

NOTE

Chytridiomycosis in endemic amphibians of the mountain tops of the Córdoba and San Luis ranges, Argentina

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ABSTRACT: Chytridiomycosis is a major threat to amphibian conservation. In Argentina, the pathogenic fungus *Batrachochytrium dendrobatidis* has been recorded in several localities, and recently, it was registered in amphibians inhabiting low-elevation areas of mountain environments in Córdoba and San Luis provinces. In the present study, we searched for *B. dendrobatidis* in endemic and non-endemic amphibians on the mountain tops of Córdoba and San Luis provinces. We collected dead amphibians in the upper vegetation belt of the mountains of Córdoba and San Luis. Using standard histological techniques, the presence of fungal infection was confirmed in 5 species. Three of these species are endemic to the mountain tops of both provinces. Although there are no reported population declines in amphibians in these mountains, the presence of *B. dendrobatidis* in endemic species highlights the need for long-term monitoring plans in the area.

KEY WORDS: *Batrachochytrium dendrobatidis* · Argentina · Anurans · Mountains

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INTRODUCTION

Amphibians are undergoing an unprecedented conservation crisis (Houlahan et al. 2000, Stuart et al. 2004). Several factors are related to this global phenomenon, including emergent diseases such as chytridiomycosis, which are a major threat to amphibian conservation (Collins & Storfer 2003, Daszak et al. 2003, Skerratt et al. 2007). Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and it has been proposed as the proximal cause of several declines of amphibian populations around the world (Berger et al. 1998, Bosch et al. 2001, Rohr & Raffel 2010). The origin and spread of this emergent disease is still unclear, and 2 contrasting hypotheses are being discussed (Rachowicz et al. 2005). The first has been called the

'novel pathogen hypothesis' or 'spreading pathogen hypothesis' (Rachowicz et al. 2005, Skerratt et al. 2007) and postulates that the introduction and spread of *Bd* among naive populations of frogs has caused the emergence of chytridiomycosis worldwide. The second hypothesis, the 'emerging endemic hypothesis', postulates that *Bd* was already a widespread pathogen that has become highly pathogenic and virulent because of abnormal environmental changes (e.g. climate change, pollution) (Rachowicz et al. 2005). Although there is no consensus, there is more evidence in favor of the first hypothesis (Skerratt et al. 2007).

In Argentina, *Bd* has been recorded in eastern (Herrera et al. 2005), northwestern (Barrionuevo & Mangione 2006), southern (Fox et al. 2006), and northeastern (Arellano et al. 2010) regions of the

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country, and recently it was recorded in amphibians inhabiting low-elevation areas (around 800 m above sea level [a.s.l.]) of mountain environments in central Argentina in the Córdoba and San Luis provinces (Ghirardi et al. 2010, Gutierrez et al. 2010).

The tops of the mountain systems Sierras Grandes, Sierras de Comechingones, and Sierras de San Luis in the Córdoba and San Luis provinces have a high degree of geographic isolation (Fig. 1A) and therefore have several endemic species of plants (Vischi et al. 2004), birds (Nores 1995), and fungi (Robledo et al. 2003, 2006, Robledo & Renison 2010). Seven species of amphibians inhabit the mountain tops (between 1800 and 2800 m a.s.l.) of Córdoba and San Luis, and 4 of them are endemic to these environments (Ceï 1972, 1980, di Tada & Bucher 1996, Valletti et al. 2009, Verga et al. 2012). About 400 yr ago, domestic animals, such as cattle, sheep, and horses, were introduced into the Córdoba mountains (García et al. 2008). Long-term livestock grazing combined with frequent anthropogenic fires has led to the degradation, fragmentation, and decline of habitats, such as tussock grasslands and *Polylepis australis* woodlands (Cingolani et al. 2004, Renison et al. 2006). Due to these human factors, the endemic amphibians of the mountains tops of Córdoba and San Luis are listed as Near Threatened in the IUCN Red List of Threatened Species (Lavilla & di Tada 2004a,b, Lavilla et al. 2004) and as Vulnerable and Threatened in Argentina (Vaira et al. 2012).

It has been suggested that the decline in amphibian populations related to epizootics of chytridiomycosis is most common at higher elevations (Alford et al. 2001, Young et al. 2001), and that the most susceptible species have restricted altitudinal ranges (Lips et al. 2006, Kriger & Hero 2007). Aiming to explore the presence of pathogenic fungi in amphibians of the mountain tops of Córdoba and San Luis provinces, Argentina, we searched for *Bd* in the amphibians of the area. Here we present the first record of the disease in threatened and endemic amphibians of the mountain tops of Córdoba and San Luis.

MATERIALS AND METHODS

Study area

The study was conducted in the upper vegetation belt of the mountain system of the Sierras Grandes in Córdoba province and at one locality of the Sierras de San Luis in San Luis province. The Sierras

Grandes continue into the mountain range called Comechingones, located in the provinces of Córdoba and San Luis (Fig. 1). The area comprises different landscapes, including valley bottoms and ravines, plateaus with different degrees of dissection, rocky hilly uplands, and steep escarpments (Cabido et al. 1987). Vegetation consists of a mosaic of tussock grasslands, grazing lawns, granite outcrops, *Polylepis australis* woodlands, and eroded areas with exposed rock surfaces (Cabido 1985, Cingolani et al. 2003, 2004). The climate is typical of mountain areas, with mean annual winter and summer temperatures of 5 and 11.4°C, respectively. Precipitation is concen-

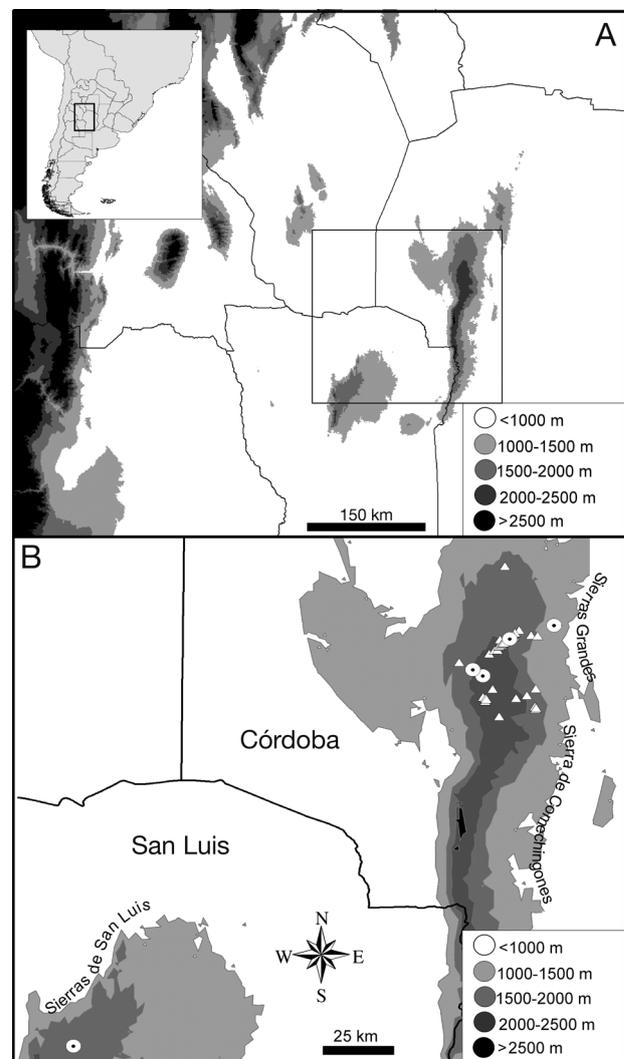


Fig. 1. (A) Location of the mountains of Córdoba and San Luis (area within the box) in central Argentina (inset: South America). Note the degree of isolation of these mountain ranges. (B) Study area and sampling sites. Surveyed streams and ponds (Δ) and localities with the presence of infected frogs (\odot) are indicated

trated between November and March and reaches an annual mean of 850 mm (Cabido 1985).

Sampling

Amphibian samples were obtained in stretches of 500 m length in 30 permanent streams. We also sampled 5 small temporary ponds (between 25 and 100 m²) (Fig. 1B). Surveys were conducted over 3 yr in months of anuran activity (August to April, 2007 to 2010). Twenty-seven stretches were located in the upper vegetation belt of the Sierras Grandes (between 1800 and 2400 m a.s.l.), and 3 were located in streams at lower altitudes (between 1200 and 1650 m a.s.l.). All ponds were located in the upper vegetation belt of the mountain areas. At each stream stretch or pond, we recorded dead amphibians, in both diurnal and nocturnal surveys.

Dead individuals collected were immediately fixed in 10% neutral formalin. Abdominal and hind limb ventral skin patches (approximately 5 × 10 mm) were excised from the anurans, stored in 70% ethanol, and dehydrated to be embedded in paraffin. Then, tissues were sectioned at 5 µm thickness with a microtome for histology, and stained with hematoxylin and eosin (H&E). With a compound light microscope, we searched for spores and sporangia in the corneous epithelium of the tissue samples, following Berger et al. (2000) and Pessier et al. (1999). In some cases, periodic acid Schiff (PAS) staining was employed to

highlight morphologic features of *Bd* and then confirm the diagnosis.

The Achala toad *Rhinella achalensis* was not found during the first 3 yr of surveys (2007 to 2009). In order to obtain tissue of this species, we extracted samples of 5 previously collected specimens kept at Cátedra de Ecología, Universidad Nacional de Río Cuarto, Argentina. Four of these specimens had been collected at Sierras Grandes (Córdoba province) and one at La Carolina, Sierras de San Luis (San Luis province). Finally during the surveys of 2010, we detected one population of Achala toads, and one juvenile individual belonging to this population was found dead.

All specimens collected in the present study are kept in the amphibian collection of the Centro de Zoología Aplicada (Universidad Nacional de Córdoba, Argentina).

RESULTS

Sixteen individuals belonging to 6 species were found dead in 7 streams and 1 pond surveyed (Table 1). We confirmed the presence of chytrid fungal infection in 5 individuals belonging to 5 of the 6 species surveyed. Infected individuals were collected at 4 different streams of Sierras Grandes (Table 1, Figs. 1B & 2A). In addition, one of the individuals analyzed from the Universidad Nacional de Río Cuarto collection, from La Carolina, San

Table 1. Individuals analyzed to assess the presence of *Batrachochytrium dendrobatidis* (*Bd*). Numbers in parentheses indicate number of individuals (if > 1) analyzed at each locality in the same year. Individuals with confirmed presence of *Bd* are marked in **bold**. CZAA: collection of amphibians of the Centro de Zoología Aplicada, Universidad Nacional de Córdoba, Argentina. UNRC-ECO: collection of Cátedra de Ecología, Universidad Nacional de Río Cuarto, Argentina. nd: no data available

Species	Locality	Coordinates	Year of collection	Collection no.
<i>Hypsiboas cordobae</i>	Río Yuspe	31° 22' 48.60" S, 64° 46' 22.02" W	2008	CZAa0009
<i>H. cordobae</i>	Los Gigantes	31° 24' 4.06" S, 64° 47' 14.40" W	2009	CZAa0141
<i>H. cordobae</i>	Copina	31° 34' 15.13" S, 64° 42' 33.74" W	2008	CZAa0146
<i>Melanophryniscus stelzneri</i>	Los Gigantes	31° 23' 35.62" S, 64° 46' 32.52" W	2010	CZAa0148
<i>M. stelzneri</i>	Los Gigantes	31° 23' 35.62" S, 64° 46' 32.52" W	2007	CZAa0014
<i>Odontophrynus achalensis</i> (2)	Río Yuspe	31° 22' 48.60" S, 64° 46' 22.02" W	2008	CZAa0007, CZAa0008
<i>O. achalensis</i> (2)	Los Gigantes	31° 24' 4.06" S, 64° 47' 14.40" W	2009	CZAa0142, CZAa0143
<i>O. achalensis</i>	La Ventana	31° 31' 39.74" S, 64° 52' 5.64" W	2009	CZAa0147
<i>O. achalensis</i>	El Volcán	31° 30' 24.14" S, 64° 53' 54.63" W	2010	CZAa0155
<i>Pleurodema kriegi</i>	El Volcán	31° 30' 24.14" S, 64° 53' 54.63" W	2010	CZAa0154
<i>P. kriegi</i>	Cerro blanco	31° 21' 15.41" S, 64° 39' 14.37" W	2008	CZAa0002
<i>P. kriegi</i>	La Ventana	31° 31' 39.74" S, 64° 52' 5.64" W	2010	CZAa0154
<i>Rhinella arenarum</i>	Los Gigantes	31° 24' 4.06" S, 64° 47' 14.40" W	2009	CZAa0144
<i>R. achalensis</i>	El Volcán	31° 30' 24.14" S, 64° 53' 54.63" W	2010	CZAa0164
<i>R. achalensis</i> (5)	Pampa de Achala	nd	1995	UNRC-ECO667 to 671
<i>R. achalensis</i>	La Carolina	nd	1999	UNRC-ECO644

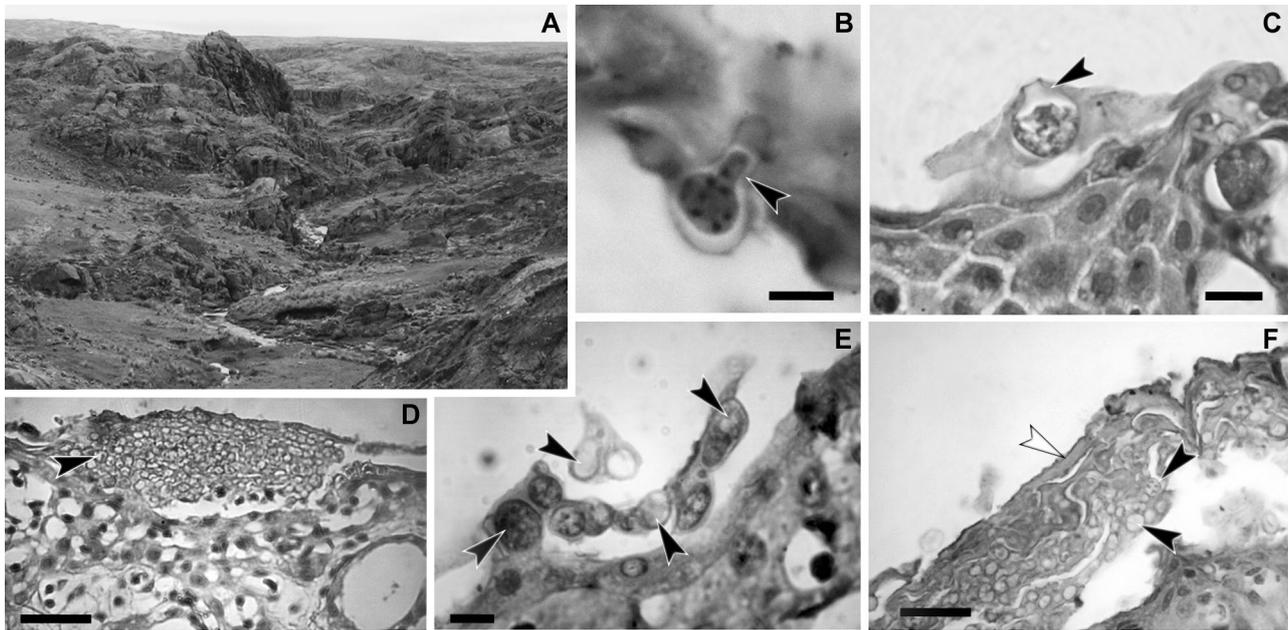


Fig. 2. (A) A stream in the upper vegetation belt of Sierras Grandes. (B) Zoosporangium with a discharge papilla (arrowhead) in the skin of *Hypsiboas cordobae*; scalebar = 10 µm. (C) Zoosporangia with a discharge papilla (arrowhead) in the skin of the endemic amphibian *Odontophrynus achalensis*; scalebar = 10 µm. (D) Severe epidermal hyperplasia and moderate hyperkeratosis in the skin of the toad *Rhinella arenarum*. Note the large numbers of *Batrachochytrium dendrobatidis* (*Bd*) zoosporangia (arrowhead) in the stratum corneum; scalebar = 50 µm. (E) Zoosporangia at different developmental stages (arrowheads) in the skin of the endemic frog *Pleurodema kriegi*; scalebar = 10 µm. (F) Different developmental stages of *Bd* in the skin of the endemic toad *R. achalensis*. Note the large numbers of *Bd* zoosporangia (black arrowheads) at different developmental stages in the stratum corneum and the severe hyperkeratosis of the skin (white arrowhead); scalebar = 50 µm

Luis, was infected with *Bd* (Table 1, Fig. 1B). Confirmed infected specimens belong to species with wide distribution ranges (*Rhinella arenarum*), as well as endemic species of the mountains of Córdoba and San Luis (*Hypsiboas cordobae*), and 3 endemic species of the mountain tops of Córdoba and San Luis (*R. achalensis*, *Pleurodema kriegi*, *Odontophrynus achalensis*). There were no signs of infection in the 2 *Melanophryniscus stelzneri* individuals collected.

Zoosporangia at different developmental stages were observed in the stratum corneum of infected individuals (Fig. 2B–F). We observed zoosporangia with discharge papillae to release the zoospores oriented toward the skin surface in *Hypsiboas cordobae* and *Odontophrynus achalensis* (Fig. 2B,C). Zoosporangia containing rounded basophilic zoospores were observed in these species. In individuals with an advanced stage of disease, the presence of empty zoosporangia was detected (Fig. 2D–F). Moreover, some colonial thalli were observed in the skin of *Pleurodema kriegi* and *Rhinella arenarum*.

Histological analyses also revealed different skin changes in infected individuals. Some species had mild epidermal hyperplasia (*Hypsiboas cordobae*),

while others (*Rhinella arenarum* and *R. achalensis*) had severe epidermal hyperplasia and moderate to severe hyperkeratosis (Fig. 2D,F).

The furthest apart sites with infected amphibians were at a straight-line distance of 252 km (Fig. 1B). The average altitude of sites with the presence of *Bd* was 1805 m a.s.l. (range: 1250 to 2200 m a.s.l.).

DISCUSSION

Threats such as disease, introduced predators, or habitat destruction are more likely to cause declines in an isolated, endemic, or rare species than in a widespread species with numerous subpopulations (Davies et al. 2000, Green 2003). Our results show that most amphibian species inhabiting the mountain tops of Córdoba and San Luis are infected with *Bd*. Because complete necropsy examination could not be performed, the possibility that the death of these frogs was due to a factor other than *Bd* infection could not be excluded. However, the magnitude of *Bd* infection and the severity of associated epidermal hyperplasia and hyperkeratosis in *Rhinella* spp. suggests that chytridiomycosis was the likely cause of

death in these species. Since the susceptibility of frogs of this area to fungal infection is still unknown, we highlight the presence of *Bd* as a potential major threat for the amphibian assemblages of this isolated mountain system, in addition to the threats already mentioned by Lavilla & di Tada (2004a,b) and Lavilla et al. (2004).

Previous records of *Bd* in Córdoba and San Luis were made at lower altitudes (near 800 m a.s.l.) and in widely distributed species (*Hypsiboas cordobae*, *Odontophrynus occidentalis*, *Leptodactylus gracilis*, *L. latrans*) (Ghirardi et al. 2010, Gutierrez et al. 2010). The observed distance between more distant points with infected amphibians recorded in the present study (Fig. 1B) in addition to the previous records suggests that *Bd* may be widely distributed over the mountains of Córdoba and San Luis, and that the presence of fungal infection in adjacent areas would also be expected, since the headwaters of numerous rivers and streams that run through other ecoregions, such as the Pampas Grasslands and Chaco dry forests, originate in these mountains. Moreover, our results not only show that the fungal pathogen is widely distributed on the tops of the mountains of Córdoba and San Luis but also represents the first record of the disease in endemic amphibians of the area (*Rhinella achalensis*, *Odontophrynus achalensis*, and *Pleurodema kriegi*).

Even though there are no studies demonstrating declines in amphibian populations of the mountains of Córdoba and San Luis provinces, personal observations of the authors during the last 5 yr suggest that some specific populations of the threatened and endemic toad *Rhinella achalensis* have declined (Lescano 2012, J. N. Lescano & S. Longo unpubl. data). Although there is no evidence to link these apparent declines with *Bd* infection, the presence of the fungal disease in the mountain tops of Córdoba and San Luis shows the urgent need to start a long-term monitoring plan to evaluate population trends in endemic amphibians of the area.

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