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Morphological and molecular sequence analysis of the harmful shell boring species of *Polydora* (Polychaeta: Spionidae) from Japan and Australia

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ABSTRACT

Accurate species identification is required to trace, monitor and limit the distribution of harmful shell boring species of the genus *Polydora*, which have been transported worldwide accompanying commercially important mollusk species. Morphological identification of species in the polydorid complex is difficult because of close similarities, therefore, nuclear 18S rRNA gene sequences were used for the first time to distinguish among the three serious aquaculture pests *Polydora brevipalpa*, *Polydora uncinata*, and *Polydora aura*, from Japan and Australia. The analysis revealed new intraspecific pigmentation variation and confirmed that *P. uncinata* and *P. aura* are closely related, possessing special notochaetae on the posterior chaetigers, while *P. brevipalpa* is a sister taxon to these two species. *Polydora brevipalpa* and *P. uncinata* share characteristic black-bar pigmentation on their palps, but they apparently are not part of the same clade.

1. Introduction

Members of the genus *Polydora* (Polychaeta: Spionidae) are famous for their boring activities into various calcareous substrates, e.g., coralline algae, corals, mollusk shells, and barnacles (Blake, 1996). Although they excavate their burrows for use as a habitat, some species have been frequently reported as harmful invaders from the viewpoint of molluskan aquaculture, as they often damage the commercially important mollusk shells by decreasing their commercial value, reducing their growth rate and meat yield, and causing heavy mortality (Handley and Bergquist, 1997; Lleonart et al., 2003; Mori et al., 1985; Sato-Okoshi et al., 2008, 2012; Simon et al., 2006). Currently, species of *Polydora* and related genera that associate with mollusk shells appear to have spread outwardly by accompanying commercially important host shells that have been transported worldwide (Radashevsky and Olivares, 2005; Simon et al., 2006). These species are a source of concern not only from the perspective of aquaculture but also as invasive species (Cohen and Carlton, 1998).

The three species of the genus *Polydora* investigated in this study have caused severe damage to commercially important mollusk shells in Japan (Sato-Okoshi and Nomura, 1990), South Korea (Sato-Okoshi et al., 2012), Australia (Sato-Okoshi et al., 2008), and Chile (Radashevsky and Olivares, 2005). The scallop *Patinopecten yessoensis* in Okhotsk Sea, Hokkaido, Japan, and sown and suspended cultured scallops in Mutsu Bay, northern Tohoku District, Japan, were heavily infested by large *Polydora brevipalpa* (Mori et al., 1985; Sato-Okoshi and Nomura, 1990). Shells of the land-based tank cultured abalones *Haliotis discus hannai* and *Haliotis discus discus* from Japan (this study), the land-based tank cultured abalone *H. discus hannai* from Chile (Radashevsky and Olivares, 2005), the land-based cultured abalone *Haliotis laevigata*, and *Haliotis roei* from Australia (Sato-Okoshi et al., 2008), and the suspended cultured oyster *Crassostrea gigas* from Japan (Sato-Okoshi, 1999) and South Korea (Sato-Okoshi et al., 2012) were observed to be seriously infested by large-sized *Polydora uncinata*. Medium-to-large-sized *Polydora aura* have often been observed to bore into the shells of wild and cultured mollusks in South Korea (Sato-Okoshi et al., 2012) and suspended cultured oysters distributed in western Japan (Sato-Okoshi, 1999).

There are several species complexes within the *Polydora* group which include different species that are morphologically indistinguishable from each other. At the same time, some *Polydora* species show a high degree of intraspecific variation, particularly with respect to pigmentation patterns. Although molecular biological approaches have been suggested to be effective for species determination in various taxa, to date, there is little molecular biological information in Spionidae in either the genus *Polydora* or related genera. Moreover, there are few GenBank sequences available for *Polydora* complex species material sourced from the Pacific and Asia.

The present study is the first attempt to analyze the molecular sequences of the 18S rRNA genes of the three morphologically resemble borers that are the most serious treats to molluskan aquaculture.

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Not only interspecific differentiation but also intraspecific morphological variation was reconfirmed at the same time.

2. Materials and methods

2.1. Morphological observations

Polydora individuals of each species were collected from the shells of wild, sown, and suspended cultured *P. yessoensis*; cultured and wild *C. gigas*; cultured *Pinctada fucata*; cultured *H. discus discus*, *H. discus hannai*, *Haliotis gigantea*, *H. roei*, *H. laevigata*, and *Haliotis diversicolor supertexta* from Japan and Australia; wild *Omphalius rusticus* from Japan; and wild *Thais orbita* and *Turbo torquatus* from Australia during the period of 1985 to 2011 (Fig. 1, Table 1). The shells were collected at the intertidal to subtidal sea level. The worms inhabited self-excavated burrows inside the shells, and they were extracted by fracturing the shells with cutting pliers and a hammer.

After the worms were removed from the shells, their morphological characteristics were observed under a stereomicroscope (Leica MZ 95) in both live and preserved conditions in 10% neutral formalin in seawater.

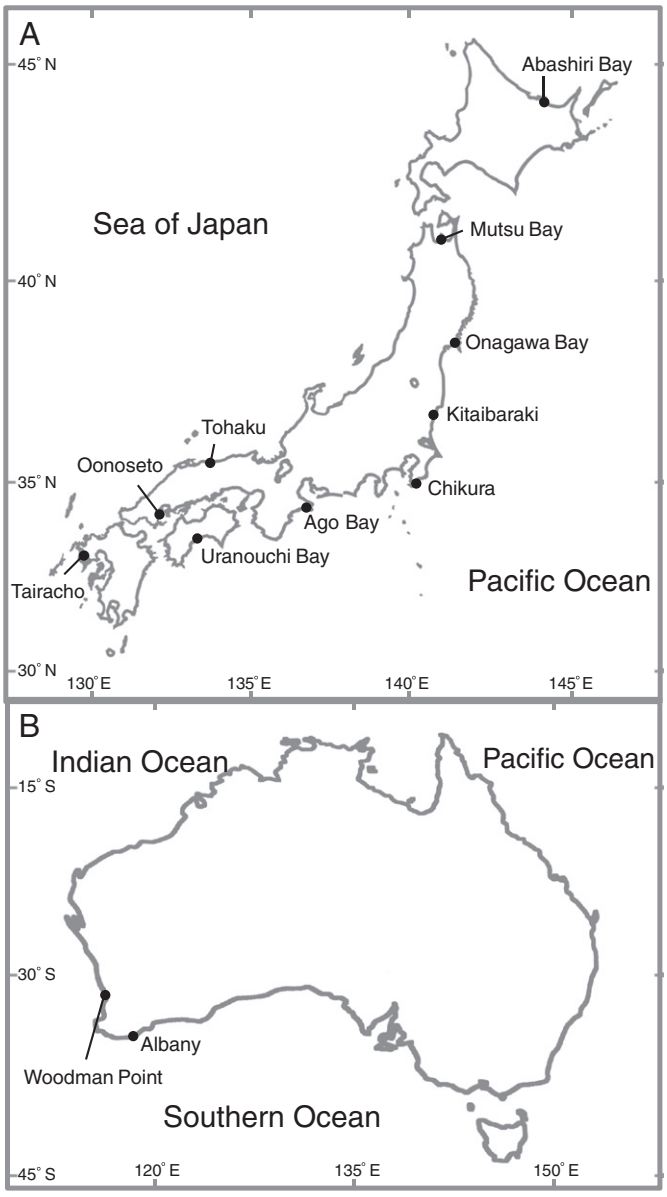

Fig. 1. Location of the sampling sites in Japan (A) and Australia (B).

Table 1
Boring *Polydora* species collected for morphological observation and gene analysis from mollusk shells in Japan and Australia.

Species	Host shell	Locality	Sampling date
Polydora brevipalpa	*Patinopecten yessoensis*[C, W]	Abashiri Bay	1985 November
	P. yessoensis[C]	Mutsu Bay*	2011 October
	P yessoensis[C]	Onagawa Bay*	2011 February
Polydora uncinata	*Haliotis discus hannai*[C]	Chikura, Chiba	2007 March
	Haliotis discus discus[C]	Tairacho, Nagasaki*	2009 April
	H. discus hannai[C]	Tairacho, Nagasaki	2009 April
	Haliotis gigantea[C]	Tohaku, Tottori	2010 September
	Haliotis laevigata[C,W]	Albany*	2005 August
	Haliotis roei[C,W]	Albany; Ocean Reef	2005 August
	Haliotis diversicolor supertexta[C]	Tairacho, Nagasaki	2009 April
	Crassostrea gigas[C]	Oonoseto, Hiroshima*	2011 July
	C. gigas[C]	Uranouchi Bay, Kochi	2011 September
	C. gigas[W]	Kitaibaraki*	2011 February
	Omphalius rusticus[W]	Onagawa Bay*	2011 August
Polydora aura	*C. gigas*[C]	Oonoseto, Hiroshima*	2011 July
	Pinctada fucata[C]	Ago Bay	2009 April
	Thais orbita[W]	Woodman Point	2005 August
	Turbo torquatus[W]	Woodman Point	2005 August

[C]: cultured, [W]: wild *: sample for rRNA analysis.

Specimens were deposited in the National Museum of Nature and Science, Tokyo (NSMT).

2.2. DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

Four specimens of *P. brevipalpa* (three from Mutsu Bay and one from Onagawa Bay), six specimens of *P. uncinata* (one each from Tairacho, Albany, Oonoseto, and Kitaibaraki and two from Onagawa Bay), and one specimen of *P. aura* from Oonoseto were used for genetic analysis (Fig. 1, Table 1). Genomic DNA was extracted from living specimens or ethanol-preserved tissues. All animal tissues were washed by several transfers in filtered (pore size 0.2 μm) seawater and distilled water to remove as much extraneous matter as possible. PCR tubes (0.2 mL) each containing 50 μL of 10% Chelex® suspension (Bio-Rad Laboratories Inc., Richmond, CA) and animal tissues were heated at 95 °C for 20 min to extract genomic DNA according to Richlen and Barber (2005). Extracted DNAs were used as templates to amplify the target regions. All PCRs were performed on a thermal cycler in a reaction mixture (25.0 μL) containing 1.0 μL of template DNA, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1× PCR buffer, 2.0 mM $MgSO_4$, 0.4 U of KOD-Plus-ver. 2 DNA polymerase (Toyobo, Osaka, Japan; with intensive 3′→5′ exonuclease activity), and 0.2 nM of each primer. For amplifying the nuclear 18S rRNA gene of the three *Polydora* species, three primer pairs (18S-1F1/18S-1R632, 18S-2F576/18S-2R1209, and 18S-3F1129/18S-R1772) were used (Nishitani et al., 2012; Teramoto et al., submitted for publication). The PCR cycling conditions were as follows: initial denaturation at 94 °C for 2 min and 38 cycles a 94 °C for 15 s, 54 °C for 30 s, and 68 °C for 45 s. The results of PCR amplification were confirmed on 1.0% agarose gels by using ethidium staining. To remove unincorporated PCR primers and dNTPs, 1.5 μL of the PCR products were then treated with 0.6 μL of exonuclease I and shrimp alkaline phosphatase (USB Corp., Cleveland, OH, USA) at 37 °C for 15 min