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Occurrence of *Batrachochytrium dendrobatidis* in the Tensift region, with comments on its spreading in Morocco

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Abstract. The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a generalist pathogen that affects many amphibian species and is responsible of chytridiomycosis onset, considered as the main causes of species extinctions and populations declines worldwide. The chytrid fungal pathogen has been first described in North Africa in 2011. The present work reported the first survey on *Bd* prevalence and intensity in the Tensift region of Morocco. The survey has been conducted on 11 different localities by collecting skin swabs and tissue samples of 97 individuals. Using a quantitative Polymerase Chain Reaction (qPCR) protocol, low-intensity of *Bd* infection has been detected in the area of study. In fact, the chytrid fungal pathogen has been identified in 10 individuals distributed in six of the 11 sites investigated, placing the 95% confidence interval for overall prevalence at 5.5-19.6%. The survey confirmed the occurrence of *Bd* at both high and low altitude localities, on four species out of seven known to inhabit the region and added two additional species (*Pelophylax saharicus* and *Sclerophrys mauritanica*) to the list of *Bd* susceptible amphibians in Morocco. The present records extended *Bd* distribution more than 400 km in the South of Morocco, indicating that the chytrid fungal pathogen is more widespread in the country than previously thought.

Keywords. *Batrachochytrium dendrobatidis,* amphibians, Tensift, prevalence, intensity.

INTRODUCTION

Habitat degradation and overexploitation are the main large-scale factors causing loss of biodiversity worldwide (Hoffmann et al., 2010). While these two factors are to blame for the rapid declines of many vertebrate species, amphibians continue to decline also in undisturbed habitats, due essentially to chytridiomycosis, an emerging infectious skin disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger, 1998; Longcore and Pessier, 1999; Bosch and Martínez-solano, 2006; Lips, 2016). *Bd* is considered as the first wildlife fungal pathogen to have caused widespread species extinctions (Skerratt et al., 2007), and has been implicated in rapid population declines and extinction of several amphibians (Retallick et al., 2004; Schloegel et al., 2006; Skerratt et al., 2007; Kolby and Daszak, 2016; Lips, 2018). It has been detected in about 48% of tested amphibian species (Olson et al., 2013), and is recently known to be rapidly expanding its global range of distribution (Fisher et al., 2009; O'Hanlon et al., 2018). In North Africa, *Bd* has been detected for the first time in Morocco at three (out of 51) tested localities, and was found in only three species (*Discoglos-*

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sus scovazzi*, *Hyla meridionalis* and *Pelobates varaldii*), with a total prevalence of 6% (El Mouden et al., 2011). The sites where *Bd* has been detected are in the northern part of the country (Tingitana peninsula and its surroundings) near South of Spain. El Mouden et al. (2011) explained this presence through a possible arrival from Spain where chytrid fungus is largely distributed, and it's responsible for severe decline of amphibian populations in mountain areas (Bosch et al., 2001; Bosch et al., 2007; Walker et al., 2010; Lips, 2016). These findings suggest further investigations in other areas were needed to collect more information on the potential extent of the spread of this fungal pathogen in Morocco, and to elucidate its potential source. Morocco is home to many endangered amphibian populations that are already threatened by habitat alteration and destruction, pollution, and climatic changes (Fahd et al., 2015; Ben Hassine and Nouira, 2012; Escoriza and Ben Hassine, 2017). The aim of this study is to evaluate the chytrid fungal presence and prevalence using qPCR test for amphibians naturally occurred in Tensift region, an area located around 400 km South from the localities where *Bd* was detected for the first time in 2011. This region is characterized by a high diversity of habitats from wetlands (High Atlas Mountains) to arid environments (Jbilets), with seven amphibians species known to inhabit the area, two of which are Moroccan endemics (Bons and Geniez, 1996; Beukema et al., 2013).

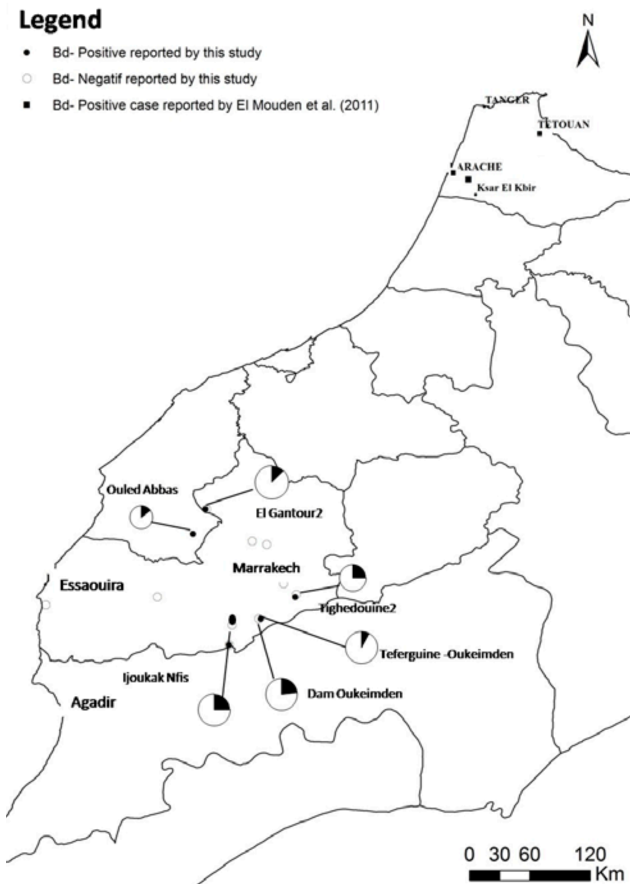

Fig. 1. Map of the Northern Morocco showing the result of *Batrachochytrium dendrobatidis* (*Bd*) sampling sites realized during the present study (circles); dark circles indicate populations with *Bd*-positive samples and open circles indicate populations with *Bd*-negative samples. Within *Bd* positive sites, pie charts indicate the proportion of infected individuals in black and uninfected individuals in white. Squares indicate infected populations as reported by El Mouden et al. (2011).

MATERIALS AND METHODS

Surveys were conducted between October 2013 and October 2017 in the Tensift region (central Morocco). The study area covers about 20,000 km^2, surrounded by the southern crest of the High Atlas Mountains (with an altitude up to 4000 m a.s.l.) and the northern Jbilet hills (separated by the Haouz plain), while the coastline of Essaouira extends in the East (Fig. 1). The climate of the region is characterized by strong annual variability, with mean temperatures ranging between a maximum of 37.7 °C and a minimum of 4.9 °C. The rainfall is generally irregular, with occasional prolonged droughts. The mean annual rainfall varies from 800 mm in the mountain to 190 mm in the plain, with significant snowfall between December and March at high elevation (up to 2000 m a.s.l.) (Alaoui Haroni et al., 2009). The semi-arid environment dominates throughout the region, while the sub-humid zones appear at high altitude (up to 1500 m a.s.l.). The Tensift region is characterized by a complex landscape and topography, including escarpments, floodplains, and a great variety of aquatic ecosystems (rivers, permanent ponds and a large network of Mediterranean temporary ponds). This diversity of water ecosystems provides a wide availability of breeding sites for seven native amphibian species: *Hyla meridionalis* Boettger, 1874, *Bufotes boulengeri* (Lataste, 1879), *Sclerophrys mauritanica* (Schlegel, 1841), *Bufo spinosus* Daudin, 1803 (Near threatened in Morocco), *Barbarophryne brongersmai* (Hoogmoed, 1972) (endemic to Morocco and Algeria), *Pelophylax saharicus* (Boulenger, 1913) (endemic to North Africa) and *Discoglossus scovazzii* Camerano, 1878 (endemic to Morocco).

Amphibians were searched opportunistically in 11 sites (Table 1; Fig. 1), during the rainy season (when detectability of amphibians is higher), during nocturnal and occasional diurnal surveys. We extensively searched in all water bodies available in the area. After capture, animals were swabbed (using Medical Wire Sterile Dryswab™ - MW100) and released immediately after sampling to the place of capture. To prevent the transmission of diseases by animals, each individual was handled with disposable gloves. In total, 86 individuals were swabbed. Swabs were then air-dried and stored at cool temperature (4 °C) until processing. Tissues samples have been collected from 11 specimens that were found dead in two sites

Table 1. Surveyed localities of the Tensift region (Morocco) with date and approximate coordinates and altitude (meters a.s.l.) of each site. Species names are abbreviated as follows: Sm, *Sclerophrys mauritanica*; Bb, *Bufotes boulengeri*; Br, *Barbarophryne brongersmai*; Ds, *Discoglossus scovazzi*; Hm, *Hyla meridionalis*; Ps, *Pelophylax saharicus*. Life history stage (LHS): Ad, adult; Juv, juvenile. P/S: number of individuals testing positive / Number of analyzed individuals. *Bd* load in genomic equivalents of zoospores. (*) Indicate individual found dead in the field and tested positive for *Bd*.

Date	Locality	Coordinates		Altitude	Species	LHS	P/S	*Bd* load
Oct 2013	Sidi Bouathman	31.9	-7.92	~ 517	Ps	Ad	0/1	
Nov 2013	**Ijoukak N'fis**	31.059	-8.164	~ 1042	**Ps**	Juv	0/1	
						Ad	1/3	26.8
Dec 2013	Sidi Bouathman	31.9	-7.92	~ 517	Ps	Ad	0/3	
Dec 2013	**Dam d'Ouled Abbas**	31.966	-8.447	~ 468	**Sm***	Ad	1/7	0.3
Dec 2013	Dam-Oukaimeden	31.208	-7.851	~ 2623	Ps	Ad	0/7	
Dec 2013	Tighedouine 1	31.4	-7.532	~ 1145	Ps	Ad	0/6	
Avr 2014	**Tighedouine 2**	31.423	-7.524	~ 1066	**Ps**	Ad	1/4	0.3
					Hm	Ad	1/7	0.4
Avr 2014	Ijoukak Tizgui	30.971	-8.125	~ 1142	Ds	Ad	0/2	
					Bb	Ad	0/1	
					Ps	Ad	0/5	
Dec 2014	El Gantour 1	32.189	-8.335	~ 402	Ps	Ad	0/5	
Dec 2014	**El Gantour 2**	32.187	-8.329	~ 398	Ds	Ad	0/2	
					Ps	Juv	0/1	
					Ps	Ad	1/8	1.3
Jan 2015	Jaidate	31.87	-7.79	~ 623	Bb	Ad	0/9	
					Br	Ad	0/3	
					Ps	Ad	0/2	
Avr 2015	**Tighedouine 2**	31.423	-7.524	~ 1066	**Ps**	Ad	1/1	1.7
Jun 2016	**Tifergine-Oukaimeden**	31.207	-7.84	~ 2565	**Ds**	Ad	1/13	1.3
Oct 2017	**Dam-Oukaimeden**	31.208	-7.851	~ 2623	**Ps***	Ad	3/6	0.6, 3.0, 0.3

(Dam-Oukaimden and Dam d'Ouled Abbas). These 11 tissue samples were preserved in ethanol (Brem et al., 2007; Hyatt et al., 2007). In total, 97 amphibian specimens were sampled belonging to four families and six species. Of these, 56 individuals were sampled in the high Atlas Mountains, 16 in the arid area of Jbilets, 23 in the El Gantour region and two in the Haouz plain (Table 1; Fig. 1).

DNA was extracted from both swabs and tissue samples using PrepMan Ultra reagent and extractions were diluted 1:10 before real-time PCR amplification, performed in duplicate with a CFX96 thermocycler (Bio-Rad), following Boyle et al. (2004). Each 96-well assay plate included samples, a negative control and standards of 100, 10, 1, and 0.1 *Bd* zoospore genome equivalents in duplicate. Samples were considered positives when both replicates were ≥ 0.1 and the amplification curves had the typical sigmoidal shape. When only one replicate from any sample amplified, we ran this sample a third time. If the third amplification did not result in an amplification profile, we considered sample as negative for infection. Samples that showed signs of inhibition (non-sigmoidal amplification) were further diluted to 1:100 and re-analyzed. If signs of inhibition remained, the samples were excluded.

RESULTS

The numbers of screened and testing positive individuals from each population and sampling event, along with geographic information on the sampling site and year of capture are reported in Table 1. The results show that 10 out of 97 screened individuals were *Bd* infected (10.3%), corresponding to a 95% confidence interval of overall *Bd* prevalence of 5.5-19.6%. Across the four species that tested positive, the infection prevalence was 11.9% (95% CI: 5.5-19.6%). This study confirmed *Bd* occurrence in both the northern and southern parts of the Tensift watershed at elevations between 468 and 2625 m a.s.l (Fig. 1).

In the study area, *Bd* was detected in six out of 11 investigated sites (Table 1), with prevalence values ranging between 0 and 25%, and lower GE (genomic equivalent) values that varied between 0.3 and 26.8. These results indicate a significant presence of *Bd* in the Tensift region, with low infection prevalence and intensities across all sites that tested positive for *Bd*. However,

Table 2. *Bd* prevalence with 95% Clopper-Pearson binomial confidence intervals for individuals grouped by species and elevation.

Group	sample size	*Bd* prevalence (%)	95% CI
Family/Species			
Ranidae			
Pelophylax saharicus	53	13.2	5.6 - 25.8%
Alytidae			
Discoglossus scovazzi	17	5.9	0.1 - 28.7%
Bufonidae			
Bufotes boulengeri	10	0	0 - 30.8 %
Sclerophrys mauritanica	7	14.3	0.4 - 57.9%
Barbarophryne brongersmai	3	0	0 - 70.8%
Hylidae			
Hyla meridionalis	7	14.3	0.4 - 57.9%
Elevation (for all species)			
Less than 700 m	41	4.9	0.6 - 16.2%
More than 700 m	56	14.3	6.1 - 25.4%

in October 2017, 50% prevalence was recorded in Dam-Oukaimden, with three testing positive samples out of the six screened individuals, which represents the maximum prevalence obtained in this study.

It seems that *Bd* prevalence can vary among species and elevations (Table 2). Consequently, 95% confidence intervals for *Bd* prevalence for individuals grouped according to these two parameters were determined. All confidence intervals were overlapping. Thus, no significant taxonomic and altitudinal difference in *Bd* prevalence has been detected given our sample sizes. However, the obtained results tend towards a more significant prevalence at higher elevations (14.3% vs 4.9%). Likewise, species infection rate varied between 0 and 14.3%. The higher percentage was observed in three species. *Bd* was found in seven out of 53 *P. saharicus* across five sampling sites. In the Dam-Oukaimden site, where a large numbers of dead *P. saharicus* were found in October 2017, we detected *Bd* on three out of six tested individuals (95% CI: 11.8-88.2%), which represents the highest prevalence recorded by species and by site during this study. For both *H. meridionalis* (Tighedouine region; April 2014) and *S. mauritanica* (Culinary dam of Ouled Abbas; December 2013), *Bd* has been detected in one out of seven individuals, corresponding to a confidence interval for the two species of 0.40-57.9%. During our investigation at Ouled Abbas dam, numerous dead specimens of *S. mauritanica* were observed in the water. Seven tissue samples were collected and screened. The results showed that one sample tested positive for *Bd*.

DISCUSSION

In our study area, four out of six amphibian species have been tested as *Bd* positive, although in comparison with other regions in Marocco, they have lower infection rate. Lower GE values are probably the result of unfavorable conditions, such as changes in the abiotic environment (Ron, 2005; Thorpe et al., 2018). It has been reported that *Bd* is temperature sensitive, with optimal growth ranging between 17 and 25 °C (Piotrowski et al., 2004; Pounds et al., 2006), with higher temperature (more than 30 °C) reported as unfavorable for its development (Watve, 2013). Previous studies have shown that lower temperature regime resulted in extended zoospore longevity and in such conditions zoospores numbers in water bodies could be expected to be greater than in warmer water (Voyles et al., 2012; Thorpe et al., 2018). The higher temperatures (more than 30 °C) recorded in the study area expected to have a negative impact on the development of this fungal pathogen. Additionally, optimum rainfall for *Bd* development has been reported to range between 1500 and 2500 mm/year (Thorpe et al., 2018), which are much higher values of the one observed in the Tensift region. Only, Oukaimden approach these rainfall values, but in general remains below to optimum parameters for the development of *Bd*.

In Morocco, *Bd* surveys were previously conducted only in the northern Tingitana area. El Mouden et al. (2011) reported *Bd* occurrence in three sampling sites. Through this study, *Bd* occurrence has been reported in six additional Moroccan localities. Considering all these data, 14.5% of the screened Moroccan localities tested positive to *Bd*. The finding that *Bd* was recorded in two separated areas suggested a possible wider distribution across the country. Similarly, *Bd* was detected in different sites ranging from 400 to over 2600 m a.s.l, indicating a probable wide distribution also along the altitudinal gradient. Sample size is still limited and should be extended to have a more robust overview of the pattern of *Bd* presence across Morocco.

The report on the occurrence of *Bd* in the Tingitana peninsula (North of Morocco) was potentially explained by the extensive commercial trade of products and animals across the straits of Gibraltar with a possible introduction of *Bd* from Spain (El Mouden et al., 2011). This hypothesis is in agreement with the explanations of O'Hanlon et al. (2018) who found *Bd* spreading out of Asia and dated its emergence on the early 20th century, coinciding with the international expansion of commercial trade in amphibians for exotic pet, medical, and food purposes. The presence of *Bd* in the Tensift region can be explained by a dissemination process from the North of the country to the South, but also by a possible independent episode of introduction through other commercial routes, such as airports and harbors. The spreading of *Bd* through the country can be carried out by potential natural vectors, including waterfowl on their feathers or feet (Johnson and Speare, 2005; Garmyn et al., 2012; Burrowes and De La Riva, 2017), water (Johnson and Speare, 2003), and non-susceptible to chytridiomycosis amphibians acting as carrier (Kolby et al., 2015).

In Morocco, as in most regions around the world, the detected *Bd* belongs to the Global Panzootic Lineage (J. Bosch, unpublished data). This is a highly virulent and highly transmissible chytrid fungus, which is currently infecting more that 700 amphibian species worldwide (Olson and Ronnenberg, 2014; Lips, 2016). We detected a mass mortality of amphibians in two localities (*S. mauritanica* in Dam d'Ouled Abbas and *P. saharicus* in Dam-Oukaimden) and some of the sampled individuals tested positive for *Bd* infection. However, in the absence of a detailed study, we have no evidence of *Bd*-associated amphibian mortality especially because mortality of individuals generally occurred with higher infection rates than those found in the present study. Other biotic and abiotic factors can be the cause of the witnessed mass mortality (e.g., Croteau et al., 2008; Hayes et al., 2010; Relyea et al., 2012; Whittaker et al., 2013; Budzik et al., 2014; De Wijer et al., 2018). However, it is worth noting that the two new species found infected by *Bd* (*P. saharicus* and *S. mauritanica*) have a wide distribution and abundance in the southern Mediterranean region (Bons and Geniez, 1996; Schleich et al., 1996; Mateo et al., 2013), which can make *Bd* dissemination faster.

A systematic survey to determine the impact of *Bd* occurrence in Morocco is crucial to better characterize its impact on the amphibian's population dynamic. In the mean time, other *Bd* positive sites located in Tensfit region have been recorded and helped extending the known *Bd* occurrence area in Morocco, both at the high and the low altitudes (486-2625 m a.s.l.). In addition, having detected *Bd* at multiple sites and in two new amphibian species contributes to the growing knowledge on the global pattern of *Bd* distribution in Morocco and North Africa.

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