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ELENA MARCO-HERRERO, SERGIO RAMÍREZ-AMARO, JULIO A. DÍAZ, FRANCESC ORDINES, ENRIC MASSUTÍ

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A new host record for the pea crab *Pinnotheres pisum* (Linnaeus, 1767) (Decapoda: Pinnotheridae) in the western Mediterranean, with an update on host species

Elena MARCO-HERRERO[1,2], Sergio RAMÍREZ-AMARO[1], Julio A. DÍAZ[1], Francesc ORDINES[1] and Enric MASSUTÍ[1]

[1] Centre Oceanogràfic de les Balears, Instituto Español de Oceanografía (IEO–CSIC), 07015 Palma, Spain
[2] Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO–CSIC), 30740, San Pedro del Pinatar, Spain

Corresponding author: Elena MARCO-HERRERO; elena.marco@ieo.csic.es

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Abstract

Pinnotheres pisum (Linnaeus, 1767) is one of the pinnotherid crab species with the highest number of reported bivalve hosts. Here we first report this species living in the bivalve *Glossus humanus* (Linnaeus, 1758), and provide an update on *P. pisum* symbiotic associations (i.e., known hosts), reaching up to 34 taxa. Partial sequences of the mitochondrial gene cytochrome c oxidase subunit I were first obtained from *P. pisum* and the *G. humanus* host from the Mediterranean Sea, confirming conspecificity with specimens from the Atlantic Ocean of the same species.

Keywords: Bivalvia; Brachyura; DNA barcoding; Heart cockle; Pea crab; Symbiont.

Introduction

The family Pinnotheridae De Haan, 1833 includes crabs that live as symbionts, commensals or parasites of various invertebrates, mostly bivalves (Schmitt *et al.*, 1973). Pinnotherid crabs are difficult to collect and identify due to their small size and cryptic lifestyle within bivalve hosts (Becker & Turkey, 2017); however a few recent reviews have advanced knowledge of this family (Pérez-Miguel *et al.*, 2019; Gier & Becker, 2020; Marco-Herrero *et al.*, 2020; Chow *et al.*, 2023).

Pinnotheres pisum (Linnaeus, 1767) is one of the pinnotherid crab species with the highest number of known bivalve hosts. It has been recorded in the Mediterranean Sea (from the Alboran Sea to the Sea of Marmara), in the north-eastern Atlantic Ocean (from the Gulf of Cadiz (Iberian Peninsula) to the North Sea and south of Scandinavia), and in the Canary Islands (d'Udekem d'Acoz, 1999; Triay-Portella *et al.*, 2018; Pérez-Miguel *et al.*, 2019). The goals of this study were to report a new bivalve host for *P. pisum*, the heart cockle *Glossus humanus* (Linnaeus, 1758), and to provide an update on available information on its symbiotic associations. Moreover, to confirm the morphological identification of *P. pisum* and *G. humanus* and check for conspecificity with Atlantic Ocean specimens, *P. pisum* and *G. humanus* were characterized genetically for the first time at the Mediterranean Sea, using a fragment of the cytochrome c oxidase subunit I (COI).

Materials and Methods

Sampling

Samples were collected in the context of the MEDITS surveys, which aim to quantitatively assess fauna, biomass, density, and population parameters of demersal fishery target species throughout the Mediterranean Sea (Spedicato *et al.*, 2019). Our samples were collected during the MEDITS_ES 2020 survey in the Balearic Islands and the northeast of the Iberian Peninsula (Fig. 1). In these surveys, the experimental bottom trawl GOC-73 was used. The bottom trawl GOC-73 was designed for experimental fishing for scientific purposes, it is 18–22 m wide, with a 2.5–3 m vertical opening, and a cod-end mesh size of 10 mm (Fiorentini *et al.*, 1999).

Morphological identification

Specimens studied in the present work were collected alive. Once bivalves were opened, the internal cavity was observed to establish the presence or absence of pea crabs. Specimens were morphologically identified on board during the trawl survey, according to Zariquiey-Álvarez (1968) and Cuesta *et al.* (2019) for brachyurans, and Gofas *et al.* (2011) for bivalves. Interesting specimens were preserved in 100% ethanol. Photographs of

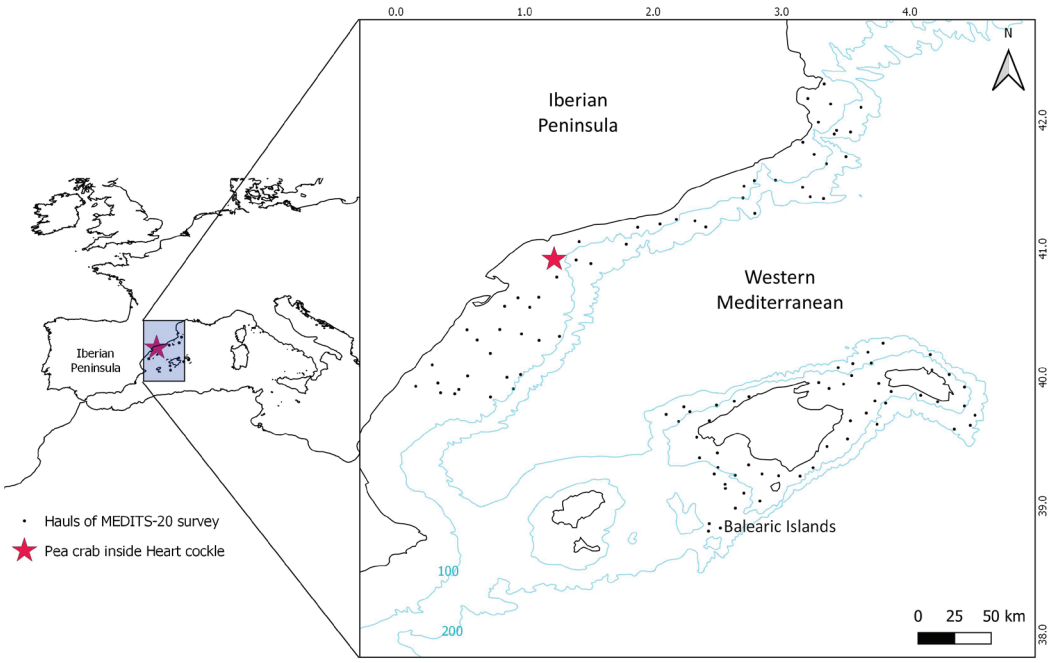

Fig. 1: Map of the study area with a red star highlighting where *Pinnotheres pisum* (Linnaeus, 1767) was found inside *Glossus humanus* (Linnaeus, 1758).

specimens were taken using a smartphone on board and a stereomicroscope Leica M165C, equipped with a camera Leica MC1 at the laboratory of C.O. de les Balears.

Morphometric measurements were performed to the nearest 0.1 mm using a Vernier calliper. Crabs' carapace length (CL = distance from the rostrum to the posterior margin of the carapace) and width (CW = maximum carapace width) were measured. Bivalves' maximum shell height (SH = dorso-ventral axes), length (SL = anterio-posterior axes), and width (SW = right to left valve) were also measured.

Hosts of pea crab Pinnotheres pisum

To update the known hosts of the pea crab *P. pisum*, a comprehensive literature review on the species was carried out.

Molecular identification

Total genomic DNA was extracted using the DNeasy Blood and Tissue Extraction kit (Qiagen, West Sussex, UK) from the muscle tissue of a pereiopod and the anterior adduct muscle for the crab and the bivalve, respectively. A polymerase chain reaction (PCR) technique was used to amplify the DNA barcoding fragment (cytochrome c oxidase subunit I) with the universal primers LCO1490 / HCO2198 (Folmer *et al.*, 1994) for both specimens. PCRs were performed in 25 µl volume containing: 17.7 µl ddH20, 2.5µl Mangobuffer (Bioline), 1 µL DNTPs, 1.75 µL $MgCl_2$, 0.5 µL for each primer (10pmol), 0.05 µL TAQ (Bioline), and 1 µL DNA. The PCR thermal profile was: an initial stage of 96 °C for 5 min; 35 cycles at 94 °C for 60 s, 50 °C for 60 s, and 72 °C for 60 s, followed by a final extension step at 72°C for 10 min. PCR products were purified using the QIAquickR PCR Purification Kit (QIAGEN) and sequenced through an ABI 3130 sequencer using the ABI Prism Terminator BigDyeR Terminator Cycle Sequencing Reaction Kit (Applied Biosystems).

Sequences were imported into BioEdit 7.0.5.2 (Hall, 1999) and checked for quality and accuracy with nucleotide base assignment. Sequences identification was validated using the BLAST function from the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.*, 1990) as well as the IDENTIFICATION function from the "Barcode of Life Data System" BOLD system (https://www.boldsystems.org; Ratnasingham & Hebert, 2007). In addition, available sequences for *P. pisum* and *G. humanus* from online repositories were retrieved and downloaded to perform sequence alignments in ClustalW (Thompson *et al.*, 1994). Genetic identity and the number of base differences between pair sequences were calculated with MEGA v.7.1 (Tamura *et al.*, 2013). The DNA sequences obtained were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Results

Sampling

A total of 106 hauls were performed between 37 and 750 m depth in the MEDITS_ES 2020 survey. Bivalves totalled 178 specimens, belonging to nine species (Table 1). The analysis of the bivalve's internal cavity revealed the presence of one single pea crab inside the only *Glossus humanus* collected alive (Fig. 2A, B), which was sampled at the following coordinates: 40.918° N, 1.228° E.

Table 1. Molluscan bivalves collected and analysed during the MEDITS_ES 2020 survey. Abbreviations: N, number of specimens; (+), presence of pea crab; (-), absence of pea crab.

Species	N	Pea crab
Acanthocardia echinata (Linnaeus, 1758)	1	(-)
Aequipecten opercularis (Linnaeus, 1758)	71	(-)
Anadara gibbosa (Reeve, 1844)	17	(-)
Anomia ephippium Linnaeus, 1758	1	(-)
Glossus humanus (Linnaeus, 1758)	1	(+)
Lima lima (Linnaeus, 1758)	2	(-)
Mimachlamys varia (Linnaeus, 1758)	69	(-)
Pteria hirundo (Linnaeus, 1758)	5	(-)
Venus nux Gmelin, 1791	10	(-)

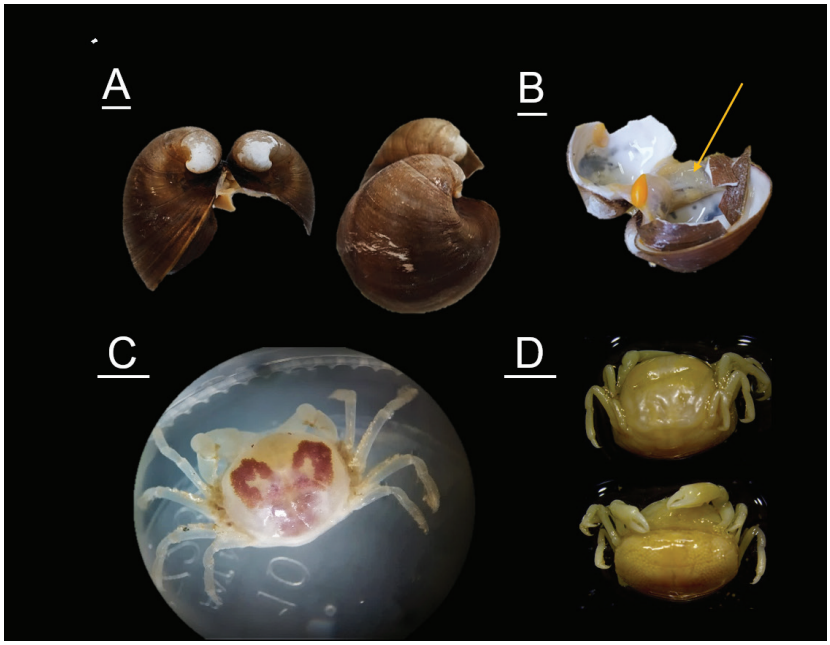

Fig. 2: The studied material from the northeast Iberian Peninsula. A-B: *Glossus humanus* (ICMCBR000480), frontal and lateral right valve view and its soft parts, with a yellow arrow pointing to the position of the pea crab. C-D: *Pinnotheres pisum* (ICMD002685), a fresh sample showing gonadal development, and dorsal and ventral view of the same specimen after fixation. Scale bars: A-B, 10 mm; C-D, 5 mm.

Systematic account

Family Pinnotheridae De Haan 1833
Genus *Pinnotheres* Bosc, 1801
Pinnotheres pisum (Linnaeus, 1767)

Material examined: one ovigerous ♀, 9.6×11.5 mm (CL×CW) (Fig. 2C, D), 2 June 2020, 88 m depth (ICMD002685, Biological Collections of Reference of the Institut de Ciències del Mar - ICM-CSIC, Barcelona, Spain).

Diagnosis: Eyes visible on dorsal view. Carapace subglobular, slightly rounded, and not projected frontal area. Equal length on all pereiopods. Dactylus of pereiopod 2–5 with short, curved tips, less than half as long as propodus. Fixed finger of pereiopod 1 without single tooth (Figs 2C, D, 3A, 4A).

Remarks: five species of pinnotherid crabs have been reported in the Mediterranean Sea: *Afropinnotheres monodi* Manning, 1993, *Nepinnotheres pinnotheres* (Linnaeus, 1758), *Pinnotheres bicristatus* García Raso & Cuesta, 2019, *Pinnotheres pectunculi* Hesse, 1872, and *P. pisum*. Soft female of *P. pisum* can be differentiated from the Mediterranean species as follows: i) *Nepinnotheres pinnotheres* has a subglobular, slightly rounded carapace with a bilobed frontal area (Fig. 3B); ii) *Pinnotheres bicristatus* has a subtrapezoidal carapace (Fig. 3C) with a characteristic pair of dorso-anterolateral turfts of curved setae; iii) *Pinnotheres pectunculi* has a subtrapezoidal carapace (Fig. 3C) and the fixed finger of the chela has a well-developed basal tooth followed by additional ones (Fig. 4B); iv) *Afropinnotheres monodi* has a subglobular carapace, and can be differentiated by the propodus short-

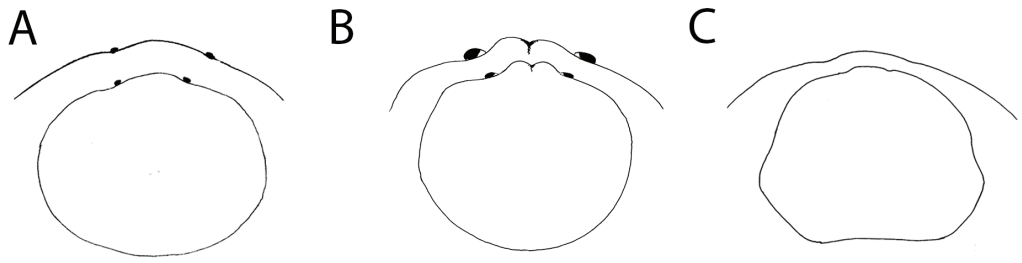

Fig. 3: Morphology of carapace and frontal area of Mediterranean Pinnotheridae species. A: *Pinnotheres pisum* (ICMD002685), and *Afropinnotheres monodi* (see Fig. 1 in Manning, 1993). B: *Nepinnotheres pinnotheres* (after Becker & Turkey, 2010), C: *P. bicristatus* (after Cuesta *et al.*, 2019), and *P. pectunculi* (see Fig. 3E in Becker & Turkey, 2017). Drawings are not to scale.

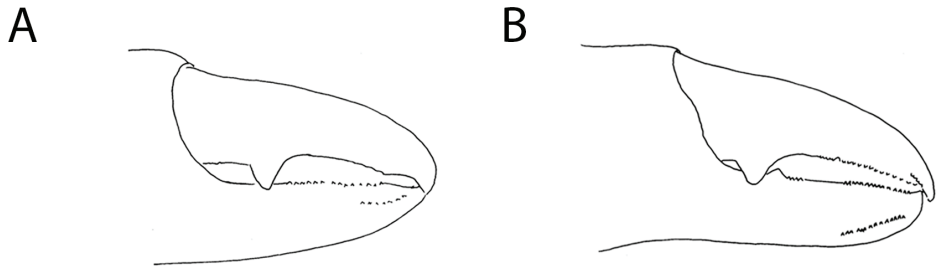

Fig. 4: Fixed finger of pereiopod I. A: *Pinnotheres pisum* (ICMD002685), B: *P. pectunculi* (after Becker & Turkey, 2010). Drawings are not to scale.

er than the carpus on the third maxilliped (see Fig. 1 in Manning, 1993, and Fig. 3C in Cuesta *et al.*, 2019).

Genetics: A 579 base pairs (bp) fragment of the COI fragment was sequenced and deposited in GenBank under the accession number OP321040. Identifiers, sampling localities, and references for the COI sequences available in the GenBank and Bold System databases for *P. pisum* and *G. humanus* are indicated in Table S1. The comparison of our sequence with those found in both genetic databases had a similar outcome, (Table S2) yielding a high level of similarity with specimens identified as *P. pisum* (99.4–99.31% identity, 4–3 bp differences) collected in the North Sea, Gulf of Bothnia, and Cantabrian Sea. Lower similarity values (85.1–84.9% identity, 81–80 bp differences) were obtained with specimens collected along the Portuguese coast reported by Lobo *et al.* (2013), which is likely due to a misidentification for *Afropinnotheres monodi*, as suggested by Pérez-Miguel *et al.* (2019).

Family Glossidae Gray, 1847 (1840)
Genus *Glossus* Poli, 1795
Glossus humanus (Linnaeus, 1758)

Material examined: one specimen 54.8×48.5×35.4 mm (SH×SL×SW), 2 June 2020, 88 m depth (ICMCBR000480, Biological Collections of Reference of the Institut de Ciències del Mar - ICM-CSIC, Barcelona, Spain).

Diagnosis: shell solid, globose, and heart-shaped in lateral view. Equivalve, inequilateral, and markedly globular with the umbos spirally enrolled, and directed anteriorly (Fig. 2A, B). Anterior lateral teeth reduced or absent. Posterior lateral teeth elongate. Surface smooth, shiny, with fine growth striations. Colour brown with some whitish areas.

Remarks: *Glossus humanus* is distributed in the western European waters and the Mediterranean Sea, inhabiting sandy, sandy-muddy, and muddy bottoms of the infralittoral and circalittoral zone. In the present study, it was associated with mollusks *Pteria hirundo* (Linnaeus, 1758), *Calliostoma granulatum* (Born, 1778), and *Galeodea rugosa* (Linnaeus, 1771); with echinoderms *Holothuria tubulosa* Gmelin, 1791, *Astropecten irregularis* (Pennant, 1777), and *Antedon mediterranea* (Lamarck, 1816); and mostly to crustaceans *Macropodia tenuirostris* (Leach, 1814), *Pagurus excavatus* (Herbst, 1791), *Palinurus elephas* (Fabricius, 1787), and *Parapenaeus longirostris* (H. Lucas, 1846).

Genetics: A fragment of 510 bp of the COI fragment was sequenced and deposited in GenBank under the accession number OQ301545. This sequence had a high similarity (97.6%, 9 bp differences) with the only *G. humanus* sequence available in both genetic databases (Table S1) and thus confirmed our morphological identification.

Pea crab Pinnotheres pisum *hosts*

The pea crab *P. pisum* is a specific host of bivalves.

To date, 33 hosts have been reported (Table 2): 15 species from European waters were reported by d'Udekem d'Acoz (1999); 17 species by Becker & Turkey (2017), accounting for three species from the Mediterranean Sea and 15 from the Atlantic Ocean; one species from the Canary Islands (Atlantic) by Triay-Portella *et al.* (2018); 26 species from Europe by in Perez-Miguel *et al.* (2019); and 1 species from the Mediterranean Sea by Marco-Herrero *et al.* (2020) (a bivalve species already reported in the Atlantic but new to the Mediterranean Sea) (Table 2).

Table 2. List of bivalve hosts known for the pea crab *Pinnotheres pisum* in European waters (EW). Mediterranean Sea (MS) and Atlantic Ocean (AO) records were separated when literature allowed it. Abbreviations: (+) present, (-) no specific data. Literature data from: [1] d'Udekem d'Acoz, 1999; [2] Becker & Turkey, 2010; [3] Versteegh & Muller, 2014; [4] Becker & Turkey, 2017; [5] Triay-Portella *et al.*, 2018; [6] Perez-Miguel *et al.*, 2019; [7] Gier & Becker, 2020; [8] Marco-Herrero *et al.*, 2020; species in bold, present work.

Host species	EW	MS	AO
Acanthocardia echinata (Linnaeus, 1758)	(+)[1,6]	(-)	(+)[4,7]
Arctica islandica (Linnaeus, 1767)	(+)[1,6]	(-)	(-)
Atrina pectinata (Linnaeus, 1767)	(+)[6]	(-)	(-)
Chamelea gallina (Linnaeus, 1758)	(-)	(+)[6,7]	(+)[4,7]
Chamelea striatula (da Costa, 1778)	(+)[1,6]	(-)	(-)
Cerastoderma edule (Linnaeus, 1758)	(+)[1,6]	(-)	(+)[4,7]
Cerastoderma glaucum (Bruguière, 1789)	(+)[6]	(-)	(-)
Clausinella fasciata (da Costa, 1778)	(-)	(-)	(+)[4,7]
Donax trunculus Linnaeus, 1758	(+)[6]	(-)	(-)
Donax variegatus (Gmelin, 1791)	(+)[6]	(-)	(-)
Donax venustus Poli, 1795	(+)[6]	(-)	(-)
Donax vittatus (da Costa, 1778)	(+)[1,6]	(-)	(+)[2,4,7]
Dosinia lupinus (Linnaeus, 1758)	(-)	(-)	(+)[4,7]
Ensis ensis (Linnaeus, 1758)	(-)	(-)	(+)[4,7]
Ensis magnus Schumacher, 1817	(-)	(-)	(+)[4,7]
Gari fervensis (Gmelin, 1791)	(+)[6]	(-)	(+)[2,4,7]
Laevicardium crassum (Gmelin, 1791)	(+)[1,6]	(-)	(-)
Lutraria lutraria (Linnaeus, 1758)	(+)[1,6]	(-)	(-)
Mactra stultorum (Linnaeus, 1758) (as *Mactra cinerea*)	(+)[1,6]	(-)	(+)[2,4]
Modiolus modiolus (Linnaeus, 1758)	(+)[1,6]	(-)	(+)[2,4,7]
Mya arenaria Linnaeus, 1758 (as *Arenomya arenaria*)	(+)[1,6]	(-)	(-)
Mytilus edulis Linnaeus, 1758	(+)[1,6]	(-)	(+)[2,4,7]
Mytilus galloprovincialis Lamarck, 1819	(+)[6]	(+)[2,4,8]	(+)[2,3,7]
Nucula nitidosa Winckworth, 1930	(-)	(-)	(+)[4,7]
Ostrea edulis Linnaeus, 1758	(+)[1,6]	(+)[2,4]	(+)[7]
Pinna nobilis Linnaeus, 1758	(+)[1,6]	(+)[2,4]	(-)
Ruditapes decussatus (Linnaeus, 1758)	(+)[6]	(-)	(-)
Spisula spp.	(-)	(-)	(+)[4,7]
Spisula solida (Linnaeus, 1758)	(+)[1,6]	(-)	(+)[2]
Spisula elliptica (Brown, 1827)	(+)[1,6]	(-)	(+)[2]
Spisula subtruncata (da Costa, 1778)	(+)[1,6]	(-)	(-)
Venerupis corrugata (Gmelin, 1791)	(-)	(-)	(+)[4]
Venus verrucosa (Linnaeus, 1758)	(+)[1,6]	(-)	(-)
Fulvia cf. *fragilis* (Forsskål, 1775)	(-)	(-)	(+)[5]
Glossus humanus (Linnaeus, 1758)	(-)	(+)	(-)

Discussion

Here we report a new host of the pea crab *Pinnotheres pisum*, the bivalve *Glossus humanus*, through an investigation that included an integrative taxonomic approach and a thorough bibliographic review. Moreover, the new bivalve host reported here is also a large infaunal bivalve mollusk (Owen, 1953), whose records as a live specimen are relatively scarce; it is more commonly found dead or even fossilized (Pons-Moya & Pons, 1999; Reynolds *et al.*, 2013), which adds additional value to the present report.

The usefulness of the approach used here was confirmed by the new insights gained both at morphological and molecular level. In fact, the five species of pea crabs that inhabit the Mediterranean Sea are very similar morphologically. Nonetheless, our observations on the diagnostic morphological characters of *Pinnotheres pisum* revealed intraspecific variability in the teeth of the fixed finger. According to Becker & Turkey (2010), and Cuesta *et al.* (2019), these teeth are always present; however, they were absent in our specimen.

The COI fragments that we report for *P. pisum* and *G. humanus* represent the first molecular data from specimens collected in the Mediterranean Sea, which confirms their conspecificity with specimens from the Atlantic Ocean of the same species. On the other hand, our bibliographic review confirmed that pea crab species recorded in the Mediterranean Sea differ in the number and species of hosts, with *P. pisum* being the most generalistic, and the other species only known within a limited number of hosts: *A. monodi*, *P. bicristatus*, and *P. pectunculi* are specific symbionts of bivalve mollusks with 12, two, and five species, respectively; *N. pinnotheres* was found in two bivalves and three ascidians (see Pérez-Miguel *et al.*, 2019; Gier & Becker, 2020; Marco-Herrero *et al.*, 2020). Thus, *P. pisum* and *A. monodi* have the widest host range, a generalist strategy that may lead to expansion success and thus to competition for space. Fortunately, *A. monodi* is restricted to the Alboran Sea, while *P. pisum* is the European pea crab with the widest distribution range, found throughout the entire Mediterranean Sea (Stevens, 1990; Becker & Türkay, 2017).

Finally, it is worth mentioning that the last new host records published for pea crabs in Europe were based on ovigerous or soft females (Triay-Portella *et al.*, 2018, Perez-Miguel *et al.*, 2019; Marco-Herrero *et al.*, 2020). Sexual dimorphism in pea crabs is marked and significant. The juveniles of both sexes remain very similar in appearance and both are good pelagic swimmers; however, after mating, females undergo a metamorphosis and never leave the host thereafter (Orton 1920; Atkins 1926; Hartnoll, 1972; Becker *et al.*, 2011). In contrast, the adult male is smaller and free-living and is only occasionally found together with a female inside the host (Becker *et al.*, 2011; Gier & Becker, 2020). This implies that the probability of finding soft or ovigerous females within bivalves is higher than finding juveniles or males, which is also in agreement with the present report. Furthermore, the small size of the pea crabs and the difficulty of finding and analyzing deep-sea bivalves, such as *G. humanus*, makes it difficult to complete all biological information on the life cycle of pinnotherids. Therefore, studies such as the one reported here not only expand species-specific knowledge but also contribute to the understanding and the improvement of ecosystems and species relationships.

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Supplementary Data

The following supplementary information is available online for the article:

Table S1. Identifiers, sampling localities, and references for the COI sequences are available in the GenBank and Bold System databases for *Pinnotheres pisum* and *Glossus humanus*. * misidentification for *Afropinnotheres monodi* (see also Pérez-Miguel *et al.*, 2019).

Table S2. Pairwise comparisons of the percent identity (below the diagonal) and numbers of base differences (above the diagonal) for COI sequences available in the GenBank and Bold System databases for *Pinnotheres pisum* (sequence generated in this study highlighted in bold). * misidentification for *Afropinnotheres monodi* (see also Pérez-Miguel *et al.*, 2019).