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On the presence of a giant bristle worm (*Eunice roussaei*) IN NW Iberian Peninsula: Comments on its taxonomy and reproductive cycle

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ABSTRACT

The presence of a giant "polychaete" annelid of the genus *Eunice* has been recently recorded in the Ría de Arousa (Galicia, NW Spain). Since *Eunice* spp. are highly appreciated as bait for recreational fishing in other regions of the world, representing a marine resource of high economic value, this finding may open up a new opportunity for the local bristle worm fishery. The present study aims to gain some insights into the biology of this largely unknown species, gathering information that may contribute to the sustainability and management of this potential fishery. Even though samples could not be collected on a monthly basis as initially planned, our results revealed some interesting features. First, as the taxonomic identity of giant specimens of the genus *Eunice* has been subject to debate, we used molecular techniques to confirm the identity of the species present in NW Spain. We also sequenced COI–5P in 9 specimens from Vrsar (Istria, Croatia), as well as in one specimen found in the facilities of the Aquarium Finisterrae of A Coruña (NW Spain) in 2015. COI–5P sequences (658 bp) revealed that Galician and Croatian individuals are conspecific and belong to the species *Eunice roussaei*, previously described in the Mediterranean Sea. Moreover, our data suggest a high haplotypic diversity (18 haplotypes were identified among the 20 sequences analyzed) but a low nucleotide diversity. Second, the observations on the 15 Galician specimens collected between October 2019 and September 2020 suggest that *E. roussaei* is a dioecious, broadcast spawner that reproduces by releasing free gametes into the water column in the autumn months of November/December. Oocytes maturated from January (mean oocyte size = 105.05 μm) to October (mean oocyte size = 202.02 μm), and oocyte size was positively correlated with water temperature ($r = 0.746$, $p < 0.05$), as well as with photoperiod ($r = 0.268$, $p < 0.05$).

1. Introduction

"Polychaete" annelids constitute a very important economic resource worldwide, mainly due to their demand as live bait for recreational fishing, leading to a sizeable market of mainly wild species (Cole et al., 2018). On the Galician coast (NW Iberian Peninsula), the commercial "polychaetes" fishery began in 2002 (Xunta de Galicia, Consellería do Mar). Currently, four species are being exploited for their use as bait in sport fishing (DOG Nº 246, 2018). However, some fishermen in the Ría de Arousa (southern Galicia, NW Iberian Peninsula) have recently reported the presence of a giant "polychaete" species of commercial importance in other regions of the world. The presence of giant "polychaetes" had not been previously documented on the Galician coasts, except for the discovery of a single specimen of *Eunice roussaei* Quatrefages, 1866, in the Ría de Ferrol (northern Galicia) in 1999 (Parapar and Harto, 2001). Later, in 2015, a second specimen was found when emptying one of the tanks of the public science centre *Aquarium Finisterrae* of A Coruña (northern Galicia).

Giant "polychaetes" belong to the family Eunicidae, and some of them have been the subject of taxonomic debate. This is, for example, the case of *E. roussaei* and *Eunice aphroditois* (Pallas, 1788) (Salazar-Vallejo et al., 2011). Fauvel (1923) was the first to identify the Adriatic giant "polychaete" as *E. roussaei*. The confusion arose after Hartman (1944) synonymised *E. roussaei* with *E. aphroditois*. Since then, studies on the Mediterranean have referred to the species as *E. aphroditois*. Years later, however, Fauchald (1992) demonstrated that *E. roussaei* and *E. aphroditois* are different species, reinstating *E. roussaei* as a valid species for the Mediterranean Sea (Zanol and Bettoso, 2006;

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Mikac, 2015). The synonymy of these two species also remained for many years in European Atlantic waters. Even though first records of giant "polychaetes" in the north Iberian Peninsula were described as *E. rousseaui* (an obvious transcription error that, however, was replicated by the World Register of Marine Species website) (Cabrera, 1909; Rioja, 1917), later records in the region were identified as *E. aphroditois* (Bellan, 2001), including a single specimen found in Asturias (North Iberian Peninsula) (Fernández-Ovies and Ortega, 1983). Still, Núñez et al. (1997) recorded the species in the Macaronesian area with its current status. In addition, in 2001, the species from the Galician coasts was identified as *E. roussaei* by Parapar and Harto (2001), while more recently Zanol and Bettoso (2006) confirmed the name *E. aphroditois* restricted to a Pacific species. This controversy exemplifies the difficulties of identifying species based only on morphological characteristics for certain taxa. Thus, in recent times, molecular tools have been proposed as necessary for delineating species boundaries and clarifying distributions in poorly studied groups, such as "polychaetes" (Nygren, 2014). In particular, cytochrome *c* oxidase subunit I (COI) sequences in mitochondrial DNA have become a useful and popular marker for the molecular identification of animals because of its rapidness and accuracy (Hebert et al., 2003).

Furthermore, some giant "polychaetes" exhibit a conspicuous way of reproduction known as epitoky, which involves a noteworthy sexual metamorphosis (Hofmann, 1974; Schulze & Timm, 2006). This process entails abrupt morphological and physiological modifications when individuals reach sexual maturity, aimed at preparing them for pelagic life. In particular, in some Eunicidae worms, the posterior end of the animal is modified. In summary, this body region, full of gametes and called the epitokous section, is detached at maturity from the anterior end (the atokous section) to spawn near the water surface. Moreover, these reproductive episodes have been observed to take place at a particular phase of the moon and always at the same time of the year (Wilson, 1991; Giangrande, 1997). This phenomenon has been long known and described for *Palola viridis* in some tropical islands of the Pacific Ocean (Stair, 1897; Caspers, 1984). However, very few studies have been carried out on the reproduction of other Eunicidae. To the best of our knowledge, the only bibliographic records on the reproduction of *Eunice* spp. correspond to the observations made by Bettoso et al. (1998) for *E. roussaei* (as *E. aphroditois*) and by Wilson (1991) for *Eunice schemacephala* Schmarda, 1861, both of them stating that these are dioecious species that reproduce by broadcast spawning into the water column.

Regarding the specimens recently observed on the coast of Galicia, neither scientific nor informal biological knowledge is available to date except for the citation by Parapar and Harto (2001). However, since these individuals reach up to 2.5 m long, they potentially represent a profitable resource for local fishermen, like in the Adriatic Sea, where they are used as bait for catching valuable fish (Gambi et al., 1994; Cetinic and Soldo, 1999). The lack of knowledge about the biology and population dynamics of "polychaetes" has been identified as one of the main limitations for the sustainability and management of bristle worm fisheries at a global level (Cole et al., 2018). Therefore, in order to gain biological knowledge on this marine resource, the taxonomic identity and reproductive biology of this species were studied. In particular, we identified the individuals from the Atlantic coast of Galicia using molecular techniques and compared them with Mediterranean individuals caught in the Adriatic Sea. In addition, we studied two aspects of its reproductive biology: spawning behaviour and sexual maturation cycle.

2. Materials and methods

2.1. Study area and sampling

Eunice specimens were collected in "O Carreiro", in the Ría de Arousa, NW Spain (midpoint of the sampled area: 42.480250N-8.934746W; Fig. 1). These "polychaetes" inhabit the subtidal zone, and specimens were collected at a depth of 1–3 m at low tide. The sampled area was characterised by a mixed rocky and sandy bottom, and

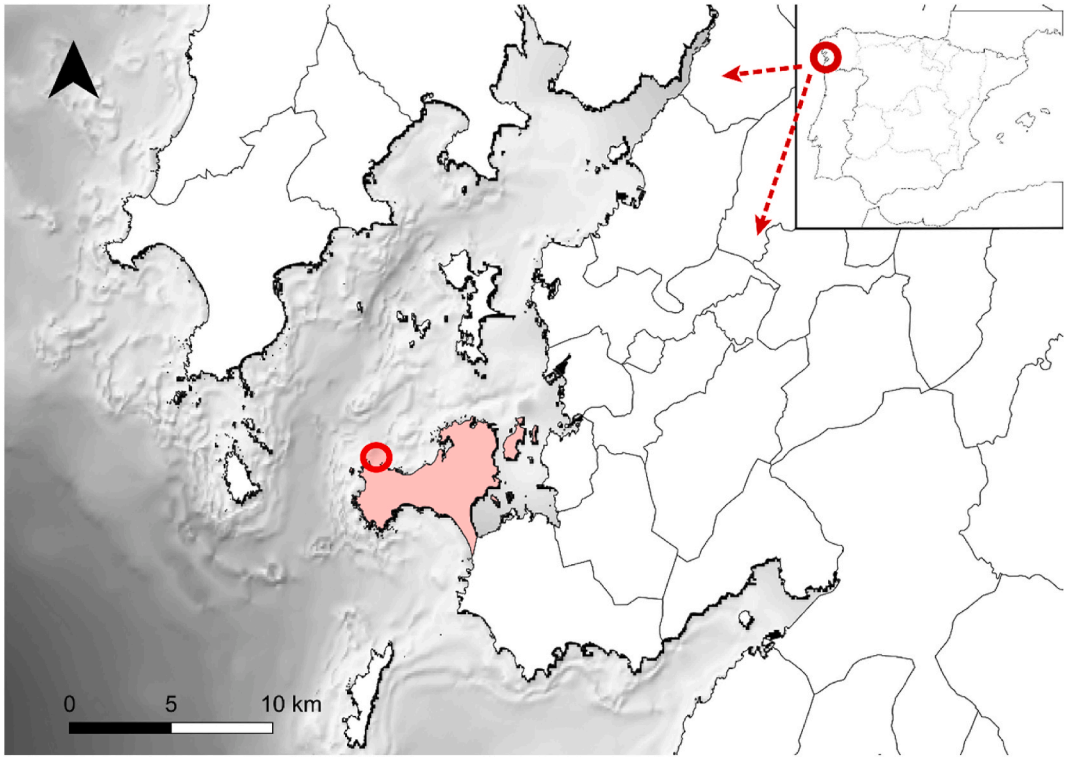

Fig. 1. Map of the Ría the Arousa with the "O Grove Peninsula" highlighted in pink. The sampled area "O Carreiro", marked with a red circle, covered an area of approximately 0.1 km2 where the E. roussaei specimens were captured (42.48025N, −8.934746W). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

"polychaete" burrows frequently occurred at the intersection of these two habitats.

Specimens were captured at monthly intervals from October 2019 to September 2020 by free diving, placing traps baited with salted anchovy over the burrows where they live (Bettoso et al., 1998). Once the animal had bitten the bait, it was slowly extracted from the burrow with the help of an air bottle attached to the trap, pulling the animal out.

2.2. Treatment of live specimens

Specimens were transported alive in sea water to the laboratory on the same day of their capture or, alternatively, were kept in an aquarium in the fishing area for up two days before being transferred to the laboratory. Once there, the individuals were anaesthetised in a 7.5% $MgCl_2$ isotonic solution with tap water, and their total length was registered using a tape measure. In addition, a preliminary morphological identification was done following Carrera-Parra (2009). Tissue samples for genetic analysis were taken, either from the posterior end of the animal or from a gill, and preserved in 100% ethanol. Finally, the whole specimens were fixed in formaldehyde 4%, and later preserved in 70% ethanol. The general appearance of the specimens can be observed in Figure A1 of the supplementary material.

2.3. Genetic analyses

Nine individuals obtained during the present study were selected for genetic analyses. Moreover, these analyses also included two additional individuals captured earlier in the region (the specimen of *Eunice* that appeared in a tank of the *Aquarium Finisterrae* in A Coruña, NW Spain, in February 2015 and another specimen caught by a fisherman at the sampling site of this study in July 2017), as well as nine individuals captured in Vrsar (Istria, Croatia) in July 2021.

DNA was extracted from a small piece of tissue using the commercial NZY Tissue gDNA Isolation kit (NZYTech). A ca. 700 bp fragment located at the 5′ end of the mitochondrial gene cytochrome *c* oxidase subunit I (COI–5P) was amplified by PCR using primers ACOIAF (5′CWA ATC AYA AAG ATA TTG GAAC 3′) (Colgan et al., 2001) and COIEUR-R (5′TCD GGR TGD CCA AAR AAT CA 3′) (Zanol et al., 2010). The PCR reactions were carried out in a final volume of 25 μL containing 12.5 μL of Supreme NZYTaq Green PCR Master Mix (NZYTech), 0.5 μM of each primer, 2.5 μL of a 1:10 dilution of the template DNA solution, and PCR-grade water up to 25 μL. Thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 42 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. Finally, PCR products were run on 2% agarose gels stained with GreenSafe (NZYTech) and, after checking correct amplicon size, were bidirectionally sequenced using the same PCR primers.

Sequences were checked and edited in Geneious 8.1.9 (Biomatters Ltd.). After alignment and trimming, the final length of our sequences was 658 bp. Sequences were first compared with those published in GenBank. Data analyses also included several estimates of DNA polymorphism calculated with the software DNASP version 6.12.03 (Rozas et al., 2017): haplotype diversity (H_E), Nei's (1987) nucleotide diversity (π), number of haplotypes (N_H), and number of segregating sites (S). Likewise, the genealogical relationship between haplotypes was inferred by the median-joining network approach implemented in Network version 10.2.0.0 (Bandelt et al., 1999).

2.4. Reproductive biology

2.4.1. External signs of epitoky

Morphological observations of the specimens were made to find possible signs of epitoky. In particular, we looked for the transformation of the posterior segments or the appearance of external structures that facilitate pelagic life (Schroeder and Hermans, 1975).

2.4.2. Oocyte maturation cycle

All specimens were dissected by making a dorsal incision along the entire length of the body to check for the presence of oocytes in their coelom. The following data were recorded: first setiger with oocytes, body interval containing oocytes (cm) and mean oocyte size (μm) in the anterior, middle, and posterior region of each individual. For the determination of the mean oocyte size, a coelomic solution with oocytes was extracted with a Pasteur pipette, mounted in a Petri dish, and examined under a stereomicroscope with a digital camera attached; the NIS-Elements image analysis software (Nikon Instruments Inc., Melville, NY) was then used to determine the diameter of 40 oocytes picked at random. We did not look for the presence of sperm because of the difficulties in detecting it once the specimens had been fixed.

In order to explore the influence of environmental variables on the oocyte maturation cycle, water temperature data were retrieved from the Meloxo oceanographic station belonging to INTECMAR (http://www.intecmar.gal/) using the available data at 3 m depth closest to our sampling dates. The photoperiod in the sampling dates was also obtained from the MeteoGalicia historical database for Vilagarcía de Arousa (https://www.meteogalicia.gal/).

2.5. Data analysis

Differences in average oocyte size depending on the body region (anterior, middle, and posterior) were checked by the Friedman test. Correlation of oocyte size with water temperature and photoperiod was evaluated by the Spearman correlation test.

Both analyses were carried out using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp. Released, 2017; Armonk, NY: IBM Corp).

3. Results

A total of 17 specimens were captured from October 2019 to September 2020 (Table 1). The catches ranged from 1 to 3 individuals per month and covered only a part of the study period (Fig. 2). Two of these specimens were transported and maintained alive in the *Aquarium Finisterrae* of A Coruña for further research; therefore, only 15 individuals were available for this study. Six out of the 17 individuals were broken during the capture process. The longest complete "polychaete" caught was 250 cm long, while the shortest complete "polychaete" was 110 cm long. The mean length of complete "polychaetes" was 179 cm.

3.1. Genetic analyses

We compared 20 sequences and found that 35 out of the 658 sites were polymorphic (5.3%). Eighteen haplotypes were identified: 8 from Vrsar in Croatia, 9 from O Grove in Galicia, and 1 from the Aquarium Finisterrae (GenBank accession numbers OL889804-OL889821; Table 2), but a NCBI BLAST search revealed that all of them showed 98.6–99.7% homology with the sequence *Eunice roussaei* voucher USNM1120728 cytochrome *c* oxidase subunit I from Funtana, Croatia (GenBank accession number GQ497543; Zanol et al., 2010). Actually, despite the fact that Vrsar and Funtana are adjacent on the coast and separated by only a few kilometres while the nearest water distance between Funtana and NW Iberian Peninsula is of the order of 4000 kilometres, the degree of similarity with this specimen (that is, GB GQ497543) was close in both groups of sequences (99.1–99.7% and 98.6–99.5% for the Croatian and Galician sequences, respectively; Table A1). On the contrary, these 20 sequences strongly diverged from the sequence *Eunice aphroditois* isolate IP0147 cytochrome oxidase subunit I from the Sisters' Islands Marine Park, Singapore (GenBank accession number MN690243; Ip et al., 2019). As shown in Table A1, pairwise comparisons between our 20 sequences and this sequence (626 bp in common) always revealed a number of nucleotide differences >119 and a sequence divergence >19%.

As mentioned above, nine different haplotypes were detected among

Table 1
For each E. roussaei individual analyzed in this study, individual code (ID), sampling data and location, total length, number of the first setiger (no), and body interval (cm) in which oocytes were detected are shown. Mean oocyte size and standard deviation (SD) for each individual, as well as mean oocyte size in the anterior, middle and posterior regions are also shown. (* incomplete specimens; ** individuals kept alive in the Aquarium Finisterrae; a individuals sequenced).

				OOCYTE PRESENCE									
							Anterior		Middle		Posterior		All	
ID	Data (m-y)	Location	Length (cm)	1st setiger (no)	Body interval (cm)	Number of oocytes analyzed	Mean oocyte ø (μm)	SD	Mean oocyte ø (μm)	SD	Mean oocyte ø (μm)	SD	Mean oocyte ø (μm)	SD
GR01[a]	jul-17	O Grove	–											
GR02	oct-19	O Grove	118	86	24–98	120	200.73	2.86	202.79	3.90	202.54	4.54	202.02	3.77
GR03[a]	jan-20	O Grove	190											
GR04[a]	jan-20	O Grove	155*	85	22–123	120	107.02	12.62	104.13	14.67	95.90	15.50	102.35	14.26
GR05	jan-20	O Grove	73*	73	27–73	80	107.34	20.14	108.15	15.12	–	–	107.75	17.63
GR06[a]	feb-20	O Grove	65*											
GR07[a]	feb-20	O Grove	225											
GR08[a]	feb-20	O Grove	155											
GR09[a]	may-20	O Grove	110	84	28–110	120	174.24	2.09	174.52	3.21	174.79	7.92	174.52	4.41
GR010	may-20	O Grove	205*	85	35–110	80	155.42	17.14	170.34	5.05	–	–	162.88	11.09
GR11[a]	may-20	O Grove	110*											
GR12[a]	jun-20	O Grove	183											
GR13[a]	jun-20	O Grove	180	89	22–112	120	153.57	5.48	166.63	2.17	162.20	3.62	160.80	3.76
GR14	jul-20	O Grove	78*											
GR15**	aug-20	O Grove	250											
GR16	sep-20	O Grove	230	77	28–198	120	205.11	1.27	204.90	1.52	207.05	2.95	205.69	1.91
GR17	sep-20	O Grove	138											
GR18**	sep-20	O Grove	–											
AC01[a]	feb-15	*Aquarium Finisterrae*	–											

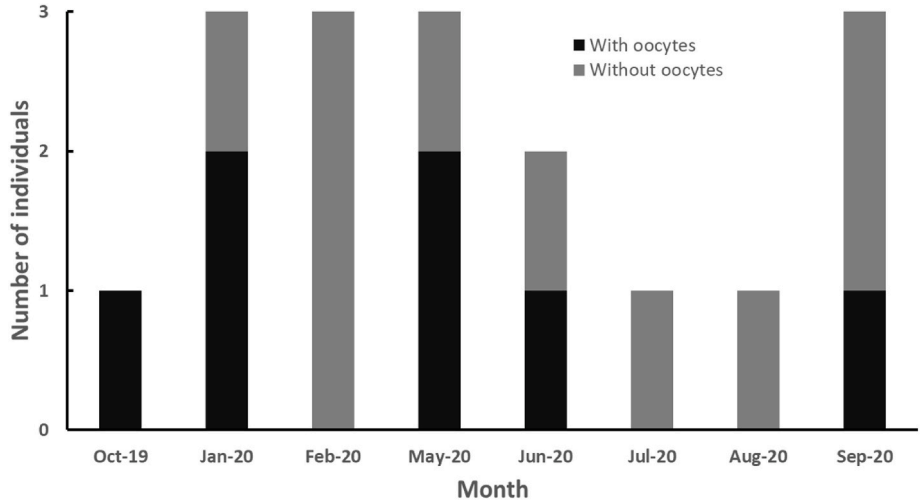

Fig. 2. Monthly distribution of the 17 "polychaetes" captured in Galicia during the present study, as well as of the subset of 7 individuals with free oocytes in their coelom.

the ten individuals sequenced from the locality of O Grove, whereas the number of haplotypes found among the nine individuals sequenced from Vrsar was eight; moreover, the specimen from the Aquarium Finisterrae of A Coruña also showed a unique sequence (Table 2). According to this, within each region, haplotype diversity (Hd) was almost 1 (Table 3). Nucleotide diversity (π) was also very similar in both groups of

Table 2
Nucleotide sequences of the 18 haplotypes found; only variable positions in the common 658 bp fragment are represented (IS01-IS09 individuals from Vrsar; the remaining codes are as in Table 1).

	Frequency	Individual	1	2	2	7	7	9	1	1	1	1	2	2	2	2	3	3	3	3	3	3	3	4	4	4	4	4	5	5	5	6	6	6	6
			7	8	0	9	9	7	0	3	8	9	3	4	6	7	0	1	3	7	0	4	5	6	7	2	8	8	0	6	1	4	0	3	4	0	9
Hap_1	2	IS01, IS08	C	G	A	G	A	G	A	G	A	C	C	G	C	A	A	G	A	T	A	G	A	G	G	G	A	G	A	A	C	A	C	C	I
Hap_2	1	IS02	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	.	.
Hap_3	1	IS03	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.
Hap_4	1	IS04	T	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_5	1	IS05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
Hap_6	1	IS06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.
Hap_7	1	IS07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
Hap_8	1	IS09	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Hap_9	1	GR11	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.
Hap_10	1	AC01	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_11	1	GR01	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.
Hap_12	1	GR03	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.
Hap_13	1	GR04	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.
Hap_14	2	GR06, GR12	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_15	1	GR07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Hap_16	1	GR08	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Hap_17	1	GR09	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Hap_18	1	GR13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.

Table 3
Genetic diversity estimates for the Mediterranean locality of Vrsar and the Atlantic locality of O Grove, as well as for NW Spain (O Grove + A Coruña). N = sample size; S = number of segregating sites; NH = number of haplotypes; HE = haplotype diversity; π = nucleotide diversity.

	Vrsar	O Grove	NW Spain
N	9	10	11
S	17	19	20
N_H	8	9	10
H_E	0.972	0.978	0.982
π	0.0060	0.0068	0.0070

sequences and revealed a mean number of 0.006–0.007 nucleotide differences per site between any two DNA sequences (Table 3).

None of the ten haplotypes found in the Atlantic were detected in the Mediterranean and, likewise, none of the eight haplotypes detected in the Mediterranean were recorded in the Atlantic (Fig. 3). Despite this, the available data did not show any particular structure or clustering, and most haplotypes diverged from a central, Mediterranean haplotype by a low number of mutations, displaying a star-like shape (Fig. 3). Moreover, the specimen obtained from the *Aquarium Finisterrae* (AC01) and the specimen captured in O Grove in 2017 (GR01) did not substantially differ from the remaining Galician individuals (Fig. 3).

3.2. Reproductive biology

External morphological observations of the 17 specimens captured did not show any of the signs of epitoky mentioned in the literature.

We found free-floating greyish-green oocytes in the coelom of seven specimens (Fig. 2). In these ovigerous females, oocytes were homogeneously distributed in virtually all setigers, except for the first 22–35 centimetres (70–90 segments) and for a similar portion at the posterior end of the animal (Table 1). Indeed, the body interval containing oocytes represented 50–75% of the total length of the animal. Mean oocyte size was 162 μm, ranging from 102.35 μm to 205.69 μm. No pattern was observed in the variation of mean oocyte size among body regions, as shown by the Friedman test (sig. = 0.549) (Fig. 4).

As for monthly variations in mean oocyte size (Fig. 4), substantially smaller oocytes were observed in the two specimens collected in January (102.35 μm; 107.75 μm) and substantially larger ones were found in the specimens collected in September and October (205.69 μm and 202.02 μm, respectively), compared with those observed in May (174.52 μm; 162.88 μm) and June (160.80 μm). Moreover, the standard deviation of oocyte size was highest in January for the two individuals sampled (14.26; 17.63) and lowest in October 2019 (3.77) and September 2020 (1.91).

Although the following conclusions are based on a small number of specimens and should be interpreted with caution, these results seem consistent with a spawning event around November–December and with new oocytes maturating and enlarging from January to October. Moreover, the standard deviation of oocyte size would suggest that at the beginning of the maturation cycle, individuals hold oocytes at different stages of maturation and, therefore, oogenesis takes place asynchronously in this species (Fig. 4).

Mean oocyte size did not correlate with the length of the specimens, but we did find a positive correlation with water temperature ($r = 0.746$; $p < 0.05$), as well as with photoperiod ($r = 0.268$; $p < 0.05$) (Fig. 5). The complete record of temperatures during the sampling year can be found in Figure A2 of the supplementary material.

4. Discussion

Sampling for *E. roussaei* specimens is a challenging and time-consuming process. Thus, collecting these "polychaetes" not only requires specific equipment for capturing the animals, namely baited traps specific for "polychaetes", but also certain skills both in search and

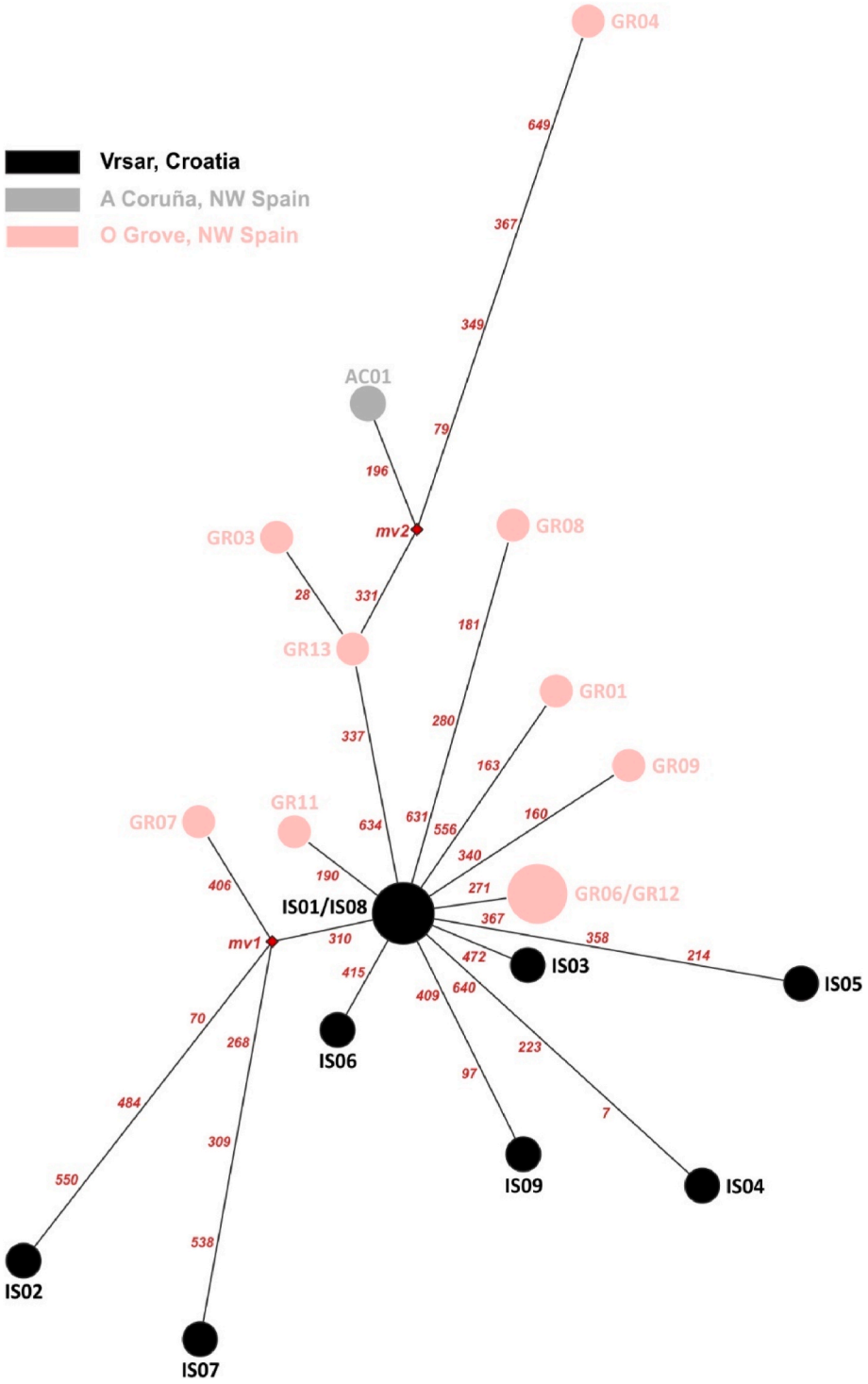

Fig. 3. Median-joining haplotypic network illustrating the evolutionary relationships between the 20 COI–5P sequences obtained in this study. Each haplotype is represented by a circle, whose area is proportional to relative frequency; the numbers in red correspond to mutational positions in the 658 bp fragment. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

recognition of "polychaete" burrows and in the extraction process.

Limitations in sampling such as low baiting success, meteorological conditions, and the Covid-19 pandemic resulted in a lower number of individuals we collected than initially expected. Despite all this, the samples captured were sufficient to determine the taxonomic identity of the giant *Eunice* species present in the Ría de Arousa and to obtain some interesting insights on its reproductive cycle.

4.1. Taxonomic identity and genetic differentiation

Our data clearly show that giant *Eunice* "polychaetes" from NW Spain (both the Ría de Arousa population and the *Aquarium Finisterrae* specimen) are conspecific with those found in the Adriatic Sea. Thus, as expected for intraspecific comparisons, the distribution of the number of pairwise differences within each group of sequences was very similar to the distribution of the number of pairwise differences between both

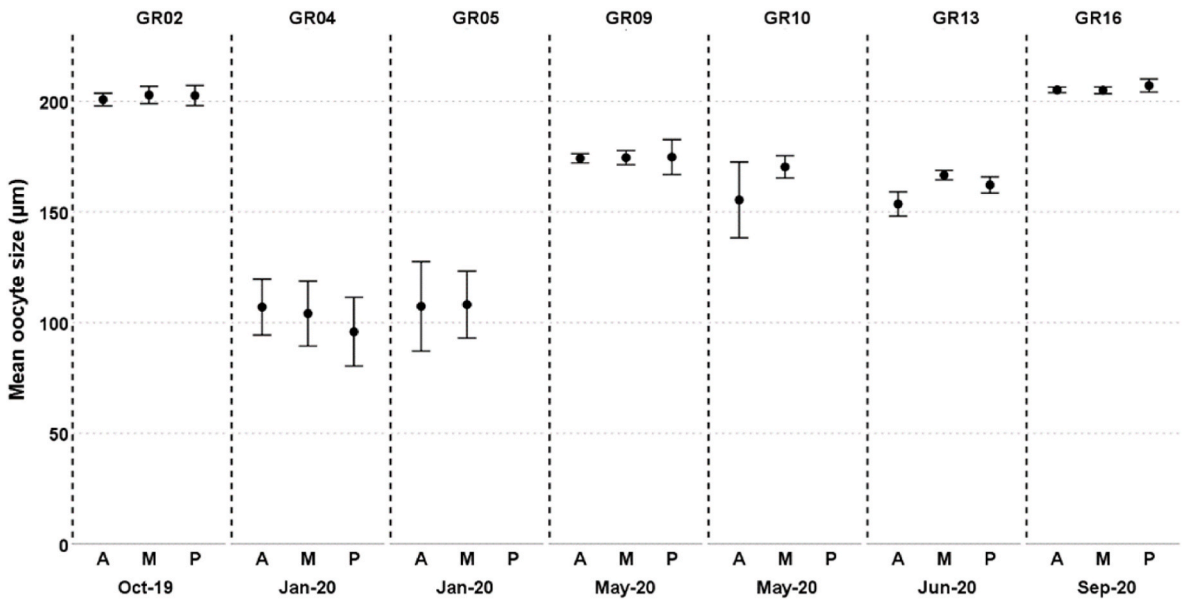

Fig. 4. Mean oocyte diameter (N = 40) in the anterior (A), middle (M), and posterior (P) body regions of seven E. roussaei specimens captured between October 2019 and September 2020. Error bars correspond to the standard deviation of mean oocyte size.

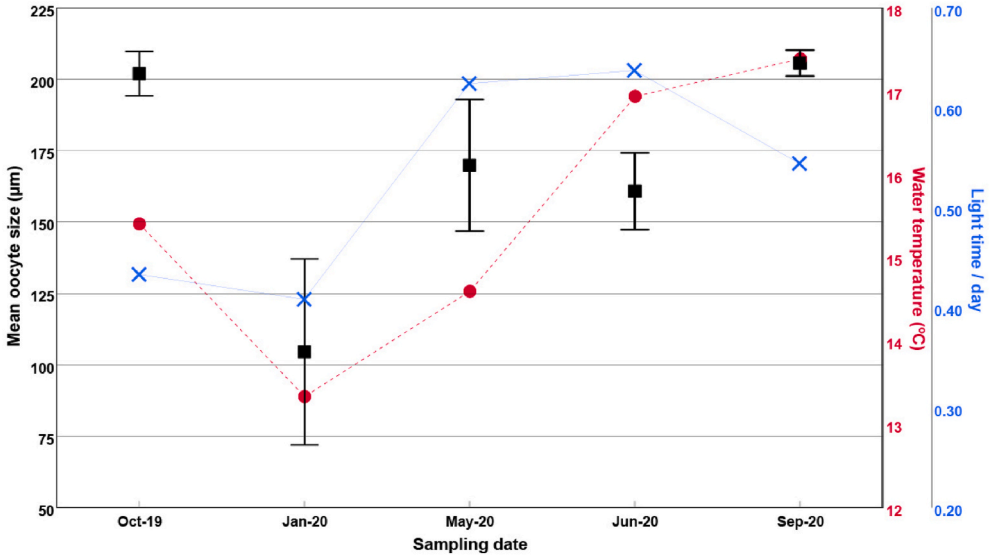

Fig. 5. Monthly mean oocyte size of E. roussaei (µm) represented by black squares; water temperature (°C) represented by red dots, and photoperiod (light time/day) represented by blue crosses.

groups of sequences (Hebert et al., 2003). In particular, the number of pairwise differences within regions ranged from 0 to 7 (Croatian sequences) or from 0 to 10 (Galician sequences), whereas the number of pairwise differences between regions varied between 0 and 11. Likewise, the average number of pairwise differences was also very similar regardless of whether the comparisons were made within regions (3.94 and 4.58 for the Croatian and the Galician sequences, respectively) or between regions (4.50). When a NCBI BLAST search was performed, the twenty sequences exhibited a high degree of similarity (98.6–99.7%) with the sequence *Eunice roussaei* voucher USNM1120728 cytochrome *c* oxidase subunit I from Funtana, Croatia (GenBank accession number GQ497543; Zanol et al., 2010). Conversely, the level of divergence with the sequence *Eunice aphroditois* isolate IP0147 cytochrome oxidase subunit I from the Sisters' Islands Marine Park, Singapore (GenBank accession number MN690243; Ip et al., 2019), always greater than 19%, was irreconcilable with intraspecific differences (Hebert et al., 2002; Carr et al., 2011).

The current knowledge on the most basic aspects of the biology and ecology of *E. roussaei*, including its natural geographical distribution range, is almost nonexistent. This knowledge gap is demonstrated by the situation in NW Spain, where only the population included in this study is known despite the long fishing tradition and the many inventories of benthic fauna carried out within this region (e.g. Besteiro et al., 2017).

Since rias in NW Spain are home to intense aquaculture activity (mostly of mussels and several species of clams) and the transfer of live animals has been a common practice for years, we cannot discard a non-native status of *E. roussaei* in this region, as occurs with many other species (e.g., Couceiro et al., 2008; Couceiro et al., 2011). However, our data are again insufficient to rule out any hypothesis. Our results suggest that both the O Grove and Vsar populations have a very similar genetic diversity pattern, characterized by high levels of haplotype diversity (>0.97) but low levels of nucleotide diversity (<0.007). Still, the two

basins did not share haplotypes, a result that is consistent with the presence of important oceanographic discontinuities between these populations, such as the Otranto Strait, the Sicily Channel, or the Almería-Oran Front, which reportedly act as barriers to gene flow in numerous species (Pascual et al., 2017).

4.2. Reproductive biology

Despite the number of epitoky records cited in the literature for Eunicidae (Hofmann, 1974; Schulze and Timm, 2006), we did not find any external signs of epitoky in the *E. roussaei* specimens analyzed in this study. Moreover, oocytes were observed along almost the entire length of the animal body, even exceeding 1 m long in one of the examined specimens. This contrasts with observations in relatively well-studied epitokous species such as *Palola viridis* or *Palola siciliensis*, where such cells develop only in the caudal segments of the animal (Hofmann, 1974). Furthermore, the lack of a clear pattern in mean oocyte size along the body seems to refute the idea that there is an oocyte production area in the anterior part of the body from which oocytes migrate as they mature to the posterior or epitokous part of the body. Therefore, although we studied a low number of specimens and did not check for the presence of male gametes, our observations suggest that this species is a dioecious, broadcast spawner, as also proposed by Bettoso et al. (1998). We also agree with these authors on the fact that, if the species were really epitokous, the phenomenon of agglomeration of caudal portions, so conspicuous in other regions of the planet (Stair, 1897; Caspers, 1984), should have been observed by local fishermen, particularly in Galicia, where an important artisanal fleet operates daily in coastal waters (do Mar, Xunta de Galicia, n.d.).

Regarding the sexual maturation cycle, our annual study suggests that the reproductive cycle of the *E. roussaei* population in the Ría de Arousa begins in January, when the oocytes observed were significantly smaller on average. Gametes mature through May and June to gain their largest size in September/October, and, finally, individuals spawn in the autumn months of November or December. These observations partially agree with those reported by Bettoso et al. (1998) for *E. roussaei* (as *E. aphroditois),* who reported a breeding season also in autumn, between September and October. Nevertheless, in contrast to our results, individuals from Croatia captured in May did not show any gonadal signs, while caudal regions showed signs of early gonadal development already in June. During this period, all the individuals sampled in our study contained intermediate-size oocytes in virtually the entire body of the animal. However, both studies were conducted in very different regions, with a 23-year time gap, used different methodologies, and were based on very small sample sizes. Thus, further studies are needed to reach conclusive results on the sexual maturity cycle of this species.

Our results also suggest that the maturation cycle and probably the spawning event(s) of *E. roussaei* seem to be driven by water temperature and, to a lesser extent, by photoperiod. This agrees with findings on other "polychaete" species (Watson et al., 2000; Nash and Keegan, 2003; Wang et al., 2020). Specifically, the analysis of the evolution of mean oocyte size with temperature records (Figure A2) suggests that spawning events could be triggered by a water temperature drop below 12.3 °C.

Many aspects of the life cycle and geographical distribution of this species still remain unknown. We do not know, for example, how this "polychaete" releases its gametes into the water or whether the fertilization takes place in the water column, inside its gallery, or elsewhere; nor do we know anything about its larval development or juvenile settlement. Our conclusion on the maturation cycle has been based solely on the study of females; therefore, little is known about males and whether or not there are similarities in the production and maturation of spermatozoa with respect to oocytes. In addition, we have no data on relevant ecological aspects of this species such as population densities, feeding habits, or distribution range. Further research on all these topics would help to better understand the species and its role on the ecosystem.

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CRediT authorship contribution statement

D. Escobar-Ortega: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **N. Fernández:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation. **R. Muíño:** Writing – review & editing, Visualization, Supervision, Methodology. **J. Parapar:** Writing – review & editing, Validation, Resources. **N. Bettoso:** Writing – review & editing, Resources. **L. Couceiro:** Writing – review & editing, Methodology, Investigation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2022.107899.

References

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.
Bellan, G., 2001. Polychaeta. In: Costello, M.J., et al. (Eds.), (2001). European Register of Marine Species: a Check-List of the Marine Species in Europe and a Bibliography of Guides to Their Identification, 50. Collection Patrimoines Naturels, pp. 214–231.
Besteiro, C., Parapar, J., Saiz Salinas, J.I., Martínez-Ansemil, E., Moreira, J., 2017. Annelida. In: Bañón, R. (Ed.), Inventario de la biodiversidad marina de Galicia: Proyecto LEMGAL. Consellería do Mar, Xunta de Galicia, Santiago de Compostela, pp. 337–370.
Bettoso, N., Faverio, G., Orel, G., 1998. Note sulla biologia e sulla pesca del verme di Rimini (*Eunice aphroditois* Pallas) in Alto Adriatico. Hydrores 16, 25–34.
Cabrera, A., 1909. Contribución al estudio de los Eunícidos de las Costas Cantábricas. La Hormiga de Oro, Barcelona, p. 46.
Carr, C.M., Hardy, S.M., Brown, T.M., Macdonald, T.A., Hebert, P.D., 2011. A tri-oceanic perspective: DNA barcoding reveals geographic structure and cryptic diversity in Canadian "polychaetes". PLoS One 6 (7), e22232.
Carrera-Parra, L.F., 2009. Eunicidae Berthold, 1827. In: de León-González, J.A., Bastida-Zavala, J.R., Carrera-Parra, L.F., García-Garza, M.E., Peña-Rivera, A., Salazar-Vallejo, S.I., Solís-Weiss, V. (Eds.), Poliquetos (Annelida: Polychaeta) de México y América Tropical. Universidad Autónoma de Nuevo León, Monterrey, pp. 165–181.
Caspers, H., 1984. Spawning periodicity and habitat of the palolo worm *Eunice viridis* (Polychaeta: Eunicidae) in the Samoan Islands. Mar. Biol. 79 (3), 229–236.
Cetinic, P., Soldo, A., 1999. Some basic characteristics of Croatian marine fisheries and its legal regulation. Acta Adriat. 40 (Suppl. P), 91–98.

Cole, V.J., Chick, R.C., Hutchings, P.A., 2018. A review of global fisheries for polychaete worms as a resource for recreational Fishers: diversity, sustainability and research needs. Rev. Fish Biol. Fish. 28 (3), 543–565.
Colgan, D.J., Hutchings, P.A., Brown, S., 2001. Phylogenetic relationships within the Terebellomorpha. J. Mar. Biol. Assoc. U. K. 81, 765–773.
Couceiro, L., Míguez, A., Ruiz, J.M., Barreiro, R., 2008. Introduced status of *Cyclope neritea* (Gastropoda, Nassariidae) in the NW Iberian Peninsula confirmed by mitochondrial sequence data. Mar. Ecol. Prog. Ser. 354, 141–146.
Couceiro, L., Cremades, J., Barreiro, R., 2011. Evidence for multiple introductions of the Pacific green alga *Ulva australis* Areschoug (Ulvales, Chlorophyta) to the Iberian Peninsula. Bot. Mar. 54, 391–402.
do Mar, Consellería, de Galicia, Xunta (n.d.). https://mar.xunta.gal/gl/o-mar/o-sector/.
DOG N° 246, 2018. ORDE do 20 de decembro de 2018 pola que se aproban os plans de xestión para recursos específicos en Galicia para o ano 2019. Consellería do Mar - Dirección Xeral de Desenvolvemento Pesqueiro. 27 of December of 2018, 53914.
Fauchald, K., 1992. A review of the genus *Eunice* (Polychaeta: Eunicidae) based upon type material. Smithsonian Contrib. Zool. 523, 1–422.
Fauvel, P., 1923. Polychètes errantes. Faune de France, 5. Librairie de la Faculté des Sciences, pp. 1–488.
Fernández-Ovies, C.L., Ortega, J.A., 1983. Captura de un ejemplar de *Eunice aphroditois* (Pallas, 1788) (Polychaeta: Eunicidae) en la costa asturiana. Boletín de Ciencias de la Naturaleza 31, 1–12.
Gambi, M.C., Castelli, A., Giangrande, A., Lanera, P., Prevedelli, D., Zunarelli Vandini, R., 1994. Polychaetes of commercial and applied interest in Italy: an overview. In: Actes de la 4eme Conference internationale des Polychetes. Memoires du Museum National d'Histoire Naturelle de Paris, 162, pp. 593–601.
Giangrande, A., 1997. Polychaete reproductive patterns, life cycles and life histories: an overview. Oceanogr. Mar. Biol. 35, 323–386.
Hartman, O., 1944. Polychaetous annelids. Part V. Eunicea. Allan Hancock Pacific Expeditions 10 (1), 1–237.
Hebert, P.D.N., Cywinska, A., Ball, S.L., DeWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. R. Soc. B: Biol. Sci. 270 (1512), 313–321.
Hofmann, D.K., 1974. Maturation, epitoky and regeneration in the polychaete *Eunice siciliensis* under field and laboratory conditions. Mar. Biol. 25 (2), 149–161.
Ip, Y.C.A., Tay, Y.C., Gan, S.X., Ang, H.P., Tun, K., Chou, L.M., Huang, D., Meier, R., 2019. From marine park to future genomic observatory? Enhancing marine biodiversity assessments using a biocode approach. Biodivers. Data J. 7, e46833.
Mikac, B., 2015. A sea of worms: polychaete checklist of the Adriatic Sea. Zootaxa 3943 (1), 1–172.
Nash, R., Keegan, B.F., 2003. Reproductive cycle of *Bispira volutacornis* (Polychaeta: Sabellidae) on the west coast of Ireland. Mar. Biol. 143 (5), 919–925.
Núñez, J., Pascual, M., Delgadoy, J.D., Brito, M.C., 1997. Anélidos poliquetos de Canarias: familia Eunicidae. Vieraea 26, 47–75.
Nygren, A., 2014. Cryptic polychaete diversity: a review. Zool. Scripta 43 (2), 172–183.
Parapar, J., Harto, I., 2001. Sobre la presencia en aguas ibéricas y variabilidad morfologica en *Eunice roussaei* Quatrefages, 1866 (Polychaeta, Eunicidae). Bol. Real Soc. Espanola Hist. Nat. Secc. Biol. 96 (34), 165–174.
Pascual, M., Rives, B., Schunter, C., Macpherson, E., 2017. Impact of life history traits on gene flow: a multispecies review across oceanographic barriers in the Mediterranean Sea. PLoS One 12 (5), e0176419.
Rioja, E., 1917. Datos para el conocimiento de la fauna de Anelidos Poliquetos del Cantábrico. Trabajos del Museo de Ciencia Naturales, Serie Zoologica 29, 1–111.
Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A., 2017. DnaSP v6: DNA sequence polymorphism analysis of large datasets. Mol. Biol. Evol. 34, 3299–3302.
Salazar-Vallejo, S.I., Carrera-Parra, L.F., de León-González, J.A., 2011. Giant Eunicid polychaetes (Annelida) in shallow tropical and temperate seas. Rev. Biol. Trop. 59 (4), 1463–1474.
Schroeder, P.C., Hermans, C.O., 1975. Annelida: Polychaeta. In: Giese, A.C., e Pearse, J.S. (Eds.), Reproduction of Marine Invertebrates. Vol. III. Annelids and Echiurans. Academic Press, New York, p. 343.
Schulze, A., Timm, L.E., 2012. *Palolo* and *un*: distinct clades in the genus *Palola* (Eunicidae, Polychaeta). Mar. Biodivers. 42 (2), 161–171.
Stair, J.B., 1897. Pālolo, a sea-worm eaten by the samoans. J. Polyn. Soc. 6, 141–144.
Wang, H., Hagemann, A., Malzahn, A.M., Handå, A., Uhre, M., Kjørsvik, E., Reitan, K.I., 2020. The role of manipulating photoperiod and temperature in oocyte development of the Polychaete *Hediste diversicolor* (OF Müller, 1976). Front. Mar. Sci. 7, 1080.
Watson, G.J., Williams, M.E., Bentley, M.G., 2000. Can synchronous spawning be predicted from environmental parameters? A case study of the lugworm *Arenicola marina*. Mar. Biol. 136 (6), 1003–1017.
Wilson, W.H., 1991. Sexual reproductive modes in polychaetes: classification and diversity. Bull. Mar. Sci. 48 (2), 500–516.
Zanol, J., Bettoso, N., 2006. Identity of *Eunice roussaei* (Eunicidae: Polychaeta: Annelida) from the Adriatic and Mediterranean seas. J. Mar. Biol. Assoc. U. K. 86 (5), 1017–1024.
Zanol, J., Halanych, K.M., Struck, T.H., Fauchald, K., 2010. Phylogeny of the bristle worm family Eunicidae (Eunicida, Annelida) and the phylogenetic utility of noncongruent 16S, COI and 18S in combined analyses. Mol. Phylogenet. Evol. 55 (2), 660–676.