



## Re-description of *Orchestia stephensi* Cecchini, 1928: designation of neotype and senior synonym to *Orchestia constricta* A. Costa, 1853 (Crustacea: Amphipoda: Talitridae) by Reversal of Precedence

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### Abstract

The beach flea *Orchestia stephensi* was originally described by Cecchini (1928), and successively by Karaman (1973). The description of this species will be herein revised by focusing on the variation of the gnathopod 2 in males, as detected during its growth period. An analysis of DNA Barcoding was performed to support the assignment of the taxonomic species to five morphotypes. As the type specimen has not yet been designated, a neotype is assigned. The name of the species is here presented as a valid name as it satisfies the requirements of a Reversal of the Principle of Priority: *Orchestia stephensi* takes precedence over the objective synonym *Orchestia constricta* A. Costa, 1853, in accordance with Article 23.9.2. of the International Code of Zoological Nomenclature. *Orchestia stephensi* Cecchini, 1928 becomes *nomen protectum*, and *Orchestia constricta* *nomen oblitum*. The results presented in this paper also support the status of *Orchestia stephensi* as a Mediterranean endemic species, thereby rejecting previous Atlantic records. The synonymies for *O. stephensi* are revised accordingly.

**Key words:** Amphipoda, Talitridae, *Orchestia stephensi*, marine beach flea, DNA Barcoding

### Introduction

A knowledge of marine talitrid amphipods is important for ecological (Davolos *et al.* 2005; Fanini *et al.* 2005; Deidun *et al.* 2007; Ugolini *et al.* 2008) and biodiversity monitoring studies (Lo Brutto *et al.* 2013), and the correct identification at the species level is essential for conservation strategies.

Historically, the talitrid species have been placed into two genera: *Orchestia* Leach, 1814 and *Talorchestia* Dana, 1853. The genus *Orchestia* was delimited as a supralittoral Atlantic-Mediterranean group (Bousfield 1982), which was arranged into approximately twenty species from different habitats.

Five *Orchestia* species inhabit the supralittoral of the Mediterranean Sea: *O. montagui* Audouin, 1826 living in the banquette of *Posidonia oceanica* (L.) Delile and on mixed sand and coarse gravel beaches (Lowry & Fanini 2013); *O. xyli* Lowry & Fanini, 2013 living on mixed sand, coarse gravel and boulder stone beaches (Lowry & Fanini 2013); *O. mediterranea* A. Costa, 1853 living under *P. oceanica* debris, far from the sea (Vecchi 1931); *O. gammarellus* (Pallas, 1766) living on sea shores, occasionally far from the sea (Chevreux 1911); and *O. stephensi* Cecchini, 1928 living on the seashore under stone, under *P. oceanica* and in the river mouth (Karaman 1973). The common Mediterranean assemblages are usually composed of three syntopic *Orchestia* species: *O. stephensi*, *O. montagui* and *O. mediterranea*; and *O. stephensi* seems to be dominant (Prato *et al.* 1995).

*Orchestia stephensi* has been described by Cecchini (1928), and the description revised by Karaman (1973). Both descriptions used specimens larger than 18 mm and, although the peraeopod 7 displayed the same shape, the gnathopod 2 in males had two different shapes. The gnathopod 2 shape described by Cecchini (1928) was the only one to be reported in the Manual by Ruffo *et al.* (1993), and commonly used for identifying species throughout the field of Mediterranean studies. Although quoted in various articles (e.g. Lowry & Fanini 2013), the work by Karaman (1973) is not widely used in the identification of Mediterranean species. As a result, there is a paucity of

information relating to the distribution of conspecifics. These two shapes have been recorded in a population sampled in the central Mediterranean (i.e. this paper) and they are referred to as the "Cecchini-shape", corresponding to the herein called Morphotype IV, and the "Karaman-shape" to Morphotype V.

A lack of previous descriptions of the mouthparts of *O. stephensi* led us to revise the description of the species, to include individuals whose lengths were from 11 to 21 mm. A morphological, taxonomic approach is integrated with DNA barcoding as a molecular tool. Within the population under investigation in this paper, a variation with growth in the gnathopod 2 male will be demonstrated and the molecular dataset which is herein presented will attempt to support species identification.

Analysing the growth stages of *O. stephensi*, a second species *O. constricta* A. Costa, 1853 was identified as being conspecific. The name *O. constricta* is not widely applied within the literature. In order to stabilize the Mediterranean talitrid taxonomy, *O. stephensi* is here presented as an accepted name as it satisfies the requirements of a Reversal of the Principle of Priority. *Orchestia stephensi* takes precedence over the objective synonym *O. constricta*, in accordance with Article 23.9.2. of the International Code of Zoological Nomenclature. *Orchestia stephensi* Cecchini, 1928 becomes *nomen protectum*, and the less circulated senior synonym *Orchestia constricta* A. Costa, 1853 is designated as *nomen oblitum*. The results presented in this paper also support the hypothesis of *O. stephensi* as a Mediterranean endemic species. Thus the previous records of *O. stephensi* from the Atlantic coast are rejected and the synonymy for the species is updated to reflect this hypothesis.



**FIGURE 1.** Sample site: banquette of *Posidonia oceanica* (L.) Delile, at the Stagnone of Marsala (western Sicily, southern Italy, central Mediterranean Sea).

## Materials and methods

**Sampling.** This study is based upon material collected from the banquette of *P. oceanica* (Figure 1) of the Stagnone

di Marsala lagoon, Sicily, southern Italy (37°55'03.83"N; 12°28'10.98"E) in May 2013. The specimens, which were collected in association with *O. montagui* and *O. mediterranea* in banquettes of *P. oceanica*, could be ascribed to *O. stephensi* Cecchini, 1928 (onward named Morphotype IV), others were ascribable to *O. stephensi* Karaman, 1973 (Morphotype V) and other specimens identified as *O. cf. stephensi*, the latter with an unusual shape of gnathopod 2 (Morphotype I, II and III) (Figure 2). The samples were collected using hand-nets and then carefully transferred into plastic containers and fixed in 95% ethanol. A neotype is preserved in the permanent archives of the Zoological Museum collections (University of Palermo, Italy) together with the organic DNA extracted, identifiable by the Voucher Number MZPA-AMPH-N\_0002.

**Biometric data and Iconography.** Fifty-three specimens were examined under a stereo-microscope and later selected for dissection. In order to ensure precise measurements, the specimens were placed on graph paper and photographed (FINEPIX S1800, FUJIFILM). The length of the specimens was measured from head to telson using ImageJ software (Rasband 2008); the flagellum articles of *antenna 2* were also counted as an estimate of growth throughout the moulting period. Differences between dry and wet specimens were observed. Some specimens were photographed in humid form and the photographs were then repeated after complete dehydration. A comparison of the measurements revealed that wet specimens were greater than dry measurements. It was, therefore, decided to proceed with wet specimens as these reporting measures were most similar to live individuals. Appendages of the dissected specimens were examined and illustrations produced using a Leica 4000B light microscope with *camera lucida*. All illustrations were drawn with pencil, then retraced with iron-gall ink and finally converted to digital form at 1200dpi resolution.

**Extraction of DNA and amplification of mtCOI gene.** Total genomic DNA extraction was performed on fixed specimens, after having dried them on paper using the Genomic DNA Extraction Kit Tissue (*RBC Bioscience*, Taiwan). A 630-bp fragment of the target mitochondrial (mt) cytochrome oxidase subunit I (COI) was amplified using the primer pairs LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer *et al.* 1994). PCR was performed in a 25 µL volume containing 1 × Incomplete NH<sub>4</sub> Reaction buffer, 4 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 2.5 U *Taq* polymerase (*Bioron GmbH*, Germany), 1 µM of each primer, 80–100 ng of DNA template.

Cycling conditions for PCR amplifications consisted of an initial 95°C denaturation step for 5 minutes followed by 35 cycles of 60 sec at 95°C, 60 sec at 46°C, and 60 sec at 72°C, with a final extension at 72°C for 8 min and a final cooling at 4°C. The resulting amplified DNA fragments were purified with the QIAquick PCR Purification Kit (*Qiagen*, Germany) and then sequenced with an *Applied Biosystems* (ABI) 3730xl DNA analysis. Sequences were deposited in GenBank with the Accession Numbers (A.N.) shown in Table 1.

**TABLE 1.** Details of COI reference sequences of *Orchestia* species used in the NJ tree.

Accession Number (A.N.)	Sampling locality	Reference
<i>Orchestia gammarellus</i>		
EU276207	Atlantic Ocean (NW Iceland)	Henzler & Ingólfsson 2007
EU276380	Atlantic Ocean (English Channel)	Henzler & Ingólfsson 2007
<i>Orchestia mediterranea</i>		
KR827689	Mediterranean Sea (Sicily, Italy)	This paper
<i>Orchestia montagui</i>		
JQ390313	Mediterranean Sea (Gozo, Malta)	Pavesi <i>et al.</i> 2012
JQ390319	Mediterranean Sea (Latium, Italy)	Pavesi <i>et al.</i> 2012
JQ390326	Mediterranean Sea (Apulia, Italy)	Pavesi <i>et al.</i> 2012
JQ390332	Mediterranean Sea (Sardinia, Italy)	Pavesi <i>et al.</i> 2012
KR827690	Mediterranean Sea (Sicily, Italy)	This paper
<i>Orchestia stephensi</i>		
JX094885	Mediterranean Sea (Tuscany, Italy)	Wildish <i>et al.</i> 2012
KR827687 (Morphotype I, III and V)	Mediterranean Sea (Sicily, Italy)	This paper
KR827688 (Morphotype II and IV)	Mediterranean Sea (Sicily, Italy)	This paper



Mitochondrial sequences of the voucher specimens of *O. stephenseni* were compared with sequences of the congeneric syntopic species which had been sampled and deposited in the GenBank (<http://www.ncbi.nlm.nih.gov>) database (see Table 1 for details). A reference sequence of *O. gammarellus* (Pallas 1766) was used as an outgroup. Sequence analyses were performed with MEGA version 6 (Tamura *et al.* 2013). Nucleotide sequences were aligned by the ClustalW model (Thompson *et al.* 1994) with default settings. Cluster analysis was performed using Kimura-2-Parameter distance (K2P; Kimura 1980) and Neighbour-Joining method (NJ; Saitou & Nei 1987).

**Terminology.** The general terminology of amphipod morphology follows that documented in Ruffo *ed.* (1993). The definition of setae and spines follows Watling (1989) which defines '*seta*' as an articulated, cuticular extension of any shape or size, and '*spine*' is a non-articulated cuticular extension which has a base generally narrower than the length of the structure. Different kinds of setae have been observed and reported in this revised description of *O. stephenseni*. Some have already been classified and clearly reviewed by Watling (1989) (i.e. '*Plumose seta*', '*Simple seta*', '*Tooth seta*'); unclassified setae include '*Bifid seta*', seta with a bifid apex, with two curved tips (Figure 3), and '*Peduncular spine-like seta*', a 'strong spine' on the hyalids peduncle of *Uropod 1*, previously observed by Bousfield & Hendrycks (2002).

**Abbreviations.** **A1**: first antenna (antennula). **A2**: second antenna (antenna). **Lbr**: upper lip (labrum). **Md**: mandible. **Lb**: lower lip (labium). **Mx1**: first maxilla (maxillula). **Mx2**: second maxilla (maxilla). **Mxp**: maxilliped. **Cx 1–7**: coxal plates of the first to the seventh peraeopod. **Gn1**: first gnathopod. **Gn2**: second gnathopod. **P3–P7**: third to seventh peraeopods. **Pl1–Pl3**: first to third pleopods. **Ep1–Ep3**: first to third epimeral plates. **U1–U3**: first to third uropods. **T**: telson.

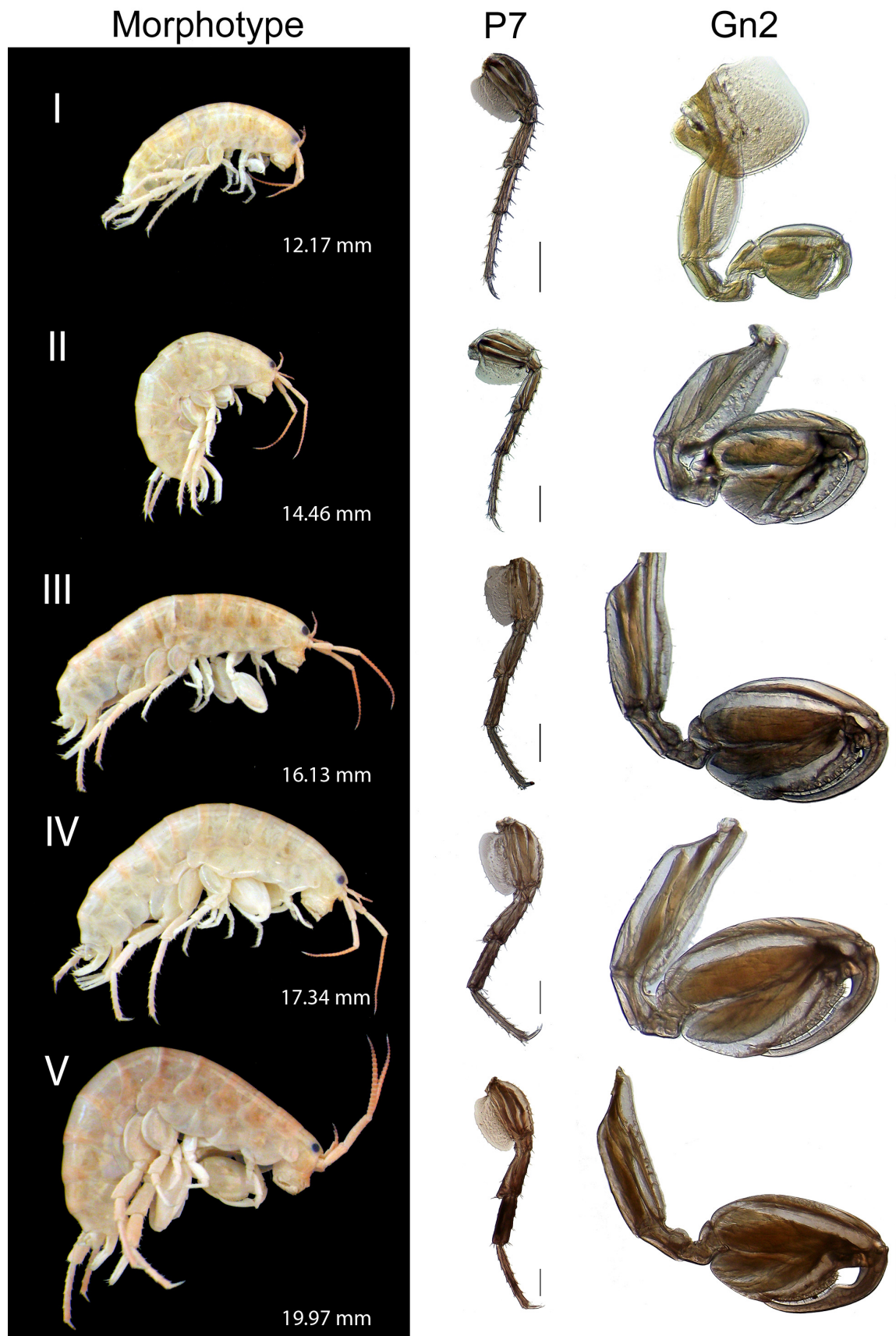
Acronym for Museums: **MZPA**, for Zoological Museum of the University of Palermo (Italy).

## Results

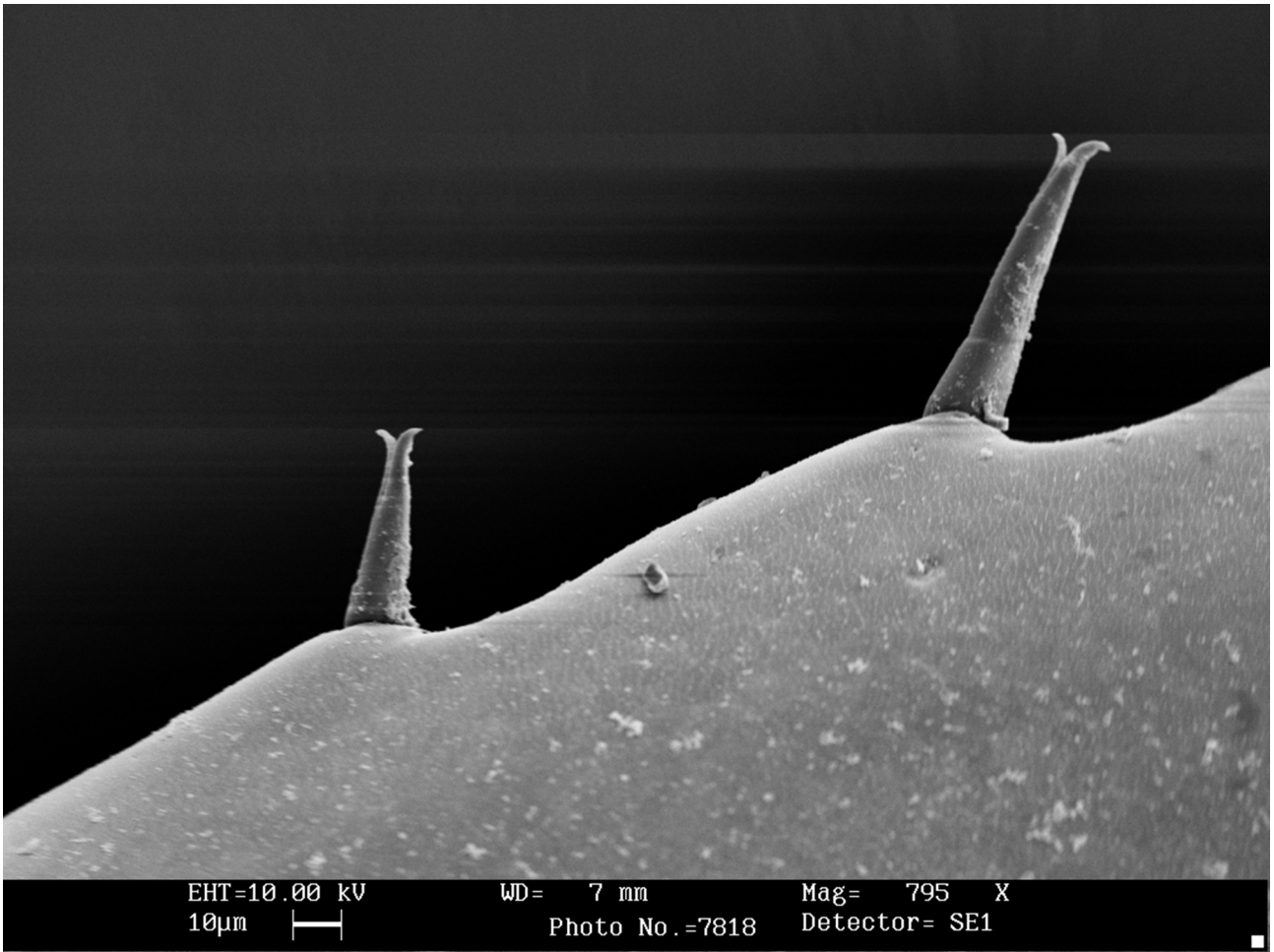
**Variation in shape.** Of a total of 53 male specimens examined, the development of *gnathopod 2* could be ascribed to five morphotypes, which were designated as: Morphotype I, Morphotype II, Morphotype III, Morphotype IV and Morphotype V (Figure 2). The specimens also displayed a very slight variation in the merus and carpus of the *peraeopod 7*: the merus and carpus were very slim in the smallest specimens whilst the merus and carpus were barely dilated in the largest specimens. Specimens also showed a variation in the shape of the '*cusps*' (*sensu* Bousfield & Hendrycks 2002) on the *coxa 2* posterior margin, which become more acute during growth. However, only the variation in the propodus and dactylus of *gnathopod 2* was significantly visible throughout the growth period. The five morphotypes corresponded to different body lengths. Morphotype I was associated to the smallest body length sampled and successive morphotypes were associated to larger specimens (Figure 2). Figure 4 shows the frequency of the five shapes in the population in relation to their body length.

In order to search for a correlation between body length and number of moults, the number of *antenna 2* flagellar articles were counted, as a discrete growth parameter. Indeed, the number of flagellar articles has often been expected to increase *x*-fold for each moult (Nair & Anger 1979). Unfortunately, no discrete size-class was discriminated (Table 2) and no association with one or more moult steps was observed. However, it cannot be excluded that the transition from one shape to another requires more than one moult. Body length varied from 11.12 to 21.31 mm and it was proportional to the number of flagellar articles of the *antenna 2*, i.e. varying from 17 to 25. Various specimens were asymmetrical in the number of flagellar articles of *antenna 2*; in these cases, we considered the highest number. A significant linear correlation between the number of flagellar articles of *antenna 2* and body-length was scored, with an  $R^2$  value of 0.7166 and  $p < 0.001$  (Figure 5). It is noteworthy to point out that a difference in body length was detected between dry and wet specimens. This measurement, performed in 95% ethanol and after complete drying, revealed differences in body-length for twenty-four specimens; a 'dry length' was found to be smaller than a 'wet length', by approximately an average of 12%.

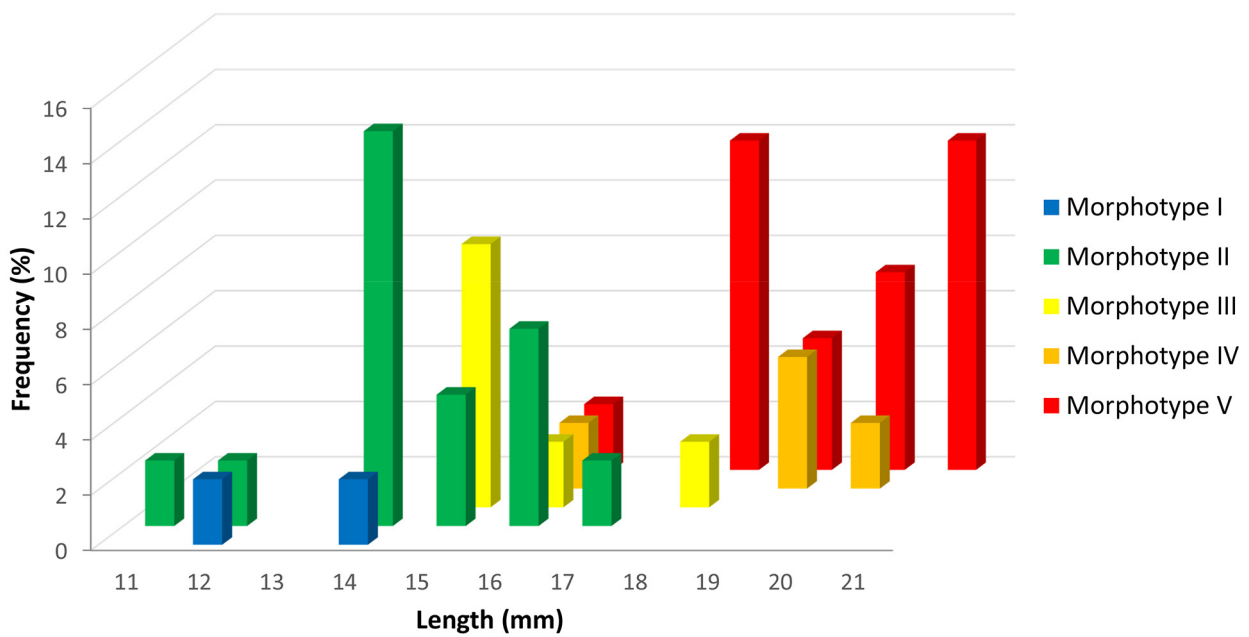
**Molecular analysis.** In order to support species identification, the mtCOI of five specimens, ascribable each to one of the five morphotypes, was sequenced. A total of 630 base pairs (bp) of COI was aligned and compared with congeneric reference species (Table 1). Intra-species K2P distances ranged from 0.0 % to 2.3 % for all COI barcodes, and the inter-species K2P distances ranged from 11.5 %, to 22.3 %, resulting in no overlap between the two levels of variation. The COI sequences of the Sicilian *O. stephenseni* specimens matched the only *O. stephenseni* reference sequence available (A.N. JX094885). Concurrently, the syntopic *O. montagui* matched the



**FIGURE 2.** The five *O. stephensi* morphotypes, named Morphotype I to V, and corresponding to the five gnathopod 2 shapes. The body-length for each photographed individual is reported. Scale bar = 1 mm.



**FIGURE 3.** *Orchestia stephensi* Cecchini, 1928. Example of *Bifid setae* on peraeopods (see text). SEM (Scanning Electron Microscope) photograph.



**FIGURE 4.** Frequency of Morphotypes along body-length (the length has been rounded to the nearest integer).

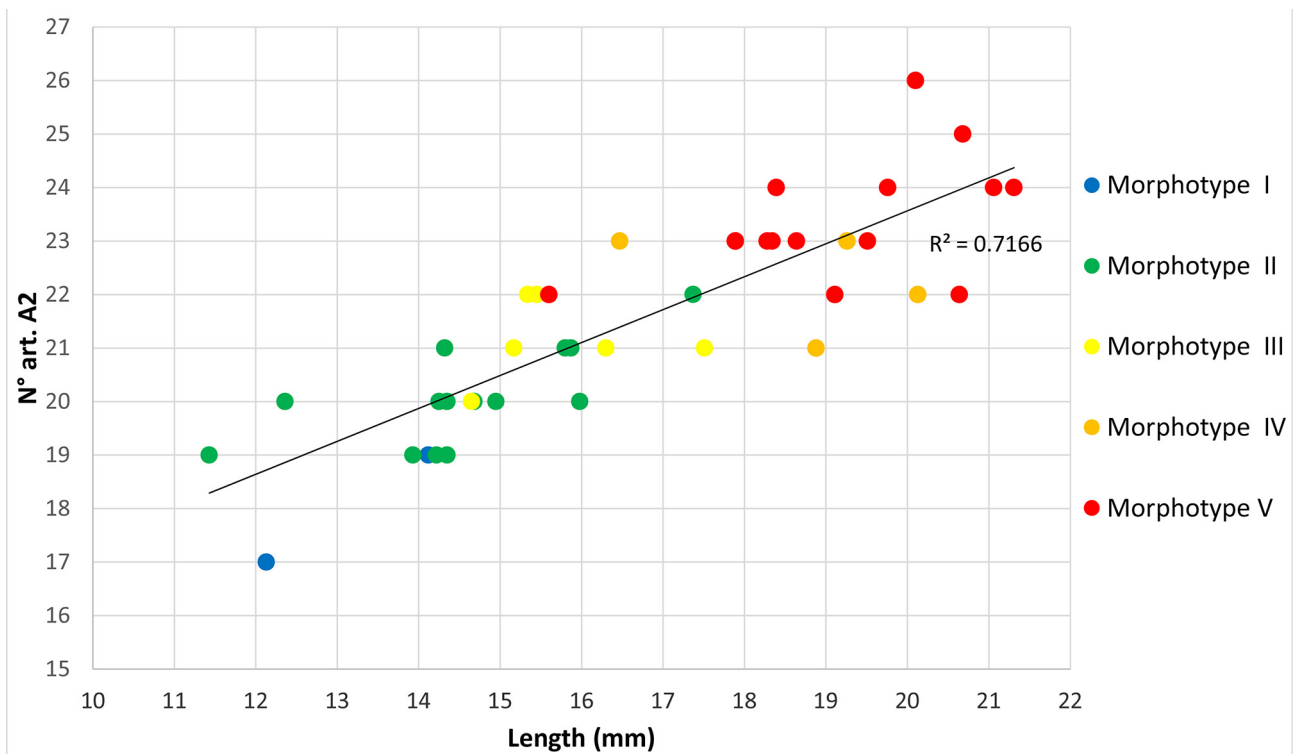


FIGURE 5. Linear regression between body length and number of *antenna 2* flagellar articles for each Morphotype.

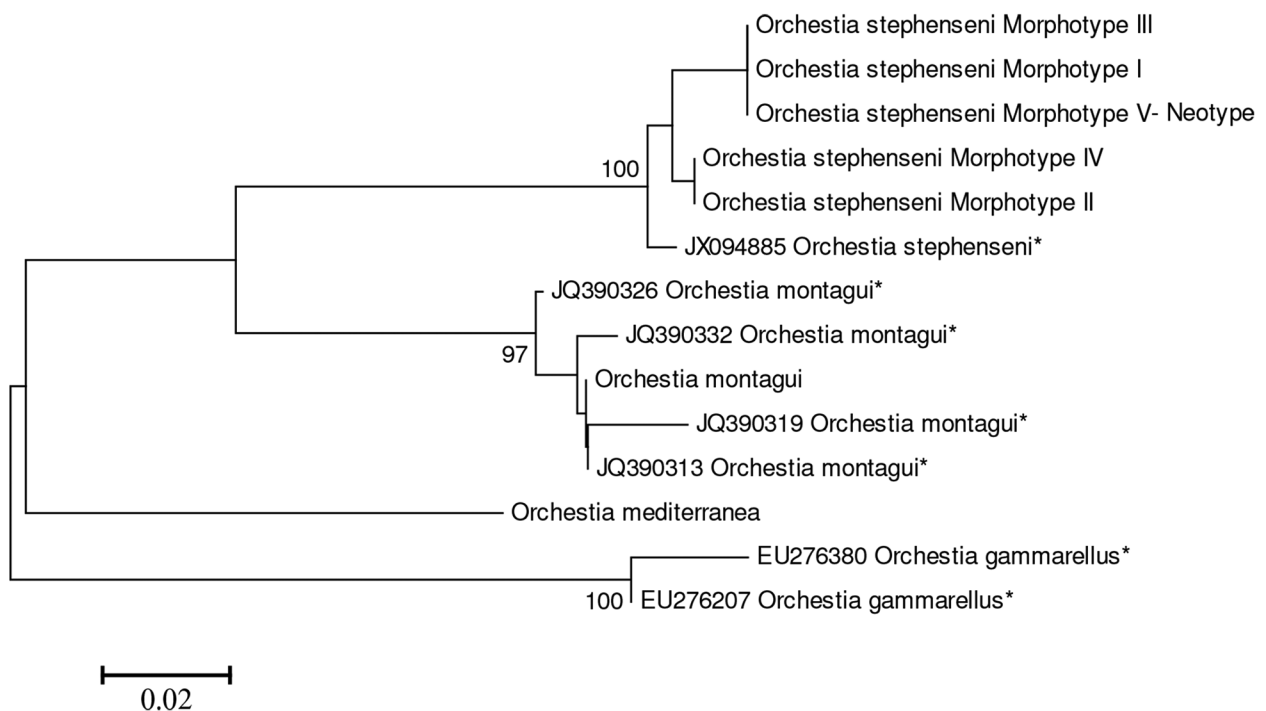


FIGURE 6. NJ tree constructed on the K2P model performed with 630-bp COI sequences, including sequences of the five *O. stephenseni* shapes, the Sicilian *O. montagui* and *O. mediterranea* samples, and sequences reference (\*) from GenBank (shown with the A.N.). The values allocated to the nodes were those calculated on 1,000 bootstrap replicates.

*O. montagui* sequences deposited in GenBank (Figure 6) while the first *O. mediterranea* sequence, here performed, set up its own separate clade (Figure 6). The NJ tree displayed the genetic cohesion of the *Orchestia* species sequenced in this research, supporting the hypothesis that the molecular discrimination approach is a powerful tool to be integrated into morphological description.

**TABLE 2.** Range of body length and number of *antenna 2* flagellar articles for the five Morphotypes.

	Body Length (mm)	N° A2 art.
Morphotype I	13.12 ± 1	18 ± 1
Morphotype II	14.56 ± 2.81	20.5 ± 1.5
Morphotype III	15.73 ± 1.77	21 ± 1
Morphotype IV	18.68 ± 1.45	22 ± 1
Morphotype V	19.15 ± 2.16	24 ± 2

## Re-description

### Suborder Senticaudata Lowry & Myers, 2013

#### Infraorder Talitrida Rafinesque, 1815

#### Superfamily Talitroidea Bulycheva, 1957

#### Family Talitridae Rafinesque, 1815

#### Genus *Orchestia* Leach, 1814

#### *Orchestia stephensi* Cecchini, 1928

(Figure 7, 8)

*Orchestia Stephensi* Cecchini, 1928: 7, pl. 2, fig. 3.—Cecchini 1929: 11.—Ruffo 1951: 1.

*Orchestia ghigii* Vecchi, 1929: 249, figs 1–5.—Maccagno 1939: 11.—Mateus & Mateus 1959: 44.

*Orchestia Ghigii*.—Ruffo 1937: 39.—Ruffo 1949: 323.

*Orchestia stephensi*.—G. Karaman 1970: 32.—G. Karaman 1973: 137, figs 1–3.—Koukouras & Russo 1991: 306, table 6.—Bellan-Santini 1993: 752, fig. 515 (key).—Prato, Pastore & Pavia 1995: 63, table 2.—Ruffo 1995: 43.—Stefanidou & Voultsiadou-Koukoura 1995: 603, table 1.—De Matthaëis, Ketmaier, Davolos & Cobolli 1999: 95 (genetics).—Ariani, Camassa & Wittman 2000: 7, 12.—De Matthaëis, Davolos, Cobolli & Ketmaier 2000: 1607 (table 1), 1611, table 4 (genetics).—Zavodnik & Kovačić 2000: 338.—Sezgin, Kocataş & Katağan 2001: 59, tables 2, 3.—Colombini, Chelazzi & Fallaci 2002: 863.—Davalos, Ketmaier, Cobolli & De Matthaëis 2002: 33 (genetics).—Fišer 2002: 38.—Davalos, Iannilli, De Matthaëis & Pietrangeli 2005: 72, fig. 4.—Deidun, Saliba & Schembri 2007: 456 (ecology).—Deidun & Schembri 2008: 19 (ecology).—Akbulut, Ustaoglu & Çelik 2009: 51, table 1.—Deidun, Saliba & Schembri 2009: 411 (ecology).—Zakhama-Sraieb, Sghaier & Charfi-Cheikhrouha 2009: 5, table 3.—Lucena-Moya, Abraín, Pardo, Hermida & Domínguez 2010: 7.—Sezgin & Aydemir Çil 2010: 9, table 1.—Grintsov 2011: 143 (morphology).—Jelassi, Khemaissia & Nasri-Ammar 2012: 383 (ecology).—Jelassi & Nasri-Ammar 2012: 437 (ecology).

*Orchestia constricta* Costa, A., 1857: 183 (type locality: Terra d'Otranto, Italy).—Heller 1866: 3.—Stebbing 1906: 542.

**Type material.** Neotype, male, 21.31 mm, MZPA-AMPH-N\_0002, Stagnone of Marsala, Sicily (37°55'03"N; 12°28'11"E), among, inside and around banquettes of *P. oceanica*, July 2013, mediolittoral, hand collected.

**Additional material examined.** Fifty-three males, MZPA-AMPH-S\_0002. Stagnone of Marsala, Sicily (37°55'03"N; 12°28'11"E), among, inside and around banquettes of *P. oceanica*, July 2013, mediolittoral, hand collected.

**Type locality.** Stagnone of Marsala, Sicily, 37°55'03"N; 12°28'11"E.

**Ecology.** Mixed sand/gravel, banquette and cobble beaches (slow-drying substrate *sensu* Pérès & Picard 1964).

**Description.** Based on neotype, male, 21.31 mm, Morphotype V, MZPA-AMPH-N\_0002.

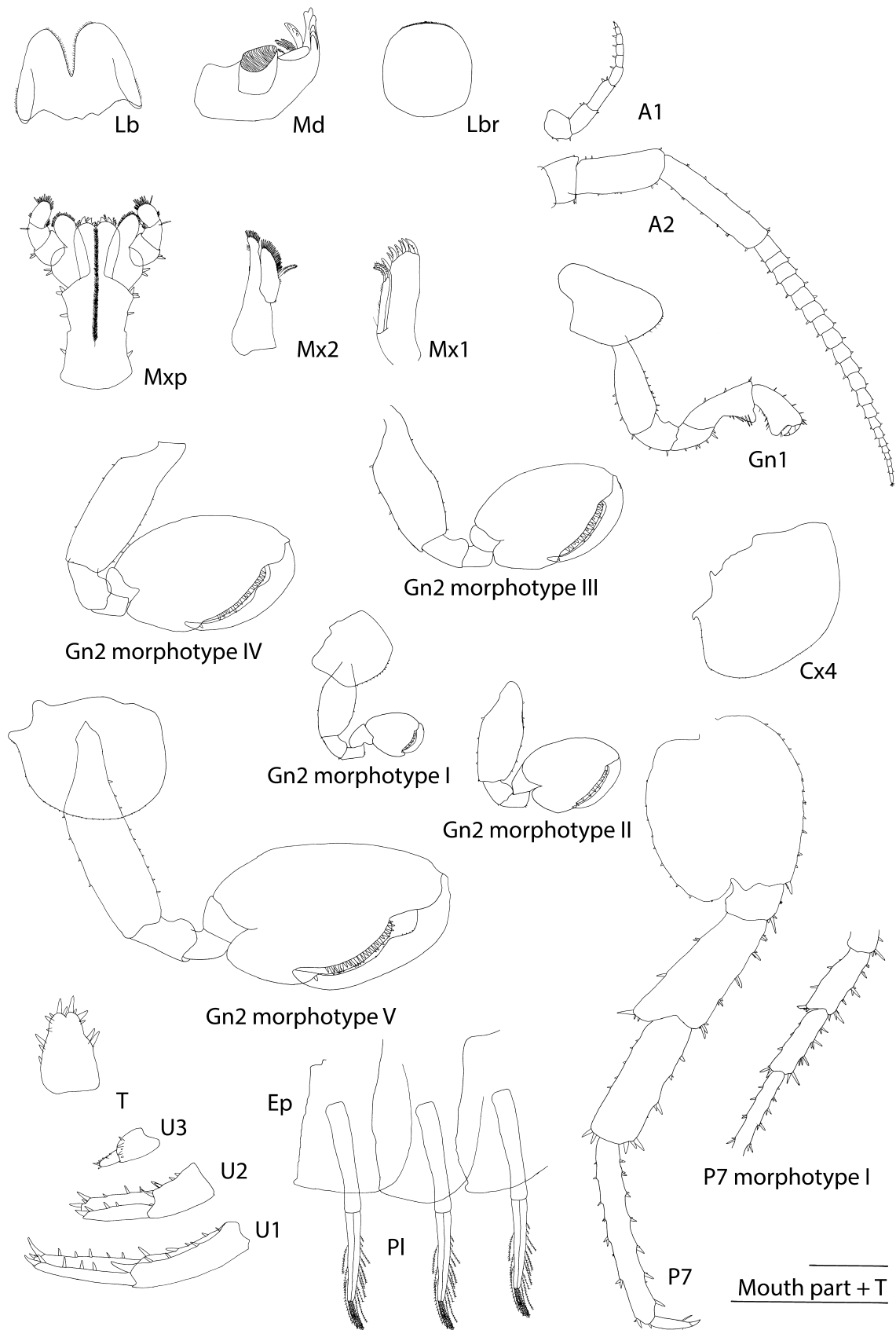
**Head** as long as peraeonite 1. Eyes present, small, circular. *Antenna 1* short, reaching peduncular article 4 of antenna 2; peduncle article 1 as long as wide, article 2 and 3, longer than wide, sub-equal in length; article 1 disto-ventral and disto-dorsal with one pairs of tooth setae, proximo-ventral with 2 setae in row; article 2 disto-ventral and disto-dorsal with one pair of tooth setae with middle 1 plumose setae, ventral margin with 2 setae; article 3 dorsal with one pairs of tooth setae with middle 1 plumose setae and disto-ventral with one pairs of tooth setae, dorsal margin with one pair of setae and ventral margin with 2 setae in row; flagellum articles 5–6 with final article cone-shaped; all articles with one pairs of tooth setae on disto-dorsal margin; last article with a group of simple setae on apex. *Antenna 2* long 1/4 of body size; peduncular articles narrow; peduncular article 5 approximately 1.5 times as long as article 4; peduncle article 3 with bifid setae on all distal margin; peduncle article 4 with sparse



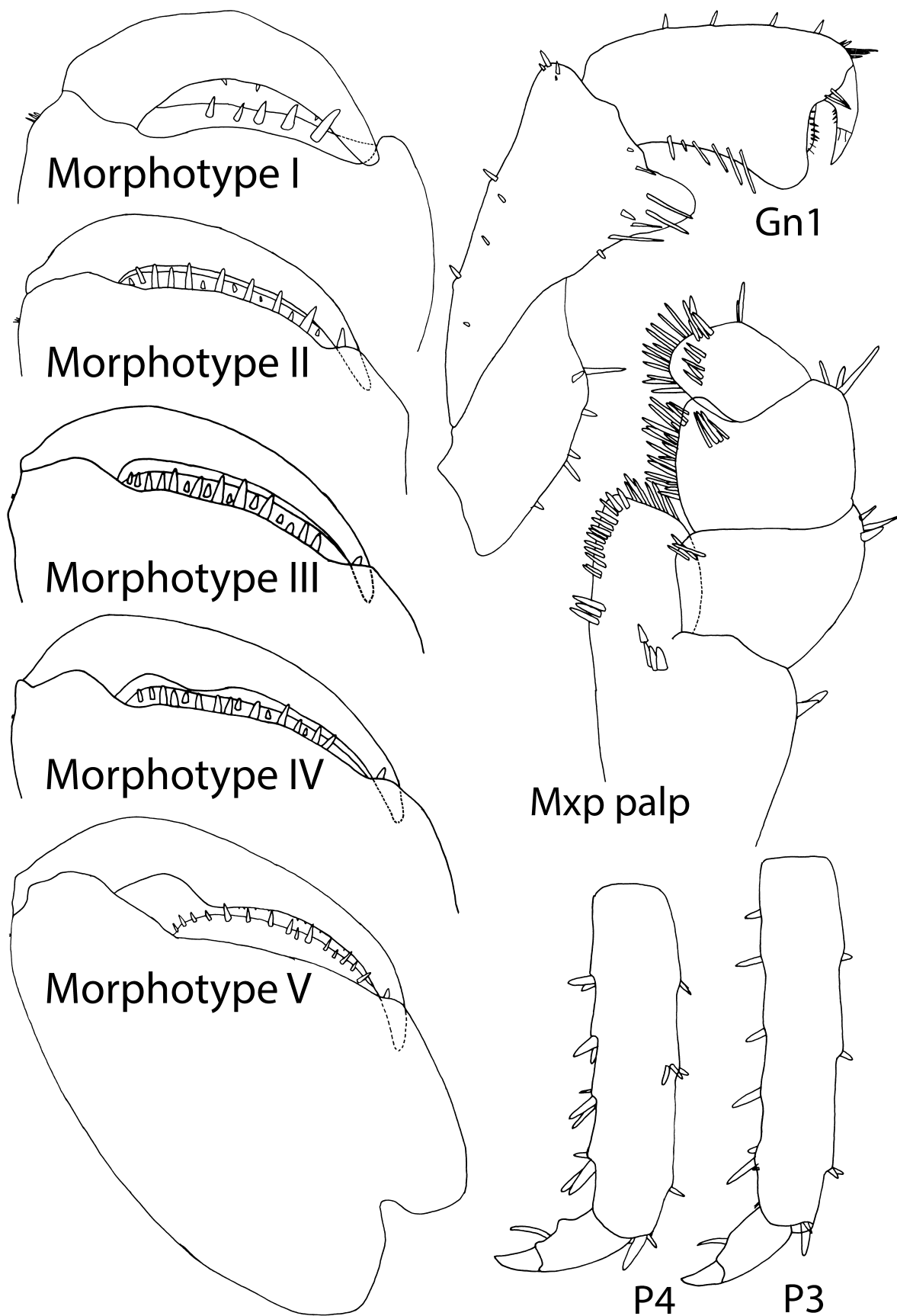
bifid setae on all margin; peduncle article 5 ventral margin with 6 groups of bifid setae in row, on dorsal margin 5 groups of bifid setae in row (2 or 3 setae for group); flagellum sub-equal than peduncles, 21-articulated final article is cone-shaped; all article with 4 tooth setae around distal margin; last article with much simple setae on apex. *Upper lip (labrum)* entire, apical margin with tooth setae. *Lower lip (labium)* with wide lobes and shoulders apically abundance tooth setae. *Mandible* stout, incisor 5-dentate (last is bi-dentate), left lacinia 4-dentate, molar finely serrated (Figure 9). *Maxilla 1* inner plate narrow with oblique apex and 2 stout apical robust plumose setae; outer plate with 8 tooth setae, medially serrate tooth setae; palp slender 1-articulate. *Maxilla 2* inner plate with 1 stout long, plumose seta medially and robust setae distally; outer plate rounded distally armed with tooth setae. *Maxilliped* inner plates with plumose setae on apical margin with 3 robust setae (tri-dentate); outer plate slightly shorter than basal height in length, with tooth setae apically; palp article 3 rounded apically and densely covered with tooth setae, article 4 absent. On external margin bifid setae are present (see Figure 5).

**Peraeon.** *Peraeonite* 1–5 subequal in length. *Gnathopod 1* coxa anterior margin straight and anterior distally sub-acute, with marginal setae; basis slightly expanded posterodistally with a row of 7 setae on anterior margin and a row of 4 setae on posterior margin; ischium shortest, rounded posteriorly, with 2 groups of setae on posterior margin and with a process on anterior margin; merus with a row of 4 groups of setae on posterior margin; merus and carpus connected by diagonal joint; carpus approximately 1.5 times as long as propodus, slightly curved posteriorly with lobe postero-distally equipped with row of setae and with a row of 3 group of setae on anterior margin; propodus expanded distally with a row of setae and a row of 5 group of setae (4 groups of setae in smallest specimens) on anterior margin, palmar margin (with setae) longer than dactylus; dactylus narrow with a group of setae on inner margin, subchelate. *Gnathopod 2* coxa slightly wider than deep, with marginal setae and with postero-proximal process and postero-distal process ('cusp' *sensu* Bousfield & Hendrycks 2002); basis sub-rectangular with a row of setae on anterior and posterior margin; ischium narrow with dorsal process; merus little sub-rectangular; carpus distinct; propodus elongate ovoid, slight protuberance near dactylar hinge, palmar margin equipped with row of setae; dactylus curved distally with proximal protuberance and near hinge with a depression; sub-chelate (see *Variation* par. below for other details). *Peraeopod 3–4* coxa sub-quadrate, with marginal setae and with pointed process posterior marginal (cusp); all anterior and posterior margin of peraeopods have group of setae; basis with straight anterior and weakly convex posterior margin; ischium the shortest with posterior marginal notch; merus slightly expanded; carpus approximately 0.7 times as long as merus; propodus slender, slightly longer than carpus; dactylus slender with single setae on inner margin. *Peraeopod 5* coxa bi-lobed, anterior lobe wider and slightly longer than posterior one, both with marginal setae; all anterior and posterior margin of peraeopods have group of setae; basis lobed; ischium wider than long with posterior marginal notch; merus as wide as ischium, slightly produced postero-distally; carpus sub-equal to merus in length; propodus narrow, sub-equal to carpus in length; dactylus with single seta enter-marginally. *Peraeopod 6* much longer than preceding peraeopods; coxa bi-lobed, anterior lobe much shorter and narrow than posterior lobe, with marginal setae; all anterior and posterior margin of peraeopod have group of setae; basis rounded, posterior margin moderately expanded; ischium with posterior marginal notch; merus slightly expanded distally; carpus sub-rectangular, sub-equal in length and narrower to merus; propodus very narrow, approximately 1.3 times of merus length, dactylus narrow with seta anterior marginal. *Peraeopod 7* longer than peraeopod 6; coxa rounded posteriorly, marginal setae; all anterior and posterior margin of peraeopod have group of setae; basis rounded, posterior margin expanded; ischium with posterior marginal process; merus slightly expanded distally; carpus sub-rectangular, longer and narrower than merus; propodus very narrow, dactylus narrow with seta anterior marginal.

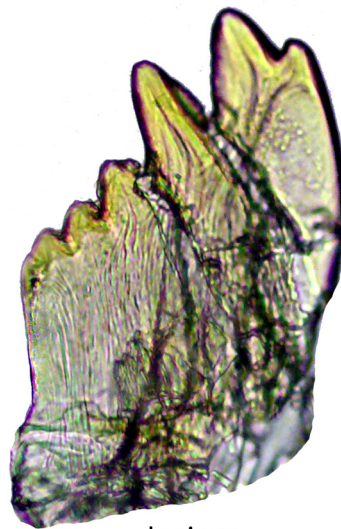
**Pleon.** *Pleopods* well developed; peduncles not expanded; bi-ramous, rami shorter than peduncle with more long plumose setae. *Epimeron 2* sub-equal in length to epimeron 3. *Epimeron 3* posterior margin smooth, postero-ventral corner. *Uropod 1* peduncle sub-equal than rami, with 4–5 (5–7) robust setae along lateral margin, disto-lateral peduncular spine-like seta; outer ramus slightly shorter than inner ramus, marginally with 2–3 marginal robust setae; inner ramus with 3–5 marginal robust setae; both rami with rounded apex with group of long stout distal robust setae. *Uropod 2* peduncle sub-equal to rami, with setae along lateral margin; inner ramus sub-equal in length to outer ramus; inner ramus with 4–5 marginal robust setae; outer ramus with 1–2 marginal robust setae. *Uropod 3* uniramous; peduncle stout, with 4–5 medial and distal robust setae; ramus shorter than peduncle, linear, with 1–4 marginal and 4–7 apical setae. *Telson* fleshly, with the 2 lobes fused on base; longer than broad, weakly cleft, with lateral and distal bifid setae.



**FIGURE 7.** Iconography of *Orchestia stephensei* Cecchini, 1928. Male. Morphotype V. *Gnathopods* 2 of specimens ascribed to Morphotype I–IV are shown. *Peraeopod* 7 of specimens ascribed to Morphotype I is shown. See text for terminology. Scale bar = 1 mm.



**FIGURE 8.** *Orchestia stephensi* Cecchini, 1928. Male. Details of shapes of palm and dactylus of *gnathopod 2*, dactylus of *peraeopod 3* and *peraeopod 4*, merus, carpus and propodus of *gnathopod 1* and palp of maxilliped.



Incisor



Lacinia mobilis

**FIGURE 9.** *Orchestia stephenseni* Cecchini, 1928. Male. Incisor and lacinia mobilis.

**Variation. Morphotype I:** *Gnathopod 2* propodus trapezoid; palm slightly convex, extending about 40% of posterior margin with a distal small protuberance, where tip of dactylus closes; dactylus smoothly arcuate. **Morphotype II:** *Gnathopod 2* propodus sub-ovate; palm slightly convex, extending about 70% of posterior margin with a distal very small protuberance, where tip of dactylus closes; dactylus slender distally. **Morphotype III:** *Gnathopod 2* propodus ovate; dactylus slender distally, as long as the palm of propodus. **Morphotype IV:** *Gnathopod 2* propodus ovate; dactylus posterior margin with postero-proximal sinus and protuberance. **Morphotype V:** *Gnathopod 2* propodus ovate; palm with postero-proximal sinus; dactylus posterior margin with well-developed postero-proximal sinus and protuberance.

**Remarks.** The material, originally described by Cecchini (1928), was collected from La Spezia (northern Italy). However, a holotype was never designated (Lowry & Fanini 2013); thus, a neotype has been designated from the collection in Sicily (southern Italy).

## Discussion

**Molecular analysis.** Over the past decade, DNA Barcoding has played a facilitator role in the accurate identification of marine fauna, thanks to the integration of molecular and traditional taxonomic methods (Costa *et al.* 2007; Landi *et al.* 2014). Molecular analysis has defined the three syntopic species in three monospecific clades, correlating with *O. stephenseni*, *O. montagui* and *O. mediterranea* respectively and, at the same time, this type of analysis has clustered the five morphotypes detected in *O. stephenseni*. It has also revealed a low divergence among specimens of *O. stephenseni* analysed, which can be described as natural intraspecific variability (Figure 6). The identification of *O. stephenseni* morphotypes was verified by the neotype mitochondrial DNA sequence, against which the reference library containing the DNA barcodes could be matched.



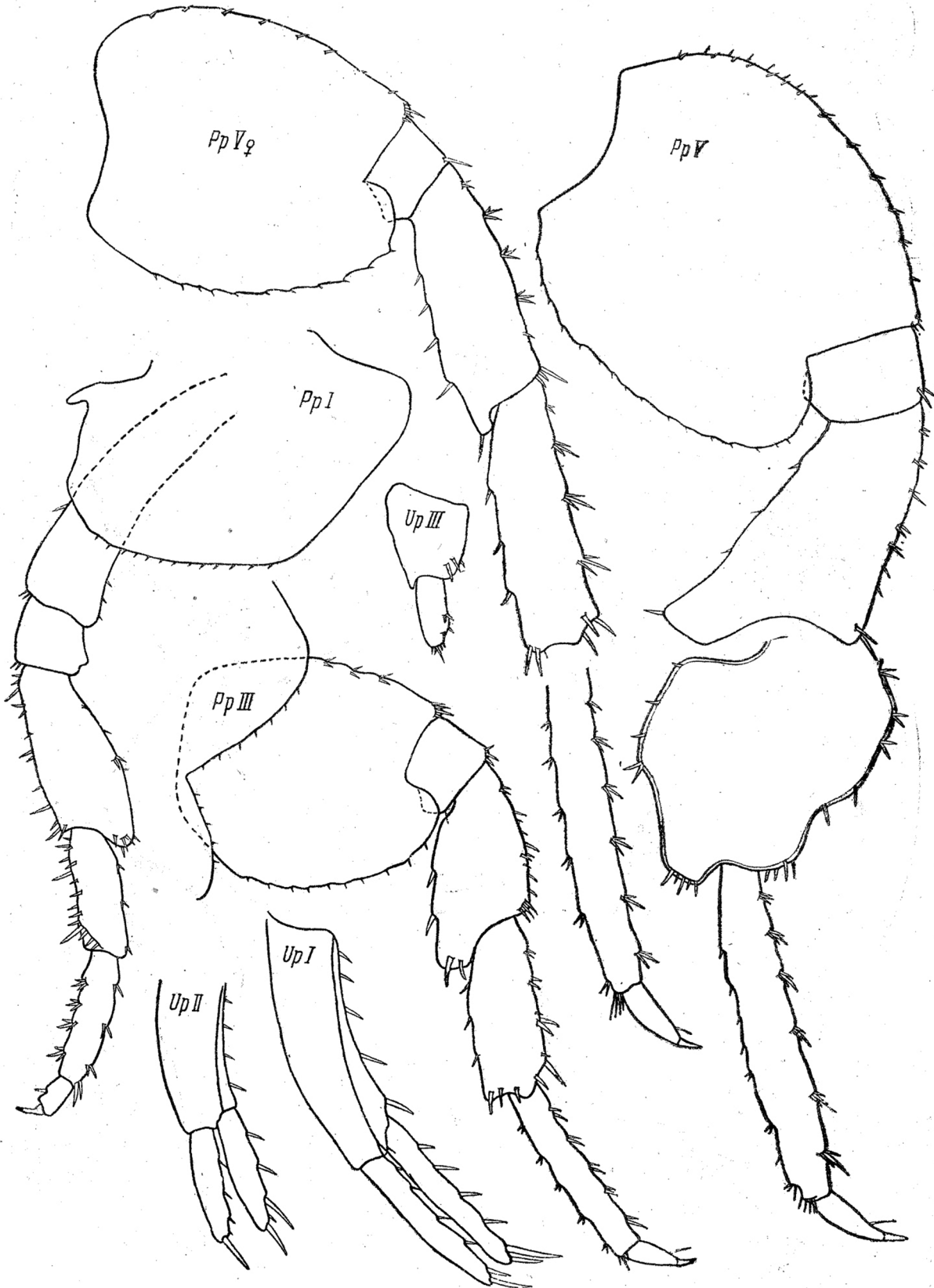


Рис. 556. *Orchestia mediterranea* Costa.

FIGURE 10. Iconography of *Orchestia mediterranea* A. Costa, male, by Bulycheva (1957); PpV, pereopod 7.

The NJ tree (Figure 6) showed a robust monophyly for the four *Orchestia* species under analysis; the branch lengths, and consequently the genetic distances, are deep enough to delimit the four species included in the tree. Few authors have performed COI sequences on talitrid species. Such data report genetic distances among Talitridae species ranging from 0.2 to 41.0 %, and values between pairs of *Orchestia* species ranging from 1.0 to 31.0 % (Wildish *et al.* 2012, and references therein), thus highlighting wide-ranging values for the whole family.

The wide inter-species COI distance is not uncommon in amphipods. Within Caprellidae, Pilar Cabezas *et al.* (2013) scored an inter-species divergence ranging from 7.6 to 15.4 %; or within Iphimediidae and Epimeriidae, Lörz *et al.* (2009) scored an inter-species divergence ranging respectively from 7.9 to 29.5% and from 8.5% to 26.2%.

As talitrids occur in different environments and they are cosmopolitan, the talitrid species can have an intra-species gene pool strongly influenced by selective pressures or by high gene flow (Pavesi & Ketmaier 2013; Pavesi *et al.* 2013); consequently various species cannot be easily discriminated by molecular techniques due to cases of homoplasy. However, here DNA Barcoding has confirmed the co-specificity of morphological variation of specimens, and it has been demonstrated as an equivalent to morphological taxonomy for species diagnosis. The clades grouping the intra-species haplotypes are well separated and the inter-species distance values do not overlap intra-species divergence values.

**Morphotype variation.** Talitrids attain the largest body-size of all the intertidal amphipods in the Mediterranean Sea, up to a maximum length of 21 mm (those reported in this paper), and the morphological characters of individuals markedly change throughout their growth period. The specimens of *O. stephensi* studied in Sicily ranged from 11 to 21 mm in length and, although they have been discriminated into five different morphotypes (I to V), definite stages of maturity have not yet been discriminated. The current classification of the Mediterranean *Orchestia* genus is based primarily on male, sexually dimorphic characters including: the shape of *peraeopod* 7 merus and carpus, and the shape of *gnathopod* 2 propodus and dactylus (Bellan-Santini 1993; Lowry & Fanini, 2013). These sexually dimorphic characters, which have been observed in this study, here are variable throughout their growth period (Figure 2).

*Orchestia stephensi* is the only Mediterranean species within the *Orchestia* genus with a *peraeopod* 7 merus and carpus which is never dilated (Bellan-Santini 1993), and the oval shaped propodus with a relatively long dactylus and short median tooth in *gnathopod* 2, here corresponding to Morphotypes I to IV. These characters (*peraeopod* 7 and *gnathopod* 2) are shared with various growth stages of other *Orchestia* species, and they have been the cause of misidentification or uncertain identification in the past. The Morphotypes III, IV and V can be misidentified with other co-generic species e.g. *gnathopod* 2 Morphotype V is similar to the *gnathopod* 2 of *O. montagui*, and *gnathopod* 2 Morphotype III and IV is similar to the *gnathopod* 2 of *O. mediterranea*.

The history of the name *O. stephensi* is overflowing with erroneous identifications and uncertainties but, in some cases, clarified by this revised description. Bulycheva (1957) has proposed the synonymy of *O. stephensi* Cecchini, 1928 with *O. mediterranea* A. Costa, 1853, and this idea was taken up by Barnard (1958) in his checklist. The description of *O. mediterranea* A. Costa 1853 was revised by Bulycheva (1957) and it has since been listed in subsequent synonymy of *O. stephensi* Cecchini, 1928 by Bellan-Santini (1993) and Lowry & Fanini (2013). However, the illustration of *peraeopod* 7 (Bulycheva, 1957; Figure 10) shows an expanded *peraeopod* 7 merus and carpus which is more likely attributable to *O. mediterranea*. Based on this iconography, the synonymy proposed by Bulycheva 1957 is rejected.

The first description of *O. stephensi* by Cecchini (1928) contained limited information regarding its features: it only described the male *gnathopod* 2 and discussed the similarity with the *gnathopod* 2 of *O. mediterranea* A. Costa, 1853, which perpetuated further confusion. The *O. ghigii* species described by Vecchi (1929) was previously described as a junior synonym of *O. stephensi* by Ruffo (1949). This placement of *O. ghigii* can be supported by this study, based on the assessment of morphology discussed in this paper, as compared to the description by Vecchi (1929). Lowry & Fanini (2013) queried the placement of several works as part of the synonymy of *O. stephensi*, including (i) the *O. stephensi* recorded by Calvario & Marques (1983) in the Atlantic Ocean; (ii) the record of *O. montagui* by Carus (1885); and (iii) *O. constricta* described by A. Costa, 1853, which can be resolved by the dataset outlined in this paper.

*Orchestia stephensi* in Calvario & Marques (1983) is here considered a misidentification. Their illustration lacks the peduncular spine-like seta in *uropod* 1, which is now considered an important character for identifying *O. stephensi*. As the record of this species by Calvario & Marques (1983) is the only one from the Atlantic Ocean, *O. stephensi* is here confirmed to be a Mediterranean endemic species.

The report of *Orchestia montagui* in Carus (1885) is here considered as a valid identification and removed from the synonymy of *O. stephenseni*: Carus described the *peraeopod* 7 merus and carpus expanded, a characteristic of *O. montagui* (“*pedes posteriores majores, articuli 4. et 5. in ♂ adultis plerumque dilatati, 4. retro in apicem productus*”, Carus 1885).

*Orchestia constricta* A. Costa, 1853 matched the *O. stephenseni* Morphotype V defined here; the author described the *O. constricta* species with a *peraeopod* 7 merus and carpus which was never dilated and the particular shape of *gnathopod* 2 corresponding to Morphotype V. Heller (1866) collected *O. constricta* in the Adriatic basin, within the Mediterranean, and described a *gnathopod* 2 like Morphotype V, separating it from *O. montagui*, which had also been collected in the same area. Thus, *O. constricta* A. Costa, 1853 can be considered as a senior synonym of *O. stephenseni*, although *O. constricta* has already been proposed as *species dubia* by Karaman (1973).

The name *O. constricta* A. Costa, 1853 occurs prior to *O. stephenseni* Cecchini, 1928, and, in accordance with the Principle of Priority, the name to be accepted by the academic community should be *O. constricta*. However, a Reversal of Precedence in Zoological Nomenclature (ICZN <http://iczn.org/>) establishes that the Principle of Priority cannot be followed and that the prevailing usage must be maintained when the following conditions are both met: “*the senior synonym or homonym has not been used as a valid name after 1899, (art. 23.9.1.1.) and the junior synonym or homonym has been used for a particular taxon, as its presumed valid name, in at least 25 works, published by at least 10 authors in the immediately preceding 50 years and encompassing a span of not less than 10 years (art. 23.9.1.2.)*”. In this case the name *Orchestia stephenseni* matches the art. 23.9.1., and is here recommended.

**Statement of the Reversal of Precedence.** The Principle of Priority (Article 23 of the International Code of Zoological Nomenclature, ICZN 2000) would require the older name to be used (i.e., *Orchestia constricta* A. Costa, 1853). In this case, the direct application of the Principle of Priority is not in the interests of nomenclatural stability because the younger name (i.e. *Orchestia stephenseni* Cecchini, 1928) is in current and widespread usage (see the references in Appendix 1). Indeed, the ICZN 2000 establishes that the Principle of Priority cannot be followed and prevailing usage must be maintained when the following conditions are both met:

*Orchestia stephenseni* takes precedence over the objective synonym *Orchestia constricta*, in accordance with article 23.9.1. of the ICZN.

*Orchestia constricta* A. Costa, 1853, the *senior synonym or homonym, has not been used as a valid name after 1899, (art. 23.9.1.1.):* since 1899, the name *Orchestia constricta* A. Costa, 1853 has been mentioned once (e.g. Stebbing, 1906), thereby filling Article 23.9.1.1 of the Code.

*Orchestia stephenseni* Cecchini, 1928, the *junior synonym or homonym has been used for a particular taxon, as its presumed valid name, in at least 25 works, published by at least 10 authors in the immediately preceding 50 years and encompassing a span of not less than 10 years (art. 23.9.1.2.):*

Appendix 1 demonstrates that the name *Orchestia stephenseni* matches the second condition. In the past 65 years (since 1951), at least 30 publications by 64 different authors have used *Orchestia stephenseni* as a valid species name, thereby fulfilling Article 23.9.1.2 of the Code.

As both requirements of Article 23.9.1 of the Code have been met, *Orchestia stephenseni* Cecchini, 1928 takes precedence over the objective synonym *Orchestia constricta* A. Costa, 1853, in accordance with Article 23.9.2. *Orchestia stephenseni* Cecchini, 1928 becomes a *nomen protectum*, and *Orchestia constricta* A. Costa, 1853 a *nomen oblitum*.

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## APPENDIX 1

The Principle of Priority is moderated by the Article 23.9.1 of the Code of Zoological Nomenclature that requires a **Reversal of Precedence** of a junior synonym when:  
 the senior synonym has not been used as a valid name after 1899 (Article 23.9.1.1);  
 and the junior synonym “*has been used for a particular taxon, as its presumed valid name, in at least 25 works, published by at least 10 authors in the immediately preceding 50 years and encompassing a span of not less than 10 years*” (Article 23.9.1.2).

The following list meets the condition Article 23.9.1.2:

30 selected publications, since 1951 treating the name *Orchestia stephenseni* as a valid species, published by 64 authors in the immediately preceding 50 years (thus, since 1965) and encompassing a span of not less than 10 years (in bold the names of the authors when are listed for the first time):

- 1 **Ruffo, S.** (1951) Studi sui Crostacei Anfipodi. XXV. Note sulle specie mediterranee del genere *Orchestia* (Amphip. Talitridae). *Atti dell'Accademia di agricoltura, scienze e lettere di Verona*, (6), 1(= 1949–50), 1–9, 1 map.
- 2 **Karaman, G.S.** (1973) XLV. Contribution to the Knowledge of the Amphipoda. *Orchestia stephenseni* Cecchini, 1928, one new Species for Adriatic Fauna (Gammaridea: Talitridae). *Fragmenta Balcanica, Musei Macedonici Scientiarum Naturalium, Skopje*, 9 (14/214), 137–145.
- 3 **Calvario, J. & Marques, J.C.** (1983) Étude systématique et écologique d'une collection d'Amphipodes de la zone intertidale de l'estuaire du Tage (Portugal). Occurrence d'*Orchestia kosswigi* Ruffo, 1949 et d'*Orchestia stephenseni* Cecchini, 1928. *Ciência biológica, ecology and systematics*, 5 (1), 79–95.
- 4 **Koukouras, A. & Russo, A.** (1991) Midlittoral soft substratum macrofaunal assemblages in the North Aegean Sea. *Marine Ecology*, 12(4), 293–316.  
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- 5 **Moreira, F., Assis, C. A., Almeida, P. R., Costa, J. L. & Costa, M. J.** (1992) Trophic relationships in the community of the upper Tagus estuary (Portugal): a preliminary approach. *Estuarine, Coastal and Shelf Science*, 34(6), 617–623.  
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- 7 **Stefanidou, D. & Voultsiadou-Koukoura, E.** (1995) An account of our knowledge of the amphipod fauna of the Aegean Sea. *Crustaceana*, 68 (5), 597–615.
- 8 **De Matthaeis, E., Davolos, D., Cobolli, M. & Ketmaier, V.** (2000) Isolation by distance in equilibrium and nonequilibrium populations of four talitrid species in the Marsala (western Sicily, southern Italy, central Mediterranean Sea). *Evolution*, 54(5), 1606–1613.  
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- 9 **Ariani, A.P., Camassa, M.M. & Wittmann, K.J.** (2000) The dolinas of Torre Castiglione (Gulf of Tarent, Italy): environmental and faunistic aspects of a semi-hypogean water system. *Mémoires de Biospéléologie*, 27, 1–14.  
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- 10 **Sezgin, M., Kocataş, A. & Katakın, T.** (2001) Amphipod fauna of the Turkish central Black Sea region. *Turkish Journal of Zoology*, 25(1), 57–61.
- 11 **Gauci, M.J., Deidun, A. & Schembri, P.J.** (2005) Faunistic diversity of Maltese pocket sandy and shingle beaches: are

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