BioInvasions Records (2022) Volume 11, Issue 4: 839–854

Research Article

Species identification and invasion pathways of an introduced snail *Macrochlamys* sp. in Japan

Kodai Kudo[1,3,*], Osamu Kagawa[2,3], Shun Ito[3,4], Shinichiro Wada[5], Hirotaka Nishi[6], Shovon Mohammad Shariar[7], Daishi Yamazaki[4,8], Takahiro Hirano[3,4] and Satoshi Chiba[3,4]

[1]*Laboratory of Aquatic Science Consultant Co., LTD., 2-30-17, Higashikamata, Ota-ku, Tokyo, 144-0031, Japan*
[2]*Shimoda Marine Research Center, University of Tsukuba, 5-10-1 Shimoda, Shizuoka 415-0025, Japan*
[3]*Graduate School of Life Science, Tohoku University, 41 Kawauchi, Aoba-ku, Sendai, Miyagi 980-0862, Japan*
[4]*Center for Northeast Asian Studies, Tohoku University, 41 Kawauchi, Aoba-ku, Sendai, Miyagi 980-0862, Japan*
[5]*Hahajima Branch of Ogasawara Ranger Office, The Ministry of the Environment, Shizukazawa, Hahajima, Ogasawara, Tokyo 100-2211, Japan*
[6]*Toyohashi Museum of Natural History, 1-238 Oana, Oiwa, Toyohashi, Aichi 441-3147, Japan*
[7]*Department of Biochemistry & Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh*
[8]*Faculty of Agriculture and Marine Science, Kochi University, 200 Monobe, Nankoku, Kochi, 783-8502, Japan*

**Corresponding author*
E-mail: *kodaikudo.1119@gmail.com*

Citation: Kudo K, Kagawa O, Ito S, Wada S, Nishi H, Shariar SM, Yamazaki D, Hirano T, Chiba S (2022) Species identification and invasion pathways of an introduced snail *Macrochlamys* sp. in Japan. *BioInvasions Records* 11(4): 839–854, https://doi.org/10.3391/bir.2022.11.4.03

Received: 22 November 2022
Accepted: 25 May 2022
Published: 7 September 2022

Handling editor: Mikhail Son
Thematic editor: Tim Adriaens

Abstract

The various problems caused by invasive species have become more serious in recent years, and thus it is important to discuss their identifications and invasion processes. Phylogenetic and population genetic methods are effective tools for solving these problems. Some land snails, including the genus *Macrochlamys*, have invaded and established themselves worldwide as invasive species. In this study, we identified the species of *Macrochlamys* sp. from Japan and estimated their invasion pathways to Japan. Phylogenetic analysis showed that the Japanese snails were closely related to *Macrochlamys indica* from Bangladesh and West Bengal. Population genetic analysis also revealed that most of the Japanese snails were concentrated in single haplotype and were close in genetic distance. Detailed anatomical investigation of genitalia and oral organs showed morphological similarities between the Japanese and Bangladeshi snails, supporting the results of the phylogenetic analysis. These findings suggest that the Japanese *Macrochlamys* sp. is recognized as *Macrochlamys indica* and may have been introduced from the vicinity of Bangladesh and West Bengal and subsequently spread within Japan.

Key words: land snail, alien species, invasive species, Ogasawara Islands, pest snail, Mollusca, *Macrochlamys indica*

Introduction

Invasive species arrive in new areas by direct human introduction or by accompanying agricultural products and other various materials, and with the advance of globalization, the various problems caused by invasive species are becoming more serious (Hulme et al. 2008; Perrings et al. 2005). Identification of invasive species and elucidation of their invasion processes are necessary for predicting future trends in order to prevent their further spread and identify control options, as well as for clarifying

the roles of the species and ecosystem features (Armstrong and Ball 2005; Colautti et al. 2006; Schaal et al. 2003). To address these problems, DNA barcoding methods provide particularly useful information for identifying species and reconstructing processes such as the non-native species' invasion and dispersal pathways and historical population dynamics (Armstrong and Ball 2005; Bryja et al. 2010; Rollins et al. 2011; Siedchlag et al. 2010).

The land snail genus *Macrochlamys* Gray, 1847 includes more than 100 species that are distributed from South Asia to Southeast Asia and southern China (Blanford and Godwin-Austen 1908; Bouchet et al. 2005; Mitra et al. 2004; Pholyotha et al. 2018; Ramakrishna et al. 2010). Some of the snails in this genus have invaded and become established all over the world as alien species. For example, the invasion of *Macrochlamys indica* Godwin-Austen, 1883 has been confirmed in the USA (Florida), Brazil, Egypt, Mauritius and Malaysia (Agudo-Padron 2017, 2018; Godwin-Austen 1905; Talamas 2020; Vermeulen and Liew 2022). In addition, *Macrochlamys hippocastaneum* Godwin-Austen, 1918 has been confirmed in Taiwan since 2000, and *Macrochlamys kalantanensis* Möllendorff, 1902 in Singapore (Sow-Yan and Wing Lup 2021; Wu and Tsai 2014). The impact by *Macrochlamys* snails on crops and ecosystems has become a problem in these areas. *Macrochlamys indica* is a pest that damages vegetables and ornamental crops in India, and therefore, the United States has registered it as a quarantine plant pest posing the greatest threat (Chanda and Mandal 2020; Jayashankar et al. 2015; Kumar and Ahmed 2000; Revynthi et al. 2020; Singh et al. 2020). Further, there is concern that the snails may have a negative impact on other snails. The alien snail *M. hippocastaneum* is a carnivorous predator that actively preys on the Asian tramp snail *Bradybaena similaris* (Férussac, 1822) (Wu and Tsai 2014). In addition, under the feeding environment used in a Japanese study, the alien snail *Macrochlamys* sp. significantly reduced the survival rate of the native snail *Bekkochlamys perfragilis* (Pilsbry, 1901) (Kimura 2015).

In *Macrochlamys* snails, taxonomic and anatomical studies have shown that morphological characters such as genitalia, penial interior sculpture, jaw plate, and radula differ among species (Godwin-Austen 1883; Pholyotha et al. 2018, 2020a; Roy 2020; Venmans 1957). Godwin-Austen (1883) provided a species description of *M. indica* based on morphological characters of the shell, genitalia, jaw plate and radula. In addition to these morphological characters, Pholyotha et al. (2018, 2020a) and Roy (2020) showed that the shape of the penial interior sculpture is an important trait for the identification of species in the genus *Macrochlamys*. On the other hand, despite molecular phylogenetic studies on several species of the family Ariophantidae Godwin-Austen, 1883, no such studies have been conducted on the genus *Macrochlamys* (Dumidae et al. 2020; Pholyotha 2020b, 2021). Therefore, these morphological classifications lack molecular phylogenetic information, and it is unclear whether molecular phylogeny is

consistent with classifications by morphological traits. Some researchers also pointed out that in Macrochlamydinae, identification based on shell morphology may result in misidentification (Pholyotha et al. 2018). Thus, approaches based on both anatomical and molecular phylogenetic methods may be effective for species identification in the genus *Macrochlamys*.

In Japan, *Macrochlamys* sp. was first identified in Okinawa Island in the early 2000s, and has since been reported from several localities in Japan (Okinawa Islands; Okinoerabujima Island; Mie Prefecture; Aichi Prefecture; and Gifu Prefecture) (Hayase et al. 2009; Hirano et al. 2018; Iwata 2015; Kimura 2011; Minato et al. 2020; Nishi and Matsuoka 2013; Ueshima 2009). Additionally, we discovered *Macrochlamys* sp. on Hahajima Island in the Ogasawara (Bonin) Islands, Japan in 2016. The Ogasawara Islands were registered as a UNESCO World Natural Heritage site in June 2011 for their unique natural ecosystems (UNESCO 2011). Meanwhile, the extinction of endemic snails due to invasive species has also been noted in the Ogasawara Islands in recent years (Chiba 2007; Ohbayashi et al. 2007). Nevertheless, *Macroclamys* sp. introduced to Japan has not yet been identified from molecular phylogeny and morphology. In addition, the invasion pathways and dispersal processes are not well understood.

In this study, we combined molecular phylogenetic and anatomical methods to identify *Macrochlamys* sp. in Japan and to evaluate the invasion pathways and spread of its distribution in Japan.

Materials and methods

Sample collection

We collected 80 samples of *Macrochlamys* sp. from Hahajima Island, the Okinawa Islands, Kagoshima Prefecture, and Aichi Prefecture in Japan and 3 samples of *Macrochlamys indica* from Rajshahi in Bangladesh (Figures 1 and 2; Table S1). A fragment of the foot muscle from each snail was excised and stored in 99.5% ethanol prior to DNA extraction. The remaining soft bodies were stored in 70% ethanol for dissection. In addition to these samples, we used the sequences of 19 snails from 6 *Macrochlamys* species found in GenBank (Ayyagari and Sreerama 2020; Hyman et al. 2007; Jirapatrasilp et al. 2021; Slapcinsky and Mulcahy 2017; Pholyotha et al. 2020b; Sutcharit et al. 2019, 2020): *Macrochlamys* sp. in Thailand and Myanmar, *M. indica* and *Macrochlamys petrosa* (Hutton, 1834) in India, *Macrochlamys aspides* (Benson, 1863) in Myanmar, *Macrochlamys tanymentula* Pholyotha & Panha, 2018 and *Macrochlamys caverna* Pholyotha & Panha, 2018 in Thailand. These are all the sequences of the genus *Macrochlamys* in GenBank whose locality is clearly described in the reference (Table S2). Three species of the family Dyakiidae such as *Bertia cambojiensis* (Reeve, 1860), *Dyakia janus* (Beck, 1837) and *Rhinocochlis nasta* (Metcalfe, 1852) were selected as outgroups (also from GenBank Data).

Figure 1. Image A shows *Macrochlamys* sp. in nature. Image B–D shows the shells of *Macrochlamys* sp. that we focused on in this study. Image B is the snail from Ogasawara Island (Hahajima Island), C is the snail from Okinawa Island and D is the snail from Aichi Prefecture. White scale bars are 1cm. Photographs by Kodai Kudo.

Molecular methods

Total DNA from the snail tissue was extracted using either of two methods. One is a method using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The other is a method modified from Sokolov (2000), which is as follows: muscle tissue homogenized in 800 µL of lysis buffer and 15 µL Proteinase K was incubated at 56 °C overnight. RNaseA (4 µL) was added as well. Saturated KCl (80 µL) was then added to the lysate, and the solution was further incubated at 56 °C for 5 min and then on ice for 5 min before centrifuging for 5 min. The supernatant (~ 700 µL) was transferred to a new tube, extracted once with a phenol/chloroform/isoamyl mixture (25:24:1), and precipitated with 600 µL of isopropanol by incubating at 4 °C for 30 min. The solution except for the visualized DNA was discarded from the incubated solution and centrifuged for 5 min. To the tube containing only DNA, 70% ethanol was added and incubated at 4°C for 5 min. Only the 70% ethanol was discarded and the solution was centrifuged for 5 min. The DNA pellets were vacuum dried to evaporate the 70% ethanol and then dissolved in 100 µL TE buffer. Fragments of the mitochondrial cytochrome subunit I gene (COI) were amplified using the primers LCO1490 (5′-GGTCAACAATCATAAAGATA TTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATC-3′) (Folmer et al. 1994). Polymerase chain reactions (PCR) were performed with the following thermal cycling conditions: 94 °C for 3 min; 34 cycles at

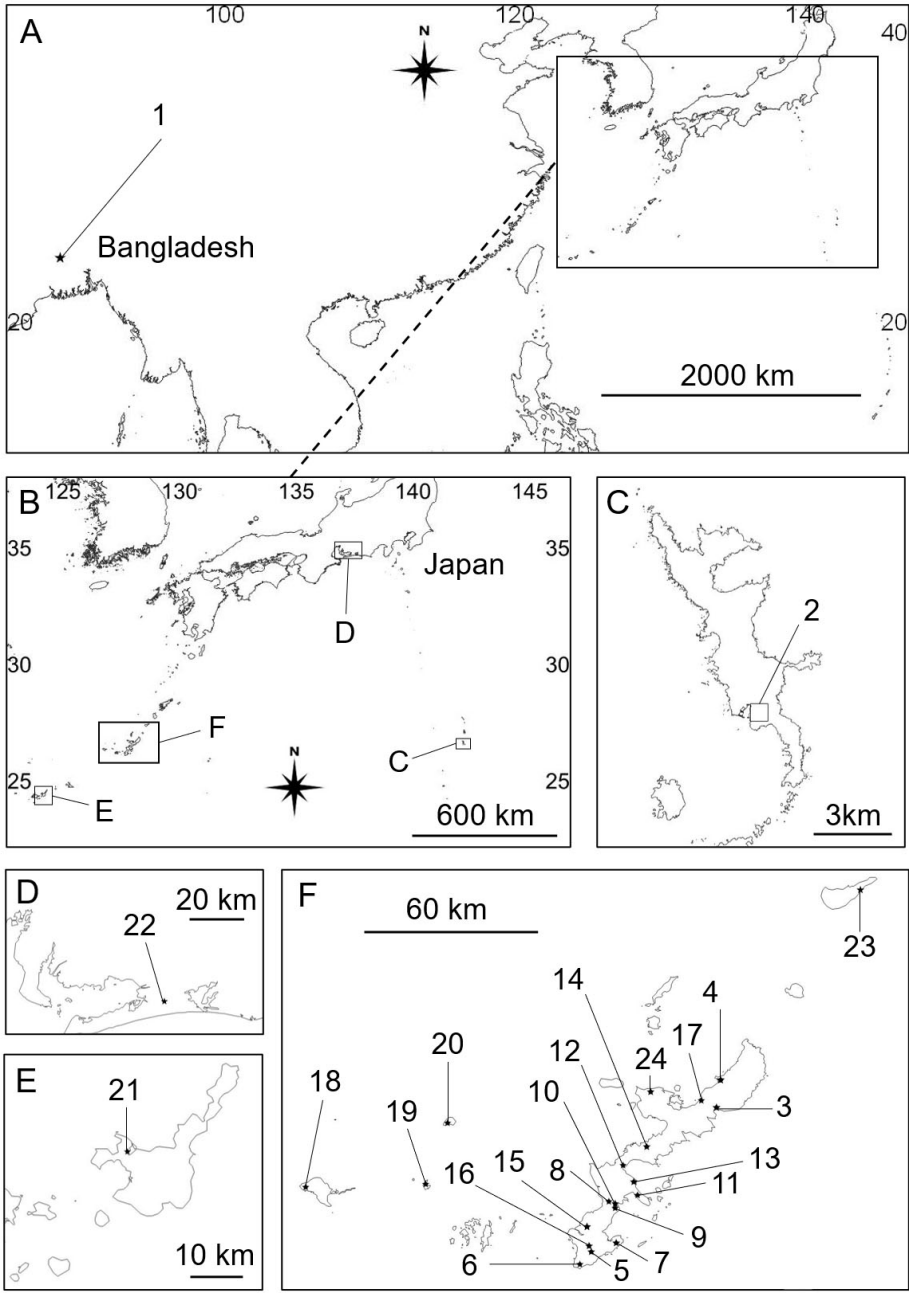

Figure 2. The maps showing the sampling sites. Map A indicates Rajshahi, Bangladesh where *Macrochlamys indica* collected. Maps C–F show the localities where *Macrochlamys* sp. was collected in Japan. Map C is Hahajima, D is Aichi Prefecture, E is Okinawa Island, Okinoerabujima Island, Kume Island, Tonaki Island, Aguni Island and F is Ishigaki Island. The dots and numbers indicate the sites where the snails were collected.

94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products were purified using Exo-SAP-IT (Applied Biosystems, Foster City, CA, USA) following the manufacturer instructions. The BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) or the SuperDye™ v3.1 Cycle Sequencing Kit (AdvancedSeq, Pleasanton, CA, USA) was used for performing cycle sequencing. The products were directly sequenced from both directions using an ABI 3130xl automated sequencer (Applied Biosystems). The sequence data has been deposited in the GenBank database (Table S1).

Construction of phylogeny and haplotype network

The obtained sequences were assembled and contigs created automatically using Phred/Phrap/Consed (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998, 2001) or manually using MEGAX (Kumar et al. 2018). All contigs were aligned using MUSCLE and manually trimmed in MEGA X (Edgar 2004). The final alignment, with a length of 452 base pairs, was obtained by combining sequences. Trimmed sequences were grouped by the same haplotype using Phylogears 2-2.0.2016.09.06 (Tanabe 2008). To estimate the phylogenetic relationship between the samples, phylogenetic trees were constructed using Bayesian inference (BI) and maximum likelihood methods (ML). Kakusan 4-4.0.2011.05.28 was used for selecting the best models based on the Bayesian information criterion (Tanabe 2011). K80+G selected for Partition 1, HKY85+G for Partition 2, and GTR+G for Partition 3. BI analysis was conducted using MrBayes v3.2.2 (Ronquist et al. 2012). We designated an initial burn-in of 5 million iterations, and 2 Markov chains were generated using 20 million Markov Chain Monte Carlo (MCMC) simulations sampled 1000 times. To evaluate the convergence of the MCMC simulations, Tracer v1.7.1 was used for examining the trace plots and effective sample sizes (>200) for all parameters (Rambaut et al. 2018). ML analysis was performed by RAxML version 8.0.0 (Stamatakis 2014). The Kakusan 4-4.0.2011.05.28 was used to select substitution models based on AICc, then GTR+G was selected in all partitions (Tanabe 2011). After selecting the best model for sequence evolution, ML analysis was performed using a bootstrap of 1,000 iterations. The estimation of phylogeny was conducted via the CIPRES Science Gateway for both BI and ML analysis (Miller et al. 2010). To visualize the geographical distribution pattern of the haplotypes, we constructed a median-joining network by using the neighbor-joining method with PopART (Bandelt et al. 1999; Leigh and Bryant 2015). Since genetic distance is also a considered in species description, pairwise distances were calculated using MEGA X (Tsai et al. 2011). Maximum Composite Likelihood was used as the model. The construction of a median-joining network and the calculation of the genetic distance were based on 80 samples of *Macrochlamys* sp. from Japan and 4 samples of *Macrochlamys indica* from 2 localities that are phylogenetically closely related to the Japanese *Macrochlamys* on the phylogenetic trees (see Results).

Anatomical methods

Dissection of the genitalia was performed to investigate the genital traits of *Macrochlamys* sp. from Japan and to compare them with those of *Macrochlamys indica*. Three samples of *Macrochlamys* sp. from Japan (K0064, K0065 and K0067, Table S1) and one sample of *M. indica* from Rajshahi, Bangladesh (K0074, Table S1) were used. Bangladesh is geographically

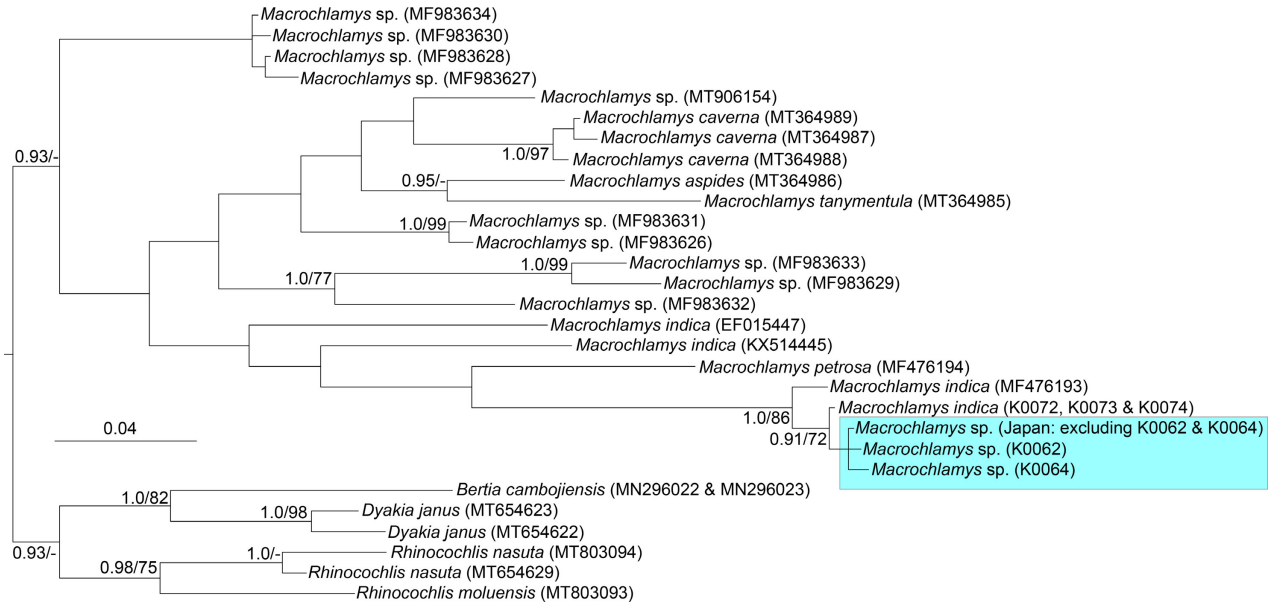

Figure 3. A phylogenetic tree based on Bayesian inference and maximum likelihood methods constructed using 452 bp of the mitochondrial COI gene from 109 snails. The same sequences were aggregated to a single OTU. *Bertia cambojiensis*, *Dyakia janus* and *Rhinocochlis nasta* are the outgroups chosen for the tree root. Each number in the branch at the end of the tree indicates the GenBank accession number (Tables S1 and S2). Numbers at the branch nodes represent BPP and bootstrap values of ML.

close to Kolkata, where the type locality of *M. indica* is located. For K0065 and K0074, the penial interior sculptures, the jaw plates and the radulae were observed. The penial interior sculptures were observed using a stereomicroscope: Nikon SMZ 1500 (Nikon, Minato-ku, Tokyo, Japan) and images were captured using a digital single lens reflex camera: Canon EOS kiss X5 (Canon, Ota-ku, Tokyo, Japan). The jaw plates and the radulae were recognized with an optical microscope: OLYMPUS CX23 (Olympus Corporation, Shinjuku-ku, Tokyo, Japan) and images were taken with a digital camera for microscopy: WRAYCAM-NOA2000 (WRAYMER, Osaka city, Osaka).

Results

The results of the analyses showed that the Japanese populations of *Macrochlamys* sp. were phylogenetically most closely related to *Macrochlamys indica* from Rajshahi, Bangladesh (K0072, K0073 and K0074, Figure 3; Table S1). *Macrochlamys indica* from Bangladesh was also phylogenetically closely related to *M. indica* from West Bengal, India, registered in GenBank (MF476193, Table S2). All these relationships showed high Bayesian posterior probability (> 0.9). On the other hand, the other two GenBank-listed samples of *M. indica* used in the analysis (EF015447 and KX514445) were found to be phylogenetically at different positions. In addition, the populations of *Macrochlamys* sp. in Japan were divided into 3 operational taxonomic units (OTU). One of the 3 OTUs contained almost all of the *Macrochlamys* sp. in Japan. Two snails (K0062 and K0064, Table S1) collected in the northern part of Okinawa Island each constituted single OTU. The first sample, K0062 was collected from Taira, Okinawa Island.

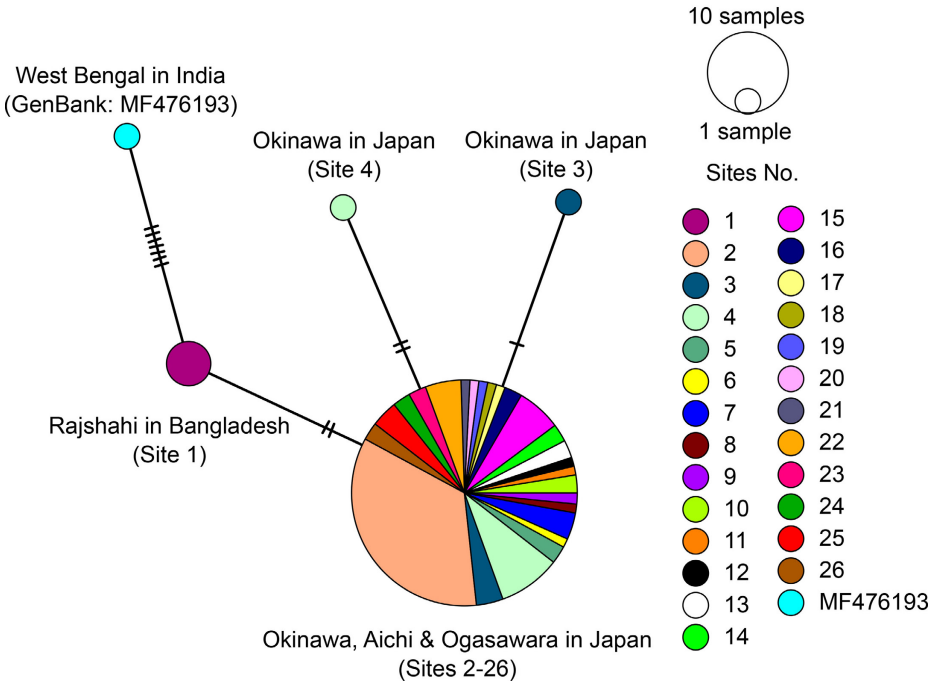

Figure 4. Haplotype network based on the mitochondrial COI genes detected in 80 *Macrochlamys* sp. collected from 25 sites in Japan and 3 *Macrochlamys indica* collected from West Begal, India and Rajshahi, Bangladesh (Tables S1 and S2). The size of the haplotype circles represents the frequency of the haplotypes, and the bars above the branches indicate the variation along the branches. The bar above the branch indicates the variation along the branch. The color indicates the region of the population in which the haplotype was detected.

Four samples from Taira were used for the analysis, and only K0062 was in a phylogenetically different position. The second sample, K0064 was collected from Okuma, Okinawa Island. Eight samples from Okuma were used for the analysis, and only K0064 was in a phylogenetically different position as well as K0062. These 3 OTUs were closely related to each other and had many identical sequences. For 3 samples of *M. indica* from Bangladesh were integrated into a single OTU.

The COI haplotype network of 80 samples of *Macrochlamys* sp. from Japan and 2 samples of *M. indica* from Bangladesh (K0072, K0073 and K0074, Table S1) and West Bengal (MF476193, Table S2) is shown in Figure 4. The population of *Macrochlamys* sp. in Japan consisted of 3 haplotypes, and 97.5% of all samples shared the same haplotype. As in the phylogenetic analysis, only 2 samples, collected in the northern parts of Okinawa Island (K0062 and K0064, Table S1), showed a haplotype that differed from the other 78 samples. The major haplotype of *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh differed by only 2 bp. In addition, the haplotype of *M. indica* from Bangladesh differed from that of *M. indica* from West Bengal by 7 bp. The calculated genetic distances among the population of *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh and West Bengal are shown in Table S3. The highest value of 2.7% was found between *M. indica* from Bangladesh and *Macrochlamys* sp. from Okinawa (K0064). The minimum value of 0.2% was found between *M. indica* from Bangladesh and West Bengal.

Figure 5. Images A and B show the genital traits of *Macrochlamys* sp. Both individuals were collected at Okuma, Kunigami-son, Kunigami-gun, Okinawa, Japan (A: K0065; B: K0067, Table S1). Image A is a post-fertilization individual, and B is a pre-fertilization individual. Image C shows the genital traits of *Macrochlamys indica* from Rajshahi, Bangladesh (K0074, Table S1). White bars are 1 cm. The name of each organ is as follows: ag: albumen gland; de: distal epiphallus; drm: dart retractor muscle; ec: epiphallic caecum; fl: flagellum; fpse: fertilisation pouch-spermatheca complex; gd: gametolytic duct; gs: gametolytic sac; mds: median dart shaft; ov: oviduct; p: penis; pe: proximal epiphallus; prm: penial retractor muscle; vd: vas deferens. Photographs by Kodai Kudo.

The genital traits of *Macrochlamys* sp. from Japan (K0065 and K0067, Table S1) are almost identical to *M. indica* from Bangladesh (K0074, Table S1), but there are a few variations in genital traits (Figures 5, S1 and S2). The variations in genitalia among the samples are as follows: the flagellum extends from the junction of vas deferens and distal epiphallus, and the length of flagellum varies among individuals even within the individuals with a same haplotype; the base of distal epiphallus forms a coil, and this twist is weaker in the sample from Bangladesh than in the samples from Japan; in epiphallic caecum, coils are identified, and this coil is smaller in the sample from Bangladesh than in the samples from Japan; the shape of the gametolytic sac is generally consistent among the samples, and this part is slightly longer in the sample from Bangladesh than in the sample from Japan; the dart of the samples from Japan is thicker and shorter, while the dart of the sample from Bangladesh is slightly thinner and has a longer tip than the samples from Japan. The internal sculptures of the penis in *Macrochlamys* sp. populations from Japan are thin elastic folds arranged in a circular pattern, where as in *M. indica* from Bangladesh, a lattice-like micro-sculpture is observed on the entire penial wall (Figure S3). In addition, the jaw plates and the radulae of both the Japanese and Bangladeshi samples are also identical that a jaw plate has a central projection and radular morphology has central tooth symmetrical tricuspid, lateral teeth asymmetrical tricuspid, and marginal teeth obliquely elongate bicuspid (Figure 6).

Discussion

We clarified the phylogenetic relationships based on COI gene and genital traits of *Macrochlamys* sp. in Japan. The results of the molecular phylogenetic

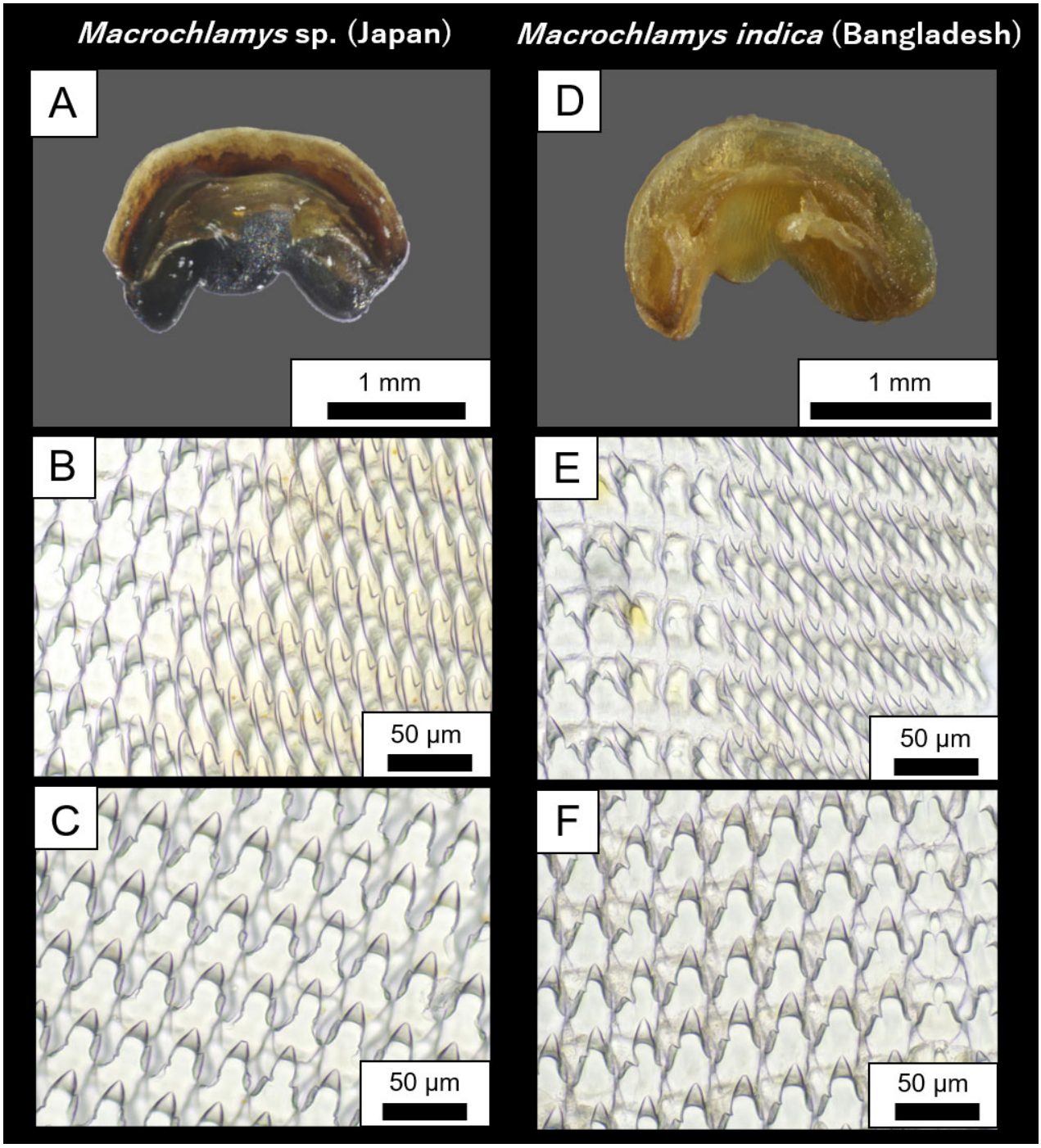

Figure 6. Images A-C are oral organs of *Macrochlamys* sp. (K0065, Table S1) and D-F are those of *Macrochlamys indica* (K0074, Table S1). Image A and D are jaw plates. The black bar indicates 1 mm. Images B and E show the outer parts of the radulae, C and F are central parts. The black bars indicate 50.00 µm in both images. Photographs by Kodai Kudo and Osamu Kagawa.

analysis showed that Japanese *Macrochlamys* sp. is phylogenetically close to *Macrochlamys indica* from Bangladesh and West Bengal, India (Figure 3). The haplotype network showed that *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh and West Bengal had mostly identical sequences, with the genetic distances among samples, ranging from 0.2% to 2.7% (Figure 4; Table S3). By comparing it to the genetic distances of other land snails using COI gene, it would be possible to evaluate the taxonomy of *M. indica* and *Macrochlamys* sp. in Japan. For example, the interspecific

genetic distance ranges from 9.5 to 21.1% in *Meghimatim*, and 3.4–4.3% within and between species in *Carychium minimum* Müller, 1774 and *Carychium tridentatum* (Risso, 1826) (Alexander et al. 2014; Tsai et al. 2011). The values of 0.2–2.7% calculated in this study are much smaller than these examples. Therefore, the results of molecular phylogenetic analysis indicate that *Macrochlamys* sp. in Japan is most likely *M. indica*.

The morphologies of the genitalia, the jaw plate and the radula we observed in *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh. Godwin-Austen (1883) described the characteristics of the genitalia of *M. indica* as follows: the distal epiphallus producing a short flagellum at the junction of the vas deferens, forming a sharp twist at the base; the proximal epiphallus forming a coil before reaching the attachment of the penial retractor muscle; the dart cylindrical, gradually tapering toward the tip where the penial retractor muscle is located; the gametolytic sac is short, slender and pear-shaped. As for the oral organs, he described as follow: the jaw plate has a moderately concave on the cutting-edge and a convex central projection; the radula is characterized by an elongated triangle in the middle with two short, pointed cusps on either side at about halfway down its length, and the lateral teeth that become progressively smaller as they reach the outer parts and finally become the short, even bicuspid teeth. These descriptions are generally consistent with the characteristics of the genitalia, the jaw plate, and the radula of *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh. Roy (2020) also showed the genital morphology of *M. indica* in Kolkata. Although the genital morphology of *M. indica* reported by Roy (2020) differs little from the sketch given by Godwin-Austin (1883), such as a longer gametolytic sac and thinner dart, Roy (2020) treated those samples as *M. indica*. Thus, some individual variations exist in the genitalia of *M. indica*, but in any case, the genitalia of *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh are similar to the sketch drawn by Godwin-Austen (1883). These findings support the identification of *Macrochlamys* sp. from Japan as *M. indica*.

The haplotype network also showed that *Macrochlamys* sp. in Japan was divided into 3 haplotypes and the major haplotype included almost all the samples in Japan, excluding only 2 samples collected from the northern regions of Okinawa Island (Figure 4, Table S1). In land snails, native species generally have a geographic genetic structure, while invasive species are often genetically homogeneous (Chuong et al. 2008; Sherpa et al. 2018; Yang et al. 2018). The detailed invasive pathways of *Macrochlamys* sp. from overseas are obscure. However, at least, the spread of the snails is likely to have originated from Okinawa Island. In particular, the population collected in Aichi was confirmed inside a greenhouse in a horticultural botanical garden. It has also been confirmed that this snail is attached to horticultural plants in Japan (Hayase et al. 2009; Iwata 2015; Kimura 2011; Minato et al. 2020; Nishi and Matsuoka 2013). According to the survey of

farmers in the Ogasawara Islands, more than half of those on Chichijima and Hahajima had introduced seedlings from outside the islands, with Okinawa Island being a highly ranked source of introduction (Ohbayashi and Fujimoto 2016). In 1996, there were some examples *Platypleura kaempferi* (Fabricius, 1794) and *Cryptotympana facialis* (Walker, 1858) transference from trees brought from Okinawa Island to Chichijima Island for planting (Ohbayashi and Takeuchi 1998). Also, in the example of soil animals, *Platydemus manokwari* De Beauchamp, 1962 may have been transferred from Okinawa Island (Ohbayashi 2006). Further, in addition to the attachment of the organisms to plants and transport materials, the eggs may be transferred by soil. Therefore, the snails may have spread their distribution by attaching to horticultural crops.

Roy (2020) cited the shape of the penial interior sculpture as a species-specific morphological feature of *M. indica*. However, the shape of the penial interior sculpture differed between *Macrochlamys* sp. (*M. indica*) from Japan and *M. indica* from Bangladesh, which are phylogenetically close and have similar genital morphology (Figure S3). For phylogenetic analysis, the sequences of 2 *M. indica* from GenBank were used in addition to *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh and West Bengal, the 2 snails from GenBank and the other *M. indica* were paraphyletic. On the other hand, the genitalia and oral organs of these 2 *M. indica* from GenBank were not investigated, and it was unclear to what extent they share morphological similarities with *Macrochlamys* sp. from Japan and *M. indica* from Bangladesh and West Bengal. The snails of the genus *Macrochlamys* have been classified based on morphology such as shells, genitalia and oral organs (Godwin-Austen 1883; Pholyotha et al. 2018, 2020a; Roy 2020; Venmans 1957), and the consistency between molecular phylogenetic distinctions and morphology-based classification has not been examined. Therefore, phylogenetic information, in addition to morphological characters such as traits of genitalia, should be considered when classifying species in the genus *Macrochlamys*. Although many invasive *Macrochlamys* species have been identified worldwide, their identification, phylogenetic position, and invasion routes are unknown. In order to discuss the processes of global spread of invasive *Macrochlamys* snails in the future, it is necessary to accumulate knowledge on the morphology and phylogenetic relationships of each species.

Anyway, the Japanese *Macrochlamys* sp. is most likely *M. indica*, introduced from the vicinity of Bangladesh or West Bengal and further dispersed in Japan. However, since in the genus *Macrochlamys*, information on phylogenetic distinctions and morphological characteristics has not been fully investigated, it is considered that taxonomic studies using more detailed genetic and morphologic information may be needed to accurately classify each species within the genus. *Macrochlamys indica* is a pest that damages crops and is registered as the most threatening quarantine plant

pest in the USA (Chanda and Mandal 2020; Jayashankar et al. 2015; Kumar and Ahmed 2000; Revynthi et al. 2020; Singh et al. 2020). In other words, there are concerns about the negative impact of this species on agriculture and ecosystems in Japan. Quarantine is the most effective way to prevent the invasion of such exotic species (Hallman 2008). Considering the possibility that *M. indica* is dispersed in Japan, quarantine should not only prevent the invasion from abroad, but also pay attention to its movement within the country.

Acknowledgements

We thank Kazuki Kimura, Yasuto Ishii, Takumi Saito and Shota Uchida for useful comments. We also thank Shizuma Yanagisawa (Ryuyo Insect Nature Observation Park) and Kiyonori Tomiyama (Kagoshima University) for useful comments on the status of *Macrochlamys* sp. in Kagoshima Prefecture. We would also like to thank Hikaru Igarashi, Fumiaki Kimura and Yumi Goto for support of field survey. We are also grateful to Dr. Mikhail Son, Dr. Tim Adriaens and anonymous reviewers.

Funding declaration

This work was supported by JSPS KAKENHI Grant Numbers 18H02506, 21H02556, 20K15872 and 20J14469.

Authors' contribution

KK, OK and SC conceived the ideas; KK, OK, SW, HN, SMS, DY and TH collected the sample; KK, OK and SI conducted phylogenetic analysis; KK led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Ethics and permits

We conducted this study in accordance with the Animal Experimentation Regulations of Tohoku University

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Supplementary material

The following supplementary material is available for this article:

Figure S1. The image shows the reproductive organ of *Macrochlamys* sp.
Figure S2. The spermathecae of *Macrochlamys* sp. from Japan.
Figure S3. Images A and B are penial interior sculptures of *Macrochlamys* sp.
Table S1. Information on the localities, collection data and GenBank accession number of each DNA sequences.
Table S2. Information on the species name, localities, GenBank accession number, and reference of the GenBank sequence.
Table S3. Genetic distance based on COI gene among the population of *Macrochlamys* sp. from Japan and *Macrochlamys indica* from Rajshahi, Bangladesh and West Bengal, India.

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