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Investigating the taxonomy and systematics of marine wood borers (Bivalvia : Teredinidae) combining evidence from morphology, DNA barcodes and nuclear locus sequences

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Abstract. Marine wood-boring teredinids, some of the most destructive wood borers in the sea, are a particularly difficult group to identify from morphological features. While in most bivalve species shell features are used as diagnostic characters, in the teredinids shell morphology shows high intraspecific variation and thus identification is based almost entirely on the morphology of the pallets. In the present study we aimed at improving 'taxonomic resolution' in teredinids by combining morphological evidence with mitochondrial and nuclear DNA sequences, respectively Cytochrome c oxidase subunit I and small subunit rRNA 18S gene, to generate more rigorous and accessible identifications.

DNA barcodes of Atlantic and Mediterranean populations of *Lyrodus pedicellatus* diverged by ~20%, suggesting cryptic species in the morphospecies *L. pedicellatus*. The low intraspecific divergence found in barcodes of specimens of *Nototeredo norvagica* (0.78%) confirms that Atlantic and Mediterranean forms of *N. norvagica*, the latter sometimes reported as *Teredo utriculus*, are the same species. *Teredothyra dominicensis* was found for the first time in the Mediterranean. A match was obtained between our 18S sequences and sequences of *T. dominicensis* from Netherlands Antilles, confirming that *T. dominicensis* in the Mediterranean is the same species that occurs in the Caribbean. There were differences in 18S sequences between *Bankia carinata* from the Mediterranean and Caribbean, which may indicate cryptic species.

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Introduction

Marine bivalves of the family Teredinidae (commonly known as shipworms) are among the most destructive organisms in the sea (Turner 1966), and were dubbed 'termites of the sea' by ship captains (Cobb 2002). Teredinids are distributed worldwide and the economic impact of the destruction they inflict in maritime structures is well documented (Kofoid and Miller 1927; Fougerousse 1971; Edmondson 1955; Distel *et al.* 2011). The fight against their ravages has been going on since early historic times (Clapp and Kenk 1963). However, in the last 50 years their profile seems to have disappeared into obscurity because metal and fibreglass replaced wood in boats (Cobb 2002) and maritime structures such as piers and pontoons have either been built with chemically treated wood or concrete (Borges *et al.* 2010). Nevertheless, recent estimates have suggested destruction by teredinids to be millions of Euros every year in single countries such as Germany (Ballast Water Convention 2004) and billions of dollars worldwide (Distel *et al.* 2011).

In the last decade the activity of teredinids such as *Teredo navalis*, *Lyrodus pedicellatus* and *Teredo bartschi* seems to be increasing in some areas in Europe (Borges 2007; Borges *et al.* 2010; Paalvast and van der Velde 2011). However, one of the great challenges of studying teredinids is that they are one of the most difficult groups of bivalves to identify (Turner 1966). Thus, it is important to improve 'taxonomic resolution' as inaccuracy or the impossibility of identification of specimens to genus or species level creates great limitations in other studies where precision relies on taxonomy (Chessman *et al.* 2007). The revision of Turner (1966), the most complete work to date on the Teredinidae, was essential to put order in the chaotic taxonomy of this family. Nevertheless there are still 'taxonomic impediments' hampering the output of taxonomy in teredinid species. One of the main problems is that in many general surveys, when teredinids are identified by non-specialists using general keys, identifications are based on shells not pallets, which has created errors that are perpetuated in the literature

(Demir 2003). In most bivalve species shell features are used as diagnostic characters. In the teredinids, however, shell morphology shows high intraspecific variation. Identification is, therefore, based almost entirely on the morphology of the pallets, a pair of calcareous structures located at the posterior end of the body, which are used as plugs to seal the tunnels inside the wood (Turner 1971). Even when teredinids are identified using pallets, these structures are quite often affected by substrate (type of wood), environmental conditions and weathering (Cragg *et al.* 2009), making identifications difficult. In addition, extracting teredinids from wood is not easy and often specimens are extracted incomplete. These are some of the reasons that have led to erroneous identifications in the past, and have created many synonyms in most accepted species (see Turner 1966).

The identification problems mentioned above highlighted the need for the use of molecular tools, which have been shown to be enormously useful to taxonomists. The use of a standard universal system remains of utmost importance for species discovery and identification. Such a system has been implemented at a worldwide scale after Hebert *et al.* (2003) proposed the concept of the DNA barcode. Given its reliable performance in species identification in comprehensive studies with diverse taxa, a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (hereafter COI-5P) has been elected the universal DNA barcode for animals (Teletchea 2010) and it has been shown to be successful in species delimitation in marine Metazoa (Bucklin *et al.* 2011) in groups such as the Mollusca (Feng *et al.* 2011) and the Crustacea (Costa *et al.* 2007; Radulovici *et al.* 2009; Fernandes *et al.* 2010). It has also revealed species complexes in groups such as the Isopoda (Raupach and Wägele 2006). One of the aims of the present study was to integrate morphological identifications and molecular markers to assist in more rigorous identification of teredinid species. We aimed also to revisit old taxonomic questions that have been left unanswered for many years. COI-5P is the most popular marker to study moderately to deep interspecific taxon relationships (Costa *et al.* 2009; Radulovici *et al.* 2009; Feng *et al.* 2011; Costa and Antunes 2012). Herein we present original DNA barcode sequences of teredinid specimens, collected from the French Atlantic coast and in the Mediterranean Sea. In addition, we sequenced the small subunit 18S rRNA gene (hereafter 18S) to avoid the possible pitfalls of using only COI sequences. The integration of molecular and morphology-based identifications will increase the chances of understanding the systematic relationships between and within teredinid species and thus facilitate the inference of biogeographical patterns of distribution. It will also improve the traceability of possible invasive species, which will be fundamental for management and protection of wooden maritime structures.

Materials and methods

Specimen collection

Specimens were collected in France from three areas (Gulf of Morbihan, Toulindac and Berder) in pine and cypress branches found in the lower part of the shore. Specimens from Mersin Bay, Turkey, were extracted from test panels of *Pinus sylvestris* exposed at 6-m depth for the period of a year (for detailed methodology, please refer to Sivrikaya *et al.* 2009). Additional specimens were obtained from a shipwreck site in Kaş, Turkey (Table 1).

For morphology-based identifications we used the following characters of the pallets: calcareous portion of the blade; shape and colour of the periostracal cap, according to the keys of Turner

Table 1. Species, number and locations of specimens examined in this study

Species name (no. of specimens)	Location	GenBank accession no. COI	GenBank accession no. 18S	Source
Bankia carinata (2)	Mersin Bay, Turkey	KC157914; KC157934	KC158195; KC158213	This study
Bankia carinata (*1*)	Bonaire, Netherlands Antilles		JF899203	Distel *et al.* 2011
Bankia carinata (*1*)	Tobago		AF120625	Giribet and Wheeler 2002
Lyrodus pedicellatus (4)	Toulindac, France	KC157917- KC157920	KC158198-KC158201	This study
Lyrodus pedicellatus (3)	Gulf of Morbihan, France	KC157915; KC157921; KC157922	KC158196; KC158202; KC158203	This study
Lyrodus pedicellatus (1)	Berder, France	KC157937	KC158216	This study
Lyrodus pedicellatus (1)	Portsmouth, United Kingdom		AM774540	Taylor *et al.* 2007
Lyrodus pedicellatus (4)	Mersin Bay, Turkey	KC157916; KC157932; KC157938; KC157939	KC158197; KC158211; KC158217; KC158218	This study
Lyrodus pedicellatus (1)	Florida, USA		JF899211	Distel *et al.* 2011
Lyrodus massa (1)	Manado Bay, Indonesia		JF899212	Distel *et al.* 2011
Martesia striata (1)	Indonesia		JF899213	Distel *et al.* 2011
Nototeredo norvagica (7)	Mersin Bay, Turkey	KC157926- KC157931; KC157936	KC158207; KC158208- KC158210; KC158215	This study
Nototeredo norvagica (2)	Kaş, Turkey	KC157923; KC157933	KC158204; KC158212	This study
Nototeredo norvagica (1)	Penerf, France	KC157924	KC158205	This study
Nototeredo norvagica (1)	Berder, France	KC157925	KC158206	This study
Pholas dactylus (1)	Charmouth, UK		JF899220	Distel *et al.* 2011
Teredothyra dominicensis (4)	Kaş, Turkey	KC157940- KC157943	KC158219- KC158222	This study
Teredothyra dominicensis (1)	Netherlands Antilles		JF899225	Distel *et al.* 2011
Pecten jacobeus (*1*)	Ankara, Turkey	JQ623969		Keskin, unpublished

(1971) and the figures and descriptions in Turner (1966). In most cases whole specimens were preserved in 96% ethanol and stored for future reference.

DNA extraction and polymerase chain reaction

Total genomic DNA was extracted using GenElute Mammalian Genomic DNA Mini Prep kit (Sigma) following the supplied protocol. The only change to the protocol was the use of ultrapure autoclaved water (50 µL) instead of gene elute solution.

A 658-bp fragment from the 5′ end of COI-5P was amplified using the primer pair LCO1490 (5′GGTCAACAAATCAT AAAGATATTGG) and HCO2198 (5′TAAACTTCAGGGTG ACCAAAAAATCA) (Folmer *et al.* 1994). Amplifications were performed in 25-µL reactions, each reaction containing 2.5 µL of 10× Promega PCR buffer, 2.5 µL of $MgCl_2$ (25 mM), 0.25 µL of dNTPs (10 mM), 0.5 µL of each primer (10 mM), 0.25 µL of Taq polymerase (5U) and 2–12 µL of DNA template, filled up with ultrapure sterile H_2O. Cycling conditions for PCR reactions were as follows: one cycle of 94°C for 1 min, 1 cycle of 94°C for 30 s, 45°C for 90 s and 72°C for 60 s, 35 cycles of 94°C for 30 s, 51°C for 90 s and 72°C for 60 s, with a final extension of 72°C for 5 min. Subsequently, 5 µL of PCR product were visualised in 1% agarose gel and successful PCR products were then purified using a mix of 10 U exonuclease I and 1 U of shrimp alkaline phosphatase. The samples were then subject to a thermal regime of 37°C for 15 min and 80°C for another 15 min.

PCR amplifications of a fragment with ~345 bp of the 18S rRNA gene were performed using 6 µL of genomic DNA template in 25 µL reaction containing identical concentrations of the reagents as the reactions above. A volume of 0.5 µL (10 mM) per reaction was used of each of the primers SSU_FO4 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and SSU_R22 (5′-GCCTGCTGCCTTCCTTGGA-3′) (Blaxter *et al.* 1998). The PCR thermal regime consisted of 2 min at 95°C, followed by 35 cycles of 1 min at 95°C, 45 s at 57°, 3 min at 72°C and a final extension of 10 min at 72°C. PCR products were cleaned up using a three-time precipitation with isopropanol.

Data analysis

Complementary strands of COI and of 18S sequences were edited and aligned using MEGA ver. 5.1 (Tamura *et al.* 2011). Specimen data, images, sequences and trace files were uploaded in the project 'Wood boring Mollusca from Europe', available in the Barcode of Life Data System (BOLD System) (Ratnasingham and Hebert 2007) and in GenBank (accessions KC157914-KC157943 for COI-5P and KC158195-KC158222 for 18S; Table 1). Following basic editing, all sequences (COI-5P and 18S) were submitted to BLAST searches against the GenBank database and, in the case of COI-5P sequences, also to homology searches in the BOLD Identification Engine (BOLD-IDS) (Ratnasingham and Hebert 2007), in order to ensure that endosymbiont bacteria and other potential contaminants had not been coamplified in error. Sequences were then aligned using Clustal W (Thompson *et al.* 1994) implemented in MEGA 5.1 (Tamura *et al.* 2011), and the amino acid translation of COI-5P sequences was used to ensure that no frameshift mutations, stop codons or unusually divergent amino acid profiles were present in the alignment.

DNA sequences consisting of 658 bp and 345 bp, for COI-5P and 18S respectively, were used for phylogenetic inference using Neighbour-joining (NJ) and Maximum-likelihood methods (ML). The program MEGA 5.1 was used to construct the NJ trees for COI-5P and 18S sequences, using the Kimura 2-parameter model (K2P), to allow comparisons with other barcoding studies where K2P is the standard genetic distance used. Bootstrap support for the nodes was determined using 10 000 replicates. Pairwise genetic distances (K2P) were calculated within and among populations from sampled sites. For the ML analysis we used jModeltest (Posada and Crandall 1998; Guindon and Gascuel 2003) to estimate the best-fit model of evolution for COI-5P (TPM1uf+G) and for 18S (TIM3ef+G) sequences. ML trees were constructed using the program PhyML (Guindon *et al.* 2009) and node support was estimated using the approximate likelihood ratio test (aLRT) with Shimodaira–Hasegawa (SH)-like support option. We also added selected GenBank sequences to compare with our dataset and to be used as outgroups both for COI-5P and 18S (Table 1). In addition, we used amino acid COI-5P sequences to construct NJ tree using Jones–Taylor–Thornton (JTT) model (Jones *et al.* 1992) implemented in MEGA 5.1 and with node support consisting of 1000 bootstrap replicates.

Results

Morphological identification of specimens

Specimens lacking pallets were identified only to family level (Teredinidae) on the basis of the following characters: worm-like long body; small shell with apophyses covering only the anterior part of the body; pallets present on the posterior end of the body, flanking the siphons. Specimens containing pallets were identified to species level using the key in Turner (1971).

Bankia carinata (Gray, 1827)

Diagnosis: pallets segmented; blade greatly elongated, composed of segments separated as distinct cones, built on a stalk that extends the length of the blade (Fig. 1*e*). Cones funnel-shaped; margin of cones not serrated with short blunt awns; periostracal margin on inner and outer face about equal; embryonic cones crowded and covered with periostracum forming compact tip.

Lyrodus pedicellatus (Quatrefages, 1849)

Diagnosis: pallets non-segmented, composed of single piece; distal half of blade composed of periostracal cap varying from light brown (Fig. 1*a*) to dark brown, almost black (Fig. 1*b*), that envelopes upper portion of calcareous base; calcareous portion of blade conical distally; periostracal cap more or less straight sided, with distal margin *U*-shaped, extending as lateral horns.

Nototeredo norvagica (Spengler, 1792)

Diagnosis: pallets composed of closely packed segments fused, indistinct and appearing as rib-like elements radiating

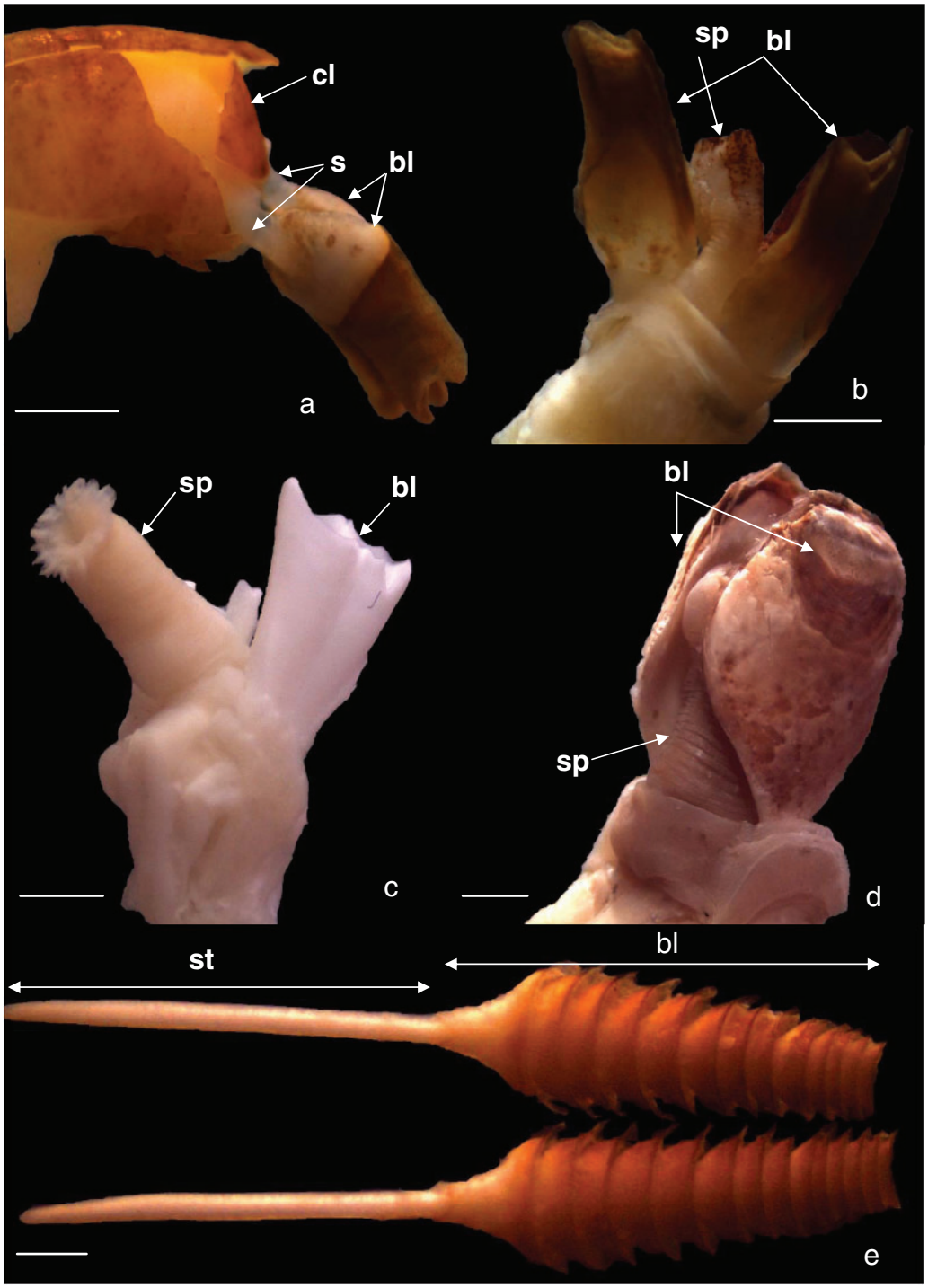

Fig. 1. Pallets of *Lyrodus pedicellatus* from (*a*) France and (*b*) Turkey, (*c*) *Teredothyra dominicensis*, (*d*) *Nototeredo norvagica*, and (*e*) *Bankia carinata*. Scale bar = 1 mm; cl, calcareous lining; sp, siphon; bl, blade of pallet; st, stalk of pallet.

from the stalk, which extends nearly length of blade; blade broadly oval, paddle shaped, broadest distally, tapering proximally (Fig. 1*d*); lateral awns evident on all segments in young specimens; in older specimens lateral awns become worn; small thumbnail depression evident at distal end.

Teredothyra dominicensis (Bartsch, 1921)

Diagnosis: pallets non-segmented and longer than wider (Fig. 1*c*); calcareous portion of the blade composed of a basal cup with a second divided cup; stalk of pallet extending into blade

only to the base of the inner cup; with distal margin of both faces of blade slightly concave, the outer more than the inner; inner cup divided, extending only a little beyond the outer basal cup; blade thick; irregular short stalk, not sheathed.

Molecular systematic analyses of COI-5P and 18S sequences

We obtained a total of 30 bidirectional COI-5P sequences. No indels or stop codons indicative of pseudogenes were found. A BLAST of the sequences revealed no close match with sequences in GenBank, probably due to the fact that most teredinid species have not been COI-sequenced yet. A COI-5P sequence of *Bankia carinata* (Teredinidae) was found in GenBank; however, the sequence has been determined to be putative bacterial symbiont or bacterial contamination in origin. Nevertheless, our COI-5P sequences aligned unambiguously (nucleotide- and amino-acid-wise) with sequences from other Bivalvia, so that aligned sequences are free from any indels or stop codons. Furthermore, before initiating the data analysis we verified the taxonomic identity of the sequences by searching for homologies in BOLD systems and GenBank's BLAST, the latter both nucleotide and amino acid sequences. The top nearest matches were Bivalvia and Gastropoda. A search of homologies in BOLD-IDS closely matched a COI-5P sequence of *Dicyathifer manni*, another species of Teredinidae, lodged in the BOLD database. This rules out any contamination by endosymbiotic bacteria or microbial eukaryotes.

Some of our unidentified specimens showed 100% match with our COI-5P sequences of *L. pedicellatus* from France, *L. pedicellatus* from Turkey, *Nototeredo norvagica* and *Bankia carinata* and therefore it was possible to use barcodes to assign them to species. Specimens identified morphologically as belonging to the same species clustered within the same groups in the case of the species *Nototeredo norvagica*, *Teredothyra dominicensis* and *Bankia carinata* (Fig. 2). After *L. pedicellatus*, the second highest COI-5P intraspecific divergence was observed in *B. carinata* (1.70%), and the least in *T. dominicensis* (0%). Specimens of the morphospecies *L. pedicellatus* formed two reciprocally monophyletic clusters, one corresponding to specimens from France and another with specimens from Turkey (Fig. 2), with an average divergence between the two clusters of 19.3% (Table 2).

We obtained 28 bidirectional 18S sequences of the same specimens from which we obtained COI-5P sequences. The BLAST search confirmed that they were 18S sequences of Teredinidae. *B. carinata*, *L. pedicellatus* and *T. dominicensis* showed close matches with sequences from the same species in GenBank. No closely matching 18S sequences were found for *N. norvagica*, although the nearest neighbours were also from the Teredinidae. Without exception, pairwise distances of 18S sequences were considerably smaller than the corresponding COI-5P distances. Despite the very low interspecific divergences the 18S sequences were able to sort all species lineages in the dataset (Fig. 3). Specimens of the same species collected in the same area showed no divergence, with the exception of specimens of *T. dominicensis* from the Mediterranean, which showed a divergence of 0.2% (Table 3). Divergence between populations of the same species collected in different areas showed values similar to that observed between different species. *B. carinata* from the Mediterranean diverged 2.5% from *B. carinata* collected in Netherlands Antilles. Populations of *L. pedicellatus* from North East Atlantic and Mediterranean diverged from *B. carinata* collected in the Mediterranean by 0.3 and 0.6%, respectively. The divergence between the forms of *L. pedicellatus* from France and from the Mediterranean was 0.3% but *L. pedicellatus* from the Mediterranean diverged by 1.5% from *L. pedicellatus* collected in Florida. The species that showed greater divergence from all the other species analysed were *T. dominicensis* and *N. norvagica* (Table 3).

Overall, COI-5P and 18S rRNA genes showed concordant tree topologies among teredinid species, although the 18S alignment was more conserved than the COI alignment. The only exception was *T. dominicensis*, in which intraspecific divergence was 0% in COI and 0.2% in 18S. Because the trees' topologies were identical independently of the evolutionary model and tree-building method (NJ of nucleotides both for COI-5P and 18S sequences and also NJ amino acids in COI-5P sequences versus ML), only the ML trees are shown here. Both methods also indicate the occurrence of putative cryptic species within the morphospecies *L. pedicellatus* and *B. carinata* (Figs 2, 3).

Discussion

Anatomical studies *per se* have not been able to clarify all taxonomic ambiguities and evolutionary relationships in the Teredinidae. In some species there appear to be selective or developmental constraints that either prevent morphological divergence (Colborn *et al.* 2001) or promote convergence (Wake 1991), complicating taxonomy. Molecular methods have been used in a few studies to investigate evolutionary relationships between the Teredinidae. Some early studies were based on allozymes (e.g. Cole and Turner 1977; Hoagland and Turner 1981) and recent ones were based on mitochondrial small subunit rRNA gene sequences (Santos *et al.* 2005) and on nuclear small and large subunits of rRNA gene sequences (Distel *et al.* 2011). However, as far as we are aware, COI-5P has not been used in taxonomic studies to assist delimitation of teredinid species.

Possible cryptic species within Lyrodus pedicellatus

One example where morphological characters alone are clearly not sufficient to delimit species is in the genus *Lyrodus* (Calloway and Turner 1983, 1987; Macintosh 2012). *Lyrodus pedicellatus* was reported to occur in Europe both in the north-east Atlantic and Mediterranean coasts of Europe (Turner 1966; Borges 2007). Morphological identification of our specimens from the Atlantic and the Mediterranean were confirmed by two specialists in *L. pedicellatus*: Laurie Cookson, Monash University, Australia (pers. comm.) and Daniel Distel, Laboratory for Marine Genomic Research, Ocean Genome Legacy, USA (pers. comm.). However, we observed very high COI-5P divergences (≈20%) and complete lineage sorting among specimens from each region. As discussed below, although considerably smaller, 18S sequence distances of 0.3% among specimens of the two

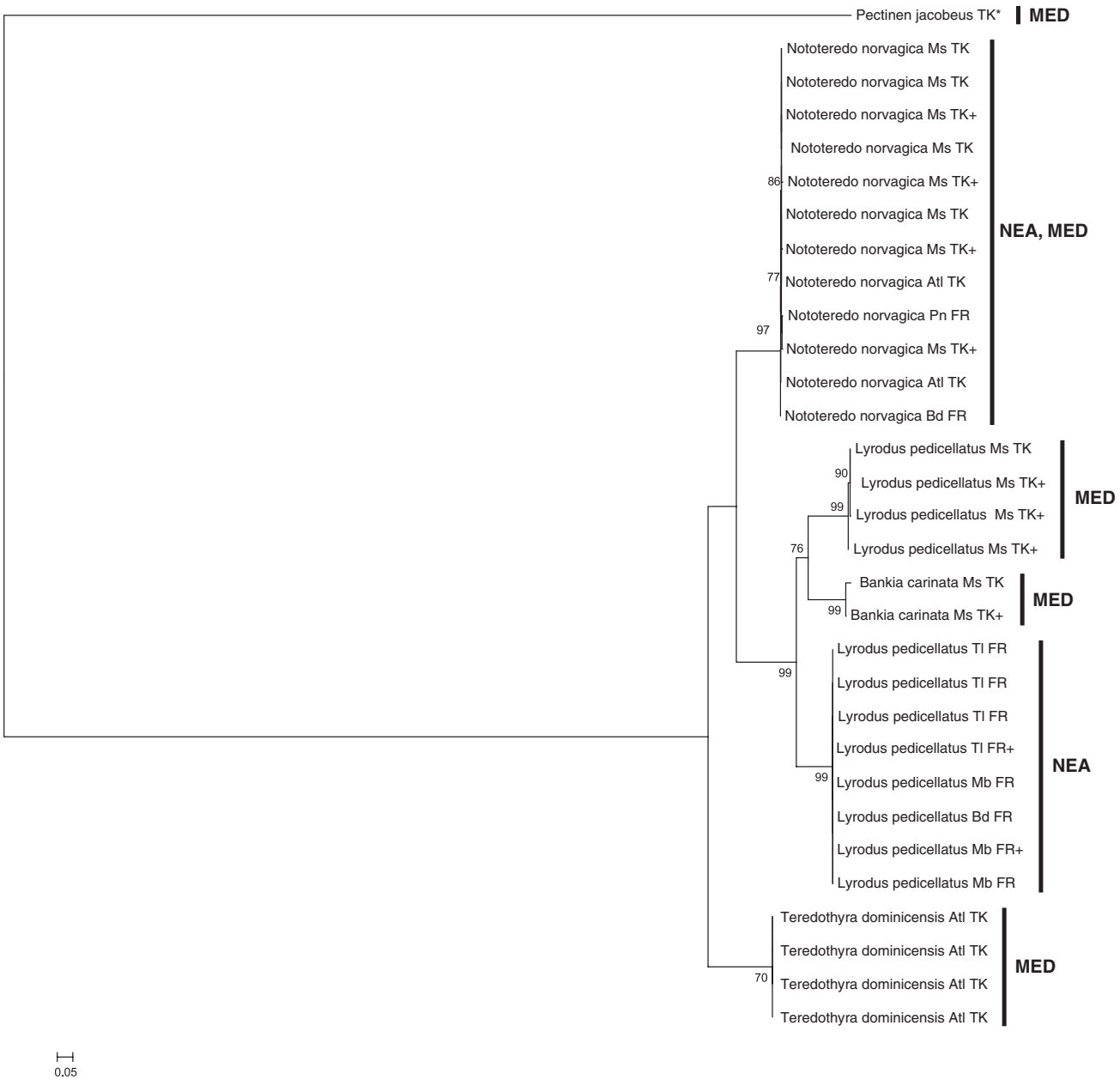

Fig. 2. Maximum-likelihood nucleotide tree of partial sequences of cytochrome c subunit I gene corresponding to the DNA barcode region (COI-5P). Only bootstrap support values (aLRT) greater than 50 are shown. Asterisks (outgroup) indicate sequence obtained from GenBank. Symbol + indicates specimens' barcode ID.

regions (which correspond to a fixed difference of one nucleotide in position 32 in our alignment), can be evolutionarily meaningful. Together with COI-5P barcodes these results suggest that the two populations of *L. pedicellatus* are cryptic species. Indeed, the level of conspecific COI-5P divergence found for *L. pedicellatus* is comparable to the average interspecific divergences found in gastropods, heteropods and pteropods (21.7 and 17.6% respectively), and much higher than the reported intraspecific divergences in these groups (Radulovici *et al.* 2010). The level of intraspecific variation in 18S sequences is largely unknown for most taxa (Fonseca *et al.* 2010). However, in a study carried out in more than 50 bivalve species, 18S sequences were characterised by the absence of intraspecific variability and low interspecific distances (Espiñeira *et al.* 2009), a pattern that was also observed in this study. The high divergence found in haplotypes from the Atlantic and the Mediterranean indicates that there is no detectable genetic exchange between these populations and suggests that this situation has been stable over a long period. This might be related to the fact that *Lyrodus* species are long-term brooders, and thus the short residence of the larvae in plankton,

Table 2. Pairwise COI barcode nucleotide for teredinid groups using K2P distances (%)

Lyrodus pedicellatus was divided in two subgroups, one with specimens from France and the other with specimens from Turkey

Taxon	Pairwise distances				
	Within species	Between species			
		1	2	3	4
1 – *Bankia carinata*	1.7				
2 – *Lyrodus pedicellatus* France	0.0	20.5			
3 – *Lyrodus pedicellatus* Turkey	0.4	19.3	19.9		
4 – *Nototeredo norvagica*	0.3	28.5	23.3	26.8	
5 – *Teredothyra dominicensis*	0.0	33.8	29.1	29.9	25.8

~36 h (Lebour 1946), might reduce gene flow among distant populations and increase the probability of allopatric speciation. Indeed, this is not the first time that cryptic species have been found in the morphospecies *L. pedicellatus*. In the past, another species of *pedicellatus*-like *Lyrodus* from Florida, *Lyrodus floridanus*, was initially synonymised with *L. pedicellatus* by Turner (1966) on the basis of pallets and shells of preserved specimens. However, later laboratory experiments showed that *L. floridanus* broods its larvae only to the straight-hinge stage and then releases them *en masse* while *L. pedicellatus* carries several cohorts of larvae at different stages of development, releasing only a few young at a time, at the pediveliger stage. This new evidence led Calloway and Turner (1983) to consider *Lyrodus*

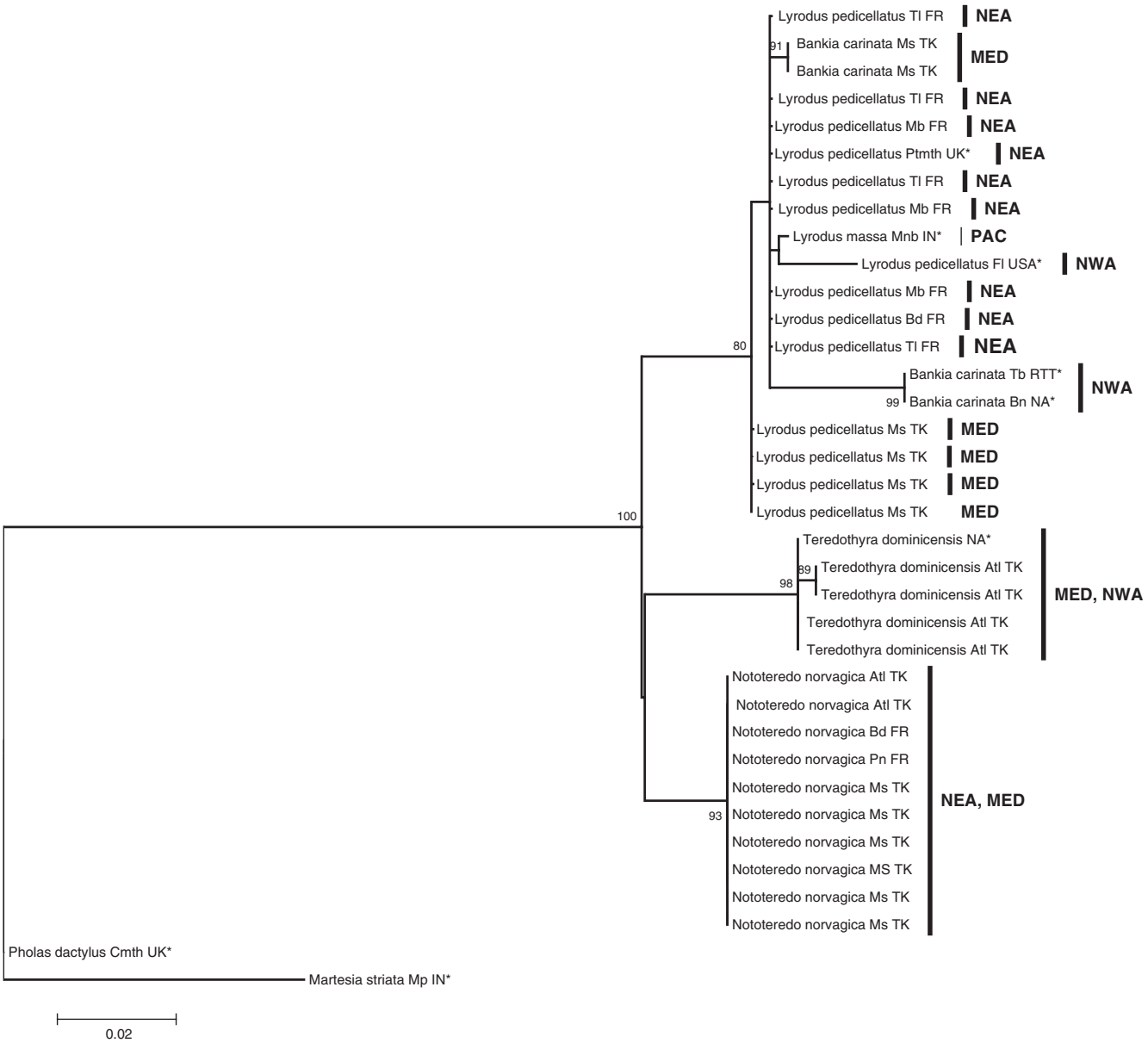

Fig. 3. Maximum-likelihood tree based on partial sequences of the 18S rRNA gene obtained from selected species of Teredinidae. Only node support values (aLRT) greater than 50 are displayed. Asterisks indicate sequences obtained from GenBank.

Table 3. Pairwise 18S rRNA sequence divergence for selected teredinids using K2P distances (%)
n.a., not applicable, group with only one specimen

Taxon	Pairwise distances							
	Within species	Between species						
		1	2	3	4	5	6	7
1 – *Bankia carinata* Mediterranean	0.0							
2 – *Bankia carinata* Netherland Antilles	0.0	2.5						
3 – *Lyrodus pedicellatus* France	0.0	0.3	2.1					
4 – *Lyrodus pedicellatus* Turkey	0.0	0.6	2.5	0.3				
5 – *Lyrodus pedicellatus* Florida	n.a.	1.5	3.4	1.2	1.5			
6 – *Lyrodus massa*	n.a.	0.6	2.5	0.3	0.6	1.2		
7 – *Nototeredo norvagica*	0.0	3.7	4.4	3.4	3.1	4.7	3.7	
8 – *Teredothyra dominicensis*	0.2	4.2	5.5	3.9	3.9	5.2	4.2	3.8

floridanus a valid species. Morphologically, however, it is not possible to distinguish young and non-breeding specimens of these two species (Calloway and Turner 1983). Although no COI-5P sequences of *L. floridanus* were found to compare with ours, the 18S sequences of *L. pedicellatus* from Florida (Distel *et al.* 2011) show divergence with both our 18S sequences of specimens from the Atlantic and from the Mediterranean and thus it seems probable that the Mediterranean form is a new cryptic species of the morphospecies *L. pedicellatus*. The divergence found in our 18S sequences of *L. pedicellatus* (Atlantic and Mediterranean forms) with the sister species *L. massa* (0.3 and 0.6% respectively) also indicates that neither forms is *L. massa*. Since the type species of *L. pedicellatus* is from San Sebastian, Spain, it is more logical to assume that our specimens from France represent the 'true' *pedicellatus*, while the specimens from Turkey are a putative new species, and the specimen from Florida (Distel *et al.* 2011) is probably *L. floridanus*.

The results above led us to reassess morphological descriptors used in *Lyrodus pedicellatus*. Pallet morphology on its own is clearly not enough for species delimitation in the morphospecies *L. pedicellatus*. Thus additional morphological characters should be considered, for instance the siphons. Specimens obtained from France all showed siphons without pigmentation. The only complete specimen from Turkey, however, showed pigmented siphons (Fig. 1). If this difference in siphons proves to be consistent among populations, it might be possible to use the siphons to distinguish species of *pedicellatus*-like *Lyrodus* from the Atlantic and the Mediterranean.

Molecular data refutes split of Atlantic and Mediterranean Nototeredo norvagica

Another example where morphological characters have not resolved the taxonomy is the case of *Nototeredo norvagica* and *Teredo utriculus.* The latter, the Mediterranean form, was considered a different species by Roch (1931). Unable to find the type species of *T. utriculus*, Turner (1966) concluded that there wasn't enough evidence to consider the Mediterranean form a different species and opted to synonymise it with *Nototeredo norvagica*. However this question was never completely settled especially because there are variations in the morphology of pallets between populations from the Atlantic and the Mediterranean (Borges 2007). Thus we decided to revisit this old taxonomic uncertainty using a combined molecular and morphological approach. Our results show that the forms from the Atlantic and the Mediterranean populations are a single species, as maximum intraspecific divergence observed in DNA barcodes varied from 0 to 0.78%, comparable with levels of the intraspecific distances reported for many marine invertebrate taxa (Radulovici *et al*. 2010) and well below the divergence range usually determined for congenerics. Nuclear 18S rRNA gene sequences corroborated COI-5P results as no divergence was observed within species, also agreeing with results obtained by Espiñeira *et al*. (2009).

Invasive teredinid species in the Mediterranean Sea

Teredothyra dominicensis has been reported as occurring consistently in the Caribbean Sea and the Gulf of Mexico (Turner 1966; Turgeon *et al.* 2009; Miloslavich *et al*. 2010), where it was initially thought to be confined. However it was also reported occurring in the Bismark Sea (Rayner 1983) although in low numbers, which was interpreted as a new introduction. In 2010 this species was found for the first time in the Mediterranean (Shipway, unpubl. data). Initial morphological identifications were later corroborated by molecular data. No match was found when performing a BLAST of our COI-5P sequences in GenBank. However a BLAST of 18S sequences matched (100%) with other sequences of 18S of *Teredothyra dominicensis* from Netherlands Antilles (Distel *et al*. 2011), confirming that it is the same species that occurs in the Caribbean. It is then possible that either adults in driftwood or larvae could have been transported in currents or in ballast water and reached the Mediterranean. Indeed, in other areas such as the Baltic Sea, teredinid larvae have been found in ballast water (Gollasch 2002), which has long been recognised as a vector for many invasive species (Carlton 1999).

Genetic divergence among populations of Bankia carinata

Bankia carinata is established around the world in tropical and subtropical waters (Turner 1966). In the Mediterranean *B. carinata* has been reported for more than a century (Graeffe 1900; Turner 1966; Borges 2007; Sivrikaya *et al.* 2009). However, our specimens from the Mediterranean were genetically distinct from specimens of *B. carinata* from the Caribbean (Fig. 3). Although extra data are needed to clarify these results, the high divergence observed seems to indicate that this might be another case of putative cryptic species. Indeed, an increasing number of taxa with disjunct geographic distributions and conserved body plans have been recognised in recent years (Bucklin *et al.* 2011).

Subfamilies Teredininae and Bankiinae not supported by molecular data

Both the results of COI-5P and 18S phylogenetic reconstructions disagree with the established subfamilies Teredininae and Bankiinae proposed by Turner (1966). All our phylogenetic trees generated with COI-5P and 18S sequences (NJ and ML for COI and 18S and NJ of amino acids for COI-5P) showed similar topologies and in all cases *Lyrodus pedicellatus* (Atlantic and Mediterranean forms) (subfamily Teredininae) was more closely related to *Bankia carinata* (subfamily Bankiinae), while *Teredothyra dominicensis* (subfamily Teredininae) was more closely related to *Nototeredo norvagica* (subfamily Bankiinae). The nearest-neighbour analysis of COI-5P sequences in the BOLD system also shows this relationship. This corroborates results of other studies in which molecular data do not support the subfamilies Teredininae and Bankiinae (Santos *et al.* 2005; Distel *et al.* 2011). Further molecular data are needed, however, to clarify the relationship among all genera of the Teredinidae, which will make possible a full assessment of the validity of Turner's three subfamilies.

Concluding remarks

The integrative approach used in the present study was useful for improving taxonomic resolution between and within teredinid species. This approach could be used in future to: (1) detect teredinid larvae in ballast water, (2) confirm the identity of invasive species, (3) identify incomplete specimens, and (4) distinguish between cryptic species. It will also promote the search for other morphological descriptors (e.g. siphons, which have been little studied), that might have been overlooked in the past, and which might prove useful in species identification in the future.

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