

Spontaneous oral chytridiomycosis in wild bullfrog tadpoles in Japan

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ABSTRACT. *Batrachochytrium dendrobatidis* (Bd) infects Anuran larvae (tadpole) mouthparts and causes oral chytridiomycosis, which can be diagnosed in tadpoles by detecting mouthparts deformities. However, oral chytridiomycosis may or may not be observable, depending on species, tadpole stage and season, and has never been reported in Japan. We aimed to observe oral chytridiomycosis characteristics in bullfrog (*Lithobates catesbeiana*) tadpoles, determine associated pathologic features and investigate the usability of bullfrog tadpoles in Japanese Bd field surveys. Wild-captured bullfrog tadpole mouthparts were examined macroscopically, histopathologically and by molecular biological examination. Macroscopic lesions were observed in 21 of 59 tadpole mouthparts. Lesions were most frequently located in the lower jaw sheaths and were mainly recognized by partial depigmentation (11 tadpoles; some were completely depigmented) and thinning of the pigmented layer (10 tadpoles). Partial defects of the tips and blunt cutting edges of the jaw sheaths were observed with severe jaw sheath depigmentation. Whitened tooth rows were observed in 7 tadpoles. Histologically, the stratified epithelium (pigmented epithelium) showed partial or diffuse hypopigmentation or pigment loss. Irregular stratified epithelium thickening with hyperkeratosis or parakeratosis was observed in the jaw sheaths. Bd infection was confirmed in 20 of 21 tadpoles presenting jaw sheath deformities, by histopathological examination and/or nested polymerase chain reaction. Depigmentation and thinning of the pigmented layers of jaw sheaths were associated with Bd infection. Thus, diagnosis of Bd infection by macroscopic observation of bullfrog tadpole mouthparts is feasible. This is the first report of oral chytridiomycosis in wild bullfrog tadpoles in Japan.

KEY WORDS: bullfrog, chytridiomycosis, mouthparts, pathology, tadpole

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Batrachochytrium dendrobatidis (Bd) is an important emerging pathogen implicated in the global decline of amphibian populations and species [1]. Thus, Bd is listed by The World Organization for Animal Health (OIE) as a pathogen that should be monitored to facilitate ecosystem conservation and biodiversity [18]. The first case of chytridiomycosis in Asia was confirmed in captive amphibians in Japan in December 2006 [29]. The Japanese Ministry of the Environment and National Institute for Environmental Studies investigated the prevalence of Bd in mainly postmetamorphic amphibians in the Japanese field [5]. However, the accuracy of the previous study is uncertain, because it was conducted between July and September, which is the hottest season and coincides with low Bd activity. More studies reporting accurate records regarding Bd distribution in Japan and its prevalence in Japanese amphibians are needed to assess the effects of Bd on native Japanese amphibians and the magnitude of the impact. Bd infects only the keratinized layer. Target organs for the detection of Bd infection must be selected according to the developmental stage, because

the distribution of the stratum corneum differs between post-metamorphic and larval amphibians [1, 10, 13]. In a post-metamorphic amphibian (frog), where the keratinized layer is distributed over the entire body, the target areas for the detection of Bd infection are the ventral, thigh and digital skin [2, 23]. In a larval amphibian (tadpole), jaw sheaths (beak) and tooth rows, the only areas with a keratinized layer, are the target areas for the detection of Bd infection [1, 17]. Additionally, Bd is known to cause macroscopic changes, such as mouthpart deformities in tadpoles [25], and the detection of Bd infection has been based on the detection of these changes [3, 4, 17, 28]. However, Bd infections manifest as mouthpart deformities in amphibians with long larval stages, but not in all amphibian species [25]. Thirty-seven species and 5 subspecies of the order Anura are found in Japan [20]. To date, neither the prevalence of oral chytridiomycosis in Japan nor its pathological features have been studied, and oral chytridiomycosis diagnoses have also not been verified in Japan. When targeting animals, we considered 3 characteristics: animals with long tadpole stages, those that are widely distributed in Japan and those that are easily caught. On the main island of Japan, only 2 species have tadpole stages with long durations: the bullfrog (*Lithobates catesbeiana*) and the wrinkled frog (*Rana rugosa*). Although both species are distributed widely across Japan, the distribution of the bullfrog is wider than that of the wrinkled frog. The bullfrog ranges in size from 120 to 150 mm, and the wrinkled frog is about 25 mm. Because bullfrogs are easier to handle than wrinkled

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frogs, the former were used in this study. The objective of the study was to confirm oral chytridiomycosis diagnoses and to determine its pathological features in bullfrogs.

MATERIALS AND METHODS

Fifty-nine wild bullfrog tadpoles (developmental stages 25 to 41 [6]) were collected randomly from a stagnant part of the Sagami River in Kanagawa, Japan (latitude, N 35.56139; longitude, E 139.323111). The collecting season was defined as between May and July 2010 so that fully grown tadpoles that had spent long durations in the larval stage could be collected. The Sagami River is a class A river, which denotes a waterway of special importance protected by the Ministry of Land, Infrastructure, Transport and Tourism. There have never been mines, factories or cultivated areas around the collection area.

All animals were euthanized by spinal cord disruption after being anesthetized by a 5-min immersion in a bath of 0.1% eugenol solution, an anesthetic drug for fishes and crustaceans (FA100, Tamura-Seiyaku Corp., Tokyo, Japan) [7, 11]. Then, the animals were measured for length and body weight, their mouthparts were inspected, and findings were recorded with a camera. Mouthparts were evaluated under macroscopic examination. These were divided into 4 sites (the upper and lower jaw sheaths and the upper and lower tooth rows) and were graded as follows. Jaw sheath depigmentation was scored as grade 1 when 1 site in the jaw sheath was involved, as grade 2 when 2 jaw sheath sites were involved, and as grade 3 when >50% of the jaw sheath was involved. Thinning of the pigmented layer on the jaw sheaths was scored as grade 1 in cases of thinning of 50% of the jaw sheath and as grade 2 in cases of thinning of >50% of the jaw sheath. Depigmentation of the tooth rows (whitening of the tooth rows) was scored as grade 1 in cases of moderate depigmentation and as grade 2 in cases of severe depigmentation. Partial defects and deformities of the jaw sheaths were recorded. The mouthparts of all tadpoles were swabbed with sterile cotton swabs (Men-tip 1P1501, Nihon-Menbo Co., Tokyo, Japan). Each sample was stored in a sterile microtube and frozen at -20°C until the molecular biological examination. Then, the mouthparts were excised from the frontal section and fixed in 10% neutral-buffered formalin.

For histopathological examination, fixed mouthparts were cut along the longitudinal midline, processed routinely in paraffin wax, sectioned at a thickness of 3 to 4 μm and stained with hematoxylin and eosin. Some samples were stained with Fungiflora Y (Biomate, Tokyo, Japan), which has a high affinity for fungal cell wall polysaccharides, as needed. Additionally, to confirm the presence of Bd zoospores and zoosporangia, some samples were immunohistochemically stained using conventional methods with diaminobenzidine as the chromogen and hematoxylin as the counterstain. The primary antibody was anti-Bd rabbit serum produced by our laboratory and diluted at 1:100. Moreover, pretreatment was performed by microwaving at 90°C for 9 min. To prepare anti-Bd rabbit serum, 3 rabbits were intradermally inoculated 3 times each. In each inoculation, the rabbits received 40 mg of

cultured and washed Bd. The immunoreactivities of the animals' sera were confirmed by immunohistochemical staining.

In the molecular biological examinations, Bd DNA was extracted from swab samples using NucleoSpin® Tissue (Macherey-Nagel, Duren, Germany). For polymerase chain reaction (PCR) testing, a nested PCR assay was performed on the extracted DNA using primers specific for Bd internal transcribed spacer (ITS) gene regions (ITS 1 and ITS 2), as described by Goka *et al.* [5]. The thermal cycling conditions were an initial 9 min at 95°C followed by 30 sec at 94°C , 30 sec at 50°C and 2 min at 72°C for 30 cycles followed by a final 7 min at 72°C . PCR products were separated on 2% agarose gels, and bands of DNA fragments were visualized by ethidium bromide staining under UV light.

The sampling of bullfrog tadpoles was performed under permits issued by the Ministry of the Environment Government of Japan. The experimental procedure of the study was approved by the Ethics Committee for Animal Experimentation of Azabu University, Sagamihara, Japan.

RESULTS

Macroscopically, mouthpart lesions were observed in 21 of 59 tadpoles. The lesions had formed on the lower jaw sheaths and were mainly observed as segmental depigmentation (pigmentation gaps) (Fig. 1c); some lesions showed substantial depigmentation (Fig. 1d) and thinning of the pigmented layer (Fig. 1b). Depigmentation of the jaw sheath was observed in 11 tadpoles; this was graded as grade 1 in 3 tadpoles, grade 2 in 2 and grade 3 in 6. Thinning of the pigmented layer of the jaw sheaths was observed in 10 tadpoles, graded as grade 1 in 6 tadpoles and as grade 2 in 4. Deformities, such as a partial defect at the tip of the jaw sheath and blunt cutting edges, were detected in severely depigmented jaw sheaths. Depigmentation of the tooth rows (loss of teeth) was observed in 7 tadpoles (Fig. 1d).

Histologically, the superficial layer of the tip of a normal jaw sheath consists of flat cells with abundant black pigment (pigmented epithelial layer) (Fig. 2a). The pigmented epithelial layer gradually thins as it advances to the base of the jaw sheath and is finally replaced by the stratum corneum. A stratified squamous epithelium exists beneath the pigmented epithelial layer and is similar to the oral mucosa. Histologically, depigmented jaw sheaths demonstrated focal or diffuse loss of the pigmented epithelial layer and were thickened by hyperkeratosis or parakeratosis. In some cases, cell debris adhered to the surface of the stratum corneum, and stratified squamous epithelial cells located under the stratum corneum proliferated out of alignment and were irregularly thickened (Fig. 2b). Tadpoles with severe depigmentation and mouthpart deformities lost the pigmented epithelial layer and demonstrated more severely proliferative stratified squamous epithelial cells. The tips to the bases of the jaw sheaths were covered with thickened stratified squamous epithelial cells, and their surfaces were observed as thin stratum corneum. Additionally, cellular degeneration, solitary cell necrosis in squamous epithelial cells and subcutaneous tissue edema were observed, but no inflammatory cell infiltration was

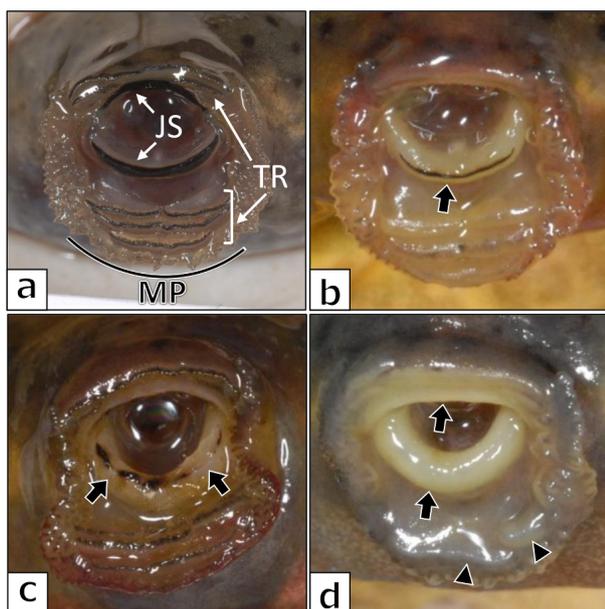


Fig. 1. Macroscopic features of normal specimens (a) and oral chytridiomycosis in the mouthparts of bullfrog tadpoles (b–d). (a) From the center to the outside, normal mouthparts consist of the upper and lower jaw sheaths (JS), upper and lower tooth rows (TR), and marginal papillae (MP). Jaw sheaths and tooth rows are highly pigmented. (b) Thinning of the pigmented layer of the lower jaw sheath (arrow). (c) Partial depigmentation and defects in the lower jaw sheath (arrows). (d) Substantial depigmentation of the upper and lower jaw sheaths (arrows) and tooth row depigmentation (loss of teeth) (arrowheads).

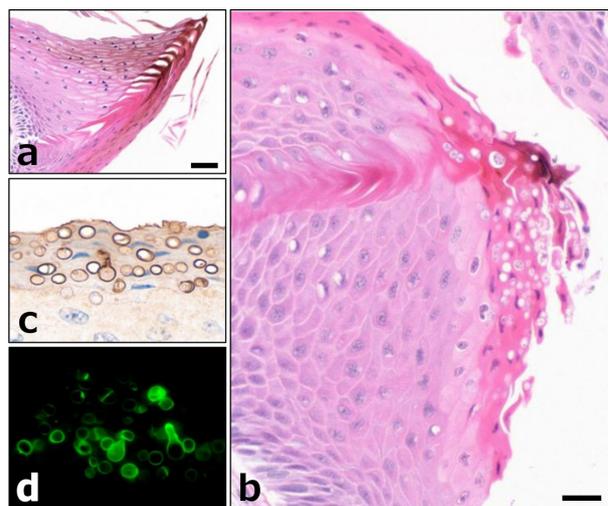


Fig. 2. Histological features of normal specimens (a) and oral chytridiomycosis in mouthparts (lower jaw sheaths) of the bullfrog tadpole (b–d). (a) The superficial layer of the tip of the lower jaw sheath consists of flat cells that contain abundant black pigment (pigmented epithelial layer), and the tip of the jaw sheath is sharp. Bar=50 μ m. (b) The cutting edges of the jaw sheath have a blunt shape, and the pigment is diffusely lost in the pigmented epithelial layer and slightly found at the tips of the jaw sheath. The lost pigmented epithelial layer is replaced by stratified corneum, which was caused by hyperkeratosis. *Batrachochytrium dendrobatidis* (Bd) zoosporangia are found in spherical and flask-like forms in the stratified corneum. Bar=25 μ m. (c, d) Bd zoosporangia are clearly observed on immunohistochemical staining (c) and Fungiflora Y staining (d).

Table 1. Numbers of *Batrachochytrium dendrobatidis* infections in tadpoles with and without mouthpart deformities from PCR tests and histopathological examinations

		Number with mouthpart deformities	Number without mouthpart deformities
Confirmation of Bd infection	Nested PCR test	3	3
	Histopathological examination	7	8
	Nested PCR test and histopathological examination	10	6
Negative		1	21
Total		21	38

noted. Thinning of the pigmented layer was seen as a diffuse decrease in pigmentation (Fig. 2b). The superficial stratum corneum was severely infected, and Bd thalli were observed to have spherical and occasionally flask-like forms (Fig. 2c and 2d). Some zoosporangia contained zoospores. These Bd structures were distinctly observed by Fungiflora Y staining and immunohistochemical staining. We could not conduct a histological examination for tooth rows and marginal papilla consistently, because these lesions were delicate and very small. However, loss of teeth was confirmed in some tadpoles with depigmentation of the tooth rows.

Bd infection was detected in 37 of 59 tadpoles, confirmed by histopathological examination in 15 tadpoles, by nested PCR in 6 and by both histopathological examination and

nested PCR in 16 (Table 1). Twenty of 21 tadpoles with macroscopic lesions were confirmed to have Bd infections (confirmed by histopathological examination in 7 tadpoles, by PCR in 3 and by both examinations in 10) (Table 1). Seventeen of 38 tadpoles that did not show obvious macroscopic findings were also confirmed to have Bd infections (by histopathological examination in 8 tadpoles, by PCR in 3 and by both examinations in 6) (Table 1). Histologically, the jaw sheaths of these tadpoles showed a slight or no decrease in the pigmented epithelial layer and proliferation of stratified epithelial cells; a small number of Bd were present in the stratified corneum of the basement of the jaw sheaths and palate along the pigmented epithelial layer. Analysis of the PCR products confirmed infection with Bd.

DISCUSSION

Characteristics, such as the number of mouthpart components and their development, vary among Anuran species. However, the basic structure of the mouthparts is generally the same; mouthparts of larval Anurans consist of the jaw sheaths (beak), tooth rows and marginal papilla. The stratified corneum is distributed on the surfaces of these structures. Bd uses keratin (stratified corneum) for growth and infects only the mouthparts of larval Anurans [4, 16, 25, 28]. Macroscopic observation of mouthpart deformities caused by Bd infection has been reported in the Mountain Yellow-Legged frog (*Rana muscosa*) and *Mixophyes sp.*; depigmentation of the jaw sheaths and tooth rows and swollen and reddish labial papillae were confirmed in *R. muscosa*, and depigmentation of the jaw sheaths and lost or shortened tooth rows were confirmed in *Mixophyes sp.* [4, 28]. Histologically, these lesions consisted of pigmented epithelial layer replacement with stratified amelanotic cells (loss of pigmentation), erosion and epithelial hyperplasia in the stratified corneum in the jaw sheaths, and loss of tooth row pigmentation (loss of teeth). In our study, the observed pathological changes were consistent with those in previous studies, with the exception that the frequency of tooth row lesions was slight. In our study, 95.2% (20/21) of tadpoles that had macroscopically visible mouthpart lesions were infected with Bd. Therefore, we concluded that mouthpart lesions were associated with Bd infection and diagnosed oral chytridiomycosis. Apart from Bd infection, larval Anuran mouthpart deformities can be caused by coal ash pollution [26]; exposure to high levels of As, Ba, Cd, Cr and Se in the environment [27]; dichlorodiphenyl-trichloroethane exposure [19]; and corticosterone exposure [9]. Additionally, mouthpart depigmentation was associated with low temperatures in the Mountain Yellow-Legged frog (*Rana muscosa*) [24]. A previous study recommended that the survey takes place at least 3 weeks after lake melt-out. However, in this study, there seemed to be no industrial facility that discharged agents known to cause mouthpart deformities around the collection site, and the survey was conducted between May and July 2010, which corresponds to the late spring to summer season. Padgett-Flohr *et al.* investigated the association between larval mouthpart deformities and Bd infection in 2,034 amphibians from 4 Anuran species in California, U.S.A., including 434 bullfrog tadpoles [21]. This previous study reported that mouthpart deformities were not associated with Bd infection, because only 11% of tadpoles with mouthpart deformities were infected with Bd. However, the prevalence of Bd infection in bullfrog tadpoles with mouthpart deformities was largely different between August 2003 and May 2004; the detection rates of Bd infection in bullfrog tadpoles with observed mouthpart defects were 0.5% and 92.6%, respectively [21]. This result may be associated with the ecological features of Bd or the life cycle of the bullfrog. The optimal temperature for Bd growth is 17–25°C; it stops growing or grows slowly above 28°C [22]. The prevalence of Bd in bullfrog tadpoles may decrease in the summer (August) when the temperature rises. Anuran species that remain in the larval stage over the

winter are thought to have long larval stages and show an increase in Bd prevalence in the early summer at the last metamorphose. However, Padgett-Flohr *et al.* [21] also collected *Bufo boreas*, *B. canorus* and *Pseudacris regilla* tadpoles during the optimal season for Bd activity and observed mouthpart deformities, but the prevalence of Bd infection was low. The reported reason for this result is unknown, because a histopathological examination of the tadpoles was not conducted. This result suggested that observation of mouthpart deformities in these 3 species was not suitable for Bd detection. The Manual of Diagnostic Tests for Aquatic Animals 2014 by the OIE [17] recommends macroscopic examination of tadpole mouthparts for the identification of Bd infection. Our study also suggests the utility of this method. However, we conclude that selection of the appropriate Anuran species and collection season are important to conduct a more thorough investigation.

The bullfrog is an introduced species that was imported from North America in 1918 [8]. This animal has been distributed throughout Japan, including the main island, Shikoku, Kyushu and part of Hokkaido [12]. It has been defined as an invasive alien species under Japanese law since 2005 and has been targeted for extirpation because of its negative impact on the Japanese ecosystem [15]. Thus, bullfrogs can be collected from every region of the country without the danger of overexploitation. Additionally, the bullfrog is globally distributed, being found in regions including North America, parts of Central America and Europe and Thailand, Taiwan and Japan in Asia [14]. A field investigation of the prevalence of Bd using the bullfrog tadpole could be conducted in multiple areas and countries worldwide; this method is better suited for general purposes than those used in previous studies [4, 16, 25, 28]. A Bd field investigation targeting tadpoles has never been conducted in Japan. This study is the first report of oral chytridiomycosis in Japanese wild bullfrog tadpoles and showed that bullfrog tadpoles could be used in Bd field investigations.

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