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## Integrating mtDNA analyses and ecological niche modelling to infer the evolutionary history of *Alytes maurus* (Amphibia; Alytidae) from Morocco

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We aimed at determining the effects of past climatic conditions on contemporary intraspecific genetic structuring of the endemic Moroccan midwife toad *Alytes maurus* using mitochondrial DNA (12S, 16S and cytochrome *b*) analysis and ecological niche modelling. Unexpectedly, our genetic analyses show that *A. maurus* presents a low level of variability in the mitochondrial genes with no clear geographical structuring. The low genetic variation in mtDNA can be explained by a much broader climatic suitability during the Last Glacial Maximum that allowed the connection among populations and subsequent homogenization as a consequence of gene flow.

**Key words:** biogeography, haplotype network, Köppen-Geiger, Maghreb, Maxent, midwife toad, Pleistocene glaciations

## INTRODUCTION

The Moroccan midwife toad (*Alytes maurus* Pasteur & Bons, 1962), endemic to northern Morocco, is the only African representative of the family Alytidae which includes only four other species: *Alytes obstetricans* Laurenti 1768 distributed across western Germany, the Netherlands, northern Switzerland, southern Belgium, Luxembourg, France and the northern half of the Iberian Peninsula; *Alytes muletensis* Sanchiz & Adrover 1979 restricted to few populations in the Serra da Tramuntana in Mallorca; *Alytes dickhilleni* Arntzen & García-París 1995 restricted to the mountainous massifs of southeastern Spain and *Alytes cisternasii* Boscá 1879 distributed across the central and southwestern Iberian Peninsula.

*Alytes maurus* was considered under the nomen *A. obstetricans* before it was described as a separate subspecies (*A. o. maurus*) by Pasteur & Bons (1962) based on divergent tadpole morphology when compared to European populations. Subsequently, the species received little scientific attention (but see e.g., Arntzen & Szymura, 1984; Libis, 1985) until the almost simultaneous publications of Bons & Geniez (1996), Schleich et al. (1996) and Salvador (1996) provided reviews of the species' geographic distribution and natural history. More recently, Donaire-Barroso & Bogaerts (2003) proposed to elevate the Moroccan midwife toad to the species level and, together with Donaire-Barroso et al. (2006), provided a substantial increase in ecological

knowledge and geographic distribution. The specific status of *A. maurus* was later confirmed by osteological, mitochondrial DNA and nuclear DNA evidence (Fromhage et al., 2004; Martínez-Solano et al., 2004; Gonçalves et al., 2007). Phylogenetic analyses of the genus *Alytes* suggest that *A. maurus*, *A. dickhilleni* and *A. muletensis*, which together compose the subgenus *Baleaphryne*, form a clade, although weakly supported by mtDNA data (Fromhage et al., 2004; Martínez-Solano et al., 2004). The results also suggest that the three species split almost simultaneously, most probably as a result of the collapse of the Gibraltar land bridge at the end of the Messinian Salinity Crisis (MSC), in the late Pliocene around 5.3 mya, which led to diversification of the *Baleaphryne*, isolating *A. maurus* on the African continent, *A. dickhilleni* in southeast Iberia and *A. muletensis* in the Balearic Islands (Martínez-Solano et al., 2004).

At present, *A. maurus* is known only from about twenty fragmented localities (200–2,050 m a.s.l.) in the Rif and Middle Atlas Mountains in northern Morocco (see Fig. 1) and it has been assessed as Near Threatened (NT) according to the IUCN Red List of Threatened Species (Donaire-Barroso et al., 2009). The species is associated with humid sites in areas with montane karst, boulders and escarpments. Adult specimens live near permanent streams, pools and other waterbodies and generally inhabit fissures and cracks in rocks, as well as under stones on clay or humus-rich soils. The

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**Fig. 1.** The study area in northern Morocco showing the specimens included in the molecular analyses (Am-01–013, white dots), all known distribution records (black triangles) as well as the distribution range according to the IUCN Red List assessment (white lines). The main locality names and the Moulouya river basin are indicated. Areas above 800 m a.s.l. are indicated in darker grey colour.

typical surrounding vegetation may consist of oak and Atlas cedar forests, scrubs and orchards. Although the species can be locally common, future threats include the destruction and fragmentation of habitat and the domestic pollution of waterbodies (see Donaire-Barroso et al., 2009; Schleich et al., 1996). Furthermore, the species may also be threatened by climate change (PdP, unpublished data) and the recently detected chytrid fungus (*Batrachochytrium dendrobatidis*) in its vicinity (El Mouden et al., 2011).

In recent years, there has been a substantial increase in the knowledge of the evolutionary biology of the genus *Alytes*, both at the interspecific and intraspecific level. Fromhage et al. (2004) and Martínez-Solano et al. (2004) used mtDNA data to provide biogeographic scenarios for the evolutionary history of the genus, while the latter also used osteological data to propose a phylogenetic hypothesis. Gonçalves et al. (2007) used both mtDNA and nuclear DNA markers to infer phylogenetic relationships in *Alytes* and to assess the possible role of recent reticulation between deeply divergent lineages in the evolutionary history of the genus. More recently, Gonçalves et al. (2009) provided a detailed study on the intraspecific genetic variation of *A. cisternasi* and reported high population subdivision resulting from multiple refugia during Pleistocene glaciations. Finally, Pinho et al. (2010) and Agata et al. (2011) developed important new nuclear and microsatellite loci for *Alytes*. The species of the genus *Alytes* therefore constitute an

interesting group for studying the effects of Pleistocene climatic oscillations. However, the intraspecific genetic variation of the Moroccan endemic *A. maurus* has never been investigated, while an interesting pattern of genetic differentiation between the Rif and Middle Atlas populations, as observed in *Salamandra algira* (e.g., Beukema et al., 2010), could be expected.

In this paper we use a modern framework of combining genetic data with ecological niche modelling (e.g., Waltari et al., 2007) to make inferences on the evolutionary history of *A. maurus* in Morocco. We specifically aim for the first time to assess the intraspecific genetic variation of *A. maurus* through mtDNA sequences, and to evaluate the potential distribution of the species in Morocco under present and past (Last Glacial Maximum) climatic conditions.

## MATERIALS AND METHODS

A total of 13 specimens of *A. maurus* covering most of its distribution range were included in the molecular analyses. Nucleotide sequences of three specimens were downloaded from GenBank. Specimen data and GenBank accession numbers of the gene fragments sequenced are listed in Table 1 and a map with all the localities of *A. maurus* included in the molecular study is shown in Fig. 1. Three mitochondrial gene fragments were sequenced: cytochrome *b* (cytb) (281 bp), 12S rRNA (12S) (298 bp) and 16S rRNA (16S) (495 bp).

**Table 1.** Codes, geographic localities (WGS 1984), mtDNA haplotypes and GenBank accession numbers in parentheses of all specimens of Moroccan *Alytes maurus* used in this study. The localities are indicated in Fig. 1.

Map	Specimen Code	Locality	Latitude	Longitude	12S	16S	cytb
Am-01	SPM000759	South of Tetouan	35,53889	-5,38639	H1 (AY333673)	H3 (KF145143)	H1 (KF145147)
Am-02	SPM000309	Chefchaouen	35,16556	-5,26138	H1 (AY333673)	-	H1 (KF145147)
Am-03	SPM000323	Chefchaouen	35,16556	-5,26138	H1 (AY333673)	H3 (KF145143)	H1 (KF145147)
Am-04	SPM001757	Chefchaouen	35,16556	-5,26138	H1 (AY333673)	H3 (KF145143)	H1 (KF145147)
Am-05	SPM002112	North Jebel Kelti	35,38214	-5,28326	H1 (AY333673)	H4 (KF145144)	H2 (KF145145)
Am-06	SPM002145(34)	North Jebel Kelti	35,38214	-5,28326	H1 (AY333673)	H3 (KF145143)	H1 (KF145147)
Am-07	SPM004484	Stream near Talassemthane	35,17614	-5,13647	H1 (AY333673)	-	H3 (KF145146)
Am-08	SPM004485	Stream near Talassemthane	35,17614	-5,13647	H1 (AY333673)	-	H3 (KF145146)
Am-09	SPM004914	Taza	34,15018	-4,00657	H1 (AY333673)	-	H1 (KF145147)
Am-10	SPM004915	Bou-Iblane	33,67165	-4,12203	H1 (AY333673)	-	H1 (KF145147)
Am-11	SPM003892	Northwest of Anasar	35,02333	-5,01530	H1 (AY333673)	-	H3 (KF145146)
Am-12	Aom01	Rif Mountains			H1 (AY333673)	H1 (AY333711)	-
Am-13	MNCN20917	Ketama	34,91263	-4,56969	-	H1 (AY442030)	H4 (AY442022)
Am-14	MNCN40768	Chefchaouen	35,16556	-5,26138	-	H1 (AY442029)	H1 (AY442021)

Primers used in both amplification and sequencing were: cb1 (5'-CCATCCAACATCTCAGCATGATGAAA-3') and cb2 (5'-CCCTCAGAACATGATATTGTCTCA-3'), both shortened at 5' from original primers L14841 and H15149 (Kocher et al., 1989), respectively, for the cytb gene; 12Sa (5'-AAACTGGGATTAGATACCCCACTAT-3') and 12Sb (5'-GAGGGTGACGGCGGTGTGT-3'), both shortened at 5' from original primers L1091 and H1478 (Kocher et al., 1989), respectively, for the 12S gene; and 16Sar-5' and 16Sbr-3' (Palumbi, 1996) for the 16S gene. PCR cycling conditions for all three gene fragments used were as follows: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute, ending with 5 minutes of final extension at 72°C. Geneious v.5.3 (Drummond et al., 2010) was used for contig assembly, visualization of sequences and as a platform for exporting into different formats. Sequences were aligned using MAFFT v.6 (Katoh et al., 2002) available online, and applying default parameters (gap opening penalty=1.53, gap extension=0.0). A median-joining haplotype network for the separate 12S, 16S and cytb gene fragments was constructed using Fluxus Phylogenetic Network Analysis software v.4.6.0.0. (Bandelt et al., 1999) and is presented in Fig. 2.

A total of 19 BioClim variables were downloaded from the WorldClim database v.1.4 (<http://www.worldclim.org/>) to form the present and past (Last Glacial Maximum; LGM) climatic datasets (Hijmans et al., 2005) at a scale of 2.5 arc minutes (nearly 5 × 5 km). Two general atmospheric circulation models (GCM) were used to generate past climate scenarios for each period: the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The two GCMs were assembled using ArcGIS v.10 (ESRI). Collinearity of the initial variables was measured with Pearson's correlation coefficient in ENMtools v.1.3 (Warren et al., 2010). A total of eight variables, all of which had a correlation degree lower than 0.75 (Pearson coefficient) were retained. Selection of predictor

variables was based on ecological understanding of the species (e.g., rainfall in the breeding season). The final set of environmental predictor variables used for ecological niche modelling (ENM) consisted of: Temperature Seasonality (BIO4), Max Temperature of Warmest Month (BIO5), Mean Temperature of Wettest Quarter (BIO8), Mean Temperature of Driest Quarter (BIO9), Annual Precipitation (BIO12), Precipitation Seasonality (BIO15) and Precipitation of Warmest Quarter (BIO18).

A total of 37 distribution records were assembled from both literature and fieldwork. The distribution records were filtered to remove duplicate records within unique grid cells in ENMtools v.1.3 (Warren et al., 2010). After filtering, a total of 26 distribution records were used for ENM (Fig. 3).

The ecological niche models were generated by the presence/background algorithm Maxent, v.3.3.3k (Phillips et al., 2006). Maxent was used with default settings (Convergence threshold=0.00001, maximum number of iterations=500 and  $\beta=1$ ) while partitioning the geographical records between training and test samples (default settings). Several studies have recently addressed the importance of selecting pseudo-absence or background locations in ENM (e.g., VanDerWal et al., 2009). We therefore followed the background approach of Webber et al. (2011) and Thompson et al. (2011) and downloaded Köppen–Geiger polygons from the CliMond database (Kriticos et al., 2011; [www.climond.org](http://www.climond.org)). Subsequently, models were projected onto a larger area (Fig. 3).

The average of ten pseudo-replicated models with randomly selected test samples was used to produce ENMs, which were plotted in logistic format. The final models were reclassified in ArcGIS v.10 (ESRI) into binary presence-absence maps based on two different thresholds: (i) following the assumption that ten percent of the records were either wrongly identified or georeferenced (Raes et al., 2009), the average ten percentile threshold (TPT) was used, meaning, the 10%

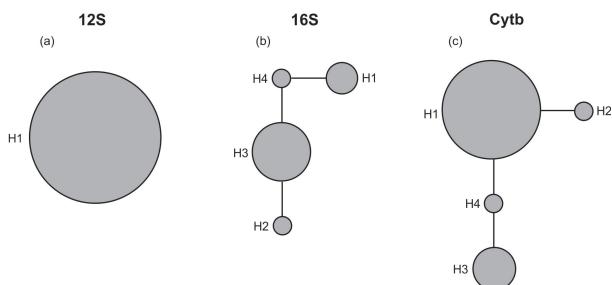
of model outputs with the lowest predicted probabilities fall into the ‘absence’ region of the thresholded model, and ‘presence’ regions include the 90% of distribution records with the highest model values, and (ii) the average lowest presence thresholds (LPT=minimum training presence threshold of Maxent software), guaranteeing that all presences are predicted as suitable (Pearson et al., 2007).

All models were tested with receiver operating characteristics (ROC) curve plots and the area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model. A comparison of the environmental variables used for projection with those used for training the model were made using visual interpretation of multivariate similarity surface pictures and the most dissimilar variable (Elith et al., 2010).

## RESULTS AND DISCUSSION

Both 16S and cytb mitochondrial gene fragments have three variable positions, and haplotype diversity is estimated at 0.75 and 0.60, respectively. The results of the network analyses are shown in Fig. 2 and indicate that among the sampled individuals, depending on the marker, up to four haplotypes are found, all interconnected with single-mutation steps, while they lack clear geographic structure. In accordance with the low level of genetic variability detected in the 16S and cytb genes, all 12 sequences of the 12S gene fragment included in this study were identical and therefore a single haplotype was detected (Table 1).

As shown in Fig. 3, Maxent produced models of high predictive accuracy, according to the average test AUC for the present ( $0.947 \pm 0.040$ ) and past (LGM) models ( $0.943 \pm 0.040$ ). The present ENM (LPT threshold; Fig. 3B) of *A. maurus* reveals a relatively large potential distribution in both south and eastward directions, with suitable areas in the Middle Atlas region (including Ifrane National Park), the area between Agadir and Marrakech and more southwards, as well as in large parts of northeast Morocco (e.g., the Beni Snassen Massif and Debou) and in northwestern Algeria (e.g., Tlemcen region). The models under the TPT reveal a more realistic potential distribution with less overprediction (Fig. 3A). The current ENM (both thresholds; Fig. 3A, B) shows a



**Fig. 2.** Median-joining network inferred for three separate mtDNA fragments (a=12S, b=16S, c=Cytb). Grey circles represent different haplotypes and have been drawn proportional to the frequency of each haplotype. Information on the samples included is shown in Fig. 1 and Table 1.

barrier of unsuitable climate fragmenting the populations of *A. maurus* from the Rif and Middle Atlas Mountains, whereas another barrier exists between the Middle Atlas and the Debdou Massif in the east.

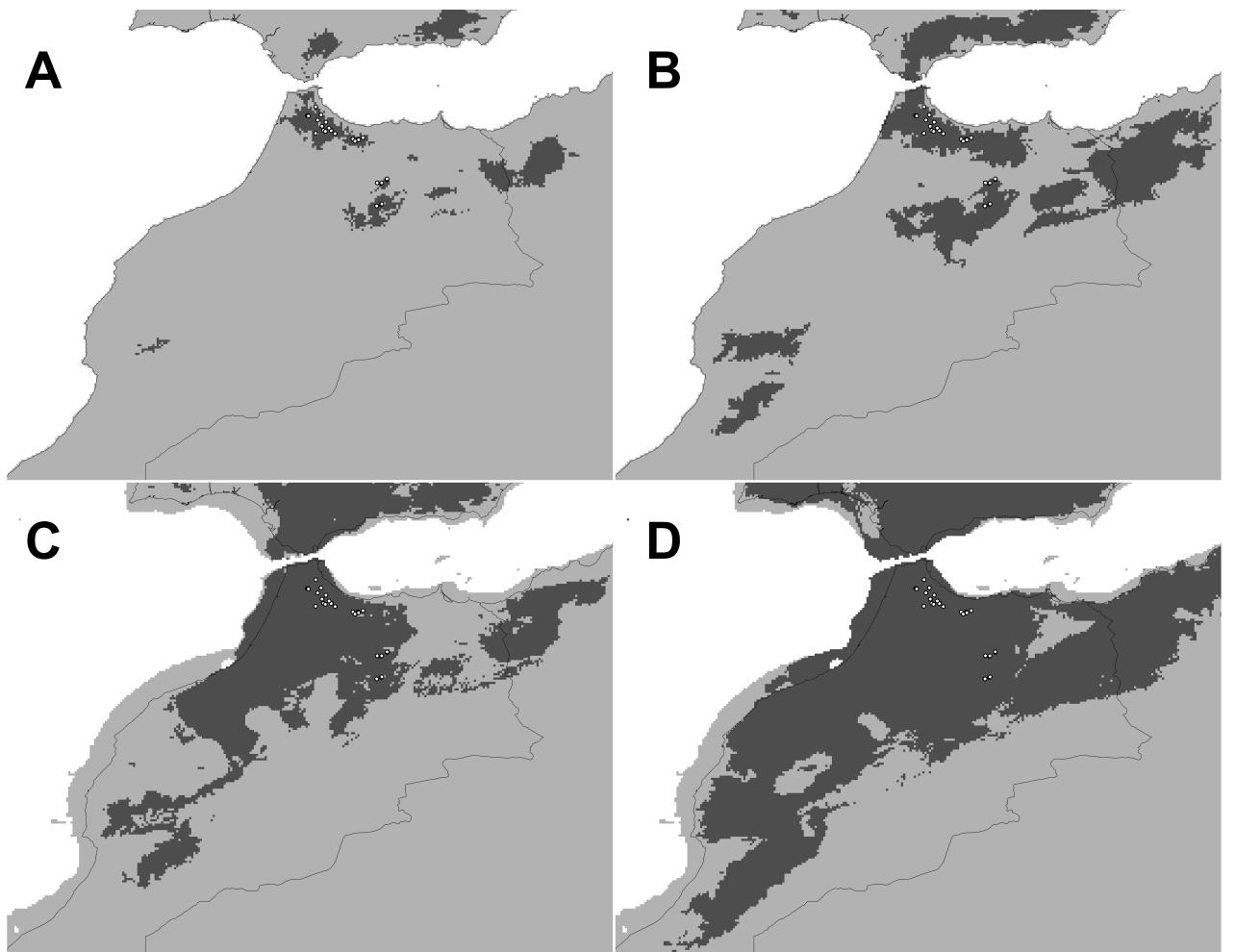
The LGM model under the LPT (Fig. 3D) shows an increase in comparison to the present potential distribution with a continuous and unfragmented suitable area towards most parts of central, southwest and northeast Morocco, and northwest Algeria. The TPT map (Fig. 3C), however, reveals that the Moulouya River basin remained a barrier between the Middle Atlas and the northeastern region.

Clamped areas can be identified in most parts of the Rif, Middle and High Atlas Mountains, and also MESS and MoD pictures reveal that these regions have a non-analogue climate. Therefore, the predictions in these regions should be treated with caution (unpublished results, available upon request).

The result of the mtDNA analysis is unexpected, especially considering that many other Moroccan herpetofauna species show much higher levels of genetic variation, which may often indicate the presence of cryptic species or species complexes (e.g., Pinho et al., 2008; Rato & Harris, 2008; Perera & Harris, 2010). On the other hand, several species of North African amphibians (e.g., Stöck et al., 2008; Harris & Perera, 2009) also present low levels of genetic variability, which suggests that the amphibians in this region have higher vagility than expected or benefited from optimal climatic periods in the past such as the LGM. The intraspecific genetic variation of the other Moroccan endemic species such as *Pelobates varaldii*, *Discoglossus scovazzi* and *Barbarophryne broningersmai* is currently under study (PdP, pers. data) and the existence of genetic structuring in these species remains to be tested.

The low level of genetic variability detected in *A. maurus* is likely to be the result of a much broader climatic suitability during the LGM that allowed for the connection among populations and subsequent gene flow. The mtDNA network reveals that the geographically most distant specimens (Tetouan and Boublane) share the same haplotype for both the 12S and cytb genes. The increased potential distribution of *A. maurus* during the LGM is a result of wetter and cooler annual climatic conditions in North Africa (Rognon, 1987; Wengler & Vernet, 1992), whereas expanded forest cover (e.g., Lubell, 2001) and an increase in shrubby vegetation (e.g., Fletcher & Sánchez-Goñi, 2008) might have also facilitated the species’ dispersal and subsequent gene flow, as was also reported in a recent study (de Pous et al., 2011a). Populations in the Rif and Middle Atlas Mountains, that show isolation under current climatic conditions, could migrate north and south and were not affected by the present unsuitable barrier between these populations.

The present potential distribution of *A. maurus*, as predicted by the ENM, is larger than the currently known species range under both thresholds. The Moroccan herpetofauna has been the subject of investigation by many researchers in the last decades, resulting in a multitude of new distribution records, often extending



**Fig. 3.** Potential distribution models of *Alytes maurus* for the present (A and B) and Last Glacial Maximum (C and D) based on the TPT (A and C) and LPT (B and D) thresholds. The 26 records with precise locality data used for the ecological niche modelling (ENM) are also indicated (white dots).

the distribution ranges of species significantly (e.g., Bons & Geniez, 1996; Barata et al., 2011). The possible presence of the species in other well communicated and hence explored parts of the Middle Atlas region (e.g., Azrou and Ifrane) therefore seems unrealistic, although the species might occur in remote areas more southwards of Bou Iblane. *Alytes maurus* is typically associated with montane karst and escarpment areas in forests or shrubby vegetated areas at an altitude ranging from 200–2050 m a.s.l. The distribution of these environmental conditions might well limit the distribution of the species as seen in *Salamandra algira* (Beukema et al., 2010), which presents a similar distribution pattern. The populations of *S. algira* from the Rif and Middle Atlas Mountains have, however, diverged approximately 0.7 mya and also show phenotypic changes likely as a result of different ecological conditions (Beukema et al., 2010).

The LGM model of *A. maurus* reveals a much wider potential distribution that continuously extends to the south and southwest in Morocco and under the LPT into northeast Algeria. The species has never been recorded outside the Rif Mountains and the Middle Atlas region, although it might have had a much larger distribution in the past. Hossini (2001) described the fossil of a member of the genus *Baleaphryne* from the Lower Pleistocene that was found in a quarry at Jebel Irhoud between Marrakech

and Safi. This fossil is likely attributable to *A. maurus* but might as well be an extinct species of *Alytes*, although this seems unlikely. The presence of a *Baleaphryne* sp. in southern Morocco during the Lower Pleistocene coincides more or less with the beginning of a period of documented cyclic fluctuations in vegetation and climate in north-western Africa, which occurred between 3.7 and 1.7 mya (Leroy & Dupont, 1994). During this time the vegetation fluctuated between humid phases that were characterized by tropical forests, and drier phases, characterized by grasslands (Thompson & Fleming, 1996). The shift towards a colder and drier climate that occurred during the upper Pliocene (2.5–1.8 mya; Webb & Bartlein, 1992) may have resulted in the extinction of the species in this region. There are currently no known fossils from the late Pleistocene or Holocene known from Morocco. The continued presence of *A. maurus* in a wider area therefore remains speculative even though optimal climatic conditions oscillated but remained present until at least the start of the cold and dry Younger Dryas (e.g., Lubell, 2001).

At present, *A. maurus* remains a relatively elusive and understudied species, despite the realistic threats that occur in the form of habitat fragmentation and destruction, pollution, climatic change and the globally spreading chytrid fungus. Furthermore, de Pous et al.

(2011b) found that only 6.96% of the total distribution range is covered by the existing protected area network. The use of additional genetic markers such as nuclear loci (Pinho et al., 2010) or microsatellites (Agata et al., 2011) to study the effects of population fragmentation on this species would therefore be advisable. Furthermore, the application of forecast ENM to assess the effects of climate change on the distribution range and wide-range sampling to detect the presence of *B. dendrobatis* would be important steps to ensure the species' survival over time.

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