

Rapid Communication**Genetic identification of *Rattus norvegicus* (Berkenhout, 1769) on the uninhabited Sasudo island provides accurate information on invasive species**Seon-Mi Park¹, Jun-Won Lee², Young-Hun Jung³, Sung-Hwan Choi³, Sang-Hyun Han⁴ and Hong-Shik Oh^{2,3,*}¹Research Institute for Basic Sciences of Jeju National University, Jeju Special Self-Governing Province, 63243, Republic of Korea²Faculty of Science Education, Jeju National University, Jeju Special Self-Governing Province, 63243, Republic of Korea³Interdisciplinary Graduate Program in Advanced Convergence Technology and Science, Jeju National University, Jeju Special Self-Governing Province, 63243, Republic of Korea⁴Habitat Conservation Division, Korea National Park Research Institute, Gyeongsangbuk-do 36015, Republic of Korea

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OPEN ACCESS**Abstract**

In the Republic of Korea, the uninhabited Sasudo island is well known as a breeding ground for seabirds; however, it is seriously threatened by the predation of invasive rats. Here, we analyzed the mtDNA *CYTB* gene sequence to identify the species of invasive rats captured on Sasudo island from August 2015 to May 2016. The *CYTB* sequence of the three invasive rats on Sasudo island was 1048 bp long. BLAST analysis showed 99.81–100% homology with the *R. norvegicus* and a monophyletic pattern in the phylogenetic tree. These results indicate that the rats found on Sasudo island belong to the species *R. norvegicus*. This study is the first to provide accurate species information and explain the invasion of *R. norvegicus* on Sasudo island through genetic identification. We conclude that genetic species identification provides reliable information regarding invasive species. This information can be used as basic data for managing invasive species to protect island biological resources.

Key words: alien species, molecular identification, mtDNA *CYTB* gene, rat, Rodentia, seabirds**Introduction**

The biodiversity of islands and the surrounding coastal waters are threatened by invasive species (Wittaker 1998; Hong 2014). Rats have been identified as the most successful invasive species on the islands. They can lead to the extinction of indigenous species on the island and trigger extreme changes in the ecosystem (Townsend et al. 2006; Duron et al. 2017). In particular, three species of the genus *Rattus*, namely *Rattus rattus* (Linnaeus, 1758), *Rattus norvegicus* (Berkenhout, 1769), and *Rattus exulans* (Peale, 1848), have been introduced to more than 80% of the world's islands (Atkinson 1985), where they pose a serious threat to seabirds (Jones et al. 2008; Smart et al. 2021). Seabirds spend most of their lives at sea, and to ensure successful breeding, they nest mainly on coastal islands that are free of terrestrial predators. Uninhabited Sasudo island is

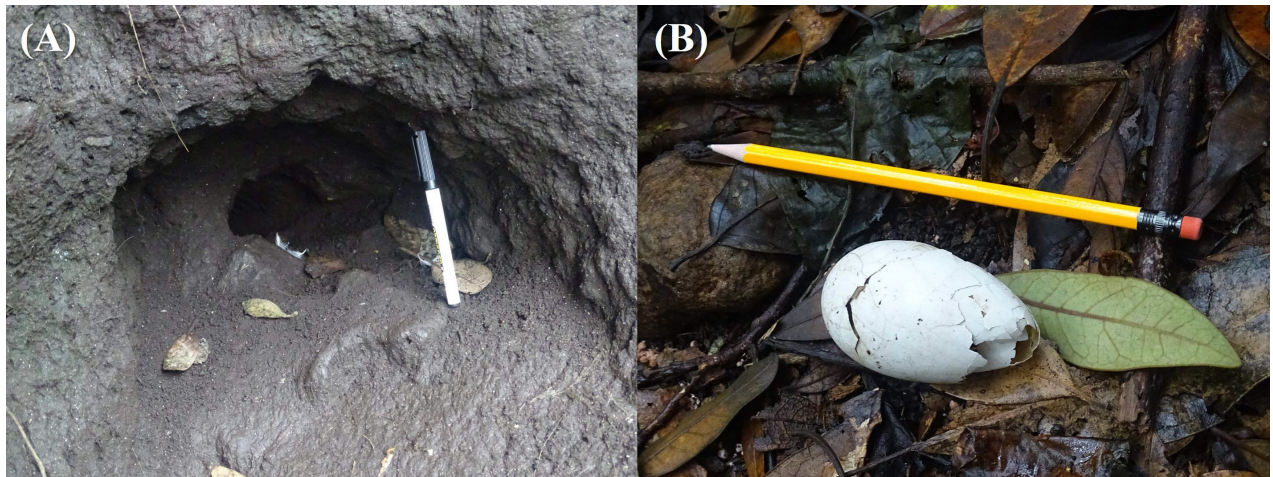


Figure 1. Signs of seabirds and rats observed on Sasudo island. (A) Nest of *Calonectris leucomelas* discovered on Sasudo island. *C. leucomelas* breed by digging burrows in the ground; (B) Egg of *C. leucomelas* that failed to hatch due to predation by rats. Photographs by Young-Hun Jung.

a major breeding ground for streaked shearwaters (*Calonectris leucomelas* Temminck, 1835) in the Republic of Korea (Lee et al. 2002; Oh et al. 2008; Nam et al. 2014). They dig burrows for breeding and spawn every summer in the area (Figure 1A). However, breeding is severely hampered on Sasudo island by rats predated on their eggs (Figure 1B).

Management strategies to reduce damage from the introduction of invasive species to island ecosystems vary depending on the situation and conditions (Duron et al. 2017). Many researchers aim to prevent introductions, which is the best way to manage invasive species (Lowe et al. 2000; Keller et al. 2011; Simberloff et al. 2013). When prevention fails, early detection and prompt response are required. With this, it is possible to respond quickly to the spread of invasive species by identifying the species and its entry route. These actions can be an effective and economical strategy for the management of invasive species (Simberloff et al. 2013).

Traditional morphological identification of invasive species can be unsuccessful in the case of poor-quality specimens and those with morphological similarities, as well as a lack of expertise of researchers. Genetic analysis is known to reduce errors in morphological analysis of invasive species and provides reliable taxonomic evidence (Thomas et al. 2016; Nagarajan et al. 2020). Genetic analysis has been used to identify a wide range of terrestrial, freshwater, and marine species (May and Marsden 1992; Van Ginderdeuren et al. 2012; Bushar et al. 2015; Castillo et al. 2021; Hassan et al. 2022). For example, mitochondrial *cytochrome b* (*CYTB*) analysis is used for DNA barcoding to identify invasive mammalian species on islands and to classify difficult-to-identify aquatic vertebrates (Bennett et al. 2011; Piaggio et al. 2014).

In the present study, we used *CYTB* sequences for accurate species identification of *Rattus* species inhabiting Sasudo island and demonstrated the importance of genetic analysis in monitoring invasive species.

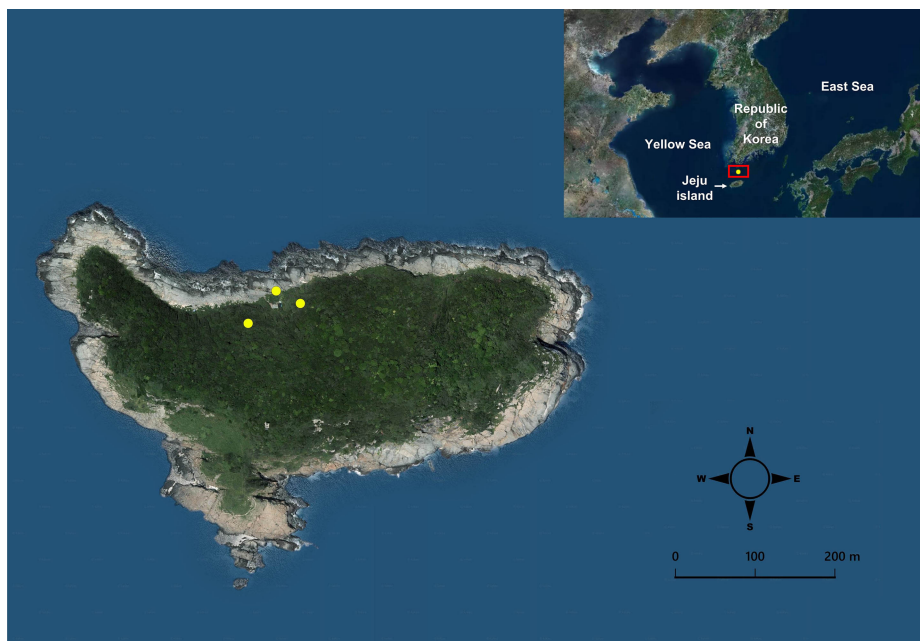


Figure 2. Map of the survey area. The sites from where the rats were captured to collect the samples are indicated using yellow circles.

Materials and methods

Sampling

Tissue samples from unknown rats inhabiting Sasudo island were collected from August 2015 to May 2016 (Figure 2). This study has been reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jeju National University. To capture unknown rats, small Sherman traps (7.62 × 8.89 × 22.86 cm, HB Sherman Traps, Tallahassee, Florida, USA) were deployed at 4 pm and were checked the next morning. The muscle and liver tissues of the captured animals were collected in the field and placed in microtubes containing 96% ethanol. The specimens were transferred to the laboratory and stored at −20 °C until DNA extraction.

Genetic analysis

Each specimen was washed with sterile water immediately before performing the experiments. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA), following the manufacturer's protocol. The extracted DNA was stored at 4 °C and used as a template for PCR. The mtDNA *CYTB* gene fragment was amplified using a pair of primer sets with the following sequence: MusCYTB_F 5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3', MusCYTB_R 5'-GCA GCC CCT CAG ATT GAT ATT TGT CCT-3'. PCR amplification was performed using the Maxime PCR Premix (iNtRON Biotechnology, Republic of Korea) and then amplified using a Thermal Cycler 2720 (Applied Biosystems, USA). Initial denaturation for one PCR cycle was performed at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 55°C for 1 min,

extension at 72 °C for 1 min; and one cycle of final extension at 72 °C for 5 min. The PCR product was confirmed by electrophoresis and purified using a QIAex II Gel Extraction Kit (Qiagen). It was then subjected to a BigDye® Terminator v3.1 cycle sequencing Kit (PE-Applied Biosystems, USA). DNA sequences were determined using an ABI 3730 lx DNA Analyzer (ABI, USA). Species were identified from the specimens by comparing them with previously reported sequences using NCBI BLAST searches. The sequences determined in this study and the highest similarity sequences detected in GenBank were aligned using the CLUSTAL W program (Thompson et al. 1994). The evolutionary analyses were conducted in MEGA X program (Kumar et al. 2018) with the most similar sequences and five species of the genus *Rattus* (Supplementary material Table S1). The sequence of *Mus musculus* (Linnaeus, 1758) was used as an outgroup. Genetic distance was calculated using Tamura-Nei model (TN93) (Tamura and Nei 1993), which was selected as the best model test. In the ML, initial tree for the heuristic search were obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the TN 93, and then selecting the topology with superior log likelihood value. a discrete gamma distribution was used to model the evolution rate differences between sites (5 categories (+G, parameter = 0.3285)). In NJ tree, the evolutionary distances were computed using the TN93 method with substitutions to include transions/transversion ratio and the rate variation among sites was modeled with a gamma distribution (shape parameter = 1). ML and NJ tree reliability were assessed by bootstrapping 1000 replicates.

Results and discussion

The *CYTB* sequence of the three rats from Sasudo island was determined to be 1048 bp. The sequence was registered at GenBank (accession number: ON160933–ON160935). The sequences of the two individuals (R75 and R77) were identical, and the remaining individual (R76) differed by 5 bp. BLAST analysis of the determined sequence showed the highest homology of 99.81–100% with the previously reported *R. norvegicus* *CYTB* sequence (Table S2). In addition, homology with *R. rattus* inhabiting the Republic of Korea was confirmed to be less than 95%. The three individuals from Sasudo island belonged to the same group as *R. norvegicus* and showed a monophyletic pattern in the phylogenetic tree (Figure 3 and Figure S1). The genetic distance between the three specimens (R75, R76, and R77) collected from Sasudo island was calculated as 0.0048 (Table S3). The two specimens (R75 and R77) shared the same sequence as the Swedish sequence (FJ919764). The value between the R76 sequence and the China sequence (MG748269) was calculated to be 0.0019. The genetic distance between all rat specimens collected from Sasudo island and *Rattus* species other than *R. norvegicus* was calculated to be 0.1505–0.2192. These results

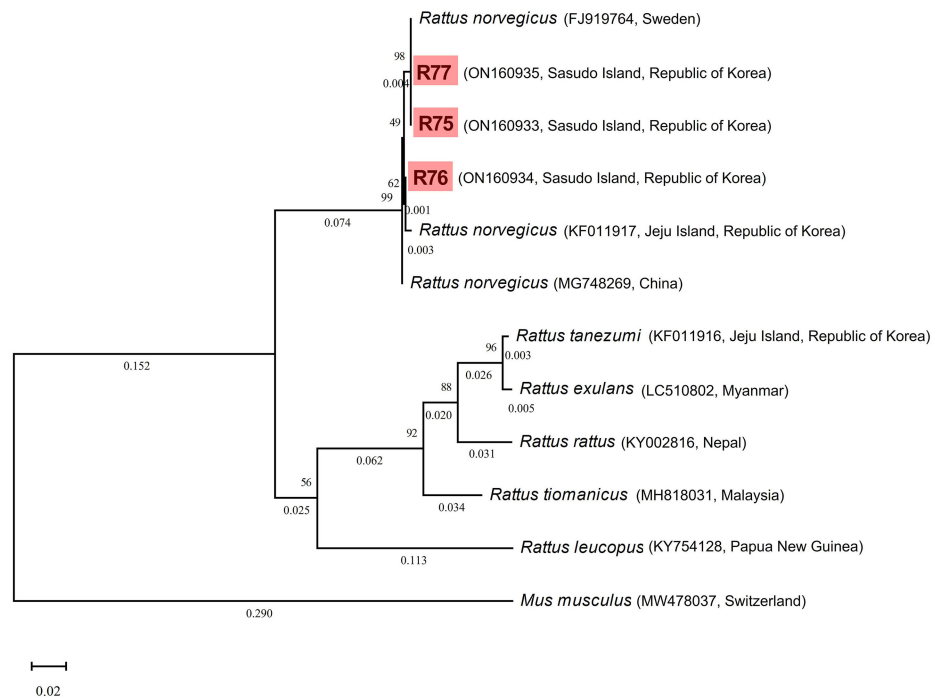


Figure 3. The phylogenetic tree for *CYTB* sequences of unknown rats on Sasudo island. The evolutionary history was inferred by using the Maximum likelihood method and the Tamura-Nei model (Tamura and Nei 1993). The numbers above the branches are the bootstrap values, and the numbers below are the length of the branches. Branch lengths less than 0.001 are not shown. Marked in red is the *CYTB* sequence of rats inhabiting Sasudo island. *Mus musculus* was used as an outgroup. The origin and GenBank accession numbers of the sequences used in the analysis are indicated in parentheses. For detailed information on each sequence, see Supplementary material Table S1.

indicate that the three rat specimens from Sasudo island analyzed in this study were identical to or closest to *R. norvegicus*. In other words, it reveals that the rat species found on Sasudo island are *R. norvegicus*.

The genus *Rattus* is a major invasive species worldwide (Shiels et al. 2014), and two species, in particular, *R. tanezumi* and *R. norvegicus*, are widespread in the Republic of Korea (Jo et al. 2018). *Rattus* species have complex phylogenetic relationships and are difficult to identify morphologically (Robins et al. 2014). In general, differences are identified by body size and tail length (Kim et al. 2013). However, these methods often misidentify the rats because they differ not only by the individual (e.g., adult or young, male or female), but also by region (Kim et al. 2013; Robins et al. 2014).

Sasudo is an uninhabited island designated as a natural reserve in the Republic of Korea. Entry to the island is possible only with permission from the Cultural Heritage Administration of the Republic of Korea. However, approximately 120 years ago, about three fishermen lived there, and it is thought that rats were introduced through ships at that time (Lee and Yoo 2002). One can enter Sasudo island with the mainland of the Republic of Korea or Jeju island, the largest inhabited island in the Republic of Korea, via various small annexed islands using boats via different routes; therefore, the entry route of *R. norvegicus* to the Sasudo island could be

very diverse. Future studies to locate the source of *R. norvegicus* in the mainland of the Republic of Korea could unravel the life history and invasion potential of *R. norvegicus* and assist in developing *R. norvegicus* eradication strategies from this island. In previous studies, the introduction of rats to Sasudo island was confirmed based on predation traces of *C. leucomelas* eggs (Nam et al. 2004, 2014; Oh et al. 2008). This was only assumed to be *R. norvegicus*, and there was no clear identification of the species responsible. This study is the first to provide evidence on *R. norvegicus* invasion through genetic identification of rats inhabiting the Sasudo island and provides accurate species information. In conclusion, we agree that genetic species identification provides reliable information on invasive species and can be utilized for invasive species management. We propose the removal of *R. norvegicus* from the Sasudo islands owing to its adverse ecological characteristics. Future studies by comparing the gene sequences and the phylogeny of *R. norvegicus* identified in Sasudo island and Korean house rats could be useful to identify the source location and introduction route of *R. norvegicus* to Sasudo island, which will help monitor the future invasions and complete eradication of *R. norvegicus* on Sasudo island. The removal of *R. norvegicus* will increase the reproductive success of seabirds and prevent disruptions in their breeding cycles.

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Authors' contributions

SMP and HSO conceived the study, field design and methodology. JWJ, YHJ and SHH carried out the field study and collected samples. SMP, SHC and SHH performed DNA extraction and data analysis. SMP wrote the original manuscript and was reviewed/edited by HSO.

Ethics and permits

This study has been reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jeju National University.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Information on the sequences used to construct the phylogenetic tree.

Table S2. BLAST search result of *CYTB* sequences of the specimens.

Table S3. Genetic distance test results based on the Tamura-Nei model.

Figure S1. The phylogenetic tree for *CYTB* sequences of unknown rats on Sasudo island was inferred using the Neighbor-Joining method.

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