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A cytogenetical study of Ischyroceridae (Amphipoda) allows the identification of a new species, *Jassa cadetta* sp. n., in the Lagoon of Venice

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Abstract

Jassa cadetta sp. n. (Amphipoda: Ischyroceridae) is described from the Venice Lagoon, northern Adriatic Sea, and a key to Mediterranean members of the genus *Jassa* Leach is provided. The new species is separated from *J. marmorata* Holmes primarily by cytogenetics, differing in chromosome number ($2n = 10$ in *J. cadetta* vs. $2n = 12$ in *J. marmorata*), karyotype morphology (FN = 20 vs. FN = 22), and chromosome location of 18S-5.8S-28S ribosomal cistrons. Cytogenetic analysis of *Ischyrocerus anguipes* Krøyer ($2n = 10$, FN = 18) gives a first insight into karyological diversity among Ischyroceridae. Analysis of random amplified polymorphic DNA markers confirms the distinction between *J. cadetta* sp. n. and *J. marmorata*.

Keywords: Amphipoda; Northern Adriatic Sea; Cytogenetics; PCR-RAPD; New species

Introduction

Libertini et al. (2000) published a monograph on the cytogenetics of *Jassa marmorata* Holmes, 1903 from the Venice Lagoon. The population was characterized by a karyotype of $2n = 12$ chromosomes, with 5 pairs of metacentrics and 1 pair of subtelocentrics. These data were consistent with those from an Atlantic population of the species in which haploid and diploid chromosome numbers had been found to be 6 and 12, respectively (Coleman 1994).

A fouling sample was collected from wooden piles at the northern mouth of the Venice Lagoon (Lido mouth) in 2000 and studied cytogenetically. Our lab regularly examines early embryos as material for karyotyping (Libertini et al. 2000); the body of the corresponding ovigerous female is usually preserved for species identification. One female from the Lido mouth, identified as *J. marmorata*, possessed a karyotype clearly different from that outlined above. This discrepancy was initially explained as a confusion in labeling of the female, because many other *J. marmorata* specimens with a 'regular' karyotype were found in the same sample.

In subsequent years, other fouling samples from the deepest part of blue mussel cultivation socks in the central mouth of the Venice Lagoon (Malamocco mouth) contained many *Jassa* specimens with a karyotype different from that of *J. marmorata*, compelling the authors to propose the presence of another species, recently recognized as new, and described below.

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The present paper reports on a genetic comparison between the two *Jassa* species from the Venice Lagoon, based on karyotypes and random amplified polymorphic DNA (PCR-RAPD), and provides the description of *Jassa cadetta* sp. n. The cytogenetic comparison is extended to *Ischyrocerus anguipes* Krøyer, 1838, considered here as an outgroup belonging to the same tribe, Ischyrocerini, according to the classification scheme by Myers and Lowry (2003).

Material and methods

The *Jassa* specimens used in this study were collected at different sites in the Venice Lagoon (NE Italy). *Ischyrocerus anguipes* was collected along the shores near the Sandgerði Marine Centre of the University of Iceland (SW Iceland), during low tide.

Chromosome preparations were made with the hot-dry method applied to early embryos as described by Libertini et al. (2000). The body of the mother carrying the embryos was preserved in 70% ethanol for identification.

For conventional karyotyping, slides were stained for 10 min in 5% Giemsa solution in phosphate buffer (pH 6.8). At least 100 chromosome plates for each species were counted to determine the diploid chromosome number. Karyotypes were arranged according to chromosome size and shape using digitized pictures of metaphase figures edited using Corel PhotoPaint. Chromosome classification is according to Levan et al. (1964); the centromeric index was evaluated following Naranjo et al. (1983). At least 20 karyotypes were examined for each species.

The chromosomal localization of major (18S-5.8S-28S) and minor (5S) rDNA sequences was performed by means of fluorescent *in situ* hybridization (FISH). A probe containing 18S-5.8S-28S genes plus intergenic spacer of the fruit fly *Drosophila melanogaster* (pDm238; Roiha et al. 1981) was used for major rDNA FISH. As the probe for 5S rDNA FISH, genomic DNA of *J. marmorata* was amplified by PCR, using two primers (5′-GAAAGCACCACTTCTCGTCC-3′ and 5′-AACG TGGTATGGCCGTTGAC-3′) obtained from the 5S rRNA sequence of the isopod *Proasellus coxalis* (Dollfus, 1892) (Pelliccia et al. 1998). Probes were labeled by nick translation with digoxigenin-11-dUTP or biotin-14-dATP (Roche Molecular Biochemicals or Invitrogen).

Chromosomes were denatured for 4 min in 70% formamide/2 × SSC at 69 °C. Denaturation of the probes was performed for 10 min at 75 °C. Hybridization was allowed to proceed overnight at 37 °C. Slides were washed three times in 50% formamide/2 × SSC at 40 °C (5 min each), twice (5 min) in 2 × SSC at room temperature (RT), once (5 min) in 2 × SSC/0.1% Tween20 at RT, and once (5 min) in PBS/0.1% Tween/0.5% skimmed milk powder at RT. Hybridization signals were detected with FITC-conjugated antidigoxigenin (Roche Molecular Biochemicals) or Cy3-conjugated extravidin (Sigma). Slides were mounted in AF1 antifade solution (Citifluor) containing 2 µg/ml DAPI (4′,6-diamidino-2-phenylindole).

Observations were made with a JenaMed 2-fluorescence microscope (Carl Zeiss Jena, Germany) equipped with the 410/450 and the 510/570 filter sets. Normal light and fluorescence images were taken with a Canon EOS 10D digital camera, processed and merged with Adobe Photoshop Elements 2.0.

For PCR-RAPD analysis, genomic DNA was extracted and purified from 10 males of *J. marmorata* and 6 males of *J. cadetta* sp. n. with the GenElute Mammalian Genomic Miniprep kit (SIGMA). The PCR-RAPD protocol was performed following Costa et al. (2004) with slight modifications, using the PCR ReadyMix kit (Sigma). PCR reactions were performed in a final volume of 25 µl, containing 0.2 mM each of dNTP, 0.75 U of Taq DNA polymerase, 1 × PCR buffer (20 mM Tris–HCl, pH 8.3; 50 mM KCl), 2.5 mM $MgCl_2$, 0.8 µM primer, and 20 ng template DNA. Primers were chosen from those successfully used by Costa et al. (2004) for *Gammarus locusta*; their respective names and sequences were (5′- to -3′): A2 (TGCCGAGCTG), A9 (GGGTAACGCC), A10 (GTGATCGCAG), A16 (AGCCAGCGAA), D2 (GGACCCAACC), D3 (GTC GCCGTCA), D5 (TGAGCGGACA), D7 (TTGGCA CGGG). All primers were purchased from MWG Biotech. Cycling conditions consisted of initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles (denaturation at 93 °C for 40 s, annealing at 37 °C for 60 s, extension at 72 °C for 60 s), and by a final extension for 6 min at 72 °C. DNA was then precipitated from the aqueous phase with ethanol and stored at −20 °C for at least 2 h or overnight. The samples were then washed in 70% ethanol. The amplification products were maintained at 4 °C until being loaded onto the gels. Electrophoresis was conducted on 2.5% agarose gels in TAE buffer (40 mM Tris, pH 7.6; 20 mM acetic acid, 1 mM EDTA), run at 90 V for 2.5 h or for 7 cm. The gels were stained in an ethidium bromide solution (30 mg/l), then photographed under UV light with a Canon EOS 10D digital camera. A molecular size standard consisting of 50–3000 bp "direct load" ladder (Sigma) was run in lanes flanking the samples, and a negative control in each gel.

For the morphological description of *Jassa cadetta* sp. n., the typical procedure for amphipods was used: direct observation of specimens in alcohol and glycerine under Reichert and Wild M5 dissecting microscopes; dissection, slide preparation (and storage) in glycerine or

Faure's medium; then drawing under a Wild M20 microscope.

Results and discussion

Genetics

According to Libertini et al. (2000), *J. marmorata* from the Venice Lagoon has a karyotype (Fig. 1A) composed of 5 pairs of metacentric chromosomes (pairs 1–5) and 1 pair of subtelocentrics (pair 6) much shorter than the other elements. The diploid chromosome number ($2n$) is 12, the fundamental number of chromosome arms (FN) is 22. In other *Jassa* specimens collected from the same lagoon, a diploid number $2n = 10$, karyotype comprising 4 pairs of metacentrics (pairs 1–3, 5) and 1 pair of submetacentrics (pair 4), and FN = 20 were found (Fig. 1B). In the latter specimens the second-longest chromosome pair has a secondary constriction on the shorter chromosomal arm (Fig. 1B, arrows), and the longest pair is almost twice as long as the others.

The karyotype of *Ischyrocerus anguipes* (Fig. 1C) is also characterized by $2n = 10$, but has 4 pairs of metacentrics (pairs 1–4) and 1 pair of subtelocentrics (pair 5), therefore FN is 18. The subtelocentric pair is much shorter than the other elements and has a secondary constriction on the shorter chromosomal arm (Fig. 1C, arrows).

The probe with major (18S-5.8S-28S) ribosomal genes hybridized in the terminal region of the shorter arm of the two subtelocentrics both in *J. marmorata* (Libertini et al. 2000; shown in Fig. 2A, for colour version see the online edition of this paper) and in *I. anguipes* (Fig. 2C). In *J. cadetta* with $2n = 10$, 18S-5.8S-28S rDNA cistrons were located at the end of the shorter arm in the second-longest metacentric pair (Fig. 2B). Therefore, in *I. anguipes* and *J. cadetta* nucleolus organizer regions (NORs) correspond to the secondary constrictions previously observed in Giemsa-stained chromosomes.

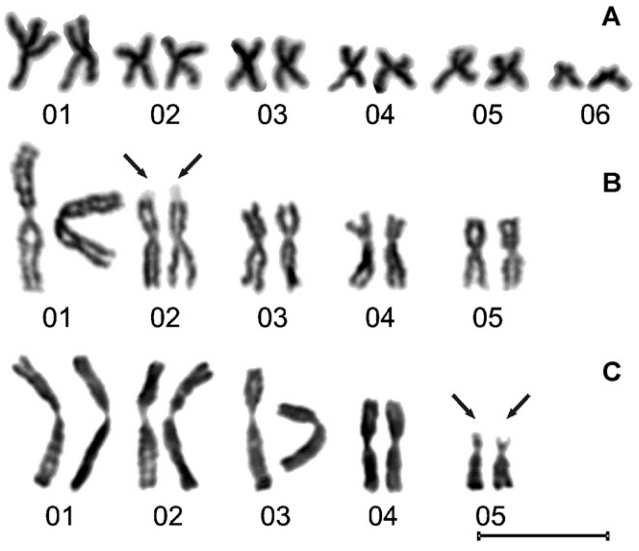

Fig. 1. Giemsa-stained karyotypes: (A) *Jassa marmorata* Holmes; (B) *Jassa cadetta* sp. n.; (C) *Ischyrocerus anguipes* Krøyer. Arrows indicate secondary constrictions. Scale bar = 10 μm.

The 5S rDNA probes hybridized in an intercalate region of the longest arm of an average-sized metacentric pair in all three species studied (Fig. 2D–F). In 6 of 7 examined metaphase figures of *I. anguipes* an additional metacentric chromosome had positive intercalate FISH signals (Fig. 2D–F). 18S-5.8S-28S rDNA and 5S rDNA cistrons are localized in two different chromosome pairs in the three Ischyroceridae (Fig. 2, arrows). The present paper is the first report on chromosomal localization of 5S rDNA genes in amphipods.

The karyological data available on Ischyroceridae are limited to species belonging to the tribe Ischyrocerini (Coleman 1994; Libertini et al. 2000; present paper). In this tribe, diploid chromosome number is 10 or 12. These numbers are among the lowest described for Amphipoda, $2n = 8$ in the aorid *Aora gracilis* (Bate, 1857) being the minimum reported for the order (Coleman 1994). Karyotypes are mainly composed of median centromere chromosomes, as in most amphipods (Libertini and Krapp-Schickel 2000), 18S-5.8S-28S rDNA cistrons are located on a single chromosome pair, and 5S and 18S-5.8S-28S rDNA genes are not co-localized.

The two *Jassa* forms from the Venice coast are clearly distinct at the cytogenetic level, showing different karyotype morphology concerning (1) chromosome number, (2) presence of subtelocentric or submetacentric pairs, (3) chromosome size classes, and (4) NOR-bearing pairs. They are similar only in location of 5S rDNA genes. The two *Jassa* were found in sympatry at some sites of the coast around Venice (Lagoon mouths of Lido and Malamocco; off of Jesolo Beach), but no hybrid karyotype was found in the analysed embryos. These results suggest that a second *Jassa* species morphologically similar to *J. marmorata* is present in the amphipod fauna of the Venice coast. This cryptic species is named *Jassa cadetta* sp. n.; its morphological description is given below.

Further support for the distinction between *J. marmorata* and *J. cadetta* comes from visual analysis of the RAPD profiles. All primers yielded amplification products. Some amplification bands were not unique or not present in all replicates, but the most informative bands clearly distinguished the two species. In addition, *J. marmorata* generally yielded the heaviest PCR products (see examples in Fig. 3).

Discoveries of cryptic diversity leading to the identification of new species have been reported in amphipods

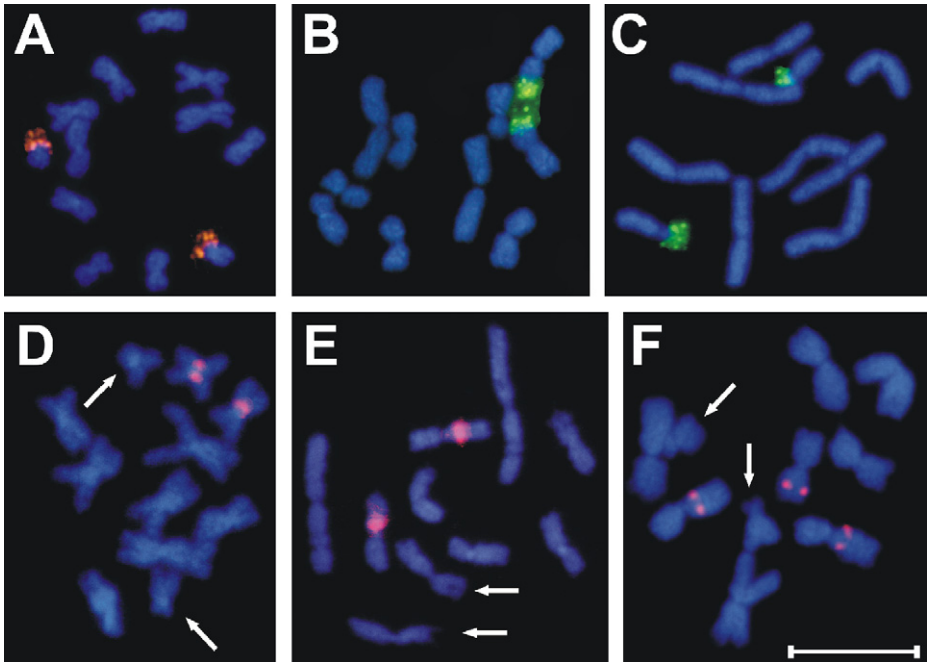

Fig. 2. Metaphase chromosomes, hybridization signals differentially stained; (A–C) after 18S-5.8S-28S rDNA FISH, (D–F) after 5S rDNA FISH. (A and D) *Jassa marmorata* Holmes. (B and E) *Jassa cadetta* sp. n. (C and F) *Ischyrocerus anguipes* Krøyer. Arrows in D–F indicate respective chromosome pair bearing major rDNA genes. Scale bar = 10 µm.

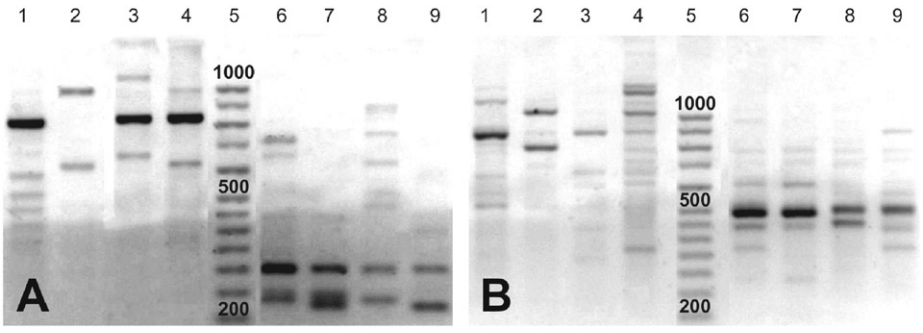

Fig. 3. Patterns of random amplified polymorphic DNA bands generated with primers A-2 (A) and D-2 (B). Lanes 1–4 contain DNA from four males of *Jassa marmorata* Holmes, lanes 6–9 from four males of *Jassa cadetta* sp. n., lane 5 the molecular weight marker.

on the basis of molecular analyses, in the genera *Eurythenes* (France and Kocher 1996), *Gammarus* (Müller 2000), *Paracorophium* (Schnabel et al. 2000), and *Hyalella* (Witt et al. 2006 and references therein). Regarding cytogenetic comparison among cryptic species, the only report so far is by Lop (1989), who found different chromosome numbers among morphologically similar *Echinogammarus* species belonging to the *E. berilloni*-group.

Jassa marmorata Holmes, 1903

Jassa marmorata Holmes. – Holmes (1903, p. 289); Holmes (1905, pp. 511–513); Lincoln (1979, p. 552, fig. 265); Myers (1989, figs. 295, 296); Conlan (1990, figs. 2–6, 17).

Jassa falcata (Montagu). – Chevreux and Fage (1925, p. 345, 346; figs. 352, 353); Sexton and Reid (1951, p. 29, "broad form" only; pls. 5, 10–17, 20–26).

Material examined

Obtained from many localities in the Venice Lagoon.

Diagnosis

See the differential diagnosis under *J. cadetta* n. sp. as well as the key below.

Jassa cadetta sp. n.

(Figs. 4–7)

Etymology

The species epithet is a Latinization of the English noun 'cadet', meaning (among others) 'little brother or sister' or 'younger family member'. It is chosen to reflect the species' similarity to the clearly bigger *Jassa marmorata*, and to be treated as an adjective for the purposes of nomenclature.

Material examined

Holotype: hyperadult male, 7 mm; Italy, Venice Lagoon, Malamocco, algae at shallow depth, 5.5.2005; deposited at Museo civico di Storia naturale, Verona, under collection no. M VR Cr 448. Additional material: 25 males (5–6 mm), 14 ovigerous females (3.5–4.5 mm); sampling data and depository as holotype.

Morphological description

Length. Average males 5–6 mm, hyperadult males up to 8 mm; average ovigerous females 3.5–4.5 mm.

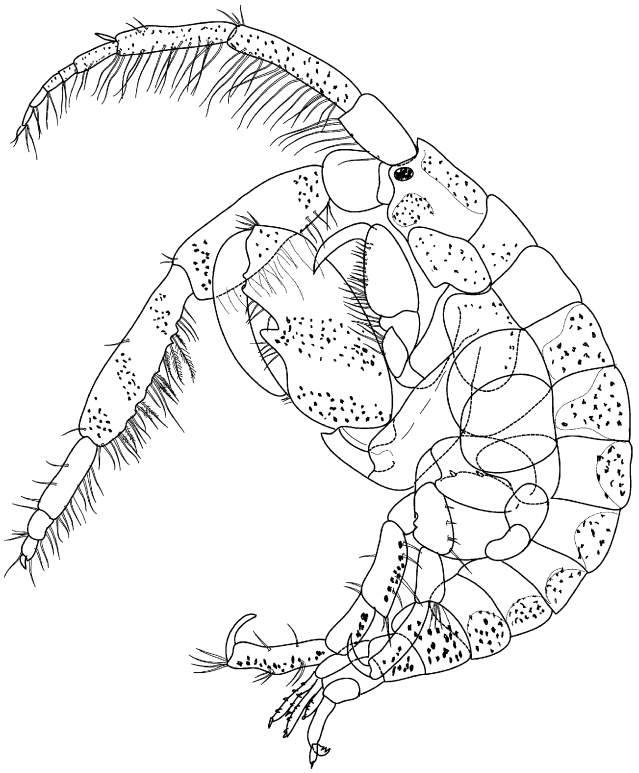

Fig. 4. *Jassa cadetta* sp. n., adult male (5 mm), habitus before thumbing.

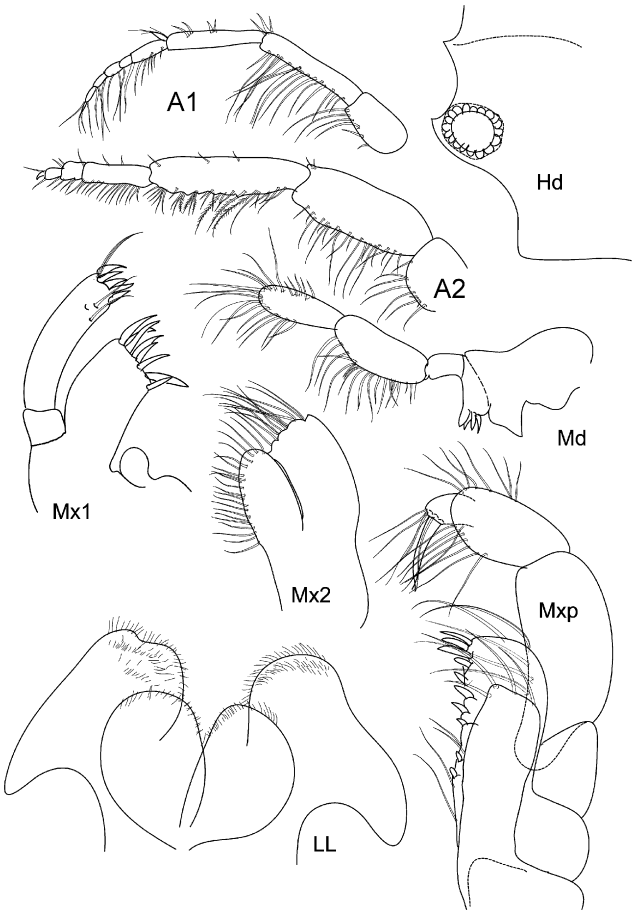

Fig. 5. *Jassa cadetta* sp. n. A1, 2 = antennae 1, 2; Hd = head; LL = lower lip; Md = mandible; Mx1, 2 = maxillae 1, 2; Mxp = maxilliped.

Antennae (Fig. 5). Antenna 1 reaching beginning of flagellum of A2. Accessory flagellum with 1 article, flagellum with 5–6. Antenna 2 with stout and thick peduncular articles, length/width of art. 4 about 2–2.5, of art. 5 about 4.5. Peduncular art. 5 with plumose setae; flagellum 4-articulate, art. 1 about half length of last peduncle article.

Mouthparts (Fig. 5). Mandibular palp segments 2, 3 with fringe of setae. Maxilliped inner plate scarcely surpassing length of ischium, outer plate reaching about half length of carpus (= second article of palp).

Gnathopods (Figs. 6 and 7). Coxa 1 rhombus-shaped with almost parallel margins. Gn1 basis rectangular, with very few and thin setae; carpus triangular, longer than wide, with anterodistal cluster of setae; propodus palm well defined by nearly right angle and spines, palm very slightly concave, nearly straight. Gn2 coxa 2 dorsal margin shorter than ventral one, ventrally convex, much wider than long, which becomes equally long in hyperadult males. Gn2 carpus without triangular prolongation, slightly longer than wide, posterior lobe

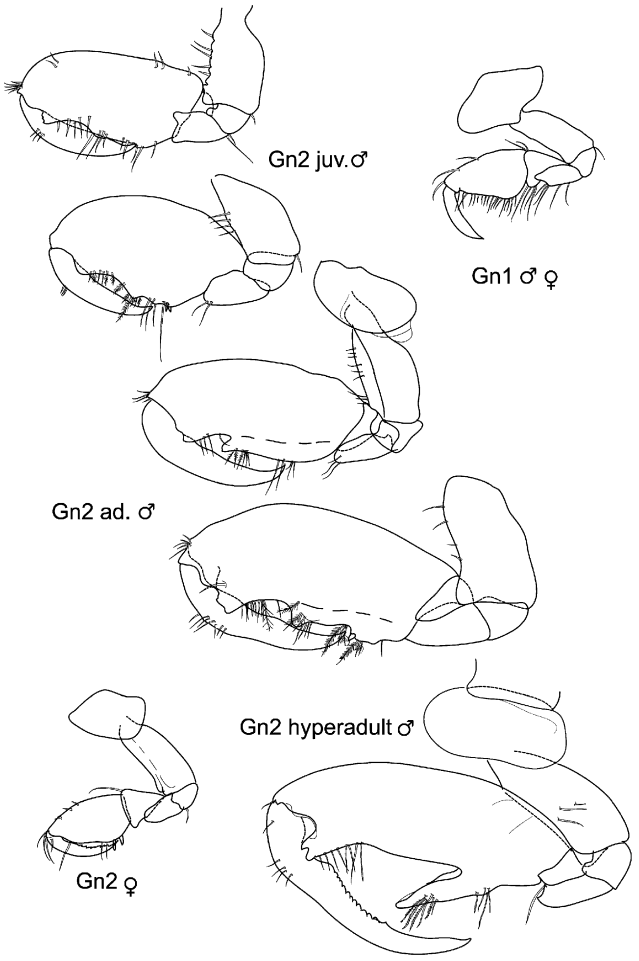

Fig. 6. *Jassa cadetta* sp. n., gnathopods (all drawn to scale) and development of male thumb. Gn1, 2 = gnathopods 1, 2.

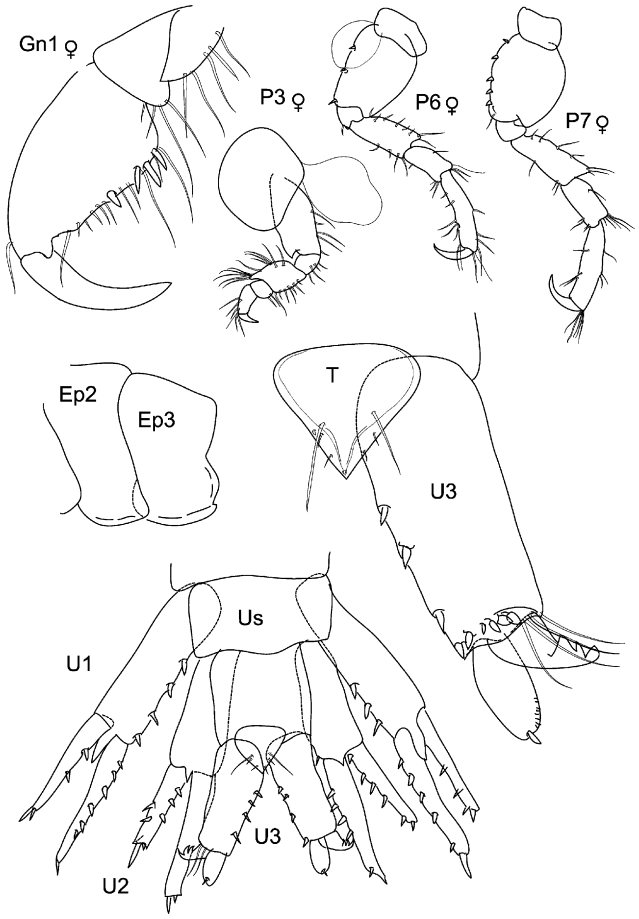

Fig. 7. *Jassa cadetta* sp. n. Gn1 = gnathopod 1 distal part enlarged; P3, 6, 7 = peraeopods 3, 6, 7; Ep2, 3 = epimeral plates 2, 3; T = telson; U1, 2, 3 = uropods 1, 2, 3; Us = urosome.

without setae. Gn2 propodus hind margin in females straight and smooth, in males changing shape with age (Fig. 6). Notably, the later 'thumb' part is already separated by a clear groove (and also differently coloured) when still fused with the main part of the propodus. Thumb distally pointed, not squared. Dactylus inner margin smooth, without protuberance, but cusped.

Peraeopods (Fig. 7). Coxal plates 3, 4 rhombus-shaped, with rounded corners, similar in size and shape. P3 merus anterodistal margin lengthened and pointed, with bundle of long setae; length and width about equal. P4 similar in size and shape, but basis and merus more widened distally. P5–7 articles distally not widened, rectangular; dactyli naked, without fringe of setae along outer margin; basis anterior margin with short spines, posterior margin concave, rounded, posterodistal corner not sharp.

Epimeral plates (Fig. 7). Ep1, 2 posterodistal margin rounded; Ep3 posterodistally with incision creating a blunt tooth.

Uropods (Fig. 7). U1 peduncle on the side near the body with many short, robust setae; distally a spine-shaped process about 1/3 of length of outer ramus, the latter a bit shorter than inner ramus. U2 similar to U1 in shape but shorter, with much shorter spine-shaped process on peduncle. U3 peduncle length/width = 2.3; inner margin beset with small, robust setae, similar setae in a row on distal margin; outer ramus with 2–3 closely approximated, dorsally recurved cusps or hooks; inner ramus egg-shaped, rounded, distally beset with many short setae and one single robust, spine-shaped seta.

Telson (Fig. 7). Triangular with sides of equal length, marginally with 2 short spine-like setae on each side, with additional very robust short setae nearby, and submarginally at about 60% of length (from insertion) on each side one long slim seta in upright position (at right angle to area of telson), thus easily overlooked when examined from dorsal. Tip of telson apparently with subapical pore, which could be the opening of a gland.

Distribution

Previous findings of *Jassa* in the Mediterranean reported under the names *Jassa falcata* (Montagu, 1808) or *J. marmorata* possibly included unrecognized specimens of *J. cadetta*, but details have not been verified. Therefore, the distribution of the latter species cannot be given more precisely than 'Mediterranean' at this time.

Differential diagnosis

Among the 21 species of *Jassa* currently recognized as valid (Myers 1989; Conlan 1990; Vader and Krapp-Schickel 2005), *J. cadetta* sp. n. is similar to *J. falcata* and *J. herdmani* (Walker, 1893) in body size (about 6 mm) and other morphological features. From the former of these species it is easily distinguished by the much stouter second antenna, a shorter and stouter body with the Gn2 propodus surpassing 1/3 of body length, by the pointed thumb (vs. squared in *J. falcata*), P5, 6 basis with posterodistal corner scarcely developed (vs. well developed and elongate), the regularly triangular telson (vs. elongate with pointed tip). In *J. herdmani* the second antenna and habitus structure are similar to *J. cadetta*, but the eyes are larger, the Gn2 dactylus inner margin has a hump, and coxa 2 is much shorter anteriorly than posteriorly (vs. more evenly shaped in *J. cadetta*). *Jassa cadetta* also shows many similarities to *J. pusilla* (Sars, 1894), from which it is distinguished by body size (*J. pusilla* male length 3 mm), the slender antennae and peraeopods, and mainly the shape of the thumb.

Jassa cadetta sp. n. shows striking similarities to *J. dentex* (Czerniavski, 1868) in the shape of A2, Gn1 and especially the female Gn2, but the male Gn2 thumb is much shorter and the dactylus carries a tooth on the inner side. Conlan (1990) advised that *J. dentex* should be removed from *Jassa*, but it has been kept in the genus, and we cannot see any reason for removing it, as *J. falcata*, *J. herdmani* and *J. oclairi* Conlan, 1990 are also described with that same tooth.

Diagnostically most important, however, are the following differences to *J. marmorata*, with which *J. cadetta* sp. n. shares biotopes at least in the Venice Lagoon. (1) Male A2 in *J. cadetta* clearly longer than half body length; flagellum with 4 articles, regularly beset with long plumose setae (in *J. marmorata* not longer than half body length; flagellum with 3 articles, the last one minute; flagellum articles densely beset with short plumose setae, which usually are heavily loaded with filtered particles). (2) Male Gn1 palmar margin in *J. cadetta* nearly as long as propodus, palmar corner next to proximal end of propodus (vs. shape of Gn1 propodus more regularly rounded in *J. marmorata*). (3) Coxa 2 rounded in male and female of *J. cadetta*, posterior margin only slightly longer than anterior one (vs. male Coxa 2 very short anteriorly, much shorter than hind margin of coxa 1, becoming nearly triangular; see Lincoln 1979, fig. 265; Myers 1989, fig. 296). (4) Gn2 thumb in hyperadult *J. cadetta* acute and pointed (vs. stout and squared). (5) Basis scarcely beset with setae in *J. cadetta* (vs. densely beset with a fringe). (6) Telson with setae about as long as telson itself in *J. cadetta* (vs. shorter than telson). (7) Male body length in *J. cadetta* usually 5.5–6 mm, rarely up to 8 mm (vs. up to 8 or even 10 mm). (9) Apart from these structural differences, members of *J. marmorata* usually are much more darkly pigmented than *J. cadetta* sp. n.

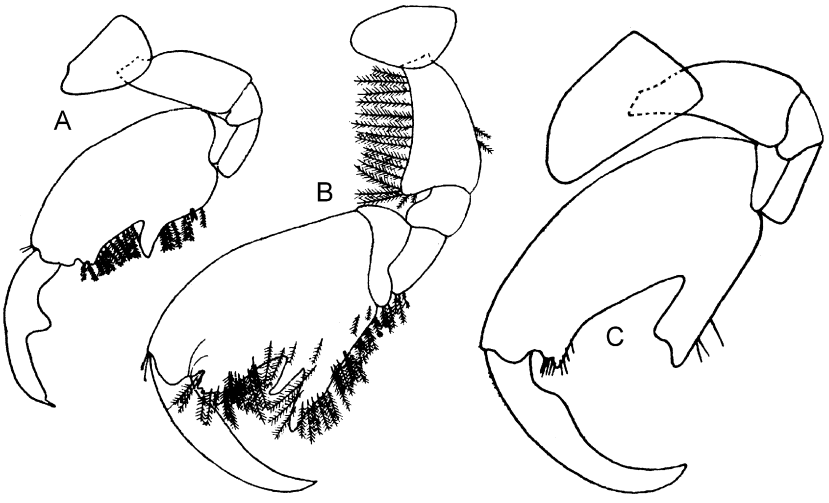

Fig. 8. Male gnathopods 2 in Ischyroceridae. (A) *Jassa dentex* (Czerniavski). (B) *Jassa ocia* (Bate). (C) *Jassa marmorata* Holmes (modified from Chevreux and Fage 1925).

Key to Mediterranean species of *Jassa* Leach

Note. *Jassa dentex* has been recorded from the Mediterranean only once, by Chevreux and Fage (1925) from algae at Sète near Montpellier, France.

1. Male and female 3 mm. Male Gn2 dactylus with strong tooth in middle of inner margin (Fig. 8A). .... *J. dentex* (Czerniavski, 1868)
– Male Gn2 dactylus without tooth on inner margin. 2
2. Male and female 4 mm. Male and female Gn2 propodus with 2 mediodistal teeth (one at palmar corner, one in middle of palm) and with two deep, U-shaped excavations (Fig. 8B). Gn1, 2 and male P3 densely fringed with plumose setae. ..... *J. ocia* (Bate, 1862)
[Note: This species probably needs to be transferred to another genus.]
– Gn2 propodus in hyperadult males with one thumb-shaped protuberance, palmar corner rudimentary or absent; in females and younger males with obtuse palmar corner and an excavation proximally and distally of a hump in middle of palm. Plumose setae, where present, never like a dense fringe. 3
3. Male up to 10 mm, female 5–8 mm. Gn2 in hyperadult males with obtuse or square-tipped thumb (Fig. 8C). Female Gn2 with palmar excavation about semicircular. Male and female coxa 2 anterior margin clearly shorter than length of coxa 1, often subtriangular. Male and female Gn1 propodus regularly rounded. .... *J. marmorata* Holmes, 1903
– Male and female 3.5–6 mm. Gn2 in hyperadult males with pointed end of thumb. Gn2 female palmar excavation shallow. Male and female coxa 2 anterior margin about as long as or scarcely shorter than coxa 1, never triangular. Male Gn1 propodus subtriangular, palmar corner near insertion. (Figs. 4–7) .... *J. cadetta* n. sp.

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