

**Rapid Communication****Record of invasive *Rana huanrenensis* Fei, Ye, and Huang, 1990 and *Pelophylax nigromaculatus* (Hallowell, 1861) on Ulleung Island, Republic of Korea**Yoonhyuk Bae<sup>1</sup>, Jongsun Park<sup>2,3</sup>, Siti N. Othman<sup>1</sup>, Yikweon Jang<sup>4</sup> and Amaël Borzée<sup>1,\*</sup><sup>1</sup>Laboratory of Animal Behaviour and Conservation, College of Biology and the Environment, Nanjing Forestry University, Nanjing, People's Republic of China.<sup>2</sup>InfoBoss Inc., room 301, 670, Seolleung-ro, Gangnam-gu, Seoul, Republic of Korea<sup>3</sup>InfoBoss Research Center, 301 room, 670, Seolleung-ro, Gangnam-gu, Seoul, Republic of Korea<sup>4</sup>Department of Life Science and Division of EcoScience, Ewha Womans University, Seoul, Republic of Korea

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**OPEN ACCESS****Abstract**

Non-native species have a significant negative impact on the environment where they have been introduced, and amphibians are among some of the worst invasives. All known amphibian introductions are linked to human activities, generally in relation with pest control or food provisioning. This is the case of all amphibians on Ulleung Island, which were originally and mistakenly thought to be “reintroduced” after extirpation, or introduced for unrealised purposes such as food provisioning and pest-control. We conducted call and visual encounter surveys in all valleys of Ulleung Island, Republic of Korea, in April and May 2021 to detect the presence of amphibian species. The call surveys and subsequent call analyses revealed the presence of two geographically independent populations of *Pelophylax nigromaculatus*, and encounter surveys resulted in the sampling of *Rana* tadpoles identified as *Rana huanrenensis* with molecular tools. These results highlight the presence of these two species at low density on this island, but do not provide data on the impact of their presence.

**Key words:** brown frog, pond frog, Northeast Asia, invasive species, amphibian**Introduction**

Invasive species are one of the prime causes of biodiversity loss because of their negative impact on native species (Kiesecker et al. 2001; Mayer et al. 2015). Within amphibians, the Cane toad (*Rhinella marina* (Linnaeus, 1758)), the African clawed frog (*Xenopus laevis* (Daudin, 1802)), the Asian black-crested toad (*Duttaphrynus melanostictus* (Schneider, 1799)) and the American bullfrog (*Lithobates catesbeianus* (Shaw, 1802)) are among the worst, and most widespread, invasive species (Andersen et al. 2021). Their presence threatens the survival of other species, as for instance *L. catesbeianus* is threatening *Rana draytonii* Baird & Girard, 1852 (Lawler et al. 1999) in North America and *Dryophytes flaviventris* Borzée & Min, 2020 (Borzée et al. 2020b) in the Republic of Korea, among many species (Li et al. 2011; da Silva Silveira and Guimarães 2021). The blame should however not be cast

on species exploiting niches made available by human activities, but on the decision made to introduce the species. These introduction are most generally linked to pest control, for instance *R. marina* in Australia (Taylor and Edwards 2005) and *Glandirana rugosa* (Temminck & Schlegel, 1838) in Hawaii, USA, (Kraus et al. 1999), or food provisioning (Groffen et al. 2019).

In the Republic of Korea, the worse invasive amphibian is *L. catesbeianus* (Groffen et al. 2019), although there are also records of *D. melanostictus* (Othman et al. 2020a) and *X. laevis*, both linked to the trade (Borzée et al. 2021). In addition, the situation could get worse due to the presence of an extremely large number of amphibian species in the pet trade (Koo et al. 2020). This is a possibly disastrous situation (Borzée et al. 2020a), although potentially addressed by the request for updated regulations in the import of non-native species (law case proposition number 7177).

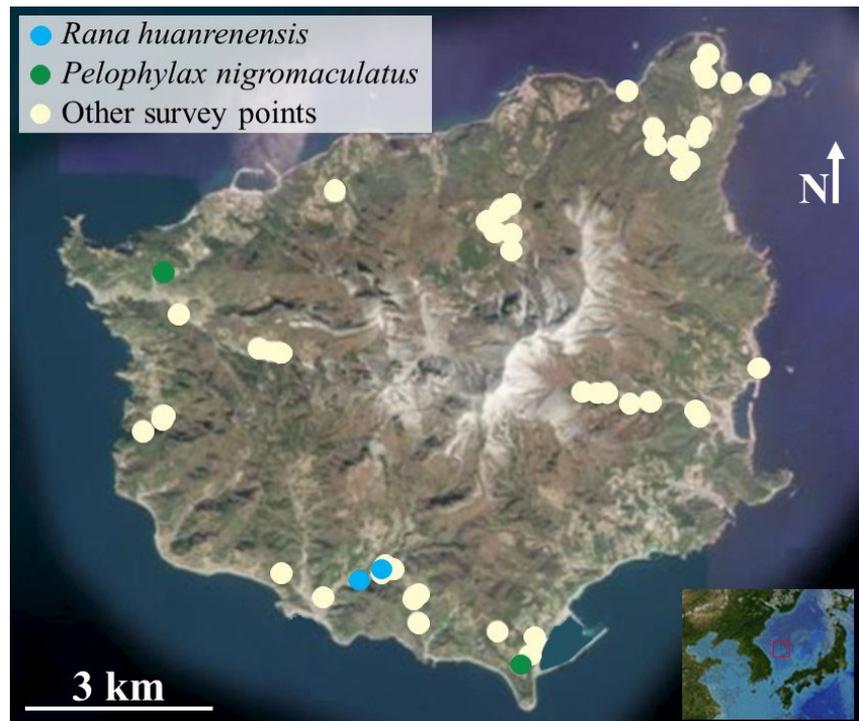
Ulleung Island is a young volcanic island dating back to the late-Pliocene and early Holocene (Kim and Lee 2008). No amphibian species is native to the island, but this knowledge was not yet determined when *Pelophylax nigromaculatus* (Hallowell, 1861) was erroneously “re”-introduced in 1937 (Shin et al. 1996), and potentially introduced in 2013 with a batch of *Rana* sp. as the species was still commonly referred to as “*Rana nigromaculata*” at that time. In addition, other amphibians have been introduced over time, and other species have been recorded but their origin is unknown, such as *Dryophytes japonicus* (Günther, 1859) (Oh 2001).

The lack of knowledge on the non-native status of amphibians of Ulleung Island is easily overcome through the grey literature, and for instance Ulleung county office released around 4,500 *Rana* sp. individuals in the Okcheon Stream for a restoration project in 2013 (Kim 2013). The fate of the reintroduced individuals was however not monitored and most frogs are expected to have been predated due to the high density of avian predators (Shin et al. 1996). We conducted call and visual encounter surveys on Ulleung Island in April and May 2021 to determine the occurrence status of early breeding amphibian species on the island, and employed acoustic and molecular analyses to identify the species.

## Materials and methods

### *Field surveys*

We conducted acoustic and visual encounter surveys between 28 April and 2 May 2021. We surveyed all streams and water bodies visible from satellite views, and conducted at least one survey in each valley (Figure 1). Surveys focused on habitat suitable for amphibians based on maps and satellite views (google.com/maps; map.naver.com). We spent a minimum of 10 min listening for calling activity at each of the wetlands. We recorded all calling individuals with a linear PCM recorder (Tascam DR-40; California,



**Figure 2.** Map of amphibian surveys conducted on Ulleung Island between 28 April and 2 May 2021. Surveys were conducted in the form of acoustic and visual encounters. Map created with arcGIS online ([www.arcgis.com](http://www.arcgis.com)). Imagery layer from Earthstar Geographics.

USA) linked to a unidirectional microphone (Unidirectional electret condenser microphone HT-81, HTDZ; Xi'an, China). We could not collect any of the individuals heard calling.

We conducted visual encounter surveys during both day time and night time to maximise the chance of finding amphibians. We focused on finding eggs or tadpoles during daytime, and adults during night time. We did not find any adults, but collected three *Rana* tadpoles that we tail-clipped following a least invasive method (Othman et al. 2020b). All individuals were captured under the permit 2021-1 issued by Ulleung county, allowing for capture of three *Rana huanrenensis* Fei, Ye & Huang, 1990 or *Rana uenoi* Matsui, 2014 for identification. All individuals were released at the point of capture following the sampling procedure.

### Call analyses

Prior to data extraction, we filtered out the background noises at 1 kHz, and the set the spectrogram configuration at a 256-sample Hann window size, 128-sample hop size with 50% frame overlap and 172-Hz frequency grid spacing. We analysed each call for both temporal and spectral domains (Raven Pro 1.4; Cornell Lab of Ornithology, New York, USA), following the recommendations of Koehler et al. (2017).

### Molecular analyses

Three *Rana* sp. tadpoles were collected on 29 April 2021 in Ulleung county (37.472778 °N; 130.848611 °E). We extracted the total DNA from the tail

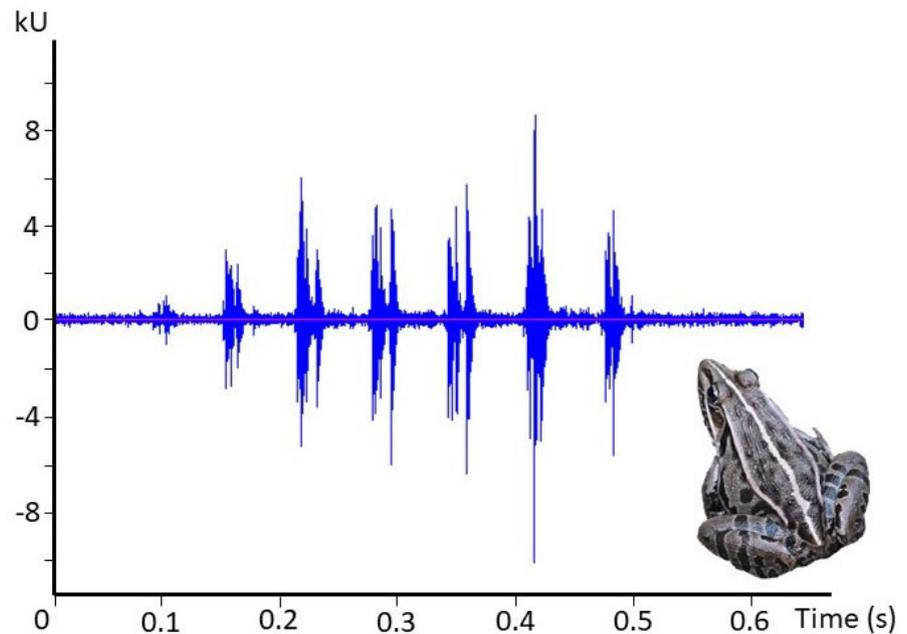
**Table 1.** Information of samples used for phylogenetic tree reconstruction.

Taxa	Locality	Accession number	Reference
<i>Rana amurensis</i>	Liaoning, Zhangwu, China	KU343216	Zhao et al. 2016 (direct submission to GenBank)
<i>Rana coreana</i>	Republic of Korea	KM590550	Jang and Hwang 2015 (direct submission to GenBank)
<i>Rana chensinensis</i>	Yellow Sea basin of North China	NC_023529	Li et al. (2014)
<i>Rana kukunoris</i>	Helan Mountains, Inner Mongolia, China	MN733918	Wang et al. 2020
<i>Rana huanrenensis</i> (Ulleung Island)	Ulleung Island, Republic of Korea	MZ779046	This study
<i>Rana huanrenensis</i> (Northern China)	Huanren, Liaoning in China	KT588071	Dong et al. (2016)
<i>Rana omeimontis</i>	Yucheng District, Sichuan, China	KU246050	Yang et al. 2018
<i>Rana uenoi</i>	Republic of Korea	MW009067	Suk and Min 2020 (direct submission to GenBank)
<i>Rana dybowskii</i>	Amur River basin of Northeast China	KF898355	Li et al. (2014)

clip of one individual (voucher number: 20RXV003) using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to manufacturer protocol to sequence the complete mitogenome of the individual. We then constructed the sequencing library using Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA) following the manufacturer's recommendations with around 350-bp DNA fragments. We obtained a total of 5.3 Gbp raw sequences from Illumina NovaSeq6000 at Macrogen Inc., Korea, and we filtered the raw sequences using Trimmomatic v0.33 (Bolger et al. 2014). We then conducted the *de novo* assembly and confirmed with Velvet v1.2.10 (Zerbino and Birney 2008), SOAPGapCloser v1.12 (Zhao et al. 2011), BWA v0.7.17 (Li et al. 2009), and SAMtools v1.9 (Song and Liang 2013) under the environment of Genome Information System (GeIS; <http://geis.infoboss.co.kr>) which has been used in previous studies (Kim et al. 2021; Jung et al. 2021; Lee et al. 2020). We used Geneious R11 v11.1.5 (Biomatters Ltd, Auckland, New Zealand) to transfer the annotation of the assembled mitogenome based on alignments with another *Rana huanrenensis* mitochondrial genome (NC\_028521; Dong et al. 2016) and MITOS (Bernt et al. 2013). We deposited the sequence to the GenBank database under the accession number MZ779046.

### Phylogenetic analysis

Here, we determined the species identity using phylogenetic analyses. We reconstructed a phylogenetic tree based on the complete mitochondrial sequence of our *Rana* sample (19,146 bp; GenBank accession number: MZ779046) with eight homologous sequences from *Rana* sp. distributed across Northeast Asia (total  $n$  taxa = 9; Table 1). We aligned the mitogenomes sequences using ClustalW2 (Larkin et al. 2007). To remove the unnecessary gaps, we trimmed both ends of the aligned sequences and obtained a final length of 13,546 kb for the aligned sequences. To infer the phylogenetic relationship, we searched for the best-fit evolutionary model using bModeltest v2.0 (Bouckaert and Drummond 2017). Here, we predicted



**Figure 2.** Call recording of *Pelophylax nigromaculatus* from Ulleung Island, Republic of Korea, recorded in April 2021.

the model K80/HKY to be the best-fit evolutionary model for our full length mtDNA dataset with a posterior probability of 74.15%. We reconstructed a maximum clade credibility (MCC) phylogenetic tree using BEAST v2.6.3 (Bouckaert et al. 2019). We then performed two independent analyses of the tree following the Markov Chain Monte-Carlo (MCMC) procedure for 20 million iterations. We ensured the adequacy of the MCMC samplings by assessing the effective sample size (ESS) values of each parameter ( $ESS > 200$ ) using Tracer v1.7 (Rambaut et al. 2018). We assembled all the generated trees in LogCombiner v2.6.1 (Bouckaert et al. 2019), and summarized the MCC tree after discarding 25% of the trees with a posterior probability limit of 0.5 using Tree Annotator v2.6.3 (Bouckaert et al. 2019). We visualised the MCC tree using Fig tree v.1.4.3 (Rambaut 2009).

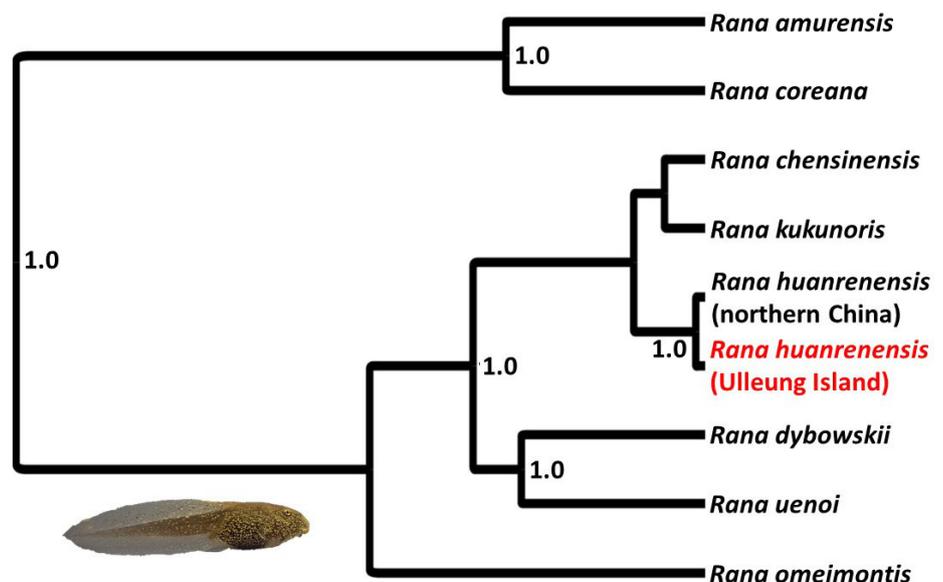
## Results

We assigned the calls we recorded (37.464155 °N; 130.869171 °E and 37.517096 °N; 130.815914 °E; Figure 1) to *Pelophylax nigromaculatus* based on the analyses (Figure 2). Determining the identity of the species is straight forward with call properties, but determining the population of origin, potentially from Japan or the Korean mainland, is impossible at the moment due to the absence of a database comparable to the one existing for genetic information.

We collected three tadpoles (37.472778 °N; 130.848611 °E; Figure 1) that were identified in the field as belonging to the *Rana* genus, in a habitat similar to what would be expected for the genus within its native range (Figure 3). Sequence similarity through BLAST showed a 98.58% match with



**Figure 3.** Pictures of the *Rana huanrenensis* individuals sampled in Ulleung Island, Republic of Korea in April 2021. (A) group picture in situ; (B) in aquarium at the site; (C) individual in situ. Photographs by YB and AB.



**Figure 4.** Maximum clade credibility (MCC) tree showing the phylogenetic placement of *Rana huanrenensis* from Ulleung Island, Republic of Korea (in red) inferred from the complete mitochondrial genome. Node values indicate the Bayesian posterior probability (BPP).

*Rana huanrenensis*. Phylogenetic analyses the full mitogenome clustered our sample with *R. huanrenensis* and showed a sister relationship between our samples and the seven other *Rana* (Figure 4). The MCC tree strongly supported the identity of the sample *R. huanrenensis*, within a monophyletic clade (posterior probability (PP) = 1.0) including the most closely related northeast Asian *Rana* such as *R. kukunoris* and *R. chensinensis* (Figure 4). The present of a shallow divergence between the two *R. huanrenensis* (BPP = 1.0; Figure 4) indicated the recent introduction of the species.

## Discussion

Our records of *Rana huanrenensis* tadpoles, the first time the species is identified on the island, and the calls of *Pelophylax nigromaculatus* clarified the presence of these two species on Ulleung Island. In addition, although putatively in the case of *P. nigromaculatus*, the two species are breeding on the island and therefore maintaining populations, which by their presence have an impact on the local ecology of species. The extent of their influence is however unknown and warrants additional research.

While originally laudable, the “re”-introduction of “extinct” *Rana* species on Ulleung island instead resulted in the introduction of a non-native species. The species was found to be restricted to small streams at comparatively lower elevation, although not all streams could be surveyed on their totality and the presence of populations at higher elevations is possible.

The case of *P. nigromaculatus* is different as the presence of the species on the island was expected. The species was introduced for pest-control of crops, and opportunistically to provide food. The absence of large scale natural or agricultural wetlands should have been a predictor of the success of the species’ introduction for pest control. The species is not currently widespread, and we detected it in two small wetlands only, one of them being a fish pond, and therefore sub-optimal for the species. These two populations are likely to be independent as they are separated by landscape barriers and a distance generally considered greater than what the species is able to disperse. We recommend conducting a genetic analysis to confirm the origin of the populations as they could originate from either the Japanese archipelago or the Korean mainland.

Current available data lists 242 insect species on Ulleung Island, among which 35 are endemics (Lim and Lee 2012). While the impact of the presence of the two anuran species cannot be assessed from the data available here, the species will clearly have access to niches that were not occupied before their introduction, with unknown impacts on local species. We recommend the implementation of a monitoring program to ensure that the non-native but established species do not spread further than their current presence, and potentially plan their eradication before the populations extend beyond control. It is also possible that these are remnant populations from the introduced individuals, and that their numbers are slowly declining, a likely hypothesis for *P. nigromaculatus*.

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## Ethical statement

The experiments conducted in this manuscript were approved by Nanjing Forestry University.

## Authors' contribution

Research conceptualization – YB, AB; sample design and methodology – YB, JP, SNO, AB; investigation and data collection – YB, SNO, AB; data analysis and interpretation – YB, JP, SNO, AB; funding provision – YJ, AB; writing (original draft) – YB, AB; writing (review and editing) – YB, JP, SNO, YJ, AB.

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