

## DNA Barcoding of Benthic Ragworms of the Genus *Nectoneanthes* (Polychaeta: Nereididae) Collected in Korean Waters

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### ABSTRACT

To provide better taxonomic information of the genus *Nectoneanthes*, the two DNA barcode regions of mitochondrial cytochrome c oxidase subunit I (*COI*) and 16S ribosomal DNA (rDNA) sequences of *Nectoneanthes oxypoda* and *N. uchiwa* were determined. In addition, the respective sequences of four nereidid species closely related to *Nectoneanthes* were retrieved from GenBank for comparison and to estimate intra- and inter-specific genetic distances. The aligned sequence lengths of *COI* and 16S rDNA were 570 bp and 419 bp long, respectively. The mean intraspecific variation in both markers was less than 1% in all species except for that in *COI* of *H. diadroma* (1.87%). The mean interspecific variation between *N. oxypoda* and *N. uchiwa* was 12.02% regarding *COI* and 1.85% regarding 16S rDNA. In contrast, the mean interspecific variation between species of other genera was comparably higher (i.e., genus *Perinereis*: 20.5% in *COI* and 8.3% in 16S rDNA; genus *Hediste*: 13.18% in *COI* and 2.64% in 16S rDNA), compared with that between the two *Nectoneanthes* species. This result indicated that these *Nectoneanthes* species are genetically more closely related than other congeneric species of different genera. The DNA barcoding information on *Nectoneanthes* species generated in this study provides valuable insights for further biodiversity studies on nereidid species.

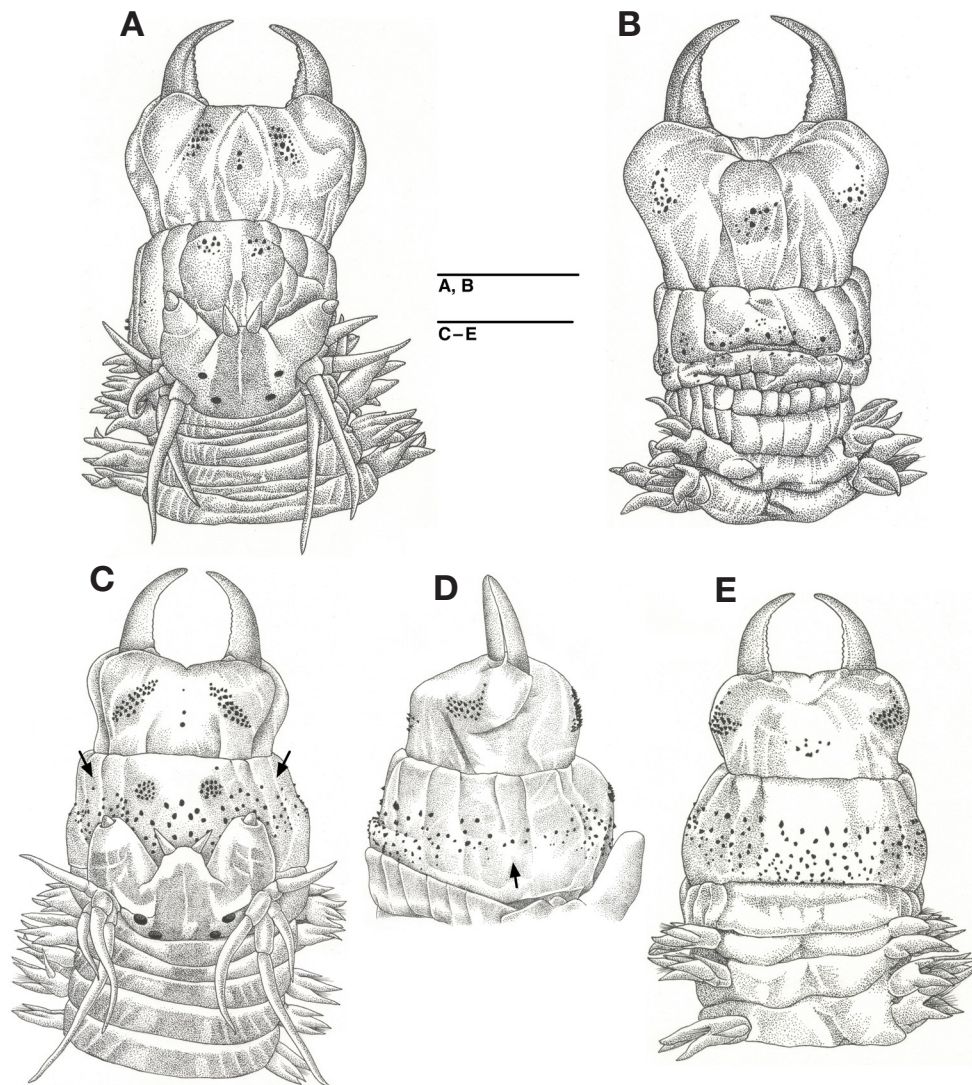
**Keywords:** Nereididae, *Nectoneanthes*, *COI*, 16S rDNA, DNA barcode

### INTRODUCTION

The genus *Nectoneanthes* Imajima, 1972, belonging to the family Nereididae de Blainville, 1818, currently comprises two species, *N. oxypoda* (Marenzeller, 1879) and *N. uchiwa* Sato, 2013. These species inhabit the sandy or muddy bottom of intertidal and subtidal areas; *N. oxypoda* is widely distributed in the Indo-Pacific region, including East Asian coasts (China, Japan, and Korea), whereas *N. uchiwa* occurs more sparsely along the East Asian coast (China, Japan, Korea, and Taiwan) (Sato, 2013; Hsueh, 2018). The taxonomic history of the genus *Nectoneanthes* is complicated. Imajima (1972) established this genus based on *Nereis (Alitta) oxypoda* Marenzeller, 1879 (the type species) and *Nereis ijimai* Izuka (1912), with their characteristics of enlarged notopodia and lack of falcigers. According to the subsequent taxonomic treatment of its type species, *Nectoneanthes* was previously considered a junior synonym of *Neanthes* Kinberg, 1865 and

*Alitta* Kinberg, 1865 by Wilson (1988), and Bakken and Wilson (2005), respectively. Thereafter, the genus *Nectoneanthes* was re-established by Sato (2013) based on clearly distinguishable characteristics such as the shape of parapodia and composition of chaetae in *Neanthes* and *Alitta*. Sato (2013) also revised several nominal species previously referred to as *Nectoneanthes*, and only two species, *N. oxypoda* and *N. uchiwa* (Fig. 1), were considered valid. In Korea, these two species had been misidentified as *N. latipoda* and *N. oxypoda* in the earliest taxonomic studies (Paik, 1972, 1973), and they are currently referred to as *N. oxypoda* and *N. uchiwa*, respectively (see Sato, 2013).

In recent taxonomic studies, DNA sequences of the mitochondrial cytochrome c oxidase subunit I (*COI*) and 16S ribosomal DNA (rDNA) regions have been used for DNA barcoding to facilitate reliable species discrimination among closely related nereidids, in addition to morphological analyses (Park and Kim, 2017; Tosuji et al., 2019; Sampieri et al., 2021;



**Fig. 1.** *Nectoneanthes oxypoda* (Marenzeller, 1879) and *N. uchiwa* Sato, 2013. A, B, *N. oxypoda*: dorsal and ventral views of the anterior end with the protruded proboscis (NIBRIV0000787923). C–E, *N. uchiwa*: dorsal, lateral, and ventral views of the anterior end with the protruded proboscis (NIBRIV0000787900). Arrows indicate continuous paragnaths in the oral ring. Scale bars: A, B=5 mm, C–E=2 mm.

Villalobos-Guerrero et al., 2021). However, DNA barcoding information is still poor for species identification of nereidid species. Thus, the two DNA barcode regions of *COI* and 16S rDNA of *Nectoneanthes* species were determined based on specimens collected on the coast of Korea. In addition, morphological observations of the two species were made, and illustrations are provided herein.

## SYSTEMATIC ACCOUNTS

Order Phyllodocida Dales, 1962

Family Nereididae de Blainville, 1818  
Genus *Nectoneanthes* Imajima, 1972

***Nectoneanthes oxypoda* (Marenzeller, 1879) (Fig. 1A, B)**  
*Nereis* (*Alitta*) *oxypoda* Marenzeller, 1879: 120–122, Pl. 2, fig. 3.  
*Nectoneanthes latipoda* Paik, 1973: 81–84, figs. 1a–j, 2; Lee et al., 2003: 191; Choi et al., 2005: 387.  
*Nectoneanthes oxypoda*: Sato, 2013: 4–24, figs. 1, 2, 3A–C, 4A, 5–8.  
(see Sato, 2013 for full synonymy)

**Material examined.** Korea: 1 ind., intertidal mud flat, Jeollanam-do: Goheung-gun, Podu-myeon, Ochwi-ri, Chwido Island, 34°33'54"N, 127°24'16"E, 28 Apr 2013, Hong SS, Kim SH, NIBRIV0000787923; 1 ind., Gyeongsangnam-do: Geje-si, Irun-myeon, Gujora-ri, intertidal mud flat, 34°48'34"N, 128°40'59"E, 24 May 2015, Park T, NIBRIV0000829796; 1 ind., Incheon-si: Ongjin-gun, Daecheong-myeon, 12 m depth, subtidal soft bottom, 37°49'23"N, 124°43'23"E, 16 May 2017, Park T, Kim KS, NIBRIV0000787898.

**Diagnosis.** Paragnaths present in all areas; relatively small number of paragnaths present in the maxillary ring (areas II, IV), compared with *N. uchiwa*; two or three rows of transverse paragnath band present in areas VII–VIII, paragnaths in distal single row relatively larger than proximal ones; proximal rows consist of large and small paragnaths, paragnath band in area VII–VIII extending to lateral and often to dorsal part of oral ring, but not reaching area V (Fig. 1A, B).

***Nectoneanthes uchiwa* Sato, 2013 (Fig. 1C–E)**

*Nectoneanthes oxypoda*: Paik, 1972: 135, fig. 4; 1973: 82–84, figs. 1k–l, 2.

*Nectoneanthes uchiwa* Sato, 2013: 24–34, figs. 4A, 9–14. (see Sato, 2013 for full synonymy)

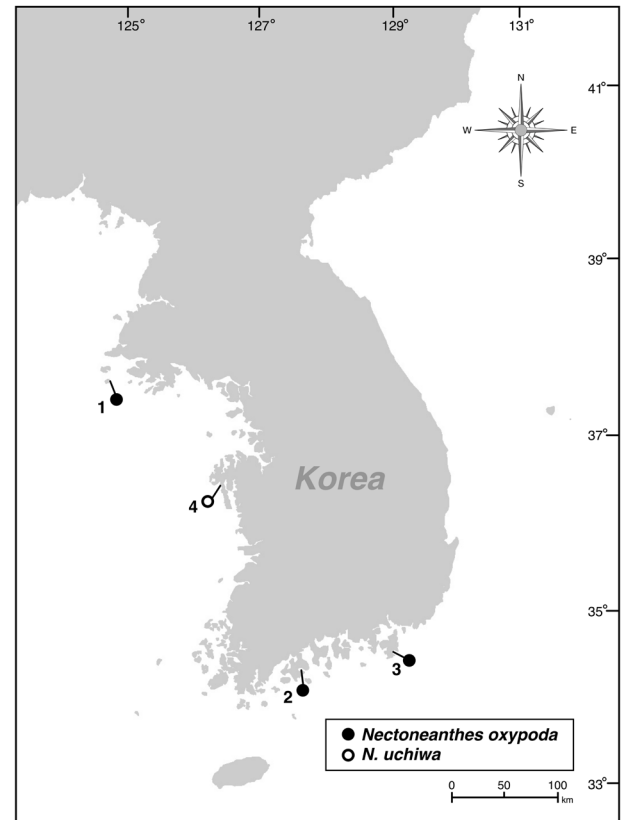
**Material examined.** NIBRIV0000787900, Korea: 3 inds., Chungcheongnam-do: Taean-gun, Nam-myeon, Mongsan-ri, intertidal mud flat, 36°40'19"N, 126°16'26"E, 11 Apr 2013, Park T, Yi S.

**Diagnosis.** Paragnaths present in all areas; relatively large number of paragnaths present in maxillary ring (areas II, IV), compared with *N. oxypoda*; many rows of continuous transverse broad paragnath band present in oral ring covering areas VII–VIII and V, transverse paragnath band in lateral region narrower than in ventral and dorsal regions; paragnaths in area VI consisting of separate clusters (Fig. 1C–E).

## RESULTS AND DISCUSSION

Specimens used in this study were collected from intertidal and subtidal muddy bottoms of Korean waters (Fig. 2) from 2013 to 2017. Three *N. uchiwa* individuals and two *N. oxypoda* individuals were collected from intertidal mud flats on the western and southern coasts using a knife to dig during low tide. A single *N. oxypoda* specimen was collected from the subtidal muddy bottom (12 m depth) on Daecheongdo Island using a grab sampler.

Specimens were observed using a stereomicroscope (SZX10; Olympus, Tokyo, Japan). Species were identified



**Fig. 2.** Collection sites of *Nectoneanthes* Imajima, 1972 species in Korea. 1, Daecheongdo Island, Daecheong-myeon, Ongjin-gun, Incheon-si; 2, Chwido Island, Ochwi-ri, Podu-myeon, Goheung-gun, Jeollanam-do; 3, Gujora-ri, Irun-myeon, Geje-si, Gyeongsangnam-do; 4, Mongsanpo Port, Mongsan-ri, Nam-myeon, Taean-gun, Chungcheongnam-do.

based on the references of Paik (1972, 1973) and Sato (2013). The observed morphological characteristics were in agreement with the redescription and original description of each species (see Sato, 2013). All voucher specimens were deposited in the invertebrate storage room of the National Institute of Biological Resources, Republic of Korea (Table 1).

Partial sequences of two DNA barcoding regions (*COI* and 16S rDNA) were produced from all collected specimens. Genomic DNA was extracted from the parapodium of each specimen using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The 658 bp of *COI* and 468–469 bp of 16S rDNA sequences from three individuals of each species were produced using the specific primer pairs polyLCO (forward; 5'-GAY-TATWTTCAACAAATCATAAAGATATTGG-3') and polyHCO (reverse; 5'-TAMACTTCWGGGTGACCAAARAAT-CA-3' (Carr et al., 2011) as well as 16sarL (forward; 5'-CG-CCTGTTTATCAAAAACAT-3') and 16sbrH (reverse;

**Table 1.** Information of taxa and sequences used to determine genetic distances and to construct neighbor-joining trees

Species	Abbreviation	Voucher No.	Collecting locality	Reference	GenBank accession No.	
					COI	16S rDNA
<i>Nectoneanthes oxypoda</i> (Marenzeller, 1879)	NecoxyG1	NIBRIV0000787923	Goheung-gun, Korea	Present study	MZ475885	MZ478251
	NecoGeo	NIBRIV0000796665	Geojedo Island, Korea		MZ475886	MZ478252
	NecoDae	NIBRIV0000787898	Daecheongdo Island, Korea		MZ475887	MZ478253
	NecoCn1	–	Taiwan Strait, China	MN256614	–	
	NecoCn2	–	–	MN256615	–	
	NecoCn3	–	–	MN256616	–	
<i>Nectoneanthes uchiwa</i> Sato, 2013	NecuM1	NIBRIV0000787900-NecuM1	Mongsanpo Port, Korea	Present study	MZ475888	MZ478254
	NecuM2	NIBRIV0000787900-NecuM2			MZ475889	MZ478255
	NecuM3	NIBRIV0000787900-NecuM3			MZ475890	MZ478256
<i>Hediste japonica</i> (Izuka, 1908)	Hjaponica1	–	Incheon, Korea	Tosuji et al. (2019)	LC323024	–
	Hjaponica2	–	Higashiyoga, Japan		LC323025	–
	Hjaponica3	–	Yongyudo Island, Korea		LC323026	–
	Hjaponica4	–	Omugagawa River, Japan		–	LC323064
	Hjaponica5	–	Yongyudo Island, Korea		–	LC323066
	Hjaponica6	–	Suncheon Bay, Korea		–	LC323067
<i>Hediste diadroma</i> Sato and Nakashima, 2003	Hdiadroma1	–	Kaminokawa River, Japan	Tosuji et al. (2019)	AB996702	AB703097
	Hdiadroma2	–	Omoigawa River, Japan		AB996695	AB703098
	Hdiadroma3	–	Kaminokawa River, Japan		AB996701	AB703100
<i>Perinereis linea</i> (Treadwell, 1936)	Pijeongok2	NIBRIV0000810291	Jeongok Port, Korea	Villalobos-Guerrero et al. (2021)	MT511711	MT540476
	Pijeongok3	NIBRIV0000810291	Possibly Korea or China		MT511712	MT540477
	Paja1	NIBRIV0000810299	–		MT511713	MT540478
<i>Perinereis euiini</i> Park and Kim, 2017	Peuiini1	NIBRIV0000502105-Pcgeoje1	Geojedo Island, Korea	Park and Kim (2017) for COI, Villalobos-Guerrero et al. (2021) for 16S rDNA	KY249122	MT540486
	Peuiini2	NIBRIV0000502105-Pcgeoje2	–		KY249123	MT540487
	Peuiini3	NIBRIV0000502105-Pcgeoje3	–		KY249124	–
<i>Alitta succinea</i> (Leuckart, 1847) <sup>a</sup>	Asuccinea	AUM45691	Florida, USA	Alves et al. (2020)	MN812981	MN812981

COI, cytochrome c oxidase subunit I; 16S rDNA, 16S ribosomal DNA; NIBR, National Institute of Biological Resources; AUM, Auburn University Museum of Natural History.  
<sup>a</sup>Taxon used as outgroup for rooting the tree.

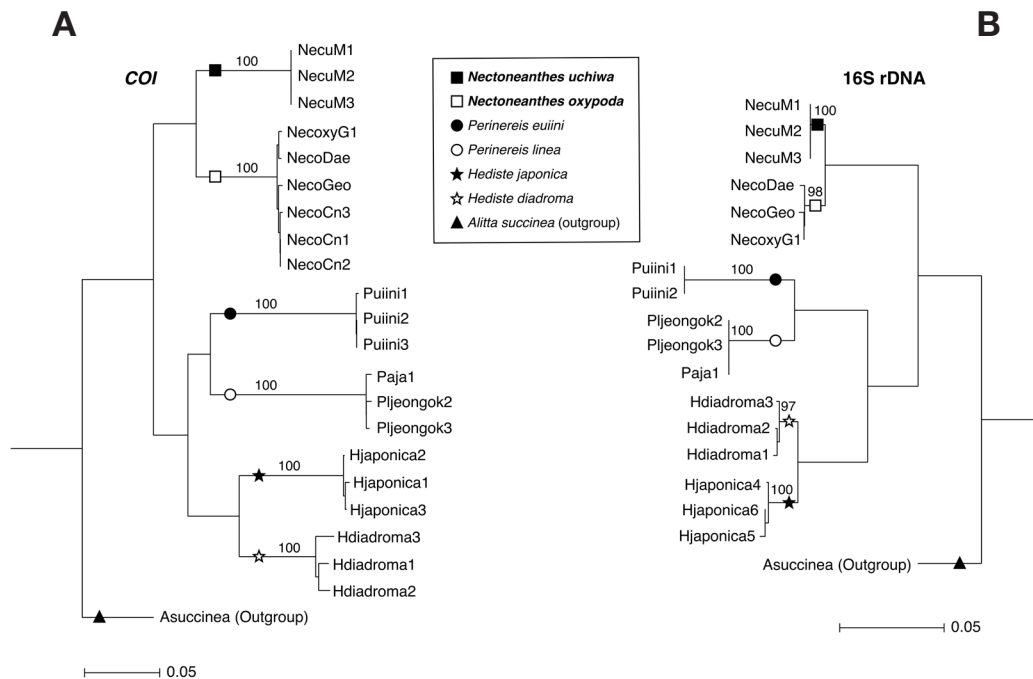
**Table 2.** Pairwise genetic distance (%; p-distance) ranges of partial *COI* (below diagonal) and 16S rDNA (above diagonal) sequences between six nereidid species

Species	<i>N. oxyioda</i> , n=3 0.24–0.48 (0.32) <sup>b</sup>	<i>N. uchiwa</i> , n=3 0 <sup>b</sup>	<i>H. japonica</i> , n=3 0.24–0.48 (0.32) <sup>b</sup>	<i>H. diadroma</i> , n=3 0.24–0.48 (0.32) <sup>b</sup>	<i>P. linea</i> , n=3 0 <sup>b</sup>	<i>P. euini</i> , n=2 0 <sup>b</sup>
<i>Nectoneanthes oxyioda</i> , n=6 0–0.7 (0.43) <sup>a</sup>	–	1.69–1.93 (1.85)	12.32–13.04 (12.64)	12.32–12.8 (12.48)	14.73	16.67
<i>Nectoneanthes uchiwa</i> , n=3 0 <sup>a</sup>	11.75–12.3 (12.02)	–	12.56–13.04 (12.8)	12.08–12.32 (12.16)	14.25	15.94
<i>Hediste japonica</i> , n=3 0.18–0.53 (0.35) <sup>a</sup>	21.58–22.14 (21.91)	21.58–21.93 (21.75)	–	2.4–2.88 (2.64)	11.3–11.54 (11.46)	13.7–13.94 (13.78)
<i>Hediste diadroma</i> , n=3 1.4–2.11 (1.87) <sup>a</sup>	19.12–20.18 (19.6)	21.4–22.11 (21.75)	12.46–14.04 (13.18)	–	10.58–10.82 (10.74)	13.22–13.46 (13.3)
<i>Perinereis linea</i> , n=3 0.53–0.7 (0.58) <sup>a</sup>	23.02–23.51 (23.37)	22.98–23.16 (23.1)	21.4–21.75 (21.64)	21.23–22.11 (21.72)	–	8.37
<i>Perinereis euini</i> , n=3 0–0.18 (0.12) <sup>a</sup>	22.14–22.63 (22.37)	21.05–21.23 (21.11)	21.05–21.4 (21.17)	22.63–22.98 (22.81)	20.35–20.7 (20.53)	–

*COI*, cytochrome c oxidase subunit I; 16S rDNA, 16S ribosomal DNA; n, number of specimens; numbers in parentheses indicate averages.

<sup>a</sup>Intraspecific distance of *COI* gene.

<sup>b</sup>Intraspecific distance of 16S rDNA.



**Fig. 3.** Neighbor-joining trees constructed using partial sequences of *COI* (A) and 16S rDNA (B) genes of six nereidid species. *Alitta succinea* was used as an outgroup for rooting the trees. Numbers on each clade indicate the bootstrap values with 1,000 replications.

5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi, 1996), respectively. PCR amplification conditions were as described by Park and Kim (2017) for *COI* and by Tosuji et al. (2019)

for 16S rDNA. In addition, *COI* and 16S rDNA sequences of species closely related to *Nectoneanthes* (*Perinereis euini* Park and Kim, 2017; *P. linea* (Treadwell, 1936), *Hediste*

*japonica* (Izuka, 1908), *H. diadroma* Sato and Nakashima, 2003) within the family Nereididae, and additional *COI* sequences of *N. oxypoda* were retrieved from GenBank (Sayers et al., 2020) to estimate intra- and inter-specific genetic distances. The sequence of *Alitta succinea* (Leuckart, 1847) was also retrieved and was used as an outgroup to root the phylogenetic trees (Table 1). The produced sequences were aligned using Geneious Prime software 2021.1.1 (Biomatters, Auckland, New Zealand). Genetic distances were calculated based on a p-distance model, and phylogenetic trees were produced using the neighbor-joining method with 1,000 bootstrap resamplings to visualize genetic distance in MEGA ver. 10.1.8. for macOS (Stecher et al., 2020).

The produced sequences were made available in GenBank (Sayers et al., 2020) under accession numbers MZ475885–MZ475890 (*COI*) and MZ478251–MZ478256 (16S rDNA) (Table 1). The lengths of the aligned sequences were 570 bp (*COI*) and 419 bp (16S rDNA). The mean intraspecific variation in both genes did not exceed 1% in all species, except for that in *COI* of *H. diadroma* (1.87%). Interspecific variation among the species pairs ranged from 11.75% to 23.51% regarding *COI* and from 1.69% to 16.67% regarding 16S rDNA (Table 2). The mean interspecific variation between *N. oxypoda* and *N. uchiwa* was 12.02% (*COI*) and 1.85% (16S rDNA). In contrast, mean interspecific variation between species in other genera was comparably higher (i.e., genus *Perinereis*: 20.5% in *COI* and 8.3% in 16S rDNA; genus *Hediste*: 13.18% in *COI* and 2.64% in 16S rDNA), compared to that of the two *Nectoneanthes* species. Moreover, according to previous studies, the mean pairwise distance between nereidid species ranged from 20% to 27% in *COI* and from 8% to 15% in 16S rDNA (Park and Kim, 2007, 2017; Villalobos-Guerrero et al., 2021). This result suggests that *Nectoneanthes* species are genetically more closely related than other congeneric species (Fig. 3).

The DNA barcoding results of the *Nectoneanthes* species presented in this study along with morphological observations and registered voucher specimens will be valuable information for further taxonomic or phylogenetic studies of this taxon, as well as for biodiversity studies on nereidid species.

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## CONFLICTS OF INTEREST

Taeseo Park, a contributing editor of the Animal Systematics, Evolution and Diversity, was not involved in the editor-

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