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On the presence of *Pontobdella muricata* (Hirudinea: Piscicolidae) on some elasmobranchs of the Tyrrhenian Sea (Central Mediterranean)

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This paper provides the first report of the leech, Pontobdella muricata *(Linnaeus, 1758), in the Tyrrhenian Sea. The leech was found on the brown ray,* Raja miraletus *(Linnaeus 1758), and on the spotted ray,* Raja montagui *(Fowler, 1910), caught by trawling during autumn 2014.*

Complete sequence of 18S rRNA gene, COI mitochondrial gene and partial sequences of the mitochondrial 12S rRNA gene corroborate the determination based on morphological characteristics.

Key words: *Pontobdella muricata*, parasite, leech, *Raja miraletus*, *Raja montagui*, Tyrrhenian Sea

INTRODUCTION

Leeches (Annelida: Clitellata: Hirudinea) are annelids that can be found in marine, estuarine, terrestrial and freshwater ecosystems. Hirudinea is a small group that includes 14 families (SKET & TRONTELJ, 2008). Out of them, members of family Piscicolidae parasitize predominantly freshwater or marine fishes (teleosts and elasmobranchs). They are found on the external body surfaces, such as the skin, mouth, gill cavity and cloaca. Piscicolids affect the health of their hosts mainly through blood-feeding activities and they can be vectors of pathogenic protozoans (CELIK & AYDIN, 2006; HAYES *et al.*, 2006). Marine fish leeches of the Mediterranean Sea have been explored mainly in the Eastern basin (SAGLAM *et al*., 2003; AKMIZA, 2004; BAKOPOULOS & KSIDIA, 2014; BULGUROĞLU *et al*., 2014). In Italian seas leeches have never been deeply studied (MIZZAN, 1994) and, up today, seven marine piscicolid species have been recorded (MINELLI, 2008).

Elasmobranchs present life–history characteristics make them a fragile resource, more susceptible to overfishing than most teleost

fishes (BOTTARI *et al*., 2013; BOTTARI *et al*., 2014; MANCUSO, 2015). The health assessment of heavily exploited stock is an essential element for the Mediterranean fish stocks assessment and management (LLORET *et al.*, 2012).

In this contest the study aims to identify leeches infesting some elasmobranchs; complete sequences of the 18S rRNA gene, COI mitochondrial gene as well as partial sequences of 28S rDNA gene and mitochondrial 12S rRNA gene were analyzed to determine the molecular identification.

MATERIALS AND METHODS

Leeches were collected from the brown ray, *Raja miraletus* (Linnaeus 1758), and from the spotted ray, *Raja montagui* (Fowler, 1910). A total of 8 brown rays and 2 spotted rays were caught by trawling in the Southern Tyrrhenian Sea (38.2° N and 15.08305° E; Central Mediterranean) during autumn 2014.

All isolated leeches (9 specimens) were identified according to the keys of LEWELLIN (1966), stored in ethanol 95-100% at room temperature until they were processed for the DNA extraction.

Tissue for the caudal sucker was used in order to minimize the possibility of contamination from host DNA. DNeasy Tissue Lyser and DNeasy Blood and Tissue Kit (Qiagen) were used for lyses and DNA purification. Subsequently electrophoresis, performed using 0.8 % of agarose gel, and quantification by Thermo Scientific NanoDrop ™ 2000 were carried out to verify the amount of extracted DNA. PCR amplification of nuclear 18SrDNA and 28S rDNA and mitochondrial 12S rDNA gene fragments were performed with the primers showed in Table 1. To obtain 18S rDNA fragments, the primer pairs “AL”, “CY”, and “BO” were used, yielding three overlapping shorted double stranded DNA fragments of approximately 600 base pair (bp) each in length (APAKUPAKUL *et al.*, 1999). Amplifications of 28SrDNA and 12SrDNA yielded fragments of approximately 365 and 400 bp, respectively (BORDA & SIDDAL, 2004).

The set of primers were used to amplify the DNA (2 µl) in 50 ml of reaction mixture containing 5 µl of 10x TaqQ-solution, 1 µl dNTPs

Table 1. Primers used for PCR amplification

Gene	Primer name	Primer sequence
Nuclear		
18SrDNA	A	5’-AACCTGGTTGATCCTGCCAGT-3’
	L	5’-CCAACTACGAGCTTTT-3’
	C	5’-CGGTAATTCCAGGTC-3’
	Y	5’-CAGACAAATCGCTCC-3’
	B	5’-TGATCCTTCCGCAGGTTCACCT-3’
	O	5’-AAGGGCACCACCAG-3’
28S rDNA	28S-A	5’-GACCCGTCTTGAAGCACG-3’
	28S-B	5’-TCGGAAGGAACAGCTACTA-3’
Mitochondrial		
12S rDNA	12S-AI	5’-AAACTAGGATTAGATACCCTATTAT-3’
	12S-BI	5’-AAGAGCGACGGGCGATGTGT-3’
Mitochondrial		
COI	LCO1490	5’-GGTCAACAAATCATAAAGATATTGG- 3’
	HCO2198	5’-TAAACTTCAGGGTGACCAAAAAATCA- 3’

(10mM), 1 µl of each primer (10 µM/ µl), 0.25 µl of Taq polymerase (Qiagen) and 0.25 RNase-free H_2O (total volume, 50 µl) Milli-Qwater (Millipore, VimodroneMI, Italy). All amplification reactions were performed in a GeneAmp PCR System. The following amplification protocol (Borda & Siddall, 2004) were used: 18S –heated to 94°C for 5 min, followed by 35 cycles of 94°C (15 s), 44 °C (20 s), and 70°C (90 s) and a final extension at 72 °C (7 min); 28S –heated to 94°C for 5 min, followed by 30 cycles of 95°C (1 min), 52 °C (1 min), and 70°C (1 min) and a final extension at 72 °C (7 min); 12S –heated to 94°C for 5 min, followed by 30 cycles of 95°C (1 min), 52 °C (1 min), and 70°C (1 min) and a final extension at 72 °C (7 min).

The amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene was performed using the primers LCO1490 and HCO2198. We used 2 µl of the DNA extracted as template for 50 µl PCR reaction, using 4 units of Taq polymerase, 2.5 µl of each primer of stock solution (10 µmol/liter), 5 µl of buffer solution (provided by the manufacturer), 5 µl of MgCl2 and 5 µl dNTPs (10mM) and 27 µl sterile distilled water. Reactions were amplified through 35 cycles at the following parameters: one minute at 95°C, one minute at 40°C, and one and a half minutes at 72°C, followed by a final extension step at 72°C for seven minutes (FOLMER *et al.*, 1994).

On the PCR products was performed the electrophoresis using a 0.8% agarose gel stained with SybrSAFE (Invitrogen), for band characterization was used U.V.. The amplicons were sent to the centre MACROGENE (Europe) for purification and sequencing. Sequences were checked for possible chimeric origin with Pintail software (ASHELFORD *et al*., 2005), identified with BLAST (ALTSCHUL *et al*., 1997).

Genes for 18S and COI genes were aligned using default parameter in MUSCLE (EDGAR *et al*., 2004) and the phylogenetic tree was inferred using the Neighbor-Joining method (SAITOU & NEI 1987) The evolutionary distances were computed using the Jukes-Cantor method (JUKES & CANTOR, 1969) and the percentage of replicate trees were done using bootstrap test (500 replicates) (FELSENSTEIN, 1985) inside MEGA7 (KUMAR *et al*., 2016).

The sequences of 18S, 12S, 28S and COI genes were concatenated and aligned using default parameter in MUSCLE (EDGAR *et al*., 2004) within Geneious software (KEARSE *et al*., 2012). The aligned file was exported in nexus format and manually edited to add morphology characteristics . Bayesian inference of phylogeny was obtained by MrBayes 3.2 (RONQUIST *et al*., 2012). The evolutionary model of GTR with gamma-distributed rate variation across sites and a proportion of invariable sites was applied on DNA data, whereas the same model with only gamma-distribute rate variation was applied for morphology characteristics. Analysis was done using 1,000,000 generations, sampling every 500th generation with 4 heated and was stopped when average standard deviation of split frequencies was 0.003154. Others parameters were setup following NYLANDER *et al*. (2004). The nucleotide sequences produced in this article were deposited in the DDBJ/EMBL/GenBank data bases under accession numbers: KY659070, KY659071, KY659072 and KY659073.

Morphological data

List of characters used in the analysis. The characters were principally adapted from Borda and Siddal (2004), Siddall and Burreson (1995) and Apakupakul et al. (1999).

Character 1
Number of jaws: (0) Agnathous, (1) Duognathous, (2) Trignathous
Character 2
Muscular jaws: (0) Absent, (1) Present
Character 3
Salivary papillae: (0) Absent, (1) Present
Character 4
Toothed jaw: (0) Monostichodont, (1) Distichodont, (2) Astichodont, (3) With stylets
Character 5
Feeding habits: (0) Macrophagous, (1) Haematophagous
Character 6

Atria: (0) Bilobed, (1) Fused
Character 7
Friction rays on caudal sucker: (0) Absent, (1) Present
Character 8
Number of annuli with eyespots: (0) None, (1) One, (2) Two, (3) Three, (4) Four, (5) Five
Character 9
Eyespots per annulus: (0) One pair, (1) Two or more pairs
Character 10
Respiratory auricles: (0) Absent, (1) Present
Character 11
Cocoons: (0) Brooded, (1) Cemented, (2) Spongy and deposited on land
Character 12
Mid-body nephropores: (0) Ventromedial, (1) Ventrolateral
Character 13
Nephridia: (0) Single funnel apparatus, (1) Multiple funnels in a ciliated organ
Character 14
Vaginal tube: (0) Absent, (1) Present
Character 15
Vaginal caecum: (0) Absent, (1) Present
Character 16
Ovisac shape: (0) Tubular, (1) Spheroid
Character 17
Common oviduct: (0) Absent, (1) Present
Character 18
Male atrium extended into elongated penis and sheath: (0) Absent, (1) Present
Character 19
Ejaculatory ducts: (0) U-shaped, (1) S-shaped
Character 20
Penis shape: (0) Straight, (1) Recurved
Character 21
Testisac per body somite: (0) One pair, (1) Two pair, (2) Four pairs
Character 22
Copulatory glands: (0) Absent, (1) Present
Character 23
Intergonadal conducting tissue: (0) Absent, (1) Present

RESULTS AND DISCUSSION

Leeches have been found on the skin ventral part**.** Local hemorrhages and swelling were evident in the skin around the attachment site of the leech. The examined parasites, showed morphologic features that, according to LLEWELLYN (1966), allowed to classify the leech as *Pontobdella muricata* (Linnaeus, 1758). Data hosts and quantitative characteristics of infection are reported in Table 2.

For 18S ribosomal RNA gene data, sequence assembly generated an unambiguous alignment of 1749 bp. The BLAST results of the sequence analysis showed that leech species present in *R. montagui* and *R. miraletus* was *Pontobdella muricata* (99%) (AF099945).

For 28S ribosomal RNA gene data, the BLAST results (AY425360) of the sequence analysis showed that leech species present in *R. montagui* was *Calliobdella vivida* (*Cystobranchus vividus* Verrill, 1872; 97%) with the sequences assembly generated an alignment of 123 bp.

For mitochondrial 12S ribosomal RNA gene data, sequence assembly generated an unambiguous alignment of 400 bp. The BLAST results of the sequence analysis showed that leech species was *Pontobdella muricata* (99%) (AF099958).

For mitochondrial COI gene data, sequence assembly generated an unambiguous alignment of 629 bp. Also in this case the BLAST results of

Table 2. Host data and quantitative characteristics of infection

Host	Number of hosts examined	Total length (mm)	Total weight (g)	Number of hosts infected	Number of leeches collected
Raja miraletus	8	430-500	260-320	2	8
Raja montagui	2	650-750	425-510	1	1

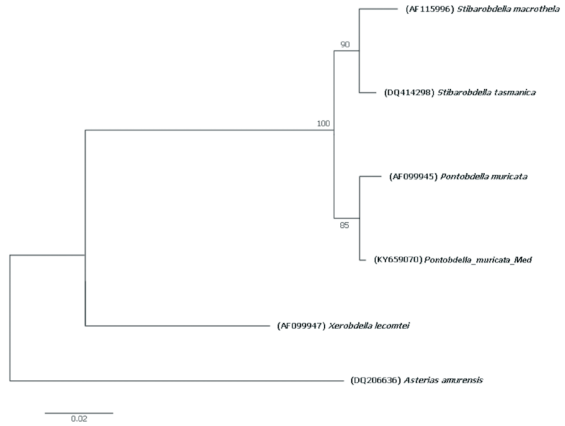

Fig. 1. Phylogenetic relationships among 18S rRNA sequences. The bootstrap values (1000 resamplings) are shown next to the branches, the scale bar represents 2% of sequence divergence. The tree was rooted with 16S rRNA gene sequence of Asterias amurensis (DQ206636)

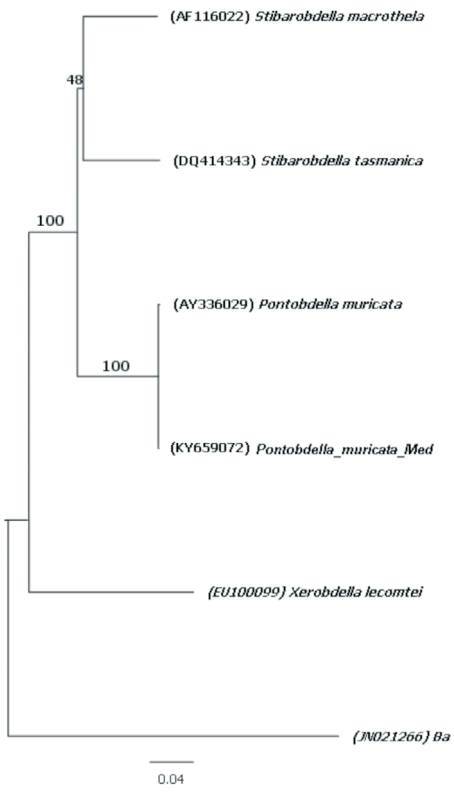

Fig. 2. Phylogenetic analyses of mitochondrial cytochrome oxidase subunit I (COI) gene. The bootstrap values (1000 resamplings) are shown next to the branches. The scale bar corresponds to 4% estimated difference in nucleotide sequence positions. The COI-phylogenetic tree was roothed with Bathymodiolus brooksi (JN021266) COI gene sequence

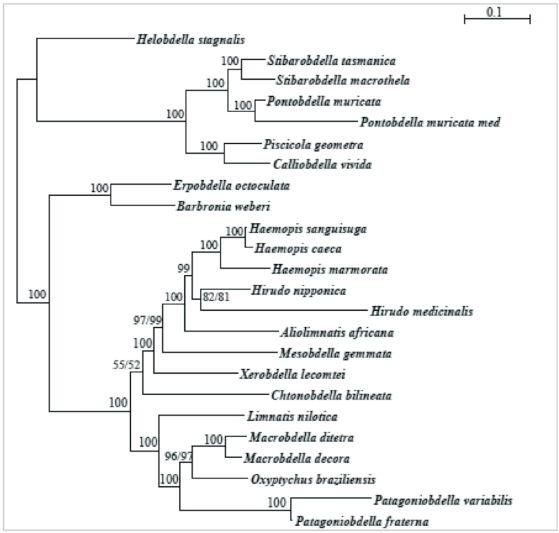

Fig. 3. Bayesian inference of phylogeny of 18S, 12S, COI and 28S genes and 23 morphological traits exposed in Table 3. The posterior probability are shown next to the branches. If 2 values are present the second one is referred to posterior probability form Bayesian inference of phylogeny of only 18S, 12S, COI and 28S genes without morphological traits. The scale bar corresponds to 10% estimated difference in nucleotide sequence positions

the sequence analysis showed that leech species was *Pontobdella muricata* (99%) (AY336029).

The Fig. 1. shows phylogenetic relationships among 18S rRNA sequences is reported. Phylogenetic analysis of 18S rRNA sequences showed a closely similarity with *Pontobdella muricata* (TRONTELJ *et al*., 2001), *Stibarobdella tasmanica* and *Stibarobdella macrothela* (WILLIAMS & BURRESON, 2006). Sequences of the mitochondrial COI gene (Fig. 2) were closely related to *Pontobdella muricata* (99% of similarity; UTEVSKY & TRONTELJ, 2004).

Bayesian inference of phylogeny corroborates these results both with only genetic data (concatenation of 18S, 12S, 28S and COI genes) and with genetic data combined with morphological data (Table 3, Table 4, Fig. 3). Both analysis showed as our organism is strictly related to *Pontobdella muricata* (TRONTELJ *et al*., 2001), *Stibarobdella tasmanica* and *Stibarobdella macrothela* (WILLIAMS & BURRESON, 2006). Until now *P. muricata* has been reported from the North-Eastern Atlantic (LLEWELLYN,

Table 3. Morphological characters and state data matrix

	Characters																						
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Aliolimnatis africana	2	1	0	2	1	1	0	5	0	0	2	0	1	1	1	1	1	1	1	1	0	0	0
Barbronia weberi	0	1	0	3	0	0	0	2	1	0	1	0	0	0	-	0	-	0	0	-	2	1	0
Calliobdella vivida	-	0	3	-	1	0	0	2	0	0	1	1	0	0	-	0	-	0	0	-	0	0	1
Chtonobdella bilineata	1	1	1	0	1	1	1	5	0	1	2	0	1	1	1	1	1	0	0	0	0	0	0
Erpobdella octoculata	0	1	0	2	0	0	0	2	1	0	1	0	0	0	-	0	-	0	0	-	-	0	0
Haemopis caeca	2	1	0	2	0	1	0	5	0	0	2	0	1	1	1	1	1	1	1	1	0	0	0
Haemopis marmorata	2	1	0	1	0	1	0	5	0	0	2	0	1	1	1	1	1	1	1	1	0	0	0
Haemopis sanguisuga	2	1	0	1	0	1	0	5	0	0	2	0	1	1	1	1	1	1	1	1	0	0	0
Helobdella stagnalis	-	0	0	-	0	0	0	1	0	0	0	0	0	0	-	0	-	0	0	-	0	0	0
Hirudo medicinalis	2	1	0	0	1	1	0	5	0	0	2	0	1	1	1	1	1	1	1	1	0	0	0
Hirudo nipponia	2	1	0	0	1	1	0	5	0	0	2	0	1	1	1	1	1	1	1	1	0	0	0
Limnatis nilotica	2	1	1	0	1	1	0	5	0	0	2	0	0	1	1	1	1	0	0	0	0	0	0
Macrobdella decora	2	1	0	0	1	1	0	5	0	0	2	0	1	1	0	1	1	0	0	0	0	1	0
Macrobdella ditetra	2	1	0	0	1	1	0	5	0	0	2	0	1	1	0	1	1	0	0	0	0	1	0
Mesobdella gemmata	2	1	0	0	1	1	1	5	0	0	2	1	1	0	1	1	1	0	0	0	0	0	0
Oxyptychus braziliensis	2	1	0	0	1	1	0	5	0	0	2	0	1	1	0	1	1	0	0	0	0	0	0
Patagoniobdella fraterna	0	1	0	2	0	1	0	5	0	0	2	0	1	1	0	1	1	1	0	0	1	0	0
Patagoniobdella variabilis	0	1	0	2	0	1	0	5	0	0	2	0	1	1	0	1	1	1	0	0	1	0	0
Pontobdella muricata	-	0	0	-	1	0	0	1	0	0	1	1	0	0	-	0	-	0	0	-	0	0	1
Pontobdella muricata Med	-	0	0	-	1	0	0	1	0	0	1	1	0	0	-	0	-	0	0	-	0	0	1
Piscicola geometra	-	0	0	-	1	0	0	2	0	0	1	1	0	0	-	0	-	0	0	-	0	0	1
Stibarobdella macrothela	-	0	0	-	1	0	0	1	0	0	1	1	0	0	-	0	-	0	0	-	0	0	1
Stibarobdella tasmanica	-	0	0	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0	-
Xerobdella lecomtei	2	1	-	0	1	1	1	5	0	0	2	1	1	1	1	1	0	0	0	0	0	0	0

(-), unknown or not applicable

1966; HAYWARD & RYLAND, 1990), Adriatic Sea (MIZZAN, 1994), Aegean Sea (BAKOPOULOS & KSIDIA, 2014), Dardanelles, Sea of Marmara and Black Sea (ERGUVEN & CANDAN, 1992; SAGLAM *et al*., 2003; OKTENER & UTEVSKY, 2010). *P. muricata* infests mainly elasmobranchs (Raja sp., *Torpedo marmorata*, *Raja clavata*; SAGLAM *et al*., 2003) and occasionally teleosts (*Pleuronectes platessa*; HAYWARD & RYLAND, 2000).

To the best of our knowledge this is the first record of *P. muricata* on *R. miraletus* and *R. montagui* in the Tyrrhenian Sea. *R. miraletus* catches should be monitored throughout its distributional range to determine existence of the subpopulations (SMALE *et al*., 2009) and, in this context, the knowledge of its parasite fauna could be useful to discriminate different stocks.

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Table 4. Taxa used for phylogenetic analysis and GenBank Accession Number

Taxon	Locality	12S	18S	28S	COI
Aliolimnatis africana	African Republic	AY425428	AY425469	AY425387	AY425451
Barbronia weberi	Austria		AF099951	HQ336356	KU553102
Calliobdella vivida	Virginia	AY425409	AF115992	AY425360	AF003260
Chtonobdella bilineata	Australia	AY425410	AF116006	AY425361	AF003267
Erpobdella octoculata	France	AF099954	AF099949	AY425368	HQ336344
Haemopis caeca	Romania	AY425419	AY040687	AY425376	AY040702
Haemopis marmorata	Michigan	FJ897509	AF116008	AY425380	FJ897515
Haemopis sanguisuga	Sweden	AF099960	AF099941	AY425381	AF462021
Helobdella stagnalis	France	AY425424	AY962416	AY425382	AF116018
Hirudo medicinalis	BioPharm,UK	AF099961	Z83752	AY425385	AY364862
Hirudo nipponica	Korea	AY425427	AY425468	AY425386	GQ368749
Limnatis nilotica	Israel	AY425430	AY425470	AY425389	AY425452
Macrobdella decora	Michigan	AY425431	AF116007	AY425390	EU100095
Macrobdella ditetra	Georgia	AY425432	AY425471	AY425391	AY425453
Mesobdella gemmata	Chile	AY425434	AY425472	AY425393	EU100097
Oxyptychus braziliensis	Brasil	AY425436	AY425473	AY425398	AY425455
Patagoniobdella fraterna	Chile	AY425441	AY425477	AY425405	AY425459
Patagoniobdella variabilis	Chile		AY425476		AY425458
Pontobdella muricata	Slovenia	AF099958	AF099945		AY336029
Pontobdella muricata_med	Italy -Med	KY659071	KY659070	KY659073	KY659072
Piscicola geometra	France	AY425437	AF099946	AY425400	AF003280
Stibarobdella macrothela	Virginia	AY425440	AF115996	AY425403	AF116022
Stibarobdella tasmanica	Tasmania Australia		DQ414298		DQ414343
Xerobdella lecomtei	Slovenia		AF099947	EU100086	EU100099

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Prisutnost pijavice *Pontobdella muricata* (Hirudinea: Piscicolidae) na hrskavičnjačama u Tirenskom moru (Srednje Sredozemlje)

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SAŽETAK

U radu se iznosi prvi nalaz pijavice, *Pontobdella muricata* (Linnaeus, 1758), u Tirenskom moru. Pijavica je pronađena na modropjegoj raži, *Raja miraletus* (Linnaeus 1758) i na crnopjegoj raži, *Raja montagui* (Fowler, 1910), iz koćarskih lovina tijekom jeseni 2014. godine. Kompletna sekvenca 18S rRNA gena, COI mitohondrijskog gena i djelomične sekvence mitohondrijskog 12S rRNA gena potvrđuju određivanje koje se zasniva na temelju morfoloških svojstava.

Ključne riječi: *Pontobdella muricata*, nametnici, pijavica, modropjega raža (*Raja miraletus*), crnopjega raža (*Raja montagui*), Tirensko more