Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians

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The current biodiversity crisis encompasses a sixth mass extinction event affecting the entire class of amphibians. The infectious disease chytridiomycosis is considered one of the major drivers of global amphibian population decline and extinction and is thought to be caused by a single species of aquatic fungus, Batrachochytrium dendrobatidis. However, several amphibian population declines remain unexplained, among them a steep decrease in fire salamander populations (Salamandra salamandra) that has brought this species to the edge of local extinction. Here we isolated and characterized a unique chytrid fungus, Batrachochytrium salamandrivoranessp. nov., from this salamander population. This chytrid causes erosive skin disease and rapid mortality in experimentally infected fire salamanders and was present in skin lesions of salamanders found dead during the decline event. Together with the closely related B. dendrobatidis, this taxon forms a well-supported chytridiomycete clade, adapted to vertebrate hosts and highly pathogenic to amphibians. However, the lower thermal growth preference of B. salamandrivorans, compared with B. dendrobatidis, and resistance of midwife toads (Alytes obstetricans) to experimental infection with B. salamandrivorans suggest differential niche occupation of the two chytrid fungi.

amphibian decline | emerging infectious disease | ecosystem health

Ampbellians have become an icon of the global biodiversity crisis (1). Although a variety of factors are involved in amphibian decline worldwide, fungal chytridiomycosis has been identified as one of the major infectious diseases involved, resulting in the extinction of >40% of amphibian species in areas in Central America and widespread losses across Europe, Australia, and North America (2, 3). Chytridiomycosis is currently considered to be caused by a single species of fungus, Batrachochytrium dendrobatidis, which is the only chytridiomycete taxon known to parasitize vertebrate hosts. However, B. dendrobatidis and other factors known to cause amphibian decline fail to explain several recent amphibian population losses (4, 5).

A dramatic and enigmatic mortality event, which has brought this species to the edge of extinction, was recently reported among fire salamanders (Salamandra salamandra) in The Netherlands (5). Since 2010, the species has declined, with only 4% of the population remaining in 2013. This rapid decline coincided with the finding of dead animals in the field (5). The recent startup of an ex situ conservation program for 39 of the remaining fire salamanders was compromised by the unexplained death of 49% of the captive animals between November and December 2012. Attempts to identify known amphibian infectious agents, including B. dendrobatidis, in these salamanders yielded negative results (5). Instead, we found, isolated, and characterized a second, highly pathogenic chytrid fungus from this decline event that occupies a different niche compared with B. dendrobatidis.

Results and Discussion

The chytrid fungus was isolated from the skin of fire salamanders from the affected population in Bunderbos (N50°54′51″, E5°44′59″). The Netherlands. Phylogenetic analyses including a broad range of representative chytrid species show that this fungus represents a previously undescribed lineage that forms a clade with B. dendrobatidis (Fig. 1; Table S1). Its considerable genetic distance from B. dendrobatidis (3.47–4.47% for the 1,513 18S + 28S rRNA base pairs) compared with the shallow divergences between B. dendrobatidis isolates (6) warrants the description of a unique species within the chytridiomycete order Rhizophysiales (family incertae sedis): Batrachochytrium salamandrivoranessp. nov. The unique chytrid represented by isolate AMPF13/1 (the holotype in liquid nitrogen at Ghent University) is the second chytrid known to parasitize and kill amphibians. In vitro, the unique taxon produces motile zoospores, which emerge from colonial (a single thallus containing multiple, walled sporangia) or monocentric thalli (Fig. 2A). The most obvious morphological differences, compared with the B. dendrobatidis type strain, are the formation of germ tubes in vitro (Fig. 2B; Fig. S1) and the abundant formation of colonial thalli both in vitro and in vivo (Fig. 3B). B. salamandrivorans grew at temperatures as low as 5°C, with optimal growth between 10°C and 15°C and death at ≥25°C, a markedly lower thermal preference compared with B. dendrobatidis (7) (Fig. 4).

Significance

Chytridiomycosis has resulted in the serious decline and extinction of >200 species of amphibians worldwide and poses the greatest threat to biodiversity of any known disease. This fungal disease is currently known to be caused by Batrachochytrium dendrobatidis, hitherto the only species within the entire phylum of the Chytridiomycota known to parasitize vertebrate hosts. We describe the discovery of a second highly divergent, chytrid pathogen, Batrachochytrium salamandrivorans sp. nov., that causes lethal skin infections in salamanders, which has resulted in steep declines in salamander populations in northwestern Europe. Our finding provides another explanation for the phenomenon of amphibian biodiversity loss that is emblematic of the current global biodiversity crisis.


The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KC762294, KX762295, and KX762296), and the description of the fungus has been deposited in MycoBank (accession no. MB803904).

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Infected fire salamanders died within 7 d after a short episode of anorexia, atrophy, and ataxia. The pathology consistently comprised multifocal superficial erosions and deep ulcerations in the skin all over the body. Keratinocytes with eosinophilic necrosis and marginalized nuclei were at the periphery of the erosions. Each of these keratinocytes contained one centrally located thallus, the majority being segmented (colonial thalli). Bacteria superficially colonized the ulcers. Additionally, anywhere in the skin, small foci of keratinocytes immediately below the damaged keratin layer were found. These presented similar eosinophilic necrosis, marginalized nuclei, and centrally located colonial thalli. The intraepidermal organisms did stain with immunohistochemistry (8) (Fig. 3A). Transmission electron microscopic examination of the skin lesions confirmed the presence of intracellular structures consistent with the colonial thalli (Fig. 3B). All animals were also screened for a wide array of other infectious diseases, but no evidence for any other pathology was found: neither PCR (9) nor quantitative PCR (qPCR) (10) suggested the presence of chytrid B. dendrobatidis DNA in the skin samples. Virological examination (including PCR for the detection of herpes viruses (11), adenoviruses (12), and ranaviruses (13) and inoculation of IgH2 (iguana heart epithelial cells) and RTG (rainbow trout gill) cell cultures for general virological investigation) was negative. Ziehl Neelsen staining, PCR for Chlamydiaceae (14), and bacterial isolation attempts did not yield any evidence of bacterial infections.

To further demonstrate that salamandrid mortality was caused by B. salamandrivorans, we performed infection experiments on healthy fire salamanders (n = 5) by exposing them to 5,000 zoospores of B. salamandrivorans for 24 h. All animals died 12–18 d after inoculation after a 1- to 2-d episode of ataxia. Isolation was attempted and succeeded from one deceased salamander. PCR (described below) showed that B. salamandrivorans DNA was present in all five infected animals, coinciding with histopathological lesions consisting of focal epidermal ulceration with very high numbers of colonial thalli of B. salamandrivorans, which matched the lesions found in wild animals. B. salamandrivorans–induced lesions are characterized by marked skin ulceration, opposed to those caused by B. dendrobatidis, which typically induces epidermal hyperplasia and hyperkeratosis (15). No clinical signs or histopathological lesions were observed in the uninfected negative control animals (n = 5). Additionally, we put two healthy fire salamanders in a terrarium with an infected individual for 2 d. One salamander died 22 d after contact and the other 27 d after being placed with the infected animal. Histology, immunohistochemistry (8), and PCR demonstrated the presence of high numbers of B. salamandrivorans in their epidermal layers, with lesions identical to those described above. Cohousing on damp toweling effectively transmitted B. salamandrivorans and caused death in <1 mo. Experimentally infected midwife toads (Alytes obstetricans), the species that is most highly susceptible to infection by B. dendrobatidis in Europe (16, 17), did not show any signs of colonization by B. salamandrivorans, as determined by immunohistochemistry and PCR, or disease, suggesting a differential amphibian host range for the two chytrids. Amphibians will clearly benefit from the rapid identification of areas in which B. salamandrivorans is present. We therefore designed diagnostic species-specific PCR primers to amplify the 5.8S ribosomal RNA gene and its flanking internal transcribed spacer regions: ITS1 and ITS2. Our set of primers STerF and STerR amplified B. salamandrivorans in all positive tissues examined. Importantly, these primers did not amplify any of the nine tested strains from all three B. dendrobatidis lineages known to infect Europe and therefore provide a rapid noninvasive method for detecting of B. salamandrivorans infections. Furthermore, by using the newly developed PCR primers, we were also able to detect B. salamandrivorans DNA in remains of the epidermises of six wild fire salamanders (from Bunderbos, The Netherlands) that were found dead in 2010 or 2011 and were stored at −70 °C. B. salamandrivorans was found present in skin swabs from all five experimentally infected and moribund fire salamanders, but in none of the midwife toads and noninfected...
fire salamanders. Additionally, 13 of 33 swabs collected from live fire salamanders from the declining population in Bunderbos, The Netherlands, in 2010 tested positive with this PCR, in contrast to 0 of 51 swabs from fire salamanders from a stable population in Belgium. Our PCR method thus allows the rapid screening of both extant populations and archived specimens for the presence of Batrachochytrium salamandrivorans–induced chytridiomycosis.

Chytridiomycosis in amphibians can no longer be attributed to a single species of chytrid, but can be caused by either Batrachochytrium dendrobatidis or Batrachochytrium salamandrivorans. Our results reveal striking similarities and differences between Batrachochytrium salamandrivorans and the behavior of the hypervirulent global pandemic lineage of Batrachochytrium dendrobatidis (18). Both fungal species share at least the following hallmarks: (1) induction of a lethal skin disease and (ii) association with mortality events and severe population decline. In contrast, it is as yet unclear to what extent Batrachochytrium salamandrivorans is capable of infecting a broad amphibian host range, as is the case for Batrachochytrium dendrobatidis (3). However, development of erosive vs. hyperplastic/hyperkeratotic skin lesions, failure to experimentally infect midwife toads, and relatively low thermal preferences of Batrachochytrium salamandrivorans suggest differential host specificity of the two pathogens and possibly a differential effect on amphibian assemblages. Because the majority of recent Batrachochytrium dendrobatidis surveillance worldwide is based on the Batrachochytrium dendrobatidis–specific qPCR (10), it is currently impossible to estimate the extent and impact of Batrachochytrium salamandrivorans on amphibian populations worldwide using the Batrachochytrium dendrobatidis mapping framework (19). However, the emergence of the pathogenic Batrachochytrium salamandrivorans chytrid fungus is worrying and warrants close monitoring, urgent risk analysis, and its inclusion in any monitoring program assessing amphibian population health.

Taxonomy. Batrachochytrium salamandrivorans Martel, Blooi, Bos-suyt and Pasmans sp. nov. MycoBank accession no. MB809904. In vitro (tryptone-gelatin hydrolysate-lactose broth). Thalli predominantly monomorphic, although some colonial. Development exogenous with sporangia forming at tip of germ tube. Rhizoids fine, isodiametric, extending from a single or several areas, lacking subsporangial swelling; Sporangium diameter 15.7–50.3 μm (average, 27.9 μm). One to several discharge papillae; cell wall at tip discharge papillae forms plugs that deliquesce resulting in release of motile zoospores. Motile zoospores roughly spherical, with highly irregular surface and cell surface projections; diameter 4.0–5.5 μm (average 4.6 μm). Resting spore not observed. Growth at 5, 10, 15, 20, and 22 °C, but not at temperatures ≥24 °C. Death of thalli after 5 d at 25 °C. Five-day generation time at 15 °C.

In Vivo. In epidermis of amphibians; forming predominantly colonial thalli that contain several walled sporangia. Thalli located inside keratinocytes; diameter 6.9–17.2 μm (average 12.2 ± 1.9 μm, n = 50).

Zoospore Ultrastructure. Ultrastructure highly similar to that of Batrachochytrium dendrobatidis. Nucleus located outside the ribosomal mass, multiple mitochondria and numerous lipid globules. Position of the nonflagellated centriole in free swimming zoospores varies from angled to parallel to kinetosome.

tDNA Sequences. Partial nucSSU tDNA GenBank accession no. KC762294, partial nucLSU tDNA GenBank accession no. KC762293, partial ITS1-5.8S-ITS2 tDNA GenBank accession no. KC762295.

Holotype. Isolate AMFP13/1 (CBS 135744) from a fire salamander (Salamandra salamandra), kept in liquid nitrogen at Ghent University.

Etymology. The species epithet salamandrivorans (sa.la.man.dri-vo.rans. L. n. salamandra, salamander; L. part. adj. vorans, eating, devouring; N.L.part. adj. salamandrivorans, salamander-devouring) refers to the extensive skin destruction and rapid mortality observed in infected salamanders.

Materials and Methods

Postmortem Examination of Fire Salamanders. Six S. salamandra that died in captivity between November and December 2012 were subject to gross necropsy, histopathology, and routine bacteriological, mycological, and virological examinations. Histological examination of liver, spleen, kidney, lung, gonad, midgut, and skin was done using microscopic examination of paraffin-embedded, 5-μm tissue sections stained with H&E, Ziehl Neelsen, or periodic acid shift. A 1:10 (vol:vol) tissue suspension of these organs in PBS was inoculated on sheep blood and tryptic soy agar and incubated at 20 °C and 30 °C. A liver suspension was inoculated on IgH2 and RTG cells. PCRs were performed to detect the presence of herpesviruses (11), adenoviruses (12), iridoviruses (13), Chlamydiales (14), and Batrachochytrium dendrobatidis (B. dendrobatidis) (9, 10). Immunohistochemistry was performed on all skin samples to detect B. dendrobatidis antigens (8). Transmission electron microscopy of epidermal samples was performed with glutaraldehyde fixation in 0.05 M sodium cacodylate buffer, followed by osmium tetroxide postfixation.
1% osmium tetroxide postfixation, and en bloc staining for 1 h in a 1% solution of uranyl acetate. Five S. salamandra specimens were found dead in the field during 2010 and 2011. Due to the severe autolysis of these animals, the postmortem examination was limited to skin histopathology and PCR for the detection of herpesviruses, adenoviruses, iridoviruses, Chlamydiaceae, and B. dendrobatidis.

**B. salamandrivorans Strain Isolation and Culture Conditions.** Chytrid isolation on tryptone-gelatin hydrolysate-lactose (TGHL) agar plates containing penicillin/streptomycin (200 μg/mL) at 20 °C was attempted from the dead S. salamandra as described previously for the isolation of B. dendrobatidis (7). Skin samples without contaminating bacterial or fungal growth were transferred to TGHL broth once zoospores were seen on the agar plates. The isolate was subsequently subcultured in TGHL broth in cell culture flasks at 15–20 °C. A 10-d-old subculture was frozen in liquid nitrogen (20). To obtain zoospores, 1 mL of a culture growing in TGHL broth was transferred to a TGHL agar plate and incubated for 5–10 at 15 °C. Zoospores were obtained by washing the agar plate with 2 mL of 0.2-μm filtered pond water. The number of zoospores in the suspension was determined using a hemocytometer.

To determine thermal growth conditions, 200 μL of a 5-d-old B. salamandrivorans culture in TGHL broth at 15 °C was transferred to the wells of a 24-well plate, and 0.8 mL of TGHL broth was added. The plates were incubated at 5 °C, 10 °C, 15 °C, 20 °C, 22 °C, 23 °C, 25 °C, and/or 30 °C ± 1 °C for 10 d. Growth was defined as a significant increase of the surface of the well covered by the fungus compared with wells incubated at 30 °C (which is above the lethal temperature for B. salamandrivorans) and the presence of motile zoospores. The surface coverage was determined by image analysis (GNU Image Manipulation Program) of pictures, taken through an inverted light microscope (Nikon Eclipse t100, 20× magnification). Each condition was tested in triplicate. If no growth was seen after 10 d of incubation, the plates were further incubated at 15 °C. Cultures were considered dead if no growth occurred within 10 d.

**B. salamandrivorans Molecular Characterization and Diagnostic PCR Development.** PCRs were done on the chytrid culture obtained to amplify the 18S, 28S, and 5.8S rRNA genes and the flanking ITS regions ITS1 and ITS2 (21). Based on the similarity of the ITS sequences, the primer set (STE f, 5′TGCCTGACCTCCCTCCTCCTCA3′ and STE r 5′TGAAGGACCTGACCTGACCTAC3′) was developed and used to detect the 5.8S rRNA gene of B. salamandrivorans in skin samples from the six S. salamandra found dead in the field, six animals that died in captivity, and 33 swabs collected from S. salamandra in Bunderbos in 2010. Amplification reactions consisted of 10 ng DNA, 1 μM of each primer, 1.5 mM MgCl2, 1× Taq buffer, 0.2 mM of each dNTP, and 0.8 units of Taq polymerase in a volume of 20 μL. PCR amplification was performed under the following conditions: 10 min at 93 °C, followed by 30 cycles of 45 s at 93 °C, 45 s at 55 °C, 60 s at 72 °C, and 10 min at 72 °C. DNA of a pure culture of B. salamandrivorans was used as a positive control. Using primer set STEF and STEr, we assessed whether DNA of nine B. dendrobatidis strains would be amplified—Cape lineage (BDCAPE) isolates: SA1D, TF5a1, and CCB1; Swiss lineage (BDCH) isolates: Allomyces arbuscula and Catenaria anguillulae were used as outgroup taxa. Alignment was done with ClustalX 2.0.10 (24), and ambiguously aligned fragments were excluded for further analysis, resulting in a 1,513-bp reliably aligned data matrix. Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* 4.0b10 (25). Heuristic MP searches were executed in 10,000 replicates, with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping. The strict consensus tree of 81 equally most parsimonious trees (tree length = 1,471) supported the (B. dendrobatidis, B. salamandrivorans) sister relationship and received an MP bootstrap support of 100. Bayesian and likelihood analyses were performed with the GTR + G + I model of DNA substitution. For the likelihood analyses, heuristic searches were performed with substitution rates, γ-shape parameter, and proportion of invariable sites estimated from neighbor joining trees. These parameters were reestimated from the best ML tree found thus far, and the tree was submitted to additional rounds of TBR swapping; this procedure was repeated several times. These maximum likelihood analyses resulted in a single best tree [ln L = 5,652,04266; pinvar = 0.031011; shape parameter α = 0.60887]. ML bootstrapping was done in 1,000 replicates with fixed parameters.

Bayesian analyses were done with MrBayes 3.1.2 (26). Two runs of four Markov chain Monte Carlo (MCMC) chains each were executed in parallel for 5,000,000 generations, with a sampling interval of 500 generations and a burn-in corresponding to the first 1,000,000 generations. Posterior probabilities for clades were obtained by combining the posterior probabilities of the parallel runs. Convergence of the parallel runs was confirmed by split frequency SDS (<0.01) and potential scale reduction factors (approximating 1.0) for all model parameters.

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