The golden mussel *Limnoperna fortunei* (Dunker) is an invasive freshwater bivalve species that has been identified as contributing towards various ecological and economic problems (Darrigran et al. 1998, Ricciardi 1998, Penchaszadeh et al. 2000, Magara et al. 2001, Boltovskoy et al. 2006, Pie et al. 2006). In 2006, this species was designated as an Invasive Alien Species of Japan due to its adverse effects on ecosystems. During the life cycle of *L. fortunei*, the planktonic stage is an important phase that facilitates the distribution of this species. Given that the process of detecting larvae in planktonic samples is time-consuming, efficient quantitative detection methods are required. Pie et al. (2006) developed a sensitive method for detecting golden mussel larvae by employing a polymerase chain reaction (PCR)-based approach using a specific primer set: LIMNO.COIR1 and LIMNO.COIF1. Furthermore, Boeger et al. (2007) reported that this method allowed for the detection of 1–5 larvae. No DNA fragments were amplified during the PCR of freshwater planktonic sample lacking golden mussel larvae which were collected from Lake Ohshio (Gunma, Japan). Real-time PCR was performed using fluorescent dye (SYBR Green) detection. Threshold cycles correlated well with the template DNA and number of larvae equivalents, and the R-square values of the standard curves were 0.99 and 0.98, respectively. These results suggest that this real-time PCR-based method can be utilized not only for the identification of *L. fortunei* in a particular habitat, but also for the quantification of larvae of this species in natural environments.

**Abstract:** A molecular based method for the quantitative detection of larvae of the golden mussel *Limnoperna fortunei* was developed. Partial nucleotide sequences of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene in several bivalve species including *L. fortunei* were analyzed. These nucleotide sequences and the CO1 gene for bivalve species found in habitats similar to *L. fortunei* (obtained from the EMBL/Genbank/DDBJ databases) were aligned. Based on these results, *L. fortunei*-specific primers were designed for amplification. DNA extracted from several bivalve species including *L. fortunei* were subjected to PCR using *L. fortunei*-specific primers, and amplification of the DNA fragment was confirmed only in PCR assays which utilized template DNA from *L. fortunei*. No DNA fragments were amplified during the PCR of freshwater planktonic sample lacking golden mussel larvae which were collected from Lake Ohshio (Gunma, Japan). Real-time PCR was performed using fluorescent dye (SYBR Green) detection. Threshold cycles correlated well with the template DNA and number of larvae equivalents, and the R-square values of the standard curves were 0.99 and 0.98, respectively. These results suggest that this real-time PCR-based method can be utilized not only for the identification of *L. fortunei* in a particular habitat, but also for the quantification of larvae of this species in natural environments.

**Key words:** golden mussel, larva, *Limnoperna fortunei*, quantitative detection, real-time PCR

*Corresponding author: Noriyuki Endo; Email, n-endo@criepi.denken.or.jp*
quencing Ready Reaction kit (Applied Biosystems, CA, USA) and the LCO1490 or HCO2198 primer. The nucleotide sequence data thus obtained were analyzed using ABI Prism® DNA Sequencing Analysis Software (Applied Biosystems). The nucleotide sequence data reported in the present study are available from the EMBL/Genbank/DDBJ databases under accession numbers AB498011–AB498018 (see Table 1). These nucleotide sequence data thus obtained were analyzed using ABI Prism® DNA Sequencing Ready Reaction kit (Applied Biosystems, CA, USA) and the LCO1490 or HCO2198 primer. The nucleotide sequence data reported in this paper.

Species accession number Collection site Collection date

L. fortunei (Dunker) AB498011* Lake Ohshio, Gunma, Japan May 2008

Xenostrobus securis (Lamarck) AB498012* Lake Hamana, Shizuoka, Japan April 2008

Mytilus edulis (Linnaeus) NCO06161 — —

Mytilus galloprovincialis (Lamarck) AB498013* Daiba, Tokyo, Japan May 2008

Mytilus trossulus (Gould) AB498014* Minamikayabe, Hokkaido, Japan January 2009

Perna viridis (Linnaeus) AB498015* Daiba, Tokyo, Japan September 2008

Modiolus modiolus (Linnaeus) DQ264392 — —

Musculista senhousia (Benson) AB498016* Daiba, Tokyo, Japan May 2008

Corbicula fluminea (Müller) AB498017* Lake Ohshio, Gunma, Japan October 2008

Corbicula japonica (Prime) AB498018* Lake Hinuma, Ibaraki, Japan February 2009

Crassostrea gigas (Thunberg) AF177226 — —

Crassostrea nippona (Seki) AF300616 — —

* Nucleotide sequence data reported in this paper.

**Table 1.** Bivalve species used for DNA analysis in the present study.
**Acknowledgements**

We thank Mr. Tsuyoshi Ohkawara of the Maisaka Oyster Cultivation Union of Lake Hamana (Shizuoka, Japan) for his assistance in the collection of several bivalve species. We also thank Dr. Takuya Kobayashi and Dr. Daisuke Nakano of the Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, for their assistance in the sampling of adult golden mussels and planktonic samples.

**References**

Boeger WA, Pic MR, Falleiros RM, Ostrenskey A, Darrigran G, Mansur MCD, Belz CE (2007) Testing a molecular protocol to monitor the presence of golden mussel larvae (*Limnoperna fortunei*) in plankton samples. J Plankton Res 29: 1015–1019.

Boltovskoy D, Correa N, Cataldo D, Sylvester F (2006) Dispersion and ecological impact of the invasive freshwater bivalve *Limnoperna fortunei* in the Rio de la Plata watershed and beyond. Biol Invasions 8: 947–963.

Darrigran G, Martin SM, Gullo B, Armendariz L (1998). Macroinvertebrates associated with *Limnoperna fortunei* (Dunker, 1857) (Bivalvia, Mytilidae) in Rio de la Plata, Argentina. Hydrobiologia 367: 223–230.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299.

Jeamougin F, Thompson DJ, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Chastal X. Trends Biochem Sci 23: 403–405.

Magara Y, Matsui Y, Goto Y, Yuasa A (2001) Invasion of the non-indigenous nuisance mussel, *Limnoperna fortunei*, into water supply facilities in Japan. Journal of Water Supply: Research and Technology—AQUA 50: 113–124.

Penchaszadeh PE, Darrigran D, Angulo C, Averbuj A, Brögger M,
Dogliotti A, Pirez N (2000) Predation of the invasive freshwater mussel *Limnoperna fortunei* (Dunker, 1857) by the fish *Leportinus obtusidens* Valenciennes, 1846 (Anostomidae) in the Rio de la Plata, Argentina. J Shellfish Res 19: 229–231.

Pie MR, Boeger WA, Patella L, Falleiros RM (2006) A fast and accurate molecular method for the detection of larvae of the golden mussel *Limnoperna fortunei* (Mollusca: Mytilidae) in plankton samples. J Molluscan Stud 23: 218–219.

Ricciardi A (1998) Global range expansion of the Asian mussel *Limnoperna fortunei* (Mytilidae): Another fouling threat to freshwater systems. Biofouling 13: 97–106.