

First record of males of *Cypridopsis vidua* (Crustacea, Ostracoda) species complex for Europe

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ABSTRACT

Until recently, only asexually reproducing populations were known in *Cypridopsis vidua*, a species complex with one of the widest geographical distributions among non-marine ostracods. The discovery of males, most likely functional, from the United States and China has called into question its obligate parthenogen status. Here we report the first report of males for Europe, found in a small artificial pond in northern Italy. It seems plausible that sexual populations are much more widespread than hitherto known, as the recognition of males requires the dissection of individuals. Compared with the available descriptions of males belonging to *Cypridopsis vidua* and related species, that of the male illustrated here show morphological differences in the antennulae and prehensile palps, which in the Cypridoidea are sexually dimorphic and often diagnostic to distinguish between related species. Adult males show significantly smaller valve sizes than adult females, while adult females in which sperm were found inside the carapace have more variable lengths than those without sperm. No differences in valve coloration or dark banding pattern were found between males and females, and differences in valve outline were mostly negligible. The limitations of an exclusively morphological approach in the case of similar morphospecies are highlighted, as well as the importance of adopting objective criteria to measure the limits of morphological variability allowing the identification of separate taxonomic entities.

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Key words: non marine ostracods; morphology; species complex; reproductive modes; artificial ponds; Italy.

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INTRODUCTION

Ostracods are an extremely diverse group of Crustacea, which occur in numerous types of aquatic and semi-terrestrial ecosystems, and for which an excellent fossil record is available from the Early Ordovician for marine forms and the Carboniferous for continental forms (Rodríguez-Lazaro and Ruiz-Muñoz, 2012).

The most updated list of the extant, non-marine ostracods includes >2300 species globally, all belonging to the order Podocopida Sars, 1866, with representatives in the superfamilies Cypridoidea Baird, 1845, Cytheroidea Baird, 1850, Darwinuloidea Brady and Robertson, 1885 and Terestricytheroidea Schornikov, 1969, with the first two clearly dominant in terms of species diversity (Meisch *et al.*, 2019). Non-marine ostracods have evolved different reproductive strategies, from fully sexual lineages to others with mixed reproduction, i.e. with sexual and asexual (parthenogenic) females (Schön *et al.*, 2003), to species with exclusive asexual reproduction attributable to multiple independent origins (Butlin *et al.*, 1998; Bode *et al.*, 2010). The Darwinulidae, the only living family of the Darwinuloidea, are putative 'ancient asexuals', i.e. species with obligate parthenogenetic reproduction that persists over geological time scales (Martens *et al.*, 2003). In several species of ostracods with both parthenogenetic and amphigonetic populations, the males may be rare in terms of occurrence and abundance compared to females, and/or with geographical distribution limited to specific areas (Martens, 1991; Martens and Meisch, 1985; Horne and Martens, 1999; Namiotko *et al.*, 2005).

Cypridopsis vidua (O.F. Müller, 1776) belongs to the subfamily Cypridopsinae Kaufmann, 1900 in the family Cyprididae Baird, 1845 and is among the most common and most studied non-marine ostracod species regarding ecological and behavioural characteristics, so that it is suitable as a model organism for a mul-

titute of fields in biological studies (Martens *et al.*, 2023). *Cypridopsis vidua* inhabits both large and small water bodies with rich vegetation, such as permanent and temporary ponds, the littoral zone of lakes, slow rivers, springs, wells, and interstitial habitats (Meisch, 2000; Roca and Danielopol, 1991). It feeds mainly on periphyton growing on macrophytes (Roca *et al.*, 1993). This species is a very active swimmer with marked exploratory behaviour (Roca and Danielopol, 1991; Uiblein *et al.*, 1994; Uiblein *et al.*, 1996). Swimming is drag-based, with thrust provided by both antennulae and antennae, resulting in a uniform and continuous movement (Hunt *et al.*, 2007). *Cypridopsis vidua* responds to predator signals on the one hand by preferring shaded areas, such as aquatic weeds, and on the other hand by increasing its swimming activity (Mbahinzireki *et al.*, 1991; Roca *et al.*, 1993; Uiblein *et al.*, 1996). In the presence of predators, *C. vidua* shows a more circular swimming mode, which allows it to move rapidly between different foraging areas and to limit the risk of being caught by predators (Uiblein *et al.*, 1994). Aggregation in this species has been reported under laboratory conditions, probably as a similar phototactic response of all individuals (Nuttall and Fernando, 1970).

Cypridopsis vidua is considered a species characterized by considerable morphological variability. Differences in the shape, size and colour of the carapace, as well as in the number of filaments of the respiratory plate of the maxillipede were often reported. Some of these differences were considered by various authors to be sufficient to erect new species, most of which, however, were eventually synonymised with *C. vidua* (Meisch, 2000; Meisch *et al.*, 2019). Molecular analyses revealed that the morphospecies *C. vidua* forms a species complex that can be differentiated into seven distinct groups with 22 molecular operational taxonomic units, and a genetic distance between these latter ranging from 7 to 15% (Gomes *et al.*, 2023). Until recently, *C. vidua* was considered to be an obligate parthenogen, as there was a lack of any finding of males for this species in extant populations and in the fossil record, the latter available from the Lower Pleistocene onwards (Griffiths, 1995; Fuhrmann *et al.*, 1997). Interestingly, like *C. vidua*, other species thought to be exclusively asexual, *e.g.*, *Darwinula stevensoni* (Brady and Robertson, 1870) and *Penthesilenula brasiliensis* (Pinto and Kotzian, 1961), both considered ancient asexual, are among the few non-marine ostracods with a cosmopolitan distribution, *i.e.* present in at least six or seven zoogeographic regions (Meisch *et al.*, 2019). However, the obligate parthenogen status for *C. vidua* must be seriously questioned after the publication of two articles reporting the presence of sexual populations, respectively in the northwest of the United States (Martens *et al.*, 2023) and in southwestern China (Zhai *et al.*, 2023), with supposedly functional males. In addition, Furtos (1936) described *Cypridopsis okeechobei* from North America, a species that Meisch (2000) considers as a probable bisexual population of *C. vidua* and that was formally synonymized with the latter species by Martens and Savatnalinton (2011).

This paper reports the first record of males of the *Cypridopsis vidua* species complex for Europe, sampled in an artificial pond in northern Italy. The male morphological characters are compared with those of the other known sexual populations of the species complex or species that are probably synonym of *C. vidua*. The limitations of an exclusively morphological approach in taxonomic studies as well as the difficulties of establishing the limits

for morphological variability within the species complex, particularly for male characters, are discussed.

METHODS

The *Cypridopsis vidua* specimens used in this study were sampled from Millepioppi pond, a small artificial pond (approx. 3 m long and 2 m wide, maximum depth 0.5 m) located within the Stirone and Piacenziano Regional Park, near the village of San Nicomede, province of Parma, coordinates 44°50'32.78"N 9°59'44.61"E, 171 m asl (Fig. 1). The pond was constructed in 2022 by digging the ground, and a plastic sheet was used to waterproof the bottom. The following aquatic plants, coming from water bodies located in different Italian provinces (Parma, Modena, Mantua, Ravenna, Ferrara, Pisa and Lucca), were introduced inside the pond: *Carex pendula* Huds., *Hydrocharis morsus-ranae* L., *Hydrocotyle vulgaris* L., *Iris pseudacorus* L., *Lythrum salicaria* L., *Marsilea quadrifolia* L., *Nymphaea alba* L., *Oenanthe lachenalii* C. C. Gmel., *Ranunculus lingua* L., *Samolus valerandi* L., *Scirpoides holoschoenus* (L.), *Sparganium erectum* L., *Stratiotes aloides* L., *Vallisneria spiralis* L. The pond was regularly replenished with tap water during the dry season to prevent it from drying out. Ostracods were collected during three surveys in 2023 (August 1, September 13, and October 17) using a 250 µm handnet and preserved in ethanol 70% immediately after collection. In the lab, sorting and dissections of ostracods were done under a stereomicroscope (Zeiss 47 50 22). Valves were stored dry in micropaleontological slides and measured under a light microscope equipped with a calibrated micrometer ocular. Digital images of valves were acquired using a Zeiss EVO MA 10 Scanning Electron Microscopy (SEM). The dissected soft parts were mounted in glycerine on microscope glass slides and sealed using nail polish. Line



Fig. 1. Millepioppi pond on September 13, 2023.

drawings of soft parts were made with the help of a camera lucida-equipped microscope (Zeiss 47 30 11-9901). Chaetotaxy of the soft parts follows Meisch (2000) and Scharf *et al.* (2020).

Asexual or sexual females were considered to be those without or with sperm inside the carapace, respectively. However, it must be taken into account that a female without sperm is not necessarily asexual, but may not yet have mated (e.g., individuals that have just moulted to adults). Furthermore, recognition mechanisms are not always flawless, so males may also copulate with asexual females, as reported for other non-marine ostracod species (Schmit *et al.*, 2013b), and might thus yield polyploid offspring.

To capture the outline of the valves, images taken under microscope were modified using GIMP (GNU Image Manipulation Program, <https://www.gimp.org>), then saved as TPS file using the TPSutil32 v. 1.76 software and digitized using the TPSdig v. 2.20 software (Rohlf, 2009). Superimposition of valve outlines was performed using Morphomatica 1.6.0.1 (Linhardt *et al.*, 2007), a program designed for the approximation of ostracod specimens using an adapted B-splines algorithm. The mean specimens of the valve outlines were calculated combining the outlines of three specimens for each category (males, females with sperm and females without sperm).

The specimens examined in this study (labelled with the abbreviation GR followed by a number) are stored in the first author's ostracod collection at the University of Parma, Italy.

RESULTS

Ostracod community of the Millepioppi pond

Cypridopsis vidua was found in all collected samples, with relatively few individuals in August and in large numbers in September and October. Other ostracod species occurring in the pond were *Herpetocypris reptans* (Baird, 1835) and *Ilyocypris* sp., both with few individuals. *Cypridopsis vidua* males were present at all dates, with a balanced, or even slightly male-skewed, sex ratio. They mostly showed a conspicuous sperm production, bundles of which were also found inside several dissected females (Fig. 2).

Taxonomic account

Class Ostracoda Latreille, 1802
Subclass Podocopa Sars, 1866
Order Podocopida Sars, 1866
Suborder Cypridocopina Baird, 1845
Superfamily Cypridoidea Baird, 1845
Family Cyprididae Baird, 1845
Subfamily Cypridopsinae Kaufmann, 1900
Tribe Cypridopsini Kaufmann, 1900
Genus *Cypridopsis* Brady, 1867

Type species (by original designation)

Cypridopsis vidua O.F. Müller, 1776.

Other species

See Meisch *et al.* (2019).

Cypridopsis vidua (O.F. Müller, 1776) (Figs. 3-8).

Synonymies

Meisch *et al.* (2019) listed 19 synonymies of this species.

Morphological description of the male of *Cypridopsis vidua* from Millepioppi pond

Valves and carapace

Carapace ovate in lateral and dorsal view (Fig. 4). Left valve (LV) slightly longer right valve (RV), LV overlaps RV antero-dorsally (Fig. 4 a,b) and ventrally (Fig. 4c).



Fig. 2. Sperm bundle found inside an adult female (GR925) on October 17, 2023.

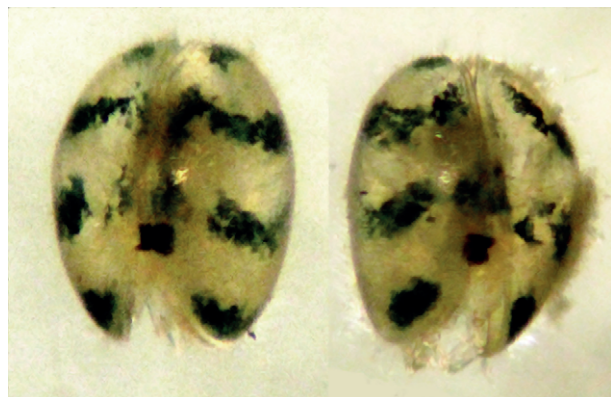


Fig. 3. Adult male (GR910, left) and female (GR912, right) of *Cypridopsis vidua* in dorsal view from Millepioppi pond (not to scale).

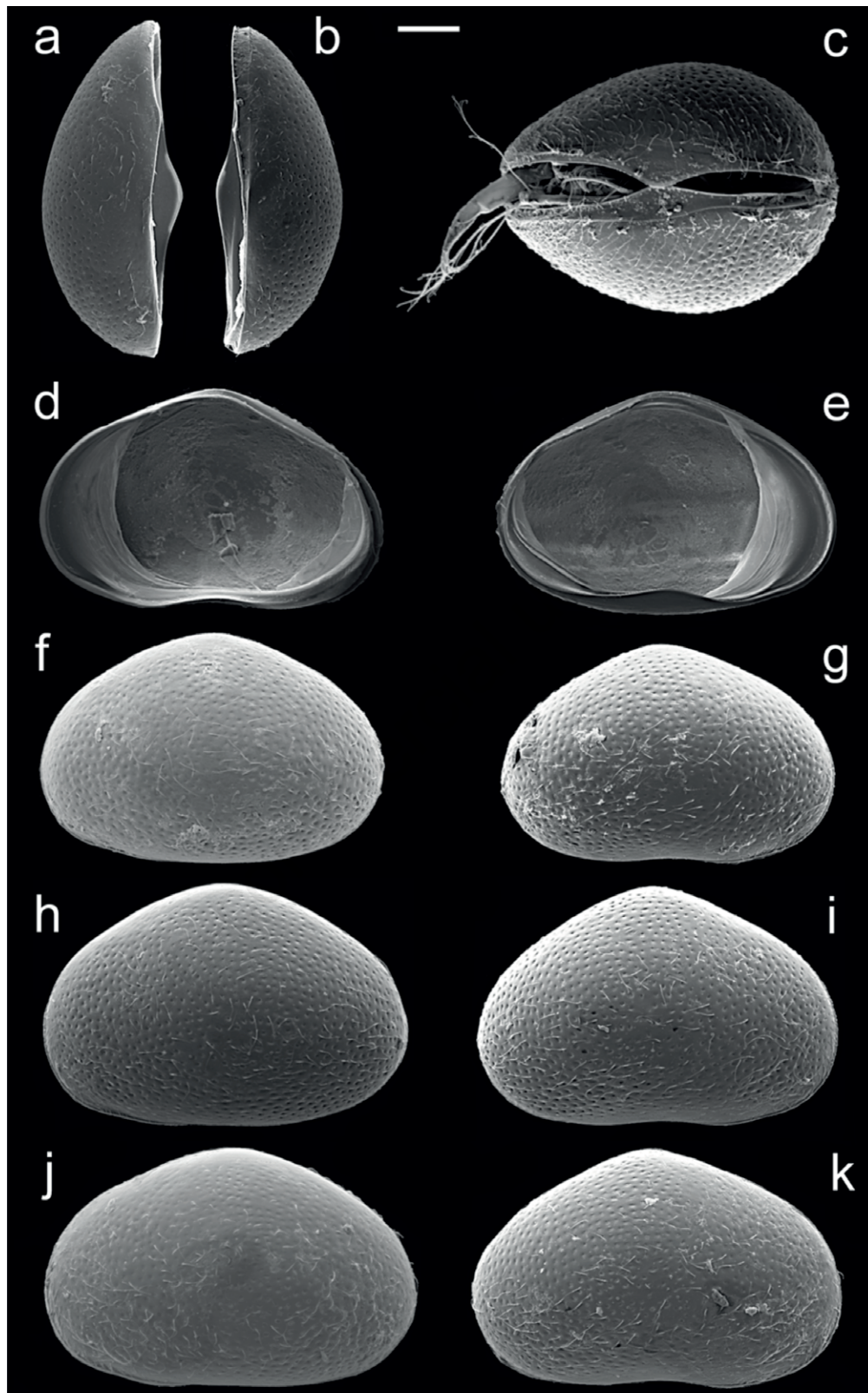


Fig. 4. SEM images of *Cypridopsis vidua* adults. **a)** Male LV, dorsal view (GR910); **b)** male RV, dorsal view (GR910); **c)** male Cp, ventral view (GR939); **d)** male RV, internal view (GR906); **e)** male LV, internal view (GR906); **f)** male LV, external view (GR904); **g)** male RV, external view (GR904); **h)** female without sperm, LV, external view (GR909); **i)** female without sperm, RV, external view (GR909); **j)** female with sperm, LV, external view (GR912); **k)** female with sperm, RV, external view (GR912). Scale bar: 100 μ m.

Maximum length at mid-height, maximum height at mid-length. In external lateral view, dorsal margin highly arched. Anterior and posterior ends rounded. Anterior external marginal zone of RV with a tiny row of pustules (Fig. 4b). Double folded inner list of RV running parallel to valve margin and to selvage (Fig. 4d). Postero ventral marginal zone of LV with double folded inner list typical of the genus, not running parallel to the valve margin (Fig. 4e). Ventral margin slightly curved (Fig. 4f) to sigmoidal, more evident on the RV (Fig. 4g). Surface ornamentation characterized by rounded pits that can be very shallow in some specimens (Fig. 4j). Central muscle scars typical of the genus.

Outline of the valves with minimal variation between males, females with sperms, and females without sperms (Fig. 5).

Remark

In adults, the carapace of males is slightly shorter than that of females, i.e., between 540 and 575 μm (median: 554 μm ; mean: 557 ± 13 μm) and between 595 and 620 μm , respectively. The size of females with and without sperm was overlapping, although those containing sperm showed slightly higher median (623 *versus* 607 μm , respectively) and mean (619 ± 23 *versus* 610 ± 23 μm) length (Fig. 6).

Soft parts

Antenna of the male (Fig. 7) with elongated appearance. Protopodite ventrally with two setae of similar length, more distally a seta approximately twice as long as the previous two. Exopodite consisting of a small plate with two short, strongly hirsute setae and a long seta reaching approximately to the distal margin of the last segment of the endopodite. First endopodal segment with a rather slender aesthetasc Y at the proximal third of the ventral margin, and sub-distally two lateral setae, the ventral one about twice as long as the dorsal one, the latter flanked by five swimming setae extending beyond the tip of the terminal claws. Second endopodal segment with setae t_1 and t_2 midway along the ventral margin, the former about $\frac{3}{4}$ as long as the latter, and more distally an aesthetasc (y_2) and a seta (G_3); dorsal margin with two setae of different lengths; claw z_1 and seta z_3 in sub-apical position, the latter surpassing the tip of terminal claws; claws G_1 and G_2 and seta z_2 apically on the distal margin of the segment. Last segment of endopodite short and narrow, distally with an aesthetasc (y_3) and a slightly longer accompanying seta, and two claws (G_M and G_m), the former long and stout and the latter short and slender.

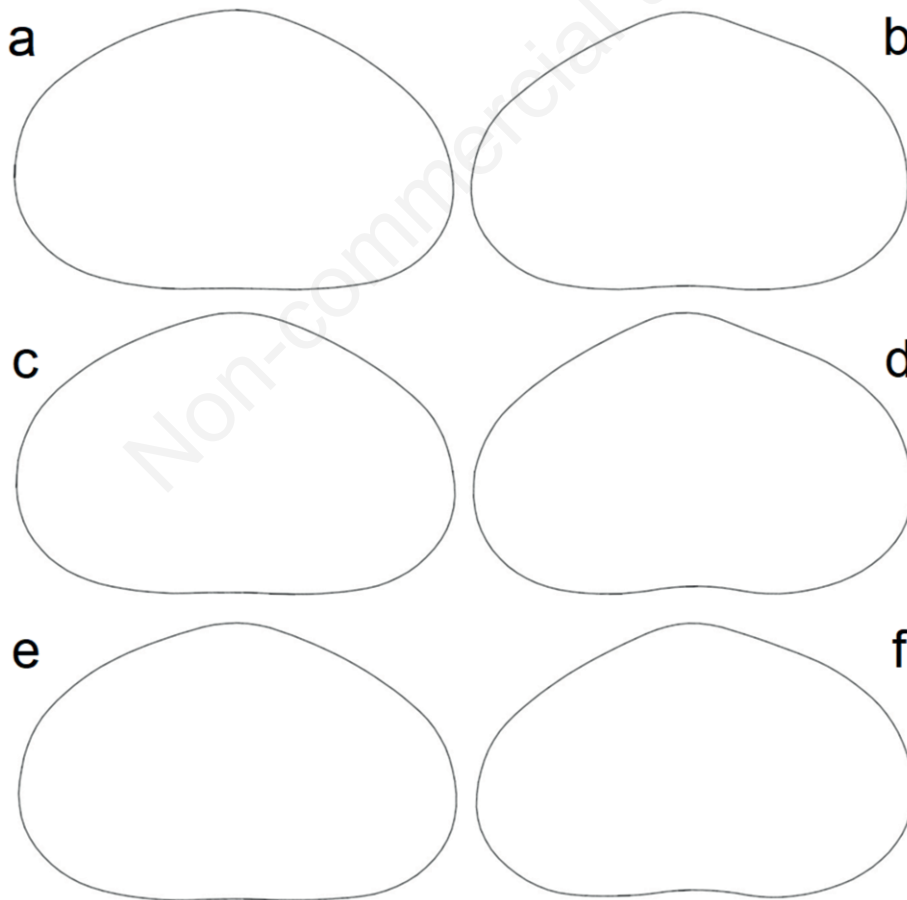


Fig. 5. Outlines of valves in lateral external view of *Cypridopsis vidua* (mean specimens, normalized for area). **a)** Male, LV; **b)** male, RV; **c)** female with sperm, LV; **d)** female with sperm, RV; **e)** female without sperm, LV; **f)** female without sperm, RV. GR918, GR923, GR931 for **a** and **b**; GR916, GR919, GR934 for **c** and **d**; GR915, GR917, GR932 for **e** and **f**.

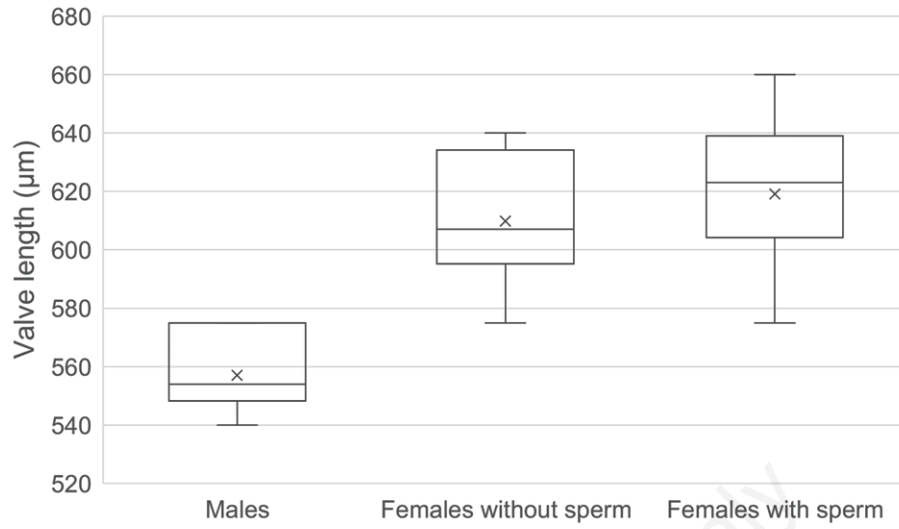


Fig. 6. Length of valves in adult males (n=10), adult females without sperm (n=8) and adult females with sperms (n=14) of *Cypridopsis vidua*. The boxes correspond to the interquartile range, the horizontal line within the box to the median, and the cross symbol to the mean; whiskers correspond to the maximum and minimum values.

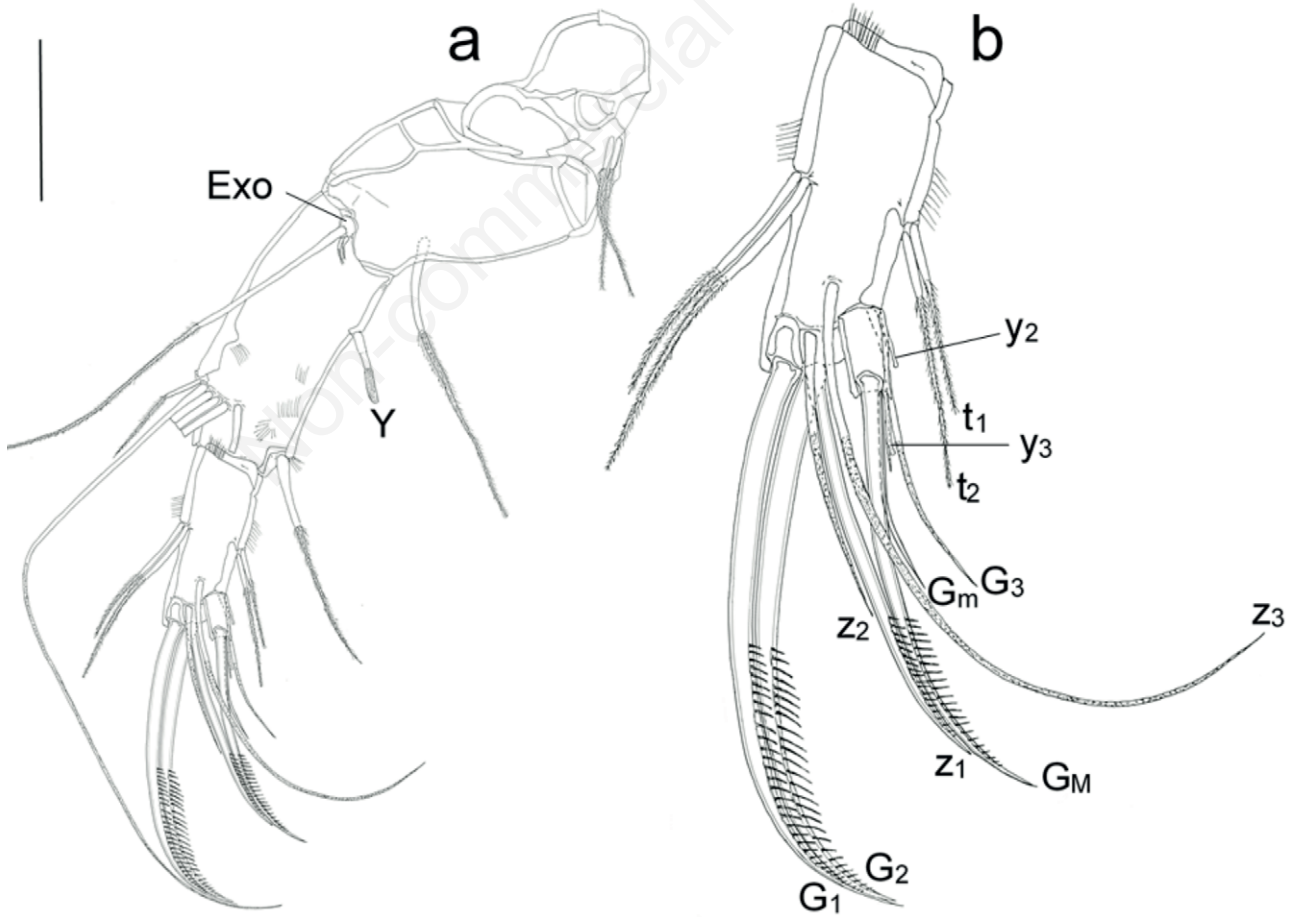


Fig. 7. *Cypridopsis vidua*, adult male, GR910. **a)** Antenna; **b)** detail of the last two endopodal segments of the antenna. Exo, exopodite. Scale bar: **a)** 50 µm; **b)** 25 µm.

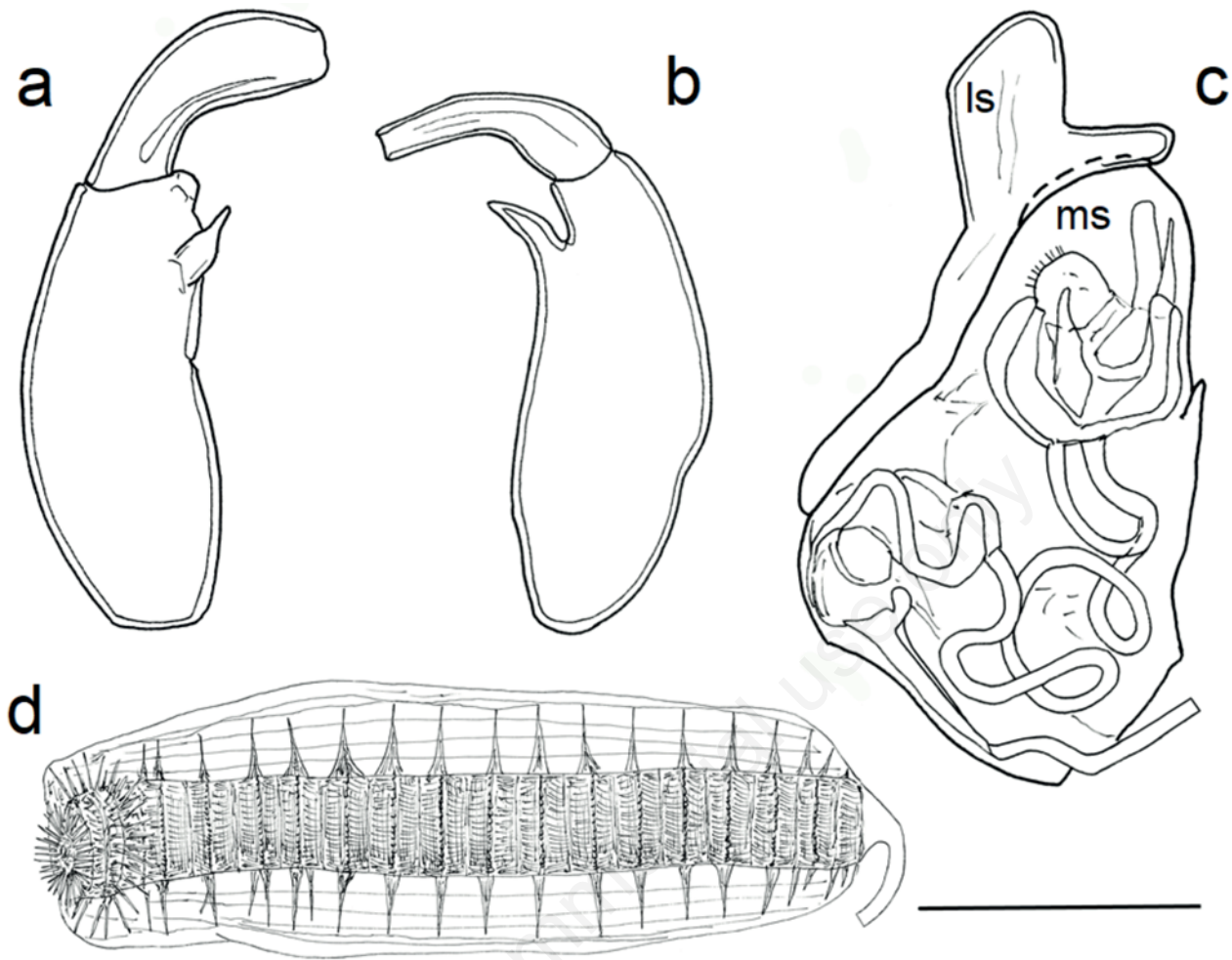


Fig. 8. *Cypridopsis vidua*, adult male, GR906. **a)** Left prehensile palp; **b)** right prehensile palp; **c)** hemipenis (ls, lateral shield; ms, medial shield); **d)** Zenker organ. Scale bar: **a-c)** 62 μ m; **d)** 50 μ m.

Prehensile palps of the first thoracopod (Fig. 8 a,b) asymmetrical. In both palps, first segment elongated and with protruding sensory organ in sub-apical position. Second segment of left palp rather stubby, with a distally rounded outer margin and a flat tip lacking sensory organ. Right palp with second segment shorter than the left one, distal part tapering with almost parallel margins; also this palp with flat tip and without distal flagellum-like expansion.

Hemipenes slightly asymmetrical. Medial shield with straight ventral margin, interrupted by a short, pointed process; dorsal margin widened in the central part; distal margin evenly rounded. Lateral shield thin and slightly curved proximally, distally with weakly rounded expansion and internally directed finger-like protrusion (Fig. 8c). Uropodal rami missing in the male as typical in Cypridopsinae. Zenker organ with 16-17 rosettes (Fig. 8d).

Remark

The morphology of the soft parts of the females and the appendages of males that are not sexually dimorphic is in agreement with what has been illustrated in the literature for *Cypridopsis*

vidua (Meisch, 2000), apart for the row of spines on the terminal claw of the second thoracopod (walking leg) which is more conspicuous in the male (*not shown*).

DISCUSSION

Males of *Cypridopsis vidua* have previously been reported for Europe, although these findings are considered doubtful (Havel and Hebert, 1989; Meisch, 2000), also due to the fact that the specimens were not figured. Weismann (1880) reported males in spring and summer and only females in autumn, with empty seminal receptacles and mature eggs from Germany. Spandl (1925) recorded the presence of males, albeit rare, in a population from an intermittent spring in northern Austria from May to October. Consequently, the description given in present paper is the first reliable one for the continent.

The condition of parthenogen for an ostracod species is not significantly affected by the presence of phenologically but non-functional males due to their incomplete sexual apparatus, or

when no sperm are observed and there is no evidence of copulation; the same applies when very rare males occur in populations with huge numbers of females (Schön *et al.*, 2009). In the sexual populations of *Cypridopsis vidua* so far described, the proportion of males is high, and sperm are present, and copulation has been documented in that of the United States (Martens *et al.*, 2023; Zhai *et al.*, 2023). Thus, it can be safely assumed that *C. vidua* is not an obligate parthenogen. However, given current knowledge, it remains to be defined what the effect may be, in evolutionary terms, of a limited number of few, isolated sexual populations surrounded by numerous asexual populations.

Certainly, recent discoveries may raise the possibility that the sexual lineages in this species complex are in fact widespread but largely overlooked. Failure to recognise males in *C. vidua* can be attributable to several factors. As pointed out by Meisch (2000), *C. vidua* is one of those ostracods that can be identified by the shape and colour of the carapace and for which dissection is not usually necessary, and consequently the soft parts are often not examined. When observed in transmitted light, the colouration of the valves and their thickness do not allow the impressions of seminiferous tubules or Zenker organs to be visualised in Recent specimens, unlike in males of most candonid and cypridinid ostracod species. In addition, the dark pattern and the morphology of the valves does not show obvious sexual dimorphism. Based on the measurements of a large number of specimens of *C. vidua*, Kesling (1951) reported average valve length values of 617 µm (range 580–660 µm) for adult females, and 528 µm (range 465–580 µm) for A-1 instar. This also makes it difficult to recognise possible males in fossil *C. vidua*, which may be misinterpreted as subadult females.

Coexisting sexual and asexual lineages have frequently been reported in non-marine ostracods (Chaplin, 1993; Rossi *et al.*, 2007). Martens *et al.* (2023) recorded the presence of sexual and asexual females (with and without sperm, respectively) in the *Cypridopsis vidua* population they studied, which could be distinguished by slight differences in the shape, size and colouring of the valves. Apart from size, such differences between females with and without sperm were not found in the Millepioppi population. Therefore, it seems unlikely from our preliminary observations that vision could be involved in mate recognition.

At this stage, we cannot say whether there are correlations between environmental gradients and the occurrence of sexual and parthenogenetic lineages, and whether these are ecologically segregated as reported, for example, for the geographic parthenogen *Eucypris virens* (Jurine 1820) (Schmit *et al.*, 2013a), a species complex with more than 40 cryptic species (Bode *et al.*, 2010).

Martens *et al.* (2023) carried out a thorough comparative analysis of the morphology of males of several species of the genus *Cypridopsis* closely related to *C. vidua*, all from North America, including one considered as valid (*C. howei* Ferguson, 1966) and two (*C. bisexualis* Cole, 1966 and *C. okeechobei* Furtos, 1936) now synonymized with *C. vidua* by Meisch *et al.* (2019), to which should be added *C. schwartzi* Kulköylüoglu *et al.* 2022 and *C. vidua* from China (Zhang *et al.*, 2023). The results of this analysis, although the male sexual characters are of high diagnostic value, show how difficult it is to taxonomically accommodate these forms with certainty.

The soft parts of the *Cypridopsis* males from Millepioppi pond shows some obvious differences with the descriptions provided by Martens *et al.* (2023) and Zhai *et al.* (2023), particu-

larly for the antennae and the prehensile palps. The antennae are the main locomotory structures for walking, climbing, digging, or swimming. In the Cypridoidea they are usually sexually dimorphic in structure and chaetotaxy and offer important taxonomic characters; the transformations observed in males are believed to play important sensory and prehensile roles before and during copulation (Meisch, 2000). The maxilliped of cypridoid males are modified into prehensile palps (or clasping organs), which are usually asymmetrical. During the mating process, an ostracod male grabs the female with the antennae and/or the prehensile palps to induce her to accept the copulation (Karanovic, 2012), and the palps are positioned at the female valve margins to keep the carapace open. The prehensile organs are and often diagnostic to distinguish between related species (Martens, 1991; Karanovic, 2019).

In the Italian population reported here, the males have the longest seta of the endopodite of the antenna exceeding the distal margin of the terminal segment, the aesthetasc Y is not bulging distally, and the t_1 and t_2 setae of the penultimate segment are of different lengths, as in the case of the males found in China; conversely, in the males reported from the United States, the endopodial seta is considerably shorter, the t_1 and t_2 setae are subequal, and the distal part of the aesthetasc Y is distinctly swollen. In the males from Millepioppi pond, the prehensile palps totally lack the long, distal membranous tip, most likely with sensorial functions (Horne *et al.*, 1998) on the distal segment, which is always present in males of all the above *Cypridopsis* species (apart from *C. howei*, for which they were neither illustrated nor described, so that no comparison is possible), albeit with different length and thickness. These differences are unlikely to be attributable to drawings that are not entirely accurate, perhaps with the only possible exception of *C. bisexualis*, where the distally displaced, small sensory organ on the first segment of the right palp is also missing. However, it should be emphasised that the detection under the microscope of small morphological details of the palps can be difficult if they are positioned unfavourably on the slide.

Current information is insufficient to assess whether the marked differences observed in the morphology of the soft parts involved in the copulation for the Millepioppi population, especially the distinctive prehensile palps, can be considered within the range of variability of the *C. vidua* complex or whether they are indicative of separate and/or reproductively isolated taxonomic entities. In this we opt for a conservative approach, considering it a sibling species within the *C. vidua* complex.

A morphological approach alone without the support of genetic data - and viceversa - is unlikely to definitively dispel doubts about the validity of morphospecies that show small differences from *Cypridopsis vidua*, and to clearly define the ranges of intraspecific variability for its characters, as well as the relationships between morphospecies and genetic species (if any, see for example Koenders *et al.*, 2017). Based on the genetic differences found in the *C. vidua* species complex (Gomes *et al.*, 2023), the presence of cryptic species can be assumed, as reported for other non-marine ostracods (Schön *et al.*, 2014, 2017; Karanovic, 2015). Interestingly, for the other two morphospecies of non-marine ostracods with cosmopolitan distribution, *Darwinula stevensoni* and *Penthesilenula brasiliensis*, the existence of cryptic diversity is also confirmed (Schön *et al.*, 2012). In these morphospecies, an increase in diversity in taxa will most likely result in their narrower geographic distribution (Baltanás and Danielopol,

2013). In general, it should be emphasized that morphological similarity is often subjectively assessed and rarely quantified to determine how similar cryptic species are (Struck *et al.*, 2018). At present, this turns out to be valid in the case of *C. vidua*. The occurrence of a bisexual population that displays homogeneity in the shape and dimensions of the valves, poses several questions about the paleontological applications such as the stability of a specie adult sex ratio in the fossil record (Martins, 2019).

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