Histology-free Descriptions for Seven Species of Interstitial Ribbon Worms in the Genus *Ototyphlonemertes* (Nemertea: Monostilifera) from Vietnam

Hiroshi Kajihara^{1,4}, Kotaro Tamura², and Shinri Tomioka³

¹ Faculty of Science, Hokkaido University, N10 W8, Sapporo, Hokkaido 060-0810, Japan
E-mail: kajihara@eis.hokudai.ac.jp

² Zukosha, W18 N1, Obihiro, Hokkaido 080-0048, Japan

³ Rishiri Town Museum, Senhoshi, Rishiri Is., Hokkaido 097-0311, Japan

⁴ Corresponding author

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Seven (including six new) species of interstitial hoplonemerteans in the genus *Ototyphlonemertes* Diesing, 1863 are described from southern Vietnam. The descriptions are based on light microscopic examination of internal structures as well as three methods of molecular species delimitation analyses, i) Automatic Barcode Gap Discovery (ABGD), ii) Poisson Tree Process (PTP), and iii) statistical parsimony, on the basis of ~658-bp cytochrome *c* oxidase subunit I gene sequences that have been i) newly determined from the Vietnamese specimens and ii) deposited in the public databases for other *Ototyphlonemertes* species. Three taxa are circumscribed as ‘species groups’ and placed at an infra-subgeneric, supra-specific rank, each represented by *O. duplex* Bürger, 1895, *O. macintoshi* Bürger, 1895, and *O. parmula* Corrêa, 1950. Of the seven Vietnamese species, one is identified as *Ototyphlonemertes* (species group *macintoshi*) *ani* Chernyshev, 2007, which is molecularly confirmed to be distributed from Vietnam (Phu Quoc and Nha Trang) to Japan (Okinawa and Wakayama). The other six are new to science, to which formal taxonomic descriptions are given: these are *O. (duplex) chernyshevi* sp. nov., *O. (duplex) envalli* sp. nov., *O. (duplex) norenburgi* sp. nov., *O. (parmula) lei* sp. nov., *O. (parmula) nakaoae* sp. nov., and *O. (parmula) tsukagoshii* sp. nov.

Key Words: COI, DNA taxonomy, Gulf of Thailand, Hoplonemertea, marine invertebrates, meiobenthos, TCS.

Introduction

Ribbon worms in the genus *Ototyphlonemertes* Diesing, 1863 are exclusively mesopsammic, living in interstices of coarse sand grains in intertidal and shallow subtidal zones almost all over the world except the Antarctica (Kirsteuer 1977; Norenburg 1988a; Leasi *et al.* 2016). They have morphological features that are likely to be the results of adaptation to the interstitial environment; these include slender body (~500 µm), statocysts in the cerebral ganglia, well-developed tactile or sensory cirri both on the head and tail, lack of eyes, and caudal adhesion plate (Norenburg 1988a). Currently recognized 27 named species (Norenburg and Gibson 2012), as well as ~100 unnamed morphospecies, can be categorized into several groups (Envall and Norenburg 2001), but reliable species discrimination cannot be achieved without DNA-sequence information in many cases (Leasi and Norenburg 2014; Leasi *et al.* 2016). However, none of the molecularly-discovered unnamed taxa have been formally described as new species in the *Ototyphlonemertes* systematics so far.

Vietnamese nemerteans have been investigated by Joubin (1905), Dawydoff (1940, 1952), and Chernyshev (2007, 2011, 2016), with about 24 named and 58 undetermined

species reported so far. Of these, *Ototyphlonemertes ani* Chernyshev, 2007 and *O. cf. martynovi* Chernyshev, 1993 are mesopsammic, meiobenthic representatives, while others are mostly macrobenthic, implying a biased sampling efforts in previous studies towards macrobenthos-sized species, with less attention to interstitial ones.

In this paper, we describe seven (including six new) species of *Ototyphlonemertes* from two, small, uninhabited islands in southern Vietnam, based on morphological characters examined *in vivo*, in combination with an approach of so-called DNA taxonomy (*e.g.*, Fontaneto *et al.* 2015). Although some of the previous taxonomic descriptions of *Ototyphlonemertes* species involved histological sectioning (*e.g.*, Chernyshev 2007; Kajihara 2007) like in many other groups of nemerteans (Gibson 1985), we establish new species without histology in this paper (*cf.* Strand and Sundberg 2011; Kajihara 2015).

Materials and Methods

Sampling and morphological observation

Sandy sediment samples were collected at two small beaches, each on the islands of Dam Trong (9°59′58.402″N, 104°02′5.914″E) and Dam Ngoai (9°59′42.533″N,

104°02'16.537"E), on 21 November 2014. The two beaches are about 400 m apart, situated to the south of Phu Quoc, southern Vietnam, facing on the Gulf of Thailand (Fig. 1). Ribbon worms were extracted from the sand following the

Fig. 1. Map showing the two sampling localities, the islands of Dam Trong and Dam Ngoai, situated to the south of the southern tip of Phu Quoc, Vietnam. Solid circles around the main island of Phu Quoc indicate the localities where interstitial nemertean worms were sought but not found.

method of Corrêa (1953): the sand is placed half in a plastic tray (30×25×6 cm), which is then slightly tilted on the beach, with the sand-filled side upward; as sea water is gently sprinkled over the sand using a polyethylene wash bottle, the worms slide down along with the sea water to the non-filled side of the tray, due to their positive geotaxis. The worms were anaesthetized in MgCl₂ solution prepared with tap water so that it had the same refractive index (or 'salinity') as that of the sea water using an IS/Mill-E refractometer (AS ONE, Japan). Of the 31 specimens collected (10 from Dam Trong, 21 from Dam Ngoai), 14 were photographed alive with a RICOH CX 5 digital camera, then mounted on a glass slide under a cover slip (Kirsteuer 1967), observed with an Olympus CH-2 compound microscope (Olympus, Japan); photomicrographs of squeezed, live specimens were taken with a Nikon D5200 digital camera attached to the CH-2 microscope by Micronet NYPIXS2-3166, HY 1S-FA, and NY 1S-1501750 adapters. The rest of the 17 specimens were also photographed alive with a Nikon D5200 digital camera attached to a Nikon SMZ 1500 stereomicroscope by the same adapters, with external strobe lighting provided by a pair of Morris Hikaru Komachi Di flash units; the specimens were likewise squeezed then examined under an Olympus BX51 compound microscope, and also photographed with the same Nikon D5200 digital camera. After examination, all the worms were preserved in 99% EtOH. Type and voucher specimens in the form of extracted DNA have been deposited in the Invertebrate Collection of the

Table 1. Museum catalogue number (ICHUM), type status, locality, and DDBJ/EMBL/GenBank accession number for COI sequences determined from 25 specimens in seven species of *Otocyphlonemertes* from Vietnam.

ICHUM	Type status	Species	Locality	Accession number
5348	holotype	<i>Otocyphlonemertes (duplex) chernyshevi</i> sp. nov.	Dam Trong	LC310990
5349	paratype	<i>Otocyphlonemertes (duplex) chernyshevi</i> sp. nov.	Dam Trong	LC310991
5350	paratype	<i>Otocyphlonemertes (duplex) chernyshevi</i> sp. nov.	Dam Trong	LC310992
5351	holotype	<i>Otocyphlonemertes (duplex) envalli</i> sp. nov.	Dam Trong	LC310993
5352	paratype	<i>Otocyphlonemertes (duplex) envalli</i> sp. nov.	Dam Trong	LC310994
5353	paratype	<i>Otocyphlonemertes (duplex) envalli</i> sp. nov.	Dam Trong	LC310995
5354	paratype	<i>Otocyphlonemertes (duplex) envalli</i> sp. nov.	Dam Trong	LC310996
5355	holotype	<i>Otocyphlonemertes (duplex) norenburgi</i> sp. nov.	Dam Ngoai	LC310997
5356	paratype	<i>Otocyphlonemertes (duplex) norenburgi</i> sp. nov.	Dam Ngoai	LC310998
5357	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC310999
5358	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311000
5359	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311001
5360	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311002
5361	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311003
5362	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311004
5363	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311005
5364	non-type	<i>Otocyphlonemertes (macintoshi) ani</i> Chernyshev, 2007	Dam Ngoai	LC311006
5365	holotype	<i>Otocyphlonemertes (parmula) lei</i> sp. nov.	Dam Ngoai	LC311007
5366	holotype	<i>Otocyphlonemertes (parmula) nakaoae</i> sp. nov.	Dam Trong	LC311008
5367	paratype	<i>Otocyphlonemertes (parmula) nakaoae</i> sp. nov.	Dam Trong	LC311009
5368	paratype	<i>Otocyphlonemertes (parmula) nakaoae</i> sp. nov.	Dam Ngoai	LC311010
5369	paratype	<i>Otocyphlonemertes (parmula) nakaoae</i> sp. nov.	Dam Ngoai	LC311011
5370	paratype	<i>Otocyphlonemertes (parmula) nakaoae</i> sp. nov.	Dam Ngoai	LC311012
5371	holotype	<i>Otocyphlonemertes (parmula) tsukagoshii</i> sp. nov.	Dam Ngoai	LC311013
5372	paratype	<i>Otocyphlonemertes (parmula) tsukagoshii</i> sp. nov.	Dam Ngoai	LC311014

Hokkaido University Museum (ICHUM), Sapporo, Japan.

No interstitial nemerteans were found at eight sites around the main island of Phu Quoc (Fig. 1) on 20 November 2014. These sites were: a beach along the western coast of the island in Duong Bao (10°08'33.1"N, 103°58'30.1"E), a small bay on the eastern coast of the island in Duong Bao north to the border between An Thoi (10°04'36.0"N, 104°01'18.1"E), two places on the eastern coast of the island near Bai Vong (10°08'44.9"N, 104°02'05.3"E; 10°07'59.8"N, 104°01'50.0"E), a small beach on the northeastern side of the island near Bai Thom (10°24'51.7"N, 104°02'31.7"E), two places on the northeastern side of the island near Hom Mot (10°24'08.8"N, 104°03'26.4"E; 10°24'11.5"N, 104°03'28.2"E), and a beach on the northwestern edge of the island near Bai bien Ganh Dau (10°22'23.3"N, 103°50'18.1"E).

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from the whole body using the silica method (Boom *et al.* 1990). PCR amplification was carried out for partial sequence of the cytochrome *c* oxidase subunit I (COI) gene by using LCO1490/HCO2190 primer pair (Folmer *et al.* 1994) with an Applied Biosystems 2720 Thermal Cycler. The reaction was done in a 10- μ l volume, containing 1–6 ng of extracted total DNA, 2 nmol of each dNTP, 5 pmol of each primer, and 0.25 U of *Ex Taq* (TaKaRa, Japan) in 1 $\times$ *Ex Taq* Buffer (TaKaRa, Japan). Thermal cycling was initiated with 1 min of denaturation at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 50°C, and 1 min at 72°C. After cycling, the reaction was completed with a final extension at 72°C for 7 min. The reaction products were visualized in a 1% agarose gel, then purified with about 5.7 mU/ μ l of shrimp alkaline phosphatase (1 U/ μ l; TaKaRa, Japan) and 28.3 mU/ μ l of *E. coli* exonuclease I (5 U/ μ l; TaKaRa, Japan) in an 18- μ l volume for 30 min at 37°C, followed by deactivation of the enzymes for 15 min at 80°C. The purified PCR products were sequenced directly with the same primer pairs used for amplification, using a BigDye Terminator ver. 3.1 Sequencing Kit (Applied Biosystems, USA) and an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, USA), following the manufacturer's protocol. For eight of the 33 specimens, either PCR amplification or DNA sequencing were unsuccessful. A total of 25 sequences generated *de novo* have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers of LC310990–LC311014 (Table 1).

Species delimitation analyses

Three different methods were used for species delimitation analyses: Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2012), Poisson Tree Processes (PTP) (Zhang *et al.* 2013), and statistical parsimony (Templeton *et al.* 1992). The analyses were based on the 25 newly determined COI sequences together with 462 others available in GenBank: HQ848613, HQ848605 (Andrade *et al.* 2012); KF935545 (Kvist *et al.* 2014); KM083821–083889, KM094175 (Leasi and Norenburg 2014); and KT36303–36314, KT722707–722370, KT730596–730678, KU230031–

230291, KU245363–245364, KU840015, KU840020–840025 (Leasi *et al.* 2016) (see Appendix). Alignment was manually inspected using MEGA ver. 6.06 (Tamura *et al.* 2013) so that the sequences could be translated to amino acids. The ABGD analysis was carried out with the aligned sequence data set and performed in a web-based interface (<http://www.wabi.snv.jussieu.fr/public/abdg/abdgweb.html>) with the following settings: Pmin, 0.001; Pmax, 0.1; Steps, 10; X (relative gap width), 1.5; Nb bins (for distance distribution), 20; distance, Kimura (K80) TS/TV 2.0. The PTP analysis was performed at the bPTP web server (<http://species.h-its.org/ptp/>) with the default settings using an input tree generated by RAXML ver. 8 (Stamatakis 2014) based on maximum-likelihood method with GTR+G+I model. The statistical parsimony analysis was done with TCS ver. 1.21 (Clement *et al.* 2000). The following sequences were excluded from the analysis due to the shortness compared with the others: KM083830 (291 bp), KM083826 (452 bp), KM083825 (543 bp), KU230098 (432 bp), KM083874 (432 bp), KM083865 (241 bp), and KM083863 (504 bp). The TCS analysis was performed with 480 sequences (478 bp) [KM083825 and KM083863 are longer than this, but they have long uncertain positions at either end in relation to the aligned sites].

Infra-generic taxonomy and nomenclature

The taxonomy and nomenclature at the infra-generic, supra-specific ranks in *Ototyphlonemertes* have been in flux. Chernyshev (1993) proposed to segregate *Ototyphlonemertes* species with polygranular statoliths and transfer to a new genus *Norenburgia* Chernyshev, 1993 (type species: *Ototyphlonemertes lactea* Corrêa, 1954). Chernyshev (1993) also established *Accirinia* as a subgenus of *Norenburgia*, but it has not received general acceptance among researchers (e.g., Gibson 1995). Envall (1996) proposed *Otohelicophora* as a subgenus for members with spiral stylets, but this name is unavailable due to the lack of type fixation in the original publication (Chernyshev 1998; Kajihara *et al.* 2008). Chernyshev (1998) recognized two subgenera *Ototyphlonemertes* and *Norenburgia*, the former consisting of species with oligo-granular statoliths and smooth central stylet, the latter with polygranular statoliths and smooth or sculptured central stylet. Subsequently, based on a combination of five characters (statolith, stylet, basis, diaphragm, and middle chamber), Envall and Norenburg (2001) put together all the ~22 named and >70 unidentified species known at the time into six groups that are represented by the first established species within each group, i.e., *Ototyphlonemertes cirrula* Mock and Schmidt, 1975, *O. duplex* Bürger, 1895, *O. fila* Corrêa, 1953 (but see below), *O. lactea* Corrêa, 1954, *O. macintoshi* Bürger, 1895, and *O. pallida* (Keferstein, 1862). These groups were variably referred to as phylogroup or morphotype, with the name of the groups indicated in non-italic face beginning with an upper-case initial letter, such as “Duplex-type” (Envall and Norenburg 2001). In a molecular phylogeographic analysis of *Ototyphlonemertes* based on four gene markers (COI, 16S rRNA, 18S rRNA, 28S rRNA), Leasi *et al.* (2016) basically followed Envall and Noren-

Fig. 2. Part of the phylogenetic tree resulting from the maximum-likelihood analysis of COI (ln L = -15264.650301), along with results of three species-delimitation analyses, ABGD, PTP, and TCS, as indicated by vertical bars. Numbers near nodes indicate maximum-likelihood bootstrap support value.

burg's (2001) nomenclature, introducing new morphological groups *Erneba* and *Santacruzensis*, although these groups were not explicitly circumscribed with morphological characteristics.

To refer to these groups of species, we invoke Article 6.2 of the Code (International Commission on Zoological Nomenclature [ICZN] 1999), which stipulates "A specific name may be added in parentheses after the genus-group name..., to denote an aggregate of species within a genus-group taxon; ...such names...must always begin with a lower-case letter and be written in full" (ICZN 1999). These infra-subgeneric, supra-specific ranks are commonly referred to as 'superspecies', 'species aggregate', or 'species group'; we adopt the last one in this paper. Recommendation 6B of the Code (ICZN 1999) states that this term (*i.e.*, 'species group' in this case) should be placed to indicate the taxonomic meaning in the same parentheses as its interpolated species-group name on the first occasion (see below).

***Ototyphlonemertes duplex* species group.** The Duplex-morph of Envall and Norenburg (2001) was corroborated by Leasi *et al.*'s (2016) molecular phylogeny as comprising a clade. This group can be diagnosed as having two-granular statoliths, smooth central stylet, thick central-stylet basis, short proboscis diaphragm, and bulbous proboscis middle chamber. It includes the recognized species *O. (duplex) antipai* Müller, 1968; *O. (duplex) aurantiaca* (Du Plessis, 1891); *O. (duplex) brunnea* Bürger, 1895; *O. (duplex) correae* Envall, 1996; *O. (duplex) duplex* Bürger, 1895; *O. (duplex) evelinae* Corrêa, 1948; and *O. (duplex) martynovi* Chernyshev, 1993.

***Ototyphlonemertes macintoshi* species group.** Leasi *et al.*'s (2016) molecular phylogeny showed that the Macintoshi and Lactea morpho-groups of Envall and Norenburg (2001) together comprised a clade, but were not reciprocally monophyletic. In this paper, we regard these groups as synonymous and refer to it as the *Ototyphlonemertes macintoshi* species group. It is because Article 6.2 of the Code (ICZN 1999) provides that the Principle of Priority applies to the names of such aggregate of species; *O. macintoshi* was established by Bürger (1895), while *O. lactea* was by Corrêa (1954). The *O. macintoshi* species group can be diagnosed as having polygranular statoliths, spirally-sculptured central stylet, long proboscis diaphragm, and short or long proboscis middle chamber. It includes the recognized species *O. (macintoshi) americana* Gerner, 1969; *O. (macintoshi) ani* Chernyshev, 2007; *O. (macintoshi) dolichobasis* Kajihara, 2007; *O. (macintoshi) lactea* Corrêa, 1953; *O. (macintoshi) macintoshi* Bürger, 1895; *O. (macintoshi) nikolaii* Chernyshev, 1998; *O. (macintoshi) pellucida* Coe, 1943; *O. (macintoshi) spiralis* Coe, 1940; and *O. (macintoshi) valentinae* Chernyshev, 2003.

***Ototyphlonemertes parmula* species group.** Envall and Norenburg (2001) established the Fila-morph to be comprised of *O. fila* and *O. parmula*. The two species, however, turned out to be synonymous by a couple of population genetic studies (Andrade *et al.* 2012; Tulchinsky *et al.* 2012). Again, as Article 6.2 of the Code (ICZN 1999) provides that the Principle of Priority applies to such infra-subgeneric, su-

Table 2. Range and average (in parentheses) of Kimura 2-parameter distances among and between 'species' that were estimated by three different delimitation analysis methods (ABGD, PTP, TCS), based on a total of 487 sequences of COI gene (~658 bp), of which 462 were available in GenBank and 25 were generated *de novo* in this study.

	Intra-specific K2P distance	Inter-specific K2P distance
ABGD	0.000–0.036 (0.007)	0.063–0.292 (0.164)
PTP	0.000–0.024 (0.004)	0.015–0.278 (0.160)
TCS	0.000–0.014 (0.004)	0.011–0.258 (0.162)

pra-specific names, we refer to this group as *O. parmula* because *O. parmula* Corrêa, 1950 has precedence over *O. fila* Corrêa, 1953. The group can be diagnosed as having polygranular statoliths, spirally-sculptured central stylet, short proboscis diaphragm, and bulbous proboscis middle chamber. It appears that in many species, the proboscis posterior chamber has a specialized anterior region, although it is not explicitly mentioned in the original descriptions of *O. fila* and *O. parmula*.

Further scrutiny is necessary to elucidate taxonomic identities for the Cirrula- and Pallida-morphs of Envall and Norenburg (2001), as well as a number of biological entities (presumably 'species') in what were identified as *Erneba* and *Santacruzensis* of Leasi *et al.* (2016), which, however, is beyond the scope of this paper. Species-group assignments of *O. brevis* Corrêa, 1948, *O. cirrula* Mock and Schmidt, 1975, *O. erneba* Corrêa, 1950, *O. pallida* (Keferstein, 1862), and *O. santacruzensis* Mock and Schmidt, 1975 remain uncertain for the time being.

Results and Discussion

Species delimitation

The ABGD analysis resulted in 46 groups, PTP in 65 entities, and TCS in 61 networks (Figs 2, 3). The average K2P distances among and between these 'species' (*i.e.*, groups, entities, networks) were 0.004–0.007 and 0.160–0.164, respectively (Table 2). So-called barcoding gaps were not found in the PTP and TCS analyses, as the maximum intra-specific values exceeded the minimum inter-specific ones (Table 2).

The 25 individuals from Vietnam, of which COI sequences were available, were classified as seven groups/entities/networks in all the three analyses. They are treated and described as distinct biological species in the following section.

Taxonomy

Ototyphlonemertes (duplex) chernyshevi sp. nov. (Fig. 4)

?*Ototyphlonemertes* (*O.*) cf. *martynovi*: Chernyshev 2007: 199, fig. 1f, g.

Material examined. Three specimens, all from Dam

Fig. 4. *Ototyphlonemertes (duplex) chernyshevi* sp. nov. A, Entire body; B, composite photomicrograph of squeezed specimen; C–E, statoliths; F, stylet apparatus, G, H, central stylets and bases; I, posterior end of body. A, C, F, I, ICHUM 5348 (holotype); B, G, ICHUM 5349 (paratype); E, H, ICHUM 5350 (paratype).

Trong; ICHUM 5348 (holotype); ICHUM 5349, 5350 (two paratypes).

Description. Body length 8–12 mm (8 mm in holotype), width 0.2–0.3 mm (0.2 mm in holotype) ($n=3$) (Fig. 4A, B). Epidermis whitish (Fig. 4A); cephalic furrow post-cerebrally; tissues around brain reddish; cirri present on cephalic region and hind end of body. Statoliths two-partite (Fig. 4C–E), 10.2–11.7 µm in diameter (10.2 µm in holotype) ($n=3$); statocyst 24.8–29.5 µm in diameter (24.8 µm in holotype) ($n=3$). Proboscis diaphragm short (Fig. 4F); gland secretion ring situated anterior to hind end of basis; accessory stylet pouches two (Fig. 4F), each containing two ac-

cessory stylets (directing either anteriorly or posteriorly), situated laterally to central-stylet basis; central stylet smooth (Fig. 4F–H), 28.2–33.6 µm long (33.6 µm in holotype); basis 26.2–30.8 µm long (30.8 µm in holotype), 9.4–11.8 µm wide (11.8 µm in holotype) ($n=3$); basis length/width ratio 2.28–2.80 (2.61 in holotype) ($n=3$); posterior chamber opaque, without specialized anterior portion. Intestinal diverticula shallow and sack-like. Posterior adhesive plate with cells containing orange contents (Fig. 4I).

Distribution. So far known only from the type locality, the island of Dam Trong, Vietnam.

Etymology. The specific name is a noun in the genitive

Fig. 5. *Ototyphlonemertes (duplex) envalli* sp. nov. A, Entire body; B, C, composite photomicrographs of squeezed specimens; D, E, statoliths; F, stylet apparatus; G, H, central stylets and bases; I, posterior end of body. A, B, D, G, ICHUM 5351 (holotype); C, E, F, ICHUM 5353 (paratype); H, I, ICHUM 5352 (paratype).

case, after the distinguished nemertean biologist Professor Dr Alexei Viktorovich Chernyshev at A. V. Zhirmunsky Institute of Marine Biology, Far East Branch of Russian Academy of Science, and concurrently at Far Eastern Federal University, in recognition of his significant contribution to the nemertean systematics in general and, in particular, that of *Ototyphlonemertes*, as well as to clarification of Vietnamese

nemertean fauna.

Remarks. *Ototyphlonemertes (duplex) chernyshevi* sp. nov. is similar to a specimen from Van Phong Bay, Vietnam (12°39'N, 109°20'E), which was uncertainly identified as *O. (duplex) martynovi* by Chernyshev (2007), in that their stylet bases are thick and stumpy, by which they can be distinguished from *O. (duplex) envalli* sp. nov. and *O.*

Fig. 6. *Ototyphlonemertes (duplex) norenburgi* sp. nov. A, B, Entire body; C, composite photomicrograph of squeezed specimen; D, statoliths; E, stylet apparatus; F, central stylet and basis; G, posterior end of body. A, C, D, E, ICHUM 5355 (holotype); B, F, G, ICHUM 5356 (paratype).

(*duplex*) *norenburgi* sp. nov.; the basis is thinner in the latter two in comparison with that of *O. (duplex) chernyshevi*. Our unpublished sequence data indicate that *O. (duplex) chernyshevi* is genetically different from *O. (duplex) martynovi* s.str. (14.89–16.29% in uncorrected *p*-distance; 0.1869–0.2109 in K2P).

Ototyphlonemertes (duplex) envalli sp. nov.
(Fig. 5)

Material examined. Four specimens, all from Dam Trong; ICHUM 5351 (holotype); ICHUM 5352–5354 (three paratypes).

Description. Body length 5.0–6.2 mm (6.2 mm in holotype), width 0.2 mm ($n=3$). Epidermis whitish (Fig. 5A); cephalic furrow post-cerebrally; tissues around brain red-

dish (Fig. 5B, C); cirri present in both anterior and posterior ends of body. Foregut-intestine junction distinct; foregut region translucent; intestine tinged with pale-yellow to whitish color (Fig. 5A). Statoliths bipartite (Fig. 5D, E), 8.5–9.2 µm in diameter (8.5 µm in holotype) ($n=3$); statocyst 23.8–25.6 µm in diameter (23.8 µm in holotype) ($n=3$). Proboscis diaphragm short (Fig. 5F); two accessory stylet pouches, lateral to central-stylet basis, each containing two accessory stylets ($n=3$); in two of three specimens examined, one of two accessory-stylet pouches contained one accessory stylet that directed backward; other accessory stylets directed forward; middle chamber bulbous (Fig. 5F); central stylet smooth (Fig. 5G, H), 23.9–31.6 µm long (23.9 µm in holotype); basis 29.6–31.6 µm long (29.6 µm in holotype), 7.4–8.1 µm wide (7.4 µm in holotype) ($n=3$); basis length/width ratio 3.90–4.00 (4.00 in holotype) ($n=3$); posterior

chamber opaque, without specialized anterior portion (Fig. 5B, C, F). Intestinal diverticula shallow. Mature oocytes up to 136 µm in diameter, accounting for ~35% of body width (Fig. 5B, C). Posterior adhesive plate well developed (Fig. 5I).

Distribution. So far known only from the type locality, the island of Dam Trong, Vietnam.

Etymology. The specific name is a noun in the genitive case, after Dr Mats Envall for his distinguished contribution to the *Ototyphlonemertes* systematics.

Remarks. *Ototyphlonemertes (duplex) envalli* sp. nov. was sister to *O. (duplex) norenburgi* sp. nov. in the phylogenetic analysis (Fig. 2) and they look similar to each other. As far as the specimens observed, *O. (duplex) envalli* may be distinguished from *O. (duplex) norenburgi* in that the ratio of the oocyte diameter to the body width is smaller than in *O. (duplex) envalli* (oocyte / body-width ratio ~0.35) than in *O. (duplex) norenburgi* (oocyte/body-width ratio >0.4). Whether or not this character is reliable in discriminating these two species should be investigated in future studies, because the degree of oocyte matureness might have been different among the specimens examined. Also, the squeezed method might have skewed the actual diameters of the body and oocytes.

Ototyphlonemertes (duplex) norenburgi sp. nov.
(Fig. 6)

Material examined. Two specimens, both from Dam Ngoai; ICHUM 5355 (holotype); ICHUM 5356 (paratype).

Description. Body length 7.8 mm (paratype) to 9.0 mm (holotype), width 0.2 mm (paratype) to 0.3 mm (holotype) ($n=2$). Epidermis whitish (Fig. 6A, B); cephalic furrow post-cerebrally; tissues around brain reddish (Fig. 6C); cirri present in both anterior and posterior ends of body. Foregut-intestine junction not distinct (intestinal caecum possibly present). Statoliths bipartite (Fig. 6D), 8.4 µm (paratype) to 9.6 µm (holotype) in diameter ($n=2$); statocyst 27.9 µm (holotype) to 30.5 µm (paratype) in diameter ($n=2$). Proboscis diaphragm short (Fig. 6E); two accessory stylet pouches, lateral to central-stylet basis, each containing 2–3 accessory stylets ($n=2$); in holotype, one of two accessory-stylet pouch contained three accessory stylets all directing forward; another accessory-stylet pouch contained two accessory stylets each directing forward and backward; paratype had two accessory stylets per pouch both directing forward; middle chamber bulbous; central stylet smooth (Fig. 6E, F), 28.3 µm (holotype) to 33.0 µm (paratype) long; basis 26.4 µm (paratype) to 29.1 µm (holotype) long, 7.5 µm (holotype) to 9.3 µm (paratype) wide ($n=2$); basis length width ratio 2.84 (paratype) to 3.88 (holotype) ($n=2$); posterior chamber opaque, without specialized anterior portion. Intestinal diverticula shallow. Posterior adhesive plate containing cells with orange contents. Mature oocytes up to 70 µm in diameter, accounting for more than 40% of body width (Fig. 6C).

Distribution. Our unpublished sequence data indicate that *O. (duplex) norenburgi* sp. nov. is also distributed in

Onnason, Okinawa-jima island, although it was recovered only from Dam Ngoai in the present study.

Etymology. The specific name is a noun in the genitive case, after the eminent nemertean biologist Dr Jon Laurence Norenburg at the Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, USA, in respect of his distinguished achievement and contribution to the ribbon-worm systematics, including that of *Ototyphlonemertes*, as well as other interstitial animals.

Remarks. In the resulting phylogenetic tree, *O. (duplex) norenburgi* was sister to *O. (duplex) envalli* (Fig. 2). Morphologically, *O. (duplex) norenburgi* may be distinguished from *O. (duplex) envalli* by the larger oocyte size relative to the body width: the oocyte/body-width ratio exceeds 0.4 in *O. (duplex) norenburgi*, while it is around 0.35 in *O. (duplex) envalli* [but see Remarks for *O. (duplex) envalli* above]. From *O. (duplex) chernyshevi*, *O. (duplex) norenburgi* may be distinguished by its thinner stylet basis: the basis length/width ratio was 2.84–3.88 in *O. (duplex) norenburgi*, while it was 2.28–2.80 in *O. (duplex) chernyshevi*.

Ototyphlonemertes (macintoshi) ani
Chernyshev, 2007
(Fig. 7)

Ototyphlonemertes (Norenburgia) ani Chernyshev, 2007: 196–199, figs 1, 2.

Ototyphlonemertes lactea (not of Corrêa 1954): Leasi *et al.* 2016: 1393, fig. 4a, network L3 (139, 160, 180).

Material examined. Eight specimens, all from Dam Ngoai; ICHUM 5357–5364.

Description. Body length 4.3–11.0 mm, width 0.3–0.4 mm ($n=8$). Epidermis whitish (Fig. 7A), containing red pigment cells sporadically scattered among other epidermal cells; cephalic furrow post-cerebrally; tissues around brain reddish; cirri not found on cephalic region ($n=5$), 5–7 cirri found near hind end of body ($n=3$). Foregut-intestine junction distinct (Fig. 7A, B). Statoliths polygranular (Fig. 7C–J), 10.9–14.7 µm in diameter ($n=8$), consisting of about 22 granules; statocyst 20.9–30.4 µm in diameter ($n=8$); additional statolith found in one specimen on single side (Fig. 7C). Proboscis anterior chamber with tactile papillae (Fig. 7L); diaphragm long (Fig. 7K, L); two accessory-stylet pouches, lateral to central-stylet basis; 2–6 accessory stylets per pouch ($n=8$ individuals $\times$ 2 pouches), with mode being 5 (three individuals having 5 accessory stylets in each pouch; one individual with 4 and 5 accessory stylets, another with 5 and 6 accessory stylets); of 69 accessory stylets in 8 specimens, 2 in one specimen directed forward, other 67 directing backward; central stylet (as well as accessory stylets) spiral (Fig. 7M), 28.5–46.2 µm long; basis 27.7–33.3 µm long, 4.1–6.5 µm wide ($n=8$), with each ridge twisting almost only once; basis length/width ratio 4.58–6.5; middle chamber antero-posteriorly elongated rectangle (Fig. 7K), with prominent diagonal muscle fibers (Fig. 7L); posterior chamber vesicular, without specialized anterior portion. Intestinal diverticula shallow and sack-like. Posterior adhesive

Fig. 7. *Ototyphlonemertes (macintoshi) ani* Chernyshev, 2007. A, Entire body; B, composite photomicrograph of squeezed specimen; C–J, statoliths (arrowhead on C indicates additional statolith); K, proboscis diaphragm and middle chamber; L, everted proboscis (arrowheads indicating rhabdoid-like papillae); M, central stylet and basis. A, E, M, ICHUM 5359; B, D, ICHUM 5358; C, K, ICHUM 5357; F, L, ICHUM 5360; G, ICHUM 5361; H, ICHUM 5362; I, ICHUM 5363; J, ICHUM 5364.

Fig. 8. *Ototyphlonemertes (parmula) lei* sp. nov., ICHUM 5365 (holotype). A, Entire body; B, composite photomicrograph of squeezed specimen; C, statolith; D, central stylet and basis.

plate with pale brown pigments.

Distribution. Tche Bay (12°35'N, 109°20'E), Hong-Long Island, Van Phong Bay, Vietnam (Chernyshev 2007); Dam Ngoai, Vietnam (present study); Gesashi, Okinawa, Japan (Leasi *et al.* 2016); Shirahama, Japan (Leasi *et al.* 2016).

Remarks. In the molecular phylogenetic tree, *O. (macintoshi) ani* was nested within a clade comprised of other OTUs representing the *O. macintoshi* species group (Fig. 3). The GenBank COI sequences KU230129 and KU230131 (from Shirahama, Japan: Leasi *et al.* 2016), as well as

KU230130 (from Gesashi, Okinawa, Japan: Leasi *et al.* 2016), all identified as *Ototyphlonemertes lactea*, turned out to represent *O. (macintoshi) ani*. It was the most abundant species in this study.

***Ototyphlonemertes (parmula) lei* sp. nov.**
(Fig. 8)

Material examined. One specimen from Dam Ngoai; ICHUM 5365 (holotype).

Description. Body length 4.9 mm, width 0.2 mm ($n=1$). Epidermis whitish (Fig. 8A); cephalic furrow post-cerebrally; tissues around brain slightly reddish; cirri present in both anterior and posterior ends of body. Foregut–intestine junction not distinct (Fig. 8B). Statolith polygranular (Fig. 8C), 9.3 μm in diameter ($n=1$), consisting of about 7 granules; statocyst 15.9 μm in diameter ($n=1$). Proboscis diaphragm short (Fig. 8B); two accessory stylet pouches, lateral to central-stylet basis, each containing 4 and 5 accessory stylets, all directing forward; central stylet (as well as accessory stylets) spiral (Fig. 8D), 54.7 μm long; basis 43.2 μm long, 8.0 μm wide (Fig. 8D), with each ridge twisting about 1.5 times; basis length/width ratio 5.4; middle chamber short; posterior chamber vesicular, without specialized anterior portion (Fig. 8B). Intestinal diverticula deep. Posterior adhesive plate with cells containing dark contents. Gonads found in posterior half of intestinal region, alternating more or less regularly with intestinal lateral diverticula.

Distribution. So far known only from the type locality, the island of Dam Ngoai, Vietnam.

Etymology. The specific name is a noun in the genitive case, after Dr Doan Dung Le at the Ho Chi Minh City University of Food Industry, Vietnam, who kindly coordinated HK's sampling trip to Phu Quoc.

Remarks. In the phylogenetic tree, *Ototyphlonemertes (parmula) lei* sp. nov. appeared to be sister to the rest of the *O. parmula* species group (Fig. 2), which was mostly represented by the Fila morpho-group of Leasi *et al.* (2016).

***Ototyphlonemertes (parmula) nakaoae* sp. nov.**
(Figs 9, 10)

Material examined. Five specimens: ICHUM 5366 (holotype) (from Dam Trong); four paratypes, ICHUM 5367 (from Dam Trong), ICHUM 5368–5370 (from Dam Ngoai).

Description. Body length 4.1–6.2 mm (6.2 mm in holotype), width 0.2–0.3 mm (0.2 mm in holotype) ($n=4$). Epidermis whitish (Fig. 9A, D, E); cephalic furrow post-cerebrally; tissues around brain slightly reddish (Figs 9B, E, F, 10A, B); cirri present in both anterior and posterior ends of body; cephalic cirrus formula (*cf.* Norenburg 1988b) observed in two specimens: A=2+2, B=0, C=9, D=6, E=4, tail=14 in ICHUM 5368 (paratype); A=0, B=2, C=7+5, D=4+5, tail=8 in ICHUM 5369 (paratype). Foregut–intestine junction not markedly distinct. Statolith polygranular (Fig. 10C–F), 8–11 μm (9 μm in holotype) in diameter ($n=4$), consisting of 7–8 (7 in holotype) granules; statocyst 19.2–26.1 μm (19.2 μm in holotype) in diameter ($n=4$). Pro-

Fig. 9. *Ototyphlonemertes (paulina) nakaoae* sp. nov. A, D, Entire body; B, E, composite photomicrographs of squeezed specimens; C, posterior end of body; E, enlargement of head, anaesthetized specimen. A–C, ICHUM 5366 (holotype); D–F, ICHUM 5368 (paratype).

boscis diaphragm short (Figs 9F, 10A, B, G); gland-secretion ring situated posterior to hind end of basis; accessory-stylet pouches two, each containing 2–5 accessory stylets (3 in

each in holotype) per pouch ($n=4$ individuals $\times 2$ pouches), directing both forward and backward; central stylet (as well as accessory stylets) spiral (Fig. 10H–K), 32–46 µm (32 µm

Fig. 10. *Ototyphlonemertes (parmula) nakaoae* sp. nov. A, B, Composite photomicrographs of squeezed specimens; C–F, statoliths; G, stylet apparatus; H–K, central stylets and bases. A, E, J, ICHUM 5367 (paratype); B, F, K, ICHUM 5369 (paratype); C, G, H, ICHUM 5366 (holotype); D, I, ICHUM 5368 (paratype).

Fig. 11. *Ototyphlonemertes (parmula) tsukagoshii* sp. nov. A, Entire body; B, C, composite photomicrographs of squeezed specimens; D, E, statoliths; F, partially everted proboscis; G, H, central stylets and bases. A, B, D, E, F, G, ICHUM 5371 (holotype); C, E, H, ICHUM 5372 (paratype).

in holotype) long; basis 39–52 µm (42 µm in holotype) long, 8–12 µm (8 µm in holotype) wide ($n=4$), with each ridge twisting 1.5–2 (1.5 in holotype) times; basis length/width ratio 4.27–5.16 (5.16 in holotype); middle chamber bulbous; posterior chamber opaque, with specialized anterior part (Figs 9B, F, 10A, B). Intestinal diverticula deep. Posterior adhesive plate containing cells with orange contents (Fig. 9C). Gonads found in posterior 1/2 to 3/4 of intestinal region, alternating more or less regularly with intestinal lateral diverticula.

Distribution. So far recorded from the islands of Dam

Trong (type locality) and Dam Ngoai, Vietnam.

Etymology. The specific name is a noun in the genitive case, after the Japanese ostracode biologist Dr Yuriko Nakao at Nihon University, Japan, who devotedly arranged our sampling trip to Phu Quoc.

Remarks. In the phylogenetic tree, *Ototyphlonemertes (parmula) nakaoae* sp. nov. was sister to *O. (parmula) tsukagoshii* sp. nov. (Fig. 2). Out of the seven species discovered in this study, *O. (parmula) nakaoae* turned out to be the only species that occurred at both the localities surveyed, the islands of Dam Trong and Dam Ngoai. Following *O.*

(*macintoshi*) *ani*, it was the second most abundant species recovered in the present survey.

***Ototyphlonemertes (parmula) tsukagoshii* sp. nov.**

(Fig. 11)

Material examined. Two specimens, both from Dam Ngoai; ICHUM 5371 (holotype); ICHUM 5372 (paratype).

Description. Body length 2.5 mm (paratype) to 4.0 mm (holotype), width 0.1 mm (paratype) to 0.2 mm (holotype) ($n=2$). Epidermis whitish (Fig. 11A); cephalic furrow post-cerebrally; tissues around brain (and even in precerebral region in one of two specimens examined) reddish (Fig. 11B, C); cirri present in both anterior and posterior ends of body; cephalic cirrus formula (*cf.* Norenburg 1988b) observed in two specimens: A=2, B=0, C=8, D=?, E=?, tail=12 in holotype; A=2, B=0, C=5+6, D=6+10, E=3, tail=10 in paratype. Foregut–intestine junction indistinct. Statolith polygranular (Fig. 11D, E), 9.5 μ m in diameter ($n=2$), consisting of 6 granules; statocyst 23.5 μ m (holotype) to 28.4 μ m (paratype) in diameter ($n=2$). Proboscis anterior chamber with wart-like papillae (Fig. 11F); diaphragm short (Fig. 11F); gland secretion ring situated posterior to hind end of basis; accessory-stylet pouches two, each containing 2–3 accessory stylets directing both forward and backward; central stylet (as well as accessory stylets) spiral (Fig. 11G, H), 35.0 μ m (paratype) to 40.2 μ m (holotype) long; basis 37.1 μ m (paratype) to 40.2 μ m (holotype) long, 8.3 μ m (paratype) to 11.2 μ m (holotype) wide ($n=2$), with each ridge twisting 3 times; basis length/width ratio 3.59 (holotype) to 4.47 (paratype); middle chamber bulbous (Fig. 11F); posterior chamber opaque, with specialized anterior part (Fig. 11B, C). Intestinal diverticula deep. Posterior adhesive plate containing cells with orange contents.

Distribution. So far only recorded from the type locality, Dam Ngoai, Vietnam.

Etymology. The specific name is a noun in the genitive case, after the Japanese ostracode systematist Professor Akira Tsukagoshi at Shizuoka University, Japan, who organized our sampling trip to Phu Quoc.

Remarks. In the phylogenetic tree, *Ototyphlonemertes (parmula) tsukagoshii* sp. nov. was sister to *O. (parmula) nakaoae* sp. nov. (Fig. 2). *Ototyphlonemertes (parmula) tsukagoshii* differs from *O. (parmula) nakaoae* by the smaller body length (2.5–4.0 mm vs. 4.1–6.2 mm), fewer number of statolith granules (6 vs. 7–8), and greater number of stylet-ridge twists (3 vs. 1.5–2). Also, *O. (parmula) tsukagoshii* tends to be more reddish in the external appearance, especially in the brain region, than *O. (parmula) nakaoae*.

Conclusion

Based on light microscopical observation of squeezed material and COI sequence data, we report seven, including six new, species of mesopsammic monostiliferans in the genus *Ototyphlonemertes* from two beaches, about 400 m apart, each in the island of Dam Trong and Dam Ngoai,

situated to the south of Phu Quoc, southern Vietnam. None of eight localities investigated for meiobenthos around the main island of Phu Quoc yielded *Ototyphlonemertes* sample. Discovered from the material include three species from Dam Trong—*O. (duplex) chernyshevi*, *O. (duplex) envalli*, and *O. (parmula) nakaoae*—and five species from Dam Ngoai—*O. (duplex) norenburgi*, *O. (macintoshi) ani*, *O. (parmula) lei*, *O. (parmula) nakaoae*, and *O. (parmula) tsukagoshii*. These species fell into three morpho-types (*cf.* Envall and Norenburg 2001), which we placed at an infra-generic, supra-specific rank as ‘species group’ in a Code-compliant manner. Except for one group that was represented by a single species, viz., *O. (macintoshi) ani*, the species within each group were hardly distinguishable from each other solely based on morphological characters. Subtle morphological differences were observed between them, but whether these characters can be consistently and reliably used to discriminate the species has not been clarified in this study due to the limited number of samples, an issue that should be investigated in future studies. For the moment, DNA taxonomy is indispensable in understanding *Ototyphlonemertes* diversity, as has been previously demonstrated (*e.g.*, Leasi and Norenburg 2014; Leasi *et al.* 2016).

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List of DDBJ/EMBL/GenBank accession numbers of COI sequences used for the species delimitation analyses performed in the present study, with the species names that are *i)* originally registered in DDBJ/EMBL/GenBank and *ii)* used in this paper based on the results of the species delimitation analyses.

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[illegible]

Appendix Continued.

[illegible]

Appendix Continued.

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Appendix Continued.

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