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Research Article

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Species delimitation and phylogenetic analyses reveal cryptic diversity within *Cerebratulus marginatus* (Nemertea: Pilidiophora)

AIDA VERDES[1,2], MARÍA BELÉN ARIAS[1], JUAN JUNOY[3], MEGAN L. SCHWARTZ[4] & HIROSHI KAJIHARA[5]

[1]Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK
[2]Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, Calle José Gutiérrez Abascal, 2, Madrid, 28006, Spain
[3]Departamento de Ciencias de la Vida, AP 20 Campus Universitario, Universidad de Alcalá, Alcalá de Henares, 28805, Spain
[4]Department Sciences and Mathematics, University of Washington, 1900 Commerce Avenue, Tacoma, 98420, WA, USA
[5]Faculty of Science, Hokkaido University, Kita-ku N10 W8, Sapporo, 060-0810, Japan

The heteronemertean *Cerebratulus marginatus* Renier, 1804 is a well-known representative of the family Lineidae (Nemertea) inhabiting sandy or muddy sediments throughout the world. Due to its wide geographic distribution and the lack of distinctive external morphological features among different populations, the species has been considered cosmopolitan. Increasing evidence indicates that nemertean species, including *Cerebratulus marginatus*, described based on morphology are often not supported by genetic data and represent complexes of cryptic species, rather than cosmopolitan species. Here, we use species delimitation and phylogenetic analyses to evaluate the speciation patterns within the putative *C. marginatus* species complex. We examined 25 specimens morphologically ascribed to *C. marginatus* and closely related species using a multilocus dataset including two mitochondrial markers (*16S rRNA*, *COI*) and three nuclear markers (*18S rRNA*, *28S rRNA*, *H3*). Our analyses identified at least four supported independent lineages among the populations analysed, indicating that samples identified as *C. marginatus* might represent indeed a complex of cryptic species. Therefore, our results do not support the cosmopolitanism of *C. marginatus* and provide additional evidence suggesting that species identifications in nemerteans should not be based exclusively on morphological or anatomical features which do not accurately reflect evolutionary patterns. Our study highlights the importance of including molecular data in addition to morphological information to accurately delimit species boundaries, thereby improving nemertean taxonomy by providing a more accurate picture of the true species diversity and geographic distribution of this typically neglected phylum.

Key words: Cryptic speciation, DNA barcoding, Heteronemertea, integrative taxonomy, marine invertebrates, phylogenetics, species complex, ribbon worms

Introduction

Nemerteans constitute a phylum of soft-bodied invertebrates also known as ribbon or proboscis worms with about 1300 species described so far (Gibson, 1995; Kajihara et al., 2008). They occur in nearly all marine benthic ecosystems and are commonly found in littoral and near-shore habitats, where they can often have an impact on the population structure of prey taxa through their voracious predatory habits (Bourque et al., 2001; Hookabe et al., 2019; Rowell & Woo, 1990).

Traditionally, taxonomic identification and classification of nemertean species has been based on morphological characters, which is especially problematic in this phylum because consistent external features of nemerteans are rare and mostly limited to the number and arrangement of the eyes, the position and shape of the cephalic furrows, and the pattern and colouration of the body surface, the latter being a highly plastic trait in many cases (Strand & Sundberg, 2005; Sundberg et al., 2010). This has led to considering many nemerteans, like *C. marginatus*, as cosmopolitan species with wide geographic distributions (Kajihara, 2020; Sundberg et al., 2010). Cosmopolitan species are common in marine ecosystems because of a lack of dispersal barriers, and therefore, a higher degree of connectivity among

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Correspondence to: Aida Verdes. E-mail: aida.verdes@mncn.csic.es; Hiroshi Kajhara. E-mail: kajihara@eis.hokudai.ac.jp

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distant populations (Álvarez-Campos, Giribet, San Martín, et al., 2017; Sanford & Kelly, 2011). However, many marine taxa considered cosmopolitan have been later shown to represent two or more morphologically indistinguishable cryptic species (Álvarez-Campos, Giribet, & Riesgo, 2017; Strand & Sundberg, 2005). In these cases, molecular tools are essential to delimit species boundaries and appropriately assess the true species diversity and distribution ranges of poorly known taxa (e.g., Ahrens et al., 2013; Carr et al., 2011; Knowlton, 2000).

Among nemerteans, recent systematic studies incorporating molecular DNA sequence data have shown that in most cases there is no correspondence between species described based on individual morphotypes and distinct evolutionary lineages, revealing in several cases the presence of morphologically indistinguishable cryptic species (e.g., Chernyshev et al., 2018; Hiebert & Maslakova, 2015; Hookabe et al., 2019; Ikenaga et al., 2021; Kajihara, 2020; Leasi & Norenburg, 2014; Rogers et al., 1995; Strand & Sundberg, 2005; Sundberg et al., 2010; Tulchinsky et al., 2012). This indicates that nemertean biodiversity has been underestimated and that species delimitation should not be based solely on morphological characters, but also combined with molecular data, and if possible, complemented with ecological, physiological, and reproductive information (Hiebert & Maslakova, 2015; Ikenaga et al., 2021).

Ribbon worm specimens identifiable as *Cerebratulus marginatus* Renier, 1804 (Fig. 1) tend to be common lineid heteronemerteans found in sandy or muddy sediments, from shallow intertidal zones to 150 m in depth (Gibson, 1982). These putative specimens of *C. marginatus* may reach up to 100 cm in length and 25 mm in width, with colouration that varies from greyish-brown, dark greyish green, slate-blue, to dull brown, with characteristic light or transparent lateral margins (Fig. 1) (Gibson, 1982, 1995; Sundberg et al., 2010). *Cerebratulus marginatus* was originally described from the Adriatic as mouse-coloured with white margins (Meneghini, 1847; Renier, 1804), and subsequently reported from various localities in the northern hemisphere, from the southern Arctic to Madeira and Cape Cod in the Atlantic Ocean, and to southern California and Singapore in the Pacific Ocean (Gibson, 1995). The short and ultimately vague description of the species' external morphology has led to the assumption that *C. marginatus* was a cosmopolitan species with a wide geographic distribution, but growing molecular evidence suggests that specimens identified as *C. marginatus* represent a complex of cryptic species (Kajihara, 2020; Sundberg et al., 2010). Unfortunately, there is no known type specimen for *C. marginatus* so a proper taxonomic assessment including a comparison to the holotype is not possible.

In this study, we use species delimitation and phylogenetic analyses to evaluate whether *C. marginatus* is a truly cosmopolitan species, or if alternatively, it represents a complex of cryptic species. We examined a multilocus dataset including two mitochondrial markers (*16S rRNA*, *COI*) and three nuclear markers (*18S rRNA*, *28S rRNA*, *H3*) from 25 specimens morphologically ascribed to *C. marginatus* collected in 10 different locations around the world and 19 specimens of closely related *Cerebratulus* species.

Materials and methods

Specimen collection and molecular analyses

Specimens were collected at tidal flats of northwestern Spain: Isla de La Toja, O Grove, 42°29′18″N, 8°51′25″W (1 specimen) and Combarro, 42°26′47″N, 8°42′18″W (6 specimens) in 2011, 2018, and 2019 (Fig. 1); and northwestern USA: False Bay, San Juan Island, WA, 48°29′15.1″N, 123°04′08.2″W (1 specimen) and Yaquina Bay, Newport, OR, 44°37′13.3″N, 124°02′21.1″W (2 specimens) (Table 1); specimens were either preserved in absolute EtOH or in RNA*later* Stabilization Solution (Invitrogen, USA). DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's protocol. DNA concentration and integrity were measured in a NanoDrop 8000 (Thermo Fisher Scientific, USA). PCR amplification and sequencing were carried out largely following methods described by previous authors (Andrade et al., 2012; Hookabe et al., 2019). For PCR, either of the three heat-resistant DNA polymerases Ex*Taq* (TaKaRa, Japan), KOD FX Neo (Toyobo, Japan), or VWR Red Taq (VWR International bvba/sprl, Belgium) were used and partial cytochrome c oxidase subunit I (*COI*) sequences were obtained for 8 specimens (Table 1) using the LCO1490 and HCO2198 primers (Folmer et al., 1994). The PCR program consisted of 5 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C; and 10 min at 72 °C. PCR products were visualized in 1.5% agarose gel electrophoresis, run at 90 V for 40 min. Sequencing was conducted on an ABI 3730XL DNA Analyzer (Applied Biosystems, USA) at the Molecular Core Labs (Sequencing Facility) of the Natural History Museum of London, using the forward and reverse primers mentioned above. For four of the specimens, partial sequences of the *16S rRNA* were sequenced, and for two specimens *18S rRNA*, *28S rRNA*, and *histone H3* genes were also determined (Table 1). In addition

Figure 1. Specimens identified as *Cerebratulus marginatus* based on morphological features, collected in northwestern Spain. (A) Anterior, dorsal view of a dark brown coloured specimen. (B) Anterior end, dorsal view of a specimen with lighter greyish-brown colouration. (C) Anterior end, dorso-lateral view of a light brown coloured specimen, showing lateral cephalic slits extending to mouth. (D) Anterior end, dorsal view, and posterior intestinal region, ventral view, of a lightly coloured specimen. (E) Anterior end, ventral view of lightly coloured specimen, showing mouth opening, lateral cephalic slits, and translucent edges. (F) Dorsal view of anaesthetized specimen; posterior end missing.

to the primers listed in Andrade et al. (2012), the following additional primers were used in sequencing reactions for the *18S rRNA* gene: 3 F, 5 R (Giribet et al., 1996), F2 and F4 (Yamaguchi & Endo, 2003). Newly determined sequences have been deposited in DDBJ/EMBL/GenBank and the corresponding accession numbers are listed in Table 1.

Phylogenetic analyses

To evaluate the phylogenetic relationships between *C. marginatus* and closely related species, we analysed a multilocus dataset including newly generated sequences for two mitochondrial markers (*16S rRNA*, *COI*) and three nuclear markers (*18S rRNA*, *28S rRNA*, *H3*) of 10 specimens of *C. marginatus*. The newly generated

Table 1. List of species, sample localities, and DDBJ/EMBL/GenBank accession numbers for the five gene markers used in the phylogenetic and species-delimitation analyses. Shaded rows indicate newly generated sequences.

Species	**Locality**	***16S***	***COI***	***18S***	***28S***	***H3***
Cerebratulus cf. *marginatus 1*	Combarro, Spain	—	MT808210	—	—	—
Cerebratulus cf. *marginatus 2*	O Grove, Spain	MT809106	MT808211	MT809112	MT809114	MW582893
Cerebratulus cf. *marginatus 3*	Combarro, Spain	—	MT808212	—	—	—
Cerebratulus cf. *marginatus 4*	Combarro, Spain	—	MT808213	—	—	—
Cerebratulus cf. *marginatus 5*	Combarro, Spain	—	MT808214	—	—	—
Cerebratulus cf. *marginatus 6*	Combarro, Spain	—	MT808215	—	—	—
Cerebratulus cf. *marginatus 7*	Combarro, Spain	MT809107	MT808216	MT809113	MT809115	MW582894
Cerebratulus cf. *marginatus 8*	Washington, USA	—	MW553859	—	—	—
Cerebratulus cf. *marginatus 9*	Oregon, USA	MW553279	—	—	—	—
Cerebratulus cf. *marginatus 10*	Oregon, USA	MW553280	—	—	—	—
Cerebratulus cf. *marginatus 11*	Oregon, USA	KU197408	KU197740	—	KU365683	—
Cerebratulus cf. *marginatus 12*	Oregon, USA	KU197409	KU197741	—	—	—
Cerebratulus marginatus 1	Wasington, USA	AJ436821	AJ436931	—	AJ436876	AJ436978
Cerebratulus marginatus 2	Wasington, USA	JF277576	HQ848575	JF293042	HQ856858	JF277729
Cerebratulus marginatus 3	Tjärnö, Sweden	—	FJ811493	—	—	—
Cerebratulus marginatus 4	Uddevalla, Sweden	—	KU839799	—	—	—
Cerebratulus marginatus 5	Uddevalla, Sweden	—	KU839798	—	—	—
Cerebratulus marginatus 6	Bonden, Sweden	—	FJ811496	—	—	—
Cerebratulus marginatus 7	Väderöarna, Sweden	—	FJ811495	—	—	—
Cerebratulus marginatus 8	Bua, Sweden	—	KU839958	—	—	—
Cerebratulus marginatus 9	Bua, Sweden	—	KU839906	—	—	—
Cerebratulus marginatus 10	Kristineberg, Sweden	—	FJ811497	—	—	—
Cerebratulus marginatus 11	Kristineberg, Sweden	—	FJ811499	—	—	—
Cerebratulus marginatus 12	Bua, Sweden	—	KU839908	—	—	—
Cerebratulus marginatus 13	Kristineberg, Sweden	—	FJ811498	—	—	—
Cerebratulus albifrons 1	Oregon, USA	KU197370	KU197714	—	—	—
Cerebratulus albifrons 2	Oregon, USA	KU197371	KU197715	—	—	—
"*Cerebratulus* cf. *bengalensis*" *1*	Sundarbans, India	—	KY991481	—	—	—
"*Cerebratulus* cf. *bengalensi*" *2*	Sundarbans, India	—	KY991482	—	—	—
Cerebratulus californiensis 1	Oregon, USA	KU197395	KU197728	—	KU365682	—
Cerebratulus californiensis 2	Oregon, USA	KU197396	KU197729	—	KU365714	—
Cerebratulus fuscus 1	Sweden	—	KU840067	—	—	—
Cerebratulus fuscus 2	Sweden	—	KU840068	—	—	—
Cerebratulus herculeus 1	USA	—	EF124964	—	—	—
Cerebratulus herculeus 2	USA	—	EF124991	—	—	—
Cerebratulus herculeus 3	Washington, USA	MW621009	MW621973	—	—	—
Cerebratulus lacteus 1	Florida, USA	JF277575	HQ848576	JF293044	HQ856857	JF277728
Cerebratulus lacteus 2	Montpellier, France	KX261740	KX261791	—	EF124924	KR336528
Cerebratulus leucopsis	Panama	KF935467	KF935517	KF935300	KF935356	KF935412
Cerebratulus lineolatus	Florida, USA	—	MK047689	—	EF178493	—
Cerebratulus longiceps	Oregon, USA	KX296733		—	KX342095	—
Cerebratulus orochi	Hokkaido, Japan	LC538101	LC538102	LC538103	LC538104	LC538105
Cerebratulus sp.	Washington, USA	MW621008	MW621974	—	—	—
Outgroups						
Dushia cf. *nigra*	—	LC389832	LC389867	LC389840	LC389844	LC389851
Kulikovia montgomeryi	Washington, USA	KU197410	KU197742	—	EF178489	—
Micrura chlorapardalis	—	KF935459	KF935512	KF935292	KF935348	KF935404
Micrura rubramaculosa	—	KF935460	KF935513	KF935293	KF935349	KF935405
P. zhanjiangensis	—	KC407657	KC602686	—	KC522310	—

sequences were combined with data from 15 additional specimens available in GenBank. We included species from the "*Cerebratulus* clade" as proposed by Chernyshev and Polyakova (2019) in addition to *Polydendrorhynchus zhanjiangensis* (Yin & Zeng, 1984), which was also suggested as a member of this clade (Hookabe et al., 2019, 2021), *Kulikovia montgomeryi*, which was recently transferred from the genus *Cerebratulus* (Chernyshev et al., 2018), and several outgroups. GenBank accession numbers for all taxa included in the analysis are listed in Table 1.

Sequences were aligned with MAFFT v7 under default parameters (Katoh & Standley, 2013) and all mitochondrial and nuclear datasets were concatenated in SeaView v4.5.4 (Gouy et al., 2010). The best-fitting model of nucleotide substitution for each marker was selected under the Akaike information criterion (AIC) as implemented in jModeltest v2 (Darriba et al., 2012). Datasets were partitioned for each marker and a maximum likelihood analysis (ML) was run in RAxML v7.4.2 (Stamatakis, 2006) with 1,000 replicates to estimate bootstrap support values.

Species delimitation analyses

Species delimitation analyses were used to infer putative species boundaries and test the validity of *C. marginatus* cosmopolitanism, using the *COI* dataset and including all specimens. We employed four different methods: Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), Poisson Tree Processes (PTP) (Zhang et al., 2013), Bayesian implementation of the PTP model (bPTP) (Zhang et al., 2013), and statistical parsimony (Templeton et al., 1992). The ABGD analysis was performed on the online server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using the *COI* multiple sequence alignment and default settings. The PTP and bPTP analyses were carried out with default parameters on the bPTP web server (http://species.h-its.org/ptp/) using a ML tree generated with the *COI* dataset. The statistical parsimony analysis was done using TCS v1.2.1 (Clement et al., 2000), with the connection limit set to 95%. Genetic distances between all taxa included in the analyses were calculated with MEGA ver. 7.0.26 (Kumar et al., 2016) using uncorrected *p*-distance and the Kimura 2-parameter model (Kimura, 1980).

Results

Phylogenetic reconstruction

Final alignments of the nuclear markers *28S rRNA*, *18S rRNA*, and *histone H3* contained 1187 bp, 1826 bp, and 330 bp, respectively. The final alignments for the mitochondrial markers *16S rRNA* and *COI* had 523 bp and 656 bp, respectively. The best-fitting nucleotide substitution model for the nuclear and mitochondrial datasets under AIC was the General Time Reversible (GTR) with gamma-distributed rates across sites and a proportion of invariant sites (GTR + G + I). Results from the ML analysis (Fig. 2) of the five concatenated loci show that *C. marginatus* is a polyphyletic group since *C. albifrons* Coe, 1901, '*C.* cf. *bengalensis*', *C. californiensis* Coe, 1905, *C. fuscus* (McIntosh, 1874), *C. herculeus* Coe, 1901, *C. lacteus* (Leidy, 1851), *C. leucopsis* (Coe, 1901), *C. lineolatus* Coe, 1905, *C. longiceps* Coe, 1901, *C. orochi* Kajihara, 2020, *Dushia* cf. *nigra* (Stimpson, 1855), *Kulikovia montgomeryi* (Coe, 1901) (previously *Cerebratulus montgomeryi*) and *Polydendrorhynchus zhanjiangensis* are all found nested within the putative *C. marginatus*. Our analysis recovered seven groups among the species identified as *C. marginatus* which are labelled Lineages 1–7, although only four lineages (1, 4, 5 and 6) are well supported and are represented by several specimens (Fig. 2). Lineage 1 contains species identified as *C. marginatus* distributed in the Pacific Ocean and collected from the west coast of the USA, closely related to the recently described *C. orochi* from northern Japan. Lineages 2, 3, 4, and 5 include specimens ascribed to *C. marginatus* collected around the coasts of Sweden, distributed in the North Sea (all lineages) and the Baltic Sea (Lineage 5) (Fig. 2). Lineage 2 is represented by a single individual and falls in a well-supported clade with the species *C. californiensis* and *C. herculeus* from the Pacific Ocean, whereas Lineage 3, also represented by a single specimen, forms a well-supported clade with *C. fuscus* from Sweden (Fig. 2). Lineages 4 and 5 form well-supported distinct clades and appear closely related to the species *C. longiceps* and *C. montgomeryi* from the Pacific Ocean, as well as *C. lineolatus* from the Atlantic Ocean (Fig. 2). Lastly, Lineages 6 and 7 correspond to species assigned to *C. marginatus* from the Atlantic Ocean, collected in the northwest coast of Spain and appear to be closely related to each other, and to a specimen of *C. lacteus* from the Atlantic coast of Florida (Fig. 2).

Species delimitation in the *Cerebratulus marginatus* species complex

To determine putative species boundaries and evaluate whether *C. marginatus* represents a complex of cryptic species, we used the complete *COI* dataset and four different species delimitation methods (i.e., ABGD, bPTP, PTP, and TCS). All four methods provided congruent results identifying seven distinct lineages within the putative *C. marginatus* (Fig. 3), which corresponded to the lineages obtained in the phylogenetic analysis: one lineage from the Pacific Ocean (Lineage 1), four lineages from the North and Baltic Seas along the Swedish coast (Lineages 2–5), and two lineages from the Atlantic Ocean (Lineages 6, 7), although similarly to the phylogenetic results, only lineages 1, 4, 5 and 6 are well supported with bootstrap values > 0.95 (Fig. 3). The majority of the methods identified seven lineages, although there were a few discrepancies. For example, all methods recognized Lineage 4 as recovered in the phylogeny comprised by a single species, except for bPTP which identified two different species within Lineage 4, one represented by *C. marginatus GB5* and *C. marginatus GB6*, and the other one represented by C. *marginatus GB7* (Fig. 3). Similarly, all analyses

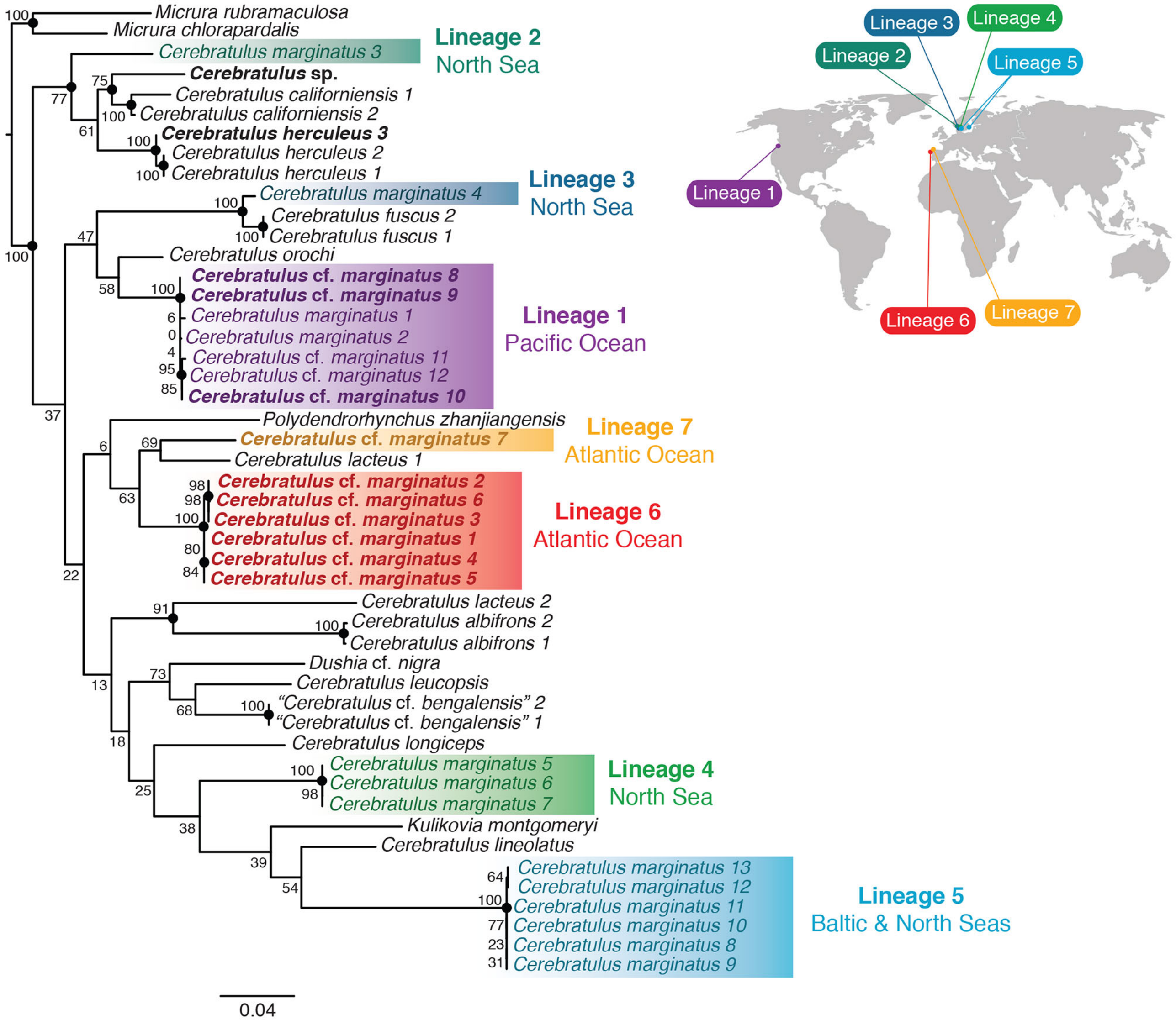

Figure 2. Phylogenetic tree of the genus *Cerebratulus* inferred from the maximum likelihood analysis of the concatenated dataset (*18S rRNA*, *28S rRNA*, *H3*, *16S rRNA*, *COI*). Newly sequenced specimens are denoted in bold and closed circles indicate bootstrap support values > 75%. Inset shows geographic distribution of the lineages identified in the phylogenetic analysis.

identified Lineage 5 as recovered in the phylogenetic analysis except bPTP which found each of the specimens comprising the clade as a distinct species. Lineage 3, represented in the phylogeny by *C. marginatus GB4*, was also recovered as a single species by all methods except ABGD, which identified this individual as belonging to the same species as two specimens classified as *C. fuscus* (Fig. 3). Additionally, we calculated the genetic distances among all specimens using the *COI* dataset (Supplementary Material Table S1). The p-distance and K2P values among any two specimens comprising the lineages 1, 4, 5 and 6 are very low (0–0.012) while the distances between any two specimens from two different lineages are much higher (0.18–0.29) and similar to the distances observed between different *Cerebratulus* species and species belonging to different genera (see Supplementary Material Table S1). This provides further evidence that lineages 1, 4, 5 and 6 represent independent lineages that might correspond to cryptic species.

Discussion

It is common practice to assign species names to nemerteans based almost exclusively on morphological

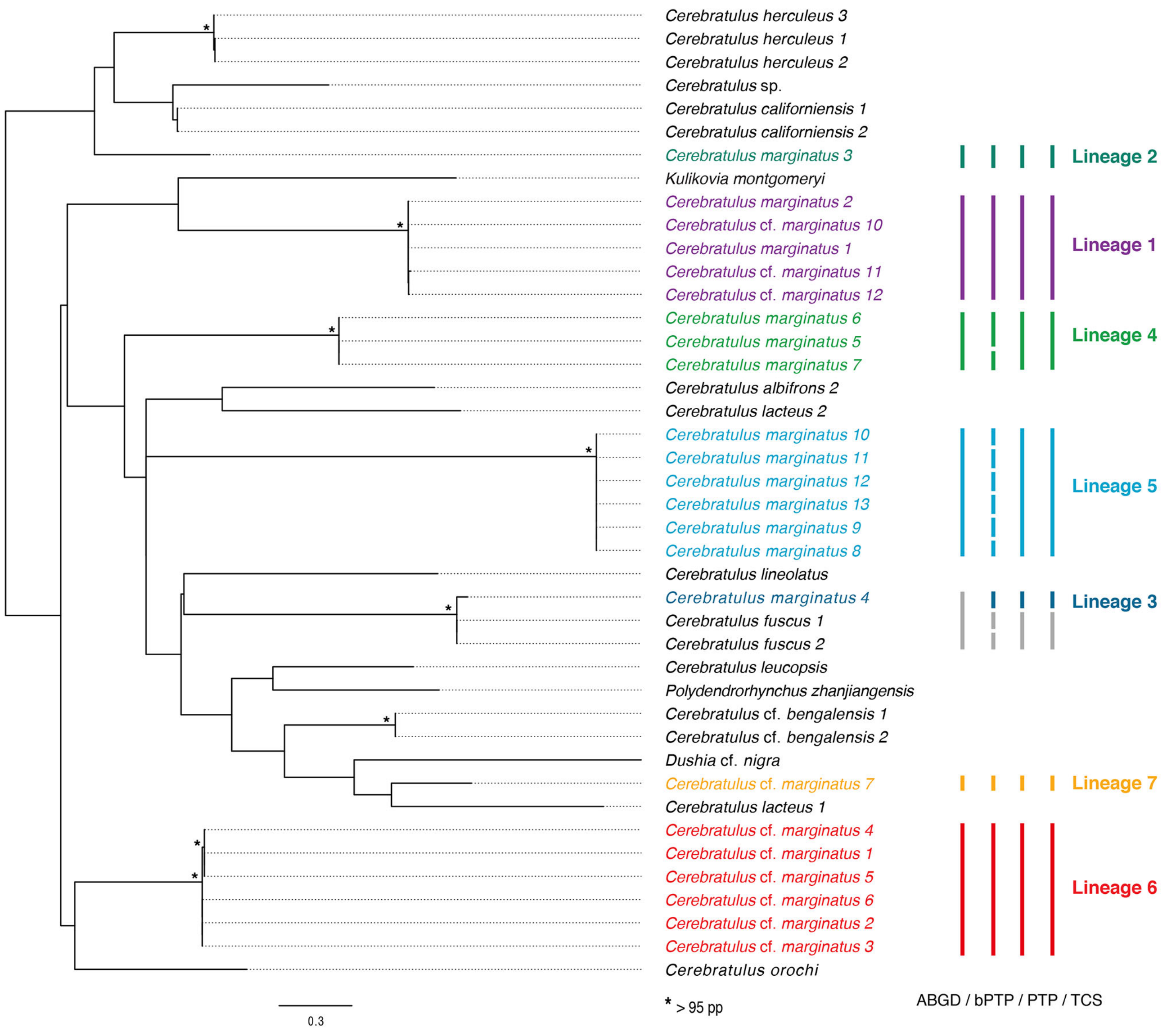

Figure 3. Maximum likelihood tree generated based on 508-bp partial *COI* sequences from 42 *Cerebratulus* specimens; asterisks indicate bootstrap support values > 95%. Vertical bars show results from the four species-delimitation analyses (ABGD, bPTP, PTP, and TCS).

examinations of their external anatomy. In particular, specimens with a slightly pointed head, horizontal lateral cephalic slits extending to the mouth, no eyes, and a dorsoventrally flattened body with translucent or white edges and a caudal cirrus, have been commonly assigned to the species *C. marginatus* (e.g., Chernyshev, 2008; Iwata, 1957; Yamaoka, 1940). However, previous authors have pointed out that many external characters, including colouration, cannot be used to differentiate species as these traits are either highly variable among populations and even throughout the life cycle of a single individual (Berg, 1985; Kang et al., 2015; Leasi & Norenburg, 2014; Manchenko & Kulikova, 1996; Strand & Sundberg, 2005; Sundberg et al., 2009, 2010; Tulchinsky et al., 2012), or conversely, are not variable enough to distinguish cryptic species (Hiebert & Maslakova, 2015; Ikenaga et al., 2021; Krämer et al., 2017). Our results combining phylogenetic and species delimitation analyses suggest that samples seemingly identifiable as *C. marginatus* are not a single cosmopolitan species or a distinct unique evolutionary lineage, but rather a complex of at least four well-supported lineages that might represent cryptic species with narrower geographic ranges distributed in different areas (Figs 2, 3). In addition, the phylogenetic analysis shows that *C. marginatus* as currently delineated, is a polyphyletic grouping with several other species of the genus *Cerebratulus* and species from other genera nesting within the clade (Fig. 2).

Previous studies using statistical parsimony analysis with *COI* sequences suggested that several species in the genus *Cerebratulus*, including *C. marginatus* specimens from different localities along the coast of Sweden represent a complex of cryptic species, and furthermore, that the Swedish samples represent three or four different biological entities (Sundberg et al., 2010). Although these results cannot be interpreted as species limits, they clearly show that many diagnostic morphological characters commonly used to differentiate *Cerebratulus* species do not correspond to evolutionary lineages, suggesting these morphological differences might be due to intraspecific variation or developmental stage (Sundberg et al., 2010). Our study combining phylogenetic analysis and species delimitation methods confirms these hypotheses, revealing that specimens ascribed to *C. marginatus* available in GenBank from the coasts of Sweden might correspond to four genetically distinct species. Our results identified two additional putative lineages among the newly collected material from the northwestern coast of Spain, and another genetically distinct entity with distribution restricted to the northwestern coast of the USA (Fig. 2). Although our results identified seven putative independent lineages within the *Cerebratulus marginatus* samples, only four had enough representative specimens and were well supported in the phylogenetic and species delimitation analyses to be considered as putative cryptic species, namely lineages 1, 4, 5 and 6 (Figs 2, 3).

After identifying a complex of cryptic species and delimiting species boundaries, the newly identified species should be formally named and described in the literature, so they are available for future research. However, we consider that a new species should meet certain requisites before it can be officially instated as a new taxon. Specifically, any newly identified species should form a distinct evolutionary lineage, with robust support in a phylogenetic analysis with a large number of specimens sequenced, as well as demonstrate identifiable, distinctive diagnostic features (e.g., morphological, ecological) (Álvarez-Campos et al., 2017a; Kawauchi & Giribet, 2010). Unfortunately, the putative *Cerebratulus* species identified here do not meet these requirements and therefore, we prefer not taking any taxonomic action until new material from the type locality (i.e., Northern Adriatic Sea) and from additional distribution areas becomes available for molecular and morphological examination.

The newly discovered lineages may not necessarily be new to science but may represent taxa that were once named but excessively synonymized with *C. marginatus*. According to Gibson (1995), these nominal species include [type locality in square brackets]: *Avenardia priei* Giard, 1878 [Pouliguen, France]; *Cerebratulus grandis* Sars in Jensen, 1878 [Florø]; *Cerebratulus spraguei* Girard, 1893 [Atlantic coast of the USA]; *Gordius fragilis* Dalyell, 1853 [Scotland]; *Lineus beattiaei* Gray, 1857 [Scotland]; *Meckelia olivacea* Rathke, 1843 [Norway]; *Meckelia somatotomus* Leuckart, 1828 [Étang de Thau, France]; and *Serpentaria beryi* Baird, 1866 [Singapore]. Future studies, especially with topotype material of these nominal species, may 'resurrect' these names (cf. Mendes et al., 2021).

Our results have important implications for nemertean taxonomy, biological surveys, and biodiversity management projects. Identifications based only on external characters will often be erroneous, and importantly, identifications based on comparison to publicly available DNA barcodes may also be inaccurate. There are currently 25 specimens in GenBank (15 previously published, 10 newly sequenced in this study) identified as *C. marginatus*, but in fact they seem to represent a complex of at least four cryptic species (Figs 2, 3). Therefore, until new material from the type locality becomes available to identify the true *C. marginatus* and clarify the taxonomic status of the genus, we recommend barcoding and depositing a voucher of all putative *C. marginatus* specimens, labelling them as *C.* cf. *marginatus* and indicating its affinity to one of the lineages described here. This will allow us to identify possible diagnostic features and formally describe the new species identified here in the future, thereby advancing nemertean taxonomy.

Our study represents yet another example of cryptic speciation in ribbon worms (Hookabe et al., 2019; Rogers et al., 1995; Sundberg et al., 2010), which is in fact increasingly becoming the rule rather than the exception among marine invertebrates in general (Hutchings & Kupriyanova, 2018; Kawauchi & Giribet, 2010). It also highlights the importance of including molecular data when inferring species boundaries, since the interpretation of morphological characters alone from a putative species often leads to underestimating real diversity (Hiebert & Maslakova, 2015; Hookabe et al., 2019; Kajihara, 2020; Rogers et al., 1995; Strand & Sundberg, 2005; Sundberg et al., 2010). Therefore, in addition to morphological data, the incorporation of molecular information, as well as ecological, physiological, or reproductive data when possible, should be essential to accurately delimit species in nemertean taxonomic research (Hiebert & Maslakova, 2015; Hookabe et al., 2019; Leasi & Norenburg, 2014; Rogers et al., 1995; Strand & Sundberg, 2005). Adopting this practice will improve nemertean taxonomy and provide a much more accurate picture of the actual species diversity and geographic distribution of this typically neglected phylum.

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No potential conflict of interest was reported by the author(s).

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ORCID

Aida Verdes http://orcid.org/0000-0002-9193-9253
Hiroshi Kajihara http://orcid.org/0000-0001-6510-9355

References

Ahrens, J., Borda, E., Barroso, R., Paiva, P., Campbell, A., Wolf, A., Nugues, M. M., Rouse, G. W., & Schulze, A. (2013). The curious case of *Hermodice carunculata* (Annelida: Amphinomidae): evidence for genetic homogeneity throughout the Atlantic Ocean and adjacent basins. *Molecular Ecology*, *22*, 2280–2291. https://doi.org/10.1111/mec.12263

Álvarez-Campos, P., Giribet, G., & Riesgo, A. (2017). The *Syllis gracilis* species complex: A molecular approach to a difficult taxonomic problem (Annelida, Syllidae). *Molecular Phylogenetics and Evolution*, *109*, 138–150. https://doi.org/10.1016/j.ympev.2016.12.036

Álvarez-Campos, P., Giribet, G., San Martín, G., Rouse, G. W., & Riesgo, A. (2017). Straightening the striped chaos: systematics and evolution of *Trypanosyllis* and the case of its pseudocryptic type species *Trypanosyllis krohnii* (Annelida, Syllidae). *Zoological Journal of the Linnean Society*, *179*, 492–540. https://doi.org/10.1111/zoj.12443

Ament-Velásquez, S. L., Figuet, E., Ballenghien, M., Zattara, E. E., Norenburg, J. L., Fernández-Álvarez, F. A., Bierne, J., Bierne, N., & Galtier, N. (2016). Population genomics of sexual and asexual lineages in fissiparous ribbon worms (*Lineus*, Nemertea): hybridization, polyploidy and the Meselson effect. *Molecular Ecology*, *25*, 3356–3369. https://doi.org/10.1111/mec.13717

Andrade, S. C. S., Strand, M., Schwartz, M., Chen, H., Kajihara, H., von Döhren, J., Sun, S., Junoy, J., Thiel, M., Norenburg, J. L., Turbeville, J. M., Giribet, G., & Sundberg, P. (2012). Disentangling ribbon worm relationships: multi-locus analysis supports traditional classification of the phylum Nemertea. *Cladistics*, *28*, 141–159. https://doi.org/10.1111/j.1096-0031.2011.00376.x

Baird, W. (1866 *Description of a new species of monoecious worm, belonging to the class Turbellaria and genus Serpentaria* [Paper presentation].Proceedings of the Zoological Society of London, 101–102.

Berg, G. (1985). Studies on *Nipponnemertes* Friedrich (Nemertini, Hoplonemertini). II Taxonomy of *Nipponnemertes pulcher* (Johnston) and some other species. *Zoologica Scripta*, *14*, 239–246. https://doi.org/10.1111/j.1463-6409.1985.tb00194.x

Bourque, D., Miron, G., & Landry, T. (2001). Predation on soft-shell clams (*Mya arenaria*) by the nemertean *Cerebratulus lacteus* in Atlantic Canada: Implications for control measures. *Hydrobiologia*, *456*, 33–44. https://doi.org/10.1023/A:1013061900032

Carr, C. M., Hardy, S. M., Brown, T. M., Macdonald, T. A., & Hebert, P. D. N. (2011). A tri-oceanic perspective: DNA barcoding reveals geographic structure and cryptic diversity in Canadian polychaetes. *PLoS One*, *6*, e22232. https://doi.org/10.1371/journal.pone.0022232

Chernyshev, A. V. (2008). Fauna of nemerteans of Amursky Bay and the adjacent areas. In: Lutaenko, K. A., & Vaschenko, M. A. (Eds) *Ecological studies and the state of ecosystem of Amursky Bay and the estuarine zone of the Razdolnaya River (Sea of Japan)*. (Vol.1 pp. 267–278). Dalnauka.

Chernyshev, A. V., & Polyakova, N. E. (2019). Nemerteans from the deep-sea expedition KuramBio II with descriptions of three new hoplonemerteans from the Kuril-Kamchatka Trench. *Progress in Oceanography*, *178*, 102148. https://doi.org/10.1016/j.pocean.2019.102148

Chernyshev, A. V., Polyakova, N. E., Turanov, S. V., & Kajihara, H. (2018). Taxonomy and phylogeny of *Lineus torquatus* and allies (Nemertea, Lineidae) with descriptions of a new genus and a new cryptic species. *Systematics and Biodiversity*, *16*, 55–68. https://doi.org/10.1080/14772000.2017.1317672

Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, *9*, 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Coe, W. R. (1901 *Papers from the Harriman Alaska Expedition. XX. The nemerteans* [Paper presentation].Proceedings of the Washington Academy of Sciences, *3*, 1–110.

Coe, W. R. (1905). Nemerteans of the west and northwest coasts of America. *Bulletin of the Museum of Comparative Zoölogy at Harvard College*, *47*, 1–318.

Dalyell, J. G. (1853). *The powers of the Creator*. Van Voorst.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). JModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, *9*, 772 https://doi.org/10.1038/nmeth.2109

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Giard, A. (1878). Sur l'*Avenardia priei*, némertien géant de la côte occidentale de France. *Compte Rendu Hebdomadaire Des Séances de L'académie Des Sciences*, *87*, 72–75.

Gibson, R. (1982). *British nemerteans: keys and notes for the identification of the species*. Cambridge University Press.

Gibson, R. (1995). Nemertean genera and species of the world: an annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats and recorded zoogeographic distribution. *Journal of Natural History*, *29*, 271–561. https://doi.org/10.1080/00222939500770161

Girard, C. (1893). Recherches sur les planariés et les némertiens de l'Amérique du Nord. *Annales Des Sciences Naturelles, Septiéme Série, Zoologie*, *15*, 145–310.

Giribet, G., Carranza, S., Baguñà, J., Riutort, M., & Ribera, C. (1996). First molecular evidence for the existence of a Tardigrada + Arthropoda clade. *Mol Biol Evol*, *13*, 76–84. https://doi.org/10.1093/oxfordjournals.molbev.a025573

Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*, *27*, 221–224. https://doi.org/10.1093/molbev/msp259

Gray, J. E. (1857). *Notice of a large species of Lineus?, taken on the coast near Montrose* [Paper presentation]. Proceedings of the Zoological Society of London, 210. https://doi.org/10.1111/j.1096-3642.1857.tb01228.x

Hiebert, T., & Maslakova, S. (2015). Integrative taxonomy of the *Micrura alaskensis* Coe, 1901 species complex (Nemertea: Heteronemertea), with descriptions of a new genus *Maculaura* gen. nov. and four new species from the NE Pacific. *Zoological Science*, *32*, 615–637. https://doi.org/10.2108/zs150011

Hookabe, N., Schwartz, M. L., Kajihara, H., & Norenburg, J. L. (2019). Molecular systematics of the heteronemertean genus *Dushia* (Nemertea, Pilidiophora), with descriptions of *D. wijnhoffae* sp. nov. and *D. nigra* species complex comb. nov. *Zootaxa*, *4691*, zootaxa.4691.4.2–358. https://doi.org/10.11646/zootaxa.4691.4.2

Hookabe, N., Xu, C.-M., Tsuyuki, A., Jimi, N., Sun, S.-C., & Kajihara, H. (2021). A new nemertean with a branched proboscis, *Gorgonorhynchus citrinus* sp. nov. (Nemertea: Pilidiophora), with molecular systematics of the genus. *Invertebrate Systematics*, *35*, 350–359. https://doi.org/10.1071/IS20057

Hutchings, P., & Kupriyanova, E. (2018). Cosmopolitan polychaetes - Fact or fiction? Personal and historical perspectives. *Invertebrate Systematics*, *32*, 1–9. https://doi.org/10.1071/IS17035

Ikenaga, J., Kajihara, H., & Yoshida, M. (2021). *Kulikovia alborostrata* and *Kulikovia fulva* comb. nov. (Nemertea: Heteronemertea) are sister species with prezygotic isolating barriers. *Zoological Science*, *38*, 193–202. https://doi.org/10.2108/zs200112

Iwata, F. (1957). Nemerteans from Sagami Bay. *Publications from the Akkeshi Marine Biological Station*, *7*, 1–31.

Jensen, O. S. (1878). *Turbellarier ved Norges Vestkyst*. J. W. Eides.

Kajihara, H. (2020). Redescription of *Cerebratulus marginatus* auct. (Nemertea: Pilidiophora) from Hokkaido, Japan, as a new species. *Zootaxa*, *4819*, zootaxa.4819.2.4–315. https://doi.org/10.11646/zootaxa.4819.2.4

Kajihara, H., Chernyshev, A. V., Sun, S.-C., Sundberg, P., & Crandall, F. B. (2008). Checklist of nemertean genera and species published between 1995 and 2007. *Species Diversity*, *13*, 245–274. https://doi.org/10.12782/specdiv.13.245

Kang, X. X., Fernández-Álvarez, F. Á., Alfaya, J. E., Machordom, A., Strand, M., Sundberg, P., & Sun, S. C. (2015). Species diversity of *Ramphogordius sanguineus*/*Lineus ruber*-like nemerteans (Nemertea: Heteronemertea) and geographic distribution of *R. sanguineus*. *Zoological Science*, *32*, 579–589. https://doi.org/10.2108/zs150064

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, *30*, 772–780. https://doi.org/10.1093/molbev/mst010

Kawauchi, G. Y., & Giribet, G. (2010). Are there true cosmopolitan sipunculan worms? A genetic variation study within *Phascolosoma perlucens* (Sipuncula, Phascolosomatidae). *Marine Biology*, *157*, 1417–1431. https://doi.org/10.1007/s00227-010-1402-z

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*, 111–120. https://doi.org/10.1007/BF01731581

Knowlton, N. (2000). Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, *420*, 73–90. https://doi.org/10.1023/A:1003933603879

Krämer, D., Schmidt, C., Podsiadlowski, L., Beckers, P., Horn, L., & von Döhren, J. (2017). Unravelling the *Lineus ruber*/*viridis* species complex (Nemertea, Heteronemertea). *Zoologica Scripta*, *46*, 111–126. https://doi.org/10.1111/zsc.12185

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*, 1870–1874. https://doi.org/10.1093/molbev/msw054

Leasi, F., & Norenburg, J. (2014). The necessity of DNA taxonomy to reveal cryptic diversity and spatial distribution of meiofauna, with a focus on Nemertea. *PLoS One*, *9*, e104385.10.1371/journal.pone.0104385 https://doi.org/10.1371/journal.pone.0104385

Leidy, J. (1851 *Helminthological contributions—No. 3* [Paper presentation]. Proceedings of the Academy of Natural Sciences of Philadelphia, *5*, 239–244.

Leuckart, F. S. (1828). *Breves animalium quorundam maxima ex parte marinorum descriptiones*. A. Osswald.

Manchenko, G. P., & Kulikova, V. I. (1996). Enzyme and colour variation in the hoplonemertean *Tetrastemma nigrifrons* from the Sea of Japan. *Hydrobiologia*, *337*, 69–76. https://doi.org/10.1007/BF00028507

McIntosh, W. C. (1874). *A monograph of the British annelids. Part I. The nemerteans*. Ray Society.

Mendes, C., Delaney, P., Turbeville, J. M., Hiebert, T., & Maslakova, S. (2021). Redescription of *Emplectonema* *viride*—a ubiquitous intertidal hoplonemertean found along the West Coast of North America. *ZooKeys*, *1031*, 1–17. [in press]. https://doi.org/10.3897/zookeys.1031.59361

Meneghini, G. (1847). *Osservazioni postume di zoologia Adriatica del professore Stefano Andrea Renier*. G. Checchini.

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, automatic barcode gap discovery for primary species delimitation. *Mol Ecol*, *21*, 1864–1877. https://doi.org/10.1111/j.1365-294X.2011.05239.x

Rathke, H. (1843). Beiträge zur Fauna Norwegens. *Nova Acta Academiae Caesareae Leopoldino-Carolinae Germanicae Naturae Curiosorum*, *20*, 1–264.

Renier, S. A. (1804). *Prospetto della Classe dei Vermi, nominati el ordinati secondo il Sistemo de Bosc*. [Page proofs of introductory book text in folio sheets, never published]

Rogers, A. D., Thorpe, J. P., & Gibson, R. (1995). Genetic evidence for the occurrence of a cryptic species with the littoral nemerteans *Lineus ruber* and *L. viridis* (Nemertea: Anopla). *Marine Biology*, *122*, 305–316. https://doi.org/10.1007/BF00348944

Rowell, T. W., & Woo, P. (1990). Predation by the nemertean worm *Cerebratulus lacteus* Verrill, on the soft-shell clam *Mya arenaria* Linnaeus, 1758, and its apparent role in the destruction of a clam flat. *Journal of Shellfish Research*, *9*, 291–297.

Sanford, E., & Kelly, M. W. (2011). Local adaptation in marine invertebrates. *Annual Review of Marine Science*, *3*, 509–535. https://doi.org/10.1146/annurev-marine-120709-142756

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics (Oxford, England)*, *22*, 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Stimpson, W. (1855 *Descriptions of some of the new marine Invertebrata from the Chinese and Japanese seas* [Paper presentation]. Proceedings of the Academy of Natural Sciences of Philadelphia, *7*, 375–384.

Strand, M., & Sundberg, P. (2005). Delimiting species in the hoplonemertean genus *Tetrastemma* (phylum Nemertea): morphology is not concordant with phylogeny as evidenced from mtDNA sequences. *Biological Journal of the Linnean Society*, *86*, 201–212. https://doi.org/10.1111/j.1095-8312.2005.00535.x

Sundberg, P., Thuroczy Vodoti, E., & Strand, M. (2010). DNA barcoding should accompany taxonomy - the case of *Cerebratulus* spp (Nemertea). *Molecular Ecology Resources*, *10*, 274–281. https://doi.org/10.1111/j.1755-0998.2009.02774.x

Sundberg, P., Vodoti, E. T., Zhou, H., & Strand, M. (2009). Polymorphism hides cryptic species in *Oerstedia dorsalis* (Nemertea, Hoplonemertea). *Biological Journal of the Linnean Society*, *98*, 556–567. https://doi.org/10.1111/j.1095-8312.2009.01310.x

Templeton, A. R., Crandall, K. A., & Sing, C. F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, *132*, 619–633.

Tulchinsky, A. Y., Norenburg, J. L., & Turbeville, J. M. (2012). Phylogeography of the marine interstitial nemertean *Ototyphlonemertes parmula* (Nemertea, Hoplonemertea) reveals cryptic diversity and high dispersal potential. *Marine Biology*, *159*, 661–674. https://doi.org/10.1007/s00227-011-1844-y

Yamaguchi, S., & Endo, K. (2003). Molecular phylogeny of Ostracoda (Crustacea) inferred from *18S* ribosomal DNA sequences: implication for its origin and diversification. *Marine Biology*, *143*, 23–38. https://doi.org/10.1007/s00227-003-1062-3

Yamaoka, T. (1940). The fauna of Akkeshi Bay. IX Nemertini. *Journal of the Faculty of Science, Hokkaido Imperial University, Series VI, Zoology*, *7*, 205–263.

Yin, Z., & Zeng, F. (1984). The study of a new species of genus *Dendrorhynchus*—*Dendrorhynchus zhanjiangensis*, of lineid heteronemertean, possessing multibranched proboscis. *Marine Science Bulletin*, *3*, 51–58.

Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics (Oxford, England)*, *29*, 2869–2876. https://doi.org/10.1093/bioinformatics/btt499

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