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Notes on the ecology and distribution of a water flea complex (Anomopoda, Daphniidae) revealed by new DNA barcodes in the Iberian Peninsula

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Abstract

Available DNA barcodes of freshwater zooplankton are scarce in regions like the Iberian Peninsula, which harbours many rare and endemic species and is considered a hotspot of crustacean biodiversity. Recently, a new species of *Ceriodaphnia* (Cladocera: Daphniidae) was described using morphological analysis of specimens in the Mediterranean region and molecular data on a single locality in southern Spain. In our study, we detected the presence of the newly discovered taxa and here, we provide new DNA sequences on the barcoding region mitochondrial cytochrome c oxidase I together with ecological information of the recently described *Ceriodaphnia smirnovi*. Additionally, we built a molecular phylogenetic tree and genetically compared these specimens with previously available mitochondrial DNA sequences and with new sequences of the genus recovered in Iberia. Our data suggest that this morphospecies might contain some cryptic taxa and might be more common than previously thought, occupying temporary to semi-permanent ecosystems, with vegetation and highly variable pH and turbidity conditions. Moreover, the existence of a non-identified clade within our phylogenetic tree requires additional morphological research. Our study highlights the need for further research on microcrustacean biota to better constrain its spatial boundaries, phylogenetic relationship and determine species hiding cryptic diversity.

KEYWORDS

Ceriodaphnia smirnovi, COI, cryptic cladocerans, Spain, temporary ponds, water fleas

1 | INTRODUCTION

Morphological identifications of freshwater zooplankton are technically challenging and time-consuming because of their small size and the need for high taxonomic specialization and expertise (Elías-Gutiérrez et al., 2008). Even so, taxonomic assignments in cladocerans require sometimes the examination of males and mictic females, which extremely rises the efforts and sampling costs to obtain both parthenogenetic and gamogenetic specimens. Concerning this, molecular studies using DNA barcode regions have proved in the last years to be a valuable tool to identify cryptic diversity (e.g. Bekker et al., 2016; Kotov et al., 2016; Neretina et al., 2020, 2021; Zuykova et al., 2019) and to produce more precise estimates of regional biodiversity of these organisms.

The case of the cladoceran *Ceriodaphnia* Dana, 1853 (Cladocera: Daphniidae) perfectly illustrates the current state of research in many zooplankton groups. Nowadays, the genus *Ceriodaphnia* is considered to contain 12 valid species but the number of species of doubtful identification that require further investigations ('*species inquirenda*') is almost twice (Kotov, 2013). The high number of *species inquirenda* has boosted in recent years molecular studies using DNA barcodes not only to find cryptic species but also to establish an accurate phylogenetic relationship between the known members of the group (Abreu et al., 2010; Alonso et al., 2021; de Waard et al., 2006; Deng et al., 2022; Jeffery et al., 2011; Sharma et al., 2014; Sharma & Kotov, 2013). However, the phylogenetic tree of *Ceriodaphnia* is still unresolved as well as the number of taxa within the genus.

Alonso et al. (2021) recently described a new species using both molecular and morphological tools. The new *Ceriodaphnia smirnovi* (Alonso et al., 2021) has an undetermined distribution but is most likely restricted to the Mediterranean region. This species can be distinguished from other *Ceriodaphnia* by examining the ephippium structure and other fine morphological features (Alonso et al., 2021), so as stated before, high expertise and sampling effort are needed to identify this taxon based on morphology. Yet, Alonso et al. (2021) also published a DNA barcode of this new species from a single locality in Spain (Lucio del Cangrejo) in the National Park of Doñana, which allows nucleotide comparison and species identification by bioinformatic tools (i.e. BLAST, basic local alignment search tool). However, as the sample size increases the confidence interval narrows, more DNA sequences of this new taxon and other underrepresented *Ceriodaphnia* spp. are needed to decrease the width of confidence intervals of each node in the phylogenetic tree of *Ceriodaphnia* and therefore, to resolve the tree structure and increase the support of each clade.

As a part of a survey using DNA sequences to study the species diversity of microcrustaceans in the Iberian Peninsula, we detected the presence of *C. smirnovi*, an unknown *Ceriodaphnia*, and other underrepresented taxa in our sampling points. The aims of our study are: (1) To provide new sequences and ecological information of the recently discovered *C. smirnovi*, (2) To publish the DNA barcodes of the unknown *Ceriodaphnia* sp. and (3) To make available cytochrome c oxidase I (COI) sequences of other underrepresented taxa within the genus *Ceriodaphnia*. We publish here more than 40 new sequences of the COI gene from 19 different locations throughout the Iberian Peninsula that will contribute to improving our understanding of phylogenetic relationships of the genus, as well as making more accurate estimations of regional biodiversity by DNA cross-validation of their barcodes in the public genetic database, which will contribute to set appropriate conservation measures of this freshwater fauna.

2 | MATERIALS AND METHODS

2.1 | Study sites

2.1.1 | Lavajos de Sinarcas

The Lavajos of Sinarcas (Lavajo de Arriba and Lavajo de Abajo; Valencia, eastern Iberian Peninsula) are temporary ponds situated at 869 m a.s.l, on an alluvial plain whose lithology is composed of conglomerates and clays (Garzón, 2015). The ponds are located on a natural depression made up of Plio-Quaternary siliciclastic deposits (rañas) lying on top of Miocene sediments (Sahuquillo & Miracle, 2010). The ponds are situated in the centre of a flooded meadow surrounded by vineyards and cereal fields. They have a circular shape and the hydroperiod is relatively long (6–8 months) but depends on interannual rainfall variability. During the wettest years, Lavajo de Abajo reaches a maximum surface of ca. 1 ha and a maximum depth of 1.5 m (Sahuquillo & Miracle, 2010).

The Lavajo de Arriba (or Lavajo del Tío Bernardo) together with the Lavajo de Abajo (or Lavajo del Jaral) take part in the Site of Community importance 'Lavajos de Sinarcas' due to the ecological and biogeographical value of the site and the species that it harbours. Microcrustacean community is very rich and rare, namely the copepod *Hemidiaptomus ingens* (Gurney, 1909) and the cladoceran *Alona anastasia* Sinev et al. (2012), whose type locality is the Lavajo de Abajo (Sinev et al., 2012). The inclusion of this ecosystem within the Nature 2000 Network indicates its significant contribution to the maintenance of the biological diversity within the Mediterranean region and its priority status for conservation purposes. The 'lavajos' are the refuge for very rare plants such as *Eleocharis multicaulis* (Sm.) Desv. or *Marsilea quadrifolia* L. and of different species of amphibians and reptiles such as *Pelobates cultripes* (Cuvier, 1829), *Pleurodeles waltl* Michaelles, 1830 or *Natrix maura* (Linnaeus, 1758). They are also protected at the regional level by the figure Micro reserves of the flora of the Valencian Community.

They have a continental Mediterranean climate, characterized by dry and hot summers, with the main rainfall occurring in autumn and spring. Mean annual rainfall (years 1999–2008) was around 500 mm and monthly evapotranspiration rates ranged from 26 mm in December to 181 mm in July (monthly means, years 1999–2008, IVIA Irrigation Technology Service).

2.1.2 | Laguna del Tesorillo

The Laguna del Tesorillo is situated on the top of the Sierra de Tiros (Extremadura, western Iberian Peninsula), which is a small Palaeozoic quartzite mountain range with a Hercynian orientation (NW-SE). This mountain separates the great plains of the Serena (schist-greywackes lithology) from the oak forest of Monterrubio de la Serena (granitic lithology). Between this last town and the pond, there is a slightly depressed surface occupied by rañas (siliciclastic conglomerates) and dedicated today to olive cultivation. As the pond is located on the top of the mountain, the lithology is dominated by gravel, sands and clays in the more depressed parts and by Palaeozoic Armorican quartzite in the highest and steepest areas (see the background in Figure 1c).

The original Mediterranean forest and scrubland were replaced in the last decades by olive fields. The pond itself is surrounded by *Cistus* spp, mainly *Cistus ladanifer* L., *Asparagus* Tourn. ex L., *Retama sphaerocarpa* (L.) Boiss and *Quercus* L. spp. The shores are dominated by Poaceae and mosses. Laguna del Tesorillo has a circular shape and the hydroperiod is very short, typically from December to March–April.

2.1.3 | Laguna del Pilar

Laguna del Pilar (Extremadura, western Iberian Peninsula) is situated on Precambric reliefs characterized by very small slopes (0.4%) on schist-greywackes lithology only altered by the fluvial net. It is only 16 km apart from Laguna del Tesorillo. It is fed by a small stream on the southern part of the pond and it has an outlet on the northern part probably connected with Arroyo de Fuente Lengua, which is one of the affluent of the Guadalefra River.

This pond has been used for a long time as a drinking trough for sheep, as a cattle trail called Cordel del Pilar passes through. In the last years, artificial drinking troughs were placed on the shores to favour this use. The

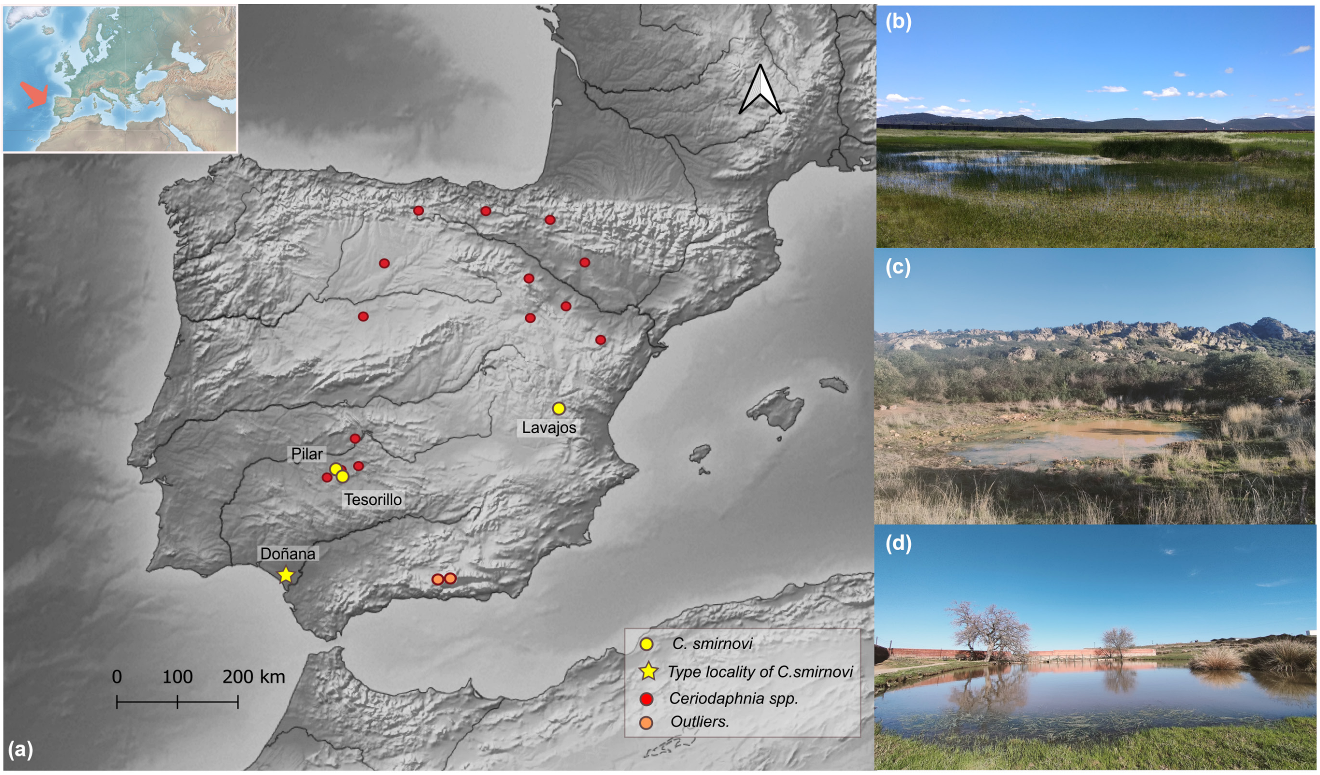

FIGURE 1 (a) Sampling localities of cladoceran in the Iberian Peninsula and type locality of *Ceriodaphnia smirnovi*. (b)–(d) photographs of sampling sites of *C. smirnovi*, (b) Lavajo de Arriba; (c) Laguna del Tesorillo and (d) Charca El Pilar. The location of the sampling sites where the outliers (*Simocephalus* and *Daphnia*) were used in the phylogenetic analysis is also indicated.

surrounding landscape was highly modified by human activity and the pond is now enclosed by a pig farm, some cereal fields and secondary residences.

The southern shore is dominated by *Juncus* L. and *Typha* L. while most of the shore perimeter is generally occupied by grasses. In spring, *Ranunculus* L. covers a large part of the surface of the pond. The northern part of the pond was highly modified in the last decades by the construction of a recreational walkway. Two specimens of *Morus alba* L. and a single elm are the only leftovers of arboreal vegetation.

The climate in these two last ponds is continental Mediterranean with great oscillations between summer and winter temperatures. The coldest months are usually December–January, with a mean temperature of 2°C and the warmest month is July with mean temperature of 34°C. Precipitation is concentrated between October and May, being October–November the rainiest month. Annual precipitation is generally lower than 400 mm (Source: weatherspark.com).

El Tesorillo and El Pilar take part in one of the largest protected areas in Extremadura, the special protection area for birds La Serena and Sierras Periféricas. This is one of the steppe areas of greatest value in the Iberian Peninsula due to its size and the importance of habitats and species present (https://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=ES0000367). The protected and very rare plants *Marsilea strigosa* Willd and *Marsilea batardae* Launert were also cited in wetland areas of this Natura 2000 site, although associations to specific ponds are unknown.

2.2 | Ecological and geological characterization

At each site, electric conductivity, turbidity and pH were measured in situ with portable instruments (Pancellent E-1 TDS & EC meter, Pancellent pH meter). Samples for nutrients were preserved cool and measured by a specialized company (Innoagral Laboratories) within 5 days. Aquatic vegetation was characterized and identified by visual examination of the shore and by making a transect from the shoreline to the deepest point of the pond. Species were identified according to Cirujano et al. (2014).

2.3 | Sampling methods and cladoceran identifications

Samples were retrieved by pulling a 53 μm-hand net from the shores by wading on sampling points shown in Figure 1 and Table 1. The material was preserved in a 96% ethanol solution and stored at 4°C for further analyses. Additionally, some samples were also preserved in 2.5% glutaraldehyde and 4% formaldehyde and used for scanning electron microscope (SEM). All Cladocera individuals were identified at the species level according to Alonso (1996) and Alonso et al. (2021).

For SEM examination, specimens were submitted to critical point drying (CPD300 Leica), coated with gold–palladium and studied under SEM (QuemScan650F) at the Centro de Intrumentación Científica from the University of Granada.

In light of the high number of cryptic species observed among *Ceriodaphnia* and to explore the affinities between the investigated populations, molecular characterization was carried out in parallel to morphology-based identifications. When possible, three specimens from each of the populations were analysed genetically by amplifying a barcoding fragment of the mitochondrial gene encoding for cytochrome b (COI). This molecular characterization comprised specimens that were morphologically identified under *C. smirnovi* and also other populations of *Ceriodaphnia* spp. recovered in different locations around the Iberian Peninsula (see Figure 1; Table 1). For comparative purposes and to guarantee the identification of our samples, more than a hundred sequences of *Ceriodaphnia* spp. were downloaded from Genbank and used in our phylogenetic analysis (see Table S1). The selection of the sequences was made using as a reference the COI phylogenetic tree published by Alonso et al. (2021) and then, selecting ca. 10–15 sequences of each clade from different locations around the world. This number was chosen to have a compromise between the number of sequences and the readability of the resulting tree.

2.4 | Molecular and phylogenetic analyses

Total genomic DNA was extracted from single individuals following a modified DNAeasy Blood & Tissue (Qiagen) protocol. Each specimen was energetically crushed with needles once in the lysis buffer to assure that the carapace and tissues break down. Following DNA extraction, 4–5 μL of DNA was added to a PCR (final volume of 20 μL consisting of 2 μL of 10× Thermopol Buffer, 1.4 μL of $MgCl_2$ [25 mM], 1.4 μL of dNTP [2.5 mM], 6.6 μL of ddH_2O, 0.2 μL of TMAC [0.5 M], 1.2 μL of BSA [10 mg/mL], 1.4 μL of forward and reverse primers [10 μM] and 0.4 of Taq Polymerase [5 U/μL]). Fragments of the mitochondrial COI were amplified and sequenced using Zplank-F and Zplank-R (Prosser et al., 2013). The polymerase chain reaction amplification conditions followed the methods of Prosser et al. (2013): five cycles of (94°C for 40 s, 45°C

TABLE 1 List of specimens sequenced in this study, location of the sampling sites and Genbank accession number for cytochrome c oxidase subunit I gene.

Sample ID	Species	Site	Latitude	Longitude	Elevation (m asl)	Genbank accesion number
CeSm_E146	*Ceriodaphnia smirnovi*	Laguna El Tesorillo, La Nava (Badajoz)	38°41′10.39″N	5°24′35.13″W	780	OQ713649
CeSm_E147	*Ceriodaphnia smirnovi*	Laguna El Tesorillo, La Nava (Badajoz)	38°41′10.39″N	5°24′35.13″W	780	OQ713650
CeSm_E148	*Ceriodaphnia smirnovi*	Laguna El Tesorillo, La Nava (Badajoz)	38°41′10.39″N	5°24′35.13″W	780	OQ713651
CeSm_E92	*Ceriodaphnia smirnovi*	Lavajo de Arriba, Sinarcas (Valencia)	39°38′49.51″N	1°14′03.76″W	860	OQ713642
CeSm_E93	*Ceriodaphnia smirnovi*	Lavajo de Arriba, Sinarcas (Valencia)	39°38′49.51″N	1°14′03.76″W	860	OQ713643
CeSm_E94	*Ceriodaphnia smirnovi*	Lavajo de Arriba, Sinarcas (Valencia)	39°38′49.51″N	1°14′03.76″W	860	OQ713644
CeSm_E76	*Ceriodaphnia smirnovi*	Lavajo de Abajo, Sinarcas (Valencia)	39°45′13.04″N	1°1419.64″W	860	OQ713641
CeSm_E33	*Ceriodaphnia smirnovi*	Charca El Pilar, Castuera (Badajoz)	38°44′12.27″N	5°31′27.83″W	466	OQ713638
CeSm_E34	*Ceriodaphnia smirnovi*	Charca El Pilar, Castuera (Badajoz)	38°44′12.27″N	5°31′27.83″W	466	OQ713639
CeSm_E35	*Ceriodaphnia smirnovi*	Charca El Pilar, Castuera (Badajoz)	38°44′12.27″N	5°31′27.83″W	466	OQ713640
CeSp_E116	*Ceriodaphnia* sp.	Embalse del Ebro, Villanueva de las Rozas (Cantabria)	42°58′27.69″N	4°01′11.67″W	835	OQ713645
CeSp_E117	*Ceriodaphnia* sp.	Embalse del Ebro, Villanueva de las Rozas (Cantabria)	42°58′27.69″N	4°01′11.67″W	835	OQ713646
CeSp_E118	*Ceriodaphnia* sp.	Embalse del Ebro, Villanueva de las Rozas (Cantabria)	42°58′27.69″N	4°01′11.67″W	835	OQ713647
CeSp_E134	*Ceriodaphnia* sp.	Embalse de la Serena, Peñalsordo (Badajoz)	38°50′30.27″N	5°06′20.40″W	350	OQ713648
CeSp_E161	*Ceriodaphnia* sp.	Embalse de Valdecaballeros, Valdecaballeros (Badajoz)	39°17′10.34″N	5°10′46.34″W	375	OQ713652
CeDu_E162	*Ceriodaphnia dubia*	Embalse de Valdecaballeros, Valdecaballeros (Badajoz)	39°17′10.34″N	5°10′46.34″W	375	OQ713653
CeDu_E163	*Ceriodaphnia dubia*	Embalse de Valdecaballeros, Valdecaballeros (Badajoz)	39°17′10.34″N	5°10′46.34″W	375	OQ713654
CeSp_E169	*Ceriodaphnia* sp.	Charca de Zalamea, Zalamea de la Serena (Badajoz)	38°38′57.72″N	5°42′04.29″W	474	OQ713655

(Continues)

TABLE 1 (Continued)

Sample ID	Species	Site	Latitude	Longitude	Elevation (m asl)	Genbank accesion number
CeSp_E173	*Ceriodaphnia* sp.	Charca Pizarras, Castuera (Badajoz)	38°46′31.07″N	5°25′56.26″W	459	OQ713656
CeSp_E174	*Ceriodaphnia* sp.	Charca Pizarras, Castuera (Badajoz)	38°46′31.07″N	5°25′56.26″W	459	OQ713657
CeSp_E175	*Ceriodaphnia* sp.	Charca Pizarras, Castuera (Badajoz)	38°46′31.07″N	5°25′56.26″W	459	OQ713658
CePu_E225	*Ceriodaphnia pulchella*	Embalse de Sotonera, Alcala de Gurrea (Huesca)	42°07′35.62″N	0°38′22.75″W	428	OQ713659
CePu_E226	*Ceriodaphnia pulchella*	Embalse de Sotonera, Alcala de Gurrea (Huesca)	42°07′35.62″N	0°38′22.75″W	428	OQ713660
CePu_E227	*Ceriodaphnia pulchella*	Embalse de Sotonera, Alcala de Gurrea (Huesca)	42°07′35.62″N	0°38′22.75″W	428	OQ713661
CePu_E228	*Ceriodaphnia pulchella*	Embalse de Oliana, Oliana (Lérida)	42°07′04.04″N	1°18′21.08″E	531	OQ713662
CeSp_E229	*Ceriodaphnia* sp.	Embalse de Oliana, Oliana (Lérida)	42°07′04.04″N	1°18′21.08″ E	531	OQ713663
CeSp_E231	*Ceriodaphnia* sp.	Embalse del Val, Los Favos (Zaragoza)	41°52′47.83″N	1°48′07.59″W	621	OQ713664
CeSp_E232	*Ceriodaphnia* sp.	Embalse del Val, Los Favos (Zaragoza)	41°52′47.83″N	1°48′07.59″W	621	OQ713665
CeSp_E233	*Ceriodaphnia* sp.	Embalse del Val, Los Favos (Zaragoza)	41°52′47.83″N	1°48′07.59″W	621	OQ713666
CeSp_E234	*Ceriodaphnia* sp.	Embalse de la Tranquera, Nuévalos (Zaragoza)	41°14′36.03″N	1°47′09.80″W	678	OQ713667
CeSp_E235	*Ceriodaphnia* sp.	Embalse de la Tranquera, Nuévalos (Zaragoza)	41°14′36.03″N	1°47′09.80″W	678	OQ713668
CeSp_E236	*Ceriodaphnia* sp.	Embalse de la Tranquera, Nuévalos (Zaragoza)	41°14′36.03″N	1°47′09.80″W	678	OQ713669
CeSp_E237	*Ceriodaphnia* sp.	Embalse de Mezalocha, Mezalocha (Zaragoza)	41°25′15.98″N	1°4.618′W	471	OQ713670
CeSp_E238	*Ceriodaphnia* sp.	Embalse de Mezalocha, Mezalocha (Zaragoza)	41°25′15.98″N	1°4.618′W	471	OQ713671
CeSp_E239	*Ceriodaphnia* sp.	Embalse de Mezalocha, Mezalocha (Zaragoza)	41°25′15.98″N	1°4.618′W	471	OQ713672
CeSp_E240	*Ceriodaphnia* sp.	Embalse de Gallipuen, Berge (Teruel)	40°52′24.13′N	0°24′51.49″W	690	OQ713673
CeSp_E241	*Ceriodaphnia* sp.	Embalse de Siurana, Siurana (Tarragona)	41°15′08.32″N	0°54′52.94″ E	471	OQ713674
CeSp_E242	*Ceriodaphnia* sp.	Embalse de Siurana, Siurana (Tarragona)	41°15′08.32″N	0°54′52.94″ E	471	OQ713675
CePu_E243	*Ceriodaphnia pulchella*	Embalse de Itoiz, Aoiz (Navarra)	42°48′49.20 N	1°21′57.93″W	517	OQ713676
CePu_E244	*Ceriodaphnia pulchella*	Embalse de Urrunaga, Vilareal (Álava)	42°58′20.74″N	2°39′18.39″W	545	OQ713677

TABLE 1 (Continued)

Sample ID	Species	Site	Latitude	Longitude	Elevation (m asl)	Genbank accesion number
SiVe_E32	*Simocephalus vetulus*	Laguna del Padul (Granada)	37°01′07.36″N	3°35′51.46″W	752	OQ713637
Dap_E10	*Daphnia* pulex group	Laguna de Río Seco (Granada)	37°03′07.73″N	3°20′43.810″W	3020	OQ713636

for 40 s, 72°C for 1 min), then 35 cycles of (94°C for 40s, 51°C for 40s and 72°C for 1 min) and a final extension of 72°C for 5 min. PCR products were visualized on a 1% agarose gel and visually positive PCR products were selected for sequencing. The amplified PCR products were Sanger sequenced by a specialized company (Microsynth AG, Balgach · Switzerland) using the same forward and reverse primers as in the PCR. The chromatograms for each DNA sequence were checked using the software Sequencer 5.4.6 (Gene Codes, Ann Arbor, MI). The primer sequences were removed before any further analysis. Sequences were deposited in GenBank, accession numbers OQ713636-OQ713677.

The authenticity of the sequences was verified using BLASTN 2.10 (Zhang et al., 2000) against the National Center for Biotechnology Information (NCBI) nucleotide database and then sequences were compiled in one dataset. Protein-coding COI sequences were aligned in Bioedit 7.2.5 (Hall, 1999). *Daphnia* O.F. Müller, 1785 (Accession number: OQ713636) and *Simocephalus* Schoedler, 1858 (Accession number: OQ713637) were employed as suitable outgroups in phylogenetic tree reconstruction. The software jModelTest 2.1.7 (Darriba et al., 2012) of the Cyber Infrastructure for the Phylogenetic Research project (CIPRES; www.phylo.org) was used to select the best-fit model of sequence substitution (COI: TrN + I + G) under the corrected Akaike Information Criterion (AICc) (Cavanaugh, 1997). Uncorrected and Kimura2-parameter (K2P) genetic Kimura 2-parameter distances (K2P distance) (550 replicates) were calculated in MEGA 7.0.14 (Kumar et al., 2016). On *C. smirnovi* sequences, a haplotype network analysis was performed using POPART (Barrett et al., 2005; Leigh & Bryant, 2015) and an ASAP analysis (Assemble Species by Automatic Partitioning) (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) based on K2P distances (7%) was used to detect putative species within the *C. smirnovi* dataset (Puillandre et al., 2021).

Phylogenetic analyses were performed using Bayesian Inference (BI) performed in MrBayes 3.2.2 (Ronquist et al., 2012) with the substitution models selected by jModelTest. Markov chain Monte Carlo (MCMC) methods used random starting trees and employed four independent runs, each with one cold chain and three incrementally heated chains. Trees were sampled every 100 generations for 2 million generations and the first 25% of all the trees sampled before convergence (i.e. average standard deviations of split frequencies <0.01) were discarded as burn-in. The 50% majority-rule consensus tree was generated from the remaining trees. Node support of the inferred tree was evaluated by Bayesian posterior probabilities (BPPs). Species clusters and supports were visualized in FigTree 1.4.3 (Rambaut, 2010).

3 | RESULTS

3.1 | Aquatic environments and co-occurring fauna and flora

Morphological identifications and detailed SEM examinations on differential diagnosis characters (i.e. dorsal head pore and specific ornamentation of the ephippium) (Figure 2) revealed the existence of *C. smirnovi* in our samples. Apart from differential characters, polygons with tiny warts were also visible on the headshield surface (Figure 2b). Ecological data on the four new sites where *C. smirnovi* was found are compiled in Table 2. Our data indicated that this species occurred in shallow freshwater ponds from temporary to semi-permanent hydroperiods, fed by rainfall or surface waters. These ponds showed high variability in conductivity values but ranged from 104 to 525 μS/cm, depending on the geological substrate where they lie and on other possible anthropogenic impacts derived from livestock influence. The highest conductivity values were registered in El Pilar and the lowest in El Tesorillo, both lying on metamorphic rocks but El Pilar is more exposed to recreational contamination as well as nearby livestock and agricultural activities. Turbidity ranged from low (0NTU) to high values (100NTU). The highest values were shown in El Tesorillo and El Pilar, the first one was impacted by a recent dredge and the second was affected by cattle bioturbation on the shore, as at the time of the sampling there were many recent cattle footprints. However, the water of Lavajos showed high transparency at the time of the sampling. This might indicate that *C. smirnovi* can live in both turbid and crystal-clear water.

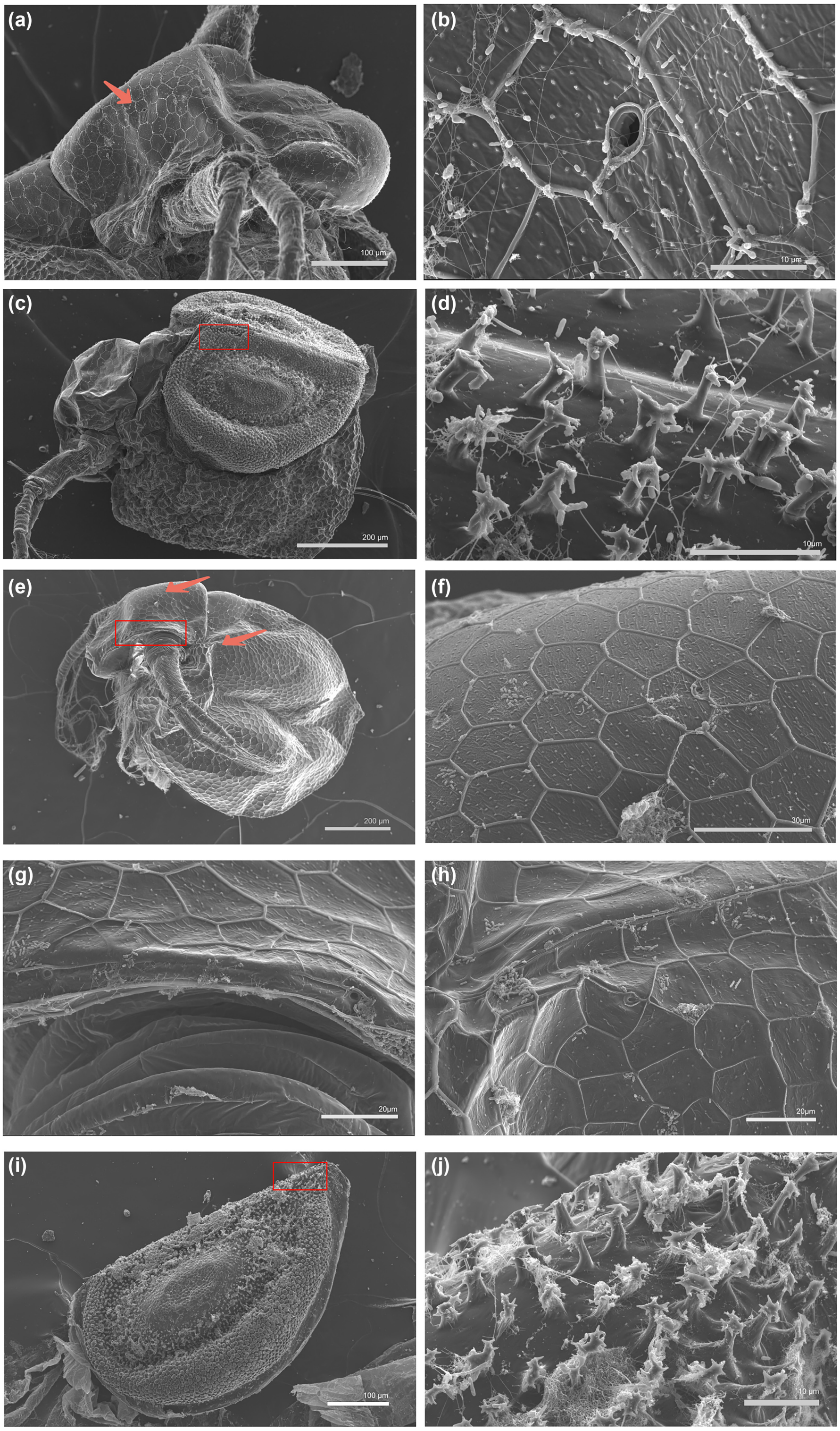

FIGURE 2 *Ceriodaphnia smirnovi* from El Pilar, Badajoz, Spain. (a) and (b), parthenogenetic female, lateral view and dorsal portion of the head with the dorsal head pore. (c) and (d), ephippial female, lateral view and ornamentation of the dorsal surface of the egg locule. (e)–(h), parthenogenetic female, lateral view with dorsal pore (f) and location of pseudopores in the edge of the headshield (g) and carapace (h). (i) and (j), ephippium and ornamentation of the dorsal surface of the egg locule.

pH in these ecosystems was also very variable, ranging from slightly acidic (pH = 6.4) to slightly basic (pH = 8.1). Nutrient determinations showed generally low values, with nitrate concentrations between 0.015 and 2.51 ppm. A common feature of all the studied ponds was that they were characterized by the presence of aquatic vegetation. In some cases, like in the Lavajos, there were well-developed rings of vegetation and in some others like in Tesorillo, vegetation was restricted to mosses on the shore. In the rest of the ponds, but Tesorillo, either *Ranunculus* L. *or Myriophyllum* L. were frequent, as well as in the type locality.

Cladoceran assemblages in all the studied ponds were typical of shallow and vegetated habitats (Table 3). Common species accompanying *C. smirnovi* were *Simocephalus vetulus* Müller, 1776; *Dunhevedia crassa* King, 1853; and *Chydorus sphaericus* O.F. Müller, 1785. Then, depending

TABLE 2 List of samples localities where *Ceriodaphnia smirnovi* was found and their chemical and physical features and vegetation communities at the sampling date.

	Lavajo de Abajo	Lavajo de Arriba	Charca del Pilar	Laguna del Tesorillo	Lucio del Cangrejo[a]
Coordinates (latitude)	39°45′10.70″N	39°45′41.93″N	38°44′12.27″N	38°41′10.39″N	37°02′25.37″N
Coordinate (longitude)	1°14′19.74″W	1°14′25.97″W	5°31′27.83″W	5°24′35.13″W	6°16′26.00″W
Municipality/Province	Sinarcas, Valencia	Sinarcas, Valencia	Castuera, Badajoz	La Nava, Badajoz	Huelva, Huelva
Sampling date	11/05/2021	11/05/2021	05/01/2021	04/01/2021	—
Included in a Nature 2000 site	SAC ES5232005	SAC ES5232005	SAC ES4310010	SAC ES4310010	SAC ES0000024
Altitude (m a.s.l.)	860	860	466	780	0
Maximum surface	1000 m^2	600 m^2	710 m^2	300 m^2	2.4 km^2
Hydroperiod	Temporary	Semi-permanent	Temporary	Temporary	Generally temporary
Water supply	Rainfall	Rainfall	Surface water	Rainfall	Surface water
Conductivity (μS/cm)	220 (80–1106)	161	525	104	5000–157,000
Maximum depth (m)	1 (2)	1 (1.5)	1	0.5	2
TDS (ppm)	141	103	224	52	-
Turbidity (NTU)	0 (0–20)	0	40	100	Wide variations in turbidity
pH	7.8	8.1	6.8	6.4	8.5–7.8
Nitrate (ppm)	0.015	0.018	0.049	2.51	1.2
Ammonia		(0.30–0.34)	0.184	0.427	
Total phosphorus	(0.05–0.4)	(0.85–2.35)	6.25	1.25	179.6
Total nitrogen	(1.5–2)	(2.41–7.76)	0.331	5.69	
Aquatic vegetation	*Chara*, *Ranunculus* and *Myriophyllum*	*Myriophyllum*	*Ranunculus*	Mosses	*Chara*, *Callitriche*, *Ranunculus*, *Ruppia*
Other vegetation of interest	*Marsilea strigosa*, *Isoetes velatum*	*Marsilea strigosa*, *Isoetes velatum*	*Marsilea*[a]	*Marsilea*[a]	

Note: Physico-chemical parameters from Lucio del Cangrejo were obtained from Reyes et al. (2007); Serrano et al. (2006). Data in Brackets in the Lavajos correspond to data from a longer-time ecological survey in Sahuquillo (2012).

[a] *Marsilea* cited in the protected area where the ponds are situated area but the precise association with specific waterbodies is unknown.

TABLE 3 Cladoceran species listed in the studied ponds at the sampling date.

Taxa	Lavajo de Arriba	Lavajo de Abajo	El Pilar	El Tesorillo
Ceriodaphnia smirnovi	X	X	X	X
Simocephalus vetulus		X	X	X
Moina sp.	X	X		
Chydorus sphaericus	X	X	X	X
Dunhevedia crassa		X	X	X
Alona anastasia		X		
Macrotrix cf. *hirsuticornis*				X
Leydigia sp.			X	
Daphnia magna			X	
Anostraca	X	X		X

on the pond and probably on specific ecological characteristics and/or microenvironments, species that thrive well on benthos appeared: *Leydigia* Kurz, 1875 in El Pilar, *Macrotrix* Baird, 1843 in Tesorillo and *Moina* Baird, 1850 in Lavajo de Abajo. Anostraca were also detected in the Lavajos and El Tesorillo.

TABLE 4 Genetic distances (%) of the main clades of *Ceriodaphnia*. Mean estimated Kimura2-parameter distances are shown below the diagonal and the standard deviation (SD) is shown above the diagonal.

	Ceriodaphnia rotunda	***Ceriodaphnia quadrangula***	***Ceriodaphnia laticaudata***	***Ceriodaphnia dubia***	***Ceriodaphnia silvestri***	***Ceriodaphnia spinata***	***Ceriodaphnia reticulata***	***Ceriodaphnia smirnovi***	***Ceriodaphnia pulchella***	***Ceriodaphnia cornuta-rigaudi***	***Ceriodaphnia megops***	***Ceriodaphnia* sp. 1**
Ceriodaphnia rotunda		2.04	1.87	1.82	1.84	1.71	2.02	1.78	1.95	1.57	1.89	1.98
Ceriodaphnia quadrangula	22.16		1.88	1.98	2.03	1.89	2.37	1.97	1.87	1.82	1.82	2.26
Ceriodaphnia laticaudata	19.99	19.69		1.26	1.53	1.62	1.90	1.78	1.77	1.54	1.60	1.89
Ceriodaphnia dubia	18.28	18.78	11.81		1.56	1.69	2.02	1.83	1.80	1.58	1.66	1.95
Ceriodaphnia silvestri	20.01	23.09	15.60	15.63		1.57	1.89	1.66	1.73	1.41	1.72	1.83
Ceriodaphnia spinata	21.22	23.93	21.03	20.05	20.21		1.75	1.53	1.56	1.36	1.66	1.83
Ceriodaphnia reticulata	20.40	25.81	18.95	18.82	18.46	19.52		1.88	1.97	1.79	1.94	2.48
Ceriodaphnia smirnovi	20.22	22.92	21.92	20.61	20.63	19.88	18.63		1.78	1.47	1.66	2.00
Ceriodaphnia pulchella	22.05	21.25	20.76	21.11	21.02	20.04	21.25	21.09		1.56	1.69	1.88
Ceriodaphnia cornuta-rigaudi	20.03	23.11	20.07	19.60	18.27	19.79	20.23	19.58	20.21		1.56	1.73
Ceriodaphnia megops	23.10	22.42	20.63	20.07	22.15	22.78	21.56	21.89	20.95	20.28		2.00
Ceriodaphnia sp. 1	20.39	23.57	20.81	19.27	19.75	23.23	23.67	23.76	20.87	21.09	23.87	

3.2 | Phylogenetic analysis

The sequencing of 40 individuals together with 100 sequences retrieved from public databases gave rise to a phylogenetic tree based on 140 sequences, which showed the existence of 13 different clades. Twelve of these clades were already identified by Alonso et al. (2021) (see Figure 15) and 10 corresponded to the currently described species. One clade (*Ceriodaphnia* sp. 1) was not identified before (Figure 2) and it was separated from the others by a K2P genetic distance of 21.84% ± 1.99% (mean ± SD) (Table 4). Its phylogenetic position within the tree is not clear due to the low to moderate support of deep branches, as expected for a tree only based on COI sequences. However, there is a genetic differentiation based on the lowest genetic distance obtained between this clade and the closest ones (mean K2P distance: *Ceriodaphnia dubia* 19.3% ± 2% and *Ceriodaphnia silvestri* 19.7% ± 1.8%) (Table 4).

On the other hand, the position in the tree of the specimens morphologically identified in our study as a *C. smirnovi* was grouped with the same specimen recovered from Lucio del Cangrejo (Figure 3). Here, we found five different haplotypes that corresponded with five distinct localities, only the sequences from the Lavajos were classified to the same haplotype, while the samples from El Pilar contained two different haplotypes (Figure 4). Mean within group K2P distance of the *C. smirnovi* clade was 6.53% ± 0.74% while the mean genetic distance with other groups was 20.7% ± 1.77% (Table 4). K2P distance of different haplotypes reached maximum values between the haplotypes of Lavajos-El Pilar (11.34% ± 1.39%) and Tesorillo-El Pilar (10.31% ± 1.30%). ASAP delimited three subsets or putative species within the *C. smirnovi* group with reliable support (*p*-value = .000038) using the COI sequences. One subset was composed of specimens from El Pilar (OQ713638-40) and Doñana (MW201517), a second subset was integrated by the specimens from the Lavajos (OQ713641, OQ713642-44) and the third one corresponded to the specimens from El Tesorillo (OQ713649-51). These relationships among the DNA sequences of different populations were also visualized in the network of haplotypes (Figure 4).

The phylogenetic analysis presented here supported most of the morphological identifications presented in Table 1 and helped to correctly place and classify specimens recovered throughout the Iberian Peninsula whose identity at the species level was not assured by morphological identifications. Sequences of *Ceriodaphnia laticaudata* P.E Müller, 1867; *Ceriodaphnia dubia* Richard, 1894; and *Ceriodaphnia pulchella* Sars, 1862 were in general recovered from reservoirs or deepwater bodies. *Ceriodaphnia laticaudata* and *Ceriodaphnia pulchella* were present in the Ebro watershed while the sequences of *Ceriodaphnia dubia* belonged to the

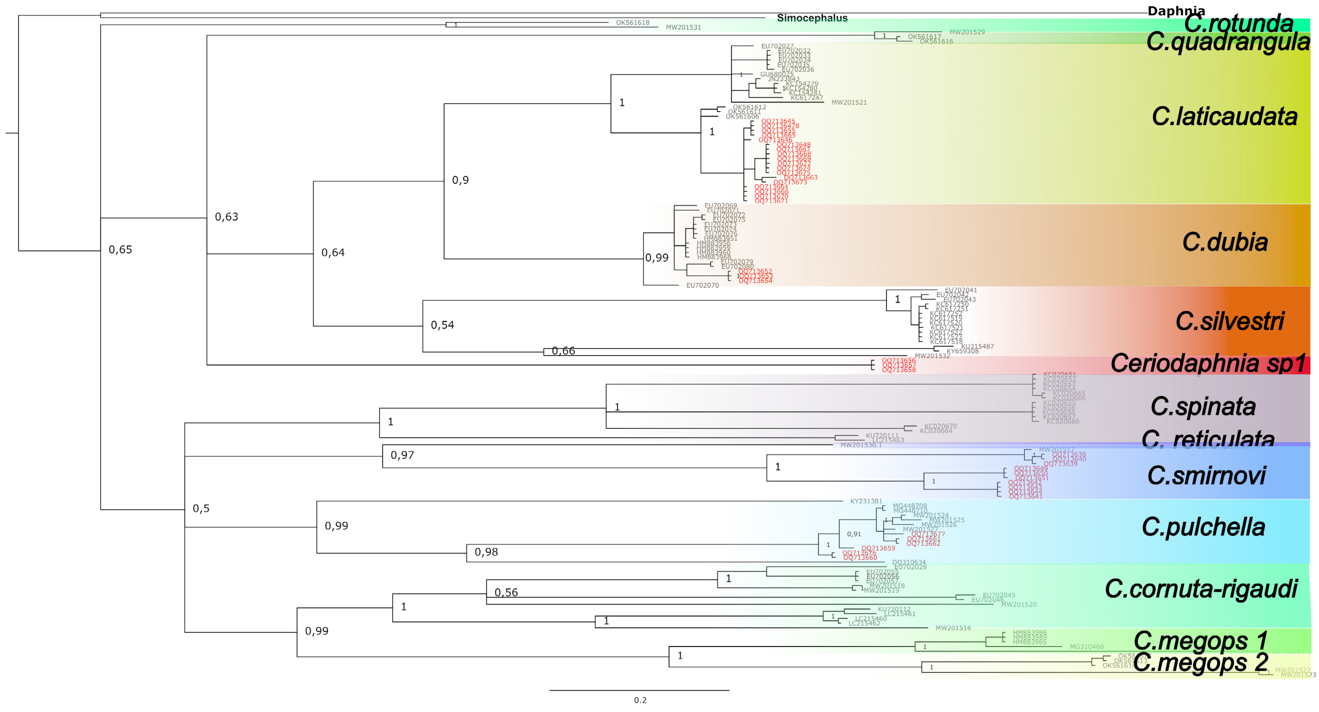

FIGURE 3 Bayesian Inference phylogram based on partial sequence of mitochondrial cytochrome c oxidase subunit I gene. The number in each node indicates the support provided by the Bayesian Inference. Codes in red indicated new sequences of *Ceriodaphnia* provided by this study.

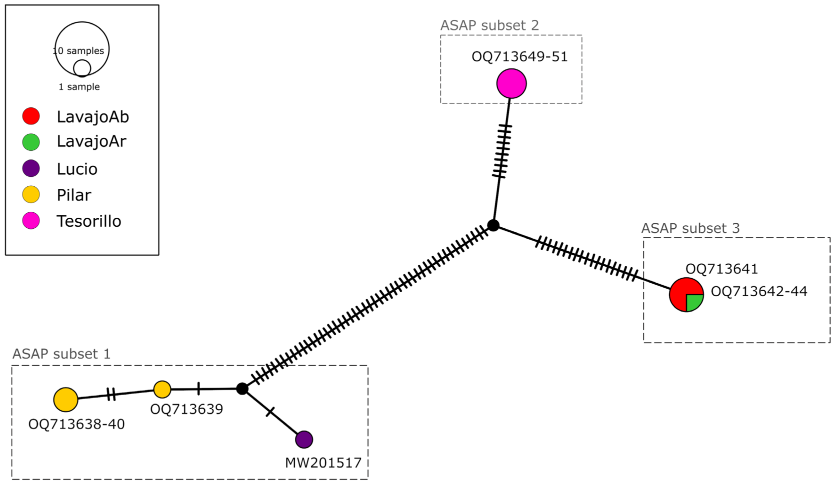

FIGURE 4 Network of haplotypes of a fragment of the mitochondrial gene for cytochrome c oxidase subunit I of *Ceriodaphnia smirnovi*. Each circle indicates a unique haplotype and its size reflects the number of individuals carrying that haplotype. Colour codes allow easy discrimination of localities. Combs in each line denote the number of mutations separating haplotypes. Subsets according to the ASAP analysis are also indicated.

Guadiana watershed. The clades of *Ceriodaphnia* sp. 1 and *Ceriodaphnia smirnovi* corresponded with specimens collected from small water bodies.

4 | DISCUSSION

Our genetic results support the existence of a species complex containing several cryptic taxa within the *C. smirnovi* morphospecies. The network of haplotypes (Figure 4) and the K2P distance show a relatively high number of mutations and COI divergences of some sequences exceeding 7%, which has been used as a threshold to establish different species within cladocerans according to Alonso et al. (2021) and de Almeida Castilho et al. (2015). This is further corroborated by the ASAP analysis, which identified with a high level of confidence three putative groups within the COI dataset of *C. smirnovi*. Alonso et al. (2021) also found large intraspecific variability (>7% for COI) in other groups of *Ceriodaphnia* (i.e. *C. megops* Sars, 1862; *C. cornuta* Sars, 1885/*C. rigaudi* Richard, 1894, *C. spinata* Henry, 1919 and *C. quadrangula* O.F. Müller, 1785), which was also interpreted as the presence of several species within each clade of their phylogenetic analysis. Such as species complexes have been recently proved to be common in many other Cladocera genera such as *Alona* Baird, 1843 (Sinev, 2020; Sinev & Dumont, 2016), *Chydorus* Leach, 1816 (Kotov et al., 2016), *Alonella* G.O. Sars, 1862 (Neretina et al., 2021) or *Moina* Baird, 1850 (Bekker et al., 2016; Montoliu-Elena et al., 2019).

The *C. smirnovi* group is thought to be restricted to the Mediterranean region (Alonso et al., 2021), where it might occupy shallow, temporary to semipermanent ponds, with littoral vegetation and highly variable turbidity and conductivity conditions. According to our data, *D. crassa* and *S. vetulus*, which are commonly associated with vegetation and/or algal mats (Błędzki & Rybak, 2016) might be shared accompanying species. Another common cladoceran in these habitats is the eurytopic *C. sphaericus*. A longer-time survey of Lavajo de Abajo during two hydroperiods also showed the presence of *Ceriodaphnia* together with very rare and endemic crustaceans such as *Ephemeroporus pinthonicus* Margaritora, 1969 and *Alona anastasia* Sinev et al., (2012) in the middle and more stable phase of water filling (Sahuquillo & Miracle, 2010).

Ceriodaphnia smirnovi complex might be more common than thought, as these environmental conditions are relatively usual in semi-arid ecosystems such as those in the southern Iberian Peninsula. In fact, this morphospecies was already detected by Sahuquillo (2012), and Sahuquillo and Miracle (2010, 2013) in many sampling points in the western Iberian Peninsula but named *Ceriodaphnia* sp. *nova*. Lavajo de Arriba was one of the sampling points where this taxon was found and whose identity is confirmed by our molecular results here. Sahuquillo (2012) reported that according to preliminary morphological determinations, the main difference between this new *Ceriodaphnia* and *C. quadrangula* was the cervical fenestra, which has been recently confirmed by the formal description of *C. smirnovi* in Alonso et al. (2021) and by identification of these specimens by the same author (Alonso, personal communication; Figure 2a,b). This species was relatively common in the studied ponds (27.6%; 47 out of 170) and was associated with temporary waters (46%) and some semi-permanent ponds (Sahuquillo, 2012). It appeared from February to June, males and ephippial females were found in spring and in two temporary ponds two morphotypes were detected, one of them very reticulated (Sahuquillo, 2012). Despite being common, the *C. smirnovi* group was probably mistaken for years as *C. quadrangula* due to the high morphological resemblance and similar outer morphology. This circumstance was also found in other cryptic cladocerans (i.e. *C. begoniae* Sinev & López-Blanco, 2018 was unrecognized and misidentified by the very common *C. rectangula* G.O. Sars, 1862 in ancient Lake Ohrid) (López-Blanco et al., 2020; Sinev & López-Blanco, 2018). Alonso et al. (2021) informed about errors in *Ceriodaphnia* identifications in 7.3% of the retrieved sequences in their study. Likewise, in 28.7% of the sequences that they recovered, specimens were only identified at the genus level.

Surprisingly, in our simple network of haplotypes (Figure 4), the population of El Pilar is more closely related to the population of Doñana than to the population of the geographically close El Tesorillo. The last one resulted to be relatively more similar to the populations of the Lavajos. Despite the obvious limitations of our study, our results showed distinct ecological conditions between these two groups; the first cluster of ponds (El Pilar-Doñana) contains waters with higher conductivity values and are fed by rivers while in the second cluster (Tesorillo-Lavajos), main source of water is rainfall, conductivity values are lower and Anostraca are common. Steppic lagoons in endorreic depressions of the Iberian Peninsula have been suggested to serve as a refuge for old groups such as Anostraca, Concostraca and some cladocerans (Miracle, 1982). These ponds originated mainly from Pleistocene river valleys and might have had lagoons since the Tertiary, which explains the current disjunct distribution and the degree of endemicity of the harbouring taxa (Miracle, 1982). This might be the case of *C. smirnovi*, with a circum-Mediterranean distribution and absent or rare in most parts of Europe, as explained by restricted distribution in temporary ponds. Regarding the similarity between haplotypes, Doñana and the steppes of La Serena are some of the most important birding localities in Europe. On the other hand, birds like *Oenanthe hispanica* Linnaeus, 1758 and *Circus pygargus* Linnaeus, 1758 are also common in La Serena and Sinarcas. Bird corridors might have facilitated the gene flow of populations with disjunct distributions. However, further COI sequences and less conservative genetic markers are needed to elucidate the ecology of different groups within the complex and to infer the recent evolutionary history and dispersal mechanisms in the Mediterranean region.

In this context, on the Iberian Peninsula, which hosts many endemic and rare crustaceans (Alonso, 1988; Alonso & Pretus, 1989; Kotov & Alonso, 2010; Sinev et al., 2012), and has a long tradition in crustacean taxonomy and ecology (i.e. Alonso, 1991, 1998, 1996; Boronat, 2003; Boronat et al., 2001; López-Blanco et al., 2013; Margalef, 1947, 1955, 1958; Miracle, 1978, 1982), there are very few studies using molecular techniques to clarify taxonomy, phylogeny and diversity (i.e. Alonso et al., 2021; Miracle et al., 2013; Montoliu-Elena et al., 2019). A recent comparison of available molecular data for the COI gene in public repositories shows that the available sequences only cover ~60% of the formally described Iberian freshwater cladocerans (López-Blanco et al., 2021). Crustacean diversity in this region of important biodiversity hotspots might be, thus, underestimated, especially in temporary ponds. Our phylogenetic tree (Figure 3) suggests that *Ceriodaphnia* sp. 1 found in the southwestern Iberian Peninsula, did not correspond to any currently described species. The phylogenetic position of this cluster is not resolved, due to the low-moderate level of support of deep branches in the phylogenetic tree. However, molecular data support a strong genetic differentiation between this clade in comparison to other clades (lowest K2P distance: 19.3% ± 2%; lowest p-distance: 16.87% ± 1.42%). Elías-Gutiérrez et al. (2008) provided data based on 507 individuals of zooplankton and summarized the sequence divergence for cladocerans, obtaining that K2P distance between cogeneric species averaged 17.64%. If we applied other threshold values (p-distance) for the invertebrates (i.e. 15.4% in Hebert et al., 2003), the average distance obtained for *Ceriodaphnia* sp. 1 in this study exceeds this value, which justifies the need for further morphological descriptions of these specimens to formally know its identity.

Overall, our study contributes to better-determining cladoceran biodiversity by offering new barcodes from poorly surveyed ecosystems. Determination of cryptic species based on barcodes will ultimately contribute to a better delimitation of species boundaries, to understand zooplankton distributions and to orientate management actions to promote its conservation.

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SUPPORTING INFORMATION

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