

# Multigene phylogeny of the Indo–West Pacific genus *Enosteoides* (Crustacea, Decapoda, Porcellanidae) with description of a new species from Australia

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## Abstract

The porcellanid genus *Enosteoides* Johnson, 1970, currently containing six species, was raised in the 1970s to contain aberrant Indo–West Pacific forms of the diverse and cosmopolitan genus *Porcellana* Lamarck, 1801. Here, we describe the most aberrant form as *Enosteoides spinosus* sp. nov., from the northeast and northwest coasts of Australia and present results on phylogenetic reconstructions of the genus, based on an 1,870 bp alignment of concatenated DNA sequences of three mitochondrial and one nuclear gene. The new species is peculiarly spiny and has a higher morphological affinity to the type species of the genus, *E. ornatus* (Stimpson, 1858), than to the other congeneric species. Our molecular results indicate that *Enosteoides* is not monophyletic. The new species and *E. ornatus* are encompassed in a clade, which does not share immediate common ancestry with the clade containing the other species of *Enosteoides*. This clade is more closely related to species of *Porcellana* and *Pisidia*. Relatively large interspecific genetic distances between and within the two clades, as compared to distances estimated in American pairs of species on each side of the Panama Isthmus, suggest ancient divergence, probably followed by extinction events or low speciation rate. Relatively large intra-specific distances between Australian populations of the new species of *Enosteoides* from geographically distant locations suggest some level of phylogeographic structure.

## Key Words

comparative morphology, marine biodiversity, mitochondrial and nuclear markers, molecular systematics, porcelain crabs, systematics, taxonomy

## Introduction

Porcellanid crabs comprise a morphologically and ecologically diverse family of decapod crustaceans containing over 300 species in 29 genera with littoral or sublittoral distributions in tropical and temperate regions of all oceans (Haig 1960; Werding et al. 2003; Osawa and Chan 2010; Osawa and McLaughlin 2010; Hiller and Werding 2016; Hiller and Lessios 2017, 2019; Werding and Hiller 2017; Osawa and Ng 2018; Hiller

and Werding 2019; Osawa and Sato 2022). While some porcellanid genera are relatively diverse, with more than 100 species (e.g. the globally-distributed *Petrolisthes* Stimpson, 1858), others contain few species (e.g. the American *Megalobrachium* Stimpson, 1858) and some are monospecific (e.g. the American *Ulloaia* Glassell, 1938).

The Indo–West Pacific (IWP) genus *Enosteoides* Johnson, 1970 was first established by Johnson (1970) as a subgenus of the cosmopolitan *Porcellana* de Lamarck, 1801 (currently with 15 species) to receive only one

species from Singapore, *Porcellana corallicola* (Haswell, 1882). The description by Haswell (1882), based on material from Port Molle, Queensland, Australia, is quite superficial, but allows unequivocal identification of *Enosteoides ornatus*. Later, Miers (1884) synonymised Haswell's species with "*Petrolisthes? corallicola?*", based on a single female specimen from the same Australian locality. His description also matches *E. ornatus*.

In her review of the genus *Porcellana*, Haig (1978, p. 709) acknowledged Haswell's *Porcellana corallicola* as a junior synonym of *Enosteoides ornatus*, described by Stimpson (1858) as *Porcellana ornata* from Hong Kong. Haig elevated Johnson's subgenus to generic rank to receive the "aberrant *Porcellana* forms", including two additional species from Palau, *E. melissa* (Miyake, 1942) and *E. palauensis* (Nakasone & Miyake, 1968). Osawa (2009) described *E. lobatus* from Japan, stating that the genus contained four IWP species. More recently, two additional species were described from the Philippines, *E. philippinensis* Dolorosa & Werding, 2014 and *E. turkayi* Osawa, 2016. Osawa (2009) stated that *E. lobatus* is morphologically closer to *E. melissa* and *E. palauensis* than to *E. ornatus*, as this later species bears distinctive spines on the margin of the carapace, on the antennular peduncle and on the surface of the cheliped's palm. Osawa (2016) also emphasised the morphological affinity amongst *E. melissa*, *E. philippinensis* and *E. turkayi*, as they share a similar shape and structure of rostrum and chelipeds and have slender walking legs. However, *E. turkayi* is clearly distinguished by the bright red colouration of the distal segments of all walking legs and by the shape of the third thoracic sternite, which resembles that of *E. lobatus* (Osawa 2016). All species, so far described, have a telson composed of seven plates.

Here, we describe *Enosteoides spinosus* sp. nov. from Australia, which, at first glance, looks quite different from all known species of *Enosteoides* because of its extremely spiny carapace and remarkably spiny and sculptured chelipeds. Nevertheless, the new species agrees with the diagnosis for *Enosteoides* by Haig (1978), with one exception: the telson is composed of five instead of seven plates. Through the reconstruction of a molecular phylogeny, based on DNA sequences of three mitochondrial and one nuclear gene, we tested the monophyly of *Enosteoides* and explored intra- and interspecific boundaries within the genus, as well as evolutionary relationships with morphologically similar genera, such as *Porcellana* and *Pisidia* Leach, 1820 and with more distantly related genera, such as *Petrolisthes* and *Pachycheles* Stimpson, 1858. For comparison purposes, we refer to previously published dated molecular divergence between geminates (sister lines on each side of America) of *Petrolisthes* and *Megalobrachium*, assumed to have diverged during the final stages of the rising of the Isthmus of Panama (Hiller and Lessios 2017, 2019) throughout the Pliocene, approximately 5 to 3 million years ago (MYA).

## Materials and methods

We collected or obtained specimens of *Enosteoides* and other porcellanids from the following museums (see Table 1 and Acknowledgements): Western Australian Museum, Welshpool, Australia (**WAM**), Muséum National d'Histoire Naturelle, Paris, France (**MNHN**), Natural History Museum Los Angeles County (**LACM**), Lee Kong Chian Natural History Museum, Singapore (**LKCNHM**, formerly known as the Raffles Museum of Biodiversity Research–ZRC) and the Western Philippines University, Puerto Princesa (**WPU**). The type material of the new species is listed below, under the Systematic Account section. One WAM paratype was kindly donated by A. Hosie (**WAM**) to be deposited in the crustacean collections of the Naturmuseum Senckenberg, Frankfurt, Germany (**SMF**). Carapace length and width (in mm) of type specimens follow locality and collection information.

## Molecular techniques

Specimens and GenBank sequences used in the molecular analyses are listed in Table 1. In order to test the monophyly of *Enosteoides*, we included in the phylogenetic reconstruction specimens representing four other genera of Porcellanidae Haworth, 1825: The East Atlantic *Porcellana platycheles* (Pennant, 1777), *P. africana* Chace, 1956 and *Pisidia bluteli* (Risso, 1816), which are morphologically close to *Enosteoides* and the more distantly related *Petrolisthes armatus* (Gibbes, 1850), *Pachycheles monilifer* (Dana, 1852) and *P. pilosus* (Milne–Edwards, 1837). We used the galatheid *Galathea squamifera* Leach, 1814 as an outgroup.

DNA was extracted from muscle tissue of chelipeds or walking legs using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturers protocol for animal tissues. A 540 bp (base pair) fragment of the ribosomal 16S rDNA was amplified using primers 16Sar (5' CG-CCTGTTTATCAAAAACAT) and 16Sbr (5' CCGGTCT-GAACTCAGATCACGT) (Palumbi 1996) and trimmed to 524 bp in the alignment. A 680 bp fragment of cytochrome oxidase I (COI) was amplified using primers jgL-CO1490 (5' TITCIACIAAYCAYAARGAYATTGG) and jgHCO2198 (5' TAIACYTCIGGRTGICCRAARAAY-CA) (Geller et al. 2013) and trimmed to 644 bp in the alignment. A 450 bp of cytochrome b (Cytb) was amplified using primers UCYTB151F (5' TGTGGRCNA-CYGTWATYACTAA) and UCYTB270R (5' AANAG-GAARTAYCAYTCNGGYTG) (Merritt and Shi 1998) and trimmed to 361 bp in the alignment. A 370 bp fragment of Histone 3 (H3) was amplified using primers H3F (5' ATGGCTCGTACCAAGCAGACVGC) and H3R (5' ATATCCTTRGGCATRATRGTGAC) (Colgan et al. 1998) and trimmed to 338 bp in the alignment. Double-stranded amplifications were performed in 25 µl reactions containing 5.0 µl of GoTaq–Flexi™ DNA Taq

**Table 1.** Species of porcellanids included in the molecular analyses, sampling localities and collection data. Taxa are listed in alphabetical order. Collection data are followed by DNA codes. GenBank (GB) sequences of each gene used in the molecular analyses are shown with respective accession numbers. A species of galatheid squat lobster was used as outgroup (OG); See text for museum codes.

Species	n	Sampling localities	Collection Data and GB
<i>Enosteoides ornatus</i>	2	Indian Ocean, Arabian Sea, India, Goa, Anjuna Beach	Under rocks, 1.5 m (mid-tide), coll. S. Harkantra, A. Hiller, B. Werding, Nov. 2006; DNA-W2A
		Indian Ocean, Arabian Sea, India, Goa, Bogmolo Beach	Under rocks, 5 m (mid-tide), coll. S. Harkantra, A. Hiller, B. Werding, Nov 2006; DNA-W3A
<i>Enosteoides palauensis</i>	2	West Pacific Ocean, Vanuatu, Espiritu Santo Island	MNHN-IU200813587, Santo Marine Biodiversity Survey, 2006; Sta. VM53, coll. Tropical Deep Sea Benthos, 15 Sep 2006; DNA-POR46
		West Pacific Ocean, Vanuatu, Espiritu Santo Island	MNHN-IU200813588, Santo Marine Biodiversity Survey, 2006; Sta. FB61, coll. Tropical Deep Sea Benthos, 15 Sep 2006; DNA-POR91
<i>Enosteoides philippinensis</i>	2	West Pacific Ocean, Philippines, Puerto Princesa Bay	WPU-01; in mangrove forest with coral rubble, coll. R. Dolorosa, 14 Jun 2004; DNA-929A
		West Pacific Ocean, Philippines, Puerto Princesa Bay	WPU-02; in mangrove forest with coral rubble, coll. R. Dolorosa, 14 Jun 2004; DNA-929B
<i>Enosteoides spinosus</i> sp. nov.	4	Indian Ocean, West Australia, Kimberley District, Beagle Reef	WAM C54779, 1M, 13 m, coll. A. M. Hosie, 20 Dec 2011; DNA-s46
		Indian Ocean, West Australia, Kimberley District, Patricia Island	WAM C54781; 1M, 13 m, coll. A. M. Hosie, 22 Oct 2011; DNA-s47
		West Pacific Ocean, East Australia, Queensland District, Heron Island, NE side of Wistari Reef	LACM Acc. No. F.P.2.2003-43; mixed dead coral, 5 m, coll. R. Wetzer, N.L. Bruce, N.D. Pentcheff, 13 Apr 2003; DNA-s24
		West Pacific Ocean, East Australia, Queensland District, Heron Island, NE side of Wistari Reef	LACM Acc. No. F.P.2.2003-43; rubble from edge of spur, 3.5 m, coll. R. Wetzer, N.L. Bruce, N.D. Pentcheff, 13 Apr 2003; DNA-s25
<i>Enosteoides turkayi</i>	1	West Pacific Ocean, Philippines, Biking, Panglao Island	LKCNHM (ex ZRC) 2RC2016.0063, paratype female, stn T29, 77–84 m, mud, coll. PANGLAO 2004 Marine Biodiversity Project, 1 Jul 2004; DNA-8280
<i>Galathea squamifera</i> (OG)	1	East Atlantic Ocean, France, Saint Maló Bay, Saint Maló	Under rocks, intertidal, coll. A. Hiller, Sep. 2000; DNA-41
<i>Pachycheles biocellatus</i>	1	East Pacific Ocean, Ecuador, Salinas	GB MN715753 (16S), MN711998 (Cytb), MN712184 (H3); DNA-D29A
<i>Pachycheles monilifer</i>	1	West Atlantic Ocean, Venezuela, Cubagua Island	GB MN715754 (16S), MN711999, (Cytb), MN712185 (H3); DNA-q9A
<i>Pachycheles pilosus</i>	1	West Atlantic Ocean, U.S.A., Florida, Key Biscayne	GB MN715755 (16S), MN712000 (Cytb), MN712186 (H3); DNA-138B
<i>Petrolisthes armatus</i>	2	West Atlantic Ocean, U.S.A, Florida, Fort Pierce, intertidal	GB KY857020 (16S), KY857297 (COI), MN711994 (Cytb), MN712180 (H3); DNA-135
		East Pacific Ocean, Colombia, Nuquí	GB KY857243 (16S), KY857520 (COI), MN711997 (Cytb), MN712183 (H3); DNA-011A
<i>Pisidia bluteli</i>	2	East Atlantic Ocean, Balearic Sea, Spain, Catalonia, Costa Brava	Under rocks, intertidal, coll. B. Werding, Sep 2001; DNA-51
		East Atlantic Ocean, Adriatic Sea, Croatia, Rovinje	Under rocks, intertidal, coll. J. Medenbach, Sep 2001; DNA-1119
<i>Porcellana africana</i>	1	East Atlantic Ocean, Senegal, Ngor Island	Under rocks, 1 m, coll. P. Wirtz, Oct 2009; DNA-9165C
<i>Porcellana platycheles</i>	2	East Atlantic Ocean, France, Saint Maló Bay, Saint Maló	Under rocks, intertidal, coll. A. Hiller, Sep 2000; DNA-48
		East Atlantic Ocean, Strait of Gibraltar, Spain, Andalucía, Tarifa, Torre de la Peña	Under rocks, intertidal, coll. S. Sereda, Sep 2007; DNA-58

buffer (5×), 3.4 µl of dNTP mix (8 mM), 1.2 µl of each primer (10 µM), 2.4 µl of MgCl<sub>2</sub> (25 mM), 0.5 µl of Go-Taq-Flexi DNA Taq Polymerase (Promega), 1.5 µl of DNA template, 10.0 µl of ddH<sub>2</sub>O and 1–1.2 µl of DNA (4–10 ng/µl). Thermal cycling for all amplifications, except those performed for the COI fragment, consisted of an initial denaturation step at 96 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. An extension step at 72 °C for 5 min followed the last cycle. Amplifications of the COI fragment followed Geller et al. (2013).

PCR product amplifications were cleaned using the ExoSap-IT kit (USB Corporation). When more than one PCR product were amplified, the one of proper size was cut out of a 2% low-melt agarose gel after electrophoresis in 1× TAE buffer. Samples were incubated at 70 °C for 10 min and then, after adding 1.5 µl of GELase™ (Epicentre Biotechnologies), they were incubated at 45 °C for 5 hours. We used the BigDye™ Terminator version 3.1 Cycle Sequencing Kit to cycle-sequence clean PCR prod-

ucts in both directions and an Applied Biosystems3130 Genetic Analyzer to electrophorese resulting fragments.

The BIOEDIT Sequence Alignment Editor (Hall 1999) was used to view sequences and chromatograms and to trim primers. The programme CLUSTALW (Thompson et al. 1994), implemented in BIOEDIT, was used to view and align forward and reverse sequences and to aid in the alignment of the protein-coding DNA regions (COI, Cytb and H3). The ribosomal fragment (16S) was aligned with MAFFT version 7 (Katoh and Standley 2013) using the profile alignment method to align sequences according to levels of divergence. Sequences of the four DNA regions of each individual were concatenated, resulting in a 1,870 bp alignment. Redundant haplotypes were removed from alignments of each gene and of the concatenated set using TCS version 1.21 (Clement et al. 2000). For each unique-haplotype gene set, the best model of evolution was evaluated with the programme jModelTest2 (Darriba et al. 2012), according to the Akaike Information Criterion (AIC) (Akaike 1974). The concatenated data-set was

subjected to partitioned phylogenetic analyses applying the appropriate model to each partition. Maximum Likelihood (ML) reconstructions were generated with RAxML (Stamatakis 2014) using the options for rapid bootstrap and automatic halting. Support values of nodes were estimated from 200 bootstrap replicates. MrBayes version 3.2.7a (Ronquist et al. 2012) was used to conduct Bayesian reconstructions, using as priors the models found by jModelTest2, and run in four chains for 5 million steps, needed for the average standard deviation of split frequencies to fall below 0.01. Credibility values of nodes were estimated by sampling every 500<sup>th</sup> tree after a burn-in discard of 1,250 trees. Phylogenetic analyses were conducted on the CIPRES Science Gateway (Miller et al. 2010). Intra- and interspecific percent two-parameter distances (K2P; Kimura 1980) were estimated using MEGA version 7.0 (Kumar et al. 2016) for each gene separately and for the concatenated alignment, within *Enosteoides*, *Pisidia* and *Porcellana* and between Atlantic and Pacific individuals of *Petrolisthes armatus* (Gibbes). Gamma corrections, estimated by jModelTest2, were implemented in these calculations.

Distances between Atlantic and Pacific individuals of *Petrolisthes armatus* have been reported as the smallest between members of American geminate Porcellanidae (Hiller et al. 2006; Hiller and Lessios 2017) and have been assumed here as reference values of relatively recently diverged lines, separated during the final stages of the rising of the Central American Isthmus, approximately 3 MYA. Additionally, 16S and Cytb sequence divergence, estimated and dated by Hiller and Lessios (2019) between American geminate species of *Megalobrachium*, was also used as reference of recent events of speciation predating the complete emergence of the Isthmus.

## Results

### Systematic account

Family Porcellanidae Haworth, 1825.

#### *Enosteoides spinosus* sp. nov.

<https://zoobank.org/24604764-3E13-43BD-AB51-719E24927467>

Figs 1, 2a–e, 3

**Material examined. Holotype:** WAM C54778, ♂, 3.5 × 3.4 mm. INDIAN OCEAN, WESTERN AUSTRALIA, KIMBERLEY DISTRICT: Beagle Reef, 15°19.60'S, 123°32.15'E, Station 73/K11–T1, intertidal, 19 Oct 2011, A.M. Hosie leg.

**Paratypes:** INDIAN OCEAN, WESTERN AUSTRALIA, KIMBERLEY DISTRICT: White Island, 15°04.58'S, 124°20.40'E, Station 68/K11–T1, 14 m depth, 17 Oct 2011, A.M. Hosie leg., WAM C54777, 1♂, 4.2 × 4.1 mm; White Island, 15°04.58'S, 124°20.40'E, Station 68/K11–T1, 14 m depth, 17 Oct 2011, A.M. Hosie leg., WAM C77600, 1♀, 3.3 × 3.4 mm; Mavis Reef, 15°30.32'S, 123°36.50'E, Station 77/

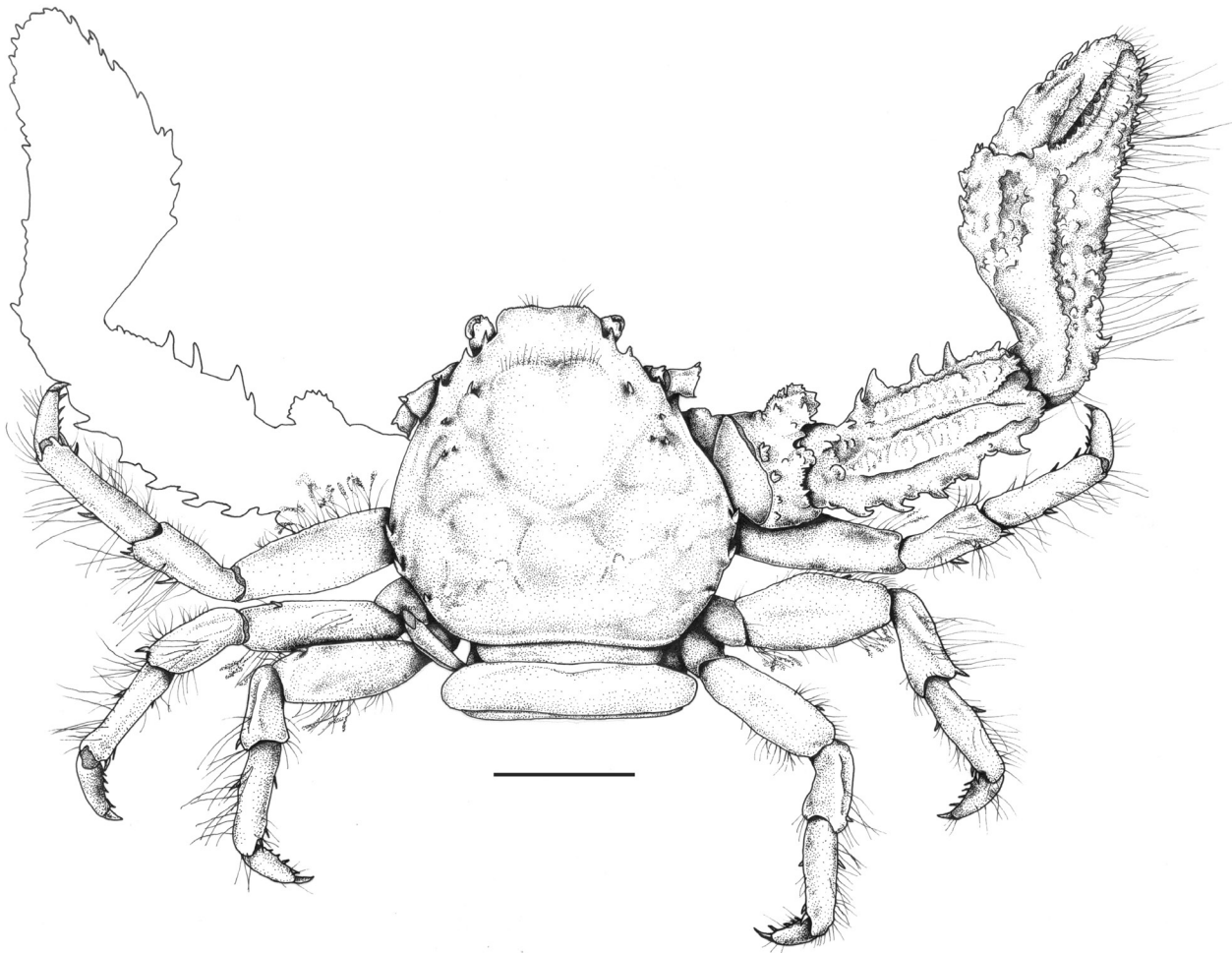
K11–T1, 12 m depth, 20 Oct 2011, A.M. Hosie leg., WAM C48628, 1♂, 3.2 × 3.0 mm; Jamieson Reef, 14°10.32'S, 125°32.95'E, Station 111/K12–T2, 4 m depth, 20 Oct 2011, A.M. Hosie leg., WAM C54780, 1♀, 2.2 × 2.2 mm; Patricia Island, 14°17.98'S, 125°22.43'E, Station 114/K12–T2, 13 m depth, 22 Oct 2011, A.M. Hosie leg., WAM C54781, 1♂, 3.0 × 2.6 mm; Beagle Reef, 15°21.13'S, 123°32.20'E, 75/K11–T1, 13 m depth, 20 Oct 2011, A.M. Hosie leg., WAM C54779, 1♂, 2.3 × 2.3 mm; Long Reef, 13°53.37'S, 125°44.56'E, Station 44/K10–T1, 12 m depth, 20 Oct 2010, A.M. Hosie leg., WAM C45725, 1♀, 4.6 × 4.8 mm; Beagle Reef, 15°19.60'S, 123°32.15'E, Station 73/K11–T1, Intertidal, 19 Oct 2011, A.M. Hosie leg., SMF58470 (ex-WAM C54778b), 1♀(ov), 3.6 × 3.7 mm; WEST PACIFIC OCEAN, AUSTRALIA: Queensland, Heron Island, NE side of Wistari Reef, 23°26.93'S, 151°53.41'E, rubble from edge of spur, 3.5 m depth, 11 Apr 2003, R. Wetzer, N.L. Bruce, N.D. Pentcheff leg., LACM CR–21354 (RW03.121), 1♀, 4.0 × 3.9 mm, 1♀(ov), 3.9 × 4.2 mm.

**Diagnosis.** Carapace hexagonal, broadest at mesobranchial level; dorsal surface strongly areolate, with spines on hepatic and epibranchial regions; acute spines on orbital, epibranchial and mesobranchial borders; front prominent, trilobed in frontal view, median lobe pronounced, lateral lobes each with a sharp terminal spine. Cheliped carpus about three times as long as wide, dorsal surface heavily eroded, with two broad longitudinal ridges, anterior margin straight with a row of three or more slender spines, posterior margin with five or six strong teeth; manus broad, depressed dorsoventrally, dorsal surface with irregular granules and a prominent crest on midline, inner border with strong, upright tooth; outer border concave, with row of sparse strong spines, dactylus with rounded median crest on dorsal surface and strong spines on inner border. Telson broad, composed of five plates.

**Description.** Carapace about as long as wide, broadest at mesobranchial level; dorsal surface strongly areolate, regions distinct and separated by deep grooves; proto-gastric crest blunt, but steep, with scattered, stiff setae. Front prominent, truncate in dorsal view, trilobed in frontal view, lateral lobes subparallel, each with a sharp, forwardly directed spine terminally, followed inwards by a smaller, rounded tooth; median lobe pronounced exceeding lateral lobes, outer borders with a row of small, acute spines, decreasing in size posteriorly; frontal margin with long, stiff setae.

Orbits relatively shallow, each with one prominent supraorbital spine and a smaller spine at outer orbital angle. Hepatic region with a strong, forwardly directed spine above elevation of median part; hepatic margin with a prominent spine. Epibranchial region with small spines on elevation. Mesobranchial border with three spines, anterior two spines strong, third spine smallest, located near metabranchial region.

Sidewalls broad, surface granulated and eroded, with transverse ridge, partly covered with long, feathered setae; anterior margin ventrally with a row of forwardly directed blunt spines.



**Figure 1.** *Enosteoides spinosus* sp. nov., female paratype WAM C45725, Indian Ocean, West Australia, Kimberly District. Left cheliped absent, symmetrically complemented in the figure. Scale bar: 2 mm.

Anterior margin of third thoracic sternite slightly convex, lateral lobes prominent, resembling forwardly directed horns. Anterior margin of fourth thoracic sternite concave.

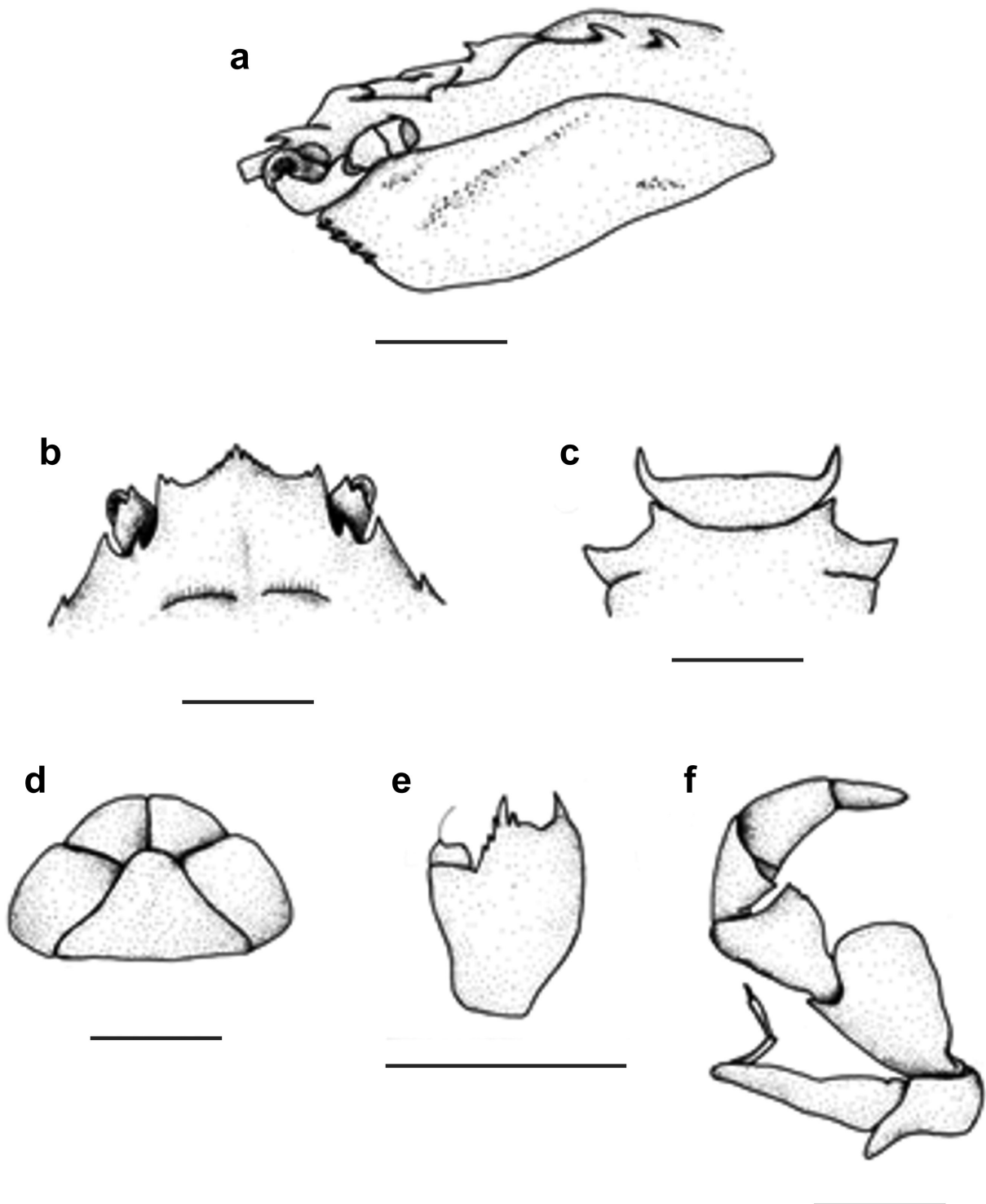
Eyes moderately large, ocular peduncles largely visible from dorsal side, distally with a distal, forwardly directed stiff seta, dorsal extension into cornea rounded.

Basal segment of antennular peduncle elongate, inner and outer lobes of anterior margin each with a terminal strong spine, inner lobe with a row of smaller spines on inner border. First segment of antennal peduncle strongly produced forwardly, broadly in contact with orbital margin, anterior margin bent upwards with a bifurcated, upwardly directed lobe; second to fourth segments movable, second segment short, with small spine at posterior distal end; third segment elongated with strong anterodistal spine; fourth segment rounded with small anterodistal spine. Antennal flagellum about 2.5 times as long as carapace, articulations thickened distally, bearing some stiff setae.

Ischium of third maxilliped broad, rounded distally; merus triangular, slightly concave distally; inner margin with some small spinules on distal edge; carpus with a triangular, spine-tipped projection on inner margin; propo-

dus broad at proximal end, narrower distally; dactylus elongate, rounded on distal margin.

Chelipeds subequal, slender, dorsal surface heavily eroded. Merus granulated with scattered, irregular, scale-like and acute protuberances on dorsal surface, anterodistal margin produced into a broad, rounded lobe with irregular protuberances and squarrose outer border. Carpus about three times as long as wide; dorsal surface with two broad longitudinal ridges, one running along mid-line, ending distally in a serrated lobe; another ridge along anterior border, separated from median ridge by a deep, steep groove; anterior margin straight, with row of three or more slender, distally somewhat curved spines of different size; posterior margin slightly convex, separated from dorsomedian ridge by a steep slope, bordered with five or six massive, distally curved teeth. Manus broad, depressed dorsoventrally, outer border concave; surface of propodus with large, irregular granules and a prominent, granulated longitudinal crest; outer border concave on median part, with a row of sparse, massive spines bearing long, simple setae. Dactylus with rounded median crest on dorsal surface and a row of massive spines on outer border.



**Figure 2.** *Enosteoides spinosus* sp. nov., female paratype WAM C45725, Indian Ocean, West Australia, Kimberly District. **a.** Left lateral view; **b.** Front, anterior dorsal view; **c.** Third and fourth thoracic sternites, ventral view; **d.** Telson, ventral view; **e.** Basal segment of left antennular peduncle, ventral view; **f.** Third maxilliped, ventral view. Scale bar: 1.0 mm.

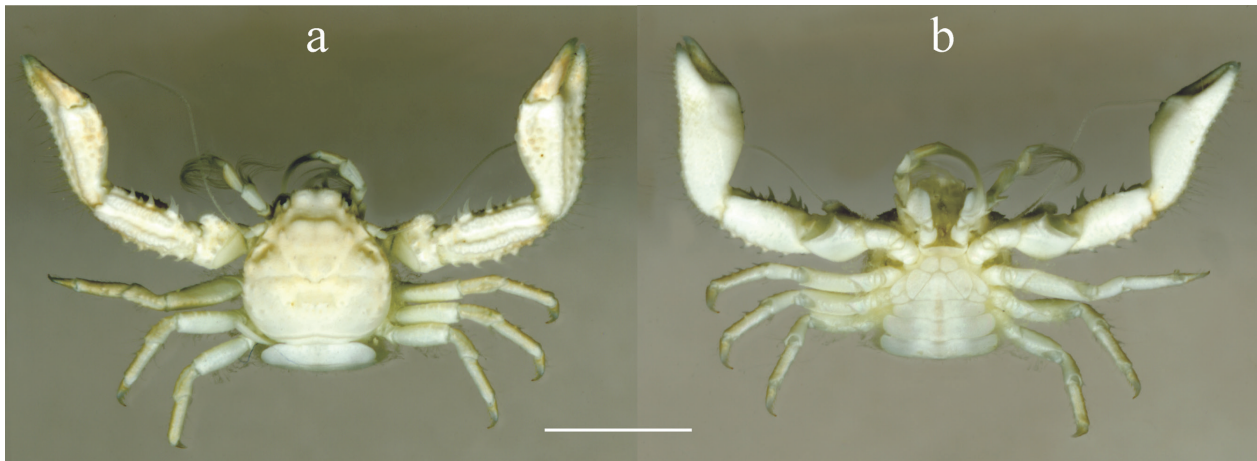
Merus of walking legs smooth with scattered, simple and feathered setae; upper border with an acute spine near distal end, additional spines sometimes present. Carpus with longitudinal depression and some stiff setae on upper side, with a strong spine; additional spines sometimes present on median part. Propodus slender, dorsal margin with one to three spines on different positions. Dactylus with four movable spines ventrally.

Telson broad, composed of five plates.

**Etymology.** The specific name *spinosus* refers to the extremely spiny appearance of the new species.

**Distribution.** *Enosteoides spinosus* sp. nov. has been so far reported from the Australian coasts of the Kimberly and Queensland Districts.

**Ecology.** The species was found in the intertidal region to a depth of 14 m, in patchy reef structures with inver-



**Figure 3.** *Enosteoides spinosus* sp. nov., female paratype LACM CR-21354, habitus, Pacific Ocean, East Australia, Queensland, Heron Island. **a.** Dorsal view; **b.** Ventral view. Scale bar: 4.0 mm.

tebrates such as sponges, hydroids, hard and soft corals and in areas with coral rubble, coarse sediment and a fine dusting of silt.

**Colouration.** The specimens from Kimberly had been recently preserved at the time of examination and colouration was greyish-brown.

**Remarks.** The new species gives a first impression of being morphologically distant from the other species of *Enosteoides* as currently defined, mainly due to the excess of sharp spines ornamenting the carapace and chelipeds. The new species is morphologically closer to *E. ornatus* than to any other species in the genus. Common characters to the two species are the spiny basal article of the antennular peduncle, the distinct spines on supra-orbital, hepatic and branchial regions and the spiny or tuberculate surface of the outer half of the palm of the chelipeds. The two main diagnostic characters of the new species is the telson, which is composed of five plates, instead of seven, a condition present in all other congeneric species and the proximal margin of the carpus, which bears sharp teeth in the new species, while it bears small denticles in *E. ornatus*.

## Molecular phylogeny and genetic distances

The topologies of the phylogenetic trees of *Enosteoides* and other porcellanid taxa produced by Maximum Likelihood (ML) and Bayesian Inference (BI), based on concatenated sequences of three mitochondrial and one nuclear gene, were congruent. The consensus tree (Fig. 4) shows nodes supported by values larger than 80% bootstrap iterations (ML) and posterior probabilities (BI). Nodes with lower support values were collapsed. The phylogeny shows three main clades: clade A containing American species of *Petrolisthes* and *Pachycheles*, clade B encompassing *Enosteoides ornatus* and *E. spinosus* sp. nov. and clade C gathering *Enosteoides palauensis*, *E. turkayi*, *E. philippinensis* and the species of *Porcellana* and *Pisidia* included in these analyses. The inclusion of *Pisidia bluteli* and of *Porcellana platycheles* and

*P. africana* in clade C (subclades C2 and C3, respectively) and the molecular divergence between this clade and clade B confirm that *Enosteoides* is not monophyletic. Since the type species of the genus, *E. ornatus*, is included in clade B together with *E. spinosus* sp. nov., all other species of *Enosteoides*, included in subclade C1, warrant their own generic status.

Table 2 lists mean percent two-parameter distances (K2P) estimated between and within species of *Enosteoides*, *Pisidia* and *Porcellana* and between Atlantic and Pacific individuals of *Petrolisthes armatus*, for each mitochondrial gene fragment (16S, COI and Cytb) and for the concatenated set (Conc). Distances between American geminate species of *Megalobrachium*, published by Hiller and Lessios (2019), are also listed in Table 2 and were also used as reference of molecular lines recently diverging in allopatry as the barrier comprised by the Central American Isthmus gradually finished emerging. Given that Hiller and Lessios (2019) published COI distances, based on a different fragment of this gene, we refer to 16S and Cytb comparisons only.

The smallest concatenated distances between Atlantic and Pacific individuals of *Petrolisthes armatus* are close to 3% and those based on 16S and Cytb sequences are around 2% and 5%, respectively. Interspecific concatenated distances within *Enosteoides* are remarkably large, with the smallest values (around 10–13%) corresponding to comparisons between *E. palauensis*, *E. philippinensis* and *E. turkayi* (Clade C1). Distances between these species, based on the 16S and Cytb fragments are, respectively, almost three and two times larger than those estimated between American geminates of *Megalobrachium*.

Concatenated distances between *E. spinosus* sp. nov. and *E. ornatus* show divergence close to 18% and those estimated between these two species and the rest of *Enosteoides* range between 23% and 26%. Such large distances, along with the topology of the phylogeny depicting independent clades, one conformed by the new species and *E. ornatus* (Clade B) and the other by the other species of *Enosteoides* (Clade C1), confirm that the genus is not monophyletic.

**Table 2.** Mean percent Kimura two-parameter distances within *Enosteoides* and between sister taxa of other porcellanid genera. Distances were estimated for each mitochondrial gene fragment (16S, COI and Cytb) and for the concatenated set (Conc) of mitochondrial and nuclear genes (H3) and are listed in ascending order of divergence. EM = East Mediterranean; EP = East Pacific; G = Gibraltar; WInd = West India; K = Kimberley District, Australia; NF = Northern France; Phil = Philippines; Q = Queensland District, Australia; S = Senegal; Vanu = Vanuatu; WA = West Atlantic; WM = West Mediterranean; NA = non-applicable because no COI sequences of the fragment used in the present analyses are available (see text).

Species	16S	COI	Cytb	Conc
<b>Between species</b>				
<i>Porcellana platycheles</i> (NF+G)– <i>P. africana</i> (S)	1.32	6.20	3.31	2.21
<i>Enosteoides palauensis</i> – <i>E. philippinensis</i>	8.77	12.44	21.06	10.37
<i>Enosteoides turkayi</i> – <i>E. philippinensis</i>	8.90	12.24	28.45	11.51
<i>Enosteoides palauensis</i> – <i>E. turkayi</i>	11.34	16.58	27.66	13.51
<i>Enosteoides ornatus</i> – <i>E. spinosus</i> sp. nov.	17.15	23.37	30.65	18.44
<i>Enosteoides philippinensis</i> – <i>E. spinosus</i> sp. nov.	24.78	24.57	36.05	23.50
<i>Enosteoides philippinensis</i> – <i>E. ornatus</i>	26.86	24.26	37.84	23.98
<i>Enosteoides turkayi</i> – <i>E. ornatus</i>	31.53	25.51	35.91	25.07
<i>Enosteoides turkayi</i> – <i>E. spinosus</i> sp. nov.	28.95	26.37	39.80	25.67
<i>Enosteoides palauensis</i> – <i>E. ornatus</i>	31.67	26.54	38.16	25.75
<i>Enosteoides palauensis</i> – <i>E. spinosus</i> sp. nov.	32.22	26.00	40.08	26.14
<b>Between geminate species</b>				
<i>Petrolisthes armatus</i> (WA)–(EP)	1.74	5.29	4.41	3.21
<i>Megalobrachium poeyi</i> (WA)– <i>M. pacificum</i> (EP)	3.41	NA	13.65	NA
<i>Megalobrachium mortenseni</i> (WA)– <i>M. lemaitrei</i> (EP)	4.21	NA	13.71	NA
<i>Megalobrachium roseum</i> (WA)– <i>M. festai</i> (EP)	5.27	NA	15.58	NA
<b>Within species</b>				
<i>Porcellana platycheles</i> (NF)–(G)	0.20	0.64	0.34	0.34
<i>Enosteoides ornatus</i> (WInd)	0.40	0.79	1.41	0.66
<i>Enosteoides philippinensis</i> (Phil)	0.00	0.67	2.88	0.78
<i>Enosteoides palauensis</i> (Vanu)	0.60	1.15	1.99	0.95
<i>Enosteoides spinosus</i> sp. nov. (K)–(Q)	0.00	2.93	2.45	1.31
<i>Pisidia bluteli</i> (WM)–(EM)	0.21	3.24	6.49	2.29

Although few specimens of the East Atlantic species of *Porcellana* and *Pisidia* were included in our analyses, comparisons of their concatenated distances serve as reference of relatively recent speciation events. Individuals of *Porcellana platycheles* from the North and the Mediterranean Seas differ from *P. africana* from Senegal by distances close to 2%, which are smaller than those found between the Western Atlantic and Eastern Pacific populations of *Petrolisthes armatus*. The concatenated distance estimated between the two Australian populations of *Enosteoides spinosus* sp. nov. from distant localities on the northeast and northwest coasts of Australia averaged at around 1.3%. This value is smaller than the transisthmian distance of *P. armatus* and the distance estimated between individuals of *Pisidia bluteli* from opposite coasts of the Mediterranean (approximately 2.3%). However, the distance within *Enosteoides spinosus* sp. nov. is larger than that found within *E. palauensis* (approximately 1%) and *E. philippinensis* (approximately 0.8%), suggesting some level of restriction of gene flow between the Kimberley and Queensland regions.

## Discussion

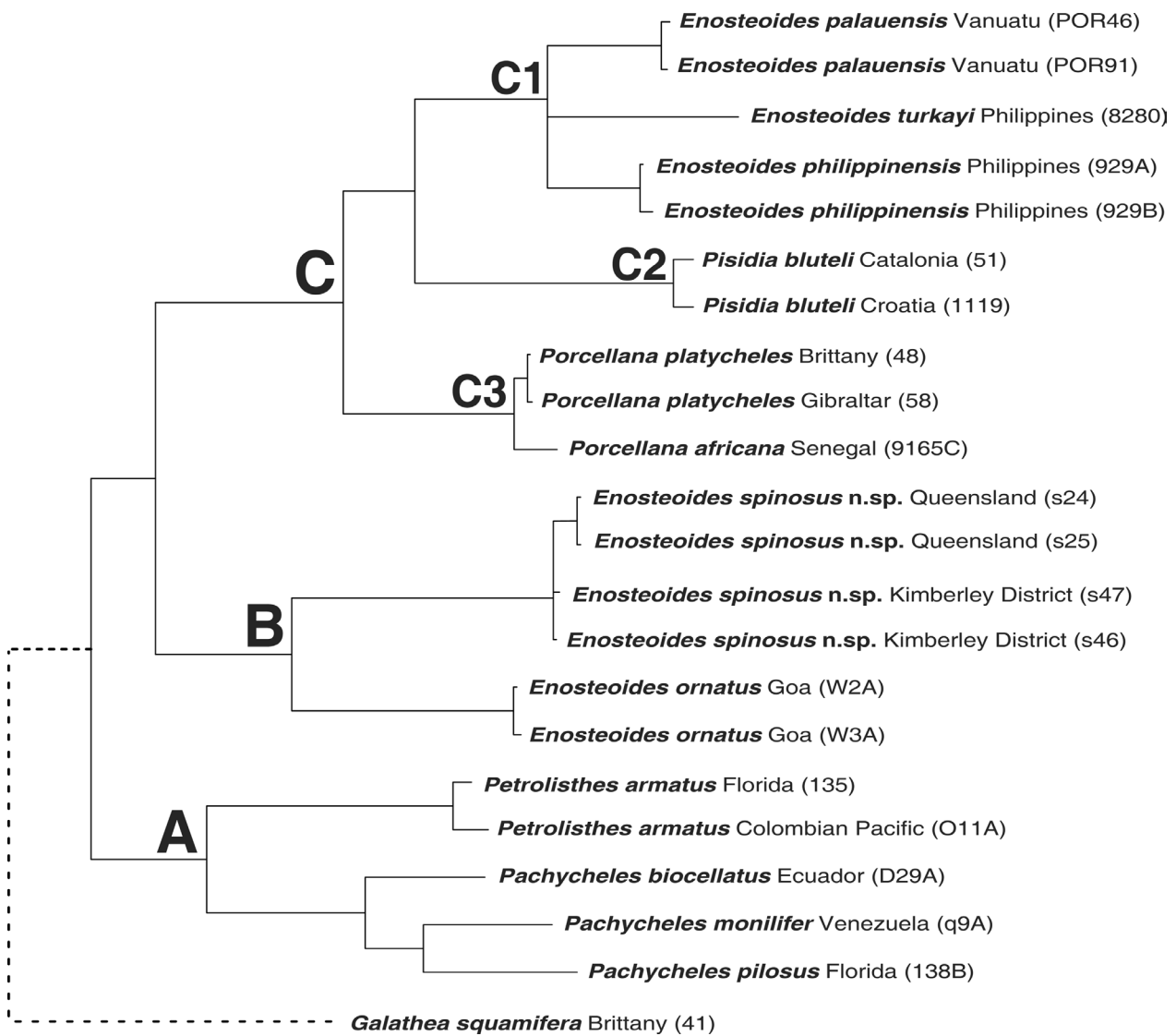
Our phylogenetic reconstructions of *Enosteoides*, based on three mitochondrial and one nuclear gene, depict two distantly-related lines independently leading to similar diagnostic morphologies: one line (clade B in Fig. 4), containing the type species *E. ornatus* and *E. spinosus* sp. nov. and another line (clade C1) encompassing the rest of species of *Enosteoides*. Although we could not include *E. lobatus* and *E. melissa* in this study, we expect these species to join the morphologically homogeneous group comprised by *E. palauensis*, *E. turkayi* and *E. philippinensis* (as defined by Osawa 2009, 2016). Our results justify retention of *E. ornatus* and *E. spinosus* sp. nov. in *Enosteoides* and a future designation of a new genus to contain all other species.

Our results rely on few samples of each species and, therefore, our phylogeographic deductions should be taken with caution and be confirmed or rejected in a future study including larger samples from different populations. The large interspecific genetic distances within each of the independent clades of *Enosteoides* suggest either ancient speciation events probably followed by high rates of extinction or a low rate of speciation within these evolutionary lines. Our reference to small interspecific genetic distances and to relatively-recent dates of divergence in other genera relies on values estimated between the extant American transisthmian *Petrolisthes armatus* and the geminate pairs of *Megalobrachium*. The lowest divergence values between geminates of *Megalobrachium* date from the late Miocene (approximately 8.9 million years ago–MYA) to the late Pliocene (circa 3 MYA), when the Isthmus of Panama was completed (Hiller and Lessios 2019 and references herein). Due to a limited sample size of *Enosteoides*, we have not placed dates of divergence in our phylogeny. Distances estimated between *Enosteoides ornatus* and *E. spinosus* sp. nov. suggest an older speciation event, as early as the mid–Miocene, over 12 MYA.

Relatively-large intraspecific genetic distances between *E. spinosus* sp. nov. from the northeast and northwest coast of Australia provide a first glance into a possible phylogeographic break along the coastline separating the Kimberley and Queensland regions, a geographic distance of over 5,000 km. Convolutional patterns of water circulation between the Indian and Pacific Oceans (Gordon 2005) may constitute a contemporary barrier restricting larval dispersal in the Indo–Australian Archipelago (Barber et al. 2006).

Despite low sample size, comparisons within the East Atlantic species of *Porcellana* and *Pisidia* included in this study, allow predictions of recent speciation events and phylogeographic breaks. The highly similar *Porcellana platycheles* and *P. africana* were first designated by Chace (1956) as two subspecies, *P. platycheles platycheles* from the European Atlantic coast and the Mediterranean Sea, with an extra-limital distribution in the Canary Islands and *P. platycheles africana*, restricted to the East African coast, from Western Sahara to Senegal. Our results confirm those published by Griffiths et al. (2018), who based on morphological and molecular data, validated the African variant





**Figure 4.** Phylogeny of concatenated mitochondrial (16S, COI and Cytb) and nuclear (H3) haplotypes of *Enosteoides* illustrating the consensus tree inferred by Maximum Likelihood (ML) and Bayesian (BA) analyses. Clades with < 80% bootstrap support (ML reconstruction) or posterior probability (BA reconstruction) were collapsed. Tip labels show names of species, sampling locality and DNA code.

as a separate species, *Porcellana africana*. Concatenated distances between these two species are smaller than those found between the American *Petrolisthes armatus*, suggesting a Late Pliocene (< 2.5 MYA) disruption of gene flow between the North Atlantic and Mediterranean populations and those on the southward African coast.

Relatively high intraspecific divergence within *Pisidia bluteli* from opposite coasts of the Mediterranean is indicative of either isolation by distance or the presence of a species complex.

### Data availability statement

DNA sequences are available in GenBank with accession numbers ON521708–ON521724 (for 16S rDNA), ON521170–ON521189 (for COI), ON548209–ON548225 (for Cytb) and ON548226–ON548242 (for H3). Input files used in analyses: Dryad <https://doi.org/10.5061/dryad.ksn02v77q>.

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