A Quick Method for Species Identification of Japanese Eel (*Anguilla japonica*) Using Real-Time PCR: An Onboard Application for Use During Sampling Surveys

Shun Watanabe, Yuki Minegishi, Tatsuki Yoshinaga, Jun Aoyama, and Katsumi Tsukamoto

Ocean Research Institute, The University of Tokyo, 1-15-1 Minamidai, Nakano-ku, Tokyo 164-8639, Japan

Abstract: To compensate for the limited number of morphological characteristics of fish eggs and larvae, we established a convenient and robust method of species identification for eggs of the Japanese eel (*Anguilla japonica*) using a real-time polymerase chain reaction (PCR) that can be performed onboard research ships at sea. A total of about 1.2 kbp of the mitochondrial 16S ribosomal RNA gene sequences from all species of *Anguilla* and 3 other anguilliform species were compared to design specific primer pairs and a probe for *A. japonica*. This real-time PCR amplification was conducted for a total of 44 specimens including *A. japonica*, *A. marmorata*, *A. bicolor pacifica*, and 6 other anguilliform species. Immediate PCR amplification was only observed in *A. japonica*. We then tested this method under onboard conditions and obtained the same result as had been produced in the laboratory. These results suggest that real-time PCR can be a powerful tool for detecting Japanese eel eggs and newly hatched larvae immediately after onboard sampling during research cruises and will allow targeted sampling efforts to occur rapidly in response to any positive onboard identification of the eggs and larvae of this species.

Key words: Japanese eel, *Anguilla japonica*, eggs, leptocephali, mtDNA, real-time PCR.

INTRODUCTION

The spawning area of the Japanese eel *Anguilla japonica* was determined to be in waters west of the Mariana Islands from the distribution patterns of morphologically identified leptocephali during a series of surveys in the Philippine Sea (Tsukamoto, 1992). However, no eggs or newly hatched larvae have been observed near the estimated spawning area; therefore, direct evidence for the precise location of the spawning area of *A. japonica* has not yet been obtained (Tsukamoto et al., 2003). Relatively large leptocephali of *A. japonica* (>30 mm TL) can be easily identified by morphologic characteristics such as the total number of myomeres (Tabeta and Mochioka, 1998). However, during the early developmental stages, it is difficult to identify them because of their undeveloped morphological characteristics. Furthermore, the present knowledge regarding the morphological characteristics of Japanese eel eggs and newly hatched larvae has been obtained only from artificially induced spawning (Yamamoto et al., 1975; Tanaka et al., 2001); those in nature are still unknown.

Molecular techniques have been used recently for the identification of various marine organisms whose morphologies during their early life stages are not well known (Coffroth and Mulawka, 1995; Medeiros-Bergen et al., 1995; Hare et al., 2000). Aoyama et al. (2001) used a DNA nucleotide sequencing technique to identify fish eggs collected to the west of the Mariana Islands as part of an effort to locate the precise Japanese eel spawning area. However, these methods require facilities that are only available in a laboratory setting, and investigators must transport the specimens collected offshore back to the laboratory for genetic species identification. This means that useful information about the presence of eggs or small larvae of *A. japonica* collected during long oceanic surveys is not available to modify the sampling strategy during the cruise. For example, if *A. japonica* eggs were collected at a particular location, more tows of the net could be made and a small-scale grid survey could be performed in that area.

To overcome this problem, a method needs to be developed that can be performed onboard research vessels during sampling surveys. However, conventional polymerase chain reaction (PCR) techniques that require electrophoresis to observe the amplicon cannot be conducted under the unstable swaying conditions on a ship at sea. Therefore, we focused on applying a recently developed real-time PCR method to solve this problem. Real-time PCR has the following advantages for species identification at sea. First, amplification is detected by real-time monitoring of fluorescence so that electrophoresis is not necessary. Second, specificity is guaranteed by both specific primers and an oligo probe that are specific to the amplicon. Third, a maximum of 384 samples can be examined simultaneously, and the procedure can be completed in less than 3 hours. Finally, obtaining immediate results on the species identification of some samples during longer sampling surveys can influence whether or not further sampling efforts are made in a particular area.

The objective of the present study was to establish an onboard experimental protocol for genetically identifying Japanese eel eggs or small leptocephali using real-time PCR. This method of onboard real-time PCR includes specific primer pairs and a probe. It may enable great advances in ecological studies of *A. japonica* to be made when the results obtained onboard can affect the sampling strategy during the cruise. This methodology also can be adapted for use with many other types of marine taxa that inhabit the open ocean and whose early life histories are yet not known.

MATERIALS AND METHODS

Design of Primer Pairs and Probe

Sequence data of the mitochondrial 16S ribosomal RNA gene (16SrRNA) from the 18 species and subspecies of freshwater eels of the genus *Anguilla* (DDBJ/EMBL/GenBank nucleotide sequence database accession numbers AB021748 to AB021764) and *Conger japonicus* (Congridae, AB021766) were obtained from the DDBJ database. In addition, sequence data of 16SrRNA for *Synahobranchus kaupii* (Synaphobranchidae) and *Serrivomer sector* (Serrivomeridae) were obtained from J. G. Inoue (unpublished data). We compared all these sequences and selected a segment of about 300 bp that contained species-specific sites for *A. japonica*. The segments of *A. japonica* were analyzed using Primer Express 2.0 (Applied Biosystems Japan Ltd.) in order to design specific primer pairs and a probe.

Samples

A total of 44 specimens of 9 species of the Anguilliformes were used to test the primer pairs and probe. A total of 25 specimens of *A. japonica* were collected at 5 different localities in Japan, including Nagasaki ($n = 3$), Tokushima ($n = 3$), Aichi ($n = 6$), and Chiba (Tone River, $n = 3$; Minato River, $n = 10$) prefectures. In addition, 10 specimens of *A. marmorata* (Okinawa Island, $n = 1$; Sulawesi Island, $n = 1$; Ambon Island, $n = 4$; Guam Island, $n = 4$) and 2 specimens of *A. bicolor pacifica* (Philippines) were used as sympatric species because the leptocephali of these two species have been collected in the general spawning area of *A. japonica* (Aoyama et al., 1999). Furthermore, 2 specimens of *C. myriaster* (Nagasaki, $n = 1$; Fukushima, $n = 1$), and 1 specimen each of *Strophidon ui*, *Rhinomuraena quaesita*, *Uropteriguius* sp. 1, *Stemonidium hypomelas*, and *Serrivomer sector* were used as genetically distant taxa. Anguilliform specimens were collected from the North Pacific Ocean except for *C. myriaster*. A small portion of liver or muscle was excised from live specimens and minced in 95% ethanol.

DNA Extraction

Two methods for DNA extraction were tested. Total genomic DNA from 34 specimens of *A. japonica* and other

specimens was isolated and purified according to the standard protocol, using phenol–chloroform–isoamyl alcohol (25:24:1 v/v) twice with diethyl ether, then concentrated by ethanol precipitation (Aoyama and Tsukamoto, 1997). Separately, for 10 specimens of *A. japonica* from the Minato River, total genomic and mitochondrial DNA was extracted by incubation at 95°C in 500 µl of a 5% chelex (BioRad) solution. The amplification of DNA templates for all specimens was confirmed by universal primer pairs in the 16SrRNA as follows: L1854, 5′-AAACCTCGTA CCTTTTGCAT-3′; and H3059, 5′-CCGGTCTGAACTCAGATCACGT-3′ (Miya and Nishida, 1996).

Real-Time PCR

To check matching to specific primer pairs designed by Primer Express 2.0 (Applied Biosystems Japan Ltd.), 3 real-time PCR using the Japanese eel-specific primers was carried out by means of the ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan Ltd.), in a 20 µl reaction volume containing 10 µl of SYBR Green PCR master mix (Applied Biosystems Japan Ltd.), 2 µl of each primer at 5 µM, 5.0 µl of sterile distilled water, and 1.0 µl of template DNA of 3 specimens of *A. japonica* collected from Nagasaki. After activation at 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of denaturation at 95°C were performed for 15 seconds, and annealing and extension at 60°C for 1 minute.

To confirm amplification in 25 specimens of *A. japonica* in 44 specimens, real-time PCR using the Japanese eel–specific probe and primers was carried out by means of the ABI PRISM 7000 Sequence Detection System in a 50 µl reaction volume containing 25 µl of *Taq*Man 2× universal PCR master mix (Applied Biosystems Japan Ltd.), 4.5 µl of each primer at 5 µM, 4.5 µl of sterile distilled water, 1.5 µl of probe (*Taq*Man probe, Applied Biosystems Japan Ltd.), and 10 µl of template DNA (2.5 to 25.0 ng/µl) of 44 specimens. After activation at 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of denaturation at 95°C were performed for 15 seconds, and annealing and extension at 60°C for 1 minute.

PCR-RFLP

To confirm the results of real-time PCR, the species identification method of polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) (Aoyama
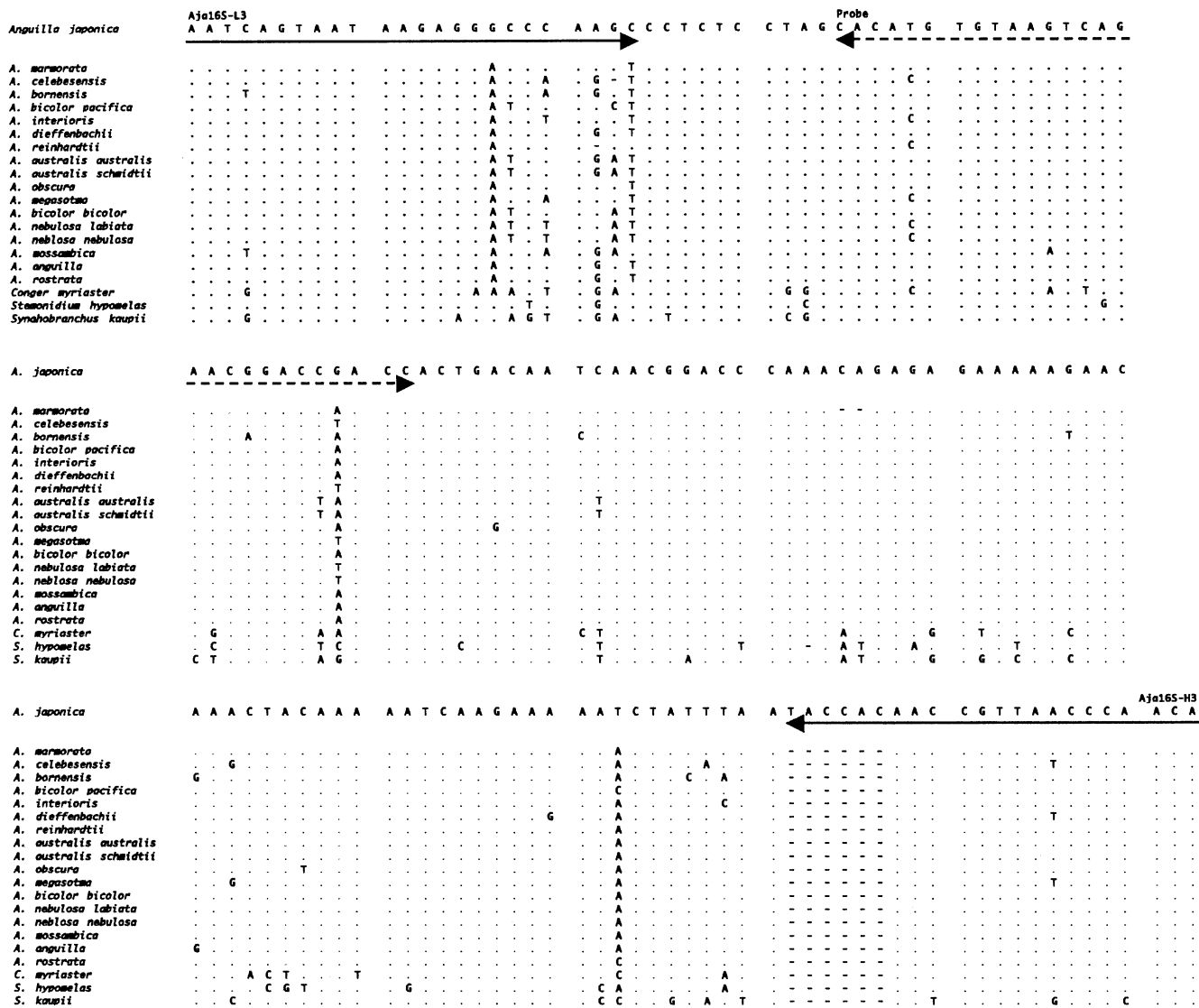
et al., 2000) was applied to all 35 specimens that were amplified by real-time PCR. A fragment of the 16SrRNA was amplified using PCR with L1854 and H3059 (Miya and Nishida, 1996). The PCR was carried out with the Gene-Amp PCR system 2400 (PerkinElmer, Inc.), with a 25-µl reaction volume containing 13.8 µl sterile distilled water, 2.5 µl 10× PCR buffer (PerkinElmer, Inc.), 2.5 µl (deoxynucleotide triphosphate at 2 mM, 5 µl of each primer at 5 µM, 0.2 µl of *Taq* DNA polymerase (PerkinElmer, Inc.) and 1 µl of total DNA. Amplification parameters were 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 60 seconds. The PCR products were cleaved by two restriction enzymes: *Bsp* 1286 and *Mva*I (Takara Shuzo Co., Ltd.). Restriction procedures were carried out in a 20-µl final volume containing 5 µl of PCR product, 10 units of restriction enzyme, and 2 µl of restriction enzyme buffer supplied by the manufacturers and incubated at 37°C overnight. RFLP was detected and compared with the positions of size markers (ϕX174-*Hinc*II digest, Takara Shuzo Co., Ltd.) using electrophoresis on 1% agarose gels with ethidium bromide staining.

Cluster Analysis

The results of real-time PCR for all 35 of the successfully amplified specimens were evaluated using cluster analysis (SPSS, Stat soft, Version 6.1) with the squared Euclidian distance measure and unweighted pair-group method using arithmetic averages (UPGMA) clustering. This analysis compared the number of cycles at which the Delta Rn fluorescence values of the PCR products of each specimen reached the 0.1, 0.5, and 1.0 levels. Therefore, the clustering grouped the specimens based on the rate at which amplification occurred.

Shipboard Trial

To test the availability of real-time PCR onboard ships, the same procedure as that performed in the laboratory was occasionally applied from October 29 to November 6, 2002, during a research cruise of the R/V *Tansei Maru* of the Ocean Research Institute, the University of Tokyo (604 tons, 50 m in length, and 9.2 m in width). Testing in rough sea conditions was conducted at 34°30′N, 136°30′E, on November 5, 2002, when the ship sailed at 8 knots per hour against a wind velocity of 15 to 20 m/s.

Figure 1 (sequence alignment)

Aja16S-L3 / Probe

Anguilla japonica:
AATCAGTAAT AAGAGGGCCC AAGCCCTCTC CTAGCACATG TGTAAGTCAG

A. japonica:
AACGGACCGA CCACTGACAA TCAACGGACC CAAACAGAGA GAAAAAGAAC

A. japonica (Aja16S-H3):
AAACTACAAA AATCAAGAAA AATCTATTTA ATACCACAAC CGTTAACCCA ACA

Species compared in alignment:
A. marmorata, A. celebesensis, A. bornensis, A. bicolor pacifica, A. interioris, A. dieffenbachii, A. reinhardtii, A. australis australis, A. australis schmidtii, A. obscura, A. megastoma, A. bicolor bicolor, A. nebulosa labiata, A. neblosa nebulosa, A. mossambica, A. anguilla, A. rostrata, Conger myriaster, Stemonidium hypomelas, Synahobranchus kaupii

Figure 1. Sequence alignment of the 153-bp amplicon (L strand) from 18 species and subspecies of *Anguilla* and 3 other species of the Anguilliformes. Beat-matched specific primer pair of *A. japonica* is shown by the two solid lines, and the specific probe of *A. japonica* is shown by a dashed line. Sequence data from the mitochondrial 16S ribosomal RNA gene (16SrRNA) of the 18 species or subspecies of *Anguilla* and *Conger japonicus* were obtained from the database of DDBJ. In addition, sequence data from the 16SrRNA of *Synahobranchus kaupii* (Synaphobranchidae) and *Serrivomer sector* (Serrivomeridae) were obtained from J. G. Inoue (unpublished data).

RESULTS

Design of Specific Primer Pairs and Probe for *A. japonica*

Three sets of Japanese eel–specific primer pairs were designed as follows: Aja16S-L1, 5′-AGCGTTAAAGCTCCGGTAAATA-3′ and Aja16S-H1, 5′-GCTAGGAGAGGGCTTGGGC-3′; Aja16S-L2, 5′-CCACGAAACCCGACGTAAAC T-3′, and Aja16S-H2, 5′-GAT GTCTCTCTTGGCTTAGGGATT-3′; Aja16S-L3, 5′-AATCAGTAATAA GAGGGCCCAAGC-3′), and Aja16S-H3, 5′-TGTTGGGTTAAC GGTTTGTGGTA-3′). The 3′ ends of these specific primer pairs were targeted against some bases unique to *A. japonica* and are not found in all the other anguillid species, or *S. hypomelas*, *C. japonicus*, and *S. kaupii*. The amplification efficiency of Aja16S-L3 and H3 was the best of the 3 pairs using SYBR Green as a result of a preliminary experiment. An *A. japonica*–specific probe was then designed within Aja16S-L3 and H3 as follows: 5′-CACATGTGTAAGTCAGAACG GACCGACC-3′ (Figure 1).
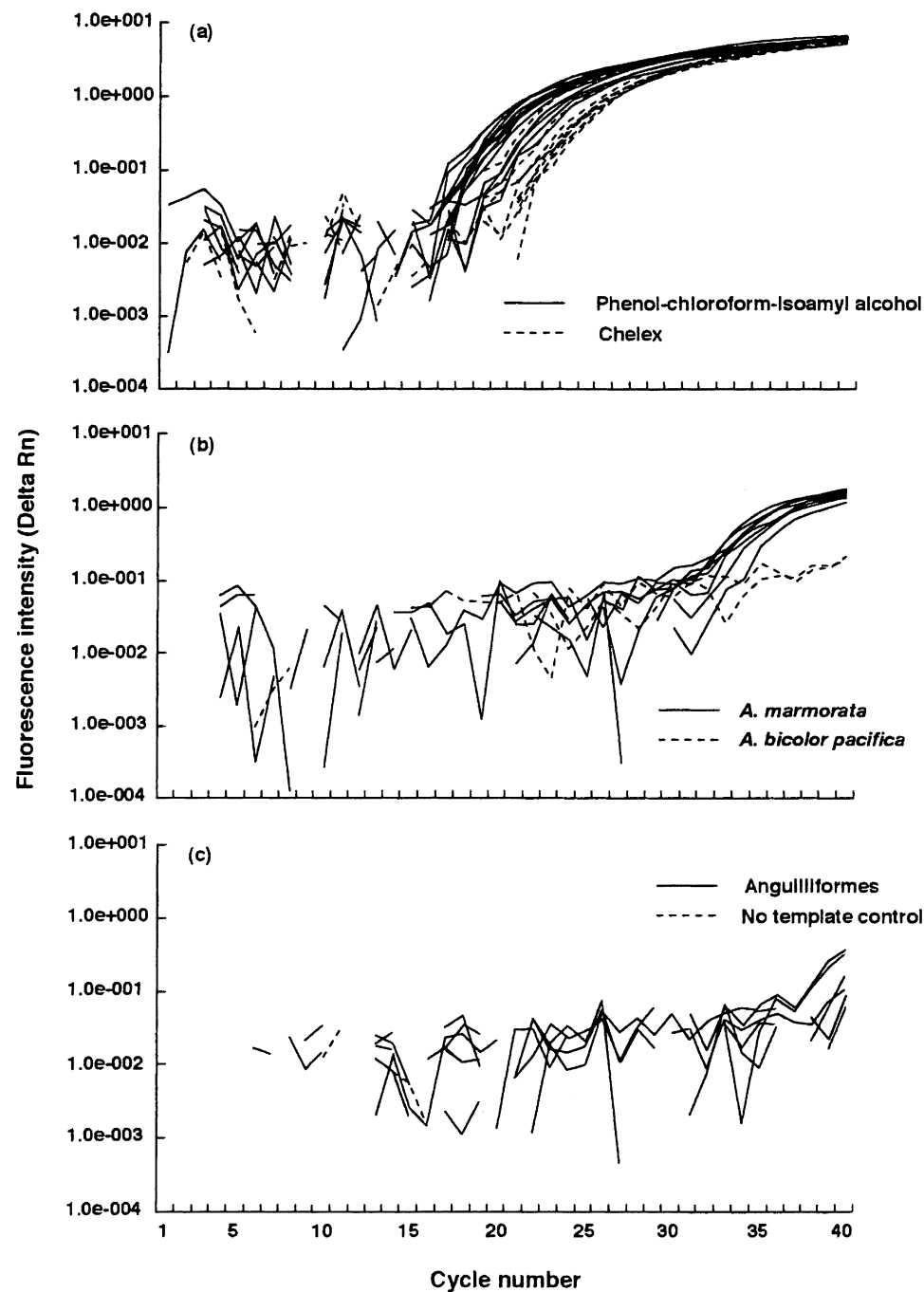
Figure 2. Results of amplification of 44 specimen templates and a no-template control in 40 cycles of annealing and extension using Japanese eel–specific primer pairs and probe by real-time PCR. **a:** Twenty-five specimens of *A. japonica*, including 10 specimens from which DNA was extracted using the chelex method. **b:** Ten specimens of *A. marmorata* and two specimens of *A. bicolor pacifica*. **c:** Seven specimens of other species of the Anguilliformes except for *Anguilla* and a no-template control.

Amplification Using Specifically Designed Primer Pairs and Probe

The real-time PCR reaction for 44 specimens of 9 taxa showed successful amplification only in the 25 specimens of *A. japonica*. This suggested that the specifically designed primers and probe worked well for detecting *A. japonica* among the morphologically similar Anguilliformes (Figure 2). Both phenol–chloroform–isoamyl alcohol and 5% chelex extractions were successful in the amplifications.

However, for the *A. japonica* specimens that had their DNA extracted by the 5% chelex method, the increase in fluorescence intensity (indicating amplification) was slower than it was for those that were extracted using phenol–chloroform–isoamyl alcohol (Figure 2, a). All specimens of *A. marmorata* were also amplified, but at a clearly slower rate and to much less degree (Figure 2, b). The amplification in *A. marmorata* occurred at almost the end of the PCR reaction cycles (35 cycles), and it was quite different from the amplification pattern observed in *A. japonica*
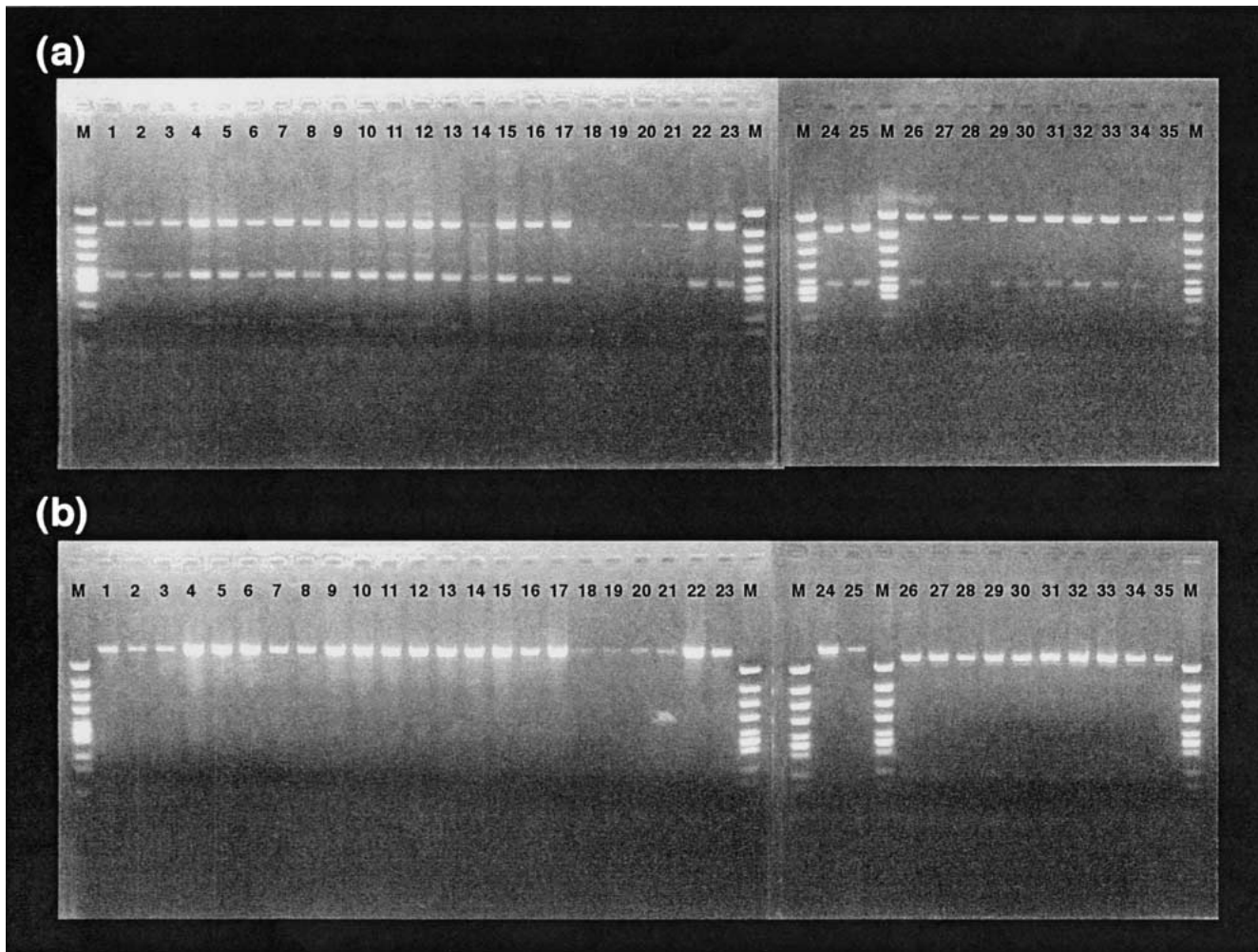
Figure 3. The species identification fragment patterns of the PCR-RFLP analyses of 35 specimens. Lanes labeled M are the size markers (φX174-*Hinc*II digest, Takara Shuzo Co., Ltd.). Fragment patterns from lanes 1 to 25 were *A. japonica* and those from lanes 26 to 35 were *A. marmorata*. **a:** Patterns resulting from using the restriction enzyme *Bsp*1286. **b:** Patterns resulting from using the restriction enzyme *Mva*I.

(Figure 2, a and b). Accordingly, these two species could be clearly distinguished by the distinct efficiencies of amplification (Figure 2, a and b). The real-time PCR reaction for the 2 specimens of *A. bicolor pacifica*, 6 other anguilliform species, and the control did not show any amplification (Figure 2, b and c).

Confirmation of Real-Time PCR Species Identifications

The results of the PCR-RFLP with two restriction enzymes (*Bsp*1286 and *Mva*I) showed that the 35 specimens amplified by the real-time PCR were identified as 25 specimens of *A. japonica* and 10 specimens of *A. marmorata* (Figure 3). The fragment patterns of these

specimens were the same as those shown by Aoyama et al. (2000) for these two species. The samples that were rapidly amplified in the real-time PCR were therefore confirmed to be *A. japonica* using a PCR-RFLP analysis, and those that were amplified much more slowly also were confirmed to be *A. marmorata*.

Cluster Analysis

The result of the cluster analysis showed that the 25 specimens of *A. japonica* and 10 specimens of *A. marmorata* were completely separated based on their rates of amplification (Figure 4). This result entirely confirmed that the real-time PCR was able to clearly distinguish *A. japonica* from *A. marmorata*. The 25 specimens of *A. japonica* were divided
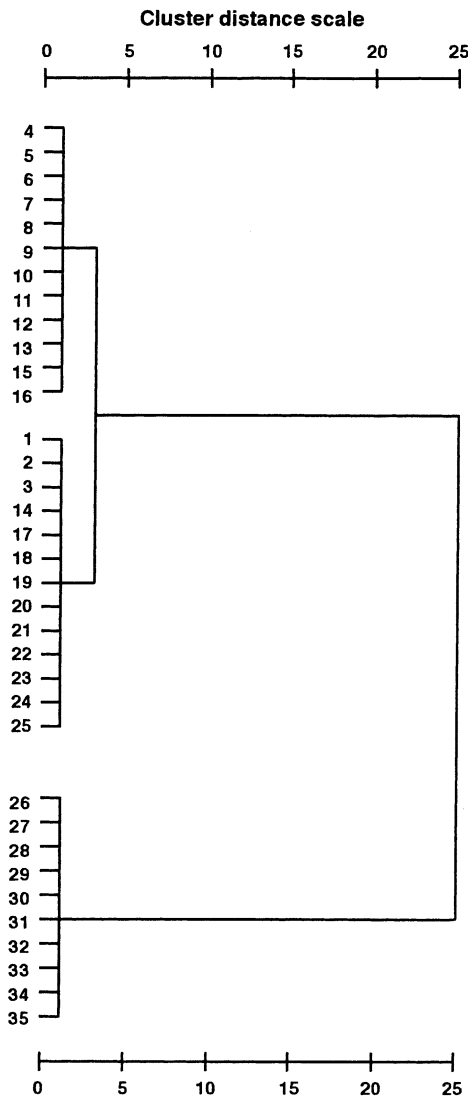
Figure 4. Dendrogram obtained from cluster analysis of rates of PCR amplification of 25 specimens of *A. japonica* and 10 specimens of *A. marmorata*. The specimen numbers are the same as in Figure 3 (1–25, *A. japonica*; 26–35, *A. marmorata*).

into two clusters, which corresponded to whether the start of the amplification of each specimen was early or late.

Onboard Trial

Using the same specimens and protocol as those used in the laboratory trial, specific amplification of *A. japonica* was successfully confirmed both in normal and rough sea conditions onboard (Figure 4). This suggests that the fluorescence marker detection system using the ABI 7000 system can work not only under stable laboratory conditions but also onboard the ship, in a wind velocity of at least 20 m/s.
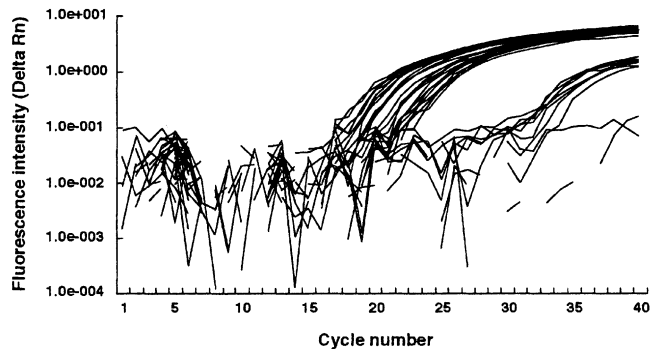
Figure 5. Results of amplification of 44 specimen templates and a no-template control in real-time PCR onboard the R/V *Tansei Maru* using 40 cycles of annealing and extension with Japanese eel–specific primer pairs and probe.

DISCUSSION

Genetic techniques have been increasingly used in recent years to identify closely related taxa such as the different species of anguillid eels, but a limitation of these techniques is that they cannot be used at sea during sampling surveys. Molecular markers such as mtDNA nucleotide sequences and restriction enzyme fragment patterns have been developed using strictly identified adult specimens and have proved to be a powerful tool for the identification of anguillid eggs and leptocephali (Aoyama et al., 1999, 2003). Although several methods of genetic species identification of *A. japonica* have been reported, they all require a sequencing technique (Aoyama et al., 1999), or PCR amplification and subsequent restriction fragment analysis (Zhang et al., 1999; Aoyama et al., 2000). Their protocols are relatively complicated and require substantial time for sequencing or restriction processing after the PCR amplification. Furthermore, it is obviously inconvenient to perform these techniques on marine organisms such as anguillid eggs and larvae onboard research vessels, because they were designed to be conducted in the laboratory.

The present study successfully provided a simple and automated species identification method that required only 3 hours to detect *A. japonica* after sorting eggs from the plankton samples. Specific primer pairs and a probe for the species of interest were easily designed by the software that comes with the real-time PCR equipment (Primer Express 2.0, Applied Biosystems Japan Ltd.). In addition, this method using real-time PCR was demonstrated to be successful onboard a research ship, even in rough seas.

In the present study we focused on the mitochondrial 16SrRNA gene for identification of *A. japonica*. This

relatively conservative gene is frequently used for evolutionary studies at the species or genus level in fish (Meyer, 1993). Aoyama et al. (2000) suggested that the 16SrRNA was appropriate for identifying eels at the species level. We also found only a few variations between most of the 153-bp mitochondrial 16SrRNA sequences amplified by the Aja16S-L3 and Aja16S-H3 primers designed by this study, and most of the variations were concentrated at the 3′ end (Figure 1). In particular, Aja16S-H3 was designed in the area of a 6-base deletion, and it was clear that the species-specificity of these primers was very high. Moreover, the melting temperature of the primers was high, and annealing could be set up at 60°C when the chance of species mismatch is lower than at cooler temperatures.

A weak point of species identification using real-time PCR is that it may be relatively expensive if used on a large number of samples, such as those collected in coastal areas with high egg and larvae densities. However, the cost of using the real-time PCR technique to ensure the success of a cruise is much less than the cost of using the research ship for another survey to clarify the findings of the first cruise. Alternatively, other newly developed techniques such as capillary electorophoresis and loop-mediated isothermal amplification (LAMP) have the potential to be useful and less expensive for onboard genetic species identification in the future. The costs of real-time PCR can be minimized, though, by selecting a smaller number of samples for analysis, which may still be adequate to determine the presence or absence of the targeted species.

When onboard identification of A. japonica could be of critical importance to the outcome of cruise, we present the following working protocol for searching for their spawning site. First, all eggs and leptocephali morphologically resembling those of A. japonica are sorted from the plankton samples. Second, total genomic and mtDNA of these specimens is extracted individually by 5% chelex solution. Third, real-time PCR is performed using the specific primer pairs and probe presented here. This protocol enables the direct identification of any A. japonica specimens that are present in the samples within 3 to 4 hours, before returning to the laboratory. If the MicroAmp optical 384-well reaction plate (Applied Biosystems Japan Ltd.) is used in conjunction with real-time PCR, up to 384 eggs or leptocephali can be simultaneously identified. The advantages of this method are that large quantities of samples can be processed in a short time, the sample does

not have to be preserved for the sake of being transported to a laboratory, and research can be conducted more efficiently because the species composition of samples can be immediately monitored onboard ship, and the cruise plan adjusted accordingly.

The method presented here provides an important genetic technique for the onboard identification of species of marine organisms that will be particularly advantageous during long sampling surveys when immediate knowledge of the results of the sampling can be used to target certain areas where species of interest are collected. Garland and Zimmer (2002) suggested that the greatest challenge in the development of techniques for the identification of marine species is to streamline the technology involved in the application of molecular probes. It is no exaggeration to say that this method fully solves that problem, and it may be able to facilitate the determination of the exact location of the spawning area of the Japanese eel in the future.

Acknowledgements

References

Aoyama, J., and Tsukamoto, K. (1997). Evolution of the freshwater eels. *Naturwissenschaften* 84:17–21.

Aoyama, J., Mochioka, N., Otake, T., Ishikawa, S., Kawakami, Y., Castle, P.H.J., Nishida, M., and Tsukamoto, K. (1999). Distribution and dispersal of anguillid leptocephali in the western Pacific revealed by molecular analysis. *Mar Ecol Prog Ser* 188:193–200.

Aoyama, J., Watanabe, S., Nishida, M., and Tsukamoto, K. (2000). Discrimination of catadromous eel species, genus *Anguilla*, using PCR-RFLP analysis of the mitochondrial 16SrRNA domain. *Trans Am Fish Soc* 129:873–878.

Aoyama, J., Ishikawa, S., Otake, T., Mochioka, N., Suzuki, Y., Watanabe, S., Shinoda, A., Inoue, J., Lokman, P.M., Inagaki, T., Oya, M., Hasumoto, H., Kubokawa, K., Lee, T.W., Fricke, H., and Tsukamoto, K. (2001). Molecular approach to species identification of eggs with respect to determination of the spawning site of the Japanese eel *Anguilla japonica*. *Fish Sci* 67:761–763.

Aoyama, J., Wouthuyzen, S., Miller, M.J., Inagaki, T., and Tsukamoto, K. (2003). Short-distance spawning migration of tropical freshwater eels. *Biol Bull* 204:104–108.

Coffroth, M.A., and Mulawka, J.M. III (1995). Identification of marine invertebrate larvae by means of PCR-RAPD species-specific markers. *Limnol Oceanogr* 40:181–189.

Garland, E.D., and Zimmer, C.A. (2002). Techniques for the identification of bivalve larvae. *Mar Ecol Prog Ser* 225:299–310.

Hare, M.P., Palumbi, S.R., and Butman, C.A. (2000). Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. *Mar Biol* 137:953–961.

Medeiros-Bergen, D.E., Olson, R.R., Conroy, J.A., and Kocher, T.D. (1995). Distribution of holothurian larvae determined with species-specific genetic probes. *Limnol Oceanogr* 40:1225–1235.

Meyer, A. (1993). Evolution of mitochondrial DNA in fishes. In: *Biochemistry and Molecular Biology of Fishes*, Hochachka, P.W., and Mommsen, T.P. (eds.). Amsterdam, Netherlands: Elsevier, pp 1–38.

Miya, M., and Nishida, M. (1996). Molecular phylogenetic perspective on the evolution of the deep-sea fish genus *Cyclothone* (Stomiiformes: Gonostomatidae). *Ichthyol Res* 43:375–398.

Tabeta, O., and Mochioka, N. (1988). Leptocephali. In: *An Atlas of the Early Stage of Fishes in Japan*, Okiyama, M. (ed). Tokyo, Japan: Tokai Univ. Press, pp 15–64.

Tanaka, H., Kagawa, H., and Ohta, H. (2001). Production of leplocephali of Japanese eel (*Anguilla japonica*) in captivity. *Aquaculture* 201:51–60.

Tsukamoto, K. (1992). Discovery of spawning area for Japanese eel. *Nature* 356:789–791.

Tsukamoto, K., Otake, T., Mochioka, N., Lee, T.W., Fricke, H., Inagaki, T., Aoyama, J., Ishikawa, S., Kimura, S., Miller, M.J., Hasumoto, H., Oya, M., and Suzuki, Y. (2003). Seamounts, New Moon and Eel Spawning: the search for the spawning site of the Japanese eel. *Env Biol Fish* 66:221–229.

Yamamoto, K., Yamauchi, K., and Kasuga, S. (1975). On the development of the Japanese eel, *Anguilla japonica*. *Bull Jpn Soc Sci Fish* 41:21–28.

Zhang, H., Mikawa, N., Yamada, Y., Horie, N., Okamura, A., Uto, T., Tanaka, S., and Motonobu, T. (1999). Foreign eel species in the natural waters of Japan detected by polymerase chain reaction of mitochondrial cytochrome *b* region. *Fish Sci* 65:684–686.