Extremophile at a Glance: Ostracod Crustacean From a Chemoautotrophic Sulphidic Cave Ecosystem

Sanda Iepure (sanda.iepure@academia-cj.ro)
Emil Racovita Institute of Speleology

Anna Wysocka
University of Gdańsk

Serban M. Sarbu
Emil Racovita Institute of Speleology

Michalina Kijowska
University of Gdańsk

Tadeusz Namiotko
University of Gdańsk

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Extremophile at a glance: ostracod crustacean from a chemoautotrophic sulphidic cave ecosystem

Sanda Iepure[1,2*], Anna Wysocka[3], Serban M. Sarbu [1,4], Michalina Kijowska[3], Tadeusz Namiotko[3]

[1] "Emil Racoviţă" Institute of Speleology, Clinicilor 5, 400006 Cluj Napoca, Romania
[2] Institutul Român de Știință și Tehnologie, str. Virgil Fulicea nr. 3, 400022 Cluj-Napoca, Romania
[3] University of Gdańsk, Faculty of Biology, Department of Genetics and Biosystematics, Wita Stwosza 59, 80-308 Gdańsk, Poland
[4] Department of Biological Sciences, California State University, Chico, CA 95929, USA

* Corresponding autor: sanda.iepure@academia-cj.ro

Sulphidic cave ecosystems are remarkable evolutionary hotspots that have witnessed adaptive radiation of their fauna represented by extremophile species having particular traits. Ostracods, a very old group of crustaceans, exhibit specific morphological and ecophysiological features that enable them to thrive in groundwater sulphidic environments. Herein, we report a peculiar new ostracod species *Pseudocandona movilaensis* sp. nov. thriving in the chemoautotrophic sulphidic groundwater ecosystem of Movile Cave (Romania). The new species displays a set of homoplastic features specific for unrelated stygobitic species, for e.g., triangular carapace in lateral view with reduced postero–dorsal part and simplification of limb chaetotaxy (i.e., loss of some claws and reduction of secondary male sex characteristics), driven by a convergent or parallel evolution during or after colonization of the groundwater realm. *P. movilaensis* sp. nov. thrives exclusively in sulphidic meso-thermal waters (21°C) with high concentrations of sulphides, methane, and ammonium. Based on the geometric morphometrics-based study of the carapace shape and molecular phylogenetic analyses based on the COI marker (mtDNA), we discuss the phylogenetic relationship and evolutionary implication for the new species to thrive in groundwater sulphidic groundwater environments.

Sulphidic ecosystems, such are thermal vents in deep sea or continental karst springs and caves, are inhabited by some of the most extraordinary extremophile organisms on the planet[1-3]. In these ecosystems, the absence of light precludes photosynthesis—the chemical process by which green plants draw energy from sunlight to build carbohydrates from water and carbon dioxide. Here, chemoautotrophic bacteria are at the bottom of the food chain and use a different strategy to extract energy by oxidizing hydrogen sulphide as a replacement for radiant energy from sunlight[4-6]. They not only survive in this challenging environment but also provide food for organisms of higher trophic levels.

In the last decade, deep sea thermal vents have attracted much attention due to their high diversity of chemoautotrophic bacteria and marine invertebrates that have adapted to wide thermal gradients, high pressure, and chemically extreme environments[7]. Biota in these environments live in association with chemosynthetic microbes, enabling them to cope with toxic waters rich in hydrogen sulphide and methane[8-9]. In contrast, in continental sulphidic cave ecosystems (SCE), which share their highly unusual nature with sulphuric deep-sea hydrothermal vents, fundamental studies on invertebrate biota are still scarce[5,10-15]. An exception is the first discovered cave ecosystem of this type, the Movile Cave in Romania, the microorganism communities of which have been exhaustively studied since its discovery[5-6, 16-17].

Caves are generally considered extreme on their own account, but SCE are even more extreme, characterized by warm waters; high levels of sulphide, methane, and ammonium; heavy metals (iron, zinc, and copper); and low oxygen concentrations up to hypoxia[5, 18-20]. Hydrogen sulphide is significantly high in SCE, being abundant in the ancient, anoxic oceans of the Proterozoic era, and serves as an energy source for early forms of life by being involved in biochemical and physiological processes of organisms[21]. Likewise, in thermal vents, organisms from various phyla have colonized this toxic environment, giving rise to unique ecological communities supported entirely by chemoautotrophic bacteria and particularly by sulphate-reducing bacteria and forming complex trophic networks[1,5,22-24]. Although sulphide is generally highly toxic to most organisms, SCE host extremophile life forms that show a combination of morpho–ecological traits and metabolic and physiologic adaptations, enabling them to cope with such extreme conditions that are considered lethal for most species. In this regard, SCE and their fauna are considered remarkable 'evolutionary hotspots'[25], which can be a model for extra-terrestrial life on Mars[26].

The few available studies on SCE aquatic invertebrates indicate that crustaceans show high diversity in different groups of copepods, amphipods, isopods, and ostracods[5,10-11,14-15].

They retain some archetypal features that appeared adaptive to cope with hypoxia and high concentrations of sulphide and methane in a similar way as their marine relatives[27-31]. Moreover, they show high capacity for long-term anaerobiosis[32,33] and well-developed mechanisms for sulphide detoxification[34-36]. However, being subterranean forms, even members of different phyla attain striking similarity[37]. Stygobite species[38] show typical 'regressive' characters, for e.g., reduction or absence of eyes, loss of pigment, impermeability of the cuticle, elongated body shape and appendages, modifications of sensory organs, and slow metabolism[39].

Among crustaceans, ostracods are only mentioned as being present in SCE[5,11,14], but no taxonomic descriptions of new species or species exclusively associated with hydrogen sulphide-rich continental groundwaters are currently available. Ostracoda are a primitive class dating back to the Early Ordovician or Late Cambrian period (~505–485 Mya)[40-41]. They are small crustaceans with the body enclosed in a calcified bivalve shell that completely covers the entire animal[41]. They generally feed on aquatic bacteria, fungi, algae, and detritus[42].

Studies on ostracod evolution and adaptive radiation have a long history. In particular, the functional morphology of calcitic carapaces, which easily fossilize and wherein evolution is often expressed as an adaptive response to environmental conditions, has been studied intensively from empirical and theoretical viewpoints[43] (references cited therein). The shape, ornamentation, and size of the ostracod carapace have often been subjected to evolution in the same direction in distinct and unrelated species but sharing similar environmental pressures[44-48]. Such similarity between organisms or their parts for reasons other than inheritance from a common ancestor is termed homoplasy and is caused by either convergent or parallel evolution[49-50]. Convergence may lead to homeomorphy, which is defined as similarity affecting the whole outer appearance to such a degree that one organism may be mistaken for the other[51].

Homoplasy (or its special case homeomorphy) is an important issue in ostracod evolutionary biology as homoplastic similarities, particularly occurring in reasonably close phylogenetic groups, can make phylogenetic analysis more challenging[44,48,52-54]. The expression of homoplasy in the design of an external morphological feature in unrelated species is supposed to arise independently, often as a result of a similar functionality of a trait or due to similar environmental constrains[55]. Morphological homoplasy is assumed to act with preference on those structures that have the highest probability to become advantageous for a species living in a certain environment.

Here, we describe a new cave ostracod species thriving in sulphidic waters of the Movile Cave (southeast Romania). We use a) geometric morphometrics related to the carapace shape of the new species in comparison with its closest relatives of the genera *Pseudocandona* Kaufmann and *Typhlocypris* Vejdovský as well as b) DNA sequences of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene to infer the phylogenetic relationships of the new species.

Results

Taxonomic account

Family: Candonidae Kaufmann, 1900

Subfamily: Candoninae Kaufmann, 1900

Genus: *Pseudocandona* Kaufmann, 1900

Pseudocandona movilaensis sp. nov.

Type material. Holotype (NSMT-Pol H-837) female and one paratype female (ICHUM-6177) are deposited in the National Museum of Nature and Science, Tsukuba (NSMT). Allotype male is dissected in glycerin with limbs mounted on a permanent slide, and left valve stored dry in a micro-paleontological cell. A paratype female deposited at the Institute of Speleology "Emil Racoviţă" (Cluj Napoca, Romania) dissected in glycerin and limbs mounted on a permanent slide; left valve stored dry in a micro-paleontological cell. Other paratypes (ca. 40 specimens in tubes with alcohol, one male and one female dissected on permanent glass slides with valves kept dry in micropaleontological slides as well as empty valves mounted on SEM stubs) are housed in ostracod collection in the Institute of Speleology "Emil Racovita" (Cluj Napoca, Romania) (SI) and Department of Evolutionary Genetics and Biosystematics of the University of Gdansk (TN). Three paratype specimens were used for DNA analysis. Sequences originated from these paratypes are deposited at GeneBank (see Table S1 for accession numbers).

Type locality and habitat. Cave sulfidic lake, Movile Cave (Romania) (43°49'36.38"N, 28°33'43.48"E, 24 m a.s.l.).

Etymology. The name of a new species is an adjective derived from the name of the type locality, Movile Cave near Mangalia, Constanţa Count, south-east Romania.

Diagnosis. *Pseudocandona movilaensis* sp. nov. is distinctive from its congeners by the following set of morphological characters: 1) Carapace and left valves of both males and females as well as juveniles with high dorsal arch, giving a triangular shape in lateral view (Figs. 1, S1, S2). 2) Female antennal claws G_1 = 1.6× and G_M = 1.3× the length of penultimate endopodial segment (Fig. 2A). 3) Male antenna with divided 2nd endopodial segment but with no male bristles (Figs. 2B, S4). 4) Male antennal claws G_2 = 1.6×, G_M = 1.3× and z_1 = 0.94× the length of (undivided) penultimate segment (Figs. 2B, S4). 5) Mandibular palp 2nd segment with 3 (setal group) + 1 + beta setae on the inner edge, and penultimate segment with gamma seta smooth (not plumose) (Figs. S3, S4). 6) Male fifth limb endopodite palps (clasping organs) asymmetrical (Figs. 2D–E, S4). 7) Seventh limb (cleaning leg) 4-segmented, protopodite with three setae, penultimate segment lacking f seta and terminal segment with two long setae h_2 and h_3, and one short and slightly curved h1 seta (Figs. S3, S4). 8) Uropodal ramus of both sexes with posterior claw G_p remarkably reduced, less than half of anterior claw G_a (Figs. 2C, S4, S5). 9) Inner lobe (b) of hemipenis distally broadly rounded, with a distinct, acuminated expansion oriented to the postero-ventral end of the body (Figs. 2F, S5).

Description (for the full description see Supplementary material)

Geometric morphometrics of the valve shape. To predict the genus/species-group to which our *Pseudocandona movilaensis* sp. nov. may be classified based exclusively on the valve shape, we used Canonical Analysis of Principal Coordinates (CAP) on the distance matrix from the B-spline geometric morphometrics analysis. The results of CAP of the discrimination between three groups of species which were selected as morphologically the closest based on the limb morphology (*Typhlocypris* vs. *Pseudocandona* ex gr. *compressa* vs. *Pseudocandona* ex gr. *rostrata*, see Table 1) show that the first squared canonical correlation was relatively large (0.904) and indeed the first canonical axis clearly separated the stygobitic species of the genus *Typhlocypris*, all having triangular valve shape in lateral view. This was the most distinct group, which had 100% allocation success under cross-validation. The other two groups of the genus *Pseudocandona* (gr. *compressa* and gr. *rostrata*) were hardly distinct from one another (Fig. 3), although their allocation success rates were still considerably large (at 80.0% and 85.7%, respectively). The second canonical axis had a much smaller eigenvalue

(0.127) and there is actually no separation of the three groups along the second axis. When our new species *Pseudocandona movilaensis* sp. nov. was introduced to the existing CAP model to classify this species into one of the three existing groups specified above, it was clearly located within the cloud of the triangular *Typhlocypris* species, close to *T. marmonieri* (Fig. 3) with the distance to the centroid of this group 0.029, compared with distances of 0.399 and 0.352 to the centroids of the groups of *Pseudocandona* gr. *compressa* and *P*. gr. *rostrata*, respectively. Although uneven number of species was included in the three studied groups (Table 1), the distance-based test for homogeneity of multivariate dispersion (PERMDISP) showed no statistically significant differences (F = 2.557; P(perm) = 0.170) in the within-group multivariate dispersion among the three groups. To conclude, based on the valve shape in lateral view *Pseudocandona movilaensis* sp. nov. resembles to a great extent the stygobitic species of the genus *Typhlocypris*.

Molecular phylogenetic analysis. In the NJ tree generated based on the haplotype COI data set (Table 1, Table S1), only the shallow branches were well-resolved (Fig. 4). The deep nodes remained poorly supported as the COI marker is unsuitable for the phylogenetic reconstructions of deep evolutionary histories. Nevertheless, focusing on well-supported terminal branches, our results showed that *Pseudocandona movilaensis* sp. nov. appeared to be closely affiliated to *Pseudocandona* species (Fig. 4). Furthermore, the new species is close to the clade formed by species of the *Pseudocandona rostrata* group (*P. marchica* and *P. hartwigi*) with the mean K2P pairwise genetic distances at the level of 0.15 (Table S2). The mean genetic distance between *P. movilaensis* sp. nov. and the *P. compressa* species-group (*P. albicans* and *P. compressa*) was 0.20, whereas between *P. movilaensis* sp. nov. and species of the genus *Typhlocypris* 0.24.

Habitat characteristics, ecology and distribution. *Pseudocandona movilaensis* sp. nov. was reported from sulfidic thermal groundwaters (21˚C) characterized by slightly alkaline waters (pH of 7.2) and high concentrations of sulfide (0.25 mol/l^{-3}), methane (0.02 mol/l^{-3}) and ammonia (0.28 mol/l^{-3})[5]. The species is known exclusively from the sulfide waters of the Movile Cave, but empty carapaces have been also found in a hand-dug well 1 km from the cave and assumed to be passively transported with the groundwater flow (Fig. 5). *In situ* live specimens were observed to move down to the bottom lake (which is almost hypoxic) and return to the surface after few seconds or crawling on the walls. They probably live at the redox interphase between the oxygenated and the cross-formational rising anoxic water,

where the chemosynthetic sulfide-oxidizing bacteria thrive. The examination of live and dead specimens immediately after sampling revealed that all living specimens bear bacterial filaments on the shells, whereas none of the carapaces of dead animals presents these attachments.

Discussion

First taxonomic description of an ostracod from a SCE. SCE species are examples of extremophiles dwelling in habitats subjected to high environmental pressure due to water toxicity. Non-marine ostracods reported from sulphidic groundwaters are very rare, although non-marine ostracods generally thrive in a large array of extreme habitats, such as hot springs (with temperatures exceeding 50°C), cold (up to freezing temperature), acidic (with pH as low as 3.4), and hypersaline waters (at salinities in excess of 100‰) as well as in temporary ponds prone to frequent complete drying or in deep groundwaters[55-59].

As it stands, there are few well-documented SCE continental sites where ostracods are essential contributors to species diversity and an important functional group in the food web network. Among them are Movile Cave in Romania, Frasassi Cave in Italy, Ayalon Cave in Israel, and the more recently discovered Melissotrypa Cave in Greece[5,10-11,15]. Ostracods in SCE, however, are yet to be taxonomically studied to determine their species–specific adaptations to SCE or to investigate the environmental conditions in sulphidic waters that govern species spatial distribution. This is the first taxonomic description of an endemic non-marine ostracod species thriving exclusively in sulphidic cave waters, which enables other biological studies and generalization of the conclusions beyond this study species.

Homoplasy, phylogeny, troglomorphic features, and adaptation to subterranean realm. *P. movilaensis* sp. nov. has three distinctive morphological traits, which we consider homoplastic, i.e. gained or lost independently by species representing separate phylogenetic lineages: 1) triangular shape of the carapace and left valve in lateral view, 2) lack of so-called male bristles on the second antenna (setae t_2 and t_3 are not transformed in males into thick sensory bristles and remain similar to their counterparts in females), and 3) reduction of the posterior claw of the uropodal ramus in both sexes (with stronger reduction in males).

There is a striking and absorbing resemblance in general triangular carapace shape between several subterranean species belonging to various genera of the subfamily Candoninae. This triangular shape is one of the diagnostic traits (coupled with fine valve

ornamentation and narrow inner lamellae in both adult and juvenile stages) of the exclusively subterranean genus *Typhlocypris*[60], which shares this trait with some species of morphologically distinct genera containing species having mostly different (non-triangular) carapace shapes. Examples include subterranean *Fabaeformiscandona aemonae*, *Mixtacandona tabacarui*, *Schellencandona triquetra*[60], and *Candonopsis mareza* but also epigean species living in ancient lakes of Ohrid (e.g., *Neglecandona goricensis* or *N. litoralis*[61]) and Baikal (e.g., *Baicalocandona navitarum* or *B. zenkevichi*)[62]. These 'triangular' species, however, can be morphologically easily distinguished from the lineage constituting the genus *Typhlocypris* based on differential diagnostic characters (of both carapace and limbs) of the genera to which they belong. *P. movilaensis* sp. nov. also possesses the carapace of triangular shape in lateral view (Fig. 1A, D), which suggests a close affinity with species of the genus *Typhlocypris* (Fig. 3). The only character of the valve morphology of *P. movilaensis* sp. nov. differing this species from species of the genus *Typhlocypris* is slightly wider calcified inner lamella in female LV, amounting anteriorly to c. 10%–11% of the valve length and being c. 2.4× as wide posteriorly (in *Typhlocypris* usually it is ≤10% and < 2.0×, respectively). *P. movilaensis* sp. nov. shows a blend of morphological characters shared with species belonging to the genus *Typhlocypris* and to the *rostrata*-group of the genus *Pseudocandona* (see Diagnosis above and Description in Supplementary Materials). For these reasons, closer relationship of this ostracod either to *Typhlocypris* or *Pseudocandona* gr. *rostrata* based on morphological characters may be considered tenuous at best. The phylogenetic placement of *P. movilaensis* sp. nov. on the *COI* sequence tree (Fig. 4) and genetic distances with other studied species (Tables S2 and S3), however, supports hypothesis of its closer affinity with the species of the *rostrata*-group of the genus *Pseudocandona*, which typically develop carapaces of rectangular shape when viewed laterally. Thus, more distant relationship of *P. movilaensis* sp. nov. and *Typhlocypris* implies homoplastic evolution of the triangular carapace shape in the studied ostracods. The only species of the genus *Pseudocandona* with triangular carapace (except for some Baikalian species) is *P. punctata* known from lakes in Ohio[63-64], but this species has an isolated position in the genus (with possible affinities with Baikalian candonids) and differs from *P. movilaensis* sp. nov. in ornamented valves, shape of male prehensile palps, straight (not curved) h_1 seta on the cleaning leg, and the shape of lobes of hemipenis[64]. Although denser species sampling is needed for genetic data (but see position of *P. movilaensis* sp. nov. in the wider phylogenetic context)[65], where this species is marked as *Pseudocandona* sp. nov.), our new findings add to previous morphological evidence for morphological homoplasy of the triangular carapace

shape among species of various genera of the subfamily Candoninae, further disassociating a polyphyletic group of 'triangular' Candoninae into different genera.

Typically, in the subfamily Candoninae, the second antenna (A2) is sexually dimorphic. In males, among other dimorphic traits, the penultimate segment is subdivided and bears the so-called male bristles, which play important prehensile and sensorial roles during courtship and copulation. These bristles are believed to be modified setae t_2 and t_3, which in females remain untransformed and are set on the undivided penultimate segment[66]. *P. movilaensis* sp. nov. has rare morphological combination, i.e. lacks male bristles but the penultimate segment of A2 is at least slightly separated by a thin chitinous septum. Within Candoninae, in some species or entire genera, male bristles are absent (with the penultimate segment divided or undivided), and it seems such cases are more frequent in subterranean lineages than in species inhabiting surface waters. Subterranean examples include, for e.g., all or most species of several endemic Nearctic genera of the tribe Cabralcandonini[67]; several Australian genera (e.g., *Leicandona* and all genera of the tribe Humphreyscandonini); Neotropical genus *Danielocandona*; Afrotropical genus *Namibcypris*; Palaearctic *Marococandona* and *Marmocandona*[68] (and references therein); as well as some or single species of the genera *Mixtacandona*, *Trajancandona*, and *Typhlocypris*. Examples from surface waters are rarer and include some or single species of the genera *Baicalocandona*, *Candona*, *Cubacandona*, and *Paracandona* as well as few species of *Pseudocandona*[68]. As for the latter genus, to which we assigned our new species, thus far, males of only four species have been known to lack male bristles, viz. European *P. insculpta* and *P. regnisnicolai* of the *compressa*-species group[66,69], north American *P. punctata* of the *carribeana*-species group[64], and Japanese *P. atmeta* of the *rostrata*-species group[70]. All these species (except for *P. punctata*, see above) have non-triangular carapace shape, clearly distinguishing them from *P. movilaensis* sp. nov. Our new species differs also from 'triangular' *Typhlocypris pretneri* (the single species of its genus lacking male bristles) in the morphology of the hemipenis and appearance of a uropod. In any case, the lack of male bristles in different genera (or even tribes) within Candoninae indicates signatures of homoplastic evolution, implying that developmental transformation of t-setae into male bristles may be caused by recurrent mutations across not closely related taxa. This does not, however, preclude that lack of male bristles shared by all species of some genera is a result of a common descent and may indicate synapomorphy.

In the subfamily Candoninae, uropod commonly consists of two rod-shaped rami, each bearing distally two claws and two setae[66,68]. A number of various reductions of this

chaetotaxic scheme have been described within separate genera and tribes. The common reductions include the absence of a posterior seta (e.g., some genera of the tribe Candonopsini[54]) or reduction of size, transformation to seta, or complete lack of a posterior claw G_p (e.g., several subterranean genera endemic to Australia or *Meischcandona*[68]; Karanovic 2012). In some species, the uropodal ramus is strongly reduced with only one apical claw or seta (e.g., some genera of the tribe Cabralcandonini[67] of the tribe Namibcypridini[71]) or even the ramus is reduced to a flagellum without any setae or claws (as in *Cabralcandona*)[67]. Beyond doubt, simplification of the uropodal ramus has occurred several times within the subfamily Candoninae, and if the similarity in the form of the caudal ramus exists in different lineages, it presents another example of homoplasy, which may create difficulties in phylogenetic analysis. In the genus *Pseudocandona* and three most closely related genera *Typhlocypris*, *Schellencandona*, and *Marmocandona*, the uropodal ramus is well-developed, with two claws and two setae. To our knowledge, *P. movilaensis* sp. nov. is the unique species of its genus with evidently reduced G_p claw in both sexes. The only other species of *Pseudocandona* with reduced G_p, but only in males, is *P. marchica*, which can be easily distinguished from our new species by having a non-triangular carapace shape and well-developed male bristles[66].

Although traditionally homoplasies are considered to be caused by convergence (when arising by different developmental pathways) or parallelism (if similar developmental mechanisms are involved)[72], some evolutionary biologists argue that convergent and parallel evolution are difficult to distinguish as there is a continuum between these, and thus, propose to use a single term—convergent evolution[73]. Nevertheless, at this stage, it is entirely speculative if the three above-mentioned homoplastic traits have evolved independently in *P. movilaensis* sp. nov. by changes in the same or different genes using similar of distinct ontogenetic modifications related to those causing similar phenotypes in other species within Candoninae.

Stygobitic cavernous crustaceans belonging to different phylogenetic groups evolve independently with similar suits of traits termed troglomorphic[74-76]. For example, several amphipods and isopods inhabiting cave waters show increased appendage length or setation and advanced development of chemo-sensorial organs[39]. As presented above, the three homoplastic characters of *P. movilaensis* sp. nov. can be also considered troglomorphic. We hypothesize that at least two of these traits (lack of male bristles and triangularly-shaped carapace) may have resulted from paedomorphosis, a well-known heterochronic evolutionary process of the retention of youthful ancestral features by adult descendants[77]. There are two

distinct processes explaining paedomorphosis: acceleration of sexual maturation relative to the rest of development (progenesis) and retardation of somatic development with respect to the onset of reproductive activity (neoteny). We believe that paedomorphic characters of *P. movilaensis* sp. nov. results from neoteny rather than progenesis, i.e. slowing or arrested growth during post-embryonic development, either from the last juvenile stage to the adult (as in the case of untransformed male bristles) or from the early juvenile stages (as in the case of triangular carapace resulting from underdevelopment of the postero–dorsal part). The pressures that cause either of these types of paedomorphic evolution remain unclear. Future investigations should address this problem through *in situ* and/or laboratory studies on developmental changes in morphology and on the life history of our new species.

The question arises whether the troglomorphic characters of *P. movilaensis* sp. nov. are adaptations to general cavernous conditions and specifically to SCE. Reduced posterior claw G_p on the uropodal ramus has probably no adaptive value because the energetic expense connected with the reinforcement of that seta into a claw would be minimal. It is, at present, also hard to deduce if uropod, with one 'normally' developed long anterior claw and short G_p, could be more effectively used to glide over the mat of sulphidic bacteria compared to the use of typical Candoninae uropod with two long claws. At this stage, it is also entirely speculative if 'immaturity' of retarded development of the t_2 and t_3 setae (resulting in the absence of chemo-sensorial male bristles) has been caused by any specific selection for such morphology, which should then have adaptive advantages in SCE. Unfortunately, clear comparative experimental data on mating or pre-copulatory behaviour of species with and without developed male bristles are so far lacking, thus, preventing an opportunity to contrast possible differences and function of this sexually dimorphic feature within the subfamily Candoninae.

The carapace of ostracods acts as an interface between the organism and environment and is more likely to be subjected to selection pressures and to have an adaptive value[78-81]. The adaptive significance of the valve shape among the true subterranean ostracods is still a debated issue[83-84]. If the evolutionary process is not driven by the selective pressure of cave conditions, 'triangularization' of the ostracod carapace shape may start either outside the cave (being already present in the ancestor or gained during the colonization of the cavernous environment) or inside the cave (gained after successful colonization of the subterranean realm). We hypothesize that triangular carapace shape may be an adaptive feature selected under environmental conditions in caves, where underdevelopment of the postero–dorsal section of the carapace may provide energetic solution to the oligotrophic cave conditions

(less material needed, see below) coupled with low reproduction rate (less space needed for lower number of eggs).

Engineering construction of the carapace. The carapace in ostracods has functional implications, and it is viewed as an efficient 'engineering construction' adopting a shape and a structure design according to the environmental conditions in which the species lives with the use of minimal amounts of material [85]. The first author advancing the idea that the ostracod carapace 'is a static frame structure with a shape, which, during evolution, can be deformed following specific rules' was Benson[86]. He stated that a hint to understand the solutions adopted by ostracods to obtain the most advantageous carapace shape can be traced by making analogies with the techniques used in architecture constructions. Hence, the ostracod carapace is seen as having similar design to a dome with a double walled cupola with the exterior part being thicker and more resistant and the internal one thin (Fig. 6). Later on, Danielopol[79] advanced the idea that the triangular shape of the ostracod valves is a benefit and fitness solution for species thriving in subterranean environment. The triangular shape of carapace is viewed in a similar way as a tripod, wherein the weight is distributed more efficiently among the three faces (Fig. 6). In agreement with the principles of geometry and mechanics, it is well-known that a triangular shape structure in general has two advantages: 1) deformation is more difficult and is able to balance the stretching and compressive forces inside the structure and 2) is less costly as it requires less material to make the three sides of the triangle.

The triangular shape of the carapace can also have an ecological meaning. In an environment with a high concentration of sulphide and methane, the species must take protective measures against the diffusion of these elements from water into the body[87]. For example, marine ostracods from thermal vents have a waterproof shell[88]. Moreover, an appropriate shell shape can also help the animal to reduce the surface area and volume so that diffusion of toxic elements is minimal. According to Fick's law[89], a triangle is a geometric shape with the smallest ratio of area to volume compared to a rectangular shape. Hence, the triangular shape remarked in *P. movilaensis* sp. nov. vs. the typical rectangular shape of the *Pseudocandona* species from the *rostrata*-group may offer a selective advantage for the species that developed or retained this solution, which was already present in its ancestor.

Ancientness. Some insight into the early evolution of the triangular-shaped Candoninae lineages can be gleaned from fossil record. The oldest records of triangular Candoninae

assigned to the phylogenetic lineage of the genus *Typhlocypris* can be traced to *T. pechelbronnensis* from Oligocene to Early Miocene deposits in eastern France and western Germany as well as *T. roaixensis* and *T. ratisbonensis* from Miocene in southern France and Czech Republic. These three species were recorded from epigean fresh or brackish water paleo-habitats and may be hypothetical ancestors of the *Typhlocypris* lineage[60,90]. The triangular shape of the valves in lateral view, which resembles that of various living triangular stygobitic European species, has been also observed in a number of fossil species of Late Miocene to Quaternary deposits in Europe and Western Asia, for e.g., *Candona (Typhlocypris)* aff. *eremita* from the Late Miocene (ca. 11.6 Mya) in (paleo) Lake Pannon[91] or *Caspiocypris schneiderae* and *Caspiocypris ola* from the Pliocene and Quaternary deposits in Azerbaijan[92]. Some of these species may represent either the lineage of *Typhlocypris* or are ancestors of other recent triangular European species, including our new *P. movilaensis* sp. nov. In order to account for such relationships forming a basis of taxonomical categorization, however, further comparative morphological studies are needed using original material. The ancientness of the potential ancestors of *P. movilaensis* sp. nov. may be further deduced with the onset of the underground system of the Movile Cave dating back to the Late Miocene (ca. 12.5 Mya)[5].

Finally, it is noteworthy to mention the recent studies on fossil ostracods from Late Pleistocene sediments (ca. 15,000 yrs. ago) in the north-west part of the Black Sea (ca. 4 m depth)[93]. Furthermore, in the sediments deposited under oxygenated bottom-water conditions, abundant populations of Candoninae (a group consisting of mostly freshwater species) were found, with few specimens showing triangular left valve[94]. The presence of Candoninae in marine sediments of the Black Sea is associated with a shift in geochemical settings and a sharp rise in the carbonate content about 15,000 yr. ago in the area. This suggests an increase of melt-water pulses that were discharged into the Black Sea basin from the Eurasian region and a passive transport of freshwater ostracods into the sea[95].

Conclusions. We assume that the triangular valve shape in the newly described cave species *P. movilaensis* sp. nov. is a paedomorphic trait, which could be advantageous in the groundwater environment. The new species had a limited range of options to shift into a beneficial trait in the reduced or even lacking environmental fluctuations in the subterranean realm. We further assume that phenotypic similarity in the valve shape of *P. movilaensis* sp. nov. with the stygobite species of the genus *Typhlocypris* (as well as with other triangular-shaped stygobitic species of other Candoninae genera) is a homoplasy caused by convergent

or parallel evolution, attributable to similar constrains of the subterranean realm as well as a developmental solution to survive in an extreme environment, such as groundwater. The position of the new species within the *rostrata* group of the genus *Pseudocandona*, as indicated by the *COI* phylogeny, confirms the homoplastic nature of also other traits that are shared with species morphologically assigned to separate lineages. These results suggest that some traditional characters used to unite certain non-marine ostracods (such as triangular carapace shape) evolved more than once, indicating extensive morphological homoplasy in groundwater ostracods of the subfamily Candoninae.

Methods

Sampling and description. More than 40 specimens of ostracod crustaceans were collected from the sulfidic waters of the Lake Room in the Movile Cave, Romania (43°49.611' N, 28°33.684' E) in three sampling campaigns in 1990, 2012 and 2015 (Fig. 5). Specimens were taken from the top 10 cm of the Lake Room by filtering the water through a planktonic net of 63 µm and preserved in 90% ethanol. Appendages were dissected and mounted in glycerol on the glass slides, while disarticulated carapace valves of the dissected specimens were stored dry in micropaleontological slides slides[96]. Undissected specimens are stored in 90% ethanol. Appendages and valves were drawn with the aid of a camera lucida. Scanning electron images of valves of selected specimens were obtained with a JEOL SM-31010 Scanning Electron Microscope at the Zoological Museum, University of Copenhagen, Denmark.

Geometric morphometry analyses. For geometric morphometrics, the left female valve of *Pseudocandona movilaensis* sp. nov. was photographed in external view using an Olympus light microscope and an Olympus digital camera and processed Adobe Photoshop[97]. To compare the valve shape of a new species with other 25 species of the genera *Pseudocandona* and *Typhlocypris*, we used published descriptions and illustrations (Table 1). The obtained outlines were digitized with TpsDig2 software, version 1.37 for further morphometric analyses[98]. The geometric analyses of the outlines were performed using the Linhart B-spline algorithm in Morphomatica v. 1.6 using 32 control points points[99-101]. The obtained Mean Delta Quadrat distances were used as morphological disparities between the obtained valve outlines. The distance matrix was subsequently used to discriminate between species belonging to three groups: A) 13 species of the genus *Typhlocypris*, B) seven species of the *rostrata*-group of the genus *Pseudocandona*, and C) five species of the *compressa*-group of

the genus *Pseudocandona* (Table 1). We used Canonical Analysis of Principal Coordinates (CAP) implemented in the PERMANOVA+ add-on to PRIMER v7 software[102] to predict the genus/species-group to which individual species belong based on the valve shape and to diagnose misclassification error. Having the CAP model, *Pseudocandona movilaensis* sp. nov. was placed onto the obtained canonical axes to classify this species into one of the three existing groups specified above. In addition, we performed a test of the null hypothesis of no differences in the within-group multivariate dispersion among the three groups by PERMDISP routine in PERMANOVA+[102].

Molecular phylogenetic analysis. Genomic DNA was extracted from 38 specimens, representing nine selected ostracod species of the subfamily Candoninae (Table 1, Table S1). Details of the DNA extraction, amplification and sequencing procedure were described previously[65]. The DNA barcoding fragment of Cytochrome-c-Oxidase subunit I gene (COI)[103] was amplified using standard primers LCO1490/HCO2198[104]. The cleaned PCR products were directly sequenced in both directions with the BigDyeTM terminator cycle sequencing method using the PCR primers by Macrogen Inc. BLAST[105] searches against the non-redundant database of the National Center for Biotechnology Information (NCBI) were performed to verify the sequence similarity of the amplified region. The 38 newly obtained sequences of COI were aligned with MAFFTv7.405[106-107] using the automatic algorithm and trimmed in GENEIOUS 10.0.2 (available at: http://www.geneious.com) leading to 567 bp long alignment. The number of unique haplotypes was calculated in DnaSp[108]. As a potential outgroup, three haplotypes of *Candona candida* and two haplotypes of *Candona weltneri* were used. All sequences were deposited in GenBank with the accession numbers (Table S1). Mean genetic distances under the Kimura 2-parameter model (*K2p*)[109] between COI data set obtained from the nine ostracod species were calculated in MEGA X 10.0.3[110] (Tables S2 and S3). For graphic presentation of the relationships among the studied species, Neighbour-Joining (NJ) tree for COI data was generated using K2P distances with 1000 bootstrap replicates[111] in MEGA.

1. Engels, A. E. Observations on the biodiversity of sulfidic karst habitats. *J Cave Karst Stud*. **69**(1), 187–206 (2007).

2. Mulec, J. & Engels, A. S. Karst spring microbial mat microeukaryotic diversity differs across an oxygen-sulphide ecocline and reveals potential for novel taxa discovery. *Acta Carsologica* **48**(1), 129 - 143 (2019).
3. Mulec, J., Ooarga – Mulec, A., Schiller, E., Persoiu, A., Holko L. & Šebela, S. Assessment of the physical environment of epigean invertebrates in a unique habitat: the case of a karst sulfidic spring, Slovenia. *Ecohydrology*, **8**(7), 1326-1334 (2015).
4. Brazelton, W., Hydrothermal vents. *Curr. Biol*. 27, 431–510 (2017).
5. Sarbu, S. M. Movile Cave: A chemoautotrophically based groundwater ecosystem. In Subterranean Ecosystems (eds. Wilken, H., Culver, D.C. & Humphreys, W.F.) 319–343 (Elsevier: Amsterdam, The Netherlands 2000).
6. Brad, T., S. Iepure & S. Sarbu. The Chemoautotrophically Based Movile Cave Groundwater Ecosystem, a Hotspot of Subterranean Biodiversity. *Diversity* **13**(3), 128 (2021).
7. Schreier, J. E. & Lutz, R. A. Hydrothermal vent biota. In: Encyclopedia of Ocean Sciences (Third Edition) 308 – 319 (Academic Press 2019).
8. Kalenitchenko, D., Le Bris, N., Dadaglio, L., Peru, E., Besserer, A. & Galand, P. E. Bacteria alone establish the chemical basis of the wood-fall chemosynthetic ecosystem in the deep-sea. *ISME J* **12**, 367–379 (2018).
9. Hourdez, S. & Jollivet, D. Metazoan adaptation to deep-sea hydrothermal vents. In Life in Extreme Environments (eds. di Prisco, G., Howell, G., Edwards, G. M., Elster, J. & Huiskes H. L.) 42 – 68 (Cambridge University Press, 2020).
10. Por, F. D., Dimentman, Ch., Frumkin, A. & Naaman, I. Animal life in the chemoautotrophic ecosystem of the hypogenic groundwater cave of Ayyalon (Israel): A summing up. *Nat. Sci*., **4**, 7-13 (2013).
11. Peterson, D. E., Danielopol, D., Finger, K., Iepure, S., Mariani, S., Montanari, A. & Namiotko, T. Reconnaissance of ostracode assemblages in the Frasassi Caves, the adjacent sulfidic spring and the Sentino River in the northeastern Apennines (Marche region, Italy). *Cave Karst Sci*., **75**(1): 11–27 (2013).
12. Flot et al., 2010. Unsuspected diversity of Niphargus amphipods in the chemoautotrophic cave ecosystem of Frasassi, central Italy. *BMC Evol. Biol*. **10**:171.
13. Bauermeister, J., Ramette, A. & Dattagupta. S. Repeatedly Evolved Host-Specific Ectosymbioses between Sulfur-Oxidizing Bacteria and Amphipods Living in a Cave Ecosystem. *PLoS ONE* 7 (2012): e50254.

14. Galassi, D. M. P., Fiasca, B., Di Lorenzo, T. et al. Groundwater biodiversity in a chemoautotrophic cave ecosystem: how geochemistry regulates microcrustacean community structure. *Aquat. Ecol.* **51**, 75–90 (2017).
15. Popa et al., 2020 Rich and diverse subterranean invertebrate communities inhabiting Melissoreypa cave in central Greece. *Trav. Institut de Spéol. "Émile Racovitza"* **58**, 65–78 (2019).
16. Chen, Y., Wu, L., Boden, R. et al. Life without light: microbial diversity and evidence of sulfur- and ammonium-based chemolithotrophy in Movile Cave. *ISME J* **3**, 1093–1104 (2009).
17. Hutchens, E., Radajewski, S., Dumont, M. G., McDonald, I. R. & Murrell, J. C. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environ Microbiol* **6**: 111–120, (2004).
18. Flot, J. F et al. *Niphargus-Thiothrix* associations may be widespread in sulphidic groundwater ecosystems: evidence from southeastern Romania. *Mol Ecol.* **23**(6), 1405-1417 (2014).
19. Kumaresan, D. et al. Microbiology of Movile Cave—A Chemolithoautotrophic Ecosystem. *Geomicrobiol.*, **31**(3), 186-193 (2014).
20. Sarbu, S.M., Kane, T. C. & Kinkle, B. K. A chemoautotrophically based cave ecosystem. *Science* **272**: 1953–1955 (1996).
21. Dahl, T.W., Siggaard-Andersen, ML., Schovsbo, N.H. et al. Brief oxygenation events in locally anoxic oceans during the Cambrian solves the animal breathing paradox. *Sci Rep* **9**, 11669 (2019).
22. Muschiol, D., Markovic, M., Threis, I. & Traunspurger, W. Predatory Copepods Can Control Nematode Populations: A FunctionalResponse Experiment with Eucyclops subterraneus and Bacterivorous Nematodes. Fundam. *Appl. Limnol. Arch. Für Hydrobiol.*, **172**, 317–324 (2008).
23. Protas, M. & Jeffery, W.R. Evolution and development in cave animals: from fish to crustaceans. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 823–845 (2012).
24. Sarbu, S. M. & Popa, R. A unique chemoautotrophically based cave ecosystem. In The Natural History of Biospeleology; Camacho, A.I., Ed. Mus. Nat. de Hist. Naturales: Madrid, Spain, 1992; pp. 637–666.
25. Boston, P. J., Hose, L. D., Northup, D. E. & Spilde, M. N. The microbial communities of sulfur caves: A newly appreciated geologically driven system on Earth and potential model for Mars. Perspectives on Karst Geomorphology, Hydrology, and Geochemistry - A

Tribute Volume to Derek C. Ford and William B. White, Russell S. Harmon, Carol M. Wicks (2006).

26. Boston, P. J. The search for extremophiles on Earth and beyond: What is extreme here may be just business-as-usual elsewhere: *Ad Astra* (Washington, D.C.), **11**(1), (1999).
27. Jahn, A., Janas, U., Theede, H. & Szaniawska. A. Significance of Body Size in Sulphide Detoxification in the Baltic Clam Macoma Balthica (Bivalvia, Tellinidae) in the Gulf of Gdańsk. Mar. Ecol. Prog. Ser. **154**, 175-83 (1997).
28. Verovnik, R., Sket, B. & Trontelj, P.. Phylogeography of subterranean and surface populations of water lice Asellus aquaticus (Crustacea: Isopoda). *Mol. Ecol.* **13**, 1519–1532 (2004).
29. Zaksek, V., Sket, B., Gottstein, S. et al. The limits of cryptic diversity in groundwater: phylogeography of the cave shrimp Troglocaris anophthalmus (Crustacea: Decapoda: Atyidae). *Mol. Ecol*. **18**, 931–946 (2009).
30. Hourdez, S. & Lallier, F. H. Adaptations to hypoxia in hydrothermal-vent and cold-seep invertebrates. *Rev Environ Sci Biotechnol.* **6**, 143–159 (2007).
31. Hand, S. C. & Somero, G. N. Energy metabolism pathways of hydrothermal vent animals: adaptations to a food-rich and sulfide-rich deep-sea environment. *Biol Bull* **165**, 167–181 (1983).
32. Theede, H., Ponat, A., Hiroki, K. et al. Studies on the resistance of marine bottom invertebrates to oxygen-deficiency and hydrogen sulphide. *Marine Biol.* **2**, 325–337 (1969).
33. Adam, N. & Perner, M. Microbially Mediated Hydrogen Cycling in Deep-Sea Hydrothermal Vents. *Front Microbiol.* **9**, 2873 (2018).
34. Somero, G. N, Childress, J. J. & Anderson, A. E. Transport, metabolism, and detoxlflcation of hydrogen sulfide in animals from sulfide-rich marine environments. *CRC Crit Rev Aquat Sci* **1**, 591-614 (1989).
35. Vismann, R. Sulfide tolerance: physiological mechanlsms and ecological implications. *Ophella,* 341-27 (1991).
36. Bagarinao, T. Sulfide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms. *Aquat Toxicol* **24**, 21-6 (1992).
37. Culver, D. C. & Pipan, T. The Biology of Caves and Other Subterranean Habitats. Oxford, UK Oxford University Press, ISBN 978-0-19-921992-6 (2009).
38. Gibert, J., Danielopol, D. L., Stanford, J. A. Groundwater Ecology. San Diego (CA): Academic Press (1994).

39. Culver, D. C. & Pipan, T. Adaptations to subterranean life. In: Culver D.C. & Pipan T. The biology of caves and other subterranean ecosystems. Oxford University Press, Oxford (2019).

40. Smith, A. J., Horne, D. J. Ecology of marine, marginal marine and non-marine Ostracodes, in: Holmes, J.A. et al. The Ostracoda: Applications in Quaternary Research. Geophysical Monograph, **131**, pp. 37-64 (2002).

41. Siveter, D. J., Tanaka, G., Farrell, Ú. C., Martin, M. J., Siveter, D. J. & Briggs, E. G. Exceptionally Preserved 450-Million-Year-Old Ordovician Ostracods with Brood Care. *Curr. Biol.*, **24** (7), 801-806 (2014).

42. Smith, A. J., Horne, D. J., Martens, K. & Schön, I. Class Ostracoda. In Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates (eds Thorp, J. & Rogers, D. C.), 757–780, Academic Press (2015).

43. Horne, D. J. Key events in the ecological radiations of the Ostracoda. *Paleont. Soc. Pap.* **9**, 181–201 (2003).

44. McKenzie, K. G. Palaeozoic-Cenozoic Ostracoda of Tethys. *Boll. Soc. Paleontol. Ital.* **21**(2–3), 311–326 (1982).

45. Danielopol, D. L. Three Groundwater Candoninae (Ostracoda) from Romania. *Int. J. Speleol.* **12**, 0 – 83 (1982).

46. Horne, D. J., Danielopol, D. L. & Martens, K. Reproductive behaviour - In K. Martens, ed.: Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine Ostracoda (Crustacea), 157-195, Backhuys Publishers, Leiden (1998).

47. Maddocks, R. F. The antennule in podocopid Ostracoda: chaetotaxy, ontogeny, and morphometrics. *Micropaleontology* **46**(2) 1–72 (2000).

48. Horne, D. J. Homology and homoeomorphy in ostracod limbs. *Hydrobiol*. **538**, 55–80 (2005).

49. Wake, D. B. Homoplasy: the result of natural selection, or evidence of design limitations? *Amer. Naturalist*, **138** (3): 543-567 (1991).

50. Owen, E. & Daintith, E. Dictionary of evolutionary biology. The Facts on File (2003).

51. Hass, O. & Simpson, G. G. Analysis of some phylogenetic terms, with attempts at redefinition. *Proc. Am. Philos. Soc.* **90**, 319–349 (1946).

52. Schallreuter, R. E. Homeomorphy, phylogeny and natural classification: case studies involving Palaeozoic ostracods. In: Evolutionary biology of Ostracoda: its fundamentals and applications (eds Hanai, T., Ikeya, N. & Ishizaki K.) 1041-1049, Elsevier (1988).

53. Ikeya, N., Tsukagoshi A. & Horne, D. J. Evolution and diversity of Ostracoda. *Hydrobiol*. **538**, 1–256 (2005).

54. Higuti, J., Martens, K. Description of a new genus and species of Candonopsini (Crustacea, Ostracoda, Candoninae) from the alluvial valley of the Upper Paraná River (Brazil, South America). *Eur. J. Taxon.* 1-31 (2012).

55. Fryer, G. Variation in acid tolerance of certain freshwater crustaceans in different natural waters. *Hydrobiol*. **250**, 119–125 (1993).

56. Griffiths, H. I. & Holmes, J. A. Non-marine ostracods and quaternary palaeoenvironments. Quaternary Research Association. Technical guide 8, London (2000).

57. Boomer, I., Horne, D. J., Smith, R. J. Freshwater Ostracoda (Crustacea) from the Assynt region, NW Scotland: new Scottish records and a checklist of Scottish freshwater species. *Bull Inst R Sci Belgique Biologie* **76**, 111–123 (2006).

58. Laprida, C., Diaz, A. & Ratto, N. Ostracods (Crustacea) from thermal waters, southern Altiplano, Argentina. Micropaleontology, **52**(2), 177-188 (2006).

59. Williams, M. et al. Ostracods: the ultimate survivors. *Geol. Today* **31**, 193-200 (2015).

60. Namiotko T., Danielopol D.L., Meisch C., Gross M. & Mori N. Redefinition of the genus *Typhlocypris* Vejdovsky, 1882 (Ostracoda, Candonidae). *Crustaceana* **87**(8-9), 952-984 (2014).

61. Mikulic, F. New species of Candona from Lake Ohrid. *Bull. Mus. Hist. Nat. Ser. B* (Beograd), **17**, 87-107 (1961).

62. Karanovic, I. & Sitnikova, T. Y. Morphological and molecular diversity of Lake Baikal candonid ostracods, with description of a new genus. *ZooKeys* **684**, 19–56 (2017).

63. Furtos, N. The Ostracoda of Ohio. Bull. *Ohio Biol. Surv.* **29**, 411-524 (1933).

64. Karanovic, I. Recent Candoninae (Crustacea, Ostracoda, Candonidae) of North America. *Rec. West. Aust. Mus., Suppl.,* **71**, 1–75 (2006).

65. Wysocka, A., Kilikowska, A., Mori, N., Iepure, S., Kijowska, M. & Namiotko, T. Monophyletic status of European morphogenera of the subfamily Candoninae Kaufmann, 1900 (Ostracoda: Candonidae) in relation to their mtDNA phylogenies. *J. Crust. Biol*., **39** (5), 567-573 (2019).

66. Meisch, C. Freshwater Ostracoda of Western and Central Europe. *In:* Schwoerbel, J.& Zwick, P. (Eds), *Süßwasserfauna von Mitteleuropa 8/3*. Spektrum Akademischer Verlag, Gustav Fischer, Heidelberg Berlin, pp. 522 (2000).

67. Külköylüoğlu, O., Yavuzatmaca, M., Akdemir, D., Yılmaz, O., Çelen, E., Dere S. & Dalkıran N. Correlational patterns of species diversity, swimming ability and ecological

tolerance of non-marine ostracoda (Crustacea) with different reproductive modes in shallow water bodies of Ağrı region (Turkey). *J. Freshw. Ecol.* **34**(1), 151-165 (2019).

68. Karanovic I. & Lee W. A review of candonid ostracods (Crustacea: Ostracoda: Podocopida) from East Asia, with descriptions of five new species from South Korea. *Zootaxa* **3368**, 7–49 (2012).
69. Karanovic, I. & Petkovski, T. K. Two new species of the subfamily Candoninae (Ostracoda) from Montenegro (SE Europe). *Crustaceana*, **72**(6), 608-616 (1999).
70. Smith, R. J. & Takahiro, K. Four new species of the subfamily Candoninae (Crustacea, Ostracoda) from freshwater habitats in Japan. *Eur. J. Taxon.* **136** (1-34) 3-11 (2015).
71. Martens, K. & Horne, D. J. Preface: Ostracoda and the four pillars of evolutionary wisdom. *Hydrobiol.* **419**, 7–11 (2000).
72. Hodin J. Plasticity and constrains in development and evolution. *J. Exp. Zool.* **28**(1), 1-20 (2000).
73. Arendt J. & Reznick D. Convergence and parallelism reconsidered: what we learned about the genetics of adaptation? *Trends Ecol. Evol.* **23**(1), 26-32 (2008).
74. Howarth F. G. & Moldovan O. T. The Ecological Classification of Cave Animals and Their Adaptations. In Moldovan O. T., Kováč L. & Halse S. (eds), Cave Ecology, Springer Link, 41 – 67 (2018).
75. Camacho A. I. A classification of the aquatic and terrestrial subterranean environment and their associated fauna. In: Camacho A. I. (ed). The natural history of biospeleology. Madrid (Spain): Museo Nacional de Ciencias Naturales, 58–103 (1992).
76. Christiansen, K. Morphological adaptations. In Culver, D. C. & W. B. White (eds), Encyclopedia of Caves. Elsevier, Academic Press, San Diego, 386–397 (2005).
77. Gould, S. J. Ontogeny and Phylogeny. The Belknap Press of Harvard University Press, Cambridge, MA, 501 pp. (1977).
78. Danielopol, D. L. Über Herkunft und Morphologie der süßwasserhypogäischen Candoninae (Crustacea, Ostracoda*). Sitz.-Ber. K. Akad. Wiss., math.* **187**, 1–162 (1978)
79. Danielopol, D. L., Olteanu R. & Lete, C. Carapace morphology of *Cytherissa lacustris* (Cytherideidae): its interest for the systematics and the phylogeny of the group. In: D.L. Danielopol, P. Carbonel & J.-P. Colin, eds: Cytherissa (Ostracoda), The Drosophila of Paleolimnology. *Bull. l'Inst. Géol. Bassin Aquitaine*, **47-48**, 27-53 (1990).
80. Horne, D. J. & K. Martens. An assessment of the importance of resting eggs for the evolutionary success of non-marine Ostracoda (Crustacea). *Adv. Limnol.* **52** 549–561 (1998).

81. Namiotko T., Meisch C., Gidó Z. & Danielopol D. L. Redescription, taxonomy, distribution and ecology of *Cryptocandona dudichi* (Klie, 1930) (Crustacea, Ostracoda). *Bull. Soc. Nat. luxemb*., **102**, 109-130 (2001).
82. Pinto R. L., Rocha C. E. F. & Martens, K. On new terrestrial ostracods (Crustacea, Ostracoda) from Brazil, primarily from São Paulo State. *Zool. J. Linn. Soc.*, **145**, 145–173 (2005).
83. Rouch, R. & Danielopol, D. L. L´origine de la faune acuatique souterraine entre le paradigme et du refuge et le modele de la colonization active. *Stygologia* **3(4)**, 345 – 373 (1987).
84. Baltanás, A., Namiotko, T. & D. L. Danielopol. Biogeography and disparity within the genus Cryptocandona (Crustacea, Ostracoda). *Vie et Milieu* **50**(4), 297-310 (2000).
85. Benson, R. A. Biomechanical Theory of Ostracode Carapace Morphology. *Paleontol. Soc. Papers*, **9**, 89-100 (2003).
86. Benson, R. H. Morphologic stability in Ostracoda. In: Swain, E. M. [Ed.]: Biology and paleobiology of" Osrracoda. Proceedings of the 4 th meeting of the Ostracod workers in Newark (Delaware) 1972. BulIetins American Paleontology, 65 (282): 13~i6; Ithaca NY (Paleont. Res. Inst.) (1975).
87. Por, F. Sulfide Shrimp? Observations on the concealed life history of the Thermosbaenacea (Crustacea). *Subt. Biol*. **14**, 63-77 (2014).
88. Tanaka, H. & Yasuhara, M. A New Deep-Sea Hydrothermal Vent Species of Ostracoda (Crustacea) from the Western Pacific: Implications for Adaptation, Endemism, and Dispersal of Ostracodes in Chemosynthetic Systems. *Zoolog Sci.* **33**(5): 555-565 (2016).
89. Wu, T. & Wang, S. A fractal permeability model for real gas in shale reservoirs coupled with Knudsen diffusion and surface diffusion effects. *Fractals*., **28**, 2050017 (2020).
90. Iepure, S., Namiotko, T. & Danielopol, D. L. Evolutionary and taxonomic aspects within the species group *Pseudocandona eremita* Vejdovský (Ostracoda, Candonidae) *Hydrobiol*. **585**, 159–180 (2007).
91. Gross, M. Zur Ostracodenfauna (Crustacea), Paläoökologie und Stratigraphie der Tongrube Mataschen (Unter-Pannonium, Steirisches Becken, Österreich). Joannea Geol. Paläontol., **5**, 49-129 (2004).
92. Agalarova, D. A., Kadyrova, Z. K. & Kulieva, S. A. Ostracoda from Pliocene and Postpliocene deposits of Azerbaijan, 1-420 (Azerbaijan State Publishers, Baku). [In Russian] (1961).

93. Boomer, I., Guichard, F. & Lericolais, G. Late Pleistocene to Recent ostracod assemblages from the western Black Sea. *J. Micropalaeontol.*, **29**, 119–133 (2010).

94. Zenina, M., Ivanova, E., Bradley, L., Murdmaa, I., Schornikov, E., & Marret, F. Origin, migration pathways, and paleoenvironmental significance of Holocene ostracod records from the northeastern Black Sea shelf. *Quat. Res.*, **87**(1), 49-65 (2017).

95. Danielopol, D. L., Marmonier, P., Boulton, A. J. & Bonaduce, G. World subterranean ostracod biogeography: dispersal or vicariance. *Hydrobiol*. **287**, 119–129 (1994).

96. Namiotko, T., Danielopol, D. L. & Baltanás, Á. Soft body morphology, dissection and slide-preparation of Ostracoda: a primer (2011).

97. Strake, A., Danielopol, D. L. & Neubauer, W. Comparative study of *Candona neglecta* valves from the shallow and deep sites of Lake Mondsee. *Ber. Inst. Erdwissenschaften K. F. Univ. Graz*, **13**: 83-88, (2008).

98. Rohlf, F. J. tpsDig digitize landmarks and outlines: Department of Ecology and Evolution, State University of New York at Stony Brook (2013).

99. Baltanas, A., Brauneis, W., Danielopol, D. L. & Linhart, J. Morphometric Methods For Applied Ostracodology: Tools for Outline Analysis Of Nonmarine Ostracodes. In: Park LE, Smith AJ, editors. Bridging the Gap: Trends in the Ostracode Biological and Geological Sciences. *Palaeontol. Soc. Papers* **9**, 101–118 (2003).

100. Brauneis, W., Linhart, J., Stracke, A., Danielopol, D.L., Neubauer, W. & Baltanás, A. Morphomatica (Version 1.6) User Manual/ Tutorial. Mondsee. User Manual/Tutorial. Limnological Institute, Austrian Academy of Sciences, Mondsee: 1–82 (2006).

101. Iepure, S., Namiotko, T. & Danielopol, D. L. Morphologi-cal diversity and microevolutionary aspects of the lineage *Cryptocandona vavrai* Kaufmann 1900 (Ostracods Candon-inae). *Ann. Limnol. Int. J. Limnol.*, **44**, 151–166 (2008).

102. Anderson, M. J., Gorley R. N. & Clarke K. R. PERMANOVA+ for PRIMER: Guide to software and statistical methods. PRIMER-E: Plymouth, UK (2008).

103. Hebert, P. D., Ratnasingham, S. & de Waard, J. R. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. Royal Soc. B, **270** (1), S96–S99 (2003).

104. Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**, 294–299 (1994).

105. Altshul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic Local Alignment Search Tool. *Mol. Biol.* **215**: 403–410 (1990).

106. Katoh, K., Misawa, K., Kuma, K. & Miyata, T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **30**, 3059–3066 (2002).

107. Katoh, K. & Standley, D. M. MAFFT Multiple Sequence Alignment Software Version 7: improvements in Performance and Usability. *Mol. Biol. Evol.* **30**, 772–780 (2013).

108. Librado, P., & Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452 (2009).

109. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120 (1980).

110. Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).

111. Felsenstein, J. Phylogenies and the Comparative Method. *Am. Nat.* **125**, 1–15 (1985).

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Author contributions

S.I. and T.N. conceived the idea, S.M.S, S.I. and T.N. collected the animals, S.I., A.W. and T.N. designed the methodology and conducted the analyses, S.I., A.W. and T.N performed data analysis, S.I. and T.N. led manuscript writing with significant contributions of A.W. and S.M.S. All authors reviewed the manuscript.

Competing Interests Statement

The authors declare no competing interests.

Figures

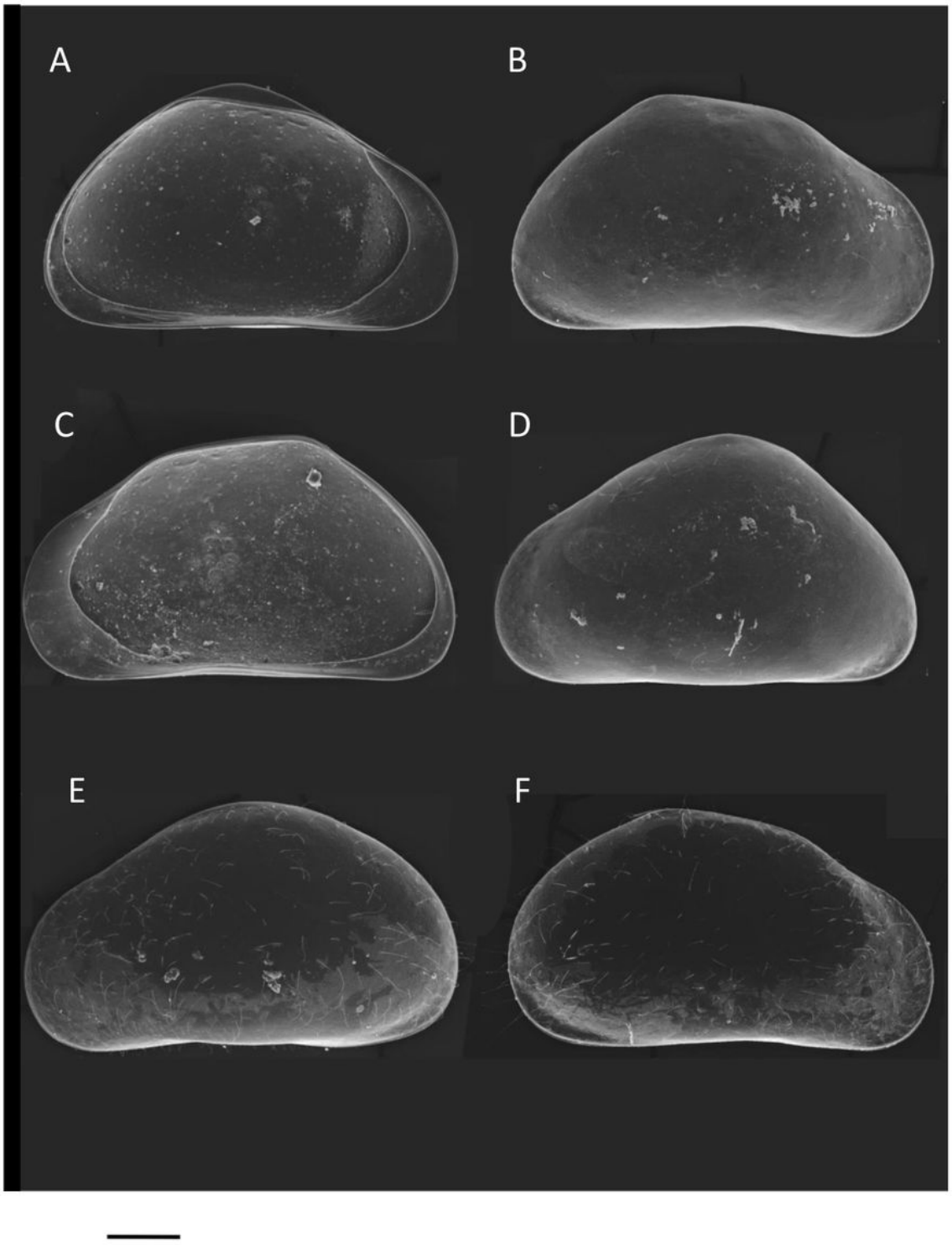


Figure 1

Pseudocandona movilaensis sp. nov. A. Internal view of female left valve. B. External view of female right valve. C. Internal view of female right valve. D. external view of female left valve. E. External view of male left valve. F External view of male right valve (scale – 200 µm).

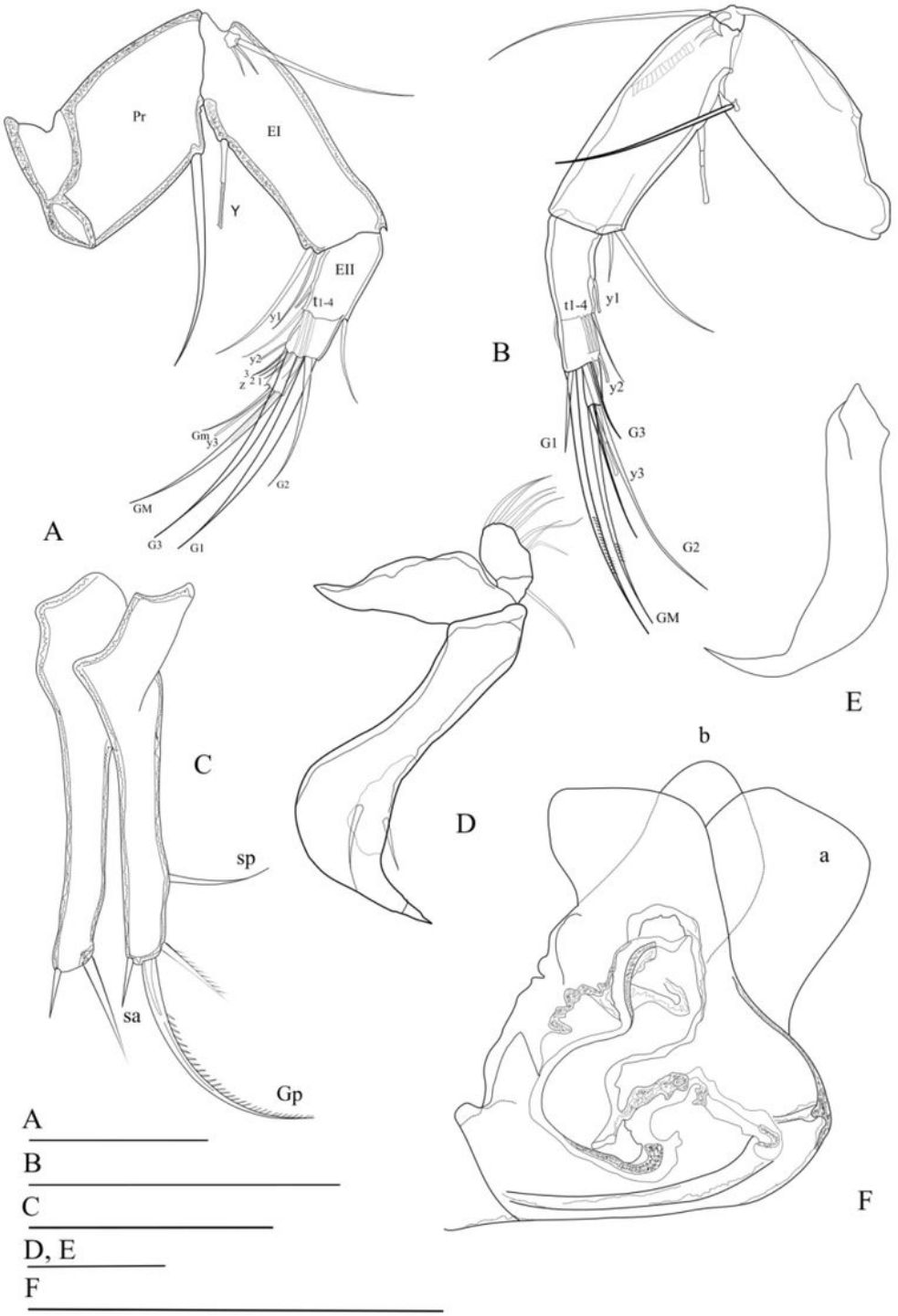


Figure 2

Pseudocandona movilaensis sp. nov. A. Female second antenna. B. Male second antenna. C. Female uropodal rami. D. Male right clasping organ (fifth limb palp); E. Male left clasping organ (fifth limb palp). F. Hemipenis. Scale bars (100 µm).

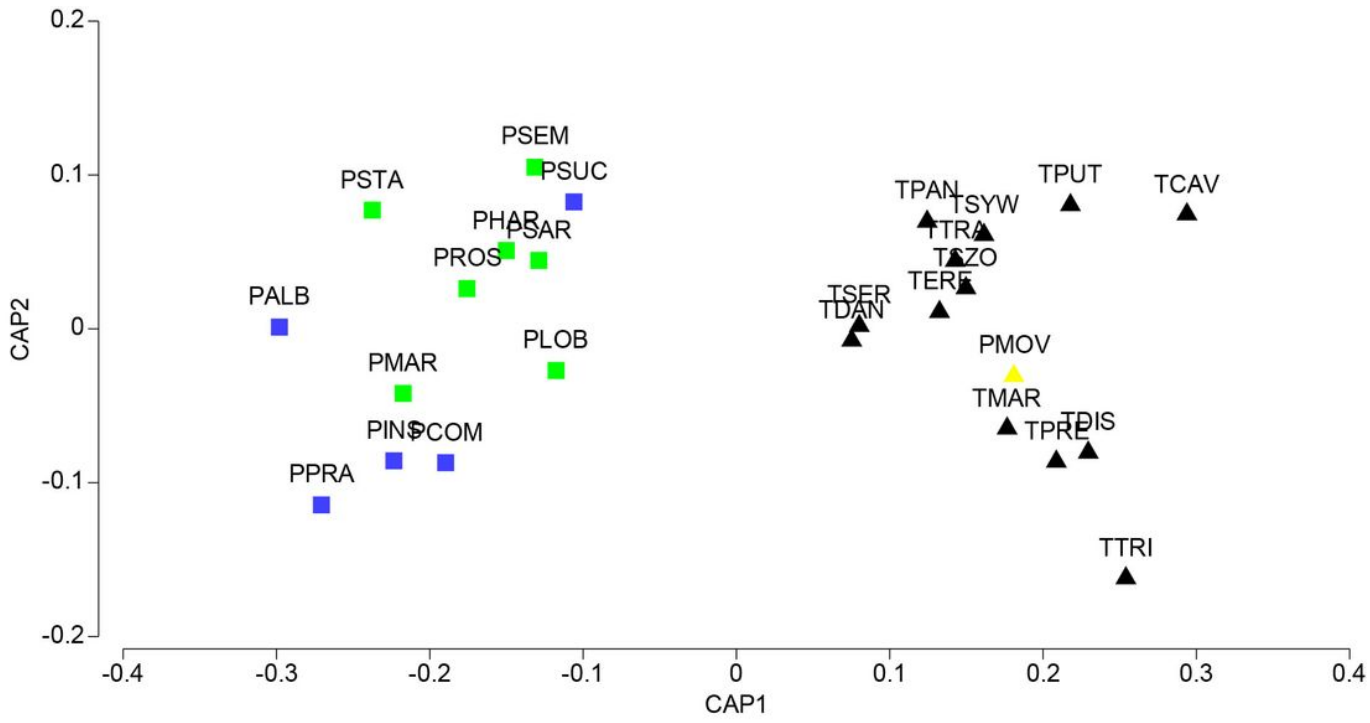


Figure 3

CAP (Canonical Analysis of Principal Coordinates) plot of geometric morphometrics data of the valve shape, showing the position of Pseudocandona movilaensis sp. nov. (yellow triangle) based on its morphometric resemblances with the species of the genus Typhlocypris (black triangles) and those of the two species-groups of the genus Pseudocandona (green squares = compressa species-group, blue squares = rostrata speciesgroup). For the species codes see Table 1.

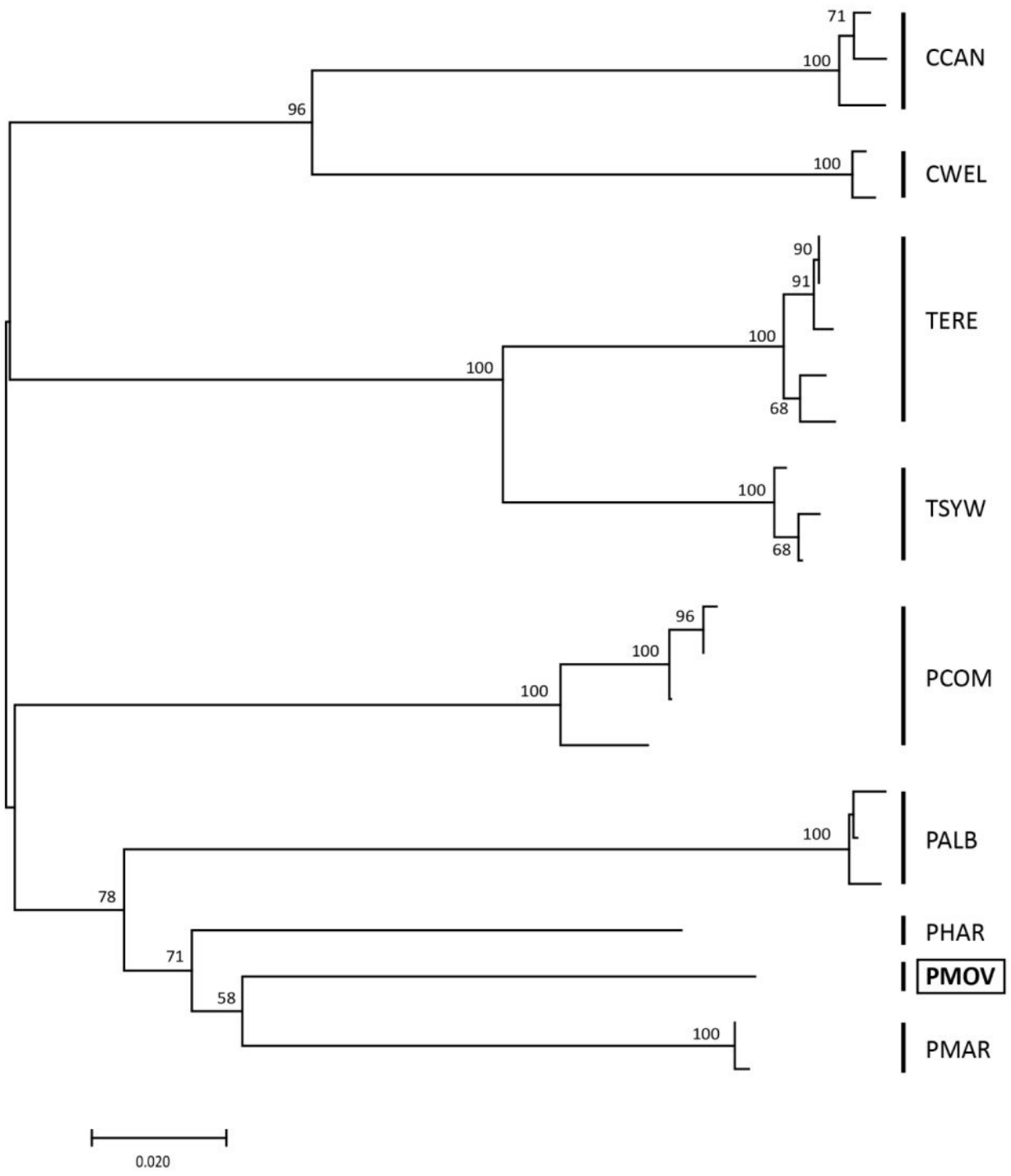


Figure 4

Neighbour-joining tree of the studied ostracod species based on the COI gene sequences (for species codes see Table 1). The distances were calculated with Kimura 2- parameter method. The numbers in front of the nodes indicate bootstrap support (1000 replicates, only values higher than 50% are presented).

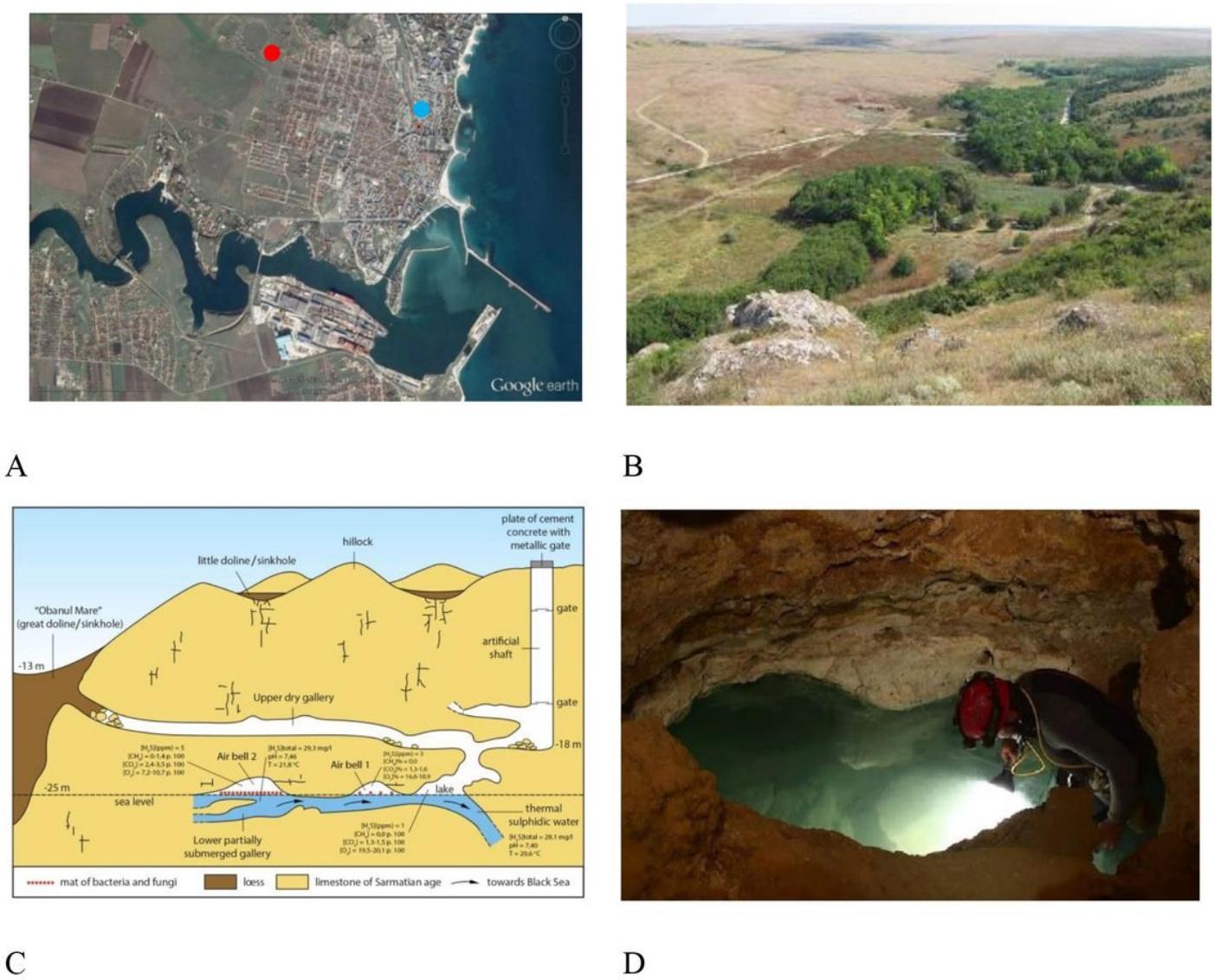
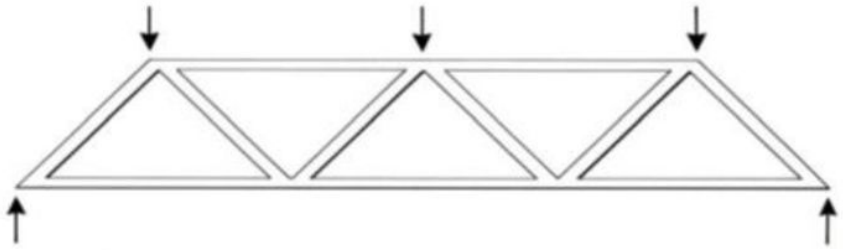
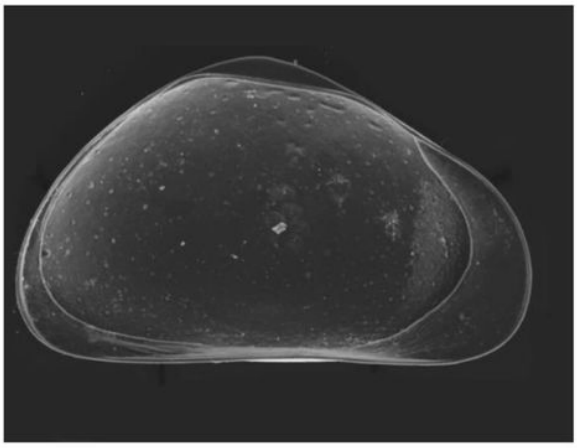


Figure 5

Location of the Movile Cave near Mangalia (south eastern Romania). A. Location of the Movile Cave (red dot) and the hand-dug well in Mangalia village (blue dot). B. Landscape around the Movile Cave. C. Profile of the Movile Cave (map after Sarbu, 2000). D. Cave lake from where the specimens were collected.



A



B

Figure 6

A. Architectural representation of the triangular shape of ostracod carapace. B. Female left valve in lateral view of Pseudocandona movilaensis sp. nov.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Supplementarymaterial.pdf