

Batrachochytrium dendrobatidis in Darwin's frog *Rhinoderma* spp. in Chile

J. Bourke^{1,*}, F. Mutschmann², T. Ohst^{3,4}, P. Ulmer⁵, A. Gutsche⁶, K. Busse¹,
H. Werning⁷, W. Boehme¹

¹Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany

²Exomed, Erich-Kurz-Strasse 7, 10319 Berlin, Germany

³Museum für Naturkunde Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin, Invalidenstrasse 43, 10115 Berlin, Germany

⁴Institut für Mikrobiologie und Hygiene (Charité), Humboldt-Universität zu Berlin, Dorotheenstrasse 96, 10117 Berlin, Germany

⁵Amselweg 1, 75417 Mühlacker, Mühlhausen, Germany

⁶Institute für Biologie, Sinnesbiologie, Humboldt-Universität zu Berlin, Invalidenstrasse 43, 10115 Berlin, Germany

⁷Reptilia, Terraria & Draco, Seestrasse 101, 13353 Berlin, Germany

ABSTRACT: The presence of the chytrid fungus *Batrachochytrium dendrobatidis* in Chile was evaluated in 2 endangered frog species of the genus *Rhinoderma*. Specimens from a captive rearing facility, wild populations and preserved collection material were analyzed using histological and molecular techniques. The fungus was identified in the rearing facility and in wild populations, but not in the archived frogs. This study confirms, for first time, the presence of chytridiomycosis in *Rhinoderma darwini* in Chile.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · *Rhinoderma darwini* · *Rhinoderma rufum* · Chile

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INTRODUCTION

Amphibian populations have been declining globally over the past few decades (Houlahan et al. 2000). Different causes have been proposed, including global climate change, increasing incidence of UV-B radiation, habitat loss or alteration, introduction of exotic species and emerging diseases (Young et al. 2001, Stuart et al. 2004). Chytridiomycosis is an amphibian disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al. 1999) that has been clearly linked to amphibian declines on every continent except Antarctica (Rachowicz et al. 2005). In South America, *Bd* has been reported in native amphibians from Ecuador (Ron et al. 2003), Colombia (Ruiz & Rueda-Almonacid 2008), Venezuela (Lampo et al. 2008), Brazil (Carnaval et al. 2006), Argentina (Barrionuevo & Mangione 2006), Perú (Seimon et al. 2007), Bolivia (Barrionuevo et al. 2008), and recently in

Uruguay (Borteiro et al. 2009). Chytridiomycosis would probably occur in Chile because the known *Bd* carrier, *Xenopus laevis*, had been introduced to Chile from South Africa and was established in the wild by 1944 (Weldon et al. 2004). Moreover, the temperate forest of Chile is 1 of the 11 regions of the New World that are most suitable for *Bd* presence (Ron 2005). This area includes the habitat of *Rhinoderma*, which is an amphibian genus that includes species that have rapidly and enigmatically declined in recent years, even in intact habitats (Young et al. 2001). Species from cooler climates (*R. darwini* and *R. rufum*) that are associated with streams (*R. rufum*) and high altitudes (*R. darwini*) are more likely to be infected by *Bd* (Ron et al. 2003, Woodhams et al. 2008), possibly because zoospores survive longer in cooler environments and moist microhabitats. In addition, lower temperatures can suppress amphibian immune systems (Woodhams et al. 2008). A preliminary survey was carried out to

*Email: johara.bourke@gmail.com

determine the presence of *Bd* in museum specimens, as well as in wild and captive populations of 2 endangered Chilean species: *R. darwinii* and *R. rufum*.

MATERIALS AND METHODS

Rhinoderma samples from wild populations, a captive rearing facility and preserved collection material were analyzed using histological and molecular methods (see Table 1). The methods and sample sizes were constrained by the availability of living and preserved amphibians. Samples from the wild and from the captive rearing facility were taken during the summer. Historical infections of *R. darwinii* and *R. rufum* were recorded using the preserved material collected from 1853 to 1981 and deposited at the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK, Bonn, Germany) and the Zoologisches Museum Hamburg (ZMH, Hamburg, Germany) (Fig. 1, Table 1).

Histologically prepared skin imprints, which were made from living animals in the field (wild population, 2005), as well as histologically prepared sections from dead individuals (museum specimens, frogs from the captive rearing facility and individuals from a wild population that were brought to Germany in 2007) were examined. Each frog was gently pressed between 2 glass slides to make skin imprints, which were then air dried (Nichols et al. 2001). Histological sections were obtained from each individual's hind leg, abdominal skin and internal organs. Samples were fixed with formaldehyde and stained with lactophenol blue or hematoxylin and eosin (H&E) using standard histological techniques (Presnell & Schreiber 1997). Detection of *Bd* was based on the method of Berger et al. (1999). Slides were observed using light microscopy. Samples were determined to be positive based on the presence of at least 1 zoosporangium inside the skin tissue. The molecular technique was applied to frogs collected from wild populations in 2008 and 2009 and to individuals brought to Germany in 2007. Then, the latter were transported by airplane in 2 ice chests and kept damp with wet paper towels. DNA was obtained from fresh toe clips or swabs that were collected using a sterile technique and preserved in individual Eppendorf tubes containing 98% alcohol. Swabs were taken by wiping a sterile cotton swab along the skin of the captured frog for ~30 s, focusing on forelimbs, back

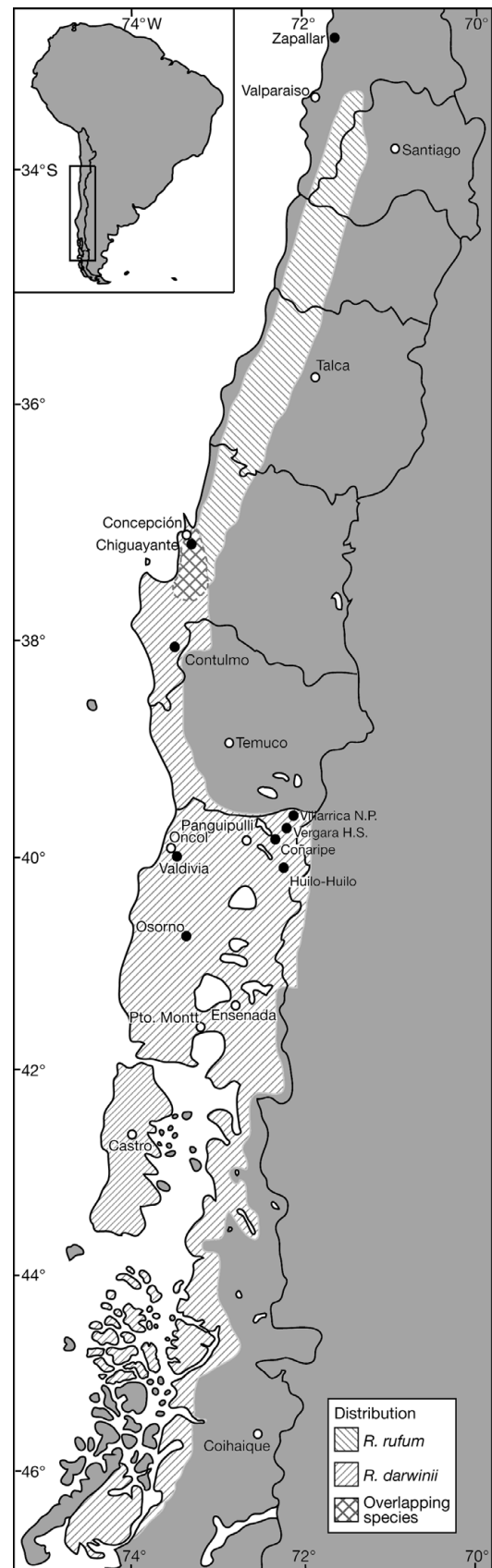


Fig. 1. *Rhinoderma* spp. The distribution of populations analyzed for the presence of *Batrachochytrium dendrobatidis* in Chile (IUCN 2009). (●) Collection locations; (○) cities for geographical reference

limbs, and the pelvic region. DNA was extracted from swabs with a blood and tissue kit (Qiagen DNeasy), following the extraction protocol. Extracted DNA samples were used in molecular detection tests. Amplification of extracted samples was conducted with standard PCR and real-time TaqMan PCR assay (Boyle et al. 2004). Real-time PCRs (rT-PCR) were run on a LightCycler 2.0 (Roche) in 20 µl reactions containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 2.5 U *Taq* polymerase (Applied Biosystems), 1.25 pmol of each dNTP, 5 pmol of each primer, 5 pmol of the TaqMan probe (with BlackBerry Quencher and 6 LNAs [locked nucleic acids]) and 2 µl template DNA. Cycling conditions were as follows:

initial denaturation for 10 min at 95°C, followed by 15 s at 95°C and 1 min at 58°C for 50 cycles. RT-PCR reactions were performed twice for each sample. The 2009 samples were also tested by normal PCR using the primers from Boyle et al. (2004). Standard PCR reactions were performed in 35 µl volumes containing the same concentrations and amounts of chemicals as for rT-PCRs (except the TaqMan probe). Samples were amplified as follows: 3 min at 96°C, followed by 50 cycles of denaturation for 15 s at 96°C, annealing for 15 s at 60°C and extension for 30 s at 72°C. This was followed by a final extension step of 3 min at 72°C. Exactly 15 µl of the PCR products were loaded on a 3% agarose gel to verify the pres-

Table 1. *Rhinoderma* spp. sampled for diagnosis of infection with *Batrachochytrium dendrobatidis*. ZMH: Zoologisches Museum Hamburg, Hamburg, Germany; ZFMK: Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany; CRF: captive rearing facility; WP: wild population; m.a.s.l.: meters above sea level; -: no voucher specimen deposited

Origin	Voucher number	Collection date	Specimens examined	Locality; Altitude (m.a.s.l.); Lat./Long.	Method of detection	Infected ind.
<i>R. rufum</i>						
ZMH	AO4439-68, AO4469-514, AO4575	1975	105	Chiguayante, Concepción; 249 m; 36° 55' S, 73° 01' W	Histological sections	0
ZFMK	ZFMK 8340/3-4/6-7/9, 8341-2/5/8	1926	10	Zapallar (labeled as Santiago) ^a ; 50–300 m; 32° 33' S, 71° 21' W	Histological sections	0
<i>R. darwini</i>						
ZFMK	ZFMK 28410-422, 36721-4/6-7, 25282-4/8-91, 21735-50, 25344, 32088-92	1853–1981	48	Contulmo; 300 m; 38° 00' S, 73° 13' W Valdivia (Panguipulli and Mehuin); 14 m; 39° 48' S, 73° 14' W Osorno; 35 m; 40° 34' S, 73° 08' W	Histological sections	0
ZFMK CRF	ZFMK no number	2005 (August)	1	Captive-bred in Bonn, Germany ^b	Histological sections	1
WP	–	2005 (Dec–Jan)	205	Villarrica National Park; 1100 m; Vergara Hot Springs; 850 m; and Coñaripe; 200–900 m; 39° S, 72° W	Skin imprints	2
WP brought to Germany	ZFMK no number	2007 (March)	30	Coñaripe; 200 m; 39° S, 72° W	PCR and histological sections	30
WP	–	2008 (March)	15	Villarrica National Park; 1100 m; 39° 30' S, 71° 52' W	PCR	0
WP	–	2008 (March)	12	Vergara Hot Springs; 850 m; 39° 30' S, 71° 52' W	PCR	0
WP	–	2009 (March)	15	Coñaripe; 200 m; 39° 33' S, 71° 59' W	PCR and histological sections	4
WP	–	2009 (March)	30	Huilo-Huilo; 617 m; 39° 52' S, 71° 54' W	PCR	1
^a Although labeled as Santiago, the sample is probably from Zapallar based on the species' absence in Santiago, the collector's residence, and mention of the Zapallar locality by Moreno in Formas et al. (1975)						
^b Original individuals brought from Panguipulli (39° 38' S, 72° 19' W), Huilo-Huilo (39° 52' S, 71° 54' W), Oncol (39° 42' S, 73° 18' W), and Ensenada (41° 10' S, 72° 32' W), Chile						

ence and length of the fragments. Each rT-PCR and standard PCR assay included control reactions containing DNA of *Bd* and no template DNA. Positive samples were determined using a positive control (PCR samples with *Bd* DNA). Contamination was also controlled by using sterilized material and isolation of individual samples. Preserved collection material was not analyzed using molecular techniques because the original fixation methods were unknown. Voucher specimens in sterile formalin were deposited in glass vials at ZFMK.

RESULTS

In June 2005, *Bd* was recognized in the histological sections from a dead individual found in the captive rearing facility at the ZFMK. This was the first record of *Bd* in *Rhinoderma darwinii*. In November of the same year, the skin imprints from 250 wild individuals from the Villarrica National Park, Vergara Hot Springs and Coñaripe, Chile, were analyzed, and 2 infected *R. darwini* individuals were found. In March 2007, 30 individuals were sent to the ZFMK captive rearing facility. All proved to be infected with *Bd*. The generalized infection of all the frogs in this group may have been the result of travel stress and the lack of isolation between individuals during transport. These frogs had pathological changes in the skin and most died between 5 and 14 d after arrival. A few survived up to 2 mo and then died. Based on this experience, massive mortality of *R. darwini* could be expected under stressful conditions.

Since no *Rhinoderma rufum* were found, they could not be used in the test for *Bd* from living animals. Histological examinations of skin from preserved *R. darwini* and *R. rufum* from the collections at ZMH and ZFMK were negative for *Bd*. Samples collected from Villarrica National Park and Vergara Hot Springs during March 2008 were *Bd* negative, but chytridiomycosis was detected from individuals sampled from Coñaripe and Huilo-Huilo during March 2009. Results are summarized in Table 1.

DISCUSSION

For the first time, the presence of *Batrachochytrium dendrobatidis* in *Rhinoderma darwini* was recorded in wild and captive populations in Chile. This expands the geographic distribution of *Bd* to one of the last countries in South America where no records of *Bd* have been published. This expands the host range to include a vocal sac breeder.

This finding serves as a warning alert for all amphibian species in Chile. Among Chilean vertebrates,

amphibians have the highest level of endemism (69%) as well as the highest rate of threatened species (36.2%). Of all countries, Chile ranks No. 11 in rates of endemism and No. 13 in percentage of endangered or extinct amphibian species (Stuart et al. 2008, IUCN 2009). Furthermore, almost one-third of Chilean amphibian species are included in the list of evolutionarily distinct and globally endangered species (EDGE 2009). Chytridiomycosis can change rate and intensity within populations—from low to massive infections, and between seasons (Woodhams et al. 2008), with infection rates usually being greater during cold seasons (Woodhams & Alford 2005, Woodhams et al. 2008). To protect the unique Chilean amphibian species and populations (especially those that are threatened with extinction) and biodiversity hotspots, it is important to monitor infected populations.

Although the presence of *Bd* in *Rhinoderma darwini* in Chile was confirmed, there was insufficient evidence to support the hypothesis that the southern introduction of *Bd* into South America was linked to the mysterious disappearance of *R. rufum* (Lips et al. 2008). Our histological results were limited to the available material collected in 1926 and 1975. Although histological methods may be less sensitive than PCR methods, the large number of specimens (105) examined from Chiguayante strongly suggests that the negative results reflect the absence of this pathogen at collection time. To determine whether the infection was present before and during the decline of *R. darwini* and the disappearance *R. rufum*, individuals collected between 1978 and 1980 need to be examined. Unfortunately, no collection specimens identified as *R. rufum* were available from these years in the museum record, but there is some chance of finding them misidentified as *R. darwini*. It would also be interesting to determine whether other Chilean amphibian species, especially populations sympatric with *R. rufum*, had been infected during that time. It was not established if chytridiomycosis, habitat alteration, climate change or all 3 factors accounted for population declines in *Rhinoderma* spp. Low *Bd* infection rates in the wild suggest that a population can exist with the infection for a long time. A dead individual found in the ZFMK captive rearing facility (August 2005) had only a slight chytrid infection, and the actual cause of death was egg retention. Some frog populations persist with stable infections, being in equilibrium with this disease and showing no evidence of decline (Retallick et al. 2004); this applies not only to tropical species (Lampo et al. 2008), but also for species from cooler environments (Longcore et al. 2007, Mutschmann 2007). This possibility provides some hope for the survival of the endangered *Rhinoderma* species.

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