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OCCURRENCE OF PATHOGENIC CHYTRID FUNGI *BATRACHOCHYTRIUM SALAMANDRIVORANS* AND *BATRACHOCHYTRIUM DENDROBATIDIS* IN THE HONG KONG NEWT (*PARAMESOTRITON HONGKONGENSIS*) AND OTHER WILD AND IMPORTED AMPHIBIANS IN A SUBTROPICAL ASIAN REGION

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ABSTRACT: One of the major threats for the massive loss in global amphibian diversity is chytridiomycosis, caused by chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). Following its discovery in 2013, *Bsal* has emerged as a severe threat to the global survival of urodelans. In 2018, a study reported a high prevalence of *Bsal* (65.6%) in the Hong Kong newts (*Paramesotriton hongkongensis*, Near Threatened) of a southern China population adjacent to Hong Kong (HK). Uncertainty regarding the *Bsal* infection status of *P. hongkongensis* inhabiting HK raised deep concern over the risk of introducing *Bsal* from that population. We screened the skin swabs from wild individuals of *P. hongkongensis*, 15 sympatric amphibian species, and 16 imported amphibian species in HK for chytrids. We found that both *Bsal* and *Bd* occur in low prevalences in *P. hongkongensis* (*Bsal* 1.7%, 5/293; *Bd* 0.34%, 1/293), Hong Kong cascade frog, *Amolops hongkongensis*, family Ranidae (*Bsal* only, 5.26%, 1/19), and Asian common toad, *Duttaphrynus melanostictus*, family Bufonidae (*Bsal* only, 5.88%, 1/17), populations of HK, with infected individuals being asymptomatic, suggesting a potential role of these species as reservoirs of *Bsal*. Conversely, *Bd*, but not *Bsal*, was present on 13.2% (9/68) of imported amphibians, indicating a high chytrid introduction risk posed by international amphibian trade. Long-term surveillance of the presence of *Bd* and *Bsal* in wild and captive amphibians would be advisable, and we recommend that import and export of nonnative chytrid carriers should be prevented, especially to those regions with amphibian populations naïve to *Bd* and *Bsal*.

Key words: Amphibian disease, *Bd*, *Bsal*, chytrid fungal pathogen reservoir, chytridiomycosis, Salamander.

INTRODUCTION

Amphibian biodiversity has been suffering a large-scale loss globally, with at least 41% of amphibian species threatened with population crash or extinction (Fisher and Garner 2020; IUCN 2022a). One of the main causes of the decline is chytridiomycosis, an infectious skin disease of amphibians that occurs in all continents where amphibians exist (Stegen et al. 2017; Olson et al. 2021). Two chytrid fungal species of the family Batrachochytriaceae, order Rhizophydiales are known to be responsible for causing chytridiomycosis. They propagate and spread by releasing motile reproductive cells, zoospores, which attach to the amphibian skin into the environment (Medina and Buchler 2020).

First discovered in 1998 (Berger et al. 1998), attention from the scientific community to the immense impact of *Batrachochytrium dendrobatidis* (*Bd*) has grown considerably across the years. By 2021, *Bd* had been detected in 93/134 (69%) countries, spanning from the Americas, Asia, Europe, and Africa, and in 1,375 amphibian species, affecting those mostly in the order Anura (frogs and toads), but some in the order Urodela (salamanders, including newts) as well as in caecilians, order Gymnophiona (Olson et al. 2021). In 2013, *Batrachochytrium salamandrivorans* (*Bsal*) was described, identified from the common fire salamander (*Salamandra salamandra*) associated with a severe outbreak of chytridiomycosis devastating populations of *S. salamandra* in the Netherlands (Martel et al.

2013). After its discovery, *Bsal* was mainly reported to occur in Asia and Europe (Spitzen-van der Sluijs et al. 2016; Stegen et al. 2017) and to cause the rapid die-offs of many European and some North American salamanders (Yap et al. 2017). Unlike with *Bd*, species in the order Urodela (salamanders and newts) seem to be the most susceptible to *Bsal*, but some anuran species can also be affected (Martel et al. 2014).

Although *Bsal* and *Bd* resulted in sporadic deaths in certain amphibian populations and 100% fatality in others, research has shown that some species are resistant to chytrid fungal infections, and some species survive with certain levels of tolerance to the infection (Lam et al. 2010; Martel et al. 2014; Voyles et al. 2018). Plummeted populations of susceptible species that rebound over time possibly have evolved resistance against the fungal pathogen or acquired traits that protect them from infection (Brucker et al. 2008). Importantly, an in vivo *Bsal* infection experiment shows that at least three species of Asian newts in the family Salamandridae, Japanese fire-bellied newt (*Cynops pyrrhogaster*), cyan newt (*Cynops cyanurus*), and Vietnam warty newt (*Paramesotriton deloustali*), may become infected with *Bsal* and survive, and may function as natural reservoirs contributing to *Bsal* transmission within and from Asia (Martel et al. 2014). Evidence from other studies supported an East Asian origin of *Bsal* (Laking et al. 2017; Fisher and Garner 2020). Similar to *Bsal*, *Bd* has also been suggested to have originated from East Asia (the Korean peninsula; O'Hanlon et al. 2018). Some studies have proposed that these pathogenic fungi might be introduced to naïve populations in other regions through movements of asymptomatic carriers (Fisher and Garner 2007; Nguyen et al. 2017). Many wild Asian newts, including some of the suggested Asian reservoir hosts, are overhunted and commonly traded to meet the growing demands from domestic and international pet markets (Rowley et al. 2016; Kitade and Wakao 2022). Therefore, understanding the prevalences of *Bsal* and *Bd* in wild amphibians and the geographical distribution of infected populations, especially those in Asia, and assessing the risk of pathogen introduction from animal trade are critical for conserving global amphibian biodiversity (Sreedharan and Vasudevan 2021).

FIGURE 1. Hong Kong newt (*Paramesotriton hongkongensis*) in its natural habitat, a stream in Hong Kong, China. Photo credit: Hon Shing Fung.

Research on *Bsal* and *Bd* in Asia has been relatively limited. One study (Yuan et al. 2018) reported *Bsal* presence across a wide geographical distribution in southern China. Remarkably, *Bsal* was highly prevalent (65.6%) in a population of Hong Kong newts (*Paramesotriton hongkongensis*; Fig. 1) in Wutongshan, Shenzhen, China (Yuan et al. 2018). This prompted us to consider the status and sustainability of other *P. hongkongensis* populations in nearby regions. Hong Kong is one of their main distribution ranges; however, the prevalence of *Bsal* and *Bd* in the amphibians in Hong Kong, including possible reservoir hosts, remains largely unknown. Hong Kong is adjacent to Wutongshan in Shenzhen (Fig. 2), and *P. hongkongensis* is the only local extant Urodela species (IUCN 2022a). This species is Red Listed as Near Threatened because of its limited distribution range and continuing decline in the extent and quality of its habitat (IUCN 2022a). We anticipated a high risk of *Bsal* introduction to Hong Kong population from Wutongshan and were concerned that if the *P. hongkongensis* in Hong Kong were naïve to the chytrid fungi, spread of *Bsal* from Wutongshan or other sources might cause a population collapse of this protected species. Moreover, some sympatric amphibian

FIGURE 2. The sites for sampling Hong Kong newts (*Paramesotriton hongkongensis*) and other amphibian species in Hong Kong, China, during 2019–2021. Green (pale) circles indicate the sampling sites with no *Batrachochytrium salamandrivorans* (*Bsal*) and *Batrachochytrium dendrobatidis* (*Bd*) detected. Red (dark) circles indicate the sampling sites with *Bsal* and/or *Bd* detected. The larger orange circle in the north of the map indicates Wutongshan in Shenzhen, Guangdong, where *Bsal* has been detected in the *P. hongkongensis* population at high prevalence (Yuan et al. 2018).

species might function as *Bsal* reservoirs and facilitate the rapid spread of *Bsal* (Fisher 2017).

Hong Kong plays a significant role as an international animal trade hub, which poses a high risk of introducing the chytrid fungi to local amphibians or to other places (Kolby et al. 2014). More than 7,000 live individuals of CITES-listed amphibians were imported during 2015–2019 (GovHK 2020), with the actual trade numbers expected to be far higher when including non-CITES-listed species. Imported amphibians may be introduced into local amphibian population through, for instance, religious mercy release, which is a common practice in Hong Kong (Lee et al. 2022). Moreover, a large number of amphibians are exported from Hong Kong. For example, >3.6 million individuals were exported to the US during 2006–2010 (Kolby et al. 2014). It is important to monitor imported amphibians to detect chytrid carriers and improve understanding of sources of amphibian chytrids.

Considering the high prevalence of *Bsal* in the *P. hongkongensis* population of Wutongshan (Yuan et al. 2018), we aimed 1) to determine the prevalences of *Bsal* and *Bd* in wild *P. hongkongensis* populations, sympatric amphibian species in Hong Kong, and imported amphibians in local pet trade market; and 2) to determine whether chytrid fungi could be detected in waters from sampling sites where *Bsal*- or *Bd*-positive individuals were found.

MATERIALS AND METHODS

To cover the distribution range of *P. hongkongensis* in Hong Kong comprehensively (Lau and Dudgeon 1999), we collected skin swab samples

from *P. hongkongensis* at 15 locations between 2019 and 2022 (Fig. 2 and Supplementary Material Table S1). Using a sterile synthetic swab (FLOQSwabs, Copan, Italy), we stroked each individual on the ventral and dorsal sides 30 times and on each leg five times. Each stroke followed a single direction. We sampled at least 10 individuals per site; all swabs were kept at 4 C in the field and stored at −80 C from that day until DNA extraction. We used the same swabbing method to collect skin swabs from other wild amphibians and from imported amphibians from local pet shops.

We first screened the samples of *P. hongkongensis* from the first sampling period (breeding season in winter, 2019–2020; Table S1) for *Bsal* and *Bd* (see the screening method in DNA extraction, nested PCR, and data analysis section). We included all samples from individuals showing signs of skin abnormalities. Based on the screening results, we found a few samples from three sites were *Bsal* or *Bd* positive. We then collected additional samples at these sites across three consecutive months during the following breeding season in winter (November 2020–January 2021; Table S1). We also collected air and water temperature data from these three sites during the second sampling period. To investigate whether zoospores of *Bsal* or *Bd* exist in the ponds of the three positive sites, we sampled the pond waters during the temporal sampling (Table S1). We collected six 600-mL water samples from each site; each water sample was filtered through a 0.22-μm polyvinylidene difluoride membrane filter (Millipore, USA). The membranes were stored at −80 C until DNA extraction.

To estimate the *Bsal* and *Bd* prevalences in other wild amphibian species in Hong Kong, we collected 81 skin swabs from wild individuals of 15 amphibian species at 11 locations. We also collected 68 skin swabs from captive individuals of 16 amphibian species (including 52 swabs from 11 species in the order Anura and 16 swabs from five species in the order Urodela) in a local pet or wet market during 2020 (Table 1). These imported amphibians originated from eight countries or regions.

Genomic DNA was extracted using the ENZA Tissue DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA). We also extracted the DNA from *Bd* (JEL423, 1,000 zoospores/μL; Rosenblum et al. 2008) and *Bsal* zoospores (AMFP13/1, 20 zoospores/μL; Martel et al. 2013) for use in positive controls and sensitivity tests.

A study comparing three molecular methods for detecting *Bd*, a nested PCR, single-round PCR, and the Boyle's real-time TaqMan PCR assay (Boyle et al. 2004), concluded that nested PCR assay was the most sensitive among the three methods (Goka et al. 2009). Zhu et al. (2014) also reported their nested PCR assay to be more sensitive than a real-time PCR assay (Blooi et al. 2013) for detecting *Bsal*. Therefore, we used nested PCRs to amplify the internal transcribed spacer sequence, the ITS1-5.8S-ITS2 region, for *Bsal* or *Bd* detection (Goka et al. 2009; Blooi et al. 2013). For *Bsal*, we designed a primer pair, Bs_ITS1F (5′AAAATCCCAACACAGTGGAA3′) and Bs_ITS 1R (5′GCGAGTTGGTTTTCTTTTGA3′), for the first PCR and a nested primer pair, STerF (5′TGCTCCATCTCCCCCTCTTCA3′) and STerR (5′TGAACGCACATTGCACTCTAC3′; Martel et al. 2013), for the second amplification. For *Bd* we used the primer pair, Bd18SF1 (5′TTTGTACACACCGC CCGTCGC3′) and Bd28SR1 (5′ATATGCTTAAGT TCAGCGGG3′; Goka et al. 2009), for the first PCR and a nested primer pair, Bd1a (5′CAGTGTGCC ATATGTCACG3′) and Bd2a (5′CATGGTTCATAT CTGTCCAG3′; Annis et al. 2004) for the second PCR. We performed sensitivity tests to determine the minimum DNA concentration of *Bsal* or *Bd* that our nested PCR assay could detect. We made 10× serial dilutions on the extracted DNA from *Bsal* (∼43–0.00043 zoospores/PCR reaction) and *Bd* (∼100–0.001 zoospores/PCR reaction) and used triplicates in the tests.

For the nested PCRs, the first PCR was carried out in 25-μL reaction, containing 5 μL 5× buffer, 3 μL $MgCl_2$ (25 mM), 2.5 μL DMSO (Sigma, Darmstadt, Germany, 10%), 0.5 μL dNTPs (10 mM), 0.125 μL bovine serum albumin (NEB, Ipswich, Massachusetts, USA, 20 μg/μL), 0.125 μL DNA polymerase (GoTaq 5U/μL, Promega, Madison, Wisconsin, USA), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), and 4 μL template DNA. The PCR condition for the first amplification started with 2 min at 95 C, followed by 35 cycles of 30 s at 95 C, 30 s at 50 C, 30 s at 72 C, and a final extension for 5 min at 72 C. Afterwards, the second PCR was conducted in 20 μL reaction, containing 4 μL 5× buffer, 1.5 μL $MgCl_2$ (25 mM), 0.4 μL dNTPs (10 mM), 0.1 μL GoTaq (5U/μL; Promega, Madison, Wisconsin, USA), 0.4 μL forward primer (10 μM), 0.4 μL reverse primer (10 μM), and 1.2 μL template DNA (the PCR product from the first amplification). The

PCR condition for the second amplification began with 2 min at 95 C, then 35 cycles of 30 s at 95 C, 30 s at 61 C, 30 s at 72 C, and an extension for 5 min at 72 C. Negative and positive controls of *Bsal* or *Bd* were included in each round of PCRs. All PCR products from the positive samples were Sanger sequenced (BGI, Hong Kong, China) to confirm the identity of *Bsal* and *Bd*.

We analyzed the amplified ITS1-5.8S-ITS2 sequences (272 base pairs [bp]) of *Bd* from all positive samples for variable sites. We compared these sequences to lineages *Bd*-Asia, *Bd*-Asia-Brazil, *Bd*-Europe, *Bd*-North America, and *Bd*-South America (GenBank accession nos.: AB435218, JQ582939, FJ010560, AY598034, and FJ232021, respectively), but not lineages such as *Bd*-GPL (global panzootic lineage) that are not available online (Byrne et al. 2019).

We estimated the prevalences of *Bsal* and *Bd* by dividing the respective numbers of positive samples by the total number of samples screened and calculated the 95% binomial confidence intervals (95% CI) using the Bayesian inference in the R package binom (Dorai-Raj 2014).

RESULTS

The results of sensitivity tests showed that our nested PCR could detect *Bsal* or *Bd* DNA equivalent to 0.043 or 1 zoospore present in a PCR reaction, respectively (Fig. S1). We detected *Bsal* in 5/293 skin swabs from *P. hongkongensis* (Table 1), confirmed by sequencing. The *Bsal* prevalence in the *P. hongkongensis* population was 1.7% (5/293; 95% CI: 0.51%, 3.44%; Fig. 3A). Individuals from three sampling sites were *Bsal* positive (Fig. 3B). One *Bsal*-positive individual was sampled at Tai Tam (prevalence 1.41%, 1/71; 95% CI: 0%, 5.38%), one at Kowloon Peak (prevalence 1.35%, 1/74; 95% CI: 0%, 5.16%), and three at Fa Sam Hang (prevalence 4.76%, 3/63; 95% CI: 0.84%, 11.02%). We did not detect *Bsal* in any of the 18 water samples collected from these sites (Table S1).

We also detected *Bsal* in 2/81 samples of other wild amphibians (Fig. 3A). These include 1/19 Hong Kong cascade frog (*Amolops hongkongensis*, Endangered, IUCN 2022b), (prevalence 5.26%; 95% CI: 0.01%, 18.83%) and 1/17 Asian common toad (*Duttaphrynus melanostictus*; prevalence 5.88%; 95% CI: 0.02%, 20.83%); both were captured from the same site, Fa Sam Hang. The overall *Bsal* prevalence in the local amphibian population was 1.9% (7/374). No *Bsal* was detected on the imported amphibians (Fig. 3C).

We detected *Bd* on 1/293 skin swabs from *P. hongkongensis*, giving a *Bd* prevalence of 0.34% (95% CI: 0%, 1.33%) in the *P. hongkongensis* population (Fig. 3A). This positive individual was one of 71 sampled at Tai Tam (prevalence 1.41%; 95% CI: 0%, 5.38%), and it was not the *Bsal*-positive individual (Fig. 3B). We did not detect any *Bd* in any of the 81 samples that we obtained from other wild amphibians (Fig. 3A).

We detected *Bd* on 9/68 imported amphibians from five pet shops and two wet markets (Fig. 3C and Table 1). These *Bd*-positive individuals include 1/11 Cranwell's horned frog, *Ceratophrys cranwelli* from the US (9.09%; 95% CI: 0.03%, 30.52%); 1/5 Perak spadefoot toads, *Megophrys aceras* from Malaysia (20.00%; 95% CI: 0.17%, 56.40%); 1/1 long-nosed horned frogs, *Megophrys nasuta* (100.00%; 95% CI: 22.85%, 100%) from Malaysia; 2/7 African bullfrogs, *Pyxicephalus adspersus* from the US (28.57%; 95% CI: 4.13%, 60.91%); 1/1 Anderson's salamander, *Ambystoma andersoni,* Critically Endangered (IUCN 2022c) from the US (100.00%; 95% CI: 22.85%, 100%); and 3/5 sword-tail newts, *Cynops ensicauda*, Vulnerable (IUCN 2022d), from Japan (60.00%; 95% CI: 23.18%, 92.32%). No *Bd* was detected from wet market samples; and *Bd*-positive samples were from two out of five pet shops. Thus, the overall *Bd* prevalence in the imported amphibians from local pet shops was 13.2% (9/68; 95% CI: 6.19%, 21.95%). For the prevalences of *Bd* in the imported amphibians from each country/region of origin, the US was 15.28% (4/26; 95% CI: 4.25%, 30.56%), Japan was 60% (3/5; 95% CI: 23.18%, 92.32%), and Malaysia was 33.33% (2/6; 95% CI: 5.42%, 68.02%) (Fig. 3D).

Our analysis of the amplified ITS1-5.8S-ITS2 sequences (272 bp) of *Bd* from all positive samples detected only two variable sites. The phylogenetic tree reconstructed using these ITS sequences was unable to resolve the lineages of

TABLE 1. The prevalences of *Batrachochytrium salamandrivorans* (*Bsal*) and *Batrachochytrium dendrobatidis* (*Bd*), in wild and captive amphibians in Hong Kong, China during 2019–2021. Skin swab samples were collected from 32 amphibian species, in which the samples of captive individuals were obtained from local pet market. Water samples were also collected from three sampling sites where several individuals of Hong Kong newt (*Paramesotriton hongkongensis*) were found to be *Bsal* positive.

	Species	Source	Sampling site or country of origin[a]	Number of samples for detection	Number *Bd* positive and prevalence, with 95% confidence interval (CI)	Number *Bsal* positive and prevalence, with 95% (CI)
Urodela						
	Paramesotriton hongkongensis	Wild	15 sampling sites	293	1 (0.34%; 0–1.33%)	5 (1.71%; 0.51–3.44%)
Anura						
	Amolops hongkongensis	Wild	TPK, PNS, TMS, MTL, HT, FSH	19	0	1 (5.26%; 0.01–18.83%)
	Duttaphrynus melanostictus	Wild	WKT, TPK, KP, PNS, TT, SLT, TMS, WLH, FSH	17	0	1 (5.88%; 0.02–20.83%)
	Eleutherodactylus planirostris	Wild	TT	3	0	0
	Fejervarya limnocharis	Wild	WKT, PNS, SLT, MTL, WLH, FSH	1	0	0
	Hoplobatrachus chinensis	Wild	SLT, HT, PNS	6	0	0
	Hylarana latouchii	Wild	TPK	1	0	0
	Kaloula pulchra	Wild	WKT, TT, SLT	1	0	0
	Limnonectus fujianensis	Wild	TPK, HT	2	0	0
	Liuixalus romeri	Wild	TT, WLH	6	0	0
	Megophrys brachykolos	Wild	KP	7	0	0
	Odorrana chloronota	Wild	WKT, TPK, KP, TT, MTL, HT, WLH, FSH	8	0	0
	Polypedates megacephalus	Wild	WKT, TPK, TT	2	0	0
	Quasipaa exilispinosa	Wild	KP, TT, TMS, MTL	4	0	0
	Quasipaa spinosa	Wild	TMS	3	0	0
	Sylvirana guentheri	Wild	WKT, TPK, TT, SLT, HT, WLH	1	0	0
	Ceratophrys cranwelli	Imported	US	11	1 (9.09%; 0.03–30.52%)	0
	Ceratophrys ornata	Imported	US	4	0	0
	Chacophrys pierotti	Imported	Europe	1	0	0

TABLE 1. Continued.

	Species	Source	Sampling site or country of origin[a]	Number of samples for detection	Number *Bd* positive and prevalence, with 95% confidence interval (CI)	Number *Bsal* positive and prevalence, with 95% (CI)
	Hoplobatrachus chinensis	Imported	Thailand	10	0	0
	Kaloula pulchra	Imported	China	3	0	0
	Lepidobatrachus llanensis	Imported	US	4	0	0
	Litoria caerulea	Imported	Indonesia	1	0	0
	Megophrys aceras	Imported	Malaysia	5	1 (20.00%; 0.17–56.40%)	0
	Megophrys nasuta	Imported	Malaysia	1	1 (100.00%; 22.85–100%)	0
	Pyxicephalus adspersus	Imported	US, Africa	7	2 (28.57%; 4.13–60.91%)	0
	Xenopus laevis	Imported	China	5	0	0
Urodela						
	Ambystoma andersoni	Imported	US	1	1 (100.00%; 22.85–100%)	0
	Ambystoma mexicanum	Imported	China	5	0	0
	Andrias davidianus	Imported	China	1	0	0
	Cynops ensicauda	Imported	Japan	5	3 (60.00%; 23.18–92.32%)	0
	Pleurodeles waltl	Imported	Europe	4	0	0
Others						
	Water samples	Streams	TT, KP, FSH	18	0	0

[a] The 15 sampling sites for free-living amphibians in Hong Kong were Kowloon Peak (KP), Pok Fu Lam (PFL), Mui Tsz Lam (MTL), Fa Sam Hang (FSH), Ma Lai Hau Hang (MLHH), Pak Ngau Shek (PNS), Sha Lo Tung (SLT), Tai Tam (TT), Tai Po Kau (TPK), Ho Chung (HC), Wu Kau Tang (WKT), Sunset Peak (SP), Wong Lung Hang (WLH), Tai Mo Shan (TMS), and Hok Tau (HT).

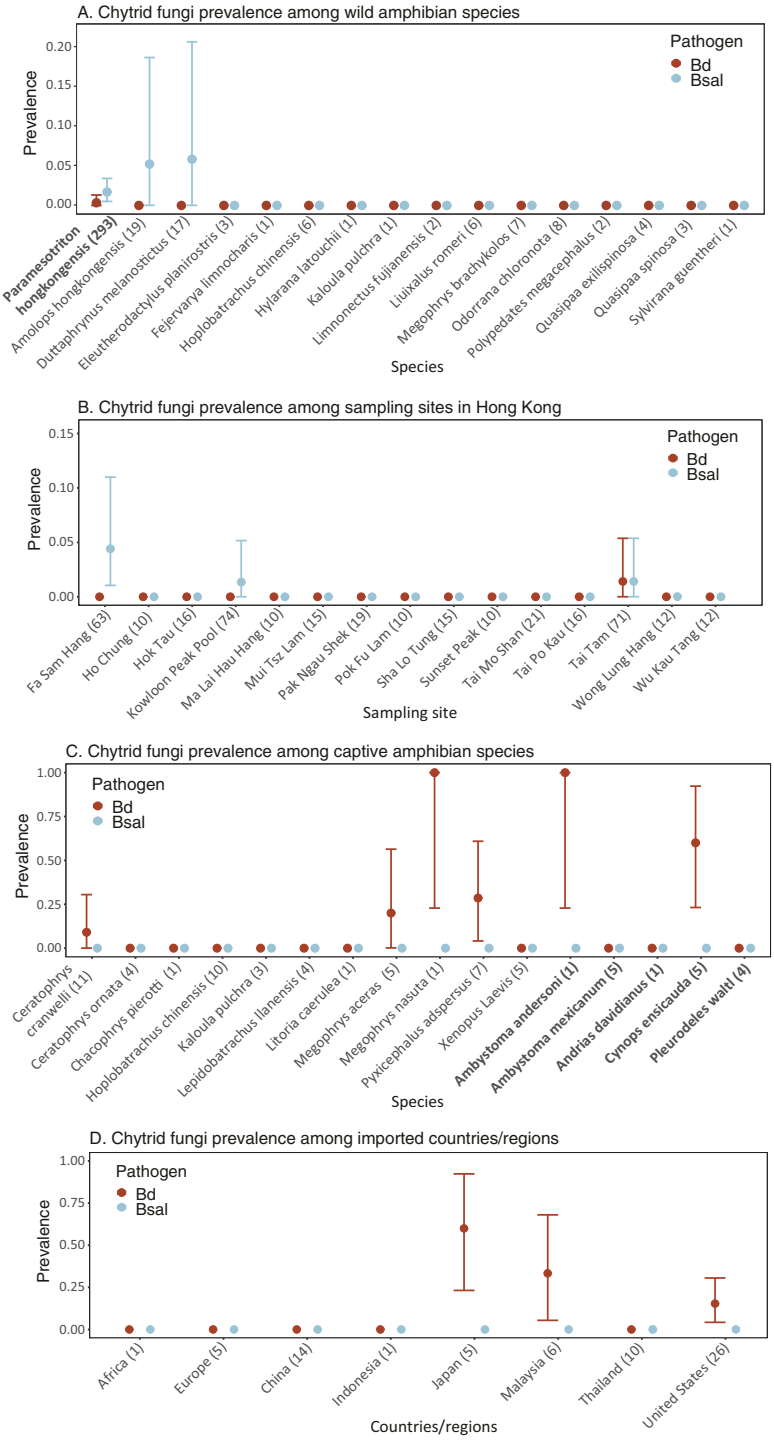

FIGURE 3. Prevalences of chytrid fungi in Hong Kong newts (*Paramesotriton hongkongensis*) and other wild and captive amphibian species in Hong Kong, China, during 2019–2021. The blue (pale) and red (dark) dots indicate the prevalences of *Batrachochytrium salamandrivorans* (*Bsal*) and *Batrachochytrium dendrobatidis* (*Bd*), respectively, with the error bars indicating the 95% confidence interval (CI). The number in each bracket indicates the sampling

Bd in Hong Kong due to insufficient variation within the ITS region.

DISCUSSION

We found that both *Bsal* and *Bd* occurred in the *P. hongkongensis* populations in Hong Kong, but with relatively low prevalences of 1.7% (5/293) and 0.34% (1/293), respectively. The prevalence of *Bsal* in the Hong Kong *P. hongkongensis* was far lower than that of the neighboring Wutongshan population (Yuan et al. 2018). We rarely encountered dead *P. hongkongensis* in the field and all chytrid-infected *P. hongkongensis* sampled were asymptomatic.

Given the uncertainty over the arrival time of locally occurring *Bsal*, it is plausible that *Bsal* might only have been introduced to the local ecosystem shortly before our sampling. Assuming that *P. hongkongensis* is naïve to *Bsal*, *Bsal* might still have been at a stage of early establishment so that the prevalence of *Bsal* remained low, and observable clinical signs of chytridiomycosis would yet to be developed among *P. hongkongensis*. However, we consider such recent establishment of *Bsal* to be very unlikely because previous studies have demonstrated that *Bsal* infection causes rapid mortality in susceptible salamanders experimentally infected with *Bsal* (e.g., *S. salamandra*), and death typically occurs within 7–54 d after *Bsal* infection (Martel et al. 2013, 2014). Responses to infection among conspecifics from a given population were also highly consistent (Martel et al. 2014). Thus, mortalities of *P. hongkongensis* with signs of disease would have been observed. Moreover, we found *Bsal*-positive *P. hongkongensis* at three localities (Fa Sam Hung, Kowloon Peak, and Tai Tam; Fig. 2B). The stream systems of these three localities are not connected to each other, nor are they closer to Wutongshan compared to other sampling localities. Therefore the chance for the locally occurring *Bsal* being recently introduced from Wutongshan was low, and the *Bsal* infections of *P. hongkongensis* discovered at these three localities were likely to be independent events. For the three localities in which individuals with positive swab samples were detected in the first sampling period (October 2019–February 2020), the swab samples collected together with water samples during the second sampling period (November 2020–January 2021) were all chytrid-negative (Table S1). The chytrid-negative water samples suggested that either chytrids were absent from the stream waters at that sampling period or the abundance of chytrid zoospores in the stream water were too low to be detected. The lack of positive samples from the second period made it impossible to establish a correlation between chytrid occurrence and environmental temperatures. It is worth noting that the study conducted at Wutongshan reported a negative correlation between *Bsal* prevalence and air and water temperatures (Yuan et al. 2018). Given the local chytrid occurrence, future studies should explore the relationship between *Bsal* or *Bd* infection events and physical parameters of the sampling sites, as well as other factors such as pollution levels.

An alternative and more likely explanation of the low prevalence of *Bsal* is that *Bsal* has been a longstanding fungus in the microbiome of local *P. hongkongensis*, and therefore the local population is not naïve to *Bsal*. Coevolution of *P. hongkongensis* and *Bsal* might result in *P. hongkongensis* exhibiting certain level of tolerance or even resistance against *Bsal*, and *P. hongkongensis* may already function as a *Bsal* reservoir (Fu and Waldman 2019). This hypothesis is consistent with the proposal that *Bsal* probably originated from Asia; that *Bsal* has been coexisting with a clade of Asian salamander hosts for millions of years; and Asian salamanders have evolved resistance to *Bsal*

size. Prevalences of *Bsal* and *Bd* are shown among (A) wild amphibian species (*P. hongkongensis* in bold), (B) all amphibians in each sampling site, (C) captive amphibian species (species in the order Urodela in bold), and (D) amphibians from different exporting countries or regions. The presence of *Bsal* or *Bd* was detected by nested PCR.

(Martel et al. 2014). Infection experiments have demonstrated that salamander species may clear *Bsal* infections, resulting in clinical cure (Stegen et al. 2017). Despite the lack of studies on *Bsal* in Asian regions, the overall prevalence of *Bsal* in mainland China is relatively low (Zhu et al. 2014; Wang et al. 2017; Yuan et al. 2018). Additionally, to date there have been no reports of clinical signs of chytridiomycosis reported for the *Bsal*-infected salamanders found in mainland China, including *Bsal*-positive *P. hongkongensis* in Wutongshan. Similarly, no decrease in the population sizes of *Bsal*-infected salamander populations have been reported, and the contributing factor underlying the high prevalence of *Bsal* in Wutongshan is poorly understood (Yuan et al. 2018). Thus, long-standing *Bsal* in local *P. hongkongensis* population seems to be more likely. The ability of *P. hongkongensis* to clear or suppress the *Bsal* infection may explain the occurrence of *Bsal*-infected individuals in a few localities separated from each other, and the low prevalence of *Bsal* in local populations with infected individuals likely being asymptomatic. Importantly, if this is the case, the defense mechanism of *P. hongkongensis* against *Bsal* will be a fruitful area to explore. The lack of knowledge on the susceptibility of *P. hongkongensis* emphasizes the importance of long-term surveillance on the chytrid fungal infection and health statuses among local populations, in order to verify the aforementioned explanations.

In addition to *P. hongkongensis*, we detected low prevalence (5–6%) of *Bsal* on two sympatric anuran species, *A. hongkongensis* (family Ranidae) and *D. melanostictus* (family Bufonidae). Similarly to *P. hongkongensis*, the individuals of these two species on which we detected *Bsal* showed no sign of disease. This supports the notion that anurans seem to be resistant to or tolerant of *Bsal* infection (Stegen et al. 2017).

Earlier evidence has suggested that amphibian species sympatric to *Bsal*-infected urodelans but less susceptible to *Bsal* may function as *Bsal* reservoirs in amphibian populations (Fisher 2017). The three *Bsal*-positive amphibian species without clinical signs that we detected have the potential to act as *Bsal* reservoirs. Because the two infected anurans were found at the same locality (i.e., Fa Sam Hung) as the three *Bsal*-positive *P. hongkongensis*, interspecific transmission events of *Bsal* might be occurring at that locality. Globally, apart from alpine newt (*Ichthyosaura alpestris*, family Salamandridae), the number of amphibian species reported to carry *Bsal* in the wild is very limited (Grear et al. 2021). European common frogs (*Rana temporaria*, family Ranidae; Schulz et al. 2020) and the small-webbed firebelly toad (*Bombina microdeladigitora*, family Bombinatoridae; Nguyen et al. 2017) are two examples of potential *Bsal* carrier species in their native range. Our discovery of *Bsal*-positive *D. melanostictus* is the first report of a species in the true toad family Bufonidae carrying *Bsal* in the wild, which expands our knowledge of the potential host range of *Bsal*. Adult Bufonidae are terrestrial most of the year outside the breeding period, and their movement is thus relatively unrestricted by water availability. In addition, true toads like *D. melanostictus* typically possess dry warty skin; hence the occurrences of *Bsal* on diverse skin types may reflect the capability of *Bsal* to survive on and spread through a wider range of amphibian host species. The presence of multispecies *Bsal* reservoirs, with variable life histories and behaviors, may affect the transmission dynamics of *Bsal* in an ecosystem, possibly promoting the transmission rate and long-term persistence of *Bsal*.

Our finding of *Bd* in local amphibians only in a single *P. hongkongensis* agrees with the general observation that no lethal outbreak of *Bd* infection has been recorded from Asia to date; Asia is referred to as a "cold spot" of *Bd* infection because *Bd* prevalence is generally low and where it is found, pathogen loads on infected amphibians are very low (Sreedharan and Vasudevan 2021). Our result is similar to that from a previous local study in 2007, which found wild individuals of four native amphibian species (*A. hongkongensis*, *Paa exilispinosa*, *P. spinosa*, and *Rana chloronota*) all negative for *Bd* (Rowley et al. 2007). Another study in 2014 that tested

multiple imported amphibian species in the US did not detect *Bd* on *P. hongkongensis* from Hong Kong, but *Bd* was detected in the water samples from the bags that carried the *P. hongkongensis* (Kolby et al. 2014).

Our finding of *Bd*, but not *Bsal*, on amphibians imported from Japan, Malaysia, and the US is consistent with the global distribution patterns of *Bd* and *Bsal*. *Bd* is globally widespread and has been reported to occur in these three countries (Olson et al. 2021). Although information on the global distribution of *Bsal* is relatively limited compared to that of *Bd* (Martel et al. 2013), current data suggests that the global distributional range of *Bsal* is more restricted than that of *Bd*; for example, *Bsal* has not been detected in the wild in North American but is prevalent in Europe (Lötters et al. 2020; Grear et al. 2021). Thus, the probability of detecting *Bd* on imported amphibians was expected to be higher than *Bsal*. Nevertheless, given that *Bsal* may be at an earlier stage of global spread than *Bd*, we must stay vigilant in preventing *Bsal* transmission by amphibian trade. Considering the presence of chytrids in local amphibian populations, it is also possible that these imported amphibians could have been infected by *Bd* at any stage after their importation into Hong Kong, such as via *Bd*-contaminated materials used in domestic animal care.

The *Bd* we detected on imported amphibians from pet shops indicates that international animal trade via Hong Kong may potentially introduce chytrid fungal pathogens to amphibian populations of different regions. The chytrid on these imported amphibians may be transmitted to other regions through re-exports, to local ecosystems through intentional release or accidental escape of individuals infected (Lee et al. 2022), or to captive environments through repeated uses of chytrid-contaminated materials for captive amphibian husbandry (Spitzen-van der Sluijs et al. 2011). To minimize the risk of spread of chytrids to local and other regions through international trade, it is crucial to quarantine imported amphibians and screen for *Bsal* and *Bd* during quarantine to gather more information on the country and breeder sources of amphibians that are chytrid carriers. Foreign chytrid carriers should be prevented from entering and being re-exported, especially to those regions with amphibian populations naïve to the chytrids.

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SUPPLEMENTARY MATERIAL

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LITERATURE CITED

Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE. 2004. A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. *J Wildl Dis* 40:420–428.

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, et al. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci U S A* 95:9031–9036.

Blooi M, Pasmans F, Longcore JE, Spitzen-van der Sluijs A, Vercammen F, Martel A. 2013. Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. *J Clin Microbiol* 51:4173–4177.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis Aquat Organ* 60:141–148.

Brucker RM, Harris RN, Schwantes CR, Gallaher TN, Flaherty DC, Lam BA, Minbiole KPC. 2008. Amphibian chemical defense: Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J Chem Ecol* 34:1422–1429.

Byrne AQ, Vredenburg VT, Martel A, Pasmans F, Bell RC, Blackburn DC, Bletz MC, Bosch J, Briggs CJ, et al. 2019. Cryptic diversity of a widespread global

pathogen reveals expanded threats to amphibian conservation. *Proc Natl Acad Sci U S A* 116:20382–20387.

Dorai-Raj S. 2014. binom: Binomial confidence intervals for several parameterizations. R Package Version 1.1-2. https://rdrr.io/rforge/binom/. Accessed June 2022.

Fisher MC. 2017. In peril from a perfect pathogen. *Nature* 544:300–301.

Fisher MC, Garner TW. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biol Rev* 21:2–9.

Fisher MC, Garner TWJ. 2020. Chytrid fungi and global amphibian declines. *Nat Rev Microbiol* 18:332–343.

Fu M, Waldman B. 2019. Ancestral chytrid pathogen remains hypervirulent following its long coevolution with amphibian hosts. *Proc R Soc Ser B* 286:20190833.

Goka K, Yokoyama J, Une Y, Kuroki T, Suzuki K, Nakahara M, Kobayashi A, Inaba S, Mizutani T, Hyatt AD. 2009. Amphibian chytridiomycosis in Japan: Distribution, haplotypes and possible route of entry into Japan. *Mol Ecol* 18:4757–4774.

GovHK. 2020. Information on imported non-CITES species for pet trade (Mammals other than cats and dogs, birds and reptiles) (2015 to 2019). https://gia.info.gov.hk/general/202010/28/P2020102800624_352846_1_1603881298200.pdf. Accessed June 2022.

Grear DA, Mosher BA, Richgels KLD, Grant EHC. 2021. Evaluation of regulatory action and surveillance as preventive risk-mitigation to an emerging global amphibian pathogen *Batrachochytrium salamandrivorans* (Bsal). *Biol Conserv* 260:109222.

IUCN. 2022a. The IUCN Red List of Threatened Species. Version 2022-1. https://www.iucnredlist.org. Accessed June 2022.

IUCN. 2022b. *Amolops hongkongensis*. The IUCN Red List of Threatened Species. Version 2022-1. https://www.iucnredlist.org/species/58210/63852284. Accessed June 2022.

IUCN. 2022c. *Ambystoma andersoni*. The IUCN Red List of Threatened Species. Version 2022-1. https://www.iucnredlist.org/species/59051/176772780. Accessed June 2022.

IUCN. 2022d. *Cynops ensicauda*. The IUCN Red List of Threatened Species. Version 2022-1. https://www.iucnredlist.org/species/59441/177225544. Accessed June 2022.

Kitade T, Wakao K. 2022. Illuminating amphibians: The amphibian trade in Japan. https://www.traffic.org/site/assets/files/17487/illuminating-amphibians-correction082022.pdf. Accessed June 2022.

Kolby JE, Smith KM, Berger L, Karesh WB, Preston A, Pessier AP, Skerratt LF. 2014. First evidence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in Hong Kong amphibian trade. *PloS One* 9:e90750.

Laking AE, Ngo HN, Pasmans F, Martel A, Nguyen TT. 2017. *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Sci Rep* 7:44443.

Lam BA, Walke JB, Vredenburg VT, Harris RN. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol Conserv* 143:529–531.

Lau MW, Dudgeon D. 1999. Composition and distribution of Hong Kong amphibian fauna. *Mem Hong Kong Nat Hist Soc* 22:1–80.

Lee WH, Fong JJ, Lee WH, Sung YH. 2022. Mercy or messy: Distribution and differentiation of native and released Chinese bullfrogs (*Hoplobatrachus rugulosus*) in Hong Kong using genetic and morphological analyses. *Manage Biol Invasions* 13:246–258.

Lötters S, Wagner N, Albaladejo G, Böning P, Dalbeck L, Düssel H, Feldmeier S, Guschal M, Kirst K, et al. 2020. The amphibian pathogen *Batrachochytrium salamandrivorans* in the hotspot of its European invasive range: Past – present – future. *Salamandra* 56:173–188.

Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt BR, Tobler U, et al. 2014. Wildlife disease. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* 346:630–631.

Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, Woeltjes A, Bosman W, Chiers K, et al. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proc Natl Acad Sci U S A* 110:15325–15329.

Medina EM, Buchler NE. 2020. Chytrid fungi. *Curr Biol* 30:R516–R520.

Nguyen TT, Nguyen TV, Ziegler T, Pasmans F, Martel A. 2017. Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. *Amphib Reptil* 38:554–556.

O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, Kosch TA, Murray KA, Brankovics B, et al. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* 360:621–627.

Olson DH, Ronnenberg KL, Glidden CK, Christiansen KR, Blaustein AR. 2021. Global patterns of the fungal pathogen *Batrachochytrium dendrobatidis* support conservation urgency. *Front Vet Sci* 8:685877.

Rosenblum EB, Stajich JE, Maddox N, Eisen MB. 2008. Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. *Proc Natl Acad Sci U S A* 105:17034–17039.

Rowley JJL, Chan SKF, Tang WS, Speare R, Skerratt LF, Alford RA, Cheung KS, Ho CY, Campbell R. 2007. Survey for the amphibian chytrid *Batrachochytrium dendrobatidis* in Hong Kong in native amphibians and in the international amphibian trade. *Dis Aquat Organ* 78:87–95.

Rowley JJL, Shepherd CR, Stuart BL, Nguyen TQ, Hoang HD, Cutajar TP, Wogan GOU, Phimmachak S. 2016. Estimating the global trade in Southeast Asian newts. *Biol Conserv* 199:96–100.

Schulz V, Schulz A, Klamke M, Preissler K, Sabino-Pinto J, Müsken M, Schlüpmann M, Heldt L, Kamprad F, et al. 2020. *Batrachochytrium salamandrivorans* in the Ruhr District, Germany: History, distribution, decline dynamics and disease symptoms of the salamander plague. *Salamandra* 56:189–214.

Spitzen-van der Sluijs A, Martel A, Asselberghs J, Bales EK, Beukema W, Bletz MC, Dalbeck L, Goverse E, Kerres A, et al. 2016. Expanding distribution of lethal

amphibian fungus *Batrachochytrium salamandrivorans* in Europe. *Emerging Infect Dis* 22:1286–1288.

Spitzen-van der Sluijs A, Martel A, Wombwell E, Van Rooij P, Zollinger R, Woeltjes T, Rendle M, Haesebrouck F, Pasmans F. 2011. Clinically healthy amphibians in captive collections and at pet fairs: A reservoir of *Batrachochytrium dendrobatidis*. *Amphibia-Reptilia* 32:419–423.

Sreedharan G, Vasudevan K. 2021. Chytridiomycosis in Asian amphibians, a global resource for *Batrachochytrium dendrobatidis* (Bd) research. *J Indian Inst Sci* 101:227–241.

Stegen G, Pasmans F, Schmidt BR, Rouffaer LO, Van Praet S, Schaub M, Canessa S, Laudelout A, Kinet T, et al. 2017. Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. *Nature* 544:353–356.

Voyles J, Woodhams DC, Saenz V, Byrne AQ, Perez R, Rios-Sotelo G, Ryan MJ, Bletz MC, Sobell FA, et al. 2018. Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. *Science* 359:1517–1519.

Wang SP, Zhu W, Fan LQ, Li JQ, Li YM. 2017. Amphibians testing negative for *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* on the Qinghai-Tibetan Plateau, China. *Asian Herpetol Res* 8:190–198.

Yap TA, Nguyen NT, Serr M, Shepack A, Vredenburg VT. 2017. *Batrachochytrium salamandrivorans* and the risk of a second amphibian pandemic. *Ecohealth* 14:851–864.

Yuan ZY, Martel A, Wu J, Van Praet S, Canessa S, Pasmans F. 2018. Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species. *Conserv Lett* 11:e12436.

Zhu W, Xu F, Bai CM, Liu X, Wang SP, Gao X, Yan SF, Li XP, Liu ZT, Li YM. 2014. A survey for *Batrachochytrium salamandrivorans* in Chinese amphibians. *Curr Zool* 60:729–735.

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