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Fatal chytridiomycosis in the Moroccan midwife toad *Alytes maurus* and potential distribution of *Batrachochytrium dendrobatidis* across Morocco

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ABSTRACT

Multiple threats, including emerging infectious diseases, are contributing to the extinction of amphibians worldwide. One of the most devastating diseases is the fatal amphibian skin disease chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). The presence of *Bd* in North Africa was described in 2011 and this included the distribution range of the endemic Moroccan midwife toad (*Alytes maurus*). Here we report new *Bd* positive occurrences across several distant regions of Morocco, augment the known number of infected species, and describe the first evidence of lethal chytridiomycosis in *A. maurus*. Although population declines in this species were not recorded, the family Alytidae has been identified as the most susceptible taxonomic group to chytridiomycosis of the Palearctic. An environmental niche model, taking into account new records of *Bd* in the country, confirms the Mediterranean coast and the Rif and Middle Atlas Mountains as very favourable areas for *Bd*. Our results suggest that the real impact of chytridiomycosis in North Africa is poorly understood, and that this continent cannot be identified as a region less impacted by chytridiomycosis, as was previously proposed.

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Introduction

Amphibians are the most endangered group of vertebrates (Stuart et al. 2004). On the IUCN Red List of Threatened Species, 41% of the known amphibian species are listed as threatened (IUCN 2019) and, accordingly, most monitored populations exhibit a decreasing trend (e.g. Houlahan et al. 2000; Baillie et al. 2010). One of the biggest threats facing amphibians is the emergence of the fatal infectious disease chytridiomycosis (Lips et al. 2006). This disease is associated with disruptions on the amphibian skin that lead to cardiac arrest and the death of the animal (Voyles et al. 2009). The causative agent, the fungus belonging to the phylum of Chytridiomycota, *Batrachochytrium dendrobatidis*

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(hereafter *Bd*; Berger et al. 1998; Longcore et al. 1999), has been recorded in more than 700 amphibian species, leading to population declines and/or extinctions of at least 500 species across the entire world (Scheele et al. 2019).

The distribution of *Bd* across Africa and the susceptibility of native species are poorly understood (Scheele et al. 2019; Zimkus et al. 2020), and consequently the impact of *Bd* in the African amphibian community could be severely underestimated, as it has pointed out recently by Doherty-Bone et al. (2020). In fact, recently Weldon et al. (2020) have shown that chytridiomycosis was the proximate cause of extinction in the wild of *Nectophrynoides asperginis*, despite population monitoring and conservation management in Tanzania. In North Africa, the occurrence of *Bd* was first reported in 2011 at three sites of northern Morocco (El Mouden et al. 2011), very close to the south of Spain where the pathogen is widely distributed (Bosch unpublished data). To date, six of the 14 known amphibian species from Morocco have tested positive for *Bd* (El Mouden et al. 2011; El Cadi et al. 2019). Penner et al. (2013) and Zimkus et al. (2020) produced fine-scale environmental niche models of *Bd* for the entire African continent. Both studies predicted a narrow region suitable for *Bd* in Morocco, ranging from its Atlantic side along the Mediterranean coast, to the Algeria frontier where it continues up to Tunisia (Penner et al. 2013; Zimkus et al. 2020). However, both models were built using only the first set of positive records in Morocco reported by El Mouden et al. (2011), and not the six additional localities in the Tensift region confirmed by El Cadi et al. (2019).

One of the *Bd* infected individuals recorded by El Mouden et al. (2011) was within the distribution range of the endemic Moroccan midwife toad (*Alytes maurus*), but infection in this species has not been reported up to now (El Mouden et al. 2011; El Cadi et al. 2019). Several population declines recorded in Europe indicate that the *Alytes* genus is highly susceptible to *Bd* with subsequent lethal consequences (e.g. Bosch et al. 2001; Walker et al. 2010; Bosch et al. 2013; Doddington et al. 2013). The rapid and significant geographic expansion of *Bd* across the entire distribution of *A. dickhilleni* in south-eastern Spain provides a relevant example of how chytridiomycosis severely affects midwife toads (Thumsová et al. 2021).

Here we report new *Bd* positive records in Morocco, increasing the number of species known to be affected, and present the first evidence of lethal chytridiomycosis in recently metamorphosed individuals of *A. maurus*. Additionally, we present a new environmental niche model performed with all available *Bd* records for Morocco.

Materials and methods

Fieldwork sampling

Sampling was conducted using a non-systematic approach across several distinct regions of Morocco between September 2012 and December 2019, and included as many amphibian taxa as possible. Individuals were searched for opportunistically at night, mainly during the rainy season to improve amphibians' detectability. Sample collection was performed by swabbing the oral disc of anuran larvae and the whole skin of adult individuals and salamander larvae with sterile dry swabs. Immediately after sampling, individuals were returned to the place of capture. To prevent transmission of pathogens, sampling was carried out following hygiene strict guidelines, all animals were handled with disposable gloves, and all field equipment, including shoes, were disinfected with a 1% Virkon solution before and after sampling each new site. Swab samples were stored dry and below 4 °C upon collection, and individuals found dead on collection were stored in 70% ethanol.

Laboratory work

DNA was extracted using PrepMan Ultra reagent and extractions were diluted 1:10 before qPCR amplification following Boyle et al. (2004). Samples were run in duplicate and against negative and positive controls (with known concentrations of 0.1, 1, 10 and 100 genomic equivalents of zoospores, hereafter referred to as GE). A sample was assigned as positive when the infection load was equal to or higher than 0.1, and the amplification curve presented a robust sigmoidal shape. When just one replicate of a sample was amplified successfully, the sample was analysed a third time and considered positive if the curve of the third amplification represented a positive result.

Environmental niche modeling

We used the software MaxEnt v3.4.1. (Phillips et al. 2020), using the maximum entropy method (Maxent) and presence-only data in conjunction with environmental data, to produce a correlative model determining those regions of Morocco that are relatively suitable to *Bd* (Phillips et al. 2006; Peterson et al. 2011). A database with current and all previously published records (El Mouden et al. 2011; El Cadi et al. 2019) of *Bd* occurrence in Morocco was compiled, but excluding identical occurrences. Thus, a total of 23 *Bd*-positive records were used in the model. Some 19 standard WorldClim Bioclimatic variables were used as environmental predictors, and elevation information was included at a spatial resolution of 30 seconds (~1 km^2; Fick and Hijmans 2017). The model was evaluated for its overall goodness of fit assessed by the 'Area under the Curve' (AUC) of the 'receiver operating characteristic' (ROC) metric. AUC ROC is a sensitive metric, which employs the presence and absence of parasites in random locations for model evaluation (Elith et al. 2006). The AUC varies from 0.5 (no-better-than-random) to 1 (perfect), and models with values over 0.8 are considered high-accuracy models (Araujo and Guisan 2006). The model was produced by 100 replicate runs employing the bootstrap function and with data divided randomly into training (75%) and validation datasets (25%). A regulation multiplier of 0.5 was used to avoid overfitting of the model with very few records (Hof et al. 2011). A first model-run was carried out with the full set of environmental data layers. The least contributed variables (<3%) were step-wise removed (Baldwin 2009), because models with all bioclimatic variables included are usually redundant and overfitted (Elith et al. 2010). The variables used were continuous and comprised two temperature and three precipitation variables (BIO2, Mean Diurnal Range; BIO7, Temperature Annual Range; BIO15, Precipitation Seasonality; BIO18, Precipitation of Warmest Quarter; and BIO19, Precipitation of Coldest Quarter). The relative contribution of each predictor to the model was calculated by MaXent. The final validation of the model was conducted using the commonly used AUC value (e.g. Elith et al. 2006). Finally, we used Jackknife tests to determine the variables with the greatest contribution to the model.

Results

We sampled 107 amphibian individuals across 50 sites in the fourteen provinces of Morocco (Table 1; Figure 1), with approximately 17% ($n = 18$) of the specimens occupying 26.0% ($n = 13$) of the sites and 50.0% ($n = 7$) of the study provinces tested positive for *Bd*. The prevalence of infection was higher in the Taounate and Berkane Taourirt provinces (38.5 and 27.8%, respectively) than in the Tiznit and Tétouan provinces (9.1 and 2.7%). The highest proportion of infected animals corresponded to *A. maurus* (4/7), followed by significantly lower values in *Discoglossus scovazzi* and *Salamandra algira* (respectively, 2/13 and 5/42).

In November 2013, a carcass of a recently metamorphosed individual of *A. maurus* was collected in the Taounate province (Taounate 01 site; Table 1). Two living specimens presenting apathy and uncoordinated movements were transported to the laboratory for observation, dying within two days. The rest of the individuals found alive ($n = 3$) were sampled by swabbing their skin. Dead individuals in the laboratory and the carcass found in the field, as well as the individuals that died in captivity tested positive for *Bd* and displayed high infection intensity (ranged from 2 710 to 4 110 000 GE), whereas the rest of the animals found alive tested negative for *Bd*. Additionally, two *Pelophylax saharicus* individuals inhabiting the same site screened positive for *Bd* a few years later (Table 1), indicating persistence of *Bd* over multiple years in that site.

The environmental niche model obtained (Figure 2), with high values of AUC (0.960 ± 0.012, mean ± SD), appears to be robust and presents a high-quality performance

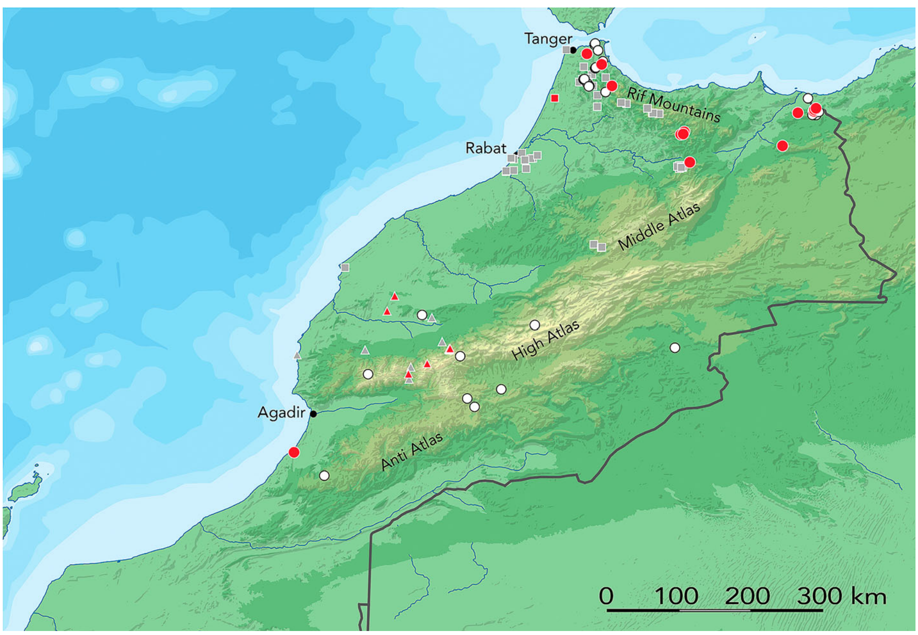

Figure 1. *Batrachochytrium dendrobatidis* (*Bd*) status of sampling sites in former and present studies. Sites where *Bd* was detected are shown in red, whereas *Bd* free sites are shown in grey (previous studies) or white (present study). Squares indicate El Mouden et al. (2011) sites; triangles El Cadi et al. (2019) sites; circles denote study sites in this work.

Table 1. Localities where amphibians were sampled for Batrachochytrium dendrobatidis. Am, *Alytes maurus*; Bab, *Barbarophryne brongersmai*; Bub, *Bufotes boulengeri*; Bs, *Bufo spinosus*; Dp, *Discoglossus pictus*; Ds, *Discoglossus scovazzi*; Hm, *Hyla meridionalis*; Ps, *Pelophylax saharicus*; Pw, *Pleurodeles waltl*; Sa, *Salamandra algira*; Sm, *Sclerophrys mauritanica*.

Province	Site	Latitude, Longitude	Altitude (m asl)	Date of sampling	Species	Life history stage	N	*Bd* +
Al Haouz	Ha 01	31.31, 7.38	2 161	Feb 2013	Ds	adult	1	0
Azilal	Az 01	31.76, 6.28	2 780	Feb 2013	Ds	juvenile	1	0
Berkane Taourirt	BT 01	35.09, 2.28	2	Feb 2013	Dp	juvenile	1	0
	BT 02	34.85, 2.21	1 288	Feb 2013	Dp	adult	1	0
	BT 03	34.83, 2.38	574	Feb 2018	Ps	adult	1	1
	BT 04	34.85, 2.14	898	Feb 2018	Sm	adult	1	1
	BT 05	34.86, 2.14	898	Feb 2018	Sm	adult	1	0
	BT 06	34.87, 2.13	747	Feb 2018	Sa	larvae	2	1
	BT 07	34.90, 2.11	425	Feb 2018	Sm	juvenile	1	0
	BT 08	34.89, 2.12	478	Feb 2018	Dp	adult	1	0
	BT 09	34.89, 2.11	456	Feb 2018	Dp	adult	1	1
	BT 10	34.39, 2.65	854	Feb 2018	Ps	adult	1	1
	BT 11	34.83, 2.15	1 006	Feb 2018	Sm	adult	1	0
	BT 12	34.84, 2.17	1 160	Feb 2018	Dp	adult	1	0
				Feb 2018	Ps	adult	1	0
				Feb 2018	Sa	larvae	2	0
				Feb 2018	Sa	adult	1	0
	BT 13	34.84, 2.16	985	Feb 2018	Sm	adult	1	0
Chefchaouen	Che 01	35.22, 5.24	865	Dec 2015	Ds	subadult	1	1
	Che 02	35.18, 5.24	1 144	Dec 2015	Sa	adults	3	0
	Che 03	35.20, 5.27	990	Dec 2019	Sa	adult	1	0
Chichaoua	Chi 01	31.04, 8.73	1 845	Feb 2013	Sm	adult	1	0
El Kelaâ des Sraghna	KdS 01	31.91, 7.93	519	Feb 2016	Bab	adult	1	0
				Feb 2016	Bub	adult	1	0
Errachidia	Er 01	31.43, 4.23	799	Feb 2013	Ps	adults	2	0
				May 2019	Sa	juvenile	1	0
				Nov 2015	Sa	adult	1	1
Larache	La 01	35.30, 5.51	1 069	Dec 2012	Sa	adult	1	0
	La 02	35.36, 5.57	729	Dec 2012	Sa	adult	1	0
	La 03	35.37, 5.55	671	Dec 2012	Sa	juvenile, adult	2	0
Ouarzazate	Ou 01	30.82, 6.77	1 257	Feb 2013	Bab	larvae	2	0
	Ou 02	30.56, 7.16	1 390	May 2014	Sm	adults	2	0
	Ou 03	30.69, 7.27	1 618	May 2014	Ps	adult	1	0
Taounate	Tao 01	34.60, 4.09	1 112	Nov 2013	Am	metamorph	6	3
				Mar 2014	Ps	juvenile	1	1
				Feb 2018	Ps	juvenile	1	1
	Tao 02	34.60, 4.08	1 112	Feb 2018	Ds	juvenile, adult	2	0
Taza	Taz 01	34.15, 4.01	1 025	Feb 2018	Ds	juvenile	1	0
				Feb 2013	Sa	adult	1	1
	Taz 02	34.56, 4.14	1 086	Feb 2018	Ds	adult	2	1
				Feb 2018	Sa	larvae	3	1
	Taz 03	34.56, 4.11	1 193	Feb 2018	Am	adult	1	1
Tétouan	Té 01	35.89, 5.40	332	Sep 2012	Sa	adult	2	0
	Té 02	35.90, 5.40	332	Sep 2012	Sa	juvenile	1	0
	Té 03	35.63, 5.28	4	Dec 2012	Ds	adult	1	0
	Té 03	35.63, 5.28	4	Dec 2012	Pw	juvenile	4	0
	Tén 04	35.79, 5.35	12	Dec 2012	Ds	adult	2	0
				Dec 2012	Hm	adult	2	0
				Dec 2012	Pw	juvenile	1	0
	Té 05	35.88, 5.40	396	Mar 2014	Ds	adult	1	0
	Té 06	35.89, 5.40	332	Mar 2014	Sa	adult	1	0
	Té 07	35.88, 5.40	396	Mar 2014	Sa	adult	2	0
				Jun 2016	Sa	adult	1	0
	Té 08	35.27, 5.48	1 361	Mar 2014	Sa	adult	1	0
	Té 09	35.53, 5.40	484	Jun 2016	Sa	adult	3	0

(*Continued*)

Table 1. Continued.

Province	Site	Latitude, Longitude	Altitude (m asl)	Date of sampling	Species	Life history stage	N	*Bd* +
	Té 10	35.54, 5.40	382	Jun 2016	Sa	adult	1	1
	Té 11	35.26, 5.49	1 307	Mar 2019	Sa	adult	5	0
	Té 12	35.54, 5.39	406	Jul 2019	Bs	adult	1	0
				Jun 2019	Ds	adult	1	0
				Mar/Apr 2019	Sa	juvenile	2	0
	Té 13	35.64, 5.67	108	Dec 2019	Pw	juvenile	1	0
	Té 14	35.81, 5.46	491	Dec 2019	Sa	adult	3	0
	Té 15	35.44, 5.42	218	Dec 2019	Sa	adult	1	0
Tiznit	Ti 01	29.80, 9.81	72	Feb 2013	Bab	larvae	7	0
				Feb 2013	Bub	larvae	3	1
	Ti 02	29.55, 9.37	1 012	May 2014	Hm	adult	1	0

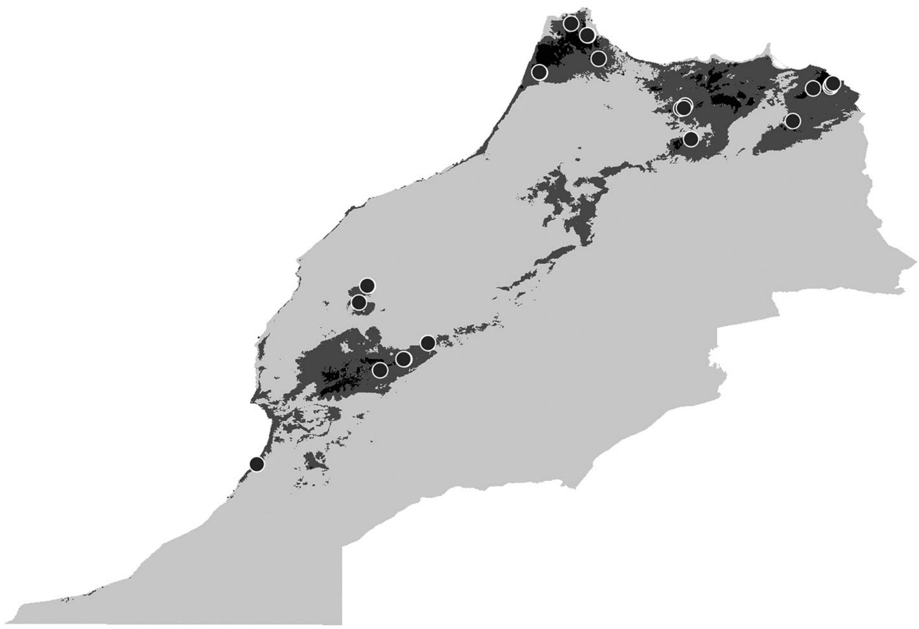

Figure 2. Relative habitat suitability for *Batrachochytrium dendrobatidis* in Morocco. Potential distribution was generated by using the maximum entropy method (Maxent) and presence-only data in conjunction with environmental data, and is based on 100-fold cross-validation replicates. Dark grey and black indicate areas of medium to high probability of occurrence, and the confirmed records of *Bd* are shown as red dots.

according to Peterson et al. (2011). The highest contribution to the final model came from the variable 'BIO19, Precipitation of Coldest Quarter' (34.5%), followed by 'BIO18, Precipitation of Warmest Quarter' (21.6%), 'BIO7, Temperature Annual Range' (19.9%), 'BIO15, Precipitation Seasonality' (15.1%), and 'BIO2, Mean Diurnal Range' (8.8%). 'BIO19, Precipitation of Coldest Quarter' appears to have the most useful information on its own, whereas 'BIO15, Precipitation Seasonality' appears to have a great amount of information that is not present in the other variables. The environmental niche model obtained indicates that the highest habitat suitability indices for *Bd* were found in the Loukkos area, the Tetouan region (called Tangitane peninsula) until Chefchaouen, and the Ketama region

(Rif mountains). These areas represent a subwet zone with an average annual rainfall between 660 and 800 mm, up to 2 000 mm towards Ketama (the wettest region of the Maghreb with Western Kabylia in Algeria), characterised by much more rainfall in winter than in summer, and annual average temperatures of 16.6–17.9 °C. The Tazzeka zone in the Middle Atlas, separated from the Rif Mountains by the Taza corridor, also represents a region of suitable habitat for *Bd*, with a model index score of 0.62–0.85. The second most suitable region in Morocco, with an index score of 0.69–0.85, is that of the mountains of the High Atlas near Marrakech (altitude >1 500 m), a subhumid area with annual rainfall up to 800 mm. The model also indicated the mountains of Beni Snassen (Oujda region) as suitable habitat, with an index score of 0.62–0.85. Within this area, the average annual rainfall is 237 mm in Taourirt. To the east (in the mountains of Beni Snassen) the rainfall is quite high (400–500 mm), and the Beni Snassen mountains are the wettest area in the eastern region (François et al. 2016). According to the model, the central plains (Gharb and Rhamna), and the remaining regions of Morocco, from the pre-Saharan zones in the south-west to the Atlantic Sahara (Eastern and Saharan domains), are not favourable to *Bd,* with an index score below 0.1. In the plain of Gharb, the average annual rainfall is approximately 550 mm in the coastal zone and 441 mm in its eastern part, with an average annual temperature of 17.1–20 °C. The climate in the Rhamna region is highly seasonal with irregular precipitation (less than 250 mm) and occasional prolonged droughts. The Sahara region is characterised by accentuated droughts (precipitation less than 250 mm) and high variation between winter and summer temperatures.

Discussion

Since the first evidence of the occurrence of *Bd* in Morocco (El Mouden et al. 2011), only one additional survey has been carried out in the country to sample this pathogen (El Cadi et al. 2019). Although a mass mortality episode was previously recorded for *Sclerophrys mauritanica* and *P. saharicus*, its association with chytridiomycosis was not confirmed due to the low infection rates found (El Cadi et al. 2019). Our results greatly extend the known occurrence of *Bd* in Morocco and provide the first evidence of the high susceptibility of *A. maurus* to *Bd*, with lethal consequences, together with the first report of a *Bd*-associated mortality in North Africa.

A positive *Bd* status has previously been recorded for five Moroccan species (El Mouden et al. 2011; El Cadi et al. 2019). In this study we add four species to the list: *A. maurus*, *Bufotes boulengeri*, *Discoglossus pictus* and *Salamandra algira*. Thus, to date, nine from the 14 known Moroccan species have tested positive for *Bd*. *Barbarophryne brongersmai*, *Pleurodeles waltl*, and *Bufo spinosus* have been poorly sampled in Morocco, testing negative despite the fact that the latter two are commonly infected in the Iberian Peninsula (e.g. Bosch et al. 2018; Oficialdegui et al. 2019). Interestingly, three adult specimens of *S. algira* tested positive for *Bd* in this study, whereas in the Iberian Peninsula adult *S. salamandra* usually only test positive during the initial stages of chytridiomycosis outbreaks (Bosch and Martínez-Solano 2006; Medina et al. 2015; Bosch, unpubl. data). Larval stages of *S. salamandra* and *B. spinosus* frequently test positive for *Bd* in the Iberian Peninsula (e.g. Medina et al. 2015; Bosch et al. 2018), so a targeted survey of amphibian larvae in Morocco is likely to yield higher rates of infection than we found in this study.

As with other midwife toads, *A. maurus* exhibits a high prevalence of *Bd* infection, probably associated with the delayed metamorphosis exhibited by tadpoles at high elevations (Bosch et al. 2001). Overwintered *Alytes* tadpoles are not likely to die as a result of infection, but maintain the infection in the aquatic environment and transmit it to hatching tadpoles. Mortality occurs following metamorphic climax (Bosch et al. 2001; Walker et al. 2010; Doddington et al. 2013). Although *A. maurus* is currently listed as Near Threatened by the IUCN (Donaire-Barroso et al. 2009), many existing threats, such as deforestation, invasive species, water pollution and climate change threaten the future of the species (Rodriguez-Rodriguez 2020). The fact that *Bd* is widely distributed throughout the *A. maurus* distribution range, and the likely high susceptibility of the species to this pathogen, greatly increases its vulnerability, and warrants the elevation of its conservation status to Vulnerable. The case of *A. obstetricans* in the Peñalara Massif in Central Spain provides an iconic example of a *Bd* driven population extirpation (Bosch et al. 2001), and provides a warning for the fate of *A. maurus*.

Two previously published studies confirmed *Bd* infection in 14.5% of screened Moroccan sites (El Mouden et al. 2011; El Cadi et al. 2019). In this study, *Bd* has been detected in thirteen additional sites in seven provinces, including the Chefchauen and Taza provinces where the infection was not detected by El Mouden et al. (2011). Currently, 19.5% of the sampled Moroccan sites ranging from 72 to >1 193 m asl tested positive for *Bd*. Our results confirm a wider distribution of the chytrid fungus across the country and along the altitudinal gradient, as was suggested by El Cadi et al. (2019). Comparing our environmental niche model with the recently published one for the whole of Africa (Zimkus et al. 2020), there are some differences in the areas predicted as suitable for *Bd* in Morocco. Our model reduces the likelihood of *Bd* being present along the Atlantic coast, and indicates probable occurrence in the regions of Tetouan, Ketama, Oujda and Tazzeka, as well as in the mountains of the High Atlas near Marrakech. Note that the model of Zimkus et al. (2020) was based on 490 unique records for a total area of 30 272 922 km^2, and included just two records from Morocco's 446 550 km^2. Our work adds 21 new Moroccan *Bd*-positive localities, resulting in a model built with three times more data per unit area than the model from Zimkus et al. (2020).

Although ecological niche modelling is increasingly used as a tool to predict the distribution of *Bd* (e.g. Lötters et al. 2010; Hof et al. 2011; Penner et al. 2013; Tarrant et al. 2013; Becker et al. 2016; Zimkus et al. 2020), it must be noted that *Bd* is a generalist fungus whose distribution is primarily based on the presence of its hosts. Therefore, and considering the infection was confirmed in the widely distributed *P. saharicus* and *B. boulengeri*, it cannot be ruled out that *Bd* could be present anywhere in Morocco where these amphibians occur. In addition, it is important to note that the prediction of *Bd* occurrence should not be associated with disease risk, as many authors incorrectly reported (e.g. Hof et al. 2011; Tarrant et al. 2013).

The recent study of Scheele et al. (2019), based on expert opinions and reported *Bd*-related population declines, identified Africa as a region relatively less impacted by *Bd* than other continents. However, given that population data of *A. maurus* are scarce and not actualised, this species was not taken into account in that study. Therefore, even when knowledge of some areas of the South of the continent is relatively good, our findings support those of Zimkus et al. (2020) and Doherty-Bone et al. (2020) indicating that the real affect of chytridiomycosis in Africa was probably underestimated.

Additional studies using quantitative data to show population trends at *Bd* locations in Morocco are needed to understand the real impact of the disease, especially since its recent rapid expansion in ecologically similar areas in the south of the Iberian Peninsula (Thumsová et al. 2021).

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