Morphological and molecular study on \textit{Yininemertes pratensis} (Nemertea, Pilidiophora, Heteronemertea) from the Han River Estuary, South Korea, and its phylogenetic position within the family Lineidae

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Abstract

Outbreaks of ribbon worms observed in 2013, 2015, and 2017–2019 in the Han River Estuary, South Korea, have caused damage to local glass-eel fisheries. The Han River ribbon worms have been identified as \textit{Yininemertes pratensis} (Sun & Lu, 1998) based on not only morphological characteristics compared with the holotype and paratype specimens, but also DNA sequence comparison with topotypes freshly collected near the Yangtze River mouth, China. Using sequences of six gene markers (18S rRNA, 28S rRNA, histone H3, histone H4, 16S rRNA, and COI), the phylogenetic position of \textit{Y. pratensis} was inferred among other heteronemerteans based on their sequences obtained from public databases. This analysis firmly placed \textit{Y. pratensis} as a close relative to \textit{Apatronemertes albimaculosa} Wilfert & Gibson, 1974, which has been reported from aquarium tanks containing tropical freshwater plants in various parts of the world as well as a wild environment in Panama.

Keywords

\textit{Anguilla japonica}, brackish-water invertebrates, freshwater invertebrates, Yellow Sea
Introduction

An explosive proliferation of unidentified, brackish-water heteronemerteans was observed in the Han River Estuary, South Korea, in the spring of 2013. Our morphological observation of the Han River ribbon worms indicated that they represent *Yininemertes pratensis* (Sun & Lu, 1998), a brackish-water heteronemertean known only by its original description from the Yangtze (Changjiang) River Estuary, China (for the nomenclature of the genus, see Sun and Lu 2008; Özdikmen 2009; Kajihara 2014). Outbreaks of *Y. pratensis* in the Han River Estuary were also observed in 2015, 2017, 2018, and 2019. Reportedly, the worms have caused severe damage (Lee 2015; Noh 2019) to local fisheries of glass eels, which are juveniles of *Anguilla japonica* Temminck & Schlegel, 1847, a valuable fishery resource in East Asian countries showing dramatic declines in recent years (Tzeng 1997; Tatsukawa 2003; Tseng et al. 2003). As causes for the eel declines, overfishing and habitat loss due to human activities (e.g., Chen et al. 2014) and oceanic–atmospheric factors such as changes in ocean circulation (Chang et al. 2018) have been suggested. To what extent the nemerteans have been contributing to the anguillid declines is not known. For glass-eel fisheries, fishermen set long, conical nets on the estuarine bottoms with apertures directing downstream. At the end of each net, ascending catches are to be concentrated mostly during flood tide. In the 2015 bloom, more than 90% of catches were worms, with none to only a few eels that were dead (Lee 2015) probably due to yet-unidentified neurotoxic substances (Kwon et al. 2017) in worm mucus within the concentrated net catches. These neurotoxins might have been discharged from epidermal cells and contained in the secreted mucus (cf. Tanu et al. 2004; Asakawa et al. 2013). To our knowledge, this is the first record of damage to fisheries directly caused by nemertean outbreaks, although a potentially indirect case is known. At certain Alaskan localities in the 1983–1984 and 1984–1985 brooding seasons of the red king crab *Paralithodes camtschaticus* (Tilesius, 1815), a widespread outbreak of the decapod-egg-predatory nemertean *Carcinonemertes regicides* Shields et al., 1989, and possibly *Oovicides paralithodis* Kajihara & Kuris, 2013 as well, caused high egg mortality (Kuris et al. 1991), which could have led to a subsequent decline in the red king crab population (e.g., Loher and Armstrong 2005). In addition, the milky ribbon worm *Cerebratulus lacteus* (Leidy, 1851) has been identified as an important threat to populations of the soft-shell clam *Mya arenaria* Linnaeus, 1758, which is one of the commercial bivalves in Atlantic Canada, although no outbreak has ever been reported for *C. lacteus* (cf. Bourque et al. 2001, 2002).

Facing a plethora of undescribed species with dwindling number of experts, some nemertean taxonomists agreed that taxonomic descriptions of ribbon worms will have to shift from traditional, internal-anatomy-based style to histology-free one with a combination of high-quality external images and molecular phylogeny (Strand and Sundberg 2011; Strand et al. 2014; Kajihara 2015; Sundberg et al. 2016). On the other hand, in the case of Heteronemertea, only about 10% of ~100 genera (Gibson 1995; Kajihara et al. 2008) have been represented by type species in terms of sequences for...
Morphological and molecular study on *Yininemertes pratensis* from...

multi-locus analysis (Thollesson and Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014, 2015). Logically, until the rest of ~90 genera are also represented in the same manner, examination of internal morphology will remain indispensable to genus-level identification (e.g., Chernyshev et al. 2018). Moreover, most heteronemertean genera currently diagnosed are non-monophyletic. This has been repeatedly pointed out in previous studies (e.g., Sundberg and Saur 1998; Schwartz 2009; Puerta et al. 2010; Hiebert and Maslakova 2015). Therefore, as many type species of genus-group names (such as *Yininemertes*) as possible should be placed in molecular phylogenetic context for proper application of genus names in many other species of heteronemerteans as long as Linnaean binominal nomenclature is employed.

In this paper, we report the identity of Han River nemerteans based on morphological characteristics in comparison to the type material of *Y. pratensis* as well as DNA barcoding data from the type locality. Also, we infer the phylogenetic position of *Y. pratensis* among Heteronemertea based on a multi-locus molecular analysis.

**Materials and methods**

**Specimen collection and processing**

Approximately 700 individuals of ribbon worms were collected from local fishermen's glass-eel nets for *Anguilla japonica*, set at about 37°36’08”N, 126°48’23”E, in Goyang, South Korea, approximately 40 km upstream of the mouth of the Han River (Figs 1A, B, 2A) on April 6, 2015 by TP. A total of 12 topotype specimens of *Y. pratensis* were collected at two sites in Shanghai by TP, Kwang-Soo Kim, Seul Yi, SS, and Guang Xi: i) Bailonggang, 31°15’40.0”N, 121°44’11.8”E, on May 13, 2016; and ii) Chongming

![Figure 1](image)

*Figure 1.* Maps showing sampling localities indicated by arrow heads. A The Han River and Yangtze River Estuaries are about 840 km apart from each other across the Yellow Sea B sampling locality in Goyang, Korea C two sampling localities, Chongming Island and Bailonggang, China.
Island, 31°34’39.4"N, 121°54’34.9"E, on May 14, 2016 (Figs 1A, C, 2C). Specimens from the Han River were anesthetized with 7% MgCl₂ solution before fixed in either 7% neutral-buffered formalin for morphological observation (~300 individuals) or 100% ethanol for DNA extraction (~300 individuals). Of these 12 specimens collected from Shanghai, nine were fixed in 70% EtOH for DNA extraction while three were used for taking photographs. Anterior portion of one formalin-fixed specimen from the Han River was dehydrated in ethanol series, cleared in xylene, embedded in paraffin (melting point: 56–57 °C), and transversely sectioned at thickness of 8 µm. Serial sections were stained with Mallory’s trichrome method (Gibson 1994). Specimens were deposited in National Institute of Biological Resources Invertebrate Collection, Incheon, Korea (NIBR IV) and Invertebrate Collection of the Hokkaido University Museum, Sapporo, Japan (ICHUM) (Table 1). For comparison, the holotype (DH005A) and a paratype (DH005C) of *Y. pratensis* deposited in Ocean University of China, Qingdao, People’s Republic of China, were also examined.

**Molecular phylogeny**

Small pieces of tissue taken from 22 specimens collected from the Han River and seven specimens from Yangtze River were used for total genomic DNA extraction using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Partial sequences of six gene markers (nuclear 18S rRNA, 28S rRNA, histone H3, and histone H4; mitochondrial 16S rRNA, and COI) were used for molecular analyses using the same primers published by Andrade et al. (2012). For PCR amplification, the following mixture was prepared in a total volume of 50 μL: 50 ng of template genomic DNA, 2.5 mM dNTPs, 5 μL of 10× Ex Taq™ buffer, 2 μL of each 10 pM primer, and 1 U (0.5 μL) of TaKaRa Ex Taq™ polymerase. Thermal cycling condition comprised an initial denaturation at 94 °C for 30 sec followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at 43–50 °C depending on primers for 30 sec, and extension at 72 °C for 1 min. A final extension step at 72 °C for 10 min was then followed. Amplified PCR products were sequenced using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA) from both directions. All sequences generated de novo in this study were deposited at GenBank (Table 2).

To assess phylogenetic affinity of the Han River nemerteans, maximum likelihood (ML) analysis and Bayesian Inference (BI) were carried out with 31 lineid heteronemertean species for which the aforementioned six gene sequences were available in public databases (Table 3). Outgroups were chosen to include *Baseodiscus mexicanus* (Bürger, 1893) and *B. unicolor* Stiasny-Wijnhoff, 1925 (cf. Andrade et al. 2012; Kvist et al. 2014). Sequence alignment was performed using MAFFT ver. 7 (Katoh and Standley 2013) with E-INS-i option for 18S, 28S, and 16S. For the protein-coding H3, H4, and COI, sequences were aligned straightforward without gaps. Sequences were edited and concatenated using MEGA ver. 5.2 (Tamura et al. 2011). Gaps and incompletely determined nucleotides accounted for 24.9% of the entire dataset of these sequences.
Morphological and molecular study on Yininemertes pratensis from...

Table 1. List of specimens identified as *Yininemertes pratensis* (Sun & Lu, 1998) in this study with catalogue numbers at the National Institute of Biological Resources Invertebrate Section, Incheon, Korea (NIBR IV) and the Invertebrate Collection of the Hokkaido University Museum, Sapporo, Japan (ICHUM) as well as their sampling date and locality.

| Catalogue number | Sampling date and locality | Remarks |
|------------------|---------------------------|---------|
| NIBR IV 0000409587–0000409590 | 6 April 2015, Goyang, South Korea | > 300 individuals fixed in 10% formalin |
| NIBR IV 0000409591–0000409595 | 6 April 2015, Goyang, South Korea | > 300 individuals fixed in 100% EtOH |
| NIBR IV 0000409596–0000409617 | 6 April 2015, Goyang, South Korea | 22 voucher specimens used for DNA extraction |
| NIBR IV 0000758851 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758852 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758853 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758854 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758855 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758856 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758857 | 13 May 2016, Bailonggang, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758858 | 14 May 2016, Chongming, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758859 | 14 May 2016, Chongming, China | 1 specimen fixed in 70% EtOH |
| NIBR IV 0000758860 | 14 May 2016, Chongming, China | 1 specimen fixed in 70% EtOH |
| ICHUM 5259 | 6 April 2015, Goyang, South Korea | Serial transverse sections of the anterior portion of a specimen, Mallory trichrome, 36 slides. |
| ICHUM 5260 | 6 April 2015, Goyang, South Korea | |

Table 2. GenBank accession numbers of sequences determined in the present study from voucher specimens of *Yininemertes pratensis* (Sun & Lu, 1998) deposited in the National Institute of Biological Resources Invertebrate Collection, Incheon, Korea (NIBR IV).

| NIBR IV | 18S rRNA | 28S rRNA | Histone H3 | Histone H4 | 16S rRNA | COI |
|---------|----------|----------|------------|------------|----------|-----|
| 0000409596 | KY274047 | KY274069 | KY274091 | KY274113 | KY274025 | KY274003 |
| 0000409597 | KY274048 | KY274070 | KY274092 | KY274114 | KY274026 | KY274004 |
| 0000409598 | KY274049 | KY274071 | KY274093 | KY274115 | KY274027 | KY274005 |
| 0000409599 | KY274050 | KY274072 | KY274094 | KY274116 | KY274028 | KY274006 |
| 0000409600 | KY274051 | KY274073 | KY274095 | KY274117 | KY274029 | KY274007 |
| 0000409601 | KY274052 | KY274074 | KY274096 | KY274118 | KY274030 | KY274008 |
| 0000409602 | KY274053 | KY274075 | KY274097 | KY274119 | KY274031 | KY274009 |
| 0000409603 | KY274054 | KY274076 | KY274098 | KY274120 | KY274032 | KY274010 |
| 0000409604 | KY274055 | KY274077 | KY274099 | KY274121 | KY274033 | KY274011 |
| 0000409605 | KY274056 | KY274078 | KY274100 | KY274122 | KY274034 | KY274012 |
| 0000409606 | KY274057 | KY274079 | KY274101 | KY274123 | KY274035 | KY274013 |
| 0000409607 | KY274058 | KY274080 | KY274102 | KY274124 | KY274036 | KY274014 |
| 0000409608 | KY274059 | KY274081 | KY274103 | KY274125 | KY274037 | KY274015 |
| 0000409609 | KY274060 | KY274082 | KY274104 | KY274126 | KY274038 | KY274016 |
| 0000409610 | KY274061 | KY274083 | KY274105 | KY274127 | KY274039 | KY274017 |
| 0000409611 | KY274062 | KY274084 | KY274106 | KY274128 | KY274040 | KY274018 |
| 0000409612 | KY274063 | KY274085 | KY274107 | KY274129 | KY274041 | KY274019 |
| 0000409613 | KY274064 | KY274086 | KY274108 | KY274130 | KY274042 | KY274020 |
| 0000409614 | KY274065 | KY274087 | KY274109 | KY274131 | KY274043 | KY274021 |
| 0000409615 | KY274066 | KY274088 | KY274110 | KY274132 | KY274044 | KY274022 |
| 0000409616 | KY274067 | KY274089 | KY274111 | KY274133 | KY274045 | KY274023 |
| 0000409617 | KY274068 | KY274090 | KY274112 | KY274134 | KY274046 | KY274024 |
| 0000754958 | KY274138 | KY274140 | KY274144 | KY274146 | KY274136 | KY274142 |
| 0000758857 | KY274137 | KY274139 | KY274143 | KY274145 | KY274135 | KY274141 |
Table 3. GenBank accession numbers of sequences used in the present phylogenetic analysis (Histone H4 sequences indicated by asterisks (*) were kindly provided by Dr Sebastian Kvist).

| Species                                      | 18S rRNA | 28S rRNA | Histone H3 | Histone H4 | 16S rRNA | COI       | Reference                  |
|----------------------------------------------|----------|----------|------------|------------|----------|-----------|----------------------------|
| Apatronemertes albimaculosa Wilfert & Gibson, 1974 | JF293030 | HQ856860 | JF277733   | JF277666   | JF277587 | HQ848584 | Andrade et al. (2012)      |
| Cerebratulus lacteus (Leidy, 1851)          | JF293044 | HQ856857 | JF277728   | JF277653   | JF277575 | HQ848576 | Andrade et al. (2012)      |
| Cerebratulus marginatus Renier, 1804         | JF293042 | HQ856858 | JF277729   | JF277652   | JF277576 | HQ848575 | Andrade et al. (2012)      |
| Gorgonorhynchus albocinctus Kajihara, 2015   | LC010650 | LC010651 | –          | –          | –        | LC010649 | Kajihara (2015)            |
| Gorgonorhynchus cf. bermudensis Wheeler, 1940 | KF935300 | KF935356 | KF935412   | *          | KF935467 | KF935517 | Kvist et al. (2014)        |
| Kulikovia alborostrata (Takakura, 1898)      | –        | AJ436877 | –          | –          | AJ436822 | AJ436932 | Thollesson and Norenburg (2003) |
| Kulikovia manchenkoi Chernyshev et al., 2018 | JF293035 | HQ856856 | JF277730   | JF277683   | JF277572 | HQ848574 | Andrade et al. (2012)      |
| Lineus acutifrons Southern, 1913             | JF304778 | HQ856855 | JF277727   | JF277681   | JF277573 | GU590937 | Andrade et al. (2012)      |
| Lineus bilineatus (Renier, 1804)             | JF293041 | HQ856844 | JF277731   | JF277682   | JF277571 | –         | Andrade et al. (2012)      |
| Lineus lacteus (Rathke, 1843)               | JF293065 | HQ856850 | JF277725   | JF277656   | JF277584 | HQ848583 | Andrade et al. (2012)      |
| Lineus longissimus (Gunnerus, 1770)          | –        | AJ436880 | –          | –          | AJ436825 | AJ436935 | Thollesson and Norenburg (2003) |
| Lineus ruber (Müller, 1774)                 | JF293040 | HQ856853 | JF277718   | JF277655   | JF277583 | HQ848580 | Andrade et al. (2012)      |
| Lineus sanguineus (Rathke, 1799)            | –        | AJ436882 | AJ436981   | –          | AJ436827 | AJ436937 | Thollesson and Norenburg (2003) |
| Maculara alaskensis (Coe, 1901a)            | –        | AJ436882 | AJ436981   | –          | AJ436827 | AJ436937 | Thollesson and Norenburg (2003) |
| Micrura chlorapardalis Schwartz & Norenburg, 2005 | KF935292 | KF935348 | KF935404   | *          | KF935459 | KF935512 | Kvist et al. (2014)        |
| Micrura dellichiiae (Hubrecht, 1879)         | KF935294 | KF935350 | KF935406   | *          | KF935461 | KF935514 | Kvist et al. (2014)        |
| Micrura fasciolata Ehrenberg, 1828          | JF293038 | HQ856846 | JF277721   | JF277660   | JF277585 | HQ848577 | Andrade et al. (2012)      |
| Micrura ignea Schwartz & Norenburg, 2005    | KF935294 | KF935350 | KF935406   | *          | KF935461 | KF935514 | Kvist et al. (2014)        |
| Micrura purpurea (Dalyell, 1853)            | JF293036 | HQ856845 | JF277726   | JF277663   | JF277577 | HQ848586 | Andrade et al. (2012)      |
|                | 18S rRNA | 28S rRNA | Histone H3 | Histone H4 | 16S rRNA | COI       | Reference               |
|----------------|----------|----------|------------|------------|----------|-----------|-------------------------|
| **Micrura verrilli** Coe, 1901a | KF935288 | KF935344 | KF935400   | *          | KF935455 | KF935508 | Kvist et al. (2014)     |
| **Micrura sp.**¹ | KF935293 | KF935349 | KF935405   | *          | KF935460 | KF935513 | Kvist et al. (2014)     |
| **Notospermus** geniculatus (Delle Chiaie, 1828) | KF935295 | KF935351 | KF935407   | *          | KF935462 | –         | Kvist et al. (2014)     |
| **Notospermus** sp. 1 (SK76) | KF935296 | KF935352 | KF935408   | *          | KF935463 | KF935515 | Kvist et al. (2014)     |
| **Notospermus** sp. 2 (SK65) | KF935297 | KF935353 | KF935409   | *          | KF935464 | –         | Kvist et al. (2014)     |
| **Notospermus** sp. 3 (SK50) | KF935298 | KF935354 | KF935410   | *          | KF935465 | KF935516 | Kvist et al. (2014)     |
| **Parborlasia** corrugata (McIntosh, 1876) | JF293037 | HQ856851 | JF277732   | JF277662   | JF277578 | –         | Andrade et al. (2012)   |
| **Parvicirrus** dubius (Verrill, 1879) | –         | AJ436885 | –          | –          | –        | AJ436830 | AJ436940                |
| **Pseudomicrura** afzelii Strand & Sundberg, 2011 | GU445924 | GU445919 | –          | –          | GU445914 | GU392013 | Strand and Sundberg (2011) |
| **Riseriullus** occultus Rogers et al., 1993 | JF293031 | HQ856848 | JF277724   | JF277679   | JF277581 | HQ848581 | Andrade et al. (2012)   |
| **Tenuilineus** bicolor (Verrill, 1892) | –         | AJ436878 | AJ436980   | –          | AJ436823 | AJ436933 | Thollesson and Norenburg (2003) |
| **Zygeupolia** rubens (Coe, 1895) | JF293045 | HQ856861 | JF277735   | JF277661   | JF277574 | HQ848585 | Andrade et al. (2012)   |
| **Yininemertes pratensis** (Sun & Lu, 1998) | KY274047 | KY274069 | KY274091   | KY274113   | KY274025 | KY274003 | Present study           |
| **Outgroup** |          |          |            |            |          |           |                        |
| **Baseodiscus** mexicanus (Bürger, 1893) | KF935281 | KF935337 | KF935393   | *          | KF935449 | KF935503 | Kvist et al. (2014)     |
| **Baseodiscus** unicolor Stiasny-Wijnhoff, 1925 | KF935284 | KF935340 | KF935396   | *          | KF935451 | KF935505 | Kvist et al. (2014)     |

¹Left unidentified in Andrade et al. (2012); the species identification herein follows that of Kajihara et al. (2016)
²Identified as *Cerebratulus lenicopis* (Coe, 1901b) in Kvist et al. (2014); re-examination of the voucher material at the Museum of Comparative Zoology at Harvard University (MCZ IZ 135331) revealed that it had a branched proboscis (Gonzalo Giribet, pers. comm. to HK)
³Transferred to *Kulikovia* by Chernyshev et al. (2018)
⁴Identified as *Lineus torquatus* Coe, 1901a in Andrade et al. (2012); the identification herein follows that of Chernyshev et al. (2018)
⁵Identified as *Ramphogordius lacteus* in Andrade et al. (2012); its generic affiliation follows that of Ament-Velásquez et al. (2016)
⁶Identified as *Ramphogordius sanguineus* in Andrade et al. (2012); the identification herein follows that of Kang et al. (2015)
⁷Identified as *Lineus* sp. in Kvist et al. (2014); the species identification herein follows that of Kang et al. (2015), and its generic affiliation follows that of Ament-Velásquez et al. (2016)
⁸Transferred to *Maculaura* by Hiebert and Maslakova (2015)
⁹Identified as *Micrura rubramaculosa* Schwartz and Norenburg, 2005 in Kvist et al. (2014); the species identification has been corrected at the database of the Museum of Comparative Zoology at Harvard University for the voucher specimen (MCZ IZ 132531).
PartitionFinder ver. 1.1 (Lanfear et al. 2012) was used to determine the best partition scheme for ML and BI. For BI, the most suitable substitution model for each partition was also selected: GTR+I+G for 16S and 28S; GTR+G for COI (1st codon), H3 (1st and 3rd codons), and H4 (1st and 2nd codons); K80+I+G for 18S and H4 (3rd codon); F81+I+G for COI (2nd codon); HKY+I+G for COI (3rd codon); and JC for H3 (2nd codon). ML analysis was performed using RAxML ver. 8.0.0 (Stamatakis 2014) with a GTR+G model of nucleotide substitution for all partitions consisting of 1000 rapid bootstraps. BI was carried out using MrBayes ver. 3.2.3 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) with two independent Metropolis-coupled analyses (four Markov chains of 10,000,000 generations for each analysis). Trees were sampled every 100 generations. Values of run convergence indicated that sufficient amounts of trees and parameters were sampled (average standard deviation of split frequencies = 0.006616; minimum estimated sample size of tree lengths = 706.26; potential scale reduction factor of tree lengths = 1.001). Run convergence was also assessed with Tracer ver. 1.6 (Rambaut et al. 2014).

Population genetics

Using 29 sequences (22 from Korea, seven from China) of 658-bp partial COI gene, haplotype network analyses were performed with Network ver. 5.0.0.1 (available at http://www.fluxus-engineering.com) using median-joining method (Bandelt et al. 1999) and TCS ver. 1.2.1 (Clement et al. 2000) using statistical parsimony (Templeton et al. 1992). Calculation of genetic distances was carried out using MEGA ver. 5.2 (Tamura et al. 2011). Calculations for haplotype diversity, nucleotide diversity, Tajima’s D, and Fu’s Fs values were done with ARLEQUIN ver. 3.5.2.2 (Excoffier et al. 2005).

Results

Morphology

The external feature of the Han River nemerteans agreed with the original description of *Y. pratensis* in that these worms were variously dark brown, brick red, and tinged with violet sometimes (Fig. 2A, B). Generally, their body color became paler posteriorly. Sun and Lu (1998) have reported that specimens from the Yangtze River Estuary sometimes show light-red, 4–10 transverse rings arranged on the body. Such ring arrangement was also found in specimens from the Han River Estuary (Fig. 2B) as well as topotype specimens (Fig. 2E, G) collected from muddy sediment with or without vegetation (Fig. 2C, D).

In specimens collected from the Han River, the proboscis was not branched, and reddish in color (Fig. 3A). Serially sectioned specimen (ICHUM 5260) showed
Figure 2. Photographs of *Yininemertes pratensis* (Sun and Lu, 1998) taken in life. **A** A haul of a glass-eel net at the Han River Estuary, South Korea, on 6 April 2015 **B** magnification of a swarm of the same worms as in **A** from the Han River Estuary taken in the laboratory; arrow heads indicating the characteristic transverse narrow rings in the intestinal region **C** a specimen dug from clayey mud sediment with vegetation at Bailonggang in the Yangtze River Estuary, China, May 13, 2016 **D** a specimen dug from non-vegetated clay sediment at Chongming Island in the Yangtze River Estuary, China, 14 May 2016 **E** topotype from the Yangtze River Estuary showing an overview of whole specimen **F** topotype from China showing magnification of head, ventral view **G** topotype from China, magnification of intestinal region, showing the characteristic narrow transverse rings, indicated by arrow heads.
Figure 3. *Yininemertes pratensis* (Sun and Lu, 1998), photograph in life (A) and photomicrographs of transverse sections (B, D, E, G, H) ICHUM 5260 C DH005C, paratype F DH005A, holotype. A Anesthetized state with proboscis partially protruded, NIBR IV 0000409596 B, C proboscis; large arrow heads indicating fibers contributing to muscle cross; small arrow heads showing rhabdoids D cerebral region showing type-3 neuron E, F foregut region, arrow heads indicating intra-epithelial somatic muscle fibers G, H cephalic region showing well-developed cephalic lacuna.
the following internal anatomical features: i) the proboscis had two muscle crosses (Fig. 3B), similar to that in the paratype of *Y. pratensis* (Fig. 3C); ii) the rhynchocoele outer circular musculature was not interwoven with the adjacent body-wall longitudinal musculature; iii) the nervous system had type-3 neurons (cf. Beckers 2015) along the inner portion of the brain (Fig. 3D); iv) the foregut wall had intraepithelial somatic muscle fibres that appeared to be circular or diagonal (Fig. 3E), similar to that observed in the holotype (Fig. 3F); v) the body-wall dermal glandular layer was not separated from the body-wall outer longitudinal muscle layer by connective tissue layer (Fig. 3E); and vi) the blood system comprised spacious cephalic lacuna (Fig. 3G, H), an alimentary vascular plexus (Fig. 3E), and a middorsal blood vessel.

**Molecular phylogeny**

Lengths of the six gene markers determined for Korean and Chinese materials were: 16S, 507–508 bp; 18S, 1000–1003 bp; 28S, 1132 bp; COI, 658 bp; H3, 331 bp; and H4, 160 bp. Resulting ML tree (\(\ln L = -51290.378661\)) and BI tree (harmonic mean of estimated marginal likelihood for two runs = -52096.68) were topologically more or less the same, with *Y. pratensis* being a sister of *Apatronemertes albimaculosa* Wilfert & Gibson, 1974 in both trees with 100% bootstrap support value and 1.0 posterior probability (Fig. 4). The inter-specific K2P distance between the COI sequences of *Y. pratensis* and *A. albimaculosa* was 0.163–0.196. More basal relations between this clade (\(= Y. pratensis + A. albimaculosa\)) and other heteronemerteans included in this analysis were poorly resolved.

**Population genetics**

Median-joining and statistical parsimony networks were identical in shape, comprising eight haplotypes with a maximal difference of five mutations (Fig. 5). From 29 specimens analysed (22 from Korea, seven from China), a total of nine haplotypes were detected, of which two were shared by Korean and Chinese populations. Eleven of 22 sequences from Korea were represented by the same haplotype, which was also the main haplotype among the Chinese population (shared by five of seven Chinese individuals analysed). Eight COI haplotypes from Korea differed by 0.000–0.006 from each other in terms of both uncorrected \(p\)-distance and K2P. The Korean population showed higher values of nucleotide diversity and haplotype diversity than the Chinese ones (Table 4). Tajima’s \(D\) and Fu’s \(F_s\) values were all negative for the Korean population, the Chinese population, and the total population, although not significantly different from zero except for the Fu’s \(F_s\) values for the Korean population and total population.
Figure 4. Maximum likelihood tree ($\ln L = -51290.378661$) for heteronemerteans based on concatenated 18S rRNA, 28S rRNA, histone H3, histone H4, 16S rRNA, and COI dataset showing phylogenetic position of *Yininemertes pratensis* (Sun and Lu, 1998). Numbers near nodes are bootstrap values for maximum-likelihood analysis and posterior probability for Bayesian inference. Scale bar indicates the number of substitutions per site.
Morphological and molecular study on Yininemertes pratensis from...

Figure 5. Median-joining network for eight haplotypes detected among 29 Yininemertes pratensis specimens (22 from Han River, Korea; 7 from Yangtze River, China; statistical-parsimony method yielded the same topology). Numbers in each circle (pie chart) indicate sample size which is proportional to the size of each pie diagram.

Table 4. Number of individuals analysed for population genetic analysis, number of haplotypes, nucleotide diversity, haplotype diversity, Tajima’s D, and Fu’s Fs based on 658-bp partial COI gene sequences from populations of Yininemertes pratensis (Sun and Lu, 1998) in the Han River and Yangtze River Estuaries.

| Locality                | Number of individuals | Number of haplotypes | Nucleotide diversity (S.D.) | Haplotype diversity (S.D.) | Tajima’s D | Fu’s Fs |
|-------------------------|-----------------------|----------------------|-----------------------------|----------------------------|-------------|---------|
| Han River Estuary, Korea| 22                    | 8                    | 0.001849 (0.001365)         | 0.7316 (0.0897)            | −0.80       | −3.87*  |
| Yangtze River Estuary, China | 7                | 3                    | 0.000868 (0.000907)         | 0.5238 (0.2086)            | −1.23       | −0.92   |
| total                   | 29                    | 9                    | 0.001632 (0.001234)         | 0.6847 (0.0899)            | −1.18       | −5.08** |

*P = 0.003; **P = 0.000

Discussion

Because fundamental biological aspects of Y. pratensis such as diet, life duration, breeding season, reproductive strategy (semelparous/iteroparous) and mode (oviparous, viviparous, and ovoviviparous), and larval ecology (if the species produces larvae in the first place) are unknown, the causes for the Y. pratensis outbreaks since 2013
in the Han River Estuary, South Korea, are open to speculation. One of the potential factors conceivable to explain the *Yininemertes* outbreaks is that the species might be capable of asexual reproduction. Until recently, asexual reproductive capacity among Heteronemertea had been confirmed only in the lineid *Lineus sanguineus* (Rathke, 1799) and *L. pseudolacteus* (Gontcharoff, 1951) (cf. Ament-Velásquez et al. 2016). However, asexual reproduction by fragmentation in the valenciniid *Baseodiscus delineatus* (Delle Chiaje, 1825) (Ikenaga et al. 2019) and *B. hemprichii* (Ehrenberg, 1831) (Kajihara and Hookabe 2019), as well as head-regenerative ability in the lineid *Cerebratulus lineolatus* Coe, 1905 (Zattara et al. 2019), *L. pictifrons* Coe, 1904 (Coe 1932), and *L. rubescens* Coe, 1904 (Coe 1930), have been documented. Asexual reproductive capacity may have evolved in more lineages than previously thought among heteronemerteans, possibly including *Y. pratensis*. Another hypothesis is that the Han River ribbon worms might have been introduced from other, unidentified localities. However, this hypothesis sounds rather unlikely, because the haplotype diversity in the Korean population (0.7316), which was greater than the Chinese one (0.5238), suggests that a stable population have existed in the Han River Estuary, probably since long before the first bloom observed in 2013. While Tajima’s *D* and Fu’s *F* values were overall negative, we cannot draw any robust conclusion about the population dynamics because most of the values were statistically not significant. Future study is needed to pinpoint possible environmental factors that are responsible for the *Y. pratensis* outbreaks, as well as to elucidate the species’ basic biology for obtaining countermeasures against the economic loss to local glass-eel fisheries caused by such blooms.

The family Lineidae McIntosh, 1874 currently contains about 90 genera and 370 species of heteronemerteans, which are morphologically characterized by having horizontal lateral cephalic slits and three apical organs. Most are marine, but six species (each in a monotypic genus) have been described from freshwater or brackish-water habitat. These are *Planolineus exsul* Beauchamp, 1928 from Indonesia; *Siolineus turbidus* Du Bois-Reymond Marcus, 1948 from Amazon; *Hinumanemertes kikuchii* Iwata, 1970 from Japan; *A. albimaculosa* from freshwater tanks in Germany (Wilfert and Gibson 1974), Austria (Senz 1993), USA (Smith 2001), and Japan (Kajihara et al. 2016), as well as in submerged logs and rocks in a pond in Panama (Kvist et al. 2018); *Amniclineus zhuijiangensis* Gibson & Qi, 1991 from Zhujiang, China; and *Y. pratensis* from China and Korea (Sun and Lu 1998; present study). Our phylogenetic tree indicates that *A. albimaculosa* and *Y. pratensis* form a highly supported clade, suggesting that the remaining fresh- and brackish-water forms, especially those in Southeast and East Asia, may also belong to the same clade. At this moment, however, neither morphological nor molecular synapomorphy between *A. albimaculosa* and *Y. pratensis* can be perceived; for instance, the characteristic outer cephalic vessels in *A. albimaculosa* are not found in *Y. pratensis*. Both species are reddish in body color, but this may be due to convergent evolution, as freshwater monostiliferous hoplonemerteans in the genus *Prostoma* Dugès, 1828 also possess reddish body. Future studies with expanded taxon sampling, along with detailed morphological examination, should clarify the evolution of these freshwater heteronemerteans.
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