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SHORT COMMUNICATION

DNA barcoding reveals a cryptic nemertean invasion in Atlantic and Mediterranean waters

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Abstract For several groups, like nemerteans, morphology-based identification is a hard discipline, but DNA barcoding may help non-experts in the identification process. In this study, DNA barcoding is used to reveal the cryptic invasion of Pacific *Cephalothrix* cf. *simula* into Atlantic and Mediterranean coasts. Although DNA barcoding is a promising method for the identification of Nemertea, only 6 % of the known number of nemertean species is currently associated with a correct DNA barcode. Therefore, additional morphological and molecular studies are necessary to advance the utility of DNA barcoding in the characterisation of possible nemertean alien invasions.

Keywords Alien invasion · DNA barcoding · Nemertea · *Cephalothrix* · Mediterranean Sea · Atlantic Ocean

Introduction

Morphology-based nemertean taxonomy is a highly specialised discipline: proper fixation and histological procedures are essential for the correct morphological identification in several groups. Most of the material collected during previous marine expeditions was of poor quality for histological studies, due to differences in fixation and anaesthetisation protocols, and thus, the correct identification of those specimens has been impeded. Within the Nemertea, the taxon Palaeonemertea is a group that often lacks external morphologically discriminant characters; thus, an anatomical analysis is usually essential. Identification of members of the genus *Cephalothrix*, composed of 28 named species (Chernyshev 2012), which we analysed here, is based on very subtle and hard-to-interpret characters. However, when morphological identification fails, molecular techniques, using frozen or ethanol-fixed tissues, can be utilised. It is also possible to identify species through DNA barcoding when previous studies with molecular markers have been performed (Darling and Blum 2007; Geller et al. 2010; Roman and Darling 2007). Due to the work of Chen et al. (2010), the genus *Cephalothrix* is the best-represented genus for cytochrome oxidase I (COI) data in GenBank databases.

In a recent survey of nemertean diversity along the Iberian Peninsula coasts, some morphospecies of *Cephalothrix* that had not been previously reported in this area were found (Fernández-Álvarez, unpublished data). Although no marine nemertean species has been demonstrated to be an alien invader, Moore et al. (2001) reviewed the role of terrestrial nemerteans in biological invasions associated with ornamental plant commerce. The dispersal capacity of these species may be crucial for understanding its present occurrence in the Iberian coasts. Therefore, understanding the larval development and population structure are important for elucidating this pattern of dispersal. Despite current efforts to study the ontogeny of nemerteans (e.g. Chernyshev 2008; Maslakova 2010a, b; Maslakova and Döhren 2009; Maslakova and Malakhov 1999), many aspects of the biology of nemerteans still remain unclear.

Here, we report a case of a marine nemertean alien invasion, which was confirmed by DNA barcoding studies.

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Materials and methods

For DNA extraction, 32 individuals of the genus *Cephalothrix* collected from Iberian Peninsula coasts were preserved in absolute ethanol: seven were from north-western coasts (Atlantic Ocean), and 25 were from north-eastern coasts (Mediterranean Sea). The sampling localities are summarised in Table 1.

The external characters such as colour, shape of the body and gonadal state were drawn, noted and photographed on live animals. In some cases, an internal examination was performed. Three selected specimens were anaesthetised in 7.5 % magnesium chloride and fixed in Bouin's fluid. Paraffin-embedded tissues were cut at 7 µm. Staining was performed using haematoxylin–eosin or Para-pak® (Meridian Biosciences) trichrome methods.

Total genomic DNA was extracted from ethanol-fixed tissue from either a fragment of the body or the entire body, depending on the size of the specimen, using the BioSprint 15 DNA kit (Qiagen), following the manufacturers' protocol. We amplified sequences from the partial mitochondrial cytochrome c oxidase I (COI) gene, using the primer pair LCO1490 (Folmer et al. 1994) and COI-H (Machordom et al. 2003). All PCRs were performed in a total volume of 50 µl that included 0.3 µl of *Taq* polymerase (5U/µl, Biotools), 5 µl of reaction buffer (Biotools), 0.8 µl of each primer (10 µM), 1 µl of dNTPs (10 mM) and 2 µl of template DNA. PCRs consisted of an initial denaturation at 94 °C (4 min), followed by 40 cycles of denaturation at 94 °C (45 s), annealing at 45 °C (90 s) and extension at 72 °C (1 min). The amplified fragments (approximately 700 bp) were purified using ethanol precipitation prior to sequencing both strands on an

Table 1 List of *C. simula* used in this work, including the sample locality, number of specimens analysed (*N*) and GenBank accession numbers

Labcode[a]	Locality	*N*	GenBank accession number	Reference
IBE-A1, IBE-A2	San Vicente do Mar, O Grove, Galicia, Spain. 42° 27′N, 8° 55′O[b]	2	JX453463 JX453464	Present work
Vlon005*	Las Represas beach, Tapia de Casariego, Asturias, Spain. 43° 34′N, 6° 56′O[b]	1	–	Present work
Vlon001*	Los Chalanos beach, Muros de Nalón, Asturias, Spain. 43° 23′N, 6° 06′O[b]	1	–	Present work
IBE-A3, IBE-A4, Vlon00A*	Aramar beach, Luanco, Asturias, Spain. 43° 36′N, 5° 46′O[b]	3	JX453465 JX453466	Present work
IBE-A5 to IBE-A7	Islares beach, Castro-Urdiales, Cantabria, Spain. 43° 24′N, 3° 17′O[b]	3	JX453467 JX453468 JX453469	Present work
IBE-M1, IBE-M2	Colera harbor, Cap de Creus, Cataluña, Spain. 42° 24′ N, 3° 09′ E[c]	2	JX453470 JX453471	Present work
IBE-M3 to IBE-M25	L′illot del Faradell, Cap de Creus, Cataluña, Spain. 42° 20′16" N, 3° 16′49" E[c]	23	JX453472 to JX453494	Present work
JAP-F30	Fukue, Japan	1	GU726622	Chen et al. (2010)
KOR2	Jeju Island, Korea	1	GU726646	Chen et al. (2010)
JAP-O	Oshoro, Japan	1	GU726619	Chen et al. (2010)
JAP-SH	Shimoda, Japan	1	GU726620	Chen et al. (2010)
JAP-SE3, JAP-SE6	Seto, Japan	2	GU726661, GU726662	Chen et al. (2010)
ITA	Trieste, Italy	1	GU733830	Chen et al. (2010)
CHI-C	Changdao, Shandong, China	1	GU726615	Chen et al. (2010)
USA-CA1	San Diego, California, USA	1	GU726639	Chen et al. (2010)

All of the Chen et al.'s (2010) specimens correspond to their so-called network 11

* Indicates specimens that were studied only for morphological characterisation

[a] Nomenclature adopted from Chen et al. (2010)

[b] Atlantic Ocean

[c] Mediterranean Sea

ABI Prism 3730. The specimens used in this analysis and their labcodes and GenBank accession numbers are summarised in Table 1.

DNA sequences were cleaned at the primer ends using Sequencher (Gene Codes Corporation) and manually aligned. Sequences were then compared with GenBank sequences using BLAST (Altschul et al. 1990). Sequences with the highest similarity were those previously analysed by Chen et al. (2010). Thus, a matrix was generated with both sets of sequences (from this study and Chen et al.'s "network 11"). The haplotype networks were constructed using the software Network 4.5 (www.fluxus-engineering.com), with default parameters with a maximum connectivity limit of 95 %. Divergence among haplotypes was calculated in PAUP v4.0a125 (Swofford 2002).

For practical reasons, we used the nomenclature of labcodes adopted in Chen et al. (2010) to name the individuals and the cephalotrichid network (Table 1).

Results

The haplotype network, with the geographical distribution of the individuals, is provided in Fig. 1. A total of nine haplotypes were found among the samples analysed, five of them in the Iberian Peninsula. The most frequent haplotype was shared by four individuals from the Pacific Ocean (CHI-C, JAP-0, KOR2 and USA-CA1), three individuals from the Atlantic Ocean (IBE-A1, IBE-A2 and IBE-A7) and 18 individuals from the Mediterranean Sea coasts of the Iberian Peninsula (IBE-M2 to IBE-M5, IBE-M7 to IBE-M14, IBE-M16, IBE-M17 and IBE-M20 to IBE-M23). Two haplotypes were present in samples from both Iberian coasts: the first haplotype was found in three individuals from the Atlantic Ocean (IBE-A3, IBE-A4 and IBE-A5) and one individual from the Mediterranean Sea (IBE-M1); the second haplotype was found in one Atlantic Ocean individual (IBE-A6) and four Mediterranean Sea individuals (IBE-M15, IBE-M18, IBE-M24 and IBE-M25). Two haplotypes were only found in individuals from the Iberian Mediterranean Sea coasts (IBE-M19 and IBE-M6), while three other haplotypes were found only in Japanese individuals (JAP-F30, JAP-SE6 and JAP-SH). The last haplotype was shared by Italian (ITA) and Japanese (JAP-SE3) individuals. The uncorrected divergences among the nine different haplotypes ranged from 0.15 to 0.61 %. The mean distance found between the Iberian and Asiatic samples was 0.46 %.

The anaesthetised living specimens (Fig. 2) showed the following habitus: sizes between 20 and 60 mm in length and 0.5 to 0.8 mm in width; cylindrical body, in some cases flattened in its posterior portion; eyes absent; and a mouth that developed into large lip forming a sucker. Overall, the body was dark yellow, dull orange or reddish yellow, with a brilliant spot of yellow or orange pigment at the tip of the head. The lateral margins were translucent. The ratio of distances from the tip of the head to the brain and the tip of the head to the mouth is between 1:2.5 and 1:3.0.

Histological analyses demonstrated that the body wall was comprised of typical cephalotrichid muscle layers (Fig. 3a, b), which are formed by an outer circular muscle, an inner longitudinal muscle layer and a thin wall of inner circular musculature formed by two to three muscle fibres around the foregut and the anterior part of the intestine. This form holds a muscle plate between the rhynchocoel and the digestive apparatus (Fig. 3c). Rhynchocoel length was variable; in some cases, it measured more than four-fifths of the total body length. The proboscis sheath was composed of an outer circular and an inner longitudinal muscle layer (Fig. 3c). A type A rhynchocoel vessel was present (Fig. 3a) (following the nomenclature of Kajihara, 2010).

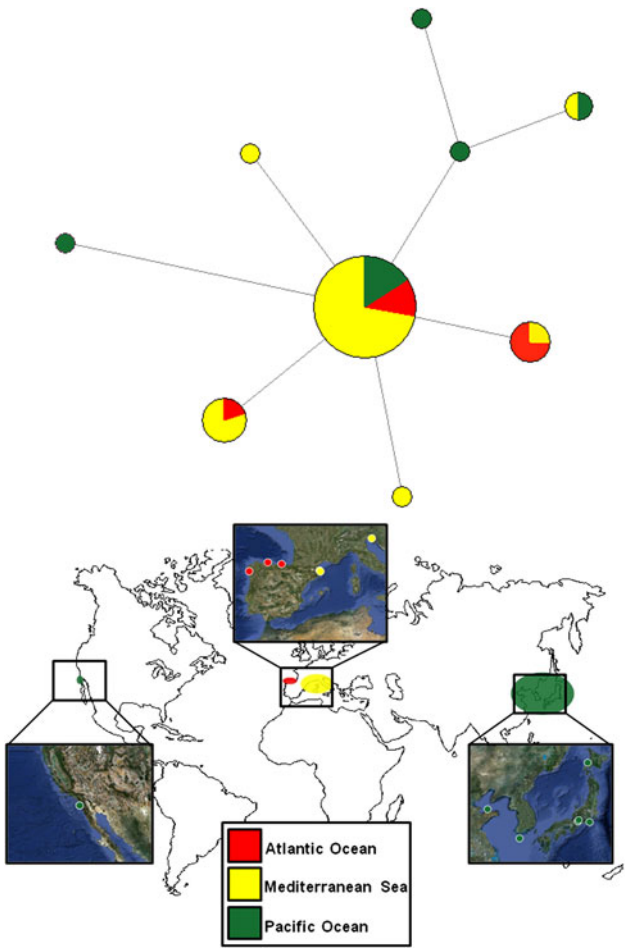

Fig. 1 Haplotype network for the COI haplotypes of *C.* cf. *simula*. The origin of each sample is indicated in the map. Colours of the haplotype network are consistent with the colours on the map

Fig. 2 Habitus of *Cephalothrix* cf. *simula*. *Scale bar* 1 mm

The unpaired buccal nerve (Fig. 3a), which originated posterior to the middle portion of the ventral commissure, ran posteriorly towards the foregut and disappeared immediately behind the mouth. The buccal nerve then branched into two nerves just anterior to the start of the foregut (Fig. 3b).

Discussion

The dispersal potential of a species is directly related to its biological features (Scheltema 1989; Shuto 1974). In the marine realm, the mode of larval development is one of the main factors that can influence such capacities. Unfortunately, little is known about the ontogeny of Cephalothrichidae; however, the characteristics of the *Cephalothrix* larvae (Smith 1935; Iwata 1960; Chernyshev 2001) suggest the presence of a lecithotrophic phase. Thus, this type of larvae is thought to have relatively limited dispersal ability. Despite the likely low dispersal ability for this group, we observed several haplotypes that are present both in the Pacific Ocean and in the Atlantic Ocean and Mediterranean Sea (Fig. 1). The observed network showed a main haplotype and several satellite haplotypes (Fig. 1). This structure is frequently associated with a population in expansion with a high level of gene flow. Therefore, this structure is difficult to couple with the supposed low dispersal capacity and the distribution of the populations analysed.

The 32 specimens analysed here clustered in clade 11 of Chen et al. (2010), which is included in a monophyletic group with clades 6 and 8, integrated by Pacific individuals assigned to different species. Chen et al. (2010) concluded that clades 6, 8 and 11 contained haplotypes from the same biological species and considered these three networks the result of "undersampling the intraspecific haplotype variation", even if each of these clades represented a separate network. The majority of the specimens analysed and included in these networks were not identified at the species level: only six of the 13 specimens included in clades 6 and 8 were considered to be *C. simula* (Iwata, 1952), while only one specimen in clade 11 was identified as *C. fasciculus* (Iwata, 1952) (Chen et al. 2010, Table S1). However, another specimen identified as *C. fasciculus* was present in clade 22. Recent unpublished taxonomic work (Kajihara et al., unpublished data) comparing the anatomy of the holotypes of *C. fasciculus* and *C. simula* with the anatomy of the proposed topogenetypes does not reveal any anatomical differences between the two species (Kajihara, pers. comm.). Furthermore, the specimen named "*Cephalothrix fasciculus* CHI-C" (network 11 in Chen et al. 2010) is from the type locality of *C. simula*, and it is designated as the topogenetype of this species, while the specimen named "*C. fasciculus* JAP-F33" (network 22 in Chen et al. 2010) is designated as the topogenetype of *C. fasciculus* (Kajihara et al., unpublished data). Moreover, the molecular divergence between networks 6, 8 and 11 (K2P = 4.6–9.2 %; Chen et al. 2010, Table 2), compared to the intranetwork divergences (K2P between 0 and 0.95 %; Chen et al. 2010, Table 1), identified a clear barcoding gap, differentiating three operational taxonomic units (OTUs), since specimens from these three networks occur in sympatry in their original distribution (thus suggesting that reproductive isolation is operating).

An uncertainty surrounds the original descriptions of *C. fasciculus* and *C. simula*: in an article from 1952, Iwata differentiated these two taxa by the presence (*C. fasciculus*) or absence (*C. simula*) of a muscle plate and by rhynchocoel length, which comprises the entire body length in *C. fasciculus*. Kajihara (2007) considers that "*this character state can be erroneously identified by misinterpretation of a body fragment as an intact specimen*". The fragile nature of Cephalothrichidae body tissues and problems related to its preservation support Kajihara's hypothesis. Subsequently, Iwata (1954) reported the presence of a muscle plate between the rhynchocoel and the gut in *C. simula*. The presence of a muscle plate was confirmed in the holotype of *C. simula* (Kajihara, pers. comm.), thus making the identification of the two species based only on morphological data impossible and the original descriptions invalid (Kajihara et al., unpublished data). Although Iberian specimens exhibited some characters that are inconsistent with the original description of *C. simula*, such as the presence of a muscle plate (Fig. 3c) and the rhynchocoel length, given that morphological descriptions for these two species are not informative for taxonomic proposes, we designate the Iberian specimens as *C.* cf. *simula*, since our samples molecularly group with the designated topogenetype (Kajihara, pers. comm.). If we consider clades 6, 8 and 11 members of the same nominal species, according to the Chen et al.'s (2010) criterion, then the presence of a clear barcoding gap between these three networks points to the phenomenon of cryptic speciation occurring in these Pacific OTUs under this specific name.

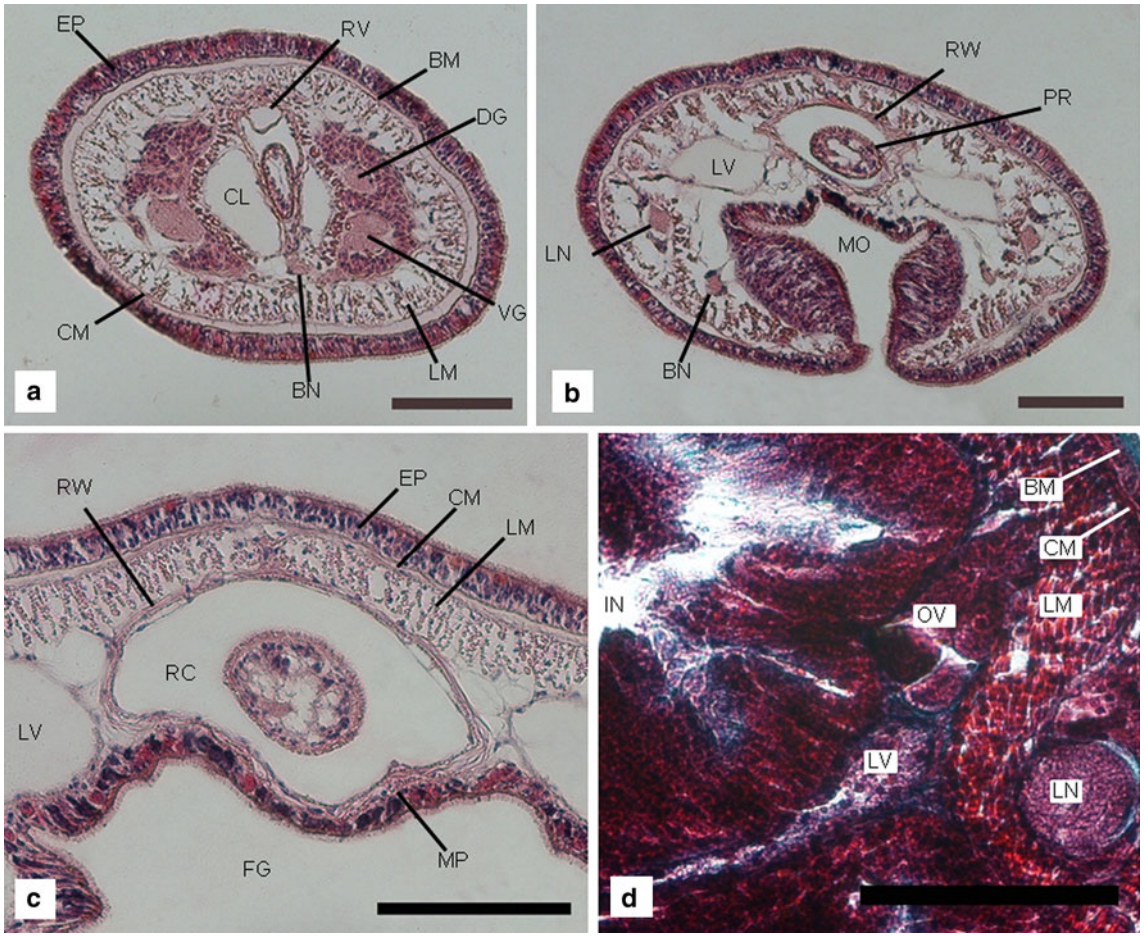

Fig. 3 *Cephalothrix* cf. *simula*. Transverse sections through (**a**) cerebral region, showing the unpaired section of the buccal nerve (*BN*), (**b**) buccal region, showing the arrangement of the branched portion of the buccal nerve, (**c**) intestinal region, showing the muscle plate (*MP*) and the rhynchocoel wall (*RW*), (**d**) intestinal region, showing the arrangement of an incipient ovary (*OV*). *BM* basal membrane, *CL* cephalic blood lacuna, *CM* circular muscle layer, *DG* dorsal ganglion, *EP* epidermis, *FG* foregut, *IN* intestine, *LM* body wall longitudinal muscle layer, *LN* lateral nerve cord, *LV* lateral blood vessel, *PR* proboscis, *RC* rhynchocoel, *RV* rhynchocoel vessel, *VG* ventral ganglion. *Scale bars* 100 μm

Although one specimen from Italy was included in the network 11 of Chen et al. (2010), it was not identified at the species level in that study. Moreover, the presence of *C. simula* has not been previously reported in the Atlantic Ocean. We consider *C. simula* to be an alien invader whose larvae could have been introduced to their allochthonous distribution area in the ballast waters of ships (e.g. *Ensis directus* in the Iberian Peninsula, Arias and Anadón 2012). The presence of the same haplotypes in different invaded areas and in its natural distribution range suggests several invasion events. The Mediterranean Sea is one of the world's regions most affected by biological invasions (Galil 2009; Zenetos et al. 2010), mainly since the opening of the Suez Canal. However, the presence of *C. simula* from the eastern Mediterranean Sea has not been reported, and the invasion of Atlantic localities cannot be explained by the hypothesis of a Lessepsian migration to the Mediterranean Sea. In this context, it is possible that environmental changes produced by climate change are currently facilitating the settlement of this species. The presence of developed gonads in one specimen (Fig. 3d) and the presence of juvenile individuals reveal that reproduction is occurring in the invaded areas.

In Colera (Cataluña, Iberian Peninsula), *C. simula* represents 28 % of the nemerteans sampled (Fernández-Álvarez, unpublished data); no other *Cephalothrix* species were found. Species of the genus *Cephalothrix* have predatory habits (Wu and Sun 2006), and their introduction into new environments can affect natural populations by competitive exclusion. This invasion has been happening cryptically, and thus, it is possible that it has had several effects on the natural environment. The lack of quantitative data along the Iberian coasts for the majority of nemertean species (e.g. García-Pérez and Anadón 2004) makes it impossible to evaluate whether competitive exclusion is operating on autochthonous *Cephalothrix* species.

Our report of *C. simula* along the Iberian coasts is the first record for this species in this local fauna. Currently, the extent of nemertean invasions has only been postulated by Moore et al. (2001) for terrestrial taxa, likely associated with ornamental plants commerce. Turbeville (2011) hypothesised the presence of a population of *Emplectonema gracile* (Johnston, 1837) in South Carolina as the result of an introduction by ballast waters. However, unlike *C. simula*, *E. gracile* has an almost cosmopolitan distribution; it is found in Europe (e.g. North Atlantic and North Sea, Madeira, Black and Mediterranean Seas), Russia (Kamchatka Peninsula), Japan and the two coasts of North America (see Turbeville 2011). This cosmopolitan distribution may indicate a greater dispersal ability for *E. gracile*, but the restricted geographical range of *C. simula* and its sister clade (network 9 in Chen et al. 2010) to Pacific localities, which is a general feature for the genus *Cephalothrix,* suggests a limited dispersal ability for *C. simula*. The proposed low dispersal ability for *C. simula* supports the hypothesis that this species was introduced outside of its typical geographical range by human-mediated means.

The use of molecular markers (such as COI) to identify cases of nemertean alien invasions is a promising method that may resolve the status of some cryptogenic marine species recorded in the bibliography (e.g. Carlton 1996). Unfortunately, the use of DNA barcoding method is hindered by the fact that only 6 % of the phylum has an associated barcode (Bucklin et al. 2011). For these reasons, we consider the combination of histological and molecular data to be essential for accurately assessing the world's nemertean biodiversity.

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