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CHALLENGES IN ANOSTRACAN RESEARCH

Chirocephalus sarpedonis sp. nov. (Branchiopoda, Anostraca, Chirocephalidae) from Turkey questions the monophyly of the traditional *Chirocephalus* species-groups

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Abstract *Chirocephalus sarpedonis* sp. nov. (Anostraca, Chirocephalidae), collected in a temporary pond in Lycia (Turkey), is described and its affinities with the other species of the genus are investigated based on both morphology and mtDNA cytochrome oxidase subunit I (COI) sequences. Male and female morphology suggests its major affinity with the species belonging to the *bairdi*-group although the morphological peculiarities of the species make it difficult to ascribe *C. sarpedonis* sp. nov. to any of the *Chirocephalus* species-groups which are currently used in the systematics of the genus. Furthermore, molecular analyses based on the comparison with available *Chirocephalus* spp. COI sequences fail to confirm the monophyly of the *bairdi*-group and exclude *C. sarpedonis* sp. nov. from the *spinicaudatus*-group also. We thus refrain from assigning the new species to any of the existing *Chirocephalus* species-groups and highlight the need for a revision of the affinities and phylogeny of the species currently ascribed to the genus. In particular, the traditional *Chirocephalus* species-groups seem to be defined based on few, sometimes shared, characters, so that the definition of a new grouping of the species based on a combined morphological and molecular approach is desirable.

Keywords Molecular taxonomy · Anostracan phylogeny · Brood pouch morphology

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Graziella Mura: Deceased on 23 March 2016.

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Introduction

Reports of Anostraca, "the most distinctive of the living crustaceans in inland waters" (Rogers, 2015), have always been frequent in Turkey, possibly due to the noteworthy climatic and physiographic diversity of the country, coupled with its large territorial extension (Brtek, 1968; Cottarelli, 1971; Cottarelli & Mura, 1974; Beladjal & Mertens, 1997, and references therein). Moreover, from the 21st century onwards, when systematic studies on Turkish large branchiopods increased, a large number of new *Chirocephalus* Prévost, 1803 species were described (Mura et al., 2005; Brtek & Cottarelli, 2006; Cottarelli et al., 2007, 2010). The speciose genus *Chirocephalus* is primarily distributed in the temperate Palaearctic, with its greatest diversity in Eurasia (Belk & Brtek, 1995; Brtek & Thiéry, 1995; Brtek & Mura, 2000) and is well represented in Asiatic Turkey, where ten species belonging to the *bairdi* and *diaphanus* species-groups as defined by Brtek (1995) are currently known (Mura et al., 2011). Conversely, no *Chirocephalus* species have been reported to occur in Turkish Thrace.

Here, *C. sarpedonis* sp. nov., a new *Chirocephalus* species from Lycia characterised by some unprecedented features both in male and female morphology, is described and compared with congeneric species based on both morphology and mtDNA sequences.

The Chirocephalus *species-groups*

After Daday de Deés (1910, 1913) and Linder (1941), a number of other works dealing with anostracan taxonomy came in succession (see references in Rogers, 2013); in this framework, some important contributions to the taxonomy of the species-rich family Chirocephalidae were made by Brtek (1966, 1995) and Brtek & Mura (2000). In particular, Brtek (1995) divided *Chirocephalus* into five species-groups based on morphology, namely *diaphanus, bairdi, spinicaudatus, Pristicephalus,* and *sinensis*. Among these, the species-groups *diaphanus*-, *bairdi*-, and *spinicaudatus*- have been accepted and widely used in the recent literature (Brancelj & Gorjanc, 1999; Brtek & Cottarelli, 2006; Cottarelli et al., 2007, 2010; Ketmaier et al., 2003, 2012). Conversely, the *Pristicephalus* and *sinensis* species-groups proved to be quite controversial, as Brtek (1995) stressed.

Pristicephalus Daday, 1910, considered a synonym of *Chirocephalus* by Brtek (1966), was re-instated as an independent genus by Weekers et al. (2002), although this choice was not shared by subsequent workers, e.g. Rogers (2002, 2013), Brtek (2005), Horváth et al. (2013), Rogers & Soufi, (2013), and Marrone et al. (2016).

The *sinensis* species-group, as defined by Brtek (1995), includes the species *C. mongolianus* Ueno, 1940, *C. nankinensis* (Shen, 1933), and *C. sinensis* Thiele, 1907. *Chirocephalus mongolianus* was ascribed to *Galaziella* Naganawa & Orgiljanova, 2000 by Naganawa & Zagas (2003); later on, further species were ascribed to this genus (Alonso & Naganawa, 2008). The genus *Galaziella* was accepted by some authors (e.g. Marrone et al., 2015) and rejected by others (e.g. Rogers, 2013). Since this genus is not supported by modern taxonomic and systematic standards, we here consider *Galaziella* a junior synonym of *Chirocephalus*.

Materials and methods

Samplings and morphological analyses

The sample was collected using a hand net with 200 μm mesh. The sample was fixed in situ in 90% ethanol for both morphological and molecular analyses. In the laboratory, the sample was examined under a dissecting microscope; *Chirocephalus* specimens were sorted out and dissected with tungsten needles; the appendages were then mounted on microscope slides in Faure's medium. In order to avoid crushing the thicker parts, small plastic plates were included between the slide and the coverslip. A Coolpix 5000 digital camera with a photo tube was used for micrographs of selected parts. Drawings were made at different magnifications using a Zeiss Axioskop phase contrast microscope and a Wild stereo dissecting microscope, both equipped with drawing tubes.

Some aspects of the morphology of the new species were also observed by scanning electron microscope (SEM) micrographs. SEM observations were performed with a JEOL JSM 6010LA as described by Mura (2001). The stubs with body parts examined by SEM are deposited at the Interdepartmental Center for

Electron Microscopy (CIME), Tuscia University (Italy).

The morphology of collected anostracans was compared to the descriptions available for all the known species of *Chirocephalus*. Nomenclature of the body parts follows Brancelj & Gorjanc (1999) and Cottarelli et al. (2010). Specimens are deposited at the Museo di Storia Naturale, Sezione di Zoologia "La Specola", Università di Firenze, Italy (MZUF); other specimens are currently stored in FM's collection at the Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche of the University of Palermo, Italy.

DNA extraction, PCR amplification, and sequencing

Single individuals of *Chirocephalus sarpedonis* sp. nov., *Chirocephalus bairdi* (Brauer, 1877) (belonging to the *bairdi* species-group sensu Brtek, 1995), and *C. croaticus* Steuer, 1899 (belonging to the *spinicaudatus* species-group sensu Brtek 1995) were processed to be included in the molecular analyses. *Chirocephalus bairdi* and *C. croaticus* were identified using Brauer (1877), Daday de Deés (1910), and Brancelj & Gorjanc (1999). Further *Chirocephalus* COI sequences representative of all the available *Chirocephalus* species and major evolutionary lineages (Ketmaier et al., 2003, 2012; Reniers et al., 2013; Zarattini et al., 2013), and the outgroups *Polyartemiella hazeni* (Murdoch, 1884) (Chirocephalidae, Polyartemiinae) and *Phallocryptus* spp. (Thamnocephalidae) were downloaded from GenBank to be included in the analyses (see GenBank Accession Numbers in Fig. 6).

Prior to DNA extraction, the selected specimens were carefully cleaned under the stereomicroscope. Two thoracic appendages from each specimen were then cut and soaked in double-distilled water for 1 h. DNA was then extracted from the thoracic appendages with the Ron's Tissue Mini Kit (BIORON) following the manufacturer's protocol.

A fragment of the mtDNA gene encoding for cytochrome c oxidase subunit I (COI mtDNA) was amplified using the universal LCO1490 and HCO2198 primers described by Folmer (Folmer et al., 1994). The PCR mix consisted of 19.4 µl double-distilled water, 2.5 µl NH_4 Reaction Buffer 10X including $MgCl_2$ mM, 0.4 µl dNTPs (10 mM of each), 0.5 µl of each primer (10 µM), 0.2 µl DFS-Taq DNA Polymerase 5u/µl, and 1.5 µl of DNA template, for a total volume of 25 µl.

The amplification consisted of an initial denaturation step of 94°C for 5 min followed by 35 cycles of 94°C for 50 s, 48°C for 50 s, and 72°C for 60 s, followed by a final extension at 72°C for 5 min. After PCR, 5 µl of each PCR product was separated by electrophoresis on a 2% agarose gel stained with Ethidium Bromide at 90 V for 20 m and visualised with a UV Transilluminator. When PCR products showed a clear and single band of the correct expected length, they were purified using the Exo-SAP-IT® kit (Affymetrix USB) and sequenced by Macrogen Inc. (Seoul, South Korea) with an ABI 3130xl (Applied Biosystems) sequencer. Both the forward and reverse primers were used for direct sequencing of the PCR product. Chromatograms were imported and edited with ChromasLite 2.01 (Technelysium Pty. Ltd., South Brisbane, Australia) and aligned with BioEdit (Ibis Biosciences, Carlsbad, CA, USA) (Hall 1999). The sequences were deposited in GenBank (Accession No. KY399030-KY399032).

Molecular analyses

The software Mega6 (Tamura et al., 2013) was used to translate in amino acids the obtained COI sequences in order to check for the possible presence of frameshifts or premature stop codons, which would indicate the presence of sequencing errors or pseudogenes, a widespread phenomenon among crustaceans (Marrone et al., 2013; Lindholm et al., 2016; Kappas et al., 2017, and references therein). The degree of substitution saturation in our dataset was tested with the entropy-based index of substitution saturation approach implemented in the software DAMBE (Xia & Xie, 2001).

Bayesian inference (BI) of phylogeny was performed on the COI dataset as implemented in MrBayes 3.2.6 (Ronquist et al., 2012). MrModeltest 2.2 (Nylander, 2004) was used to test for the best fitting model of nucleotide substitution for our dataset under the Akaike information criterion, resulting in a Hasegawa, Kishino, and Yano model of sequence evolution for molecular data with a proportion of invariable sites (Pinvar: 0.4571) and gamma-distributed rate variation among sites (shape: 0.7889) (HKY + I + G). The analyses were performed using the corresponding evolutionary

model (Prsetstatefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 6 rates = invgamma). Node supports were evaluated by their posterior probabilities in the BI tree. The BI analysis was performed with two independent runs of 2,000,000 generations and four Markov chains using default heating values. Trees and parameter values were sampled every 100 generations resulting in 20,000 saved trees per analysis. An initial fraction of 5,000 trees (25%) was conservatively discarded as "burn-in". For all analyses, standard deviation of split frequencies reached values lower than 0.019985, and values of the potential scale reduction factor (PSRF) were between 1.000 and 1.010 for all parameters, indicating convergence of the runs.

Results

Taxonomy

Class Branchiopoda Latreille, 1817
Subclass Sarsostraca Tasch, 1969
Order Anostraca Sars, 1867
Suborder Anostracina Weekers et al. (2002)
Family Chirocephalidae Daday de Deés (1910)
Subfamily Chirocephalinae Daday de Deés (1910)
Genus *Chirocephalus* Prévost, 1803

Chirocephalus sarpedonis sp. nov.
(Figures 1, 2, 3, 4, 5; Online resources A1 and A2)

Note: authorship of the new species is attributed to VC and FM and should be cited as "Cottarelli and Marrone" in "Cottarelli et al." (ICZN, 2000, Recommendation 51E)

Examined material. 12 adult males; 6 adult females; Giuseppe Ippolito legit, 14th April 2015.

Type series: Holotype: adult male, 11.5 mm, partly dissected and mounted on Faure's medium on slides marked "*Chirocephalus sarpedonis* holotype male", and numbered from 1 to 13. Undissected parts stored in EtOH 70% and glycerine in a tube labelled "*C. sarpedonis* holotype male". Holotype stored in MZUF, Firenze, registration number 617.

Allotype: one ovigerous female, 11.9 mm, dissected and mounted on Faure's medium on slides marked "*C. sarpedonis* allotype female", and numbered from 1 to 8. Undissected parts stored in EtOH 70% and glycerine in a tube labelled "*C. sarpedonis* allotype female". Allotype stored in MZUF, Firenze, registration number 618.

Paratypes: Two paratypes, an adult male and an adult female, are stored in EtOH 80% in MZUF, Firenze, registration number 619.

The stubs with body parts prepared for SEM are deposited at the Interdepartmental Center for Electron Microscopy (CIME), Tuscia University (Italy).

Further 5 females and 8 males are stored in ethanol 90% in the crustacean collection of FM at the University of Palermo, Italy.

Locus typicus. The "Sidyma pool". Coordinates WGS84: 36.410350°N, 29.194327°E; elevation: 544 m a.s.l. (Online resource fig. A1) The pond is located within a Hellenistic necropolis near the ancient Lycian town of Sidyma (Σίδυμα), close to the village of Dodurga Asari in Muğla Province, Turkey. The new species was collected in a clayey temporary pond without aquatic macrophytes. At the sampling date, the pond was experiencing its drying phase. Co-occurring crustaceans, belonging to the classes Branchiopoda, Copepoda, and Ostracoda, are currently under study and will be discussed in a later work.

Derivatio nominis. The specific epithet is the masculine singular genitive of Sarpedon, the Latin name of a mythical hero and Lycian king, son of Zeus and Laodamia, cited in the Homer's Iliad.

Diagnosis. Male antennal appendage bilamellar, with the dorsal lamella long and triangular bearing robust digitiform lateral projections. Ventral lamella long and narrow, a little shorter than the upper lamella (Fig. 1D). The antenna proximal antennomere bears a short, stout, conical, truncated apophysis on the medial surface (Fig. 1C, 4E, F). Distal antennomere with a proximal, lamellar branch, with 5–6 distal conical projections (Fig. 1C, E).

Females. The eighth and ninth thoracic somites bear on their medial line a rounded bulge. On the medial line, the tenth and eleventh somites bear an apophysis equipped with two rounded tubercles (Fig. 3E). The brood pouch is highly peculiar, with a large and rounded proximal part linked with a narrow cylindrical distal part (Fig. 3F, H). Abdominal somites I–V each bear a pair of lateral spiniform projections (Fig. 3H).

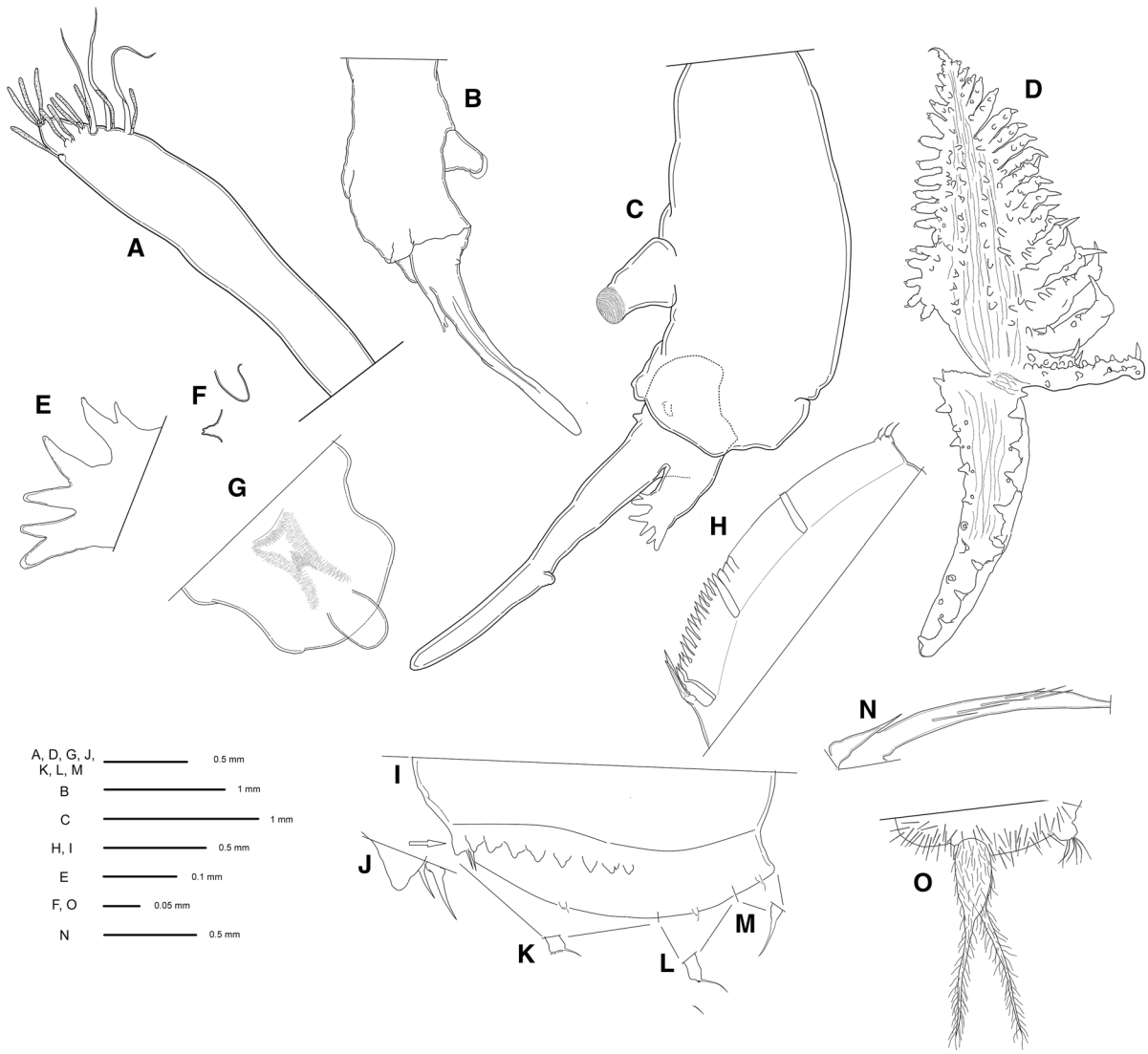

Fig. 1 *Chirocephalus sarpedonis* sp. nov. Male. **A** Apical ornamentation of the antennula; **B** antenna, dorsal view; **C** antenna, ventral view; **D** antennal appendage, dorsal and ventral lamellae; **E** apical part of the basal branch of the distal antennomere; **F** basal tubercles of the second antennomere; **G** labrum, ventral view; **H** right mandible; **I** left mandible; **J** posterior, curved spines; **K**–**M** detail of a denticle of the first (**K**), second (**L**), and third (**M**) groups (for details see text); **N** maxilla I, posteroventral, modified spine; **O** maxilla II

Description

Male. Average length of the preserved material (12 males examined), from the anterior margin of the head to the tip of cercopods: 11.4 mm. Length range: 10.7–11.6 mm. Colour of the specimens in vivo: scarlet. Head large, thorax longer than the abdomen without cercopods. Cercopods subequal in length to the last four abdominal somites combined. On different parts of the body, e.g. on the dorsal surface of the antennae, sensorial areas constituted by small chitinous denticles surrounding a central sensillum. Head typical of the genus; compound eyes and naupliar eye present. Compound eyes half the length of the proximal antennomere of the antenna.

Antennula slightly shorter than the first antennomere of the antenna, with a distal roughly claviform apex bearing three long flattened setae subapically and 11 thin aesthetascs apically (Fig. 1A).

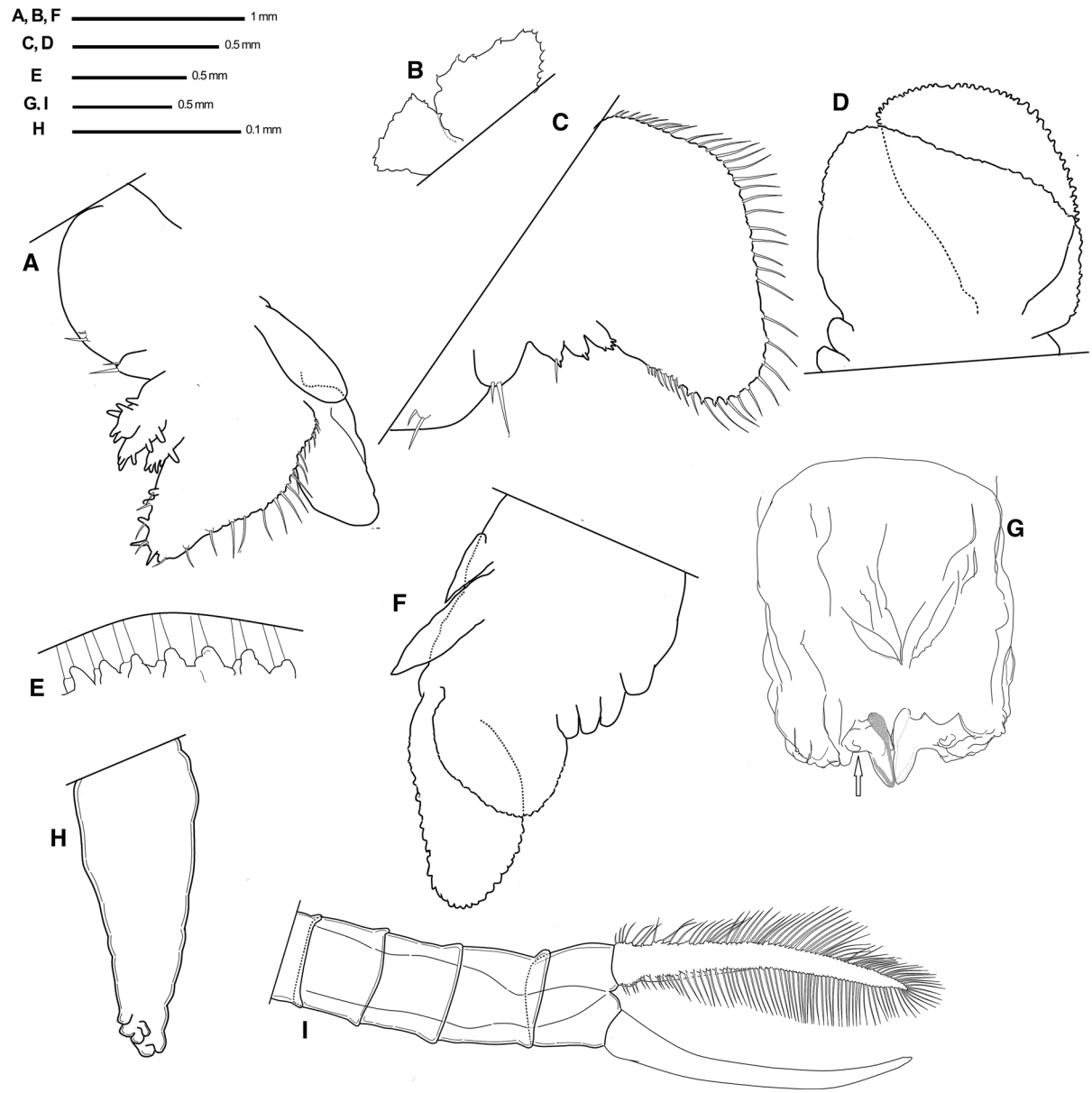

Fig. 2 *Chirocephalus sarpedonis* sp. nov. Male. **A** I thoracopod; **B** pre-epipodites of the I thoracopod; **C** endopodite and endites of the VI thoracopod; **D** exopodite and endopodite of the VI thoracopod; **E** distal margin of the exopodite of the VI thoracopod; **F** XI thoracopod; **G** gonopods, ventral view; **H** apex of the distal eversible portion of the gonopods; **I** cercopods and the last four abdominal somites

Antenna (Figs. 1B, C, 4A, E) rather long and slender. Proximal antennomere (Fig. 1C) subcylindrical, slightly expanded distally, with apophysis at 2/3 length of medial margin. Apophysis (Figs. 1C, 4E, F) subconical, truncated, with a rounded apex covered by thick chitinous denticles (length\width ratio: 0.96). Antenna distal antennomere slightly shorter than proximal antennomere, slightly curving medially, with a basal, lateral branch and a dorsally directed medial protrusion, located at approximately 60% of the antennomere length. Branch approximately one-third of the length of the distal antennomere. Branch flattened, spatulate and digitate, with digitate projections as much as one-fifth branch length (Figs. 1C, E,

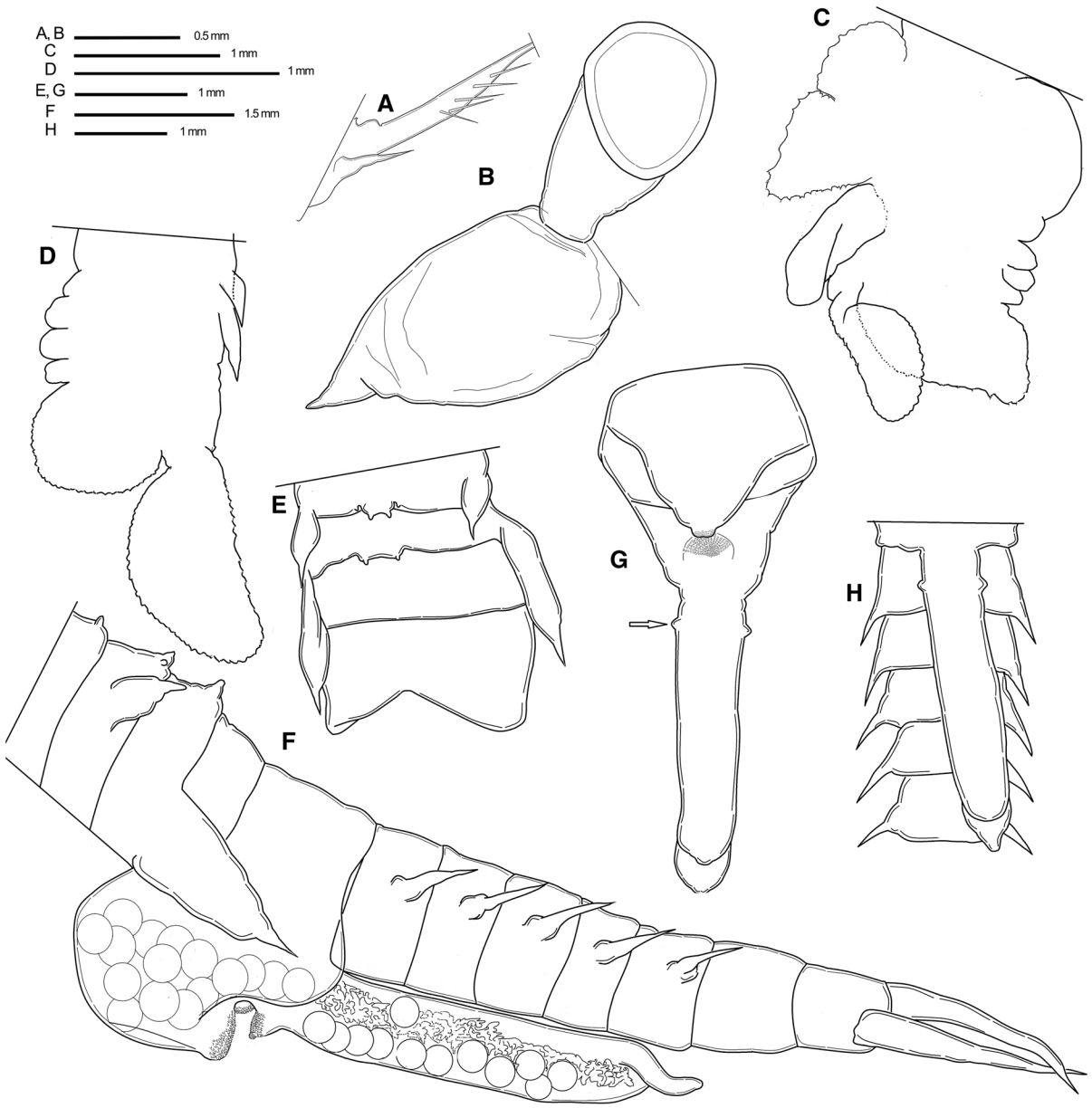

Fig. 3 *Chirocephalus sarpedonis* sp. nov. Female. **A** maxilla I, posteroventral modified spine; **B** second antenna and stalked, compound eye, lateral view; **C** I thoracopod; **D** XI thoracopod; **E** X and XI thoracic somites, and genital somites, dorsal view; **F** IX–XI thoracic somites, genital somites, brood pouch, abdominal somites, telson, and cercopods, lateral view; **G** brood pouch, ventral view; **H** brood pouch (*partim*) and abdominal somites I–V

4D). Antennomere base with small tubercles opposite or adjacent to basal branch (Figs. 1C, F, 4D). Medial protrusion rounded, covered in fine denticles (Figs. 4C, 5B). Antenna distal antennomere with medial and lateral surfaces each bearing a longitudinal groove; portions on the distal side of this antennomere with fine denticles (Figs. 4C, 5A). Antennomere apex rounded, directed distally.

Antennal appendage bilamellar (Fig. 1D). Dorsal lamella broadly triangular, 80% the length of the antenna proximal antennomere, with digitiform papillae along medial and lateral margin, with small

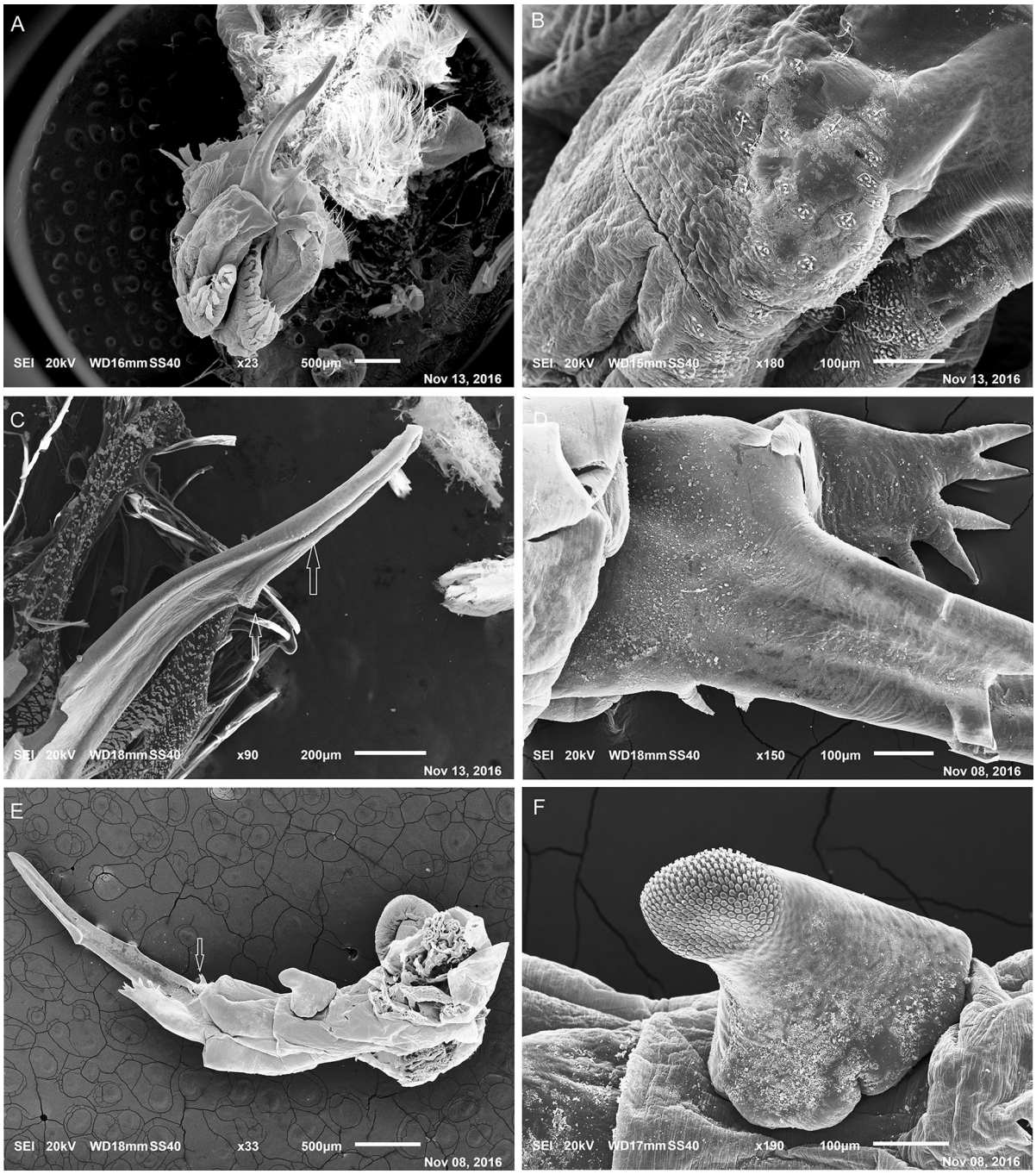

Fig. 4 SEM micrographs of *Chirocephalus sarpedonis* sp. nov. Male: figs. **A–E**. **A** antenna and antennal appendages, dorsal view; **B** antenna, sensillary areas on the proximal antennomere, dorsal view; **C** distal part of the second antennomere, ventral view; **D** second antennomere, proximal lamellar branch and diametrically opposed occurring tubercles; **E** antenna, ventral view; **F** antenna, apophysis of the proximal antennomere

scattered papillae ventrally. Medial marginal papillae with sharp points. Apical papilla tipped. Lateral marginal papillae longer proximally, becoming shorter and less complex distally. Proximolateral papillae tipped, with smaller papillae ventrally. Ventral lamella narrowly triangular, slightly curved,

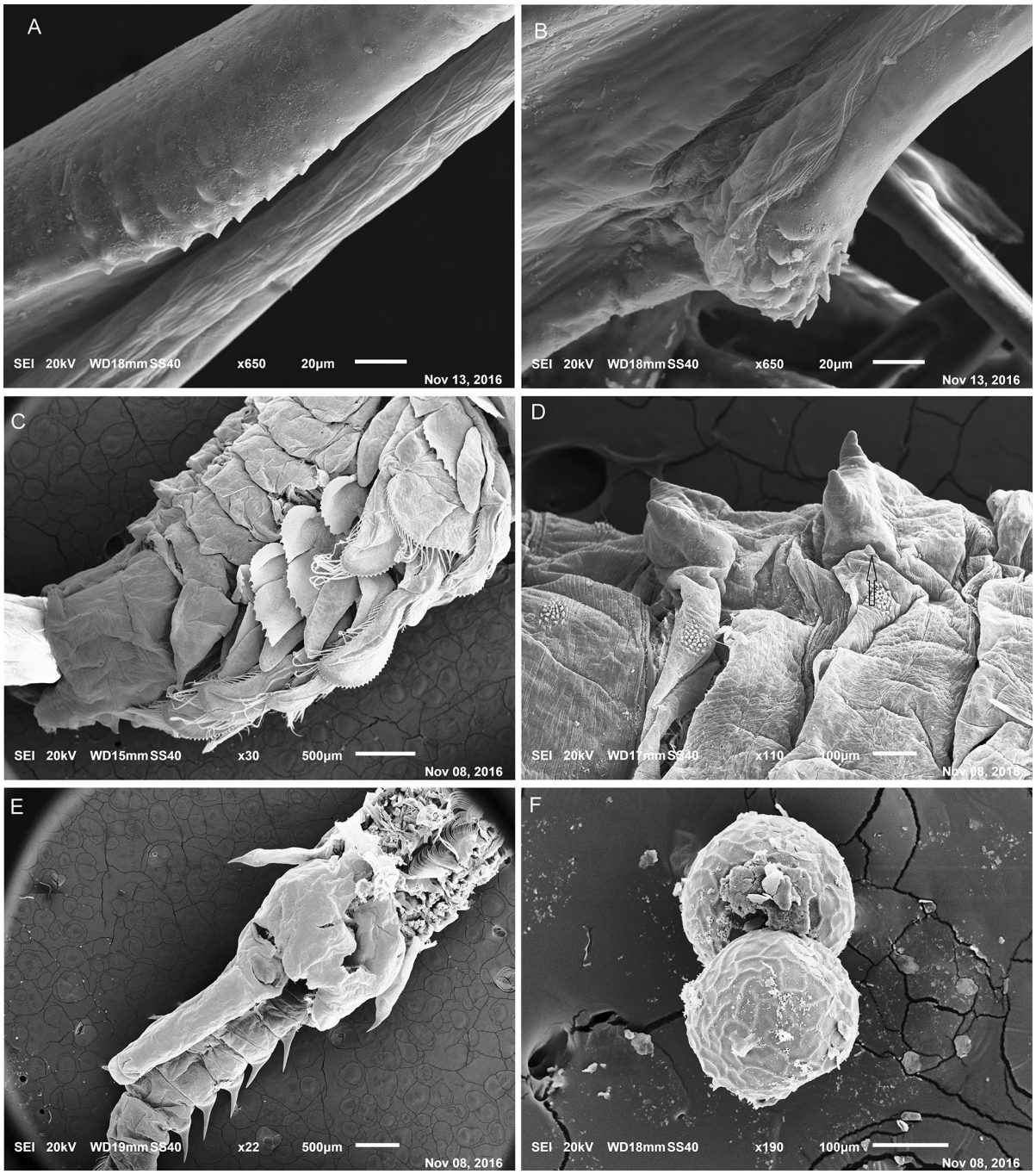

Fig. 5 SEM micrographs of *Chirocephalus sarpedonis* sp. nov. Male: figs. **A**–**B**; female: figs. **C**–**F**. **A** distal antennomere of the antenna, denticled margin, ventral view; **B** distal antennomere of the antenna, denticled medial protrusion; **C** VIII–XI thoracic somites and genital somites, lateral view; **D** medial bulge of the IX thoracic somite and apophysis of the X and XI thoracic somites, lateral view; **E** lateral laminar outgrowths of the XI thoracic somite, brood pouch, and abdominal somites, ventral view; **F** resting eggs

slightly shorter than dorsal lamella, with small, simple, dorsally directed marginal papillae.

Labrum (Fig. 1G). Ventral surface with two paired longitudinal rows of setules, arcuate medially; joined with transversal row of spinules anteriorly. Labral projection rounded, extending 50% of length beyond labral margin.

Mandibles (Fig. 1H, I) asymmetrical; right mandible molar surface with two sharp spines, subequal in length posteroapically (Fig. 1H). Left mandible molar surface with a row of nine robust denticles along free margin;; the first two posterior denticles separated by two robust, slightly curved spines (Fig. 1I, J). Left mandible molar with three groups of denticles with different morphology distally, here referred to as first (Fig. 1K), second (Fig. 1L), and third (Fig. 1M) group.

Maxilla I. Typical for the genus, with 18 setae apically. Setae aciculate and straight. Maxilla I with modified aciculate and straight spine posteroventrally (Fig. 1N).

Maxilla II (Fig. 1O) a flat, rounded setulose lobe with two subequal, robust pinnate setae, expanded proximally. Distal margin with small, rounded setulose process.

Thoracic somites typical for the genus, smooth, without ornamentation.

The thoracopod I (Fig. 2A, B) endopodite forming a right angle with the rest of the appendage. Margins of the endites IV–VI and endopodites of the thoracic appendages with some projections of different shape and size (Fig. 2A, C), acute on the endites, rounded in the endopodites,. The exopodite of all the thoracopods bears marginally some pointed projections (Fig. 2D, E). Eleventh pair of thoracopods illustrated in Fig. 2F.

Genital somites. Gonopods typical for the genus. Not-retractile portion long and narrow (Fig. 2G), with a curved tubercle distolaterally (arrow in Fig. 2G). Distal retractile portion nearly straight, ending into an apex with 5–6 tiny tubercles (Fig. 2H).

Abdominal somites smooth, without projections.

Cercopods (Fig. 2K) nearly as long as last four abdominal somites combined, lateral and medial margins with plumose setae.

Female. Average length of the preserved material (6 females examined): 11.7 mm. Length range: 11.1-11.9 mm.

Antennula, labrum, mandibles, maxilla II as in the male. Maxilla I: the posterior ventral modified spine (Fig. 3A) shorter than the homologous spine in the male.

Antenna (Fig. 3B). As long as the antennula; uniarticulate, laminar and flattened; apex pointed and curved, with tiny setae and circular sensorial areas dorsally, as in the male.

Thoracopods. The endopodite and the endites of the thoracopod I differ from those of the male: the endopodite is larger, and marginal tubercles are absent on the endites (Fig. 3C). The sixth pair of thoracopods lack the tubercles reported for the endites of the male. The eleventh pair of thoracopods (Fig. 3D) identical to that of the male.

Thoracic somites. On each somite there are sensorial patches dorsolaterally (Fig. 5D), and which occur on the abdominal somites also. Eighth and ninth somites with a rounded bulge on their medial line (Fig. 5C). Tenth and eleventh somites dorsally with a lateral outgrowth equipped with two rounded tubercles medially (Figs. 3E, 5D). On the tenth somite, each of these bulges accompanied by two lateral smaller tubercles (see arrowed tubercles in Fig. 5D). The same somites also with lateral thin and pointed laminar outgrowths, protruding externally (Fig. 3E, F). Laminar outgrowths of the tenth somite rather small, originating dorsolaterally on the somite itself; the ones of the eleventh somite large, diaphanous and rather long: in lateral view nearly reaching the length of the second genital somite. Furthermore, these are inserted lower on the somite than the laminar outgrowths occurring on the tenth somite (Fig. 3F).

Genital somites and brood pouch (Figs. 3G, H, 5A). Genital somites unadorned; the first one reaches half the length of the second; the two genital somites separated both dorsally and laterally; the amplexial groove unadorned (Fig. 3E, F).

Brood pouch: in lateral view, a large, rounded proximal part, as wide and long as the two genital somites from which it originates, followed by a long, cylindrical distal part reaching up to the sixth abdominal somite. The upper, distal part of the gonopore well-developed, protruding on the lower margin of the gonopore itself (Fig. 3F). The cylindrical part of the brood pouch with two lateral tubercles proximally (arrowed in Fig. 3G). Between the proximal and distal parts of the brood pouch there is a ventral sulcus, and the proximal ending of the distal part of the brood pouch presents a rounded apophysis (Fig. 3G); these two parts are connected ventrally by a denticled

surface, and other thick denticles are spread along the margins (Fig. 3F, G). In ventral view, the proximal part of the ovisac has a roughly pentagonal aspect.

Abdominal somites (Figs. 3H, 5E). Each of the abdominal somites following the genital somites with a couple of sharpened lateral spiniform outgrowths. On each abdominal somite several sensorial patches, as on the thoracic somites.

Cercopods (Fig. 3F). Similar to those of the male, but slightly shorter than the last three abdominal somites combined.

Eggs spherical (Fig. 5F) with an average diameter of 275 μm ($N = 10$). The outer surface appears somewhat "polygonal- reticulated", with low intersecting ridges. Tertiary shell represented by two alveolar layers, outer cortex, inner alveolar layer, separated by a subcortical space.

Observed variability and dimorphism. Studied characters proved to be constant on the studied specimens with the only exception, in the males, of the number of the apical finger-like tubercles of the proximal apophysis of the second antennomere, which varied from five to seven, and of the number of the small proximal bulges occurring on the ventral-medial surface of the second antennomere. Moreover, the digitiform expansions on the proximal part of the outer margin of the dorsal lamella varied in number from five to eight.

The sexual dimorphism pertains to, beside the antennae, (i) the shape and ornamentation of the endopodite and endites of the thoracopods (especially of thoracopod I), (ii) the dorsal and lateral ornamentation of the thoracic somites VIII–XI and of the postgenital somites I–V, (iii) the length of the cercopods (shorter in females), and (iv) the total length (females are slightly larger).

Morphological affinities of the species

Chirocephalus sarpedonis sp. nov. does not closely resemble any of the currently known *Chirocephalus* species, showing a combination of morphological features which are individually to be ascribed to different species and species-groups within the genus.

The upper lamella of the antennal processes of *C. sarpedonis* sp. nov. resembles those which can be observed in *Chirocephalus* species belonging to different species-groups, and the morphology of the lower lamella is similar to that of the single lamella occurring in *Pristicephalus* (i.e. the "*Pristicephalus* species-group" sensu Brtek, 1995). The contemporary presence of a "generalised" upper lamella and of a slender lower lamella finds a counterpart in *C. vornatscheri* Brtek, 1968, *C. murae* Brtek & Cottarelli, 2006, and *C. orghidani* Brtek, 1966, all of these taxa belonging to the *bairdi* species-group. However, the lower lamella of a Macedonian population of *C. brevipalpis* (Orghidan, 1953) has a lateral lobe, which is absent in *C. sarpedonis* sp. nov. (Petkovski, 1991).

However, it also has to be stressed that the taxonomic value of the morphology of the basal lamina in *Chirocephalus* has been recently questioned by Reniers et al. (2013), who considered that it may not be informative based on a contrast between morphological and mtDNA-based molecular identification of the samples for some members of the *diaphanus* species-group.

The morphology of the second antennae, showing an enlarged distal portion ending in a median tubercle on its inner side, is similar to that of the Turkish species belonging to the *bairdi* species-group, *C. vornatscheri*, *C. kerkyrensis* Pesta 1921, *C. brteki* Cottarelli et al. (2010), *C. murae*, and in *C. bairdi* itself. In these species, however, the second antennae are stouter and more curved than those of *C. sarpedonis* sp. nov.

The proximal apophysis of the first antennomere is similar to that of *C. murae* and *C. brevipalpis* (*bairdi* species-group). Conversely, the proximal apophysis of the second antennomere of the new species is closer to that of the species of the *spinicaudatus* species-group, such as *C. spinicaudatus* Simon, 1886, *C. chyzeri* Daday, 1890, and *C. povolnyi* Brtek, 1967. Seemingly similar proximal apophyses showing tubercles and digitiform processes are also known for other species-groups, e.g. in *C. algidus* Cottarelli et al., 2010 (belonging to the *diaphanus* species-group), and *C. brteki* (belonging to the *bairdi* species-group); however, in these last two taxa the apophysis is conical and pointed.

The thoracopods of the first, sixth, and eleventh somites are similar to those of *C. algidus* (*diaphanus* species-group) and to those of several species of the *spinicaudatus* species-group (see *C. croaticus* in: Brancelj & Gorjanc, 1999).

Although diagnostic for anostracan genera (Linder, 1941; Brendonck, 1995; Brendonck & Belk, 1997; Rogers, 2002), the gonopod morphology has poor taxonomic value to discriminate *Chirocephalus* species-groups: in the same species-group, it is often possible to find stout and short *vs.* thin and slender apophyses (see *C. brteki* vs. *C. murae* and *C. anatolicus* Cottarelli et al., 2007). *Chirocephalus sarpedonis* sp. nov. presents long and narrow projections on the gonopods, with a couple of curved tubercles at the distal margin and on their ventral surface (Fig. 2G).

In the females, the presence of dorsal bulges and tubercles on the thoracic and abdominal somites occurs in representatives of different species-groups, e.g. *C. murae* (*bairdi* species-group) shows on the VIII thoracic somite a bulge equipped with two rounded tubercles resembling that of *C. sarpedonis* sp. nov. Conversely, the lateral laminae on the X and XI thoracic somites have nearly no counterparts within the genus; only in *C. croaticus* (*spinicaudatus* species-group), 50% of the females show "wing-like projections" on the XI thoracic segment (Brancelj & Gorjanc, 1999). Moreover, *C. soulukliensis* Rogers & Soufi, 2013 (doubtfully attributed to the *Pristicephalus* species-group by the describers) females show a "thoracic segment XI with a lateral posteriorly directed spiniform lobe projecting posteriorly over the amplexial groove" (Rogers & Soufi, 2013). *Chirocephalus horribilis* Smirnov, 1948 and *C. robustus* Müller, 1966 (both belonging to the *spinicaudatus* species-group) and *C. brevipalpis* (*bairdi* species-group) present some projections on the X thoracic somite, but they are different from those occurring in *C. sarpedonis* sp. nov.

The species of the *spinicaudatus* species-group (e.g. *C. tereki* Brtek, 1984) have spiniform lobes on the genital and post-genital somites but, as already noticed by Daday de Deés (1910), they show "segmenta thoracalia omnia inermia" (i.e. the thoracic segments are all unadorned).

The strong spiniform outgrowths of the abdominal somites of the female *C. sarpedonis* sp. nov. closely resemble those present in the *spinicaudatus* species-group; similar structures are known also for other taxa, e.g. *Chirocephalus carnuntanus* (Brauer, 1877) (belonging to the *Pristicephalus* species-group according to Brtek, 1995) and *C. brevipalpis* (*bairdi* species-group), but in these taxa the outgrowths are sensibly less developed than in *C. sarpedonis* sp. nov. and in the *spinicaudatus* species-group. Other species in the *bairdi* and *diaphanus* species-groups have tubercles on the female abdominal somites, but these are small and rounded, never spiniform.

The brood pouch of *C. sarpedonis* sp. nov. is unprecedented among the Chirocephalidae and constitutes an important autapomorphy of the species.

Resting egg morphology closely resembles that of *C. tauricus* Pesta, 1921 (*diaphanus* species-group) and is similar to that of *C. brteki* (*bairdi* species-group), being thus consistent with the "*tauricus-appendicularis* pattern" (Mura, 2001; Mura et al., 2002).

Molecular results

Novel sequences were deposited on GenBank with the following accession numbers: KY399030 (*Chirocephalus bairdi*), KY399031 (*C. sarpedonis* sp. nov.), and KY39902 (*C. croaticus*). The alignment of the novel amplified COI fragment and those downloaded from GenBank did not demonstrate any gap or insertion and, after having trimmed the tails which were not present in all the individuals, led to a COI aligned fragment of 440 bp. The entropy-based index of substitution saturation evidenced little or no sequence saturation (Iss: 0.3336; Iss.c: 0.7013; P: 0), so that we used all codon positions in our phylogenetic analysis.

The obtained BI phylogenetic tree shows a moderately supported topology with a clustering of the species which does not fit with the one expected according to the species-groups defined on morphology (Fig. 6). Furthermore, *Chirocephalus diaphanus* Prévost, 1803, and *Chirocephalus ruffoi* Cottarelli & Mura, 1984, are shown to be largely paraphyletic (cf. also Reniers et al., 2013, and references therein).

Discussion

Both males and females of *Chirocephalus sarpedonis* sp. nov. cannot be unanimously attributed to any of the species-groups defined by Brtek (1995) for the genus *Chirocephalus* since they present a patchwork of morphological features currently considered diagnostic of different species-groups, coupled with peculiar characters, unprecedented in the genus. The same applies to the morphology of the resting eggs, which

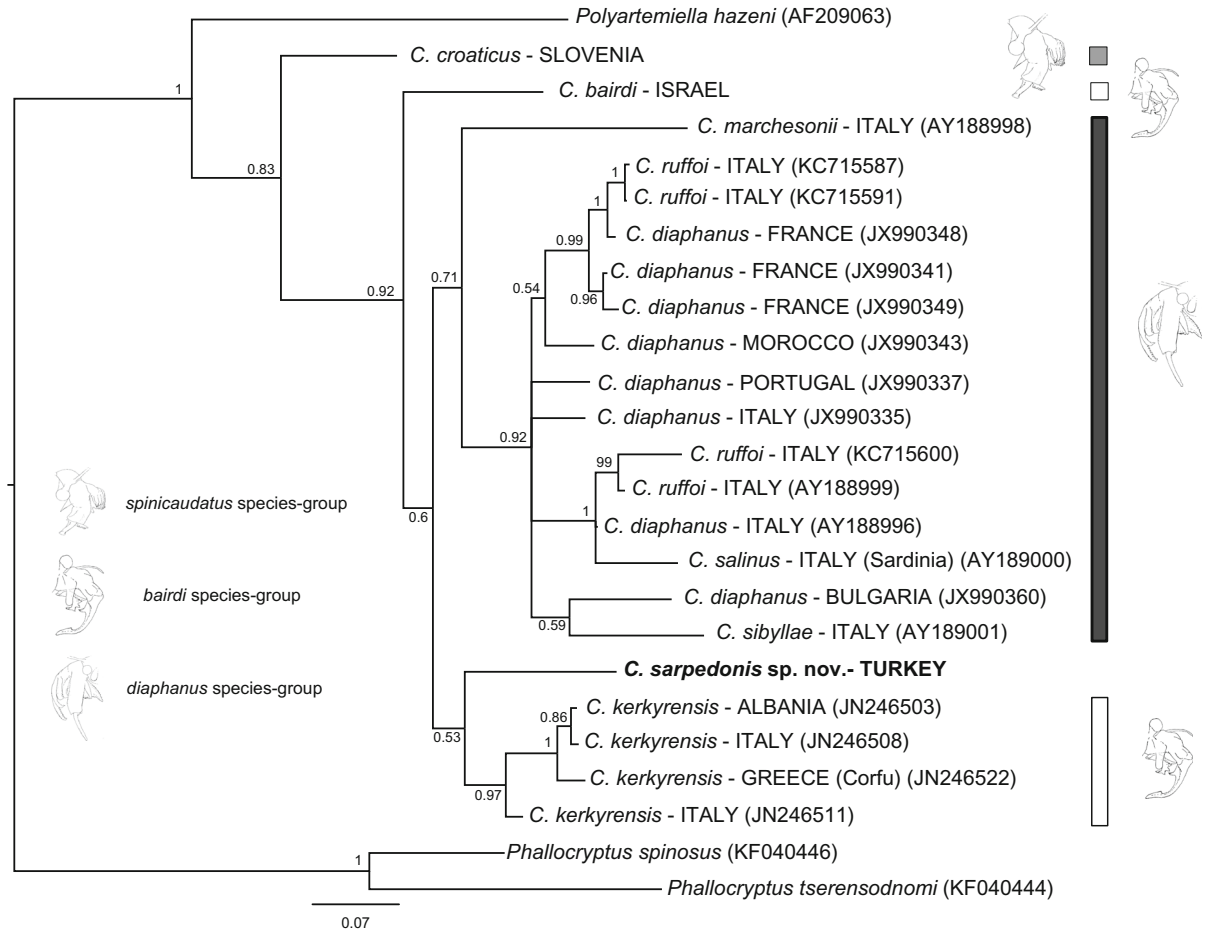

Fig. 6 Bayesian consensus phylogram based on a 440-bp-long fragment of the mitochondrial gene encoding for the cytochrome oxidase subunit I (mtDNA COI). Node support is reported as nodal posterior probabilities. Accession numbers of sequences derived from GenBank are shown in *brackets*. See the text for the GenBank accession numbers of the novel sequences. *Chirocephalus sarpedonis* sp. nov. is reported in *bold*. *Rectangles* stress the species-groups to which each taxon is traditionally attributed; *black diaphanus* species-group; *grey spinicaudatus* species-group; *white bairdi* species-group. *Drawings* represent heads of males belonging to different species-groups in lateral view (modified from Brtek & Mura, 2000)

would lead to the identification of species-groups contrasting with those based on adult morphology (Mura, 2001; Mura et al., 2002). Furthermore, the phylogenetic tree based on mtDNA COI does not support the monophyly of Brtek's species-groups and merely suggests a close relationship between the new taxon and *C. kerkyrensis* (currently ascribed to the *bairdi* species-group) (Fig. 6). However, it is also to be stressed that the used COI sequences did not allow us to obtain a strongly supported topology, so that the implementation of alternative molecular markers for investigating *Chirocephalus* phylogeny is desirable.

Our results, in accordance with the morphological works of Brtek (1995), Rogers (2005), and Rogers & Soufi (2013), and with the available molecular results (Reniers et al., 2013), thus highlight that the currently described species-groups are in need of being tested and revised, since they are in contrast one with the other, and both with the first molecular evidences we are gathering. Moreover, the new *Chirocephalus* species described here cannot be definitively ascribed to any of the existing species-groups, showing that some morphological features to date considered to be diagnostic of the species-groups are, in fact, shared by different groups.

In order to obtain a classification and arrangement of the *Chirocephalus* s.l. taxa based on their actual phylogeny, it is needed to review and update the old

species descriptions to the modern taxonomic standards for the Anostraca. In particular, attention should be paid to microcharacters, such as the presence of chitinous denticles on the antennae, and\or the morphology and ornamentation of labrum, mandibles, and first and second maxillae. Such characters were here described but could not be compared with those occurring in several other *Chirocephalus* species due to the lack of such information in the available species description.

In conclusion, *C. sarpedonis* sp. nov. seems rather close to the species of the *bairdi* species-group (e.g. for the morphology of male antenna and of the thoracic somites in females) although both males and females show some characters currently considered typical of the *spinicaudatus* species-group, and an unprecedented morphology of the brood pouch, thus preventing us from assigning the species to this group. Such results are supported by the molecular analysis, which suggests a close relationship between the new species and *C. kerkyrensis*, but also shows that these two taxa do not form a monophylum with *C. bairdi*, the eponymous species of the *bairdi* species-group.

Some adaptive and evolutionary remarks on observed morphology

Rogers (2002) and Rogers & Hamer (2012) studied the amplexial morphology of anostracans, stressing the importance of the "amplexial groove", i.e. the area comprised between XI thoracic somite and the brood pouch. With the exceptions of *Polyartemia* Fischer, 1851 and *Polyartemiella* Daday, 1910, anostracan males use the amplexial groove, which might be unadorned or complexly ornamented, to embrace the female during amplexus. In the Parartemiidae and in the Chirocephalidae, the amplexial groove shows morphological features which complement the ornamentation of the male's second antennae, creating a 'lock and key' fit unique to each species. According to Rogers (2002), based on observations made on *C. spinicaudatus*, male *Chirocephalus* amplexes the female on the thorax at the base of the brood pouch. Conversely, the peculiar morphology of the brood pouch of *C. sarpedonis* sp. nov. suggests the possibility that in this species males might embrace the females directly on the brood pouch, inserting the distal antennomeres into the sulcus of the brood pouch, where they could be held with the help of the denticles occurring on the antennomeres themselves, and of those denticles occurring on the sulcus and neighbouring areas (Fig. 3F, G). Analogous structures on the brood pouch are known also for *C. graziellae* Rogers, 2013 (syn. *Galaziella murae* Alonso & Naganawa, 2008), which presents dorsolateral verrucose outgrowths which might be useful for the amplexus (Alonso & Naganawa, 2008), and for the chirocephalid *Linderiella baetica* Alonso & García-de-Lomas, 2009, which presents lateral fields of filiform setae on the brood pouch that likely facilitate amplexus (Alonso & Garcia de Lomas, 2008). This is currently just a hypothesis, which should be tested with the observation of living specimens, and analogous observations should be made on other *Chirocephalus* species.

Another morphological peculiarity of the species is the presence of well-developed lateral laminar expansions on the X and XI thoracic somites and on some abdominal segments of the females. It can be hypothesised that these lateral laminar wings might constitute a hydrodynamic adaptive trait apt at counterbalancing the weight of the brood pouch, thus helping to save energy during the swimming.

Brief remarks on the genus Chirocephalus *in Turkey*

To date, 11 *Chirocephalus* species are known to occur in Turkey (Mura et al., 2011, present work); to this should be possibly added *C. reiseri* Marcus, 1913, whose presence in the country is doubtful (Mura et al., 2005), and an unidentified *Chirocephalus* sp. reported by Mura et al. (2011). Four species were attributed to the *bairdi* species-group, the remaining were attributed to the *diaphanus* species-group or are impossible to be attributed to any of the traditional species-groups (present work). The present work questions the monophyly of the *bairdi* species-group, thus calling into question also the hypothesis raised by Cottarelli et al. (2007, 2010), who suggested the role of Asiatic Turkey as a possible centre of origin and diversification for the species of the group. Any other hypothesis on the natural history of the genus *Chirocephalus* should be set aside pending for a revision of the phylogeny of the genus.

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