divergences, suggesting that more than one species is currently identified as *P. natalensis* (Fig. 3C). In addition, statistical parsimony analysis could not link two haplotype networks of *P. natalensis* within the limit of 95% (Fig. 4). This result supports the presence of at least two distinct species in East Africa: one including populations in the northern and central regions and the other with populations in the central and southern regions (Haplotypes A and B; Fig. 4).

Results of analyses conducted in this study reveal that additional taxonomic work is required before a number of East African *Phrynobatrachus* species can be confidently identified. In addition to those taxonomic issues mentioned previously, it is important to call attention to the fact that a number of species were represented in these analyses by only one individual, including *P. auritus* Boulenger, 1900, *P. kinangopensis* Angel, 1924, *P. mababiensis* A, *P. pallidus*, *P. versicolor* Ahl, 1924, *P. ukingensis* and *P. uzungwensis* (Fig. 2). Therefore, the inclusion of additional sequence data from the aforementioned species, as well as other East African species not represented with molecular data, including *P. asper* Laurent, 1951, *P. bequaerti* (Barbour & Loveridge, 1929), *P. breviceps* Pickersgill, 2007, *P. cryptotis* Schmidt & Inger, 1959, *P. dalcqui* Laurent, 1952, *P. irangi* Drewes & Perret, 2000, *P. petropedetoides* Ahl, 1924, *P. rouxi* (Nieden, 1913), *P. stewartae* Poynton & Broadely, 1985, *P. scapularis* (De Witte, 1933) and *P. sulfureogularis* Laurent, 1951, will improve the understanding of the relationships among puddle frogs and allow more confidence when investigating molecular systematics of East African *Phrynobatrachus* species using the 16S rRNA marker.

Use of DNA barcoding to identify puddle frog species

DNA barcoding is a tool promoted for rapid species identification in which standardized, short DNA sequences (400–800 base pairs) are compared against a reference library of sequence data (Hebert *et al.*, 2003, 2004; Hebert & Gregory, 2005; Kress & Erickson, 2008). If used correctly,

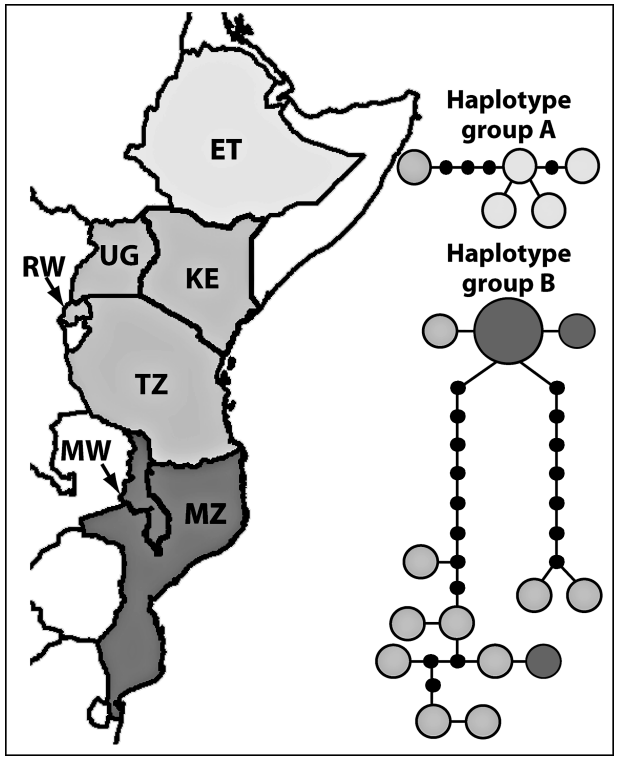

Fig. 4. Relationships among *Phrynobatrachus natalensis* 16S rRNA haplotypes using statistical parsimony networks as implemented in TCS. Each circle in a given network corresponds to one observed haplotype with the size of each circle proportional to the number of individuals corresponding to the observed haplotype (small circle, one individual; large circle, two individuals). Circles are colour-coded by region (northern, central or southern); only those countries where haplotypes were collected are colour-coded and labelled. Small black dots represent hypothetical haplotypes needed to connect the network but not observed among the samples.

DNA barcoding allows users to quickly and easily identify a species that may be difficult to recognize using morphology alone. Currently, cytochrome c oxidase subunit 1 (COI), a mitochondrial gene, is considered the standard barcode region for animals (Hebert *et al.*, 2003, 2004; Barrett & Hebert, 2005; Ward *et al.*, 2005; Smith *et al.*, 2008). However, the 16S rRNA gene has also been determined to be a useful fragment for amphibian barcoding (Vences *et al.*, 2005*a*, 2005*b*). The analysis of 16S rRNA of East African puddle frogs conducted here illustrates the need for taxonomic and phylogenetic analyses prior to implementation of DNA barcoding. Otherwise, DNA barcode sequences will reveal cryptic species without the possibility of assigning the sequence to a described species.

The benefits of DNA barcoding are undeniable, regardless of the caveats that must first be addressed. This method has accelerated the rate of taxonomic discovery by revealing cryptic species complexes and supporting morphological data in the description of new species. Specifically for amphibians, DNA barcoding allows the identification of various life history stages, including eggs, tadpoles and juveniles, as well as the association between sexually dimorphic species (Vences *et al.*, 2005*b*; Willassen, 2005; Raharivololoniainaa *et al*., 2006). The ability to identify candidate species, detect cryptic species complexes and describe new species using barcoding methods will undeniably facilitate the prioritization of conservation actions (Köhler *et al.*, 2005). However, before DNA barcoding methods can be used for species identification and conservation, taxonomic exploration is clearly necessary for lineages without complete phylogenies. In light of the global crisis of massive biodiversity loss and the ongoing worldwide amphibian decline (IUCN, 2008), we therefore strongly suggest immediate action to further investigate *Phrynobatrachus* taxonomy in accordance with the IUCN Amphibian Conservation Action Plan (Gascon *et al.*, 2007), including the construction of a complete phylogeny of sub-Saharan puddle frogs.

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