Molecular evidence of cryptic species diversity in the *Perinereis nuntia* species group (Annelida: Nereididae) with first records of *P. nuntia* and *P. shikueii* in southern Japan

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**Abstract:** Taxonomic reexamination of Japanese populations of the *Perinereis nuntia* species group, which constitutes a major polychaete component in intertidal benthic communities, was carried out by analyzing the mitochondrial 16S rDNA and the nuclear ribosomal ITS sequences of 80 specimens, newly collected from 27 sites in Japan, together with 18 museum-preserved specimens collected from southern neighboring countries outside Japan. The Japanese populations of the *Perinereis nuntia* species group could be divided into four genetically different groups, which corresponded morphologically to four nominal species (*P. mictodonta*, *P. wilsoni*, *P. shikueii*, and *P. nuntia*), with some exceptions. *Perinereis nuntia* and *P. shikueii* were recorded as new to the Japanese fauna, with their distributions restricted to southern Japan. The clade containing *P. shikueii* was subdivided into two sister clades (forms A and B), indicating that cryptic speciation has occurred within this clade. Our results revealed a remarkable variability in the number of bars in area VI of the proboscis in form B of *P. shikueii*, which appeared to be caused by breakage of the long bar into short bars during growth from a juvenile to an adult.

**Key words:** 16S rDNA, ITS, paragnaths, polychaetes, taxonomy

**Introduction**

The genus *Perinereis* Kinberg, 1865 (Annelida: Nereididae) includes approximately 66 species in the world, occurring as major components of intertidal benthic communities (Sampérguètgi et al. 2013). The *Perinereis nuntia* species group is characterized by the presence of an arc of bar-shaped paragnaths (or a mixture of bars and cones) on area VI of the eversible proboscis. The species group currently comprises 15 species, recorded from a wide range in the temperate and tropical Indo-Pacific (Wilson & Glasby 1993, Glasby & Hsieh 2006).

Only two species of the *Perinereis nuntia* species group, *P. mictodonta* (Marenzeller, 1879) and *P. wilsoni* Glasby & Hsieh, 2006, are currently recognized from Japan (Sato 2017) and Korea (Park & Kim 2007); whereas, six species have been recorded from nearby Taiwan (Glasby & Hsieh 2006). Prior to the taxonomic revision of Glasby & Hsieh (2006), the Japanese and Korean populations of *P. mictodonta* and *P. wilsoni* were known as *P. nuntia* var. *brevicirris* sensu Fauvel, 1932 and *P. nuntia* var. *val-lata* sensu Fauvel, 1932, respectively (Imajima 1972; Paik 1972). Though the two species are morphologically very
similar (Glasy & Hsieh 2006), molecular studies using the ribosomal internal transcribed spacers (ITS) (Chen et al. 2002) and the mitochondrial cytochrome oxidase I (COI) gene (Park & Kim 2007) suggested that they are distinct species. In Japan, the two species have been classified according to the key of Imajima (1972, 1996): P. mictodonta has three paragnaths in a triangle on area V, whereas P. wilsoni has a single paragnath in the same area. However, Glasby & Hsieh (2006) and Park & Kim (2007) showed that the paragnath numbers in area V are variable within each species and overlap between the two species (P. mictodonta: 1–3 in the type locality (Japan), 1–5 in Taiwan and Korea; P. wilsoni: 1–3 in Taiwan, 1–4 in Korea) (Table 1). Moreover, these two species are also morphologically very similar to another two species, P. nuntia (Savigny, 1818) and P. shikueii Glasby & Hsieh, 2006 (Glasby & Hsieh, 2006), which are known from Taiwan. Therefore, it seems that the species diversity of the Perinereis nuntia species group may be underestimated in Japan (especially in southern Japan) because some tropical and subtropical species could be easily misidentified as P. mictodonta or P. wilsoni by Imajima’s key.

Molecular approaches by DNA analyses can be of significant help in taxonomy. The most widely used molecular markers for both intraspecific and interspecific relationships in polychaetes are the 18S ribosomal RNA genes from nuclear DNA (e.g., Bleidorn et al. 2003), and the COI and the 16S ribosomal RNA genes from mitochondrial DNA (e.g., Jolly et al. 2006, Iannotta et al. 2007). The nuclear ribosomal DNA (rDNA) of eukaryotes consists of an external transcribed spacer (ETS), small-subunit ribosomal RNA (18S rRNA), first inter-transcribed spacer (ITS 1), 5.8S rRNA, second inter-transcribed spacer (ITS 2), and large-subunit rRNA (28S rRNA) (Gerbi 1986). Ribosomal ITS sequences are also used for phylogenetic construction at and below the species level, because they can provide a spectrum of signals for phylogenetic resolution, and their degree of polymorphism has been shown to vary with species (e.g., Chen et al. 2002). Chen et al. (2002) revealed that the interspecific divergence of ITS among the four species of Perinereis (P. aibuhitensis (Grube, 1878), P. floridanana (Ehlers, 1868), P. mictodonta, and P. wilsoni) is much higher than intraspecific divergence, and the interspecific divergence of ITS 2 is significantly higher than that of ITS 1.

In the present study, we examine the molecular phylogeny of 80 Japanese specimens of the Perinereis nuntia species group, together with 18 reference materials of four foreign species sourced from museum collections. Our analysis is based on molecular markers of the mitochondrial 16S rDNA and the nuclear ribosomal ITS sequences. Our results indicate that the Japanese specimens of the Perinereis nuntia species group can be divided into four genetically different groups, which correspond to four nominal species (P. mictodonta, P. wilsoni, P. nutria, and P. shikueii) and that the clade of P. shikueii can be clearly subdivided into two sister clades, suggesting the existence of a cryptic species closely related to P. shikueii.

### Materials and Methods

#### Collection and preparation of specimens for DNA analyses

A total of 80 Perinereis specimens were collected from 27 locations of the intertidal zone in Japan from June 1981 to April 2019 (Table 2 and Figs. 1–5). We also obtained an additional 18 reference materials, which were collected outside of Japan and had been deposited in the Northern Territory Museum and Art Gallery, Darwin, Australia (NTM) and the Research Museum, Research Center for Biodiversity, Academia Sinica, Taipei, Taiwan (ASIZW), including specimens examined in Glasby & Hsieh (2006) (Table 3).

The specimens, fixed in 80–99% ethanol, were used for DNA isolation, with the reference material specimens being soaked for 30 min in phosphate-buffered saline (PBS) prior to the DNA extraction. Total DNA was extracted from several millimeters of the middle section of each worm using two methods: the hexadecyltrimethylammonium bromide (CTAB) method from Winnepeninckx et al. (1993) and a combination method from Huelsken et al.
CTAB method: The specimens were transferred to a micro tube containing preheated (60°C) 2×CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl pH 8, 0.1 mg/mL proteinase K). After incubation at 60°C for at least 30 min, the samples were then purified by adding the same volume of chloroform-isooamyl alcohol (24:1), two times. Nucleic acids were precipitated by adding isopropanol (2/3 vol.). After overnight incubation at room temperature (25±2.5°C), the nucleic acids were then collected by centrifugation. DNA was washed in 75% ethanol. After air drying, the DNA

| Species | Site numbers | Code | Location (latitude and longitude) | Sampling date | Accession number |
|---------|--------------|------|-----------------------------------|--------------|-----------------|
| P. nuntia | Pnun-7 | Nagashima Island, Kagoshima Prefecture (32°06′N, 130°08′53″E) | April 29, 2014 | LC482160 |
|          | Pnun-5 | Hirakawa, Kagoshima, Kagoshima Prefecture (31°26′77″N, 130°31′03″E) | March 20, 2014 | LC482160 |
|          | Pnun-6 | Hirakawa, Kagoshima, Kagoshima Prefecture (31°26′77″N, 130°31′03″E) | March 20, 2014 | LC482160 |
|          | Pnun-8 | Tokunoshima Island, Kagoshima Prefecture (27°52′00″N, 128°53′32″E) | June 18, 2016 | LC482159 |
|          | Pnun-9 | Tokunoshima Island, Kagoshima Prefecture (27°52′00″N, 128°53′32″E) | June 18, 2016 | LC482159 |
|          | Pnun-10 | Tokunoshima Island, Kagoshima Prefecture (27°52′00″N, 128°53′32″E) | June 18, 2016 | LC482159 |
|          | Pnun-11 | Yonagunijima Island, Okinawa Prefecture (24°26′40″N, 122°58′59″E) | March 3, 2015 | LC482158 |
|          | Pnun-13 | Yonagunijima Island, Okinawa Prefecture (24°26′40″N, 122°58′59″E) | March 3, 2015 | LC482138 |
| P. shikaei (form A) | Shio-7 | Shiotagawa River, Shiroishi, Saga Prefecture (33°07′05″N, 130°07′45″E) | July 5, 2015 | LC482191 |
|          | Shio-8 | Shiotagawa River, Shiroishi, Saga Prefecture (33°07′05″N, 130°07′45″E) | July 5, 2015 | LC482191 |
|          | Tako-3 | Takorigawa River, Tara, Saga Prefecture (32°57′57″N, 130°12′83″E) | September 26, 2015 | LC482191 |
|          | Tako-4 | Takorigawa River, Tara, Saga Prefecture (32°57′57″N, 130°12′83″E) | September 26, 2015 | LC482190 |
| P. shikaei (form B) | NTO-2 | Niigawa River, Tushima Island, Nagasaki Prefecture (34°23′28″N, 129°18′36″E) | April 5, 2019 | LC482193 |
|          | NTO-3 | Niigawa River, Tushima Island, Nagasaki Prefecture (34°23′28″N, 129°18′36″E) | April 5, 2019 | LC482200 |
|          | NTO-1 | Oura Bay, Tushima Island, Nagasaki Prefecture (34°40′43″N, 129°25′70″E) | April 6, 2019 | LC482199 |
|          | Imu-1 | Imari River, Saga Prefecture (33°17′55″N, 129°51′22″E) | August 22, 2018 | LC482192 |
|          | Imu-2 | Imari River, Saga Prefecture (33°17′55″N, 129°51′22″E) | August 22, 2018 | LC482198 |
|          | Mizu-5 | Mizanoura, Ishaya Bay, Nagasaki Prefecture (32°54′42″N, 130°09′33″E) | September 26, 2015 | LC482196 |
|          | Mizu-6 | Mizanoura, Ishaya Bay, Nagasaki Prefecture (32°54′42″N, 130°09′33″E) | September 26, 2015 | LC482155 |
|          | Usu-1 | Suehirosegawa River, Usaki, Oita Prefecture (33°07′55″N, 131°47′63″E) | September 25, 2018 | LC482196 |
|          | Kedo-1 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′18″N, 130°17′20″E) | October 26, 2014 | LC482154 |
|          | Kedo-11 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | May 8, 2016 | LC482194 |
|          | Kedo-12 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | May 8, 2016 | LC482195 |
|          | Kedo-14 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | May 8, 2016 | LC482196 |
|          | Kedo-15 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | May 8, 2016 | LC482196 |
|          | Kedo-17 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | May 8, 2016 | LC482197 |
|          | Kedo-18 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | May 8, 2016 | LC482196 |
|          | Kedo-21 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | November 13, 2016 | LC482197 |
|          | Kedo-22 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | November 13, 2016 | LC482195 |
|          | Kedo-31 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482197 |
|          | Kedo-32 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482197 |
|          | Kedo-33 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482196 |
|          | Kedo-34 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482196 |
|          | Kedo-35 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482195 |
|          | Kedo-36 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482195 |
|          | Kedo-37 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482196 |
|          | Kedo-38 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | January 31, 2017 | LC482197 |
|          | Kedo-103 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | November 30, 2016 | LC482195 |
|          | Kedo-104 | Kedogawa River, Makurazaki, Kagoshima Prefecture (31°16′8″N, 130°17′45″E) | November 30, 2016 | LC482195 |
was dissolved in a buffer containing 1 mM Tris, 0.1 mM EDTA (0.1×TE).

Combination method: The DNA solution obtained with the DNeasy Blood and Tissue Kit (Qiagen) was mixed thoroughly with one volume of 2×CTAB buffer and incubated for 2–4 hours at 60°C. The sample was then purified by adding an equal volume of chloroform-isooamyl alcohol (24:1), two times. The DNA was ethanol precipitated overnight and the nucleic acids were then collected by centrifugation. DNA was washed in 75% ethanol. After air-drying, the DNA pellet was redissolved in 0.1×TE (1 mM Tris–HCl pH 8, 0.1 mM EDTA).

Sequencing of partial mitochondrial 16S rDNA

We analyzed the partial mitochondrial 16S rDNA sequences of Perinereis worms. A PCR reaction was performed using a TaKaRa Ex Taq DNA polymerase (Takara Bio). The 16S rDNA fragments were amplified using the primers 16sarL–16sbrH (Palumbi 1996) and the cycling protocol was as follows: initial denaturation at 95°C for 1 min; 38 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 1 min; with a final 7-minute extension at 72°C. On occasion, 16sarL did not work satisfactorily; in such cases, we used 12412F (Tosuji et al. 2019) combined with the forward primer 16SbrH primer. The sequences of primers are shown in Table 4. All products were separated on a 1% agarose gel and purified with the Plus gel elution kit (GMbiolab). Nu-
cleotide sequencing was outsourced to Fasmac in Kanagawa, Japan using the direct sequencing method with the amplification primers or sequencing primers (12415SF and 12897SR) (Tosuji et al. 2019) and all sequences were checked and corrected by visual inspection. The sequencing data obtained from an ABI genetic analyzer were assembled to get contig sequences using the sequence analysis software Genetyx-Mac ver. 19 for manual editing. The data have been submitted to the DDBJ database under accession numbers LC482156–LC482200.

Sequencing of ribosomal internal-transcribed spacers (ITS)

For our molecular analysis, we also used nuclear ITS sequences, which were previously used to examine the molecular phylogeny of the *Perinereis nuntia* species group in Taiwan by Chen et al. (2002).

PCR conditions to amplify the ITS were as described above. The amplicons were cleaned from the agarose gels using a Plus gel elution kit and then cloned using the Promega pGEM-T system under the manufacturer’s recommended conditions. Nucleotide sequencing was outsourced to Fasmac in Kanagawa, Japan. To facilitate DNA sequencing, the amplification primers with the internal primers r5.8S1 and r5.8S2 (Chen et al. 2002) were used. All sequences were checked and corrected by visual inspection. The sequencing data obtained from an ABI genetic analyzer were assembled to get contig sequences using the sequence analysis software Genetyx-Mac ver. 19 for manual editing. These nucleotide sequences are deposited in DDBJ (accession numbers LC482138–LC482155 and LC482304).

Sequence alignment and data analysis

The 16S rDNA sequences were aligned using the mul-
multiple sequence alignment software MAFFT ver. 7.310 (Katoh & Standley 2013) with the L-INS-i method. When samples had the same sequence, the duplicate sequences were removed. The ITS sequences were aligned using the DNA alignment software MUSCLE ver. 3.8.31 (Edgar, 2004)

The best-fitting model of nucleotide substitution for a Bayesian inference (BI) tree was selected by MrModeltest v. 2.4 (Nylander 2004). This is a general time reversible model with discrete gamma distribution and by assuming that a certain fraction of sites were evolutionarily invariable (GTR+G+I) as determined by Akaike information criterion (AIC) (Akaike 1973) for both 16S rDNA and ITS. MrBayes v. 3.1.6 (Ronquist et al. 2012) was used to obtain BI phylogenetic trees.

The sequence data of *P. aibuhitensis* (KF611806 collected from Gang-hwa Island, Korea, and AF332148 collected from Chuwei, Taiwan) were used as an outgroup for rooting the BI tree in the analysis of 16S rDNA and ITS sequences, respectively.

Eight Metropolis-coupled Markov chain Monte Carlo algorithms were run, starting with random initial trees and sampling every 100 generations. The analyses were allowed to continue until the average standard deviation of split frequencies (ASDSF) reached below 0.01. As a result, 500,000 generations (ASDSF=0.008169) for 16S rDNA and 500,000 generations (ASDSF=0.003817) for ITS were obtained. The first 25% of the sampled trees were excluded as burn-in samples and burn-in value for each analysis was assessed using the software Tracer1.6 (Rambaut et al. 2014).
We used Arlequin version 3.512 (Excoffier & Lischer 2010) to calculate pairwise fixation index ($F_{ST}$) values (Weir & Cockerman 1984) to estimate the level of genetic divergence between populations.

**Morphological observations**

Anterior maximum body width excluding parapodia (BW) was measured under a stereoscopic microscope. Selected diagnostic characteristics of the paragnaths in areas V (number of cones and their arrangement) and VI (number and length of bars) on the proboscis and of the lower (ventral fascicle) neurochaetae around chaetiger 10 (presence or absence of heterogomph spinigers) (Table 1) were examined. Another diagnostic characteristic is the relative length of dorsal cirri to the notopodial dorsal ligule (Glasby & Hsieh 2006). This characteristic was not examined in the present study. Photographs were taken with a digital camera (E3CMOS, TouTek) attached to a stereoscopic microscope.

**Results**

**Molecular phylogeny based on 16S rRNA sequences**

Both extraction methods (CTAB method and combination method) provided DNA of nucleotide sequencing quality.

The 16S rDNA dataset consisted of 45 nucleotide sequences (haplotypes) (accession numbers LC482156–LC482200) from 95 specimens containing 420–426 characters (432 characters with gaps). Nucleotide substitutions occurred at 84 sites. The dataset had 354/432 conserved characters (81.9%), 75/432 variable characters (17.4%), and 64/432 parsimony informative characters (14.8%), excluding the outgroup species.

In the BI tree (Fig. 6), 45 haplotypes were divided into three major clades, which were judged to correspond to *P. nuntia*, *P. shikueii* and *<P. wilsoni>P. mictodonta*</3> based on their species-specific morphological characteristics (Table 1) in most specimens with a few exceptions (see below). This result was supported by high posterior probabilities (>99.9%). The clade of *P. nuntia* was first divided from another large clade, which was subsequently divided into the clades of *P. shikueii* and *<P. wilsoni>P. mictodonta*</3>. The clade of *P. shikueii* was clearly subdivided into two sibling clades that were here designated as forms A and B. The posterior probability of these branches was relatively high (92.0%). The clade of *<P. wilsoni>P. mictodonta*</3> was constituted by a monophyletic clade of *P. wilsoni* and a paraphyletic group of *P. mictodonta*.

In the clade of *P. nuntia*, nucleotide substitutions occurred at eight sites within the 424–426 bp region, producing five haplotypes (accession numbers LC482156–LC482160) originating from a total of 10 specimens (seven specimens from four sites in southern Japan, and three from two sites in Australia (Fig. 1). No marked genetic differentiation was detected between specimens from Australia and Japan.

In the clade of the form A of *P. shikueii*, nucleotide substitutions occurred at one site within the 420 bp region, producing two haplotypes (accession numbers LC482190 and LC482191) originating from a total of five specimens (four specimens from two sites in the Ariake Sea, Japan, and one from Taiwan) (Fig. 2). No genetic differentiation was detected between specimens from Taiwan and Japan,
with a haplotype (LC482191) shared by a Taiwan and three Japanese specimens.

In the clade of the form B of *P. shikueii*, nucleotide substitutions occurred at 34 sites within the 421–423 bp region, producing nine haplotypes (accession numbers LC482192–LC482200) originating from 27 Japanese specimens from six sites in Kyushu and Tsushima Island (Fig. 3). The genetic differentiation was low among four haplotypes originating from 22 specimens from three sites in middle and southern Kyushu (10–12 in Fig. 3), with a single haplotype (LC 482196) shared by nine specimens collected from the three sites. On the other hand, the genetic differentiation was relatively high among five haplotypes originating from five specimens from three sites in northern Kyushu and Tsushima Island (7–9 in Fig. 3), which constituted a monophyletic subclade.

In the paraphyletic group of *P. mictodonta*, nucleotide substitutions occurred at 11 sites within the 421–424 bp

### Table 3. Reference materials of the museum-preserved specimens of *Perinereis nuntia* species group collected from the outside of Japan.

| Species         | Site numbers | Registration number | Location                        | Sampling date       | Accession number  |
|-----------------|--------------|---------------------|----------------------------------|---------------------|-------------------|
| *P. nuntia*     | 29           | NTMW 21260          | Redcliffe, SE QLD, QLD, Australia| September 21, 2005  | LC482156          |
|                 | 28           | NTMW 22580          | Casuarina Beach, Lizard Island, QLD, Australia | April 12, 2008 | LC482157 LC482141 |
|                 | 29           | NTMW 26217          | Redcliffe, SE QLD, QLD, Australia | 2016                | LC482156          |
| *P. shikueii* (form A) | 30     | ASIZW 666 *         | Tanshui Estuary, New Taipei, Taiwan | August 12, 2002    | LC482191          |
| *P. mictodonta* | 36           | ASIZW 647 *         | Beigang River, Yuanlin County, Taiwan | January 25, 2003   | LC482167          |
|                 | 37           | ASIZW 650 *         | Budai River, Chia-yi County, Taiwan | January 25, 2003   | LC482167          |
|                 | 36           | ASIZW 667 *         | Beigang River, Yuanlin County, Taiwan | January 25, 2003  | LC482162          |
|                 | 31           | ASIZW 864           | Huchia, Kinmen, Taiwan            | October 16, 2003   | LC482166          |
|                 | 32           | ASIZW 888           | Beihai, Guangxi Province, China   | July 17, 2002      | LC482164          |
|                 | 30           | ASIZW 898           | Tanshui Estuary, New Taipei, Taiwan | August 12, 2002   | LC482167          |
|                 | 35           | NTMW 19320 *        | Tonghsiao, Miaoli County, Taiwan  | November 4, 1999   | LC482167          |
|                 | 33           | NTMW 26198          | Chumphon National Park,           | September 9, 2003  | LC482167          |
|                 | 34           | NTMW 26196          | Chumphon Province, Thailand       | February 15, 2015  | LC482168          |
| *P. wilsoni*    | 32           | ASIZW 673 *         | Beihai, Guangxi Province, China   | July 17, 2002      | LC482180          |
|                 | 38           | ASIZW 674 *         | Hong Kong, China                  | July 24, 2002      | LC482171          |
|                 | 39           | ASIZW 678 *         | Shinmen, New Taipei, Taiwan      | February 21, 2003  | LC482182          |
|                 | 40           | ASIZW 679 *         | Tienihsien, Penghu County, Taiwan | March 19, 2003     | LC482181          |
|                 | 31           | ASIZW 885           | Kinmen, Taiwan                    | October 16, 2003   | LC482183          |

1) Numbers correspond to the site numbers in Figs. 1–5. 2) ASIZW: Research Center for Biodiversity, Academia Sinica, Taipei, Taiwan. NTM: Northern Territory Museum and Art Gallery, Darwin, Australia. Asterisks (*) indicate the specimens examined by Glasby and Hsieh (2006). 3) A specimen of ASIZW 667 was identified as *P. shikueii* by Glasby and Hsieh (2006).

### Table 4. List of primers used in this study.

| Target        | Name | Sequence (5′–3′) | Reference |
|---------------|------|------------------|-----------|
| **Amplification primer** | 16S  | 16SarL CGCCTGTTTCATCAAAAACAT | Palumbi et al. (1996) |
|               |      | 16SbrH CCGGTCTGAACTCAGATCACGT | Palumbi et al. (1996) |
|               |      | 12412F CAAAACATCGCCTGGTTG | Tosuji et al. (2019) |
|               | ITS  | ITS-F GGTACCCCTTT GTACACACCGCCCAGTCGCT | Chen et al. (1996) |
|               |      | ITS-R GCTTTGGGCTGCAGTCCCAAGCAACCCGACTC | Chen et al. (1996) |
| **Sequence primer** | 16S  | 12415SF AACAAYCGCCCTRTTGAAC | Tosuji et al. (2019) |
|               |      | 12897SR CATGTAGTGATTTAATGGTTG | Tosuji et al. (2019) |
|               | ITS  | r5.8S1 CGATGAAGAG CGCAGGCCAGC | Chen et al. (1996) |
|               |      | r5.8S2 CGATGTTCG AATGCTGC | Chen et al. (1996) |
region, producing eight haplotypes (accession numbers LC482161–LC482168) originating from a total of 27 specimens (18 specimens from 10 sites in western and southern Japan, and nine specimens from eight sites in Taiwan, China, Thailand, and Indonesia) (Fig. 4). The genetic differentiation among the eight haplotypes was low (2.6%), with a haplotype (LC 482167) shared by as many as 19 specimens, which were collected from 13 sites (one site in Thailand, four sites in Taiwan, and eight sites in Japan).

In the large clade of *P. wilsoni*, nucleotide substitutions occurred at 23 sites within the 421–422 bp region, producing 21 haplotypes (accession numbers LC482169–LC482189) that originated from a total of 28 specimens (23 specimens from nine sites in northern to southern Japan, and five specimens from five sites in Taiwan and China) (Fig. 5). The genetic differentiation was relatively high (5.5%) among these haplotypes; the clade of this species was subdivided into two monophyletic clades, which were subsequently divided into several small clades. Some of the small clades covered a narrow geographic range, whereas the others covered a wide geographic range (Fig. 6); a haplotype (accession number LC482177) was shared by three specimens from northern Japan (Asamushi, Aomori) and a specimen from southern Japan (Okinawajima Island).

The values of pairwise \( F_{ST} \) were calculated between all taxa (Table 5). The pairwise \( F_{ST} \) value between *P. mictodonta* and *P. wilsoni* was the lowest (0.40616). The \( F_{ST} \) value between the two forms of *P. shikueii* showed sufficiently high value (0.76373) compared to the values of *P. mictodonta* and *P. wilsoni* (0.40616), which are considered to be different species, with 29 bases substituted within the 420–423 bp region of 16S rDNA sequences.

**Molecular phylogeny based on nuclear ITS sequences**

Two previous sequences from *P. mictodonta* (accession number AF332163) and *P. wilsoni* (AF332158), which were reported by Chen et al. (2002) as P. sp. 1 and P. sp. 2, re-

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**Fig. 6.** Bayesian inference phylograms for *Perinereis* species derived from the analyses of 420–426 bp fragment of mitochondrial 16S rDNA sequence. *Perinereis aibuhitensis* (accession number KF611806) was used as an outgroup for rooting the tree. The posterior probability values are indicated on the branches and shown as a percentage. Numbers in brackets correspond to the site numbers in Figs. 1–5. Underlined numbers are the museum-preserved specimens. The scale bar corresponds to the substitutions per nucleotide site.
Table 5. Pairwise $F_{ST}$ values among the *Perinereis* species based on the dataset of 16S rDNA sequence.

|              | *P. mictodonta* | *P. wilsoni*      | *P. nuntia*      | *P. shikuei* (A) | *P. shikuei* (B) |
|--------------|-----------------|-------------------|-------------------|------------------|------------------|
| *P. mictodonta* | 0.00000         | 0.00000±0.0000    | 0.00000±0.0000    | 0.02148±0.0038   | 0.00000±0.0000   |
| *P. wilsoni*   | 0.40616         | 0.00000           | 0.00000±0.0000    | 0.00586±0.0026   | 0.00000±0.0000   |
| *P. nuntia*    | 0.93171         | 0.87071           | 0.00000           | 0.03906±0.0058   | 0.00000±0.0000   |
| *P. shikuei* (A) | 0.88156       | 0.74794           | 0.93462           | 0.00000          | 0.01855±0.0050   |
| *P. shikuei* (B) | 0.81441       | 0.74574           | 0.91212           | 0.76373          | 0.00000          |

Pairwise $F_{ST}$ (below diagonal) and $p$ values (above diagonal) for population differentiation.

Fig. 7. Bayesian inference phylograms for *Perinereis* species obtained from the 790–828 bp fragment of nuclear ITS sequence. The DNA sequence data, AF332158 (*P. wilsoni*) and AF332463 (*P. mictodonta*) were added as reference. *Perinereis aibuhitensis* (accession number AF332148) was used as an outgroup for rooting the tree. The posterior probability values are indicated on the branches and shown as a percentage. Numbers in brackets correspond to the site numbers in Figs. 1–5. Underlined number is the museum-preserved specimen. The scale bar corresponds to the substitutions per nucleotide site.

spectively, based on specimens collected from Taiwan, and later identified as coming form these species by Glasby & Hsieh (2006), were added as the reference sequences.

The whole ITS dataset including the two reference sequences consisted of 20 haplotypes (accession numbers LC482158–LC482155 and LC482304) from 20 specimens containing 790–828 characters (896 characters with gaps). The dataset had 684/896 conserved characters (76.3%), 197/896 variable characters (22.0%), and 151/896 parsimony informative characters (16.9%), excluding the outgroup
Perinereis nuntia species complex in Japan

The BI tree of the ITS dataset (Fig. 7) showed a topology similar to that of the 16S rDNA dataset; 20 haplotypes were divided into four monophyletic clades, which were judged to correspond to the four nominal species, *P. nuntia*, *P. shikueii*, *P. wilsoni* and *P. mictodonta*. This result was supported by high posterior probabilities (>99.7%). The clade of *P. nuntia* was first divided from another large clade, which was subsequently divided into the clades of *P. shikueii* and <*P. wilsoni*+*P. mictodonta*>. The clade of *P. shikueii* was subdivided into the two sibling clades of forms A and B. This result was also supported by high posterior probability (99.8%).

In the clade of *P. nuntia*, nucleotide substitutions occurred at 54 sites within the 816–825 bp region, producing five haplotypes (accession numbers LC482138–LC482142) originating from four Japanese specimens (from three sites in southern Japan) and one Australian specimen.

The clade of the form A of *P. shikueii* constituted a single haplotype (accession number LC4821502) originating from one Japanese specimen (from one site in the Ariake Sea). In the clade of the form B of *P. shikueii*, nucleotide substitutions occurred at 12 sites within the 793–795 bp region, producing five haplotypes (accession numbers LC482151–LC482155) originating from five Japanese specimens (from two sites in the Ariake Sea and one site in southern Kyushu).

In the clade of *P. mictodonta*, nucleotide substitutions occurred at 30 sites within the 788–798 bp region, producing five haplotypes (accession numbers LC482143, LC482144, and LC482304) originating from three Japanese specimens (from five sites in western and southern Japan). The sequence of a specimen from Taiwan (AF332163) was also included in the same clade.

In the clade of *P. wilsoni*, nucleotide substitutions occurred at 60 sites within the 795–828 bp region, producing three haplotypes (accession numbers LC482143, LC482144, and LC482304) originating from three Japanese specimens (from three sites in northern and southern Japan). The sequence of a specimen from Taiwan (AF332158) was also included in the same clade.

**Morphological characteristics and distributions in Japan**

(1) *Perinereis nuntia*

A total of eight Japanese specimens of the clade of *P. nuntia* were examined (Fig. 8). They were collected from intertidal flats with boulders in Kyushu (the Yatsushiro Sea, Kagoshima Bay) and the Ryukyu Islands (Tokunoshima and Yonagunijima Islands) (Table 2, Fig. 1).

They exhibited 1–3 cones in area V, which were usually situated at a more proximal position away from area VI (Fig. 8), agreeing well with the previous key for this species (Table 1). They exhibited 4–21 short even-length bars (sometimes cone-like) in area VI, almost agreeing with the previous key, with a record of an unusual high number (21 in Pnun-6) (Fig. 10A).

Heterogomph spinigers were absent in the lower neurochaetae of anterior parapodia (chaetigers 1 to 10) in most specimens, agreeing well with the previous key. A single heterogomph spinner was found in chaetigers 6 in a specimen (Pnun-8, epitokous male) from Tokunoshima Island.

(2) Form A of *Perinereis shikueii*

A total of five specimens (four from Japan, one from the type locality of *P. shikueii* in Taiwan) of the clade of the form A of *P. shikueii* were examined (Tables 2 and 3). The Japanese specimens were collected from estuarine mudflats with boulders and oyster clusters at two sites in the innermost part of Ariake Sea in western Kyushu (mouths of Shiotagawa and Takorigawa Rivers) (Fig. 2).

They exhibited 3–5 (usually 3) cones in area V, and 6–11 short even-length bars in area VI (Figs. 9A–C and 10B), agreeing well with the previous key for *P. shikueii* (Table 1). The three cones in area V were consistently arranged in a transverse line, agreeing well with the previous key.

A few heterogomph spinigers were present in the lower neurochaetae of anterior parapodia.

Epidermal pigmentation on prostomium and anterior
dorsum was relatively faint, showing as dusky brown in color.

(3) Form B of *Perinereis shikueii*

A total of 27 Japanese specimens of the clade of the form B of *P. shikueii* were examined. They were collected from estuarine intertidal flats with boulders and oyster clusters in Tsushima Island (Niigawa River and Oura Bay) and Kyushu (Imari Bay, Isahaya Bay in the Ariake Sea, Usuki and Makurazaki) (Table 2, Fig. 3). In the mouths of Kedogawa River in Makurazaki and Niigawa River in Tsushima Island, this form of the species coexisted with *P. mictodonta*.

In area V, they exhibited 2–5 (usually 3) cones; the two or three cones were consistently arranged in a transverse line or a flat inverted triangle (Fig. 9D–G), agreeing well with the previous key for *P. shikueii* (Table 1).

In area VI, most specimens contained 3–9 bars of uneven length, where the innermost or outermost bars were longest (Fig. 9D–F), not agreeing with the previous key for *P. shikueii*, but similar to the characteristics of *P. mictodonta* and *P. wilsoni* (Table 1). Two small specimens (Kedo-1, Kedo-104, with BWs of 0.6 and 0.7 mm, respectively) possessed only two long bars of even length (Fig. 9G). Segmentation of large bars was often observed (Fig. 9E). The number of bars in area VI (NB) was significantly correlated with the body width (BW), according to the following regression formula: \[ NB = 1.5 BW + 2.5 \] \( r = 0.42, P = 0.03, n = 27 \) (Fig. 10B).

A few heterogomph spinigers were present in the lower neurochaetae of anterior parapodia.

Epidermal pigmentation on prostomium and anterior dorsum was very strong, showing as dark black or dark brown except for a few specimens collected from the Ariake Sea showing faint pigmentation.
A total of 27 specimens (18 from Japan, nine from the outside of Japan) of the clade of *P. mictodonta* were examined (Tables 2 and 3). The Japanese specimens were collected from intertidal flats with boulders in the coasts of the Sea of Japan and the Seto Inland Sea in western Japan, the western and southern coasts of Kyushu, and the Ryukyu Islands (Amami-Oshima and Okinawajima Islands) (Fig. 4).

They contained 1–4 (usually 3) cones in area V, and 3–9 bars of uneven length in area VI (Figs. 10C and 11A–E), agreeing well with the previous key for this species (Table 1).

The ratio of specimens with three cones in area V was higher (93%, \(n=13\)) in specimens from a temperate region (Honshu and Kyushu in Japan) than in those from a subtropical-tropical region (the Ryukyu Islands in Japan, Taiwan, southern China, Thailand and Indonesia) (64%, \(n=11\)). The three cones were usually arranged in a regular triangle (Fig. 11A, B), agreeing with the previous key, though they were sometimes arranged in a transverse line (Fig. 11C, D) or a flat inverted triangle (Fig. 11E), which is the diagnosis for *P. shikueii* in the previous key. A specimen collected from Taiwan (ASIZW 667) (Fig. 11E), which possessed three cones arranged in a flat inverted triangle, was identified as *P. shikueii* by Glasby & Hsieh (2006).

The number of bars in area VI (NB) was significantly correlated with the body width (BW) in the Japanese specimens, according to the following regression formula: \(NB = 1.7 \times BW + 2.8\) (\(r=0.50, P=0.03, n=18\)) (Fig. 10C).

A few heterogomph spinigers were present in the lower neurochaetae of anterior parapodia of most specimens. They were, however, absent in two museum specimens with a BW of 2 mm (NTMW 26198 and 26196, collected from Thailand and Indonesia, respectively), against the previous key for this species.

(5) **Perinereis wilsoni**

A total of 28 specimens (23 from Japan, five from the outside of Japan) of the clade of *P. wilsoni*, were examined (Tables 2 and 3). Japanese specimens were collected from intertidal flats with boulders in Mutsu Bay in northern Japan, the Seto Inland Sea in western Japan, the southern coasts of Kyushu, and the Ryukyu Islands (Takarajima, Okinawajima, Kumejima and Ishigakijima Islands) (Fig. 5). In the Seto Inland Sea (Hatsukaichi) and the southern Kyushu (Sakurajima in Kagoshima Bay), this species coexisted with *P. mictodonta* in the same habitats.

Most of them exhibited 1–3 cones in area V, and 3–8 bars of uneven length in area VI (Figs. 10D and 11F, G, I, J), agreeing well with the previous key for this species (Table 1). However, a specimen (Kume-1) with a BW of 1.4 mm contained a single long bar in both the left and right of area VI (Fig. 11H). The number of bars in area VI in the Japanese specimens was not significantly correlated with the body width (\(P=0.27, n=19\)) (Fig. 10D).

The ratio of specimens with a single cone in area V was higher (77%, \(n=13\)) in specimens from the temperate region (Honshu and Kyushu in Japan) than in those from the subtropical-tropical region (the Ryukyu Islands in Japan, Taiwan, southern China) (29%, \(n=14\)). Plural cones in area V were arranged in various forms as a triangle (Fig. 11J), a transverse line (Fig. 11I), or a longitudinal line, not always agreeing with the previous key for this species.

**Discussion**

The present study revealed that the *Perinereis nuntia* species group comprises four nominal species in Japan; two species (*P. shikueii* and *P. nuntia*) were newly found from Japan, whereas two previously known species (*P. mictodonta* and *P. wilsoni*) were distributed in wider ranges, with the ranges of all of the four species overlapping in southern Japan.

*Perinereis shikueii* was previously known only from the western coast of Taiwan (Glasby & Hsieh 2006). The finding of *P. shikueii* in southern Japan is the first record of this species outside of Taiwan. Our molecular data demonstrated that there exist two sister clades (forms A and B) that were well differentiated genetically within the clade of
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Perinereis mictodonta (H.-4: A, collected from Hatsukaichi, Hiroshima Prefecture, Japan; B, Kedo-101 from Kedogawa River, Kagoshima Prefecture, Japan; C, S-oki-1 from Dogo Island, Shimane Prefecture, Japan; D, ASIZW 864 from Huchia, Kinmen County, Taiwan; E, ASIZW 667 collected from Beigang estuary, Chiayi County, Taiwan. Perinereis wilsoni (F–J): F, A-4 from Asamushi, Aomori Prefecture, Japan; G, A-3 from Asamushi, Aomori Prefecture, Japan; H, Kume-1 from Kumejima Island, Okinawa Prefecture, Japan; I, Ishi-1 from Ishigakijima Island, Okinawa Prefecture, Japan; J, Takara-1 from Takarajima Island, Kagoshima Prefecture, Japan. Scale bars: 0.5 mm.}

Fig. 11. Variation in paragnath morphology in areas V and VI on the oral ring of the proboscis in Perinereis mictodonta and P. wilsoni. Perinereis mictodonta (A–E): A, H-4 collected from Hatsukaichi, Hiroshima Prefecture, Japan; B, Kedo-101 from Kedogawa River, Kagoshima Prefecture, Japan; C, S-oki-1 from Dogo Island, Shimane Prefecture, Japan; D, ASIZW 864 from Huchia, Kinmen County, Taiwan; E, ASIZW 667 collected from Beigang estuary, Chiayi County, Taiwan. Perinereis wilsoni (F–J): F, A-4 from Asamushi, Aomori Prefecture, Japan; G, A-3 from Asamushi, Aomori Prefecture, Japan; H, Kume-1 from Kumejima Island, Okinawa Prefecture, Japan; I, Ishi-1 from Ishigakijima Island, Okinawa Prefecture, Japan; J, Takara-1 from Takarajima Island, Kagoshima Prefecture, Japan. Scale bars: 0.5 mm.

P. shikueii; the pairwise $F_{ST}$ value between the two forms of P. shikueii were sufficiently high ($F_{ST}=0.76373$) (Table 5), comparable with the interspecific values between other nereidid species (Tosuji & Furota 2016, Tosuji et al. 2019). We found also slight morphological differences between the two forms in the paragnath shape in area VI. These results strongly suggest the existence of an undescribed species closely related with P. shikueii. At present, form B including only Japanese specimens seems to be the undescribed species, whereas form A including a specimen from the type locality in Taiwan and a few specimens from the Ariake Sea in Japan seems to correspond to P. shikueii sensu stricto. Our morphological data support this idea; the morphology of the form A agrees well with that described in the original description of this species (Glasby & Hsieh 2006), whereas the morphology of the form B is slightly different from that in the original description. In the present study, both of the forms were mostly collected from the intertidal oyster beds. The taxonomic descriptions for the two forms will be presented in a following paper.

Perinereis nuntia is widespread throughout the tropical Indo-Pacific, previously known from the Red Sea (type locality) in the west, eastward to northern Australia in the south and Taiwan in the north (Glasby & Hsieh 2006) (Fig. 1). In the present study, this species was collected from the upper intertidal zone (under stones or dead corals) in a wide area of southern Japan (southern Kyushu to the Ryukyu Islands). This species is clearly distinguishable from the other species of the Perinereis nuntia species group in the region by the absence of heterogomph spinigers in the lower neurochaetae in anterior parapodia (Glasby & Hsieh 2006), though we found that one or two heterogomph spinigers are rarely present in few anterior parapodia. Wilson & Glasby (1993) also report neuropodial heterogomph spinigers in non-type specimens from the Red Sea.

Our results support the previous conclusion that P. mictodonta and P. wilsoni are morphologically very similar but distinct species (Chen et al. 2002, Glasby & Hsieh 2006, Park & Kim 2007). Their very close relationship is shown by the finding that the pairwise $F_{ST}$ value between the two species (0.40616) was lower than that between the two forms of P. shikueii (0.76373).

Our molecular data of the mitochondrial 16S rDNA revealed contrasting characteristics of genetic diversity between P. wilsoni and P. mictodonta. The genetic diversity
of *P. wilsoni* is relatively high. There are 16 haplotypes for 23 Japanese specimens (21 haplotypes for 28 specimens from Japan, Taiwan, and China). The clade of this species was subdivided into several small clades, which did not always correspond to geographical neighbors. This result indicates that a marked intraspecific genetic differentiation has occurred in *P. wilsoni* over a wide geographic range from northern Japan to Taiwan and southern China. On the other hand, the genetic diversity of *P. mictodonta* is extremely low. There are only four haplotypes for 18 Japanese specimens (eight haplotypes for 27 specimens from Japan to Indonesia). No marked intraspecific differentiation was detected even among geographically far separated populations (Indonesia, Thailand, Taiwan, and Japan). This result suggests that *P. mictodonta* may have relatively recently and rapidly expanded its distribution from a small original population to a wide range, even though this species seems to have a low capability for larval dispersal, as this species lacks a planktonic larval stage (Hardege & Bartels-Hardege 1995).

Previous certain records of *P. mictodonta* are limited to Japan, Korea, Taiwan, and China (Marenzeller 1879, Glasby & Hsieh 2006, Park & Kim 2007). The fact that the Thailand and Indonesian specimens were included in the clade of *P. mictodonta* in our analysis of the mitochondrial 16S rDNA indicates that this species may be widespread in tropical Southeast Asia. However, we cannot yet conclude this at present, because we could not find a heterogomph spiniger in the lower neurochaetae in anterior parapodia of the Thailand and Indonesian specimens, which is against the key character of *P. mictodonta*, agreeing instead with that of *P. nuntia*. Further examination is needed to clarify the possibility that the Thailand and Indonesian specimens belong to a cryptic species distinct from *P. mictodonta*. Such a close relationship is known between the forms A and B of *Hediste atoka*, which belong to the same clade in the analysis of 16S rDNA, but to the separated clades in the analysis of COI gene (Tosuji et al. 2019).

Several aspects of the paragnath morphology in areas V and VI were previously considered as reliable diagnostic characters for species identification (Table 1). However, our results indicate that the paragnath morphology is more variable than previously thought, demanding that the previous key (Table 1) be slightly revised. Because an intraspecific variation in each species largely overlapped with that of others, the exact species identification based on the paragnath morphology is rather difficult among the Japanese species of the *Perinereis nuntia* species group, though *P. nuntia* is clearly distinguishable from the others in the absence of neuropodial heterogomph spinigers in the anterior-most parapodia. Though the arrangement of three cones in a transverse line in area V seems to be exceptionally stable in both the forms A and B of *P. shikueii*, this characteristic is shared with variations of *P. mictodonta* (Fig. 11C, D) and *P. wilsoni* (Fig. 11I). However, our limited data suggest that the simple key of Imajima (1972, 1996) (viz., *P. mictodonta* with three paragnaths in a triangle on area V, whereas *P. wilsoni* with a single paragnath at that site) may be useful to some extent in temperate regions (Honshu and Kyushu in Japan).

Our data also showed that the number of bars in area VI increases with the growth of individuals, at least in *P. mictodonta* and the form B of *P. shikueii*, probably by breaking the long bar into short pieces during ontogeny. We found that the two smallest juveniles of the form B of *P. shikueii* had two elongated bars in area VI (Fig. 9G). These specimens are very similar to those described from China (Wu et al. 1985; Fig. 112A, Yang & Sun 1988; Fig. 11G, Khlebovich 1996; Pl. 47-I, Sun & Yang 2004; Fig. 104A) as *P. camiguinoides* (Augener, 1922) (type locality: Chile), and those described from Korea (Park and Kim 2007; Fig. 5A) and Taiwan (Takahasi 1933; Fig. 2b) as a variation of *P. mictodonta*, in terms of the presence of two elongated bars in area VI and three cones in a transverse line in area V. *Perinereis camiguinoides* was also recorded from Wakanoura in Wakayama Prefecture, central Japan without any description and drawing of its morphology by Uchida (2016). These previous records of *P. camiguinoides* and a variation of *P. mictodonta* may be based on the misidentification of the young of *P. shikueii*, implying that the actual distributional range of *P. shikueii* may be much wider than that shown in the present study.

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