Identification of three Asian Hediste species (Polychaeta: Nereididae) by PCR-RFLP analysis of the mitochondrial 16S rRNA gene

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Abstract: An easy polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was designed to identify the three morphologically similar nereidid polychaete species Hediste atoka, Hediste diadroma and Hediste japonica which often dominate in estuarine macrobenthic fauna in eastern Asia. Restriction digestion analysis of the PCR products of the partial mitochondrial 16S rRNA gene (approximately 600 bp) of these three species, using EcoRV and TspEI endonucleases, generated species-specific restriction patterns. The PCR-RFLP method enables easy and accurate identification of the three Hediste species.

Key words: Hediste, polychaetes, 16S rRNA, PCR-RFLP, species identification

Hediste species (Nereididae, Polychaeta, Annelida) often dominate in macrobenthic fauna in shallow brackish waters in eastern Asia (Sato & Nakashima 2003), and therefore play an important role in the activity of nutrient cycling in an estuary ecosystem (Tsuchiya & Kurihara 1979, Sayama & Kurihara 1983, Kikuchi 1986, Iwamatsu et al. 2007). The Asian Hediste involves three morphologically similar species (Hediste atoka, Hediste diadroma and Hediste japonica), and these commonly coexist in the same place, forming a species complex (Sato & Nakashima 2003, Sato 2004, Hanafiah et al. 2006). Comparisons of their ecology, life history and other biological aspects render interesting insights to clarify the evolutionary significance of sympatric or parapatric occurrence of these closely related species. However, these studies have been difficult to do because the morphologies of the three species are so similar that their identification is not easy. The three species are clearly distinguishable by complete allele substitutions at several allozyme loci (e.g., lactate dehydrogenase), which are detectable by electrophoretic analysis (Sato & Masuda 1997, Sato & Nakashima 2003). However, the analysis needs plenty of fresh materials, and complicated procedures.

The present study was carried out to develop a simple, rapid and accurate method for identification of the three Hediste species by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns of the partial mitochondrial 16S rRNA gene.

A total of 15 individuals of H. atoka, 13 individuals of H. diadroma and 10 individuals of H. japonica were examined. The collection sites in Japan are shown in Table 1 & Fig. 1. Mature worms of H. japonica and H. diadroma were collected with a scoop net during reproductive swarming near the water surface at night during the spring tides of the cold season. Mature and immature worms of H. atoka and immature worms of H. japonica were collected from sediment samples dug from intertidal flats within estuaries. Three species were morphologically identified for mature worms of all three species and immature worms of H. japonica according to the key of Sato & Nakashima (2003). H. atoka at the immature stage was identified by a diagnostic electrophoretic pattern of lactate dehydrogenase according to the method of Sato & Masuda (1997). The whole body or a part of the body of each fresh sample was frozen and stored at −50°C, or fixed in 99% ethanol and stored at −20°C prior to actual DNA isolation. The samples preserved in ethanol were placed in a PBS (phosphate-buffered saline) for 30 min before the DNA extraction. From both materials (frozen and ethanol-preserved), the same results were obtained.

Total DNA was extracted with the spin-column method (DNeasy Tissue kit, QIAGEN) using the middle part of worms 0.5–1.0 cm in length, following the DNeasy protocol for animal tissues. Polymerase chain reactions were performed using Platinum PCR SuperMix High Fidelity (Invitrogen) on a DNA Thermal Cycler 480 (PE Biosystems). Used as a primer pair were 16SbrH (CCGGTCTGAAGCTCAGTACGT) and 16SarL (CCGCTGTATGATCAAAAACAT), which were designed by Palumbi (1996). The cycling regimes were as fol-
lows: initial denaturation at 94°C for 3 min, followed by 34 cy-
clcles of strand denaturation at 94°C for 30 sec, annealing at
50°C for 30 sec and primer extension at 68°C for 90 sec, and
final 5-minute extension at 68°C.

An aliquot of the PCR products was withdrawn from each
tube and recovered by ethanol precipitation, then 1
m
DNA
digested by 2.5 U of
Eco
RV (TOYOBO, Japan) or
Tsp
EI (TOYOBO, Japan) at 37°C for 3 h. Then they were loaded into a 5%
polyacrylamide gel and electrophoresed in a mini slab gel
electrophoresis equipment at 100 V using Tris-borate-EDTA
(TBE) buffer and detected by staining with SYBR Gold nu-
cleic acid gel stain (Molecular Probes).

The amplified partial mitochondrial 16S rRNA gene before
enzyme digestion was estimated to be approximately 600 bp.
Restriction digestion analyses of the PCR product of the three
Hediste species generated species-specific restriction patterns
(Table 2 & Fig. 2). In EcoRV digestion, two fragments (ap-
proximately 330 bp and 190 bp) were produced for
H. atoka,
whereas no cleavage was observed for
H. diadroma
and
H. japonica.
In TspEI digestion, two well-defined fragments (ap-
proximately 260 bp, 100 bp and 60 bp) and several small fragments
below 60 bp were produced for
H. atoka
and
H. japonica.
The small prod-
ucts less than 60 bp digested by
Tsp
EI were not easily identi-
fied. All specimens of each species examined in the present
study showed species-specific patterns independing on their lo-
calities.

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Table 1. Collecting data for three Hediste species.

| Species         | Collection | Date     | Preservation (Sexual maturity) | Number of individuals |
|-----------------|------------|----------|-------------------------------|----------------------|
| Hediste atoka   | Takase-gawa River (1) | 1 June 1992 | frozen (M) | 3                     |
|                 | Shinjo-gawa River (2)  | 3 June 1992 | frozen (M) | 3                     |
|                 | Kumano-gawa River (4) | 3 January 1997 | frozen (I) | 3                     |
|                 | Omoi-gawa River (8) | 26 September 1991 | frozen (M) | 1                     |
|                 | Kotsuki-gawa River (9) | 23 February 2004 | ethanol-fixed (M) | 1                     |
|                 | Kotsuki-gawa River (9) | 19 March 2004 | ethanol-fixed (M) | 3                     |
|                 | Kotsuki-gawa River (9) | 3 April 2004 | ethanol-fixed (M) | 1                     |
| Hediste diadroma| Nanakita-gawa River (3) | 26 April 1994 | frozen (M) | 2                     |
|                 | Ohashi-gawa River (5) | 2 April 2007 | frozen (M) | 3                     |
|                 | Omuta-gawa River (7) | 23 December 2003 | ethanol-fixed (M) | 1                     |
|                 | Omuta-gawa River (7) | 21 January 2004 | ethanol-fixed (M) | 3                     |
|                 | Omoi-gawa River (8) | 4 April 2004 | ethanol-fixed (M) | 2                     |
|                 | Kotsuki-gawa River (9) | 23 February 2004 | ethanol-fixed (M) | 1                     |
|                 | Kotsuki-gawa River (9) | 19 May 2004 | ethanol-fixed (M) | 1                     |
| Hediste japonica| Higashiyoga (6) | 28 July 1995 | frozen (I) | 2                     |
|                 | Omuta-gawa River (7) | 4 January 1999 | ethanol-fixed (M) | 1                     |
|                 | Omuta-gawa River (7) | 23 November 2003 | ethanol-fixed (M) | 2                     |
|                 | Omuta-gawa River (7) | 6 December 2003 | ethanol-fixed (M) | 3                     |
|                 | Omuta-gawa River (7) | 11 December 2003 | ethanol-fixed (M) | 1                     |
|                 | Omuta-gawa River (7) | 5 January 2004 | ethanol-fixed (M) | 1                     |

*1 Numbers in parentheses correspond with numbers in Fig. 1.
*2 M: mature worms, I: immature worms.

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Fig. 1. Collection sites of three Hediste species. Numbers on
the map correspond with Table 1. 1. Takase-gawa River (Aomori),
2. Shinjo-gawa River (Aomori), 3. Nanakita-gawa River (Miyagi),
4. Kumano-gawa River (Wakayama), 5. Ohashi-gawa River (Shi-
mane), 6. Higashiyoga (Saga), 7. Omuta-gawa River (Fukuoka),
8. Omoi-gawa River (Kagoshima), 9. Kotsuki-gawa River
(Kagoshima).
Actually, we could identify species-specific allozyme loci (Sato & Masuda 1997). In this aspect, the present method is superior to electrophoretic analysis of fixed materials such as gametes, larvae and juveniles. In this case, difficulty in species identification in adults was worked out using PCR-RFLP analyses. It has also been a useful tool for species identification in larvae of mussels (Côrte-Real et al. 1994), asteroids (Evans et al. 1998) and spiny lobsters (Chow et al. 2006).

Our data indicate that the PCR-RFLP method using EcoRV and TspEI restriction enzymes enable easy and accurate identification of the three sympatric morphologically similar Hediste species. The EcoRV digestion of the PCR products can distinguish H. atoka from the other two species, and the TspEI digestion of the PCR products can distinguish H. diadroma from the other two species.

The present method would be applicable to species identification by use of a small amount of freshly frozen or ethanol-fixed materials such as gametes, larvae and juveniles. In this aspect, the present method is superior to electrophoretic analysis of species-specific allozyme loci (Sato & Masuda 1997). Actually, we could identify H. atoka with a specimen immersed in 75% ethanol at room temperature for 17 years (data not shown).

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### Table 2. Cleavage pattern shown by fragment sizes of PCR products of the partial mitochondrial 16S rRNA gene after digestion by one of two restriction enzymes. The size of each fragment was checked against a 100-bp DNA mass ladder.

| Restriction enzyme | Species          | H. atoka | H. diadroma | H. japonica |
|-------------------|------------------|----------|-------------|-------------|
| EcoRV             |                  | 330 bp   | 600 bp      | 600 bp      |
|                   |                  | 190 bp   |             |             |
| TspEI             |                  | 260 bp   | 210 bp      | 260 bp      |
|                   |                  | 100 bp   | 60 bp       | 100 bp      |
|                   |                  | 60 bp    | <60 bp*     | 60 bp       |
|                   |                  | <60 bp*  |             | <60 bp*     |

* Several bands were observed.

PCR-RFLP is a useful tool to identify morphologically indistinguishable species. This method has been applied in taxonomic, ecological or biogeographic studies in such marine invertebrates as mussels (Heath et al. 1995, Toro 1998), scallops (López-Piñón et al. 2002), and pearl oysters (Masaoka & Kobayashi 2005). In these cases, difficulty in species identification in adults was worked out using PCR-RFLP analyses. It has also been a useful tool for species identification in larvae of mussels (Côrte-Real et al. 1994), asteroids (Evans et al. 1998) and spiny lobsters (Chow et al. 2006).

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