Phylogeography of North African *Amietophrynus xeros* estimated from mitochondrial DNA sequences

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Amietophrynus xeros **was sequenced for part of the 16S rRNA mitochondrial region to assess genetic diversity between populations from Niger, Mali, Senegal, Mauritania and Tanzania. Although populations are currently unconnected, diversity within the Sahel region was relatively low, indicating that the species only expanded into this region relatively recently, perhaps after the last glacial maximum. Diversity was higher between samples from Tanzania. Some individuals of two species from previously published studies, *A. garmani* and *A. gutturalis*, share haplotypes with *A. xeros*, but this is likely to be due to error, possibly misidentification. Similar errors appear to exist in published studies of other North African *Amietophrynus* species such as *A. regularis*.**

Key words: ***Amietophrynus xeros*****, Mitochondrial DNA, 16S rRNA, North Africa.**

INTRODUCTION

Amietophrynus xeros is a medium sized species of toad, with a typically symmetrical patterning of dark patches across the back and red spots on the posterior part of the thighs. It has relictual populations in Saharan Mountains, such as the Hoggar and Aïr Mountains, and has been reported from the Sahelian zone of Mauritania, Niger, Chad and southwards to Tanzania (Schleich *et al.* 1996). Despite the extreme aridity of this region several bufoniids exist in isolated oases and mountains within parts of this region, including *'Bufo' mauritanicus, Pseudepidalea viridis, Amietophrynus regularis* and *A. xeros.* Of these, *P. viridis* is unrelated to the other species, and appears to be a species complex, with some authors considering the North African populations belonging to a distinct species, *P. boulengeri* (Stöck *et al.* 2006). The remaining species form part of a well-supported clade, including various other sub-Saharan species (Maxson 1981; Harris & Perera 2009), most of which have 20 chromosomes rather than the Bufoniid norm of 22. Frost *et al.* (2006) refer these to *Amietophrynus,* and although *'Bufo' mauritanicus* was not placed in this group due to lack of evidence, mtDNA sequences support it as a member of the clade (Harris & Perera 2009). Although *A. xeros* and the morphologically similar *A. regularis* are comparatively widespread, no estimates of genetic variation within either species have been performed. Such studies are essential, as genetic data have indicated various other widespread amphibians from North Africa, such as *Hyla meridionalis* and *Discoglossus pictus* are highly variable so that cryptic but distinct forms occur (Recuero *et al.* 2007; Zangari *et al.* 2006). On the other hand *'Bufo' mauritanicus* showed minimal intraspecific variation (Harris & Perera 2009). Data from more species are therefore necessary to help elucidate comparative phylogeographical patterns for North African amphibians, such as those identified in reptiles (Barata *et al.* 2008).

Two phylogenetic studies of North African bufonids indicate conflicting estimates of relationships for *A. xeros,* with this species being the sister taxon of, and genetically very similar to, *A. garmani* (Pramuk 2006; Pramuk *et al.* 2009) or in the '*gutturalis* clade' unrelated to the *A. garmani* lineage (Cunningham & Cherry 2004). At the same time distribution data for *A. xeros* are generally vague, possibly due to the logistical difficulties of sampling across the Sahara region (Brito *et al.* 2008). Thus it is unclear if it is extremely localized in a few available water bodies, or more widespread. Furthermore, specimens from the Guelta de Timia, Niger, presented skin ulcers on the dorsum, probably related to parasitic as well as

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fungus or viral infections, or even secondary opportunistic infections, suggesting that isolated populations have increased risk of localised extinction (Brito *et al.* 2005). Detailed information about the local distribution of this species is needed to assess the conservation status of the species, and as baseline data for future assessments.

The aim of this study was to collect samples from across Mauritania, Senegal, Mali and Niger of *A. xeros*, and to determine genetic diversity within the species by sequencing part of the 16S rRNA mitochondrial region. By comparing the data with published sequences from other North African bufonids the phylogenetic relationships could also be re-examined using multiple individuals.

MATERIALS & METHODS

Specimens were captured by hand in the field. A single toe was removed and stored in 96% ethanol (Table 1 and Fig. 1). Genomic DNA was extracted following standard high-salt protocols (Sambrook *et al.* 1989). A fragment of the 16S rRNA was amplified by PCR and sequenced following Harris (2001). Sequences were obtained on an automated sequencer (ABI 310). All new haplotypes were submitted to GenBank (Accession numbers ****** to ******).

Alignment was performed with ClustalW using Bioedit v. 5.0.9. (Hall 1999), and adjusted manually (available on request from D.J.H.).

Since it was immediately evident that intraspecific variation was low, sequences were joined in statistical parsimony, constructed under a 95% parsimony criterion using TCS 2.1 (Clement *et al.* 2000). For the phylogenetic analysis, sequences were imported into PAUP* 4.0b10 (Swofford 2003). Only unique haplotypes within *A. xeros* were included, as well as related African bufonid species, following Harris & Perera (2009). We used maximum likelihood (ML) analysis with random sequence addition (10 replicate heuristic searches) to estimate the evolutionary relationships. Support for nodes was estimated using the bootstrap technique (Felsenstein 1985) with 1000 replicates. The AIC criteria carried out in Modeltest 3.06 (Posada & Crandall 1998) was used to choose the model of evolution employed. Bayesian analysis was also implemented using Mr. Bayes v. 3.1 (Huelsenbeck & Ronquist 2001) with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. The analysis was run for 2×10^6 generations, saving one tree in each 100 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree, in which frequency of any particular clade represents the posterior probability (Huelsenbeck & Ronquist 2001).

RESULTS

Forty individuals from 18 populations of *A. xeros* were sequenced for a 502 bp part of the 16S rRNA. A single previously published specimen from Bamako, Mali (Pramuk 2006) and two individuals from Tanzania (Cunningham & Cherry 2004) were also included. Because of their obvious great similarity, sequences from specimens of *A. gutturalis* and *A. garmani* (both from Kenya, Pramuk 2006) were also included. In total 12 haplotypes were recovered, with *A. gutturalis* and *A. garmani* sharing haplotypes with *A. xeros* (Fig. 2).

For the phylogenetic analysis, the 12 haplotypes found for *A. xeros* were included in addition to 16 specimens from other species. The most appropriate model of evolution for this dataset was the general time reversible model, with an estimate of invariable sites (I = 0.49) and a discrete approximation of the gamma distribution ($\alpha = 0.604$). The ML analysis recovered a single tree (–ln = 1997; Fig. 3). The Bayesian analysis recovered the same tree as the ML analysis, with one minor exception (indicated by an asterisk in Fig. 3).

DISCUSSION

Within *A. xeros*, 12 haplotypes were recovered, with one common haplotype (23 individuals) and others found in one to four individuals. Within *A. xeros* maximum intraspecific divergence between the two haplotypes from Tanzania was 1.6%. This is quite high, but considerably less than the 3% cut-off value used in amphibian studies with this gene region to indicate possible cryptic taxa (Vieites *et al.* 2009). Most previous phylogeographical studies of North African herpetofauna have uncovered extensive intraspecific variation (e.g. Barata *et al.* 2008; Fonseca *et al.* 2008). The finding of only 12 closely related haplotypes across such a wide range is therefore unexpected. For example, specimens from Tanzania and the Aïr Mountains in Niger shared haplotypes, and Aïr Mountain specimens differed by just one nucleotide from specimens in Mauritania. This is despite the fact that currently there are large areas of unsuitable

Table 1. Localities of specimens included in this study. Population code refer to Fig. 1 and individual codes refer to Figs 2 and 3.

Code	Population code	Latitude	Longitude	Region	Country	Genbank no.
368	1	18.094950	8.761267	Aïr mountains	Niger	GQ868485
369	1	18.094950	8.761267	Aïr mountains	Niger	GQ868486
370	1	18.094950	8.761267	Aïr mountains	Niger	GQ868486
462	2	14.545417	-11.943033	Kayes	Mali	GQ868487
994	3	13.593750	-10.381817	Kayes	Mali	GQ868488
445	4	14.505667	-9.633000	Kayes	Mali	GQ868488
452	4	14.511500	-9.702700	Kayes	Mali	GQ868492
455	5	14.683100	-10.384917	Kayes	Mali	GQ868488
456	5	14.683100	-10.384917	Kayes	Mali	GQ868489
473	6	16.178167	-13.997233	Saint-Louis	Senegal	GQ868490
474	6	16.178167	-13.997233	Saint-Louis	Senegal	GQ868488
475	6	16.178167	-13.997233	Saint-Louis	Senegal	GQ868488
57	7	16.611667	-7.269583	Hodh Ech Chargui	Mauritania	GQ868488
2211	8	16.403148	-9.559860	Hodh El Gharbi	Mauritania	GQ868491
2212	8	16.403148	-9.559860	Hodh El Gharbi	Mauritania	GQ868491
2213	8	16.403148	-9.559860	Hodh El Gharbi	Mauritania	GQ868488
2074	9	17.249855	-10.667613	Hodh El Gharbi	Mauritania	GQ868488
2075	9	17.249855	-10.667613	Hodh El Gharbi	Mauritania	GQ868488
2076	9	17.249855	-10.667613	Hodh El Gharbi	Mauritania	GQ868488
2443	10	15.932785	-12.010887	Guidimaka	Mauritania	GQ868488
2444	10	15.932785	-12.010887	Guidimaka	Mauritania	GQ868488
2445	10	15.932785	-12.010887	Guidimaka	Mauritania	GQ868488
118	11	17.392517	-13.452850	Brakna	Mauritania	GQ868488
119	11	17.392517	-13.452850	Brakna	Mauritania	GQ868488
120	11	17.392517	-13.452850	Brakna	Mauritania	GQ868492
85	12	17.279133	-12.221450	Nema - Nouakchott	Mauritania	GQ868488
102	13	17.938317	-12.267117	Moudjeria - N'Beika	Mauritania	GQ868488
2676	14	17.737962	-12.245253	Tagant	Mauritania	GQ868493
2032	15	17.834850	-11.557833	Tagant	Mauritania	GQ868488
2033	15	17.834850	-11.557833	Tagant	Mauritania	GQ868488
2034	15	17.834850	-11.557833	Tagant	Mauritania	GQ868488
1952	16	18.818115	-11.777500	Tagant	Mauritania	GQ868488
1953	16	18.818115	-11.777500	Tagant	Mauritania	GQ868492
1954	16	18.818115	-11.777500	Tagant	Mauritania	GQ868492
1826	17	19.684525	-13.033803	Adrar	Mauritania	GQ868488
1827	17	19.684525	-13.033803	Adrar	Mauritania	GQ868494
1828	17	19.684525	-13.033803	Adrar	Mauritania	GQ868494
1699	18	20.580946	-13.136361	Adrar	Mauritania	GQ868488
1700	18	20.580946	-13.136361	Adrar	Mauritania	GQ868488
1701	18	20.580946	-13.136361	Adrar	Mauritania	GQ868488

habitat separating these regions. The 'star-like' pattern of haplotypes from Mali, Mauritania and Senegal in particular is typical in populations that have undergone recent expansions (Rogers & Harpending 1992). On the other hand the two specimens from Tanzania (*A. xeros* a and *A. xeros* b in Fig. 3) were the most divergent of the haplotypes recovered, indicating that intraspecific variation is probably highest in the southern part of the species' range.

In Europe, comparative phylogeographical patterns are relatively well elucidated for the period since the last glacial maximum. On this continent, southern areas acted as 'refugia' for many species that did not survive in northern regions (Hewitt 2000). After the last glacial maximum, as the climate warmed, these species then spread north, leading to a situation in which most genetic diversity is found in the southern regions, while the northern regions are extremely depauperate. This

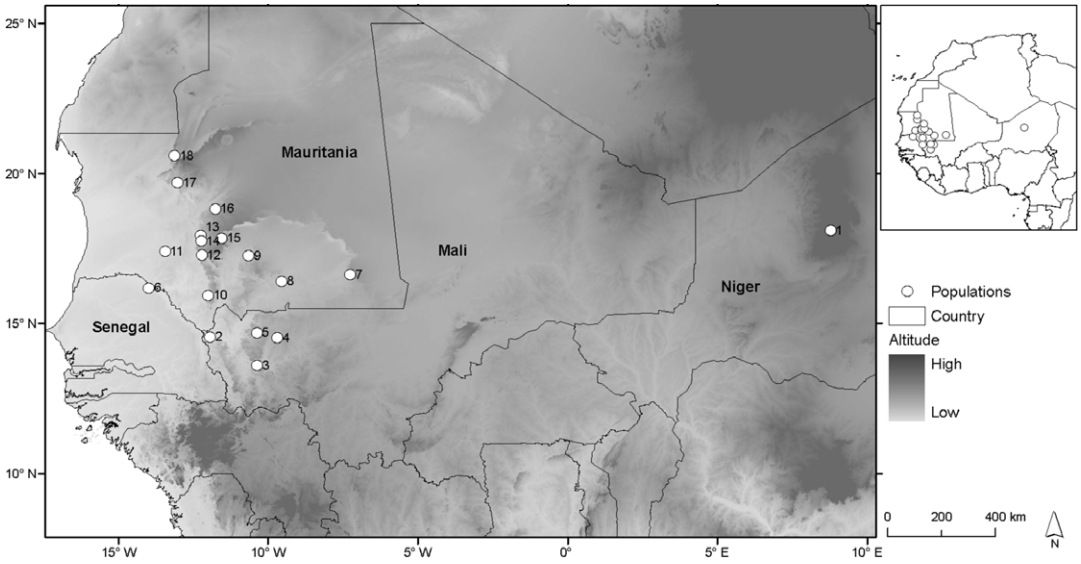

Fig. 1. Map showing the localities of the *Amietophrynus xeros* individuals sequenced for this study.

has been demonstrated for many species, for example *Epidalea calamita* (Rowe *et al.* 2006). Although the climate in North Africa would have fluctuated less than that in Europe during the last glacial cycle, considerable climatic alterations would have taken place (Elenga *et al.* 2000). The Sahelian region was particularly arid during the last glacial maximum (Gasse 2000), became more humid for approximately 10 000 years and then became progressively more arid again. It seems likely that during the more arid periods species such as *A. xeros* would have been absent, or at least highly restricted, within the Sahel region, but with large populations to the south capable of maintaining considerable diversity. During the more humid and thus favourable climatic period the species expanded across the Sahel, leading to the observed genetic structure. The current more arid phase has isolated many of these populations, which survive in the remaining watercourses, oases and more mountainous zones (Joger 1981). Although phylogeographical studies of species from this area are rare, an assessment of variation in the rodent *Mastomys huberti* recovered a similar pattern (Mouline *et al.* 2008).

Assessment of the phylogenetic relationships of *A. xeros* and other related North African bufonids using multiple individuals indicates why there was a discrepancy in relationships between earlier phylogenetic studies (Pramuk 2006; Cunningham & Cherry 2004). The specimens of *A. gutturalis* and *A. garmani* used by Pramuk (2006) share haplotypes with *A. xeros* individuals, while those specimens of the same species used by Cunningham & Cherry (2004) are very different lineages (both haplotypes were beyond the 95% connection criteria used to construct the network of Fig. 2; see also Fig. 3). Various explanations could explain this. One possibility would be contamination of the samples used by Pramuk (2006), who also analysed *A. xeros*. However, this seems unlikely, as the specimens were later sequenced for various unlinked markers that gave similar results (Pramuk *et al.* 2009). The possibility of mitochondrial introgression causing the result is also therefore unlikely. An alternative possibility is that the sequences of Cunningham & Cherry (2004) are erroneous, either through extensive sequencing errors, or perhaps due to the amplification of nuclear copies of mtDNA. Although errors have been reported for the same gene region in other published bufonids (Harris 2001) this also seems unlikely, as these sequences have been checked for evidence of possible errors and did not appear to be unusual (Pauly 2008). A third possibility is that the specimens used by Pramuk (2006) were misidentified and actually are *A. xeros.* In North Africa *A. xeros* can be easily mistaken for *A. regularis* (Schleich 1996), and this would explain the differences reported from the various phylogenetic studies. Sequencing of additional *A. gutturalis* and *A. garmani* would be useful to confirm this. Authors using the phylogeny of African bufonids to assess character evolution or biogeographical

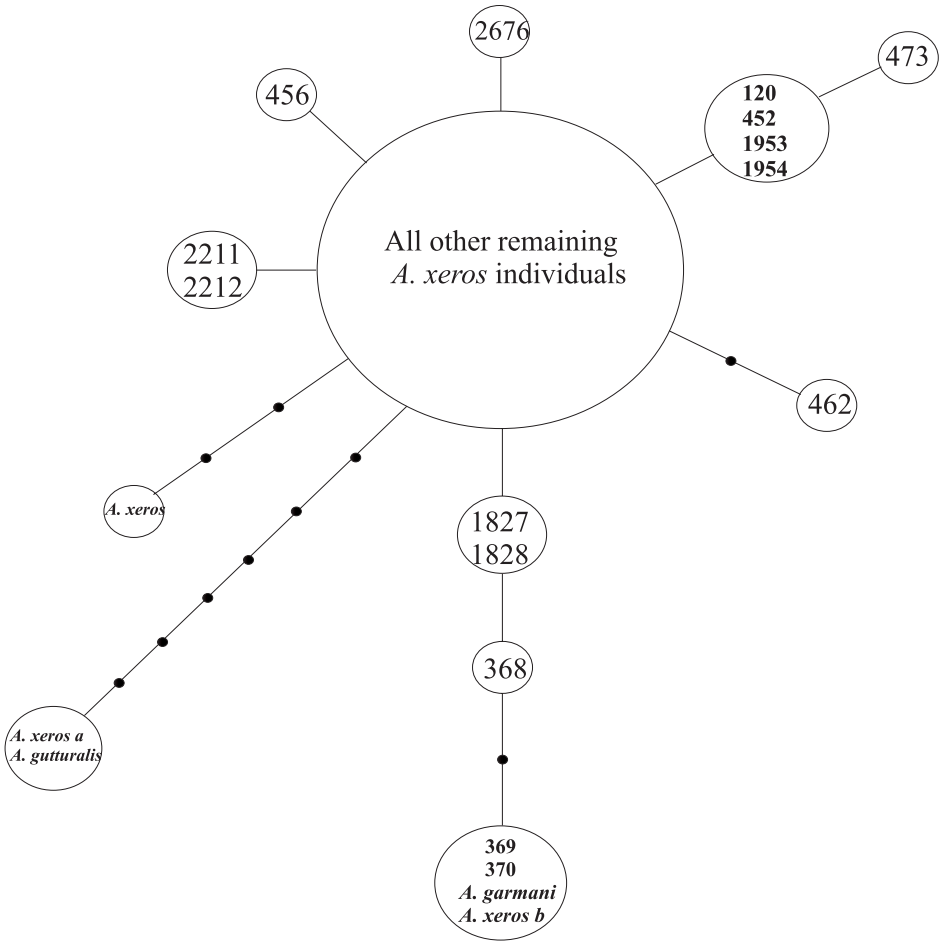

Fig. 2. Parsimony network of 16S rRNA haplotypes (treating gaps as 'missing data') observed in *Amietophrynus xeros*. For individual codes please see Table 1. Geometric figure size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates. Sequences from Genbank: *A. gutturalis* (DQ283436), *A. garmani* (DQ158453), *A. xeros* (DQ158499) *A. xeros* a (AF220887) and *A. xeros* b (AF220888).

hypotheses should be cautious in their use of these species until this issue is fully resolved.

As well as the apparent confounding of *A. gutturalis* and *A. garmani* with *A. xeros,* another suspect paraphyly occurs in *A. regularis.* One specimen of this species (from Pauly *et al.* 2004) is identical to other specimens of *A. kisoloensis,* and highly differentiated from remaining *A. regularis* sequences published in multiple different studies (Cunningham & Cherry 2004; Hoegg *et al.* 2004). Similarly a specimen identified as *A. maculatus* (Graybeal 1997) groups either within (ML analysis), or close sister taxa to (Bayesian analysis), three specimens of *A. regularis* (Fig. 3). Again a detailed reassessment of *A. regularis* with multiple individuals analysed across the range is needed to ascertain if this is due to misidentification or introgression following hybridization.

To conclude, *A. xeros* is currently widespread across much of the Sahel, and this study therefore provides important field data for assessing the future status of these populations. The species appears to have expanded into the region relatively recently, thus currently isolated and geographically dispersed populations are genetically similar although greater diversity is reported from the southern part of the species' range. Sequences published as *A. gutturalis* and *A. garmani* are identical to haplotypes from *A. xeros,* possibly due to misidentification, so that a close relationship between these species as previously proposed is no longer supported.

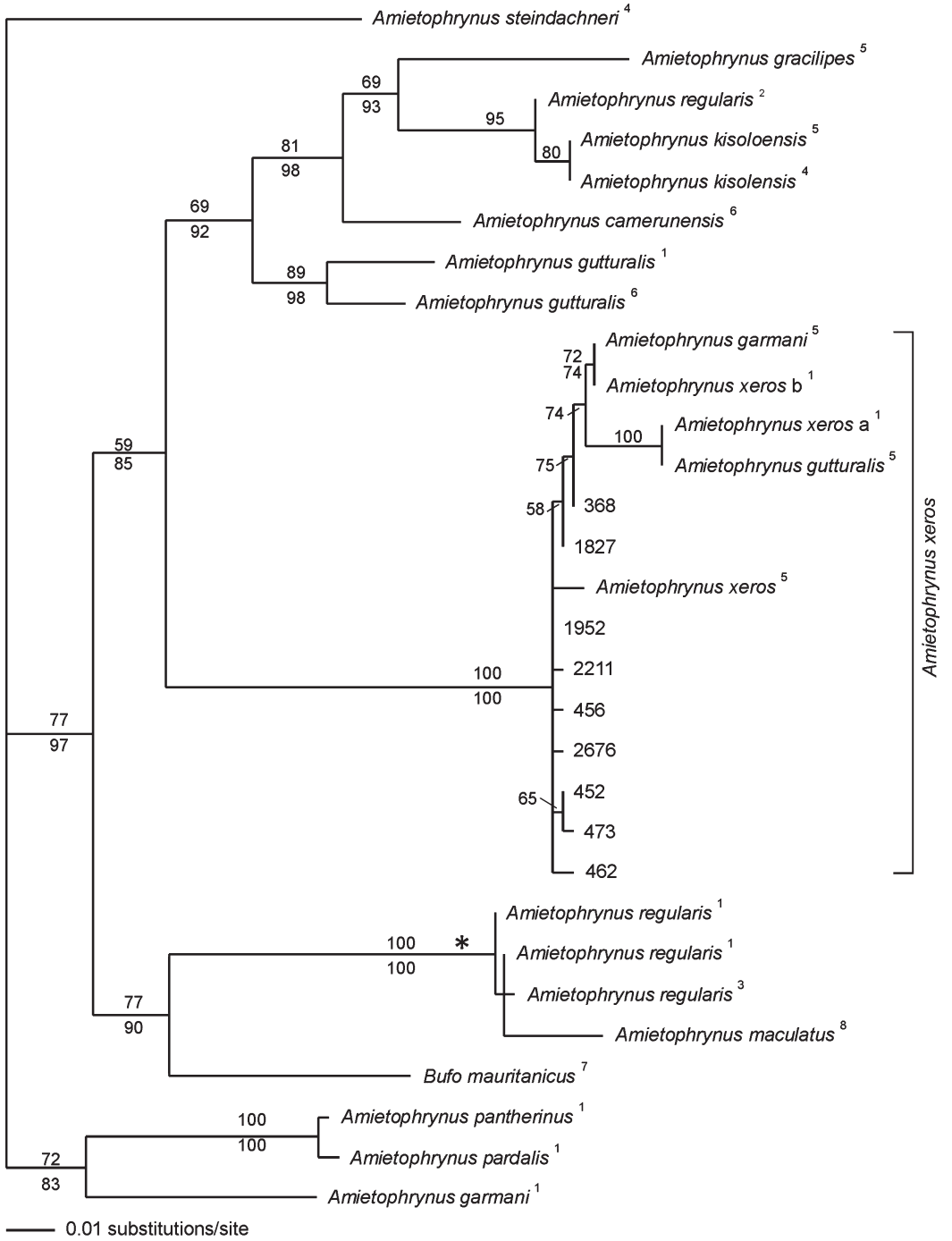

Fig. 3. Phylogenetic relationships estimated using maximum likelihood as described in the text. ML bootstrap support is indicated above nodes, Bayesian posterior probabilities below nodes. *Amietophrynus xeros* codes (this study) refer to Table 1. In the Bayesian estimates of relationships, *A. maculatus* was sister taxon to the three related *A. regularis* sequences which were weakly associated as a clade (*33%BPP). Otherwise relationships were identical. Superscripted numbers after species names indicate the source of published sequences: 1) Cunningham & Cherry 2004; 2) Pauly *et al.* 2004; 3) Hoeggs *et al.* 2004; 4) Darst & Cannatella 2004; 5) Pramuck 2006; 6) Frost *et al.* 2006; 7) Harris & Perera 2009; 8) Graybeal 1997.

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REFERENCES

BARATA, M., HARRIS, D. J. & CASTILHO R. 2008. Comparative phylogeography of northwest African *Natrix maura* (Serpentes: Colubridae) inferred from mtDNA sequences. *African Zoology* **43**: 1–7.

BRITO, J.C., REBELO, H., CROCHET, P-A. & GENIEZ, P. 2008. Data on the distribution of amphibians and reptiles from North and West Africa, with emphasis on *Acanthodactylus* lizards and the Sahara Desert. *Herpetological Bulletin* **105**: 19–27.

BRITO, J.C., REBELO, H., WELDON, C. & SOARES C. 2005. Skin ulcers in *Bufo xeros* from the Central Sahara. *Froglog* **71** (Oct. 2005): 3–4.

CLEMENT, M., POSADA, D. & CRANDALL, K.A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657–1660.

CUNNINGHAM, M. & CHERRY, M.I. 2004. Molecular systematics of African 20-chromosome toads (Anura: Bufonidae). *Molecular Phylogenetics and Evolution* **32**: 671–685.

DARST, C.R. & CANNATELLA, D.C. 2004. Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* **31**: 462–475.

ELENGA, H., PEYRON, O., BONNEFILLE, R., JOLLY, D., CHEDDADI, R., GUIOT, J., ANDRIEU, V., BOTTEMA, S., BUCHET, G., DE BEAULIEU, J.L., HAMILTON, A.C., MALEY, J., MARCHANT, R., PEREZ-OBIOL, R., REILLE, M., RIOLLET, G., SCOTT, L., STRAKA, H., TAYLOR, D., VAN CAMPO, E., VINCENS, A., LAARIF, F. & JONSON, H. 2000. Pollen-based biome reconstruction for southern Europe and Africa 18 000 yr BP. *Journal of Biogeography* **27**: 621–634.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **39**: 783–791.

FONSECA, M., BRITO, J.C., REBELO, H., KALBOUSSI, M., LARBES, S., CARRETERO, M.A. & HARRIS, D.J. 2008. Genetic variation among spiny-footed lizards in the *Acanthodactylus pardalis* group from North Africa. *African Zoology* **43**: 8–15.

FROST, D.R., GRANT, T., FAIVOVICH, J., BAIN, R.H., HAAS, A., HADDAD, C.F.B., DE SA, R.O., CHANNING, A., WILKINSON, M., DONNELLAN, S.C., RAXWORTHY, C.J., CAMPBELL, J.A., BLOTTO, B.L., MOLER, P., DREWES, R.C., NUSSBAUM, R.A., LYNCH, J.D., GREEN, D.M. & WHEELER, W.C. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History* **297**: 1–371.

GASSE, F. 2000.Hydrological changes in the African tropics since the last glacial maximum. *Quaternary Science Reviews* **19**: 189–211.

GRAYBEAL, A.M. 1997. Phylogenetic relationships of bufonid frogs and tests of alternate macroevolutionary hypotheses characterizing their radiation. *Zoological Journal of the Linnean Society* **119**: 297–338

HALL, T.A. 1999. Bioedit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.

HARRIS, D.J. & PERERA, A. 2009. Phylogeography and genetic relationships of North African *Bufo mauritanicus* Schlegel, 1841 estimated from mitochondrial DNA sequences. *Biologia* **64**: 1–5.

HARRIS, D.J. 2001. Reevaluation of 16S ribosomal RNA variation in *Bufo* (Anura: Amphibia). *Molecular Phylogenetics and Evolution* **19**: 326–329.

HEWITT, G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405**: 907–913.

HOEGGS, S., VENCES, M., BRINKMANN, H. & MEYER, A. 2004. Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. *Molecular Biology and Evolution* **21**: 1188–1200.

HUELSENBECK, J.P. & RONQUIST, F. 2001. Mr. Bayes: Bayesian inference of the phylogeny. *Bioinformatics* **17**: 754–755.

JOGER, U. 1981. Zur herpetofaunistik Westafrikas. *Bonner zoologishe Beiträge* **32**: 297–340.

MAXSON, L.R. 1981. Albumin evolution and its phylogenetic implications in African toads of the genus *Bufo*. *Herpetologica* **37**: 96–104.

MOULINE, K., GRANJON, L., GALAN, M., TATARD, C., ABDOULLAYE, D., ATTEYENE, S.A., DUPLANTIER, J-P. & COSSON, J.F. 2008. Phylogeography of a Sahelian rodent species *Mastomys huberti*: a Plio-Pleistocene story of emergence and colonization of humid habitats. *Molecular Ecology* **17**: 1036–1053.

PAULY, G.B. 2008. Phylogenetic systematics, historical biogeography, and the evolution of vocalizations in Nearctic toads (*Bufo*). Ph.D. thesis, University of Texas, Austin.

PAULY, G.B., HILLIS, D.M. & CANNATELLA, D.C. 2004. The history of a nearctic colonization: molecular phylogenetics and biogeography of the nearctic toads (*Bufo*). *Evolution* **58**: 2517–2535.

POSADA, D. & CRANDALL, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

PRAMUK, J.B. 2006. Phylogeny of the South American *Bufo* (Anura: Bufonidae) inferred from combined evidence. *Zoological Journal of the Linnean Society* **146**: 407–452.

PRAMUK, J.B., ROBERTSON, T., SITES, J.W. & NOONAN, B.P. 2008. Around the world in 10 million

years: biogeography of the nearly cosmopolitan true toads (Anura: Bufonidae). *Global Ecology and Biogeography* **17**: 72–83.

RECUERO, E., IRAOLA, A., RUBIO, X., MACHORDOM, A. & GARCÍA-PARÍS, M. 2007. Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern. *Journal of Biogeography* **34**: 1207–1219.

ROGERS, A.R. & HARPENDING, H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9**: 552–569.

ROWE, G., HARRIS, D.J. & BEEBEE, T.J.C. 2006. Lusitania revisited: a phylogeographic analysis of the natterjack toad *Bufo calamita* across its entire biogeographical range. *Molecular Phylogenetics and Evolution* **39**: 335–346.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Press, New York.

SCHLEICH H.H., KÄSTLE, W. & KABISCH, K. 1996. *Amphibians and Reptiles of North Africa.* Koeltz Scientific Books, Koenigstein, Germany.

STÖCK, M., MORITZ, C., HICKERSON, M., FRYNTA, D., DUJSEBAYEVA, T., EREMCHENKO, V., MACEY, J.R., PAPENFUSS, T.J. & WAKE, D.B. 2006. Evolution of mitochondrial relationships and biogeography of Palearctic green toads (*Bufo viridis* subgroup) with insights in their genomic plasticity. *Molecular Phylogenetics and Evolution* **41**: 663–689.

SWOFFORD, D.L. 2003. PAUP* ver 4.0.b10. *Phylogenetic Analysis Using Parsimony and Other Methods.* Sinauer Associates, Sunderland, MA.

VIEITES, D.R., WOLLENBERG, K.C., ANDREANE, F., KOHLER, J., GLAW, F. & VENCES, M. 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Science,* in press.

ZANGARI, F., CIMMARUTA, R. & NASCETTI, G. 2006. Genetic relationships of the western Mediterranean painted frogs based on allozymes and mitochondrial markers: evolutionary and taxonomic inferences (Amphibia, Anura, Discoglossidae). *Biological Journal of the Linnean Society* **87**: 515–536.

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