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Larval morphology and DNA barcodes as valuable tools in early detection of marine invaders: a new pea crab found in European waters

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Four species of Pinnotheridae inhabit European marine waters, Afropinnotheres monodi, Nepinnotheres pinnotheres, Pinnotheres pectunculi *and* Pinnotheres pisum. *For these four species there are data available on the morphology of their larval stages as well as DNA markers. This information has allowed us to detect some larvae in plankton samples from the Gulf of Cadiz (SW Iberian Peninsula) that do not belong to any of these European pinnotherid species and to be confirmed by DNA barcoding. In this study these findings are shown as a case of early detection of a newly introduced and unknown species in European marine waters.*

Keywords: Crustacea, Decapoda, Pinnotheridae, larval description, non-indigenous species

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INTRODUCTION

The introduction of non-indigenous species (NIS) poses a significant ecological and economic threat to coastal marine communities (Cohen & Carlton, 1998; Sakai *et al.*, 2001). Once introduced and established NIS proliferate and are one of the major threats to the world's biodiversity (Simberloff *et al.*, 2013; Comtet *et al.*, 2015). For this reason, the early detection of incipient invasions increases the possibilities of eradicating them or constraining their spread (Tidbury *et al.*, 2016). Early detection has been recommended as a priority in management plans and programmes of biological invasions treatment (Vander Zanden *et al.*, 2010; Xiong *et al.*, 2016).

In these strategies of early detection of NIS DNA-based methods are playing an important role (Darling & Mahon, 2011; Darling & Piranio, 2015) as it has been successfully tested (Montoliu *et al.*, 2015; Brown *et al.*, 2016). However, DNA barcoding presents a bottleneck since it needs the previous knowledge of NIS DNA sequences deposited in accessible databases such as GenBank. However, recent technological advances, such as high-throughput sequencing (HTS) or a metabarcoding approach, are solving this problem (Brown *et al.*, 2016; Xiong *et al.*, 2016).

Another way to detect NIS in aquatic ecosystems is the analysis of larval stages. An important number of aquatic taxa, including benthonic species, show a larval phase that differs in morphology and habitat to the adult. In many cases larval specimens are present in the plankton in higher numbers than the adult stage. Moreover, some species occupy cryptic habitats as adults and are easier to find as larval forms in the plankton, for example the trachelifer larvae of *Jaxea* spp. (Wear & Yaldwyn, 1966).

There are cases where larvae of NIS were detected before the adults. For example, larvae of *Palaemon macrodactylus* Rathbun, 1902 were found in the Mediterranean in 2005 and 2010 surveys (Torres *et al.*, 2012), but the first adults were observed in 2012 and 2013 (Cuesta *et al.*, 2014). In general, the use of DNA barcoding has allowed the identification of an important number of adults (Radulovici *et al.*, 2009) and planktonic larval stages of different taxa (Marco-Herrero *et al.*, 2013).

In European marine waters there are four known species of the family Pinnotheridae. Becker & Türkey (2010) reduced the number of European species from five to three, *Nepinnotheres pinnotheres* (Linnaeus, 1758), *Pinnotheres pisum* (Linnaeus, 1767) and *Pinnotheres pectunculi* Hesse, 1872, after synonymizing *Pinnotheres ascidicola* Hesse, 1872 and *Pinnotheres marioni* Gourret, 1888 to *N. pinnotheres* and validating the status of *P. pectunculi*. Later Subida *et al.* (2011) found an African species, *Afropinnotheres monodi* Manning, 1993, in the Gulf of Cadiz, elevating to four the current number of European species of pinnotherids. For all these species there are larval data available. Atkins (1954) described the complete larval development of *N. pinnotheres* (two zoeal and one megalopa stages), and *P. pisum* (four zoeal and one megalopa stages); only data of zoea I of *P. pectunculi* are included in Becker (2010), and Marco-Herrero *et al.* (2016) described the four zoeal stage and the megalopa of *A. monodi*. Also, with the exception of *P. pectunculi*, there are 16S mtDNA sequences available for all the European pinnotherids.

This previous information has allowed us to detect larvae of an unknown pinnotherid in the Gulf of Cadiz. In the

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present study these larval stages are described and DNA sequences are provided to help identify this new NIS for European waters and follow its spread, adding more value to the early detection of this aquatic invasion.

MATERIALS AND METHODS

Material studied

Larval stages: A total of 139 unidentified zoeae (stages of zoea II–IV) and 15 unidentified megalopae attributed to unknown pea crab species (Crustacea, Decapoda, Pinnotheridae), as well as eight zoeae (different stages) of *Pinnotheres pisum*, were collected in two plankton samples obtained in the context of the project 'Ecology of the early stages of the *Engraulis encrasicolus* life cycle: the role of the coupled ecosystem 'Guadalquivir estuary and its coastal influence area' in the recruitment process of this species' (ECOBOGE). The samples were collected with a suprabenthic sled equipped with two superimposed nets (200 µm mesh size) that sampled the motile fauna in the first metre near-bottom water layers. The two samples were obtained on 14 November 2013. The first one (PD10) was collected at 36,52900 (start) – 36,52978 (end) N – 6,28913 (start) – −6,29783 (end) W, 14 m depth, and the second one (MT10) at 36,57320 (start) – 36,57571 (end) N −6,3211 (start) – 6,32451 (end) W, 11 m depth (Figure 1). Both samples were fixed in ethanol (90%) for later morphological and molecular studies.

Adult stages: two specimens of *Pinnotheres pectunculi* (one male SMF34383 and one female SMF34529 both from Roscoff, France) were borrowed as a loan from the Senckenberg Museum (Frankfurt, Germany) to obtain DNA sequences, since *P. pectunculi* was the only European Pinnotheridae species without gene sequences available in GenBank.

Identification of samples

Initially all zoeae and megalopae were undoubtedly identified as larval stages belonging to Pinnotheridae crabs. Eight zoeae were identified as zoeae II and III of *Pinnotheres pisum*, and the rest of the larval stages (zoeae II, III, IV and megalopae) shared characters that clearly placed them also in the subfamily Pinnotherinae, *sensu* Palacios-Theil *et al.* (2016), and belonging to different larval stages of a single unknown species.

Molecular analysis

The DNA barcoding identification of larval stages was based on partial sequences of the 16S mtDNA gene, a molecular marker that has shown to be suitable for DNA barcoding in crustaceans (Schubart *et al.*, 2000). Cytochrome oxidase 1 (Cox1) was discarded as a marker because there are no Cox1 sequences available for all these species in GenBank, together with the difficulties found in the amplification of this gene for species of this family (Mantelatto *et al.*, 2016). Total genomic DNA was extracted from maxillipeds muscle tissue of three zoeal specimens and from pereiopods of one megalopa, following a modified Chelex 10% protocol by Estoup *et al.* (1996).

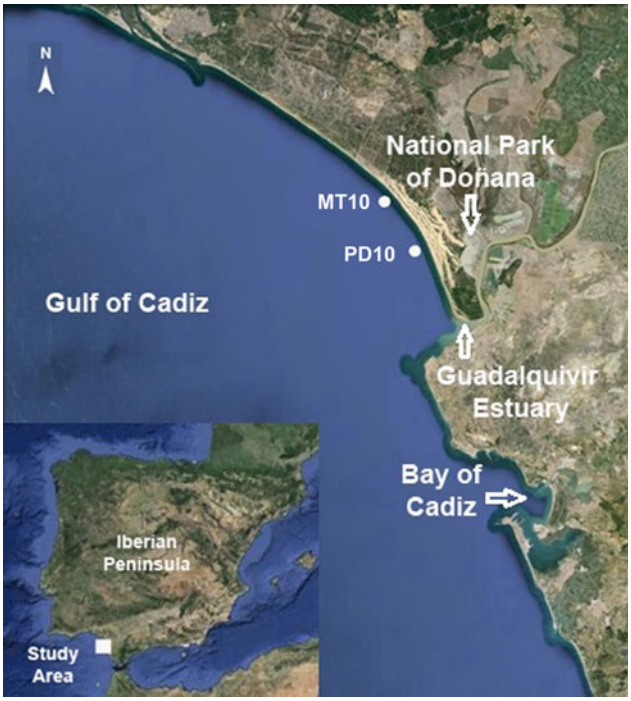

Fig. 1. Map of the Gulf of Cadiz (SW Iberian Peninsula) showing the sampling stations MT10 and PD10 where larvae of *Pinnotheres pisum* and *Pinnotheres* sp. were collected. Images from Google Earth, version 7.1.2.2041 ©2013.

Target mitochondrial DNA from the 16S mtDNA gene was amplified with polymerase chain reaction (PCR) using the following cycling conditions (with Taq polymerase): 2 min at 95°C, 40 cycles of 20 s at 95°C, 20 s at 45–48°C, 45 s at 72°C, and 5 min 72°C. Primer pairs 1472 (5′-AGA TAG AAA CCA ACC TGG-3′) (Crandall & Fitzpatrick, 1996) and 16L2 (5′-TGC CTG TTT ATC AAA AAC AT-3′) (Schubart *et al.*, 2002), and 16Sbr-H (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Palumbi, 1996) and L12 (5′-TGA CCG TGC AAA GGT AGG ATA A-3′) (Schubart *et al.*, 1998) were used to amplify 420–540 bp of 16S mtDNA gene. PCR products were sent to Stab-Vida laboratories to be purified and then bidirectionally sequenced.

Sequences were edited using the free software Chromas Lite version 2.0. The obtained final DNA sequences were compared with those from adult specimens of the four Pinnotheridae species that inhabit European waters, obtained in the context of the MEGALOPADN and AFROBIV projects, as well as downloaded from GenBank databases. New partial sequences of 16S mtDNA obtained from larvae of *Pinnotheres* sp., zoea of *Pinnotheres pisum* and two specimens of *Pinnotheres pectunculi* were deposited in GenBank under accession numbers MF069147–MF069151.

An evolutionary distances analysis was carried out in MEGA6 (Tamura *et al.*, 2013). An alignment of 16S sequences was built using the sequences obtained in the present study from the larval stages of *Pinnotheres* sp., one zoea of *Pinnotheres pisum* and two specimens of *Pinnotheres pectunculi* as well as the sequences of *Afropinnotheres monodi*, *Nepinnotheres pinnotheres*, *Pinnotheres pisum*, *Orthotheres barbatus* and *Zaops ostreus* downloaded from GenBank (http://www.ncbi.nlm.nih.gov). The phylogenetic reconstruction was inferred from neighbour-joining analyses using the *P*-distance method. The nodal confidence of the obtained topologies was assessed via 2000 bootstrap replicates.

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Larval morphology description

Dissections, drawings and measurements of larval stages of *Pinnotheres* sp. were made using a Wild MZ6 and a Zeiss Axioskop compound microscope with Nomarski interference, both equipped with a *camera lucida*. For an easier observation of larvae structures and setation under the microscope, a digestion-stain procedure was carried out. First, entire specimens were placed for 10 min on a watch glass with 2 ml of heated lactic acid. Immediately after that, 3 drops of Clorazol Black stain (0.4 g Clorazol Black powder dissolved in 75 ml 70% ethanol) were added to the heated solution. After 5–10 min, the specimens were removed from the solution and placed on a slide with lactic acid, in order to proceed with the dissection of the appendages (for more details see Marco-Herrero *et al.*, 2012).

Drawings and description were based on examinations of three zoea II, six zoea III, seven zoea IV and nine megalopae. Measurements were made with an ocular micrometer, based on three zoea II, five zoea III, five zoea IV and five megalopae. In zoeal and megalopa stages the cephalothorax length (CL) was measured from the base of the rostrum to the posterior margin of the cephalothorax, and the cephalothorax width (CW) as the maximum width of the cephalothorax.

Descriptions and figures were arranged according to the standards proposed by Clark *et al.* (1998), and updated by Clark & Cuesta (2015). Samples of larvae of *Pinnotheres* sp. and *P. pisum* are deposited at the Crustacean Decapod Collection from Cadiz Oceanographic Centre under the accession numbers IEO-CD-ECOB/1926–IEO-CD-ECOB/1928.

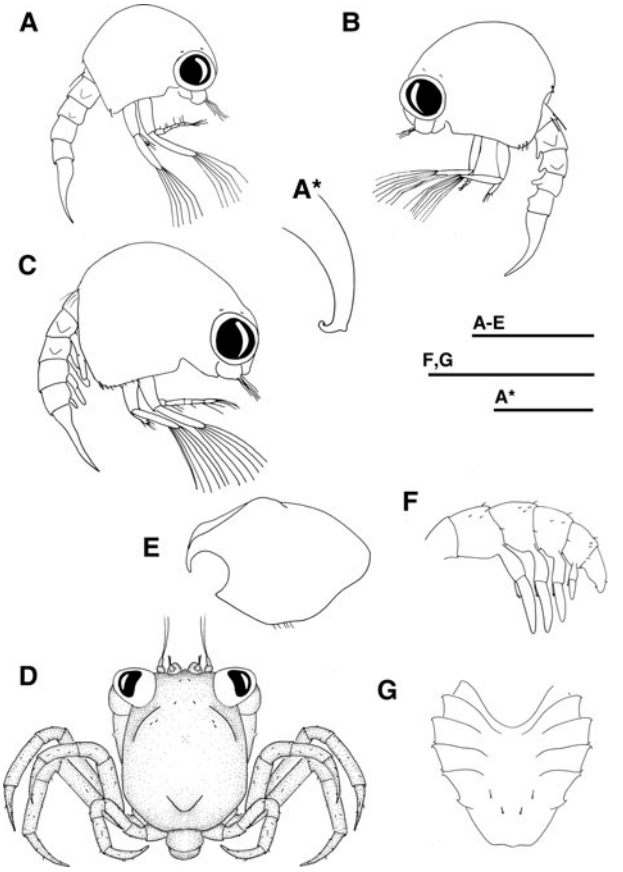

Fig. 2. *Pinnotheres* sp. Lateral view, (a) zoea II, (a*) detail of rostrum. (b) zoea III. (c) zoea IV. Megalopa, (d) dorsal view, (e, f) lateral view, (e) carapace, (f) pleon. (g) sternum. Scale bars, (a–g) = 0.5 mm, (a*) = 0.1 mm.

RESULTS

In the plankton samples were identified a total of eight zoeae (two zoea II, six zoea III) of *Pinnotheres pisum*, and three zoeae II, 97 zoeae III, 23 zoeae IV and 16 megalopae of *Pinnotheres* sp., but no zoea I attributed to this last species was found.

According to the morphology and size of the larvae identified, the larval development of *Pinnotheres* sp. consists of four zoeal stages and a megalopa.

The second zoea is described in detail, and only the main differences in subsequent stages are noted.

LARVAL DESCRIPTION

Order DECAPODA Latreille, 1802
Infraorder BRACHYURA Latreille, 1802
Family PINNOTHERIDAE De Haan, 1833
Genus *Pinnotheres* Bosc, 1801
Pinnotheres sp.
(Figures 2–5).

ZOEA II

(Figures 2a, a*, 3a–c, 4a–d, 5a)

Size: CL = 0.472 ± 0.019 mm; CW = 0.375 ± 0.039 mm, N = 3.

Cephalothorax (Figures 2a, a*): Globular, without tubercles. Dorsal and lateral spine absent. Rostrum short, elephant trunk shape with a minute tubercle (Figure 2a*). One pair of posterodorsal and two pairs of anteromedian simple setae. Ventral margin with one plumose setae. Eyes stalked and movable.

Antennule (Figure 3a): Unsegmented and conical. Endopod absent. Exopod with 5 terminal aesthetascs (3 long, 2 shorter), without setae.

Antenna (Figure 3b): Endopod present as small bud. Protopod and exopod absent.

Mandible (Figure 3c): Incisor and molar processes developed. Palp absent.

Maxillule (Figure 4a): Coxal endite with 4 plumodenticulate setae. Basial endite with 7 setae (5 terminal cuspidate, 2 subterminal plumodenticulate). Endopod 2-segmented, proximal segment without setae, and 4 terminal (2 + 2) sparsely plumose setae on distal segment. Exopod seta present.

Maxilla (Figure 4b): Coxal endite with 6 plumodenticulate setae. Basial endite bilobed, with 5 + 4 plumodenticulate setae. Unsegmented endopod with 1 + 2 long plumodenticulate setae. Exopod (scaphognathite) with 9 plumose marginal setae.

First maxilliped (Figure 4c): Coxa without setae. Basis with 10 medial sparsely plumodenticulate setae arranged as 2 + 2 + 3 + 3. Endopod 5-segmented with 1,2,1,2,4 (1 subterminal + 3 terminal) sparsely plumodenticulate setae. Exopod unsegmented, with 6 terminal plumose natatory setae.

Second maxilliped (Figure 4d): Coxa without setae. Basis with 4 sparsely plumodenticulate setae arranged 1 + 1 + 1 + 1. Endopod 2-segmented with 0, 1 subterminal serrulate + 4 terminal setae (2 long plumodenticulate, 2

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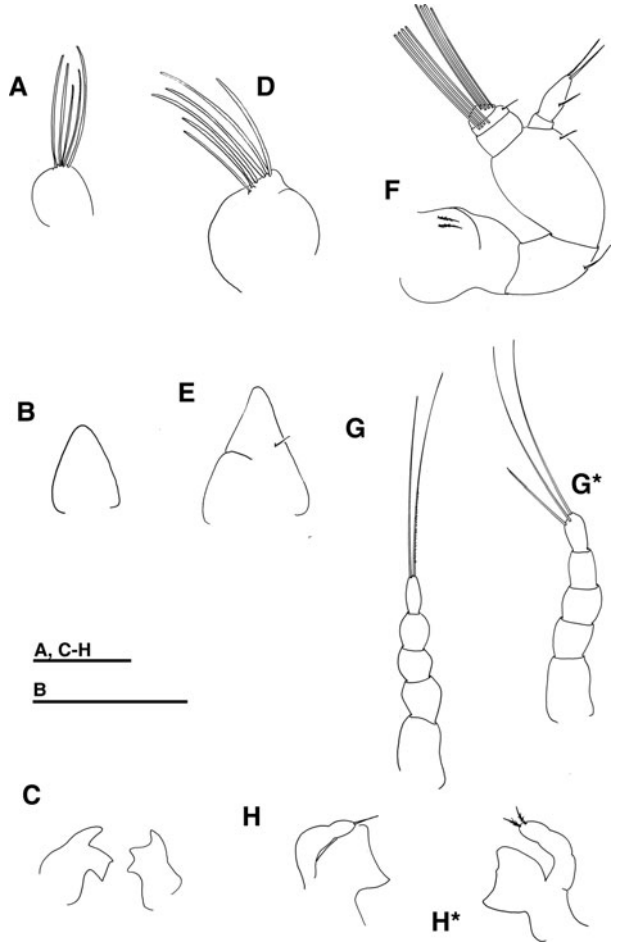

Fig. 3. *Pinnotheres* sp. Antennule, (a) zoea II, (d) zoea IV, (f) megalopa. Mandible, (c) zoea II, (h, h*) megalopa. Antenna, (b) zoea II, (e) zoea IV, (g, g*) megalopa. Scales bars = 0.1 mm.

shorter: 1 plumodenticulate and 1 sparsely plumodenticulate). Exopod unsegmented with 6 terminal plumose natatory setae.

Third maxilliped: Absent.

Pleon (Figures 2a, 5a): 5 pleonites. Pleonite 1 with one middorsal simple seta. Pleonites 2–5 with a pair of minute simple setae on posterodorsal margin. Pleonites 2–3 with pair of laterally directed dorsolateral processes, those of pleonite 3 smaller.

Pleopods: Absent.

Telson (Figures 2a, 5a): Trilobed with 2 pairs of 3 serrulate setae on posterior margin, inner setae longest; each lateral lobes crenulated distally.

ZOEA III

(Figures 2b, 5b)

Size: CL = 0.521 ± 0.020 mm; CW = 0.442 ± 0.008 mm, N = 5.

Cephalothorax (Figure 2b): Ventral margin with 1 highly plumose and 2 sparsely setose setae.

Antennule: Exopod with 6 aesthetascs.

Antenna: Endopod enlarged. Exopod present as a simple seta.

Maxillule: Basial endite with 9 setae (3 subterminal plumodenticulate, 5 terminal cuspidate and 1 proximal simple seta).

Maxilla: Basial endite with 5 + 5 plumodenticulate setae. Scaphognathite with 12 plumose marginal setae.

First maxilliped: Exopod with 7–8 terminal plumose natatory setae.

Second maxilliped: Exopod with 7–8 terminal plumose natatory setae.

Third maxilliped: Present as undifferentiated buds.

Pereiopods: All present as buds, slightly segmented, first pair chelate.

Pleon (Figure 5b): Pleonite 1 with 3 middorsal simple setae.

Pleopods: Present on pleonites 2–5 as small buds, endopods absent.

ZOEA IV

(Figures 2c, 3d, e, 4e, f, 5c)

Size: CL = 0.626 ± 0.020 mm; CW = 0.510 ± 0.033 mm, N = 5.

Cephalothorax (Figure 2c): Ventral margin with 1 highly plumose and 7 sparsely setose setae.

Antennule (Figure 3d): Endopod bud present. Exopod with 6 aesthetascs (3 subterminal and 3 terminal).

Antenna (Figure 3e): Endopod more elongated than in zoea III.

Mandible: Palp present as unsegmented bud without setae.

Maxillule (Figure 4e): Coxal endite with 5–6 plumodenticulate setae. Basial endite with 10–12 setae (4–5 subterminal plumodenticulate, 5–6 terminal cuspidate, 1 proximal simple seta).

Maxilla (Figure 4f): Coxal endite with 7 plumodenticulate setae. Scaphognathite with 16 plumose marginal setae.

First maxilliped: Exopod with 7–8 terminal plumose natatory setae.

Second maxilliped: Exopod with 7–8 terminal plumose natatory setae.

Third maxilliped: Triramous. Endopod and exopod present as slightly segmented buds, without setae. Epipod bud present.

Pereiopods: Cheliped and pereiopods slightly segmented without setae.

Pleon (Figures 2c, 5c): Pleonite 1 with 5 middorsal simple setae.

Pleopods (Figures 2c, 5c): Biramous buds more elongated with endopod bud present.

MEGALOPA

(Figures 2d–g, 3f–h, h*, 4g–k, 5d–i)

Size: CL = 0.609 ± 0.042 mm; CW = 0.483 ± 0.016 mm; N = 9.

Cephalothorax (Figures 2d, e): Slightly longer than broad, subquadrate. Rostrum small, ventrally deflected (~70°), with median longitudinal depression. Protogastric, cardiac and mid-posterior region with tubercles. Setation as illustrated. Eyes stalked.

Antennule (Figure 3f): Peduncle 3-segmented, with 2 plumose, 1 simple, and 1 simple setae respectively. Endopod 2-segmented, without setae on proximal segment, and 1 subterminal and 2 terminal simple setae on distal one. Exopod 4-segmented, with 0, 0, 4, 4 aesthetascs and 0, 0, 1, 0 simple seta respectively.

Antenna (Figures 3g, g*): Peduncle 3-segmented without setae and flagellum 2-segmented without setae on first segment, and 2 long terminal setae (and sometimes 1 shorter subterminal) on distal segment.

Mandible (Figures 3h, h*): Palp 3-segmented with 1 or 2 terminal simple or sparsely plumodenticulate setae on distal segment.

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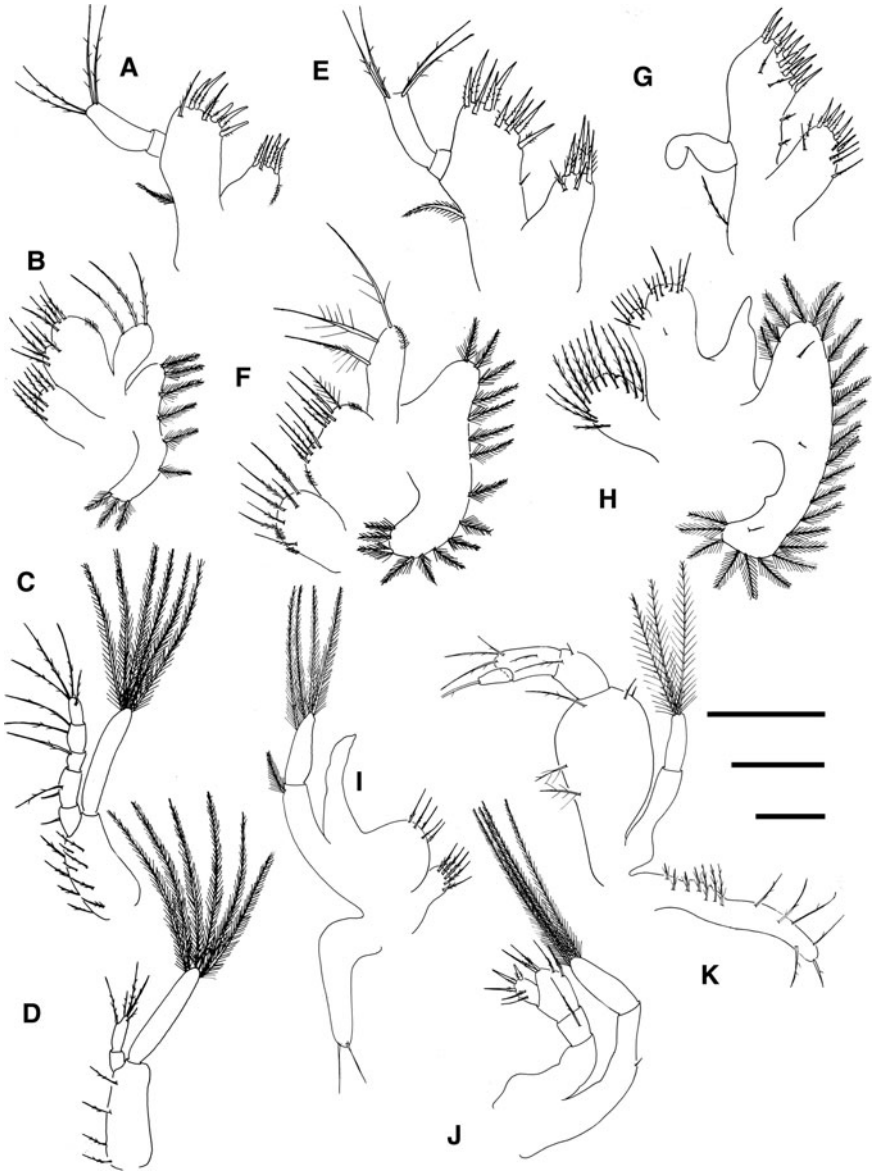

Fig. 4. *Pinnotheres* sp. Maxillule, (a) zoea II, (e) zoea IV, (g) megalopa. Maxilla, (b) zoea II, (f) zoea IV, (h) megalopa. First maxilliped, (c) zoea II, (i) megalopa. Second maxilliped, (d) zoea II, (j) megalopa. Third maxilliped, (k) megalopa. Scale bars = 0.1 mm.

Maxillule (Figure 4g): Coxal endite with 11 plumodenticulate setae. Basial endite with 15 setae (2 subterminal plumodenticulate + 5 subterminal cuspidate + 6 terminal cuspidate + 2 proximal plumodenticulate). Endopod unsegmented without seta.

Maxilla (Figure 4h): Coxal endite with 12 plumose setae. Basial endite bilobed with 6 + 7–8 plumodenticulate setae. Endopod unsegmented without setae. Scaphognathite with 23–26 marginal plumose setae plus 3 small simple setae, on the same lateral surface.

First maxilliped (Figure 4i): Epipodite with 2 terminal simple setae. Coxal endite with 4–5 plumodenticulate setae. Basial endite with 4–5 plumodenticulate setae. Endopod unsegmented without setae. Exopod 2-segmented with one terminal plumose setae on proximal segment, and 4 terminal plumose setae on distal segment.

Second maxilliped (Figure 4j): Epipodite absent. Coxa and basis undifferentiated without setae. Endopod 4-segmented with 0, 1 long sparsely setose, 4 plumodenticulate, 5 (2 subterminal sparsely setae + 3 plumodenticulate) setae respectively, dactylus inserted subterminally on propodus. Exopod 2-segmented with one medial simple setae on proximal segment and 4 terminal plumose setae on distal segment.

Third maxilliped (Figure 4k): Epipodite well developed with 7 proximal plumodenticulate setae and 6 long terminal setae. Protopod without setae. Endopod 4-segmented, ischium and merus fused with 5 setae (3 long sparsely setose + 2 small simple setae), carpus with 2 setae (1 plumodenticulate, 1 simple), propodus with 4 plumodenticulate setae (3 terminal, 1 subterminal), dactylus inserted subterminally on propodus with 2 terminal simple setae. Exopod 2-segmented, proximal segment without setae and 3 terminal plumose setae on distal segment.

Pereiopods (Figures 2d, 5e–g): All segments well differentiated. Cheliped sparsely setose as shown. Pereiopods 2–5 thin and setose, with a small spine subteminal in dactylus (Figure 5f, f*).

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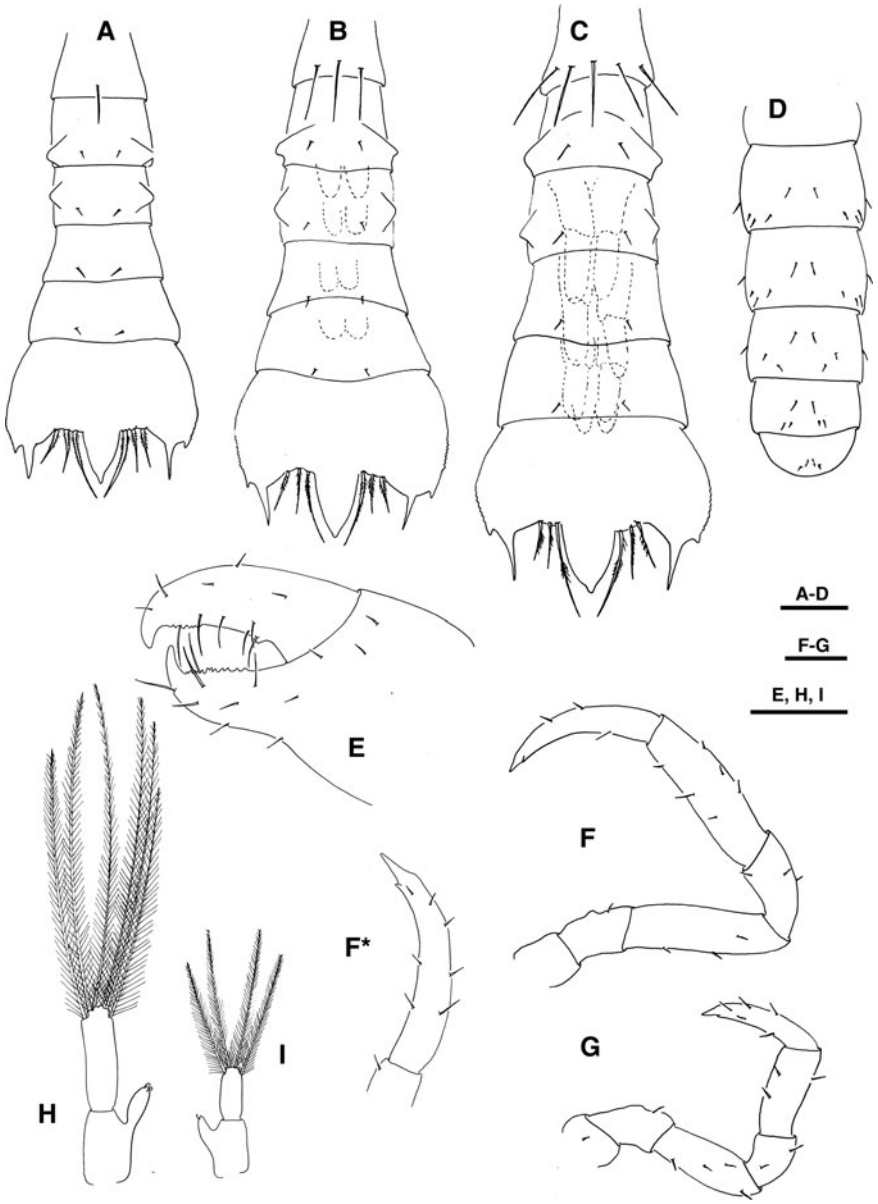

Fig. 5. *Pinnotheres* sp. Pleon, dorsal view, (a) zoea II, (b) zoea III, (c) zoea IV, (d) megalopa. Megalopa, (e) cheliped, (f, f*, g) pereiopods, (h) pleopod, (i) uropod. Scale bars = 0.1 mm.

Sternum (Figure 2g): Maxillipeds sternites fused with 2 simple setae. Chelipeds sternites with 2 simple setae. Sternites of pereiopods 2–5 without setae.

Pleon (Figures 2f, 5d): Sixth pleonite absent, setation as shown.

Pleopods (Figures 2f, 5h, i): Biramous, present on pleonites 2–5. Endopod of pleonites 2–4 with 3 cincinuli and exopod with 6 long terminal plumose natatory setae. Endopod of pleonite 5 with 1–2 cincinuli and exopod with 2–5 long terminal plumose natatory setae. Uropods absent.

Telson (Figures 2f, 5d): Rounded with one pair of simple setae on dorsal margin and one pair on ventral margin.

DNA BARCODING IDENTIFICATION

The 16S mtDNA (439 bp) of the zoea initially identified as belonging to *Pinnotheres pisum* show a 100% match with the sequences of *P. pisum* from Germany and France obtained by Schubart *et al.* (2006) and Bui *et al.* (2008) respectively. However, the sequences of larval stages (zoea and megalopa) of *Pinnotheres* sp. do not match with any sequence deposited in GenBank, or with any other European pinnotherid. The sequences obtained for zoea and megalopa are 100% identical to each other and in the obtained tree both cluster together. A major clade joins them to *Pinnotheres pisum* and *P. pectunculi*, clearly separated from *N. pinnotheres* and *A. monodi* (Figure 6).

DISCUSSION

Eradication and control of invasive species are often possible only if populations are detected when they are small and localized (Inglis *et al.*, 2006). For early detection accurate surveillance and monitoring programmes must be established, and these management programmes must take into account the last DNA-based techniques (Darling, 2015).

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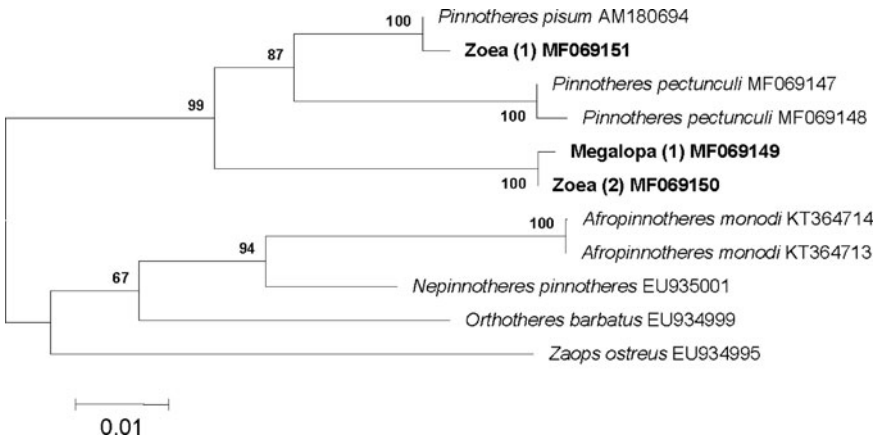

Fig. 6. Topology of neighbour-joining tree based on 540 bp of the 16S mtDNA gene sequences, showing inferred phylogenetic relationships within the European representatives of the family Pinnotheridae with *Zaops ostreus* and *Orthotheres barbatus* as outgroups. Numbers close to nodes indicate bootstrap support (only values above 60% shown). GenBank accession numbers are shown after name of species.

For successful early detection of NIS a good knowledge of the native fauna is required. This knowledge should include both adult/larval morphology and DNA barcodes. In the present study concerning European Pinnotheridae this knowledge has been the key for the detection of an unknown species.

Firstly, the morphology of these larvae caught our attention. Pinnotherid zoea are clearly distinguishable from the rest of the brachyuran larvae due to a typical trilobate telson (see Figures 5a–c), but when comparing them to known European pinnotherid (Atkins, 1954; Becker, 2010; Marco-Herrero *et al.*, 2016) these zoeae of *Pinnotheres* sp. did not exhibit dorsal and lateral spines in the cephalothorax (see Figures 3a–c). In *Pinnotheres pisum* and *P. pectunculi*, lateral spines are present, and *N. pinnotheres* and *A. monodi* show both dorsal and lateral spines. With these findings, a more accurate study of the megalopae from the same samples also indicated that these megalopae were different from those of *P. pisum* (for example in the number of antennal flagellum segments, 3 in *P. pisum* and only 2 in *Pinnotheres* sp.), and *N. pinnotheres* and *A. monodi* (pleon with 6 pleonites and only 5 pleonites in *Pinnotheres* sp.). However, due to the lack of data for the megalopa stage of *P. pectunculi*, this species cannot be discarded.

The results of comparing the fragments of 16S mtDNA confirmed that the zoeae and the megalopae here analysed belong to the same species (Figure 6) and that this species is not any of the four previously recognized European pinnotherids, or any of the Pinnotheridae currently included in the GenBank databases.

The lack of cephalothoracic dorsal and lateral spines in zoeal stages of *Pinnotheres* sp. allow to distinguish it from the rest of European pinnotherids, but this feature does not place it close to other species with these characters, for example *Zaops ostreus* (Say, 1817) (see Sandoz & Hopkins, 1947) or *Orthotheres barbatus* (Desbonne, 1867) (see Bolaños *et al.*, 2005) (Figure 3).

From the DNA analysis it was clear that *Pinnotheres* sp. is closer to species of the genus *Pinnotheres* than any other Pinnotheridae genera, but currently *Pinnotheres* comprises 64 species (Palacios-Theil, 2014) and there are DNA sequences available for only two species of this genus, *P. pisum* and *P. pectunculi*.

Regarding the origin of this species there are two main possible explanations, (1) it could have been introduced within ballast water or as part of ship hull fouling. Samples were collected in an area close to the Guadalquivir estuary, a place where several species have been introduced as a result of the transport by one or more of the many vessels entering and leaving the Sevilla Harbour, which experiences considerable international ship traffic (Cuesta *et al.*, 2004). Taking into account that *Pinnotheres* species are mainly hosted by bivalve and ascidian species common in fouling communities and allowing alive transport for long time periods, *Pinnotheres* sp. could have come from anywhere in the world. (2) Considering that *Afropinnotheres monodi* was detected in 2010 and the first samples of this species were collected in 1995 (López de la Rosa, 2002), this species could be another African species just in an initial stage of establishment (taking into account that larvae are a sign of reproduction). It could be arriving in southern Europe in a natural northward expansion of this species. One of the effects of global warming is the northward expansion of African species, and this could be another example. Manning & Holthuis (1981) listed a total of 11 *Pinnotheres* species in West Africa, including five *Pinnotheres* spp. More recently Manning (1993), studying West African *Pinnotheres*, erected six new genera (*Afropinnotheres*, *Alainotheres*, *Ernestotheres*, *Hospitotheres*, *Nepinnotheres* and *Waldotheres*) to remove 14 species from this genus. Except for *Afropinnotheres monodi* and *Nepinnotheres pinnotheres* no DNA data are available for any of these 14 species of African pinnotherid species.

The information provided here (larval morphology and DNA sequences) will help in the near future to identify this unknown species of Pinnotheridae (when adults are collected), and to follow its spread in European waters through the presence of larvae in plankton samples.

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