

Following the Phoenician example: western Mediterranean colonization by *Spirobranchus* cf. *tetraceros* (Annelida: Serpulidae)

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Summary: A newly established population of the fouling polychaete *Spirobranchus* cf. *tetraceros* is reported from the western Mediterranean (Valencia Port). Despite previous intensive surveys, this is the first record for the taxon in the Iberian Peninsula. Molecular analyses revealed that *S.* cf. *tetraceros* from Valencia are genetically identical to specimens from Heraklion, Crete, but different from those collected in the Red Sea and *S. tetraceros sensu stricto* from the type locality in Australia. Mediterranean and Red Sea *S.* cf. *tetraceros* form a well-supported monophyletic clade but are clearly distinct from New South Wales specimens of *S. tetraceros*. Our new molecular evidence supports the hypothesis that *S. tetraceros* is not a global invader of Australian origin but rather a large species complex in need of a comprehensive worldwide revision. These results highlight the importance of integrative taxonomic research for species with reported global distributions because these taxa may include cryptic invaders. An illustrated morphological account of the Valencia and Heraklion specimens and a taxonomic key for *Spirobranchus* species in the Mediterranean Sea are provided.

Keywords: cryptic species; ports; shipping; biological invasions; polychaetes; cytochrome b; identification key.

Siguiendo el ejemplo fenicio: colonización del Mediterráneo occidental por *Spirobranchus* cf. *tetraceros* (Annelida: Serpulidae)

Resumen: Una población del poliqueto incrustante *Spirobranchus* cf. *tetraceros* se reporta como establecida en el Mediterráneo occidental (Puerto de Valencia). A pesar de intensivos muestreos previos, este es el primer registro del taxón en la Península Ibérica. Los análisis moleculares revelan que los ejemplares de *S.* cf. *tetraceros* de Valencia son genéticamente idénticos a especímenes recogidos de Heraklion (Creta), pero diferentes de los muestreados en el Mar Rojo y de los *S. tetraceros sensu stricto* de la localidad tipo en Australia. Los ejemplares de *S.* cf. *tetraceros* del Mediterráneo y Mar Rojo forman un clado monofilético, pero claramente distinto de los especímenes de *S. tetraceros* de Nueva Gales del Sur. La nueva evidencia molecular respalda la hipótesis de que *S. tetraceros* no es un invasor global de origen australiano, sino más bien un gran complejo de especies que necesita una revisión exhaustiva. Estos resultados destacan la importancia de la investigación taxonómica de complejos de especies con distribución global. Se proporciona una descripción morfológica ilustrada de los especímenes de Valencia y Heraklion y una clave taxonómica para las especies de *Spirobranchus* presentes en el Mar Mediterráneo.

Palabras clave: especies crípticas; puertos; tráfico marítimo; invasiones biológicas; poliquetos; citocromo b; clave de identificación.

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INTRODUCTION

The Mediterranean Sea is a global hotspot for marine traffic under strong bioinvasion pressure (Ulman et al. 2017). A total of 821 marine non-indigenous species (NIS) have already been recorded (Zenetos et al. 2017), accounting for approximately 4.8% of its total marine biodiversity (López and Richter 2017). Shipping is the most common introduction pathway for NIS, either through hull fouling or ballast water (Çinar 2013). Marinas play a major role as invasion hubs for dispersal (Ferrario et al. 2017), and NIS appear to be more successful on artificial substrates than native species (Glasby et al. 2007, Tyrrel and Byers 2007, Megina et al. 2016). Harbour walls and floating pontoons provide ideal substrates for settlement of invasive encrusting biota (Mineur et al. 2012, Megina et al. 2013), most likely due to the enclosed nature of these specialized habitats. Sedentary tube worms belonging to the family Serpulidae are commonly found within these fouling communities along Mediterranean marinas. Invasive serpulids are of particular concern because they cause an economic burden in fuel consumption due to extra friction and professional cleaning required to remove them from hulls (Rouse 2000).

The Mediterranean Sea has shown the highest increase in NIS records (41%) since 2012 (Zenetos et al. 2017) and hosts nearly half (63/134) of the total number of polychaete NIS in the world (Çinar 2013). Polychaetes constitute up to one third of hard-bottom assemblages in both abundance and species richness in the Mediterranean (Antoniadou et al. 2004, Giangrande et al. 2004) and represent 12% of the total NIS (Zenetos et al. 2010). Artificial substrates in Mediterranean harbours are usually dominated by species of *Hydroides* Gunnerus, 1768 (e.g. Çinar 2006), but other alien calcareous tubeworms are becoming increasingly common. For example, a recent study across 50 marinas showed *Hydroides elegans* (Haswell, 1883) to be present in 66%, *Hydroides dirampha* Mörch, 1863 in 32% and *Ficopomatus enigmaticus* (Fauvel, 1923) in 14% of them (Ulman et al. 2019a). Serpulids were the most common family in boat hull biofouling communities, with *H. elegans* found on 71% (N=418) of the hulls and all serpulids combined accounting for over one-third of NIS records in relative abundance (Ulman et al. 2019b).

The serpulid genus *Spirobranchus* Blainville, 1818 currently includes 34 nominal species (Read and Fauchald 2019), 1 subspecies and 3 taxa inquirenda; five of these species have been reported from the Mediterranean: *S. lima* (Grube, 1862), *S. polytrema* (Philipp,

1844), *S. triqueter* (Linnaeus, 1758), *S. lamarcki* (Quatrefages, 1866) and *S. tetraceros* (Schmarda, 1861). Previous reports of *S. kraussii* (Baird, 1865) in the Mediterranean should be assigned to a different species, *S. cf. kraussii*, apparently undescribed (Simon et al. 2019). *Spirobranchus tetraceros* is considered an NIS of Indo-Pacific origin (Çinar 2013), with its type locality being New South Wales, Australia (ten Hove and Kupriyanova 2009). The distribution of *S. tetraceros* has been subject to debate in recent decades due to its wide range and invasive capabilities (ten Hove and Kupriyanova 2009, Ben-Eliahu and ten Hove 2011). *Spirobranchus tetraceros* is ranked among the 100 worst invasive species in the Mediterranean (Streftaris and Zenetos 2006) and has been historically considered a Lessepsian invader entering through the Suez Canal (Çinar 2013). Its first Mediterranean record is from the Lebanese coast (Laubier 1966), and it has been repeatedly collected along the eastern Mediterranean coasts since then (Ben-Eliahu 1991, Ben-Eliahu and ten Hove 1992, Ulman et al. 2017; see Fig. 1). Reported in 2016 from Siracusa (Sicily), *S. tetraceros* is considered to be undergoing a westward expansion (Ulman et al. 2017). The only previous record from western Mediterranean waters is that of six *S. tetraceros* specimens found (1979) in the biofouling community of the French aircraft carrier *Foch* arriving via the Suez Canal in Toulon after a stay of seven months in the Indian Ocean (Zibrowius 1979), but no establishment ever ensued in the area.

The first established population of *S. tetraceros* in the western Mediterranean is reported here, with specimens collected during 2015-2017 representing the first country record for Spain and the first regional record for the Marina Real (Valencia Port). Molecular evidence using cytochrome b (cytb) sequence data suggests that the nominal taxon *S. tetraceros* comprises in fact multiple species. Specimens from the *S. tetraceros* type locality (New South Wales, Australia) were genetically distinct from both Red Sea and Mediterranean material. An illustrated morphological account of the Valencia and Heraklion specimens and an updated taxonomic key for *Spirobranchus* taxa in the Mediterranean Sea are provided.

MATERIALS AND METHODS

Sampling

The Port of Valencia, Spain in the western Mediterranean Sea consists of three boathouses, the “Marina Real” and an outer harbour. Malvarrosa Beach, north

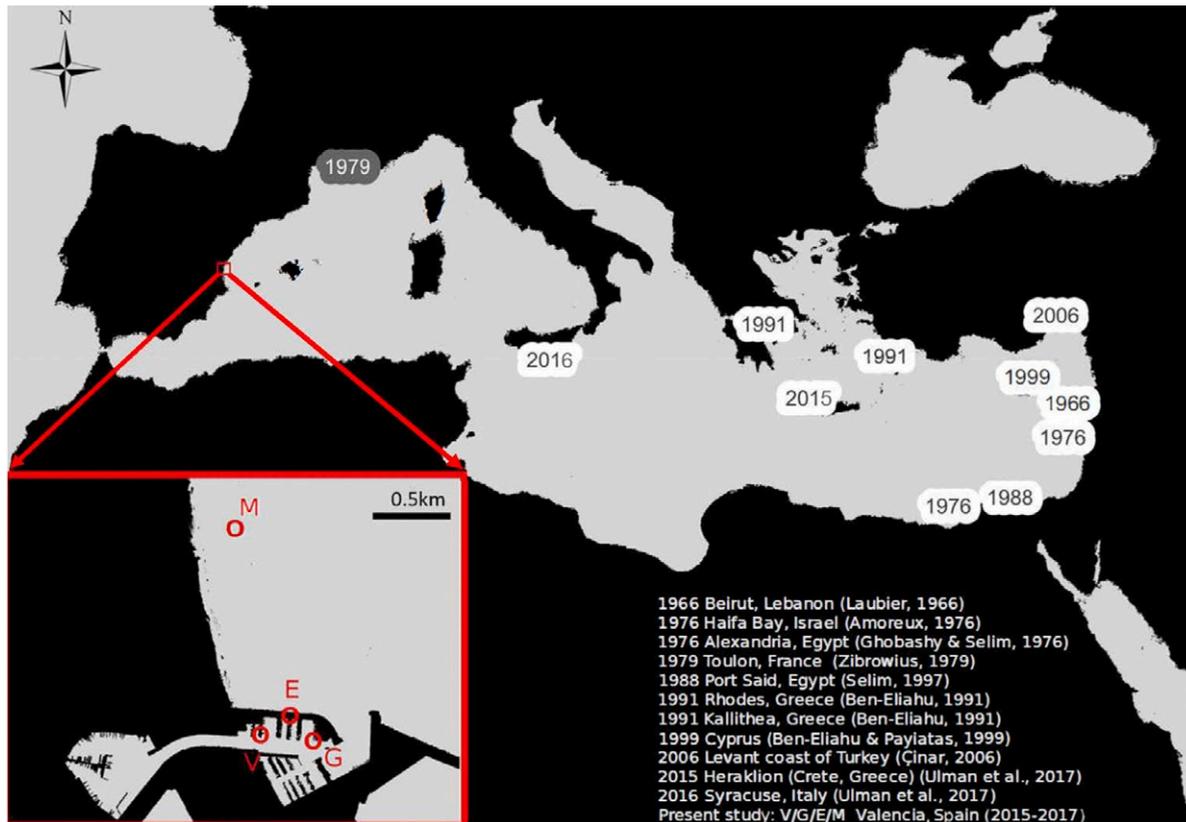


Fig. 1. – Previous records of *Spirobranchus* cf. *tetraceros* from the Mediterranean Sea and location of the new records reported in this study (red rectangle). Sampling sites (red circles) at the Malvarrosa beach artificial reef (M) and within the Valencia Port: gas station (G), sailing school (V) and breakwater (E). White labels represent the recorded year of established populations, the grey label represents a hull-fouling record (Toulon, France). Map image edited with gvSIG (<http://www.gvsig.com/es/inicio>) and GIMP (<https://www.gimp.org>) software.

of the port, is a highly-anthropized fine sand beach with several artificial concrete reefs installed in 2014 at 4 m depth, less than 200 m from the coast (Station M in Fig. 1). Sampling was carried out at three stations of the Marina Real of Valencia Port (39°26.9'N, 0°18.1'W) and one on the artificial reef (station M: 39°28'39.2"N, 0°19'13.4"W) located at Malvarrosa Beach (Fig. 1; Table 1). The Marina Real sampling was carried out at surface level (0–0.3 m) at two stations, the sailing school (V: 39°27'41.5"N, 0°19'06.5"W) and the gas station (G: 39°27'40.2"N, 0°18'45.8"W), by manual scraping using a 25×25 cm square on biological concretions located in the submerged areas of the pontoons and internal walls of the Marina. Outside the Marina, at the north breakwater (Station E: 39°27'46.1"N, 0°18'50.2"W) samples were obtained by SCUBA divers from 2–3 m depth. Samples from the Marina Old Venetian Harbour of Heraklion (35°20'51.0"N, 25°08'27.4"E) were obtained in a similar way, scraping 25×20 cm at 1.5 m depth. All biological samples were obtained from artificial substrates.

Spirobranchus cf. *tetraceros* specimens were collected in summer 2015 and in summer and winter 2016 at all three Valencia Port stations (V, sailing school; G, gas station; E, north breakwater), but not from the Malvarrosa Beach artificial reefs (see Fig. 1). Additional specimens from Valencia Port (sailing school station) were found in August and October 2016 and July 2017. Specimens were anaesthetized with 7.5% magnesium

chloride in seawater and sieved in the laboratory using a 1 mm mesh. Some individuals were removed from their tubes and fixed in 4% formaldehyde for 24 h, rinsed in seawater and transferred to 70% ethanol, while other specimens were directly preserved in 100% ethanol for later molecular analysis. Sequences were obtained for two specimens from the Valencia Port with different operculum types (simple conical and flat fully branched). To ensure a proper comparison with *S. tetraceros*, we also sequenced material collected from the type locality (New South Wales, Australia) and a previously reported population from Heraklion, Crete, Greece in the eastern Mediterranean Sea (Ulman et al. 2017). Sequences of *S. tetraceros* specimens from the Red Sea (Eilat, Israel), already available in GenBank (Perry et al. 2018), were also included in the molecular analyses (Table 1).

Morphological analyses

In order to identify and document morphological features, the specimens were examined using two Leica dissecting microscopes (models M165C and DMS 1000) and photographed using a Leica DFC420 digital camera. Chaetae and uncini were mounted under a Leica DM3000 microscope and photographed using a Leica DFC450 digital camera. Measurements were taken using the Leica Application Suite software and following Bastida-Zavala and ten Hove (2002): total

Table 1. – Specimens belonging to the *S. tetraceros* species complex analysed for the first time in the present study. Museum vouchers, GenBank accession numbers (cytochrome b sequences) and geographical origin are included. GenBank codes for *Spirobranchus* material from previous studies are shown in Figure 2. Museum abbreviations: MUVHN, Museu de la Universitat de València d'Història Natural; AM, Australian Museum.

Species	Sampling location	Museum voucher	Genbank accessions	Sampling date	Latitude	Longitude
<i>S. cf. tetraceros</i>	Spain: Valencia Port, Gas station (G)	MUVHN-ZK0000		22/07/2016	39°27'40.2"N	0°18'45.8"W
<i>S. cf. tetraceros</i>	Spain: Valencia Port, Sailing school (V)	MUVHN-ZK0001	MN631164	20/08/2016	39°27'41.5"N	0°19'06.5"W
<i>S. cf. tetraceros</i>	Spain: Valencia Port, Sailing school (V)	MUVHN-ZK0002	MN631163	05/10/2016	39°27'41.5"N	0°19'06.5"W
<i>S. cf. tetraceros</i>	Spain: Valencia Port, Breakwater (E)	MUVHN-ZK0003		04/03/2016	39°27'46.1"N	0°18'50.2"W
<i>S. cf. tetraceros</i>	Greece: Heraklion, Crete	MUVHN-ZK0004	MN631162	-/11/2015	35°20'51.0"N	25°08'27.4"E
<i>S. tetraceros</i>	Australia: New South Wales, Anchor Reef	AM W.42389	MN631161	16/03/2009	34°00'33.1"S	151°13'50.9"E

length from the tip of radioles to end of pygidium; thoracic length in ventral view from the posterior edge of the apron to the anterior edge of collar; thoracic width measured over the ventral side of the collar region across the fifth unciniger; radiolar length from the base of the radiolar crown to the tip; abdominal length from the posterior edge of the apron to the end of the pygidium in lateral view; opercular diameter; number of abdominal chaetigers; and number of radioles in each half of the crown.

DNA analyses

Total genomic DNA was extracted from samples of *Spirobranchus* collected from Heraklion, Valencia and NSW (Australia) (see Table 1 for details) using a QIAamp DNA Mini Kit (QIAGEN Inc) and following the manufacturer's instructions. DNA quality was assessed by gel electrophoresis (1% agarose) (Palero et al. 2010) and quantified using a Qubit 3.0 fluorometer (Life Technologies). A fragment (~400 bp) of the mitochondrial cytochrome b gene was amplified with ~30 ng of genomic DNA in a reaction containing 1 U of Taq polymerase (Amersham), 1 × buffer (Amersham), 0.2 mM of each primer (Cytb 424F = GGWTAYGT-WYTWCCWTGRGGWCARAT and Cytb 876R = GCRTAWGCRAAWARRAARTAYCAYTCWGG; Boore and Brown (2000)) and 0.12 mM dNTPs. The polymerase chain reaction (PCR) thermal profile was 94°C for 4 min for initial denaturation, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s and a final extension at 72°C for 4 min. Amplified PCR products were purified using QIAquick PCR Purification Kit (QIAGEN Inc.) before direct sequencing of the product. The sequences were obtained using the BigDye v3.1 (Applied Biosystems) kit on an ABI Prism 3770. Chromatograms for each PCR amplicon were checked visually and ambiguous positions were left as such using IUPAC codes. Primer sequences and flanking regions were removed from the consensus sequences created from forward and reverse strands using BioEdit ver. 7.2.5.

Sequences of several species of *Spirobranchus* were obtained from GenBank, including *S. tetraceros* from the Red Sea (MF319330, MF319331), *S. giganteus* (Pallas, 1766) from Brazil (NC032055); *S. latiscapus* (Marenzeller, 1885) from New Zealand (JX144879), *S. corniculatus* (Grube, 1862) from the Red Sea and *S. cariniferus* (Gray, 1843) from New Zealand (e.g. JX144873, JX144875) (Fig. 2). Sequences were aligned using Muscle ver. 3.6 (Edgar

2004) and conserved (ungapped) blocks of sequence were extracted using the Gblocks server with default parameters (Castresana 2000, Talavera and Castresana 2007). Estimates of p-distances (proportion of genetic differences) and Kimura 2-Parameter (K2P) evolutionary divergence between groups were obtained from the aligned cytb dataset using MEGA X (Kumar et al. 2018). Before running molecular phylogenetic analyses, the most suitable nucleotide substitution model was selected according to the BIC criterion as implemented in MEGA X (Kumar et al. 2018). The aligned sequences and selected evolutionary model were then used to estimate the maximum likelihood phylogenetic tree in RAxML (Stamatakis 2014). Node support was evaluated with 1000 bootstrap replicates.

RESULTS

Molecular identification and genetic distances

After adding GenBank data and Gblocks trimming, the final cytb alignment included 317 bp positions (from the original 400 bp). The selected DNA substitution model was the Hasegawa-Kishino-Yano model (HKY+G+I) with invariant positions (34% of the sites invariable) and heterogeneity across sites (G=1.10). The phylogenetic tree obtained by maximum likelihood (Ln=-2999.90) provides further support for the separation of the Australian *S. tetraceros* from the Mediterranean specimens, showing that these two populations are not monophyletic. Therefore, Mediterranean *Spirobranchus* are here referred to as *S. cf. tetraceros* and considered to belong to a different species, most likely undescribed, rather than to *S. tetraceros sensu stricto* from Australia. Red Sea samples clustered (with high bootstrap support) with samples from the Mediterranean (Fig. 2).

Both p-distances and K2P distances showed a similar pattern, with intraspecific genetic distances (not shown) being much lower (<0.02) than inter-specific distances (>0.14) (Table 2). Observed values for the K2P genetic distances between *S. tetraceros sensu stricto* from the type locality (NSW) and *S. cf. tetraceros* from Mediterranean (0.424±0.045) or Red Sea (0.385±0.042) were larger than distances between *S. cf. tetraceros* from Mediterranean and Red Sea (0.274±0.034). For comparison, K2P distances between those two groups of *S. cf. tetraceros* were larger than distances observed between other pairs of valid species such as *S. aloni* and *S. corniculatus* (0.197±0.028) or *S. aloni* and *S. gardineri* (0.252±0.033). Several non-

synonymous changes could be observed between the *S.* cf. *tetraceros* from Mediterranean and Red Sea when translating the DNA sequences into protein, which suggests that these two populations may correspond in fact to valid (most likely undescribed) taxa. Nevertheless, a more comprehensive revision, including more populations and genetic markers should be carried out before drawing a final conclusion on the taxonomic status of these two groups.

Morphological analyses and systematic account

Genus *Spirobranchus* Blainville, 1818 *Spirobranchus* cf. *tetraceros*

Spirobranchus tetraceros Ben-Eliahu and ten Hove 2011: 88-95, Fig. 34, Table 5

Spirobranchus tetraceros sensu lato Ulman et al. 2017: 34-35, Table 2

Material examined. Western Mediterranean Sea, Marina Real (Valencia Harbour, Spain): 14 specimens, Stations: V (7), E (6), G (1); eastern Mediterranean Sea, Heraklion (Crete, Greece) at the

Marina Old Venetian Harbour: six specimens. Measurements and detailed morphological descriptions are based on the largest complete specimens from Valencia Port (Station E; Fig. 1) and Heraklion, and complemented with details on structures (e.g. tubes) from other specimens collected at the same localities. The specimens are deposited at Department of Zoology, School of Biological Sciences, University of Valencia (Spain).

Tube. Attached to artificial substrates such as plastic pontoons, vertical cement walls, buoys, metal ladders and cement blocks. Tube outside and inside predominantly white (though occasionally slightly pinkish internally near opening), triangular to circular, with a tooth over entrance and one high, irregular longitudinal ridge, a pair of low lateral keels and many transversal ridges (Fig. 3A, B).

Total length of the largest specimen. 51.3 mm (Valencia) and 11.9 mm (Heraklion). Note that differences in size of the largest specimen depends on sampling. The number of complete specimens examined from the

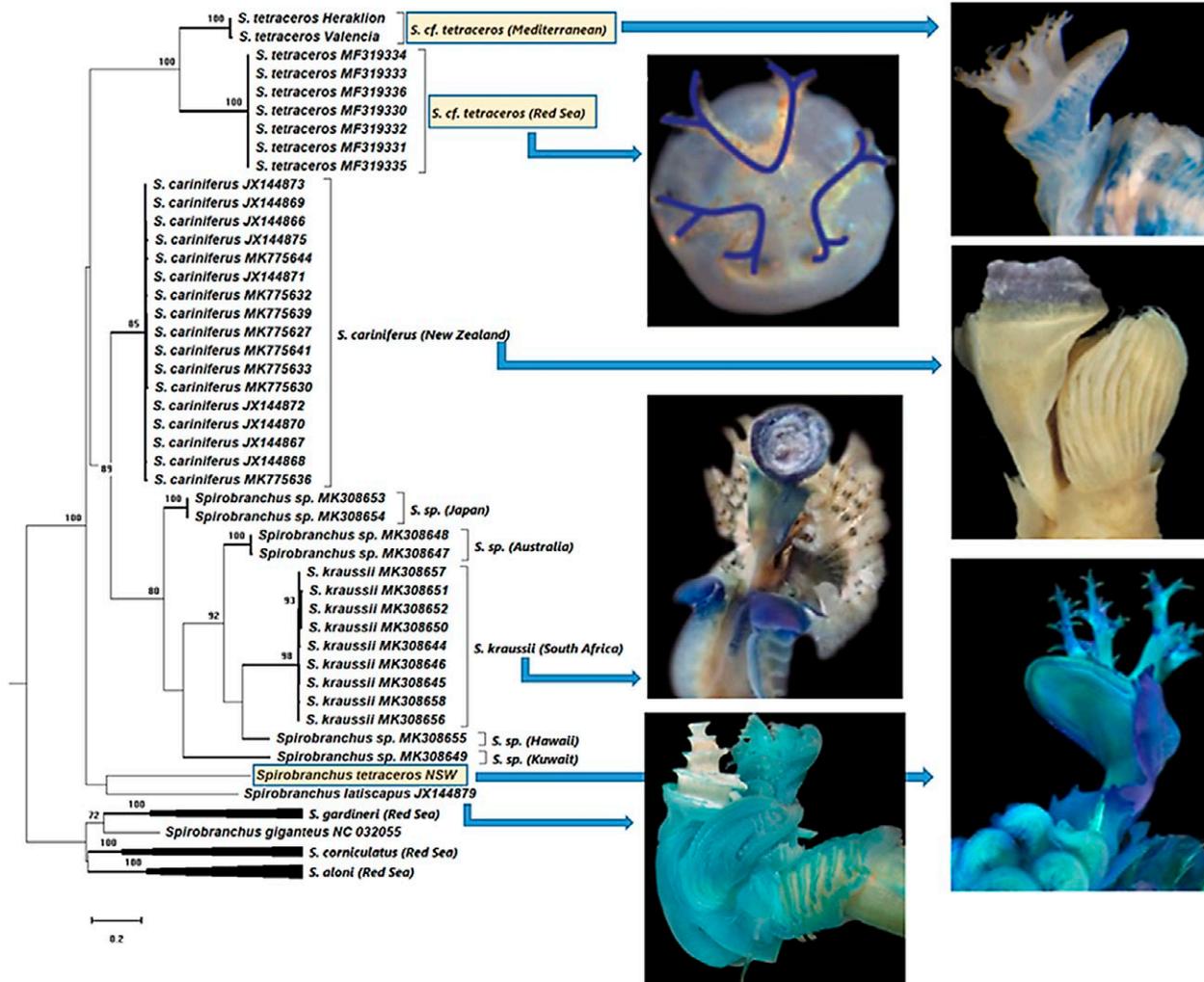


Fig. 2. – Maximum likelihood phylogenetic tree. Only bootstrap support values above 70% are shown. Images for specimens assigned to the *Spirobranchus tetraceros* complex taken by Aylin Ulman (Mediterranean clade), Orly Perry (Red Sea clade) and Eunice Wong (Australian clade). Photos of *Spirobranchus cariniferus* and *S. latiscapus* were taken by Eunice Wong and that of *S. kraussii* was taken by Carol Simon and modified for presentation here.

Table 2. – Estimates of evolutionary divergence between groups. The number of base substitutions per site (\pm standard error estimates) obtained from averaging over all sequence pairs between groups are shown. P-distances are shown above the diagonal and K2P distances below the diagonal. Analyses were conducted using MEGA X (Kumar et al. 2018).

	<i>S. cariniferus</i>	<i>S. kraussii</i>	<i>S. tetraceros</i> (NSW)	<i>S. cf. tetraceros</i> (Mediterranean)	<i>S. cf. tetraceros</i> (Red Sea)	<i>S. gardineri</i>	<i>S. aloni</i>	<i>S. corniculatus</i>
<i>S. cariniferus</i>		0.232 \pm 0.025	0.283 \pm 0.024	0.256 \pm 0.023	0.294 \pm 0.025	0.311 \pm 0.025	0.312 \pm 0.025	0.275 \pm 0.025
<i>S. kraussii</i>	0.280 \pm 0.035		0.294 \pm 0.027	0.291 \pm 0.025	0.283 \pm 0.027	0.310 \pm 0.026	0.317 \pm 0.027	0.296 \pm 0.026
<i>S. tetraceros</i> (NSW)	0.362 \pm 0.042	0.378 \pm 0.043		0.322 \pm 0.025	0.300 \pm 0.025	0.312 \pm 0.024	0.334 \pm 0.026	0.323 \pm 0.026
<i>S. cf. tetraceros</i> (Mediterranean)	0.319 \pm 0.036	0.369 \pm 0.044	0.424 \pm 0.045		0.224 \pm 0.022	0.310 \pm 0.025	0.322 \pm 0.026	0.317 \pm 0.025
<i>S. cf. tetraceros</i> (Red Sea)	0.381 \pm 0.044	0.357 \pm 0.041	0.385 \pm 0.042	0.274 \pm 0.034		0.319 \pm 0.026	0.344 \pm 0.027	0.327 \pm 0.026
<i>S. gardineri</i>	0.410 \pm 0.045	0.403 \pm 0.046	0.406 \pm 0.041	0.402 \pm 0.044	0.417 \pm 0.044		0.211 \pm 0.022	0.181 \pm 0.021
<i>S. aloni</i>	0.415 \pm 0.046	0.416 \pm 0.048	0.445 \pm 0.048	0.426 \pm 0.047	0.466 \pm 0.05	0.252 \pm 0.033		0.171 \pm 0.021
<i>S. corniculatus</i>	0.347 \pm 0.038	0.378 \pm 0.042	0.426 \pm 0.044	0.418 \pm 0.045	0.434 \pm 0.045	0.210 \pm 0.028	0.197 \pm 0.028	

Valencia Port area (N=14) is larger than those found in Heraklion (N=6). Moreover, specimens from Valencia were collected both in summer and winter and therefore it is reasonable that they show a greater variation in size.

Radiolar crown. Composed of two circular lobes each with 25 (Valencia) or 18 (Heraklion) radioles (Fig. 4A-C). Length of radiolar crown 7.1 mm (Valencia) and 2.2 mm (Heraklion). Colour blue/white.

Interradiolar membrane. Usually with unilobed, bilobed or multilobed processes (Fig. 4C-E), the shape of which may vary from complex dorsally to simple ventrally.

Peduncle. Inserted on the left of median line, pigmented with white/blue colours (Fig. 4B). Lateral distal wings clearly protruding left and right of opercular

plate with pointed tips and crenulated on their inner and outer margins (doubly fringed) (Fig. 5A).

Operculum. Peduncle joining operculum in dorsal position. Diameter 3.8 mm (Valencia) and 2.2 mm (Heraklion). Operculum with circular calcareous endplate, which may be flat, concave, convex or even conical; endplate bearing three groups of dichotomously branched (antler-like) spines, sometimes appearing as three spines only (particularly in the conical operculum) (Fig. 5B-E); position of spines always the same: one (or one group) medio-ventrally and two (or two groups) latero-dorsally. The most complex opercula showing one medio-ventral spine split thrice and two latero-dorsal

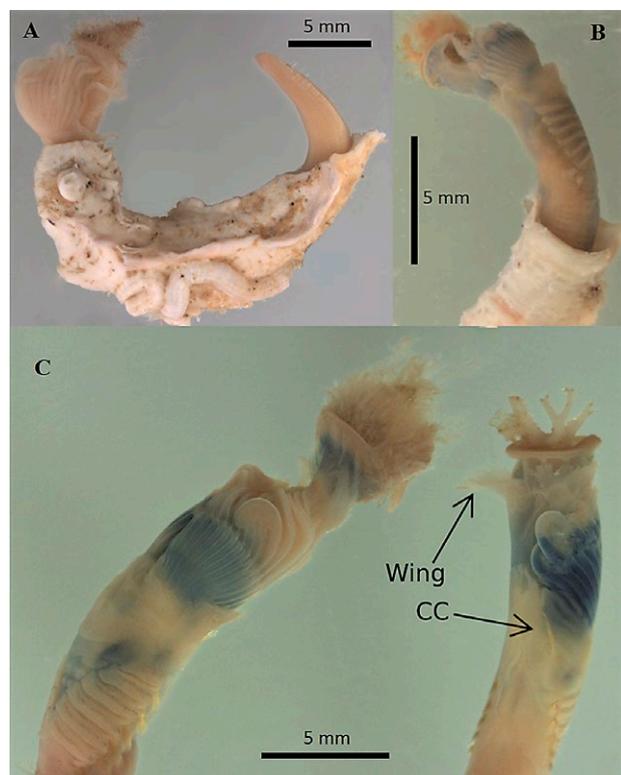


Fig. 3. – *Spirobranchus* cf. *tetraceros* from Valencia Port. A-B. Specimens in their tubes: A, lateral view; B, dorsal view. C, worms removed from their tubes (lateral and dorsal views). Abbreviation: CC: collar chaeta.

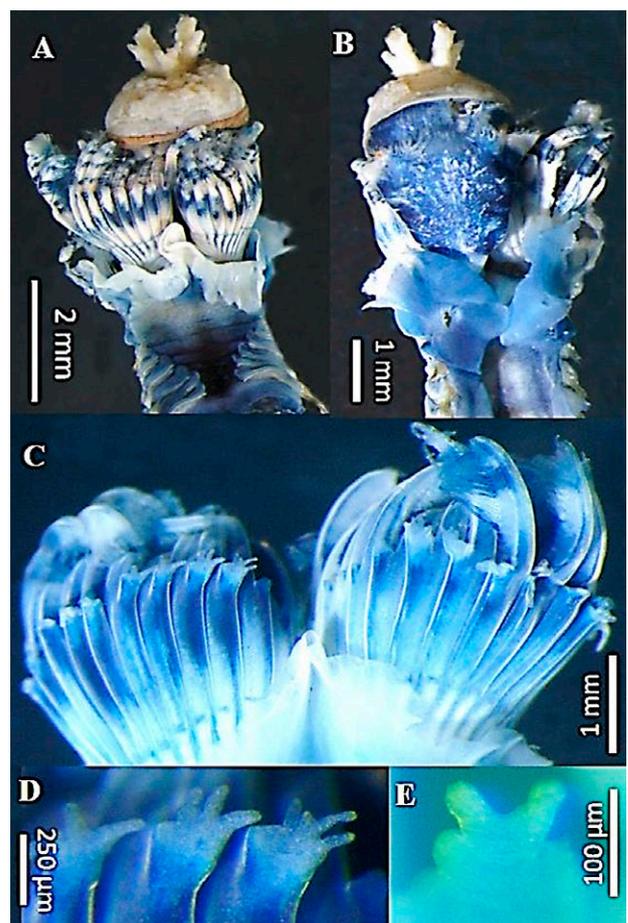


Fig. 4. – *Spirobranchus* cf. *tetraceros* (Valencia Port) removed from its tube. A, ventral view. B, dorsal view. C-D, interradiolar membrane with multilobed processes. E, details of bilobed processes.

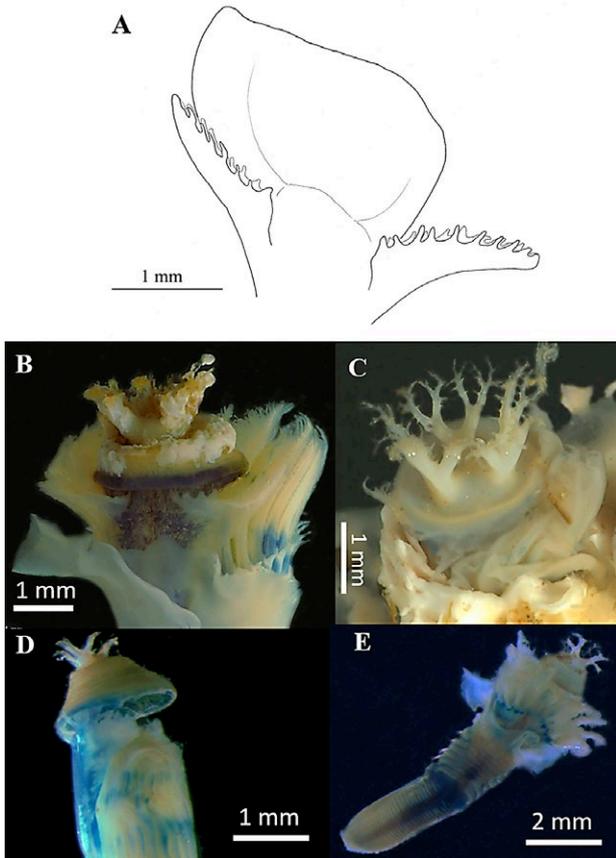


Fig. 5. – *Spirobranchus* cf. *tetraceros* from Valencia Port. A, opercular peduncle with distal wings (dorsal view); B-D, opercular morphology; D-E, ventral view of conical opercula with spines.

spines split twice to thrice, with medial spinules irregularly placed.

Polymorphism of opercula. Simple conical (Fig. 5D, E), flat and flattened fully branched (Fig. 5C) as well as intermediate forms (Fig. 5B) were found within the Valencia Port samples.

Collar and thoracic membranes. Collar divided into one ventral and two lateral lobes. Latero-dorsal lobes continuing into thoracic membranes (Fig. 4A)

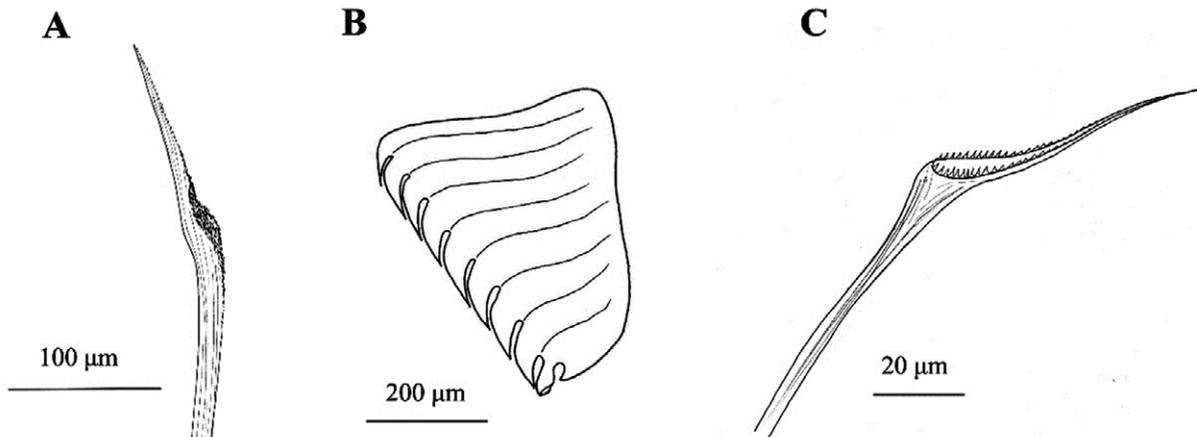


Fig. 6. – Chaetae of *Spirobranchus* cf. *tetraceros* from Valencia Port. A, *Spirobranchus*-type collar chaetae; B, thoracic uncini; C, true trumpet-shaped abdominal chaetae.

producing a short ventral apron with shallow midventral indent (Fig. 5E). Collar chaetae of two types: special *Spirobranchus*-type covered with minute denticles (Fig. 6A) and limbate-striated (not shown).

Thorax. Seven thoracic chaetigers, including six uncinigerous. Collar fascicle (CC in Fig. 3C) situated at some distance anterior to remaining thoracic chaetae (Fig. 3C). Thoracic chaetae limbate. Uncini saw-shaped with peg (anterior-most tooth) gouge-shaped (Fig. 6B). Length of thorax: 8.2 mm (Valencia) or 3.4 mm (Heraklion). Thoracic width at 5th row of uncini: 5.9 mm (Valencia) or 1.6 mm (Heraklion). Ventral ends of thoracic uncinigerous tori widely separated anteriorly, gradually approaching one another towards the end of thorax, thus leaving a triangular depression (Fig. 4A).

Abdomen. 87 (Valencia) or 37 (Heraklion) chaetigers. Length 36.2 mm (Valencia) or 7.5 mm (Heraklion). True trumpet-shaped chaetae (Fig. 6C) in a single fascicle, becoming increasingly longer posteriorly. Saw-shaped uncini with gouge-shaped pegs.

Colouration of preserved specimens. Anterior end of thorax, radioles, peduncle and operculum dark blue (Fig. 4B).

Remarks. The original description of the species by Schmarda (1861), based on material from NSW (Australia), does not follow current standards and prevents a suitable comparison with Mediterranean material. A re-description of *S. tetraceros* from the type locality could not be included here. An accurate and comprehensive morphological revision of material from multiple localities is needed before pointing out useful characters to discriminate between putative taxa.

Key to Mediterranean *Spirobranchus*, including alien species (*)

- 1. Collar chaetae absent.. *Spirobranchus* cf. *kraussii* (*)
- Collar chaetae present..... 2

2. Collar chaetae few, fine and capillary 3
 - Collar chaetae numerous, large and *Spirobranchus*-type 4
3. Opercular ampulla cup-shaped, higher than distal calcareous cap, which may be flat, concave or slightly convex, with or without distal projections (in this case, with a central cylindrical protuberance from which project 1-3 short tips); anterior margin of lateral-dorsal lobes of collar not fringed *Spirobranchus lamarcki*
 - Opercular ampulla flat as an empty balloon, thinner than the distal convex, often conical calcareous cap, with or without projections (often with three teeth); anterior margin of lateral-dorsal collar lobes finely fringed *Spirobranchus triqueteter*
4. Opercular plate typically with three groups of dichotomously branched spines, sometimes conical cap only; interradiolar membrane and anterior margin of peduncular wing with finger-like processes.. *S. cf. tetraceros* (*)
 - Opercular plate without spines; interradiolar membrane without finger-like processes.....5
5. Opercular plate flat or concave; opercular peduncle with wide wings fringed at the tip; tube rose, with about five longitudinal serrated keels..... *S. lima*
 - Opercular plate convex, often with two dorsal tubercles; opercular peduncle with narrow, rarely bifid wings; tube white, with three keels and a series of lateral alveoli..... *S. polytrema*

DISCUSSION

Several specimens of *S. cf. tetraceros* were collected from the Marina Real of Valencia Port during both winter and summer surveys in three consecutive years (2015-2017). Consequently, an established population of *S. cf. tetraceros* from the western Mediterranean is reported here for the first time, also representing a first record for the Iberian Peninsula. *Spirobranchus tetraceros* was first reported from the western Mediterranean 40 years ago, fouling the aircraft carrier “*Foch*” in Toulon (Zibrowius 1979, and see above), but did not establish then. Despite a recent intensive survey of dozens of recreational marinas along the Mediterranean coast, Ulman et al. (2017) did not find *S. tetraceros sensu lato* in the Marina of Alicante (Spain) or the OneOcean Port Vell Marina in Barcelona. The fact that this invader was present in Valencia during the same time period, but apparently not in Alicante or Barcelona, underscores the necessity of a comprehensive study of Mediterranean ports and marinas in order to identify established invasive species.

Morphology and molecular sequence analyses confirm that the specimens of *S. cf. tetraceros* from Valencia are identical to those found in the eastern Mediterranean (Heraklion, Crete). Most importantly, Mediterranean specimens are shown to be genetically different from specimens of *S. tetraceros sensu stricto* collected from the type locality (New South Wales, Australia). This result directly supports the hypothesis (in Perry et al. 2018), that *S. tetraceros* is not a single widely distributed invader of Australian origin, but

rather a complex of cryptic species. The second implication is that the Mediterranean specimens examined herein may belong to a yet undescribed species of the *S. tetraceros* complex. The identity and origin of the Mediterranean population remains uncertain, because the widely accepted hypothesis of Ben-Eliahu (1991) that *S. cf. tetraceros* is a Lessepsian migrant passively crossing the Suez Canal to the Mediterranean is not conclusively supported by our results. Genetic distances between Red Sea (Gulf of Eilat) specimens and Mediterranean *S. cf. tetraceros* seem large enough to be considered as belonging to distinct taxa. Nevertheless, a more comprehensive worldwide revision, including more populations and genetic markers, should be carried out before drawing a final conclusion on the taxonomic status of these populations.

Morphological species delimitation is particularly difficult in *Spirobranchus* because of their high intraspecific variability opercular structures, considered one of the major taxonomic characters of the genus. Several taxa were initially synonymized by ten Hove (1970) under *S. tetraceros* and a cosmopolitan distribution was hypothesized for this taxon, among other reasons because of its high opercular variation (see also Perry et al. 2018). Ben-Eliahu and ten Hove (2011) and Willette et al. (2015) also reported highly variable opercula for *S. tetraceros* specimens from the Suez Canal and the Indo-Pacific *S. corniculatus*, respectively. The molecular characterization of Valencia Port and Heraklion specimens carried out here, including specimens with either conical or fully-branched opercula, confirms that this morphological variation simply corresponds to intraspecific plasticity. This result highlights the importance of using molecular data for species delimitation and the need to further analyse the morphology of the species of *Spirobranchus*. Other characters should be used to discriminate species within the *S. tetraceros* complex, such as the shape and distribution of multilobed processes from the interradiolar membrane.

Worldwide distributed cryptic invaders are particularly difficult to track because they are often assumed to be native species or wrongly assigned to other invasive species (Morais and Reichard 2018). Our results are relevant for the management of Mediterranean NIS, showing that *S. tetraceros* represents a species complex rather than a single widely distributed species. Mediterranean specimens differ genetically from *S. tetraceros sensu stricto* from the type locality and may have a different ecology, so management practices should be planned taking this into account. Further sampling and ecological studies across both temperate and tropical areas, including populations from West Africa and the Caribbean Sea, are necessary to complete a worldwide revision of the *S. tetraceros* complex. Reliable species delimitation within this complex will require a complete re-evaluation of morphological characters and ecological and biogeographical considerations, as well as the analysis of both mitochondrial and nuclear markers (e.g. microsatellites or single nucleotide polymorphisms). The combined use of morphological and molecular data, as carried out here, should be consid-

ered of paramount importance for the study of widely distributed invasive species.

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