Phylogeography of the African common toad, *Amietophrynus regularis*, based on mitochondrial DNA sequences: inferences regarding the Cape Verde population and biogeographical patterns

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The amphibian *Amietophrynus regularis* is distributed throughout equatorial Africa, with presumed introduced populations in the Cape Verde archipelago. Portions of the 12S and 16S rRNA mitochondrial regions of 30 specimens from Kenya, Uganda, Niger, Mali, Burkina-Faso, Ghana, Guinea-Bissau and Cape Verde were used to assess genetic diversity and to identify the most probable geographic origin for the introduction of this toad on the latter archipelago. Two lineages with 1.4% genetic divergence between them were identified in western and eastern Africa. All sequences from the different Cape Verde Islands were identical to each other and to the Guinea-Bissau samples, indicating, together with other historical evidences, that an anthropogenic introduction event probably occurred, possibly from Guinea-Bissau, but further work is needed to confirm this. As previously noted, several individuals from previous genetic studies seem to have been misidentified.

Key words: ***Amietophrynus regularis*, '*Bufo*', mitochondrial DNA, 12S and 16S rRNA, Cape Verde Islands.**

INTRODUCTION

Amietophrynus is a large genus of 38 species of true toads native to Africa, with typically 20 chromosomes, with a complex and unresolved taxonomy. Originally, all *Amietophrynus* species groups were part of the genus *Bufo,* but were separated primarily based on molecular analyses by Frost *et al.* 2006 (although see criticisms in Smith & Chiszar (2006); Pauly *et al.* 2009). The African common toad, *Amietophrynus regularis* (Reuss, 1833), also known as the square-marked toad, Egyptian and Reuss's toad, is listed as Least Concern by the IUCN in view of its wide distribution in a broad range of habitats and presumed large populations. It occurs in savannas, shrublands, grasslands, forests, rural gardens, urban areas, rivers and freshwater lakes, from near sea level up to 2500 m high (Tandy *et al.* 2006). Its range appears to be restricted by increasing aridity; in drier areas, away from permanent water, it is replaced by species such as *A. garmani* and *A. xeros* (Tandy *et al.* 2006), which are morphologically similar and sympatric with *A. regularis* to some extent (Tandy *et al.* 2004; Rödel *et al.* 2006).

Amietophrynus regularis ranges from Senegal to Nilotic Egypt, Sudan and Ethiopia, southwards to western Democratic Republic of the Congo, northwestern Angola, Uganda and central-southern Kenya (Fig. 1). It is also present on the Cape Verde Islands where it is thought to have been introduced deliberately in water tanks to control mosquitoes (Schleich 1987). The oldest reference concerning the presence of toads on this archipelago, where no native amphibians occur (Schleich 1987), is from 1844 by Lopes de Lima (*in* Bocage 1896). It is only known to occur in Santiago, S. Nicolau and Santo Antão Islands (López-Jurado *et al.* 2005) (Fig. 1). The introduction of *A. regularis* to the Cape Verde Islands remains unconfirmed and the geographic origin of introduced populations and the number of introduction events undetermined.

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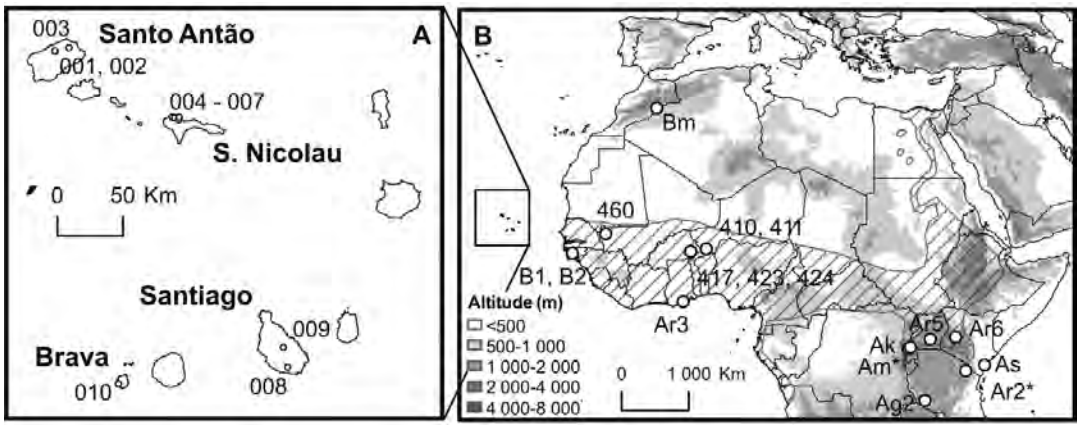

Fig. 1. Locations of the *Amietophrynus* and '*Bufo*' sequences used in this study (**A**) from the Cape Verde Islands and (**B**) North Africa. The distribution of *A. regularis* is indicated in dashed lines (adapted from Tandy *et al.* 2006). For codes with an asterisk (*), refer to the Discussion section.

With such a wide range, it is important to determine genetic diversity within the species, in order to evaluate if geographical structure is present. By sequencing part of the 12S and 16S rRNA mitochondrial region, this work will also increase the amount of data available to infer comparative phylogeographical patterns of African amphibians, as this region was already the focus of studies of '*Bufo*' *mauritanicus* (Harris & Perera 2009), present in the Maghreb, and *A. xeros* (Froufe *et al.* 2009), occurring in the subdesert belt. In addition, some sequences of *Amietophrynus* species from previous publications appear to be misidentifications, as noted by Froufe *et al.* (2009). Thus, a detailed reassessment of *A. regularis* with multiple individuals analysed across its range and including near relatives is needed to establish the recognition of the species group, considered paraphyletic by Frost *et al.* (2006) based on analyses of DNA sequences, and to ascertain if this is due to misidentification, the presence of cryptic species or because of hybridization followed by introgression. Subsequently, the objectives of this study were: 1) to determine genetic diversity across the range of *A. regularis,* 2) to ascertain the origin and number of introduction events on the Cape Verde Islands, and 3) to resolve discrepancies regarding previously published sequences.

MATERIALS & METHODS

Sampling and gathering of molecular data set

A total of 29 sequences of *Amietophrynus* and one of '*Bufo*' *mauritanicus* were used in the analysis (Fig. 1): a) 10 from new specimens collected on the Cape Verde Islands, including one specimen from Brava where this study recorded its presence for the first time; b) eight from new specimens collected in northern African countries; and c) 12 from several African countries available on GenBank (Table 1). Digital photographs of the collected specimens were taken and a piece of toe was removed and stored in 100% ethanol. Sampled animals were released immediately afterwards. Identification codes, localities and all GenBank accession numbers are listed in Table 1.

Total genomic DNA was extracted using standard methods, following Harris (2001). Polymerase chain reaction (PCR) primers used in amplification and sequencing of the two mitochondrial (*mt*DNA) genes were 12Sa and 12Sb for the 12S rRNA, 16SL and 16SH for the 16S rRNA from Kocher *et al.* (1989) and Palumbi *et al.* (1991), respectively. Thermocycling consisted of an initial 3 min at 95°C followed by 35 cycles of 30 s at 95°C, 50°C and 72°C and then a single cycle of 7 min at 72°C. Amplified mitochondrial fragments were sequenced from both strands on a 3100 Applied Biosystems DNA Sequencing Apparatus. Alignment was performed with ClustalW using Bioedit v. 5.0.9. (Hall 1999) and adjusted by hand.

Phylogenetic analyses

Sequences were imported into PAUP* 4.0b10 (Swofford 2003). Four GenBank sequences of *A. kisoloensis, A. gutturallis, A. steindachneri* and '*Bufo*' *mauritanicus* were used as outgroups (Table 1).

Table 1. Details of material and sequences used in the present study. For codes with an asterisk (*), refer to the Discussion section.

Species	Code	Country	Region/Island	Latitude	Longitude	12s/16s Genbank
'*Bufo*' *mauritanicus*	Bm	Morocco	Errachidia Province	–	–	AY680265
A. kisoloensis	Ak	Uganda	Rukungiri District	–	–	AY325995
A. steindachneri	As	Kenya	Arubuko Sokoke forest	–	–	AY325981
A. gutturalis	Ag1	Tanzania	–	–	–	FJ882851
A. gutturalis	Ag2	Tanzania	Mumba Village	–8.15000	31.85100	DQ283436
A. regularis	Ar1*	–	North Africa	–	–	AY680264
A. regularis	Ar2*	Tanzania	Kilimanjaro Region	–3.99979	37.37750	DQ283163
A. maculatus	Am*	Uganda	Rukungiri District	–0.79114	29.92490	U52762/28
A. regularis	Ar3	Ghana	Eastern Region	–	–	DQ158485
A. regularis	Ar4	–	Africa	–	–	AY330899/91
A. regularis	Ar5	Uganda	Kampala	–	–	AF220890/43
A. regularis	Ar6	Kenya	Lake Baringo	0.60923	36.01560	AF220889/43
A. regularis	410	Niger	Tapoua	12.47480	2.42760	HM769984/770002
A. regularis	411	Niger	Tapoua	12.47480	2.42760	HM769985/770003
A. regularis	417	Burkina Faso	Gourma	12.06033	0.36933	HM769986/770004
A. regularis	423	Burkina Faso	Gourma	12.06033	0.36933	HM769987/770005
A. regularis	424	Burkina Faso	Gourma	12.06033	0.36933	HM769988/770006
A. regularis	460	Mali	Kayes	14.50400	–11.09098	HM769989/770007
A. regularis	B1	Guinea-Bissau	Bissau	11.86031	–15.57870	HM769990/770008
A. regularis	B2	Guinea-Bissau	Bissau	11.86031	–15.57870	HM769991/770009
A. regularis	001	Cape Verde	S. Antão	17.11363	–25.16835	HM769992/770010
A. regularis	002	Cape Verde	S. Antão	17.11363	–25.16835	HM769993/770011
A. regularis	003	Cape Verde	S. Antão	17.13884	–25.07343	HM769994/770012
A. regularis	004	Cape Verde	S. Nicolau	16.66314	–24.36332	HM769995/770013
A. regularis	005	Cape Verde	S. Nicolau	16.65855	–24.34591	HM769996/770014
A. regularis	006	Cape Verde	S. Nicolau	16.64485	–24.32094	HM769997/770015
A. regularis	007	Cape Verde	S. Nicolau	16.66047	–24.31520	HM769998/770016
A. regularis	008	Cape Verde	Santiago	14.94691	–23.57285	HM769999/770017
A. regularis	009	Cape Verde	Santiago	15.08491	–23.60028	HM770000/770018
A. regularis	010	Cape Verde	Brava	14.83216	–24.73434	HM770001/770019

For the phylogenetic analyses, maximum likelihood (ML) and maximum parsimony (MP) methods were used with random sequence addition (100 replicate heuristic searches). Support for nodes was estimated through bootstrap techniques (Felsenstein 1985) with 1000 replicates. Modeltest 3.06 (Posada & Crandall 1998) was used to select the model of sequence evolution that fit the data set better using the Akaike information criterion (AIC). This model was implemented in the ML and Bayesian analyses. The Bayesian analysis was carried out using MrBayes v. 3.1 (Huelsenbeck & Ronquist 2001), model parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. The analysis was run for 10^7 generations, saving one tree each 1000 generations. Twenty-five per cent of the saved trees were discarded as burn-in. It was confirmed that all parameters had ESSs above 100 after burn in using the software Tracer v1.5 (Rambaut & Drummond 2007). The remaining trees (7500) were combined in a 50% majority consensus tree, in which the frequency of any particular clade represents its posterior probability (Huelsenbeck & Ronquist 2001).

Network and population analysis

The genealogical relationships within the 23 sequences of *A. regularis* were assessed with haplotype networks constructed using statistical parsimony (Templeton *et al.* 1992). This analysis was implemented in the program TCS v1.21 (Clement *et al.* 2000) with a connection limit of 95% and deletions treated as a fifth state. Other sequences available on GenBank only for 12S or 16S were not included in this analysis: AY028486

from Ghana (Pramuk *et al.* 2001) and GQ183570 from Uganda, Rwenzori Mountains, Bundibuyo (Siow *et al.,* in press), respectively.

Haplotype (Hd) and nucleotide diversity (π) values, number of haplotypes (h) and segregating sites (S) were calculated with DnaSP v.4 (Rozas *et al.* 2003). Estimates of average evolutionary divergence over sequence pairs within and between groups, using *p*-distances (*p*-dist), were calculated based on the number of base differences per site from averaging over all sequence pairs within each group and estimation of net average between groups of sequences, respectively. Analyses were conducted in MEGA 4.0.2 (Tamura *et al.* 2007). Standard error estimates were obtained by a bootstrap procedure (1000 replicates).

RESULTS

In total, including outgroups, 30 individuals were analysed with the combined data set including 904 bp (379 bp from 12S and 525 bp from 16S rRNA), of which 106 positions were variable and 73 parsimony-informative (27 and 19 for 12S and 79 and 54 for 16S rRNA, respectively).

The general time reversible model (GTR), with an estimate of invariable sites (I = 0.7795), was the most appropriate model of evolution for this dataset. A single tree (-ln = 2192.36412) was recovered from the ML analysis. Two MP trees were recovered (191 steps), the consensus of which differed from the ML tree only in some minor arrangements of taxa or individual samples. The Bayesian analysis recovered the same tree as the ML analysis. The results of the MP, ML and Bayesian analyses of the combined 12S+16S rRNA data are shown in Fig. 2 and indicate that two lineages, western and eastern, exist within *A. regularis.* The genetic distance between these lineages is 1.4 ± 0.4% (Table 2). In addition, two sequences identified as *A. regularis* in previous studies, Ar1* and Ar2* (with GenBank codes AY680264 and DQ283163, respectively) cluster with *A. kisoloensis* and *A. gutturalis,* respectively, whereas one sequence assigned to *A. maculatus* (Am*, U52728/62) groups within the eastern lineage of *A. regularis* (Fig. 2).

According to the network analyses, the western and eastern *A. regularis* lineages are 13 mutational steps apart (Fig. 3). In the western lineage, all sequences from the four Cape Verde Islands are identical to each other and to the Guinea-Bissau sequences. By contrast, sequences from individuals from Mali, Ghana, Burkina Faso and Niger are between one and three mutational steps apart from those (Fig. 3). After preliminary analysis, the sample Ar4 (AY330899/91) of an unknown locality (Table 1) was assumed to belong to the eastern lineage due to its affinities with this clade. As a result, in the eastern lineage, four closely connected haplotypes were recovered (Fig. 3).

DISCUSSION

The phylogenetic analyses showed that two distinct mitochondrial lineages of *A. regularis* appear to exist, one in the western and another in the eastern part of Africa with a genetic divergence of 1.4% (1.5 % based only on 16S rRNA). Divergence levels between these western and eastern lineages could be explained by isolation through geographic distance alone or together with geographical barriers such as high mountains (Fig. 1). Few anuran phylogeographic studies from northern Africa exist and the ones using the same molecular markers, the Maghrebian *B. mauritanicus* and the sub-Saharan *A. xeros,* recovered very different patterns. The former presents minimal genetic variation within its range (Harris & Perera 2009) and the latter exhibits maximal divergence between samples from the same country (Froufe *et al.* 2009). However, an assessment of variation in the rodent *Mastomys erythroleucus* with the same geographical range as *A. regularis* recovered a similar pattern to the one presented here, although with two additional central African lineages (Brouat *et al.* 2009). Thus, further sampling of *A. regularis* in Central Africa would be needed to define the ranges of the eastern and western lineages and to assess if additional lineages could be uncovered. Furthermore, it would be useful to sample isolated populations in the Sahara, such as in the Hoggar mountains of southern Algeria and in the oasis of southwestern Libya (Schleich *et al.* 1996), to confirm its presence and determine if more variation exists.

Considering the Cape Verde Islands, it seems that this species is indeed introduced there and that a single introduction event occurred, as all individuals from the four islands where the species occurs have the same mitochondrial haplotype. Nevertheless, a scenario of multiple colonizations from the same source population is also possible and further SNPs or microsatellites analyses would be needed to distinguish with certainty between the two scenarios. The network analysis indicates Guinea-Bissau as the most likely source of the introduction of *A. regularis* on the Cape Verde Islands, given that samples from both

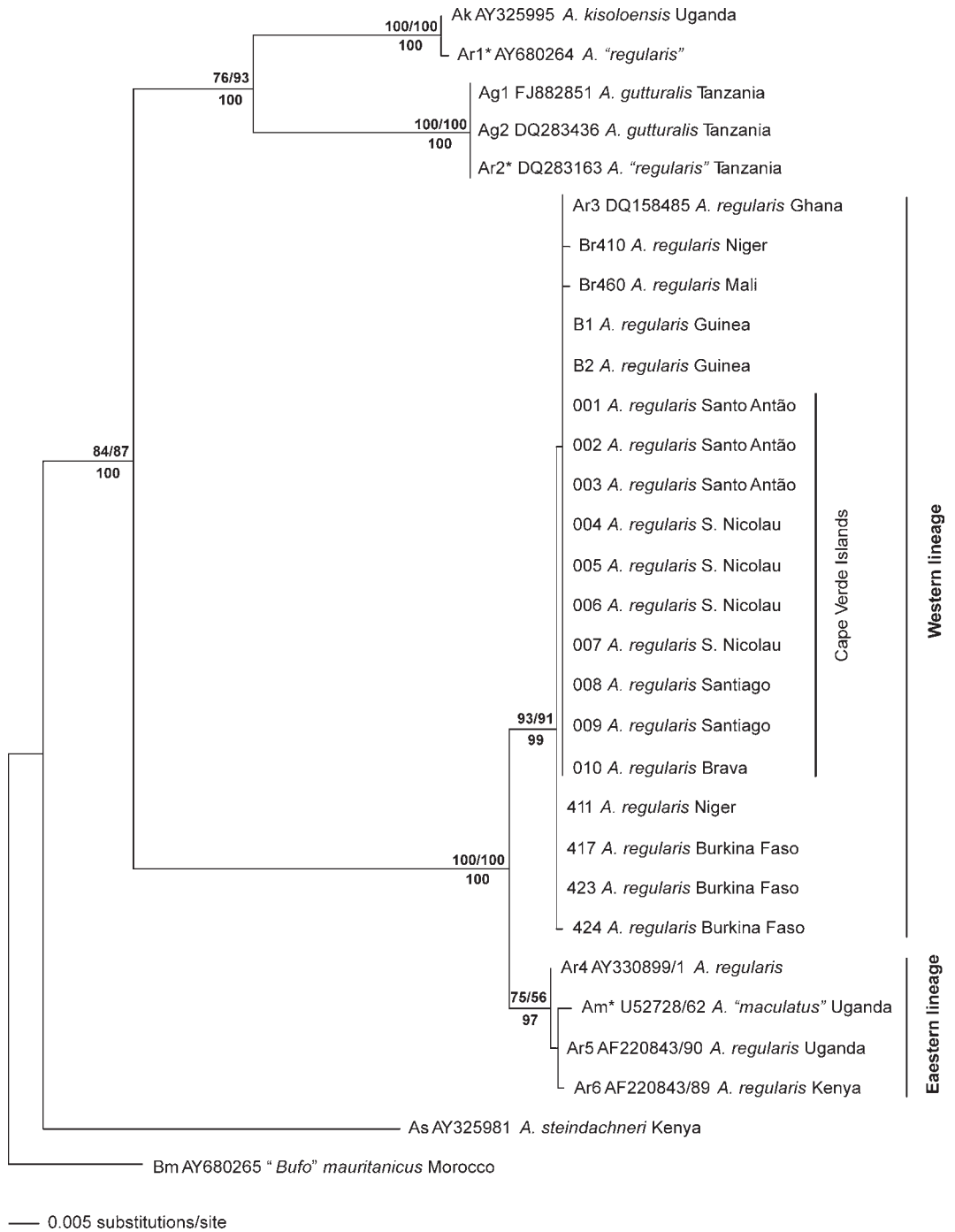

Fig. 2. Maximum likelihood (ML) tree inferred using the GTR+I model of sequence evolution showing relationships of *A. regularis* from different origins. The tree is rooted using '*Bufo*' *mauritanicus*. Bootstrap support values above 50% for the MP and ML analysis are shown above nodes, respectively, and posterior probability values for the Bayesian analysis below nodes (see Materials & Methods). Sequences downloaded from GenBank are shown in the figure with their respective GenBank accession numbers for the 16S and 12S rRNA genes separated by a slash if the accession numbers differ. For locality data and GenBank accession numbers of the new (ranging from 0000 to 0000) and previously published sequences see Table 1.

Table 2. Mitochondrial 12S and 16S diversity of the western and eastern lineages of *Amietophrynus regularis*. *n*, sample size; π, nucleotide diversity; Hd, haplotype diversity; h, number of haplotypes; S, segregating sites; evolutionary divergence within and between groups (*p*-dist ± standard error).

Lineage	*n*	π	h	Hd	S	*p*-dist	*p*-dist
Western	19	0.00095	7	0.574	4	0.2 ± 0.1%	1.4 ± 0.4%
Eastern	4	0.00194	4	1.000	3	0.1 ± 0.1%	

regions share the same haplotype. This seems feasible as the Portuguese made regular trips during the 16th and 17th centuries from Guinea-Bissau to the previously uninhabited archipelago for colonizing it with a slave workforce and as a strategic stopping point of the slavery trade route between Guinea-Bissau and the American continent (Silva 1995). However, as samples from other West African areas are lacking and other sequences from different origins are only one to three mutational

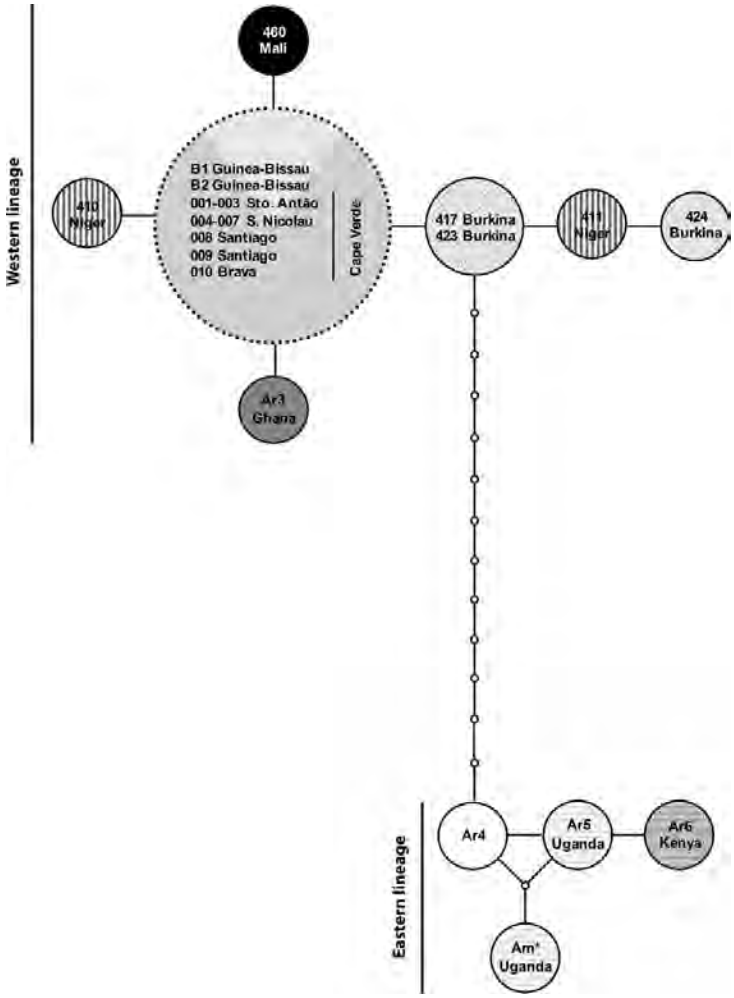

Fig. 3. Parsimony network corresponding to the 12S and 16S rRNA sequence variation in *Amietophrynus regularis*. Lines represent a mutational step, circles haplotypes and dots missing haplotypes. The size of the circle is proportional to the number of individuals. The dotted circle represents the probable ancestral haplotype. Samples from the same country are indicated using the same pattern or grey scale. For correspondences of sample and location codes see Table 1. For codes with an asterisk (*), refer to the Discussion section.

steps away from the Cape Verde sequences, an alternative origin for *A. regularis* found on the Cape Verde Islands cannot be ruled out.

Since no other native amphibian exists on the islands, the presence of *A. regularis* probably does not raise any direct conservation issues, contrary to other accidentally introduced herpetofauna occurring on the Cape Verdes, such as *Hemidactylus angulatus, H. mabouia* (Arnold *et al.* 2008) and *Agama agama* (Vasconcelos *et al.* 2009). However, the impact that *A. regularis* might have on native invertebrates or indirectly on the vertebrate community dynamics is unknown. It is known to be abundant in Santiago, S. Nicolau and S. Antão (Hazevoet 1995) and it was considered invasive by López-Jurado *et al.* (2005) on the archipelago. This study reports it for the first time on Brava. Further studies are clearly warranted to assess its impact on this insular ecosystem.

Finally, as previously noted by Froufe *et al.* (2009), the Ar1* (AY680264; Pauly *et al.* 2004) and Am* sequences (U52728/62; Graybeal 1997) are probably morphological misidentifications rather than introgression, sequencing errors, contaminations or amplification of nuclear copies of the *mt*DNA. An additional sequence of *A. 'regularis'*, Ar2* (DQ283163; Frost *et al.* 2006), appears identical to *A. gutturalis* specimens sequenced by Frost *et al.* (2006) and Van Bocxlaer *et al.* (2009). This study again emphasizes the importance of using multiple individuals of the same species in phylogenetic analyses and of including sequences from GenBank with caution. Additionally, as some *Amietophrynus* are morphologically similar, with some individuals presenting ambiguous morphological characters used in identification keys (pers. obs.), future work with nuclear genes should be done to confirm the estimates of relationships based on *mt*DNA sequence data. Also additional morphological studies should be implemented in order to try to find clearly diagnostic characters.

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