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DETECTION OF *BATRACHOCHYTRIUM SALAMANDRIVORANS* IN A U.S. MILITARY BASE IN JAPAN INDICATES OVERSEAS TERRITORIES AS POSSIBLE INTRODUCTION ROUTES

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Abstract.—**The chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) has caused severe declines in amphibian populations, particularly in Europe, yet it has not been detected in North America, a global hotspot of salamander biodiversity. Japan hosts several amphibian species that may act as *Bsal* carriers, and cases have been recently documented on Okinawa Island. The island also contains several U.S. military bases, which operate semi-independently and maintain regular personnel movement between Japan and the U.S. These facilities may represent overlooked but important points of pathogen transfer. As part of a broader *Bsal* survey in Japan, we sampled amphibians inside the U.S. Marine Corps Jungle Warfare Training Center (JWTC) on Okinawa. We detected *Bsal* in *Cynops ensicauda* (one individual) confirming the presence of the pathogen within USA jurisdiction. This finding highlights the potential for pathogen spillover via military personnel and logistics operating between overseas territories and mainland countries. Such sites should, therefore, be incorporated into invasion risk assessments and policy actions aimed at preventing *Bsal* introduction.**

Key Words.—amphibians; *Bsal*; chytrid fungus; chytridiomycosis; conservation; fungal pathogen; Salamanders; USA

INTRODUCTION

Amphibian chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), has driven global amphibian declines (Scheele et al. 2019). Although not yet detected in North America, *Bsal* poses a significant threat to salamander-rich regions (Gray et al. 2023). In Asia, the presumed native range of *Bsal*, its distribution remains insufficiently documented. Several potential carrier species inhabit Japan, which currently lacks regulations to limit *Bsal* spread (Lastra González et al. 2025). Furthermore, detections have been recorded on Okinawa Island in southwestern Japan (Martel et al. 2014; Lastra González et al. 2024), raising concerns about possible transmission routes.

In Western Europe, *Bsal* has caused severe declines in salamander and newt populations, primarily through skin infections that result in fatal lesions (Martel et al. 2013). The introduction of *Bsal* to Europe has been closely linked to the international pet trade in salamanders originating from East and Southeast Asia, which prompted the implementation of trade restrictions (EU Commission 2018). To mitigate the risk of further spread into *Bsal*-free regions, it is essential to regulate amphibian trade and enhance pathogen surveillance. Monitoring *Bsal* within its alleged native range is critical for understanding its ecological requirements and identifying high-risk areas where anthropogenic activities may facilitate future outbreaks.

Overseas territories, including military installations, represent geopolitical contexts where personnel movement may bypass border biosecurity measures. These territories often occur on islands and can have different origins and uses. Military bases typically use their own airports and officially do not cross international borders. The presence of U.S. military bases in Okinawa creates an opportunity for unintended pathogen movement. Here, we present results from targeted monitoring within a U.S. military installation and examine their implications for *Bsal* invasion-risk models, while also exploring potential overlooked transmission pathways to North America, which currently remains free of *Bsal*.

MATERIALS AND METHODS

Study site.—We conducted the sampling inside the U.S. Marine Corps Jungle Warfare Training Center (JWTC; Camp Gonsalves), a restricted 78-km² area established in 1958 and surrounded by Yanbaru National Park, where *Bsal* has been recorded. We had a day-pass access that allowed us a one-time

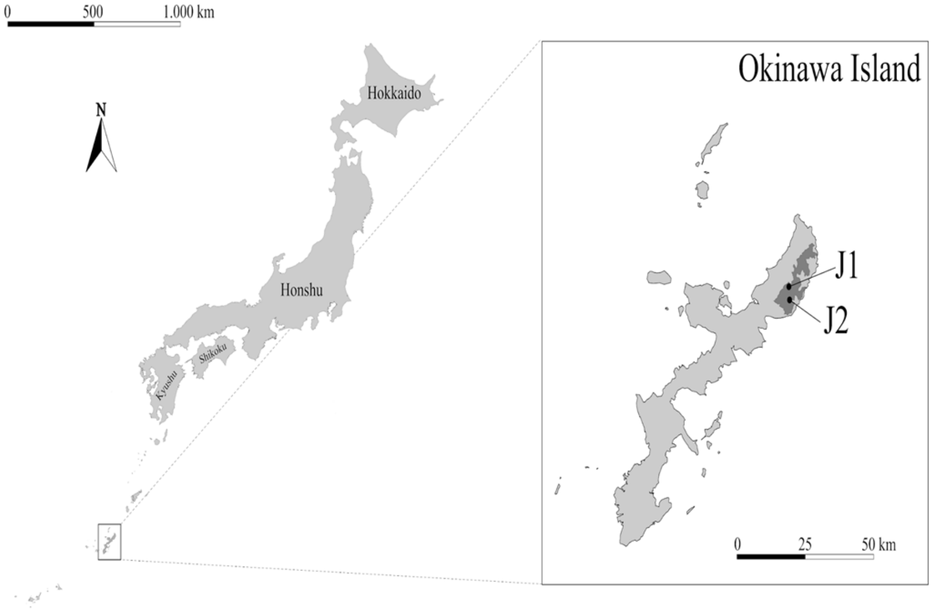

Figure 1. Location map showing Okinawa Island within its Japanese distribution (left) and Jungle Warfare Training Center of USA (in dark grey) within the Okinawa Island including the two sampled locations J1 and J2 (right).

opportunity to collect samples. We collected samples at two sites located approximately 3 km apart: site J1, a shallow pond next to a muddy training path, and site J2, a narrow creek with puddles (Fig. 1).

Field sampling.—We swabbed amphibians during March 2024 following standard chytrid sampling protocols. We captured Sword-tail Newts (*Cynops ensicauda*) using dip nets at site J1 and by hand at site J2. We swabbed each newt with one sterile dry swab (Men-tip 1P1501, Nihon-Menbo Co., Tokyo, Japan), which we stored in 1.5 ml Eppendorf tubes at -18° C with silica gel until analysis (see Lastra González et al. 2024 for further details).

Laboratory analyses.—We extracted Genomic DNA, using a protocol for the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). We used two detection assays: one based on standard polymerase chain reaction (PCR) followed by electrophoresis, and the other on duplex *Bd* and *Bsal* quantitative PCR (qPCR). We analyzed samples using standard PCR with *Bsal*-specific primers STerF and STerR (Martel et al. 2013) and qPCR methods (Blooi et al. 2013) at the University of the Ryukyus. For standard PCR, we carried out electrophoresis on amplified targets. We reanalyzed samples that produced positive or equivocal results in standard PCR by qPCR. Quantification standards were created from gBlocks (Integrated DNA Technologies Inc., Coralville, Iowa, USA) as *Bsal* and *Bd* standards. In qPCR tests, all analyses were performed with 5 µL of DNA template in duplicate in Roche LightCycler 480 II (Roche Diagnostics, Tokyo, Japan), using the Roche 480 Probes Master mix (Roche Diagnostics, Tokyo, Japan). We considered a sample to be positive only if both wells amplified, the increase in fluorescence showed a standard sigmoidal curve, and the Ct value was below 40. We retested samples with single well amplification. In all analyses, we used the calculation of the 2nd derivative maximum, which is available in LightCycler 480 software. Finally, we sequenced samples with inconsistent results using chain-terminating (Sanger) sequencing on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) targeting the 5.8S rRNA gene.

Results

We sampled 29 *Cynops ensicauda* from two JWTC sites. We collected 18 samples at site J1 and 11 at J2. Two newts displayed clinical abnormalities: a bloated male at J1 and a blistered female at J2 (Fig. 2). PCR and qPCR identified the bloated male as *Bsal*-positive with a genomic equivalent load of 1 GE per reaction. Sequencing (160 base pairs) showed 100% identity with GenBank accession KC762295. No other samples tested positive.

Figure 2. Sword-tailed Newts (*Cynops ensicauda*) found during monitoring surveys on Okinawa Island. (Photographed by Nuria Viñuela Rodríguez [left] and David Lastra González [right]).

DISCUSSION

Our detection of *Bsal* within U.S. military installation in Japan provides new information about its Asian distribution and reveals a potential pathway for long-distance pathogen movement. JWTC hosts hundreds of U.S. Marines annually, and training in muddy environments may promote spore transport (Stegen et al. 2017). Transfers to continental U.S. military bases, including regions with endemic salamanders, could elevate introduction risk. If the deadly fungal pathogen *Bsal* were to spill over into the U.S., the potential damage would be catastrophic, as the country harbors the greatest diversity of salamander species in the world, with over 200 species found nowhere else (Yap et al. 2015). Given its high virulence, *Bsal* has the potential to cause mass mortality events, severe population declines, and even local extinctions among these highly susceptible amphibians, particularly in high-risk regions like the Pacific Coast, the southern Appalachian Mountains, and the mid-Atlantic states where conditions are favorable for the fungus (Richgels et al. 2016). An example of a possible vulnerability connected with this base could be trainee movement to the Marine Corps Mountain Warfare Training Center in Bridgeport, California, USA, with access to the Sierra Nevada, home to several endemic salamander species (e.g., Ensatina, *Ensatina eschscholtzii*; Mount Lyell Salamander, *Hydromantes platycephalus*; and Sierra Newt, *Taricha sierrae*), and to the Marine Corps Base in Albany, Georgia, USA, near the Flint River, home to many salamander species (e.g., Marbled Salamanders, *Ambystoma opacum*, Mole Salamanders, *Ambystoma talpoideum*, and Southern Two-lined Salamanders, *Eurycea cirrigera*).

Existing mitigation frameworks, such as the Lacey Act (U.S. Fish and Wildlife Service 2016), and multi-agency recommendations (Gray et al. 2015; Richgels et al. 2016) aim to reduce *Bsal* spread, yet invasion models (Yap et al. 2015; Grear et al. 2021; Moubarak et al. 2022) do not include overseas territories. Future studies should survey additional overseas territories within the native range of *Bsal* to clarify the broader distribution of the pathogen and identify previously unrecognized nodes of long-distance spread. Incorporating these territories into predictive models and management frameworks would improve conservation planning and help prevent the introduction of *Bsal* into currently unaffected regions, including *Bsal*-free areas such as North America.

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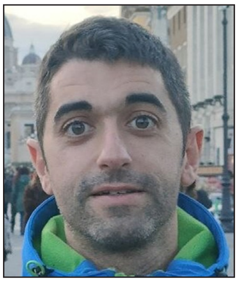

David Lastra González is a herpetologist specializing in disease ecology. He earned his B.S. and M.S. in Environmental Sciences from the University of Salamanca, Spain, and a B.S. in Forestry Sciences from the University of León, Ponferrada, Spain. He later obtained his Ph.D. in Ecology from the Czech University of Life Sciences in Prague, Czech Republic. His research focuses on diseases affecting amphibians, particularly the presence of *Batrachochytrium salamandrivorans* (Bsal) in Europe and Japan. He has conducted extensive studies on the distribution of Bsal and the role of the pet trade in facilitating its spread. Currently, he serves as a Scientific Officer in the Natural Resources and Biodiversity section at Cesefor Foundation, a non-profit organization headquartered in Soria, Spain. He is based at the Biotechnology Institute in León, where he carries out research and participates in conservation projects related to forest restoration and forest ecology. (Photographed by Nuria Viñuela Rodríguez).

Atsushi Tominaga is a herpetologist specializing in the evolution and speciation of amphibians. He received his Bachelor of Agriculture from Kyushu University, Fukuoka, Japan, and obtained his M.Sc. and Ph.D. from the Graduate School of Human and Environmental Studies at Kyoto University, Kyoto, Japan. His research focuses on evolutionary processes in amphibians, particularly speciation and hybridization in salamanders and frogs. He has conducted extensive studies on the phylogeography, population genetics, and evolutionary history of East Asian amphibians, including the Japanese Fire-bellied Newt (*Cynops pyrrhogaster*). His work also addresses conservation issues, including emerging infectious diseases such as chytridiomycosis, as well as the ecological impacts of invasive aquatic vertebrates. Atsushi has authored more than 60 peer-reviewed publications on amphibian evolution, ecology, and conservation. He is currently Professor in the Faculty of Education and the Graduate School of Science and Engineering at the University of the Ryukyus, where he conducts research and teaches courses in Ecology, Evolutionary Biology, and Conservation Science. (Photographed by Atsushi Tominaga).