

Article

# On the Occurrence of *Metadiaptomus chevreuxi* (Calanoida, Diaptomidae, Paradiaptominae) in the Iberian Peninsula, With Notes on the Ecology and Distribution of its European Populations

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**Abstract:** Temporary ponds are one of the most peculiar ecosystems in the world, being characterized by an extraordinarily rich crustacean fauna, with a high degree of endemism. Among them, diaptomid copepods are among the most biogeographically interesting taxa. However, the present knowledge on diaptomid distribution is still far from being exhaustive, even in the relatively well-studied western European countries. In this study, we report the first record of the diaptomid calanoid copepod *Metadiaptomus chevreuxi* for the Iberian Peninsula, where it was collected in five temporary ponds in Andalusia (Spain). The characteristics of the new sites are described, the literature dealing with the European localities of the species is reviewed, and a molecular phylogenetic tree has been built, based on new and previously available mitochondrial DNA sequences, thus expanding the knowledge on the ecology and phylogeography of this rare species. The species mainly occupies small isolated temporary ponds in (semi-)arid regions, suggesting adaptations to unpredictable aquatic habitats. The existence of two molecular clades separating the Iberian from the Sicilian and Tunisian populations supports the existence of a longitudinal long-term disjunction, whereas the north-south flow is probably facilitated by migrating birds. Further research on the biota of the small water bodies of the western Mediterranean area may help to expand our knowledge on rare aquatic species, such as *M. chevreuxi*, and to better interpret their natural history.

**Keywords:** Andalusia; Diaptomidae; Mediterranean wetlands; *Metadiaptomus chevreuxi*; pond conservation; temporary ponds

## 1. Introduction

The inland waters of the Iberian Peninsula host an extraordinarily rich crustacean fauna, which is characterized by a high degree of endemism, and the occurrence of relict taxa with disjunct distributions [1–6]. Among them, diaptomid calanoid copepods are known to be excellent biogeographic markers, due to their high degree of endemism and relatively limited distribution ranges, which are significantly constrained by historical biogeographic events [7–9].

Although the diaptomid fauna of the Iberian Peninsula has been the object of extensive study [1,3,10–14], the known distribution of species in this group is under continuous update [15,16], new species for the fauna of the peninsula and associated archipelagos are still being found [17,18], and the actual identity of some taxa is in need of confirmation. Accordingly, the recent synopses published by different authors [19–21] are only tentative, and the compilation of an updated checklist and distribution atlas of the Iberian diaptomid fauna is desirable. For this reason, sampling campaigns aimed at providing new data on diaptomid presence and distribution in hitherto undersampled areas of the Iberian Peninsula are currently underway.

In this frame, the paradiaptomin copepod *Metadiaptomus chevreuxi* (Guerne & Richard, 1894) is one of the five Paradiaptominae species currently known to occur out of Africa (where the diversity hotspot of this diaptomid subfamily is located: 19). Its distribution includes arid and semi-arid areas of the western Palearctic biogeographical region, in an area roughly comprised between 23° and 39° of latitude N and 9° W to 53° E of longitude, where it is a conspicuous denizen of temporary ponds and pools. Within this wide geographical belt, *M. chevreuxi* has a scattered distribution, with three isolated clusters of occurrence localities located in the western Mediterranean area (Maghreb, Majorca and Sicily), in some central Sahara oases (Tassili N'Ajjer and Ahaggar National Parks, southern Algeria, and Brach oasis, Libya), and in the Middle East (Jordan, Iraq, and Iran) (19). These distribution clusters are separated by vast uninhabited areas, where the species has never been collected; moreover, even within each of these distribution clusters, *M. chevreuxi* is not homogeneously distributed, with a succession of densely-inhabited areas and areas where the species is absent.

In the light of the rarity of the species out of Africa, and of its puzzling distribution pattern, the identity and affinities of the new findings of Iberian populations are hereby discussed. The currently known European occurrence localities of the species are reviewed and briefly characterized, with the explicit aim of contributing to a better understanding of the factors determining *M. chevreuxi* distribution.

## 2. Materials and Methods

### 2.1. Study Areas

In the present paper we discuss samples and data obtained from the three European areas where *M. chevreuxi* is currently known to occur: Andalusia (southern Spain; this work), Majorca (Balearic Islands, Spain) and Sicily (southern Italy).

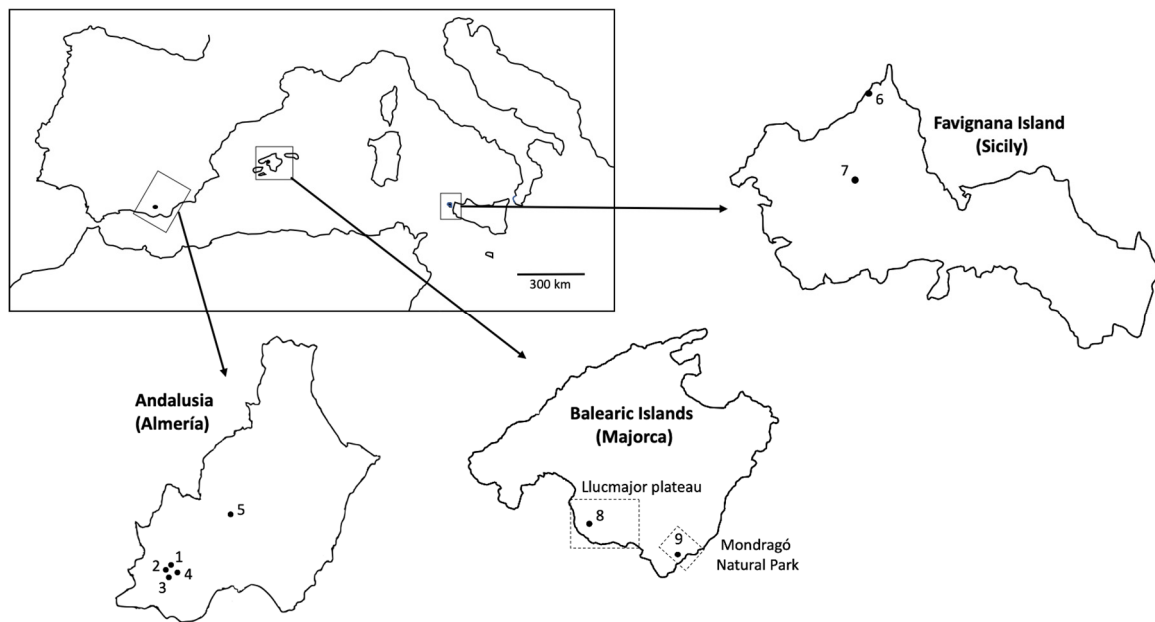
#### 2.1.1. Andalusia

The five ponds under study are located in the internal zone of the Betic Cordillera (southeastern Iberian Peninsula), all of them occurring in the province of Almería (Table 1; Figures 1 and 2). Four tectonically super imposed complexes form this internal zone; they are the: (i) Nevado-Filábride; (ii) Alpujárride; (iii) Malaguide and (iv) Dorsal complexes zones; all of them are affected by the Alpine orogeny [22]. Four ponds (i.e., Balsa Blanca, Balsa de Caparidán, Balsa de Barjalí and Balsa del Sabinar) are located in Sierra de Gádor (Almería, Spain), in the Alpujárride complex. The geologic materials in Sierra de Gádor are limestone and dolostone rocks [23], and the origin of these ponds is related with a pluvio-nival karstification process (*sensu* [24]). All ponds are traditionally used as cattle troughs and have possibly been modified and managed to favor this use. Conversely, the fifth pond (Charca de Filabres) is located in Sierra de los Filabres (Almería, Spain), in the Nevado-Filábride complex. The geologic materials in Sierra de los Filabres are principally quartzites, schists and mica schists with marble intrusions [25].

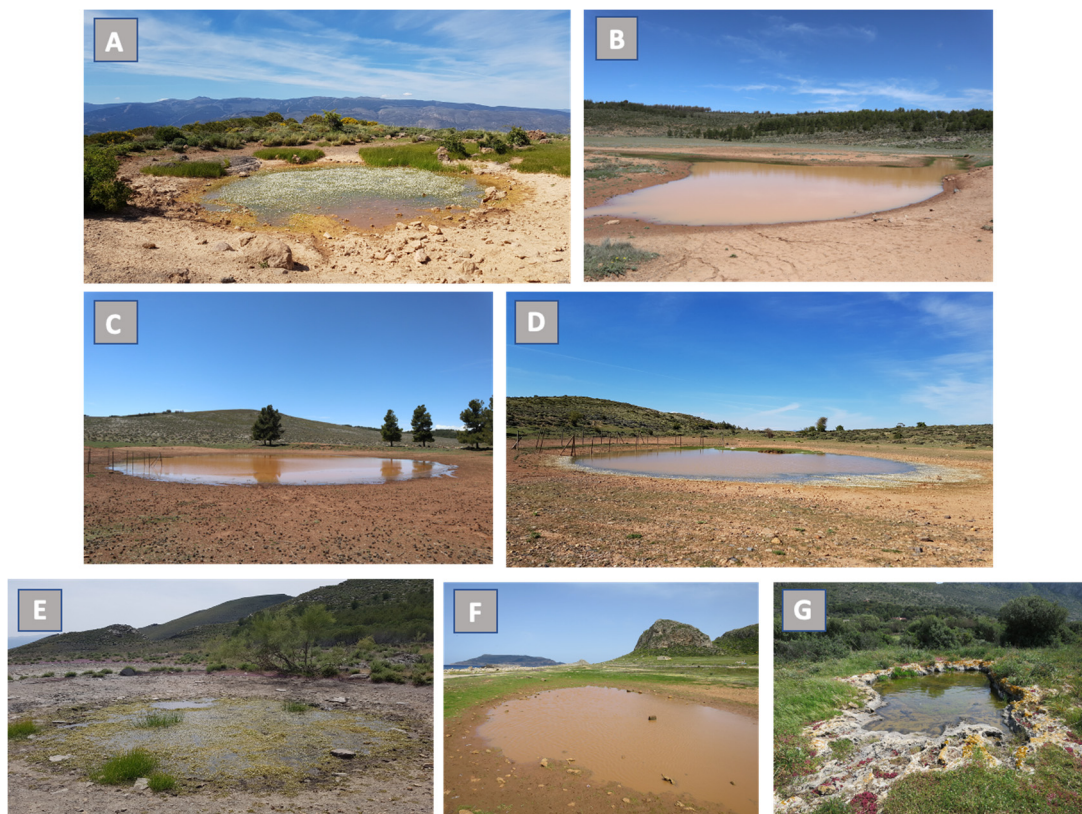
**Table 1.** List of the sampled localities and their chemical and physical features and biological communities at the sampling date. n.a.: not available. Aquatic vegetation: I: *Tolypella prolifera* (Ziz ex Braun) Leonhardi, 1863 (\*) new locality for this species (the first record outside Sierra de Gádor); II: *Ranunculus peltatus* subsp. *saniculifolius* (Viv.) C.D.K. Cook, 1984; III: *Eleocharis palustris* (L.) Roem. & Schult., 1817; IV: *Coronopus navasii* Pau, 1922; V: *Callitriche* sp.; VI: *Elatine macropoda* Guss., 1827. Vertebrate fauna: VII: *Epidalea calamita* (Laurenti, 1768); VIII: *Bufotes boulengeri siculus* (Stöck, Sicilia, Belfiore, Buckley, Lo Brutto, Lo Valvo & Arculeo, 2008).

	Blanca	Caparidán	Sabinar	Barjalí	Filabres	Pozze dell'Ucceria	Pozza di Grotta del Piano
Coordinates (latitude)	36°56'49.55" N	36°55'53.70" N	36°52'56.11" N	36°55'8.30" N	37° 8'21.10" N	37°56'58.71" N	37°56'1.25" N
Coordinates (longitude)	2°50'13.84" W	2°54'17.48" W	2°51'35.97" W	2°47'53.79" W	2°30'49.55" W	12°18'5.17" E	12°17'59.41" E
Country/region	Spain/Andalusia	Spain/Andalusia	Spain/Andalusia	Spain/Andalusia	Spain/Andalusia	Italy/Sicily	Italy/Sicily
Municipality	Fondón	Laujar de Andarax	Berja-Dalías	Dalías-Padules-Almócita	Gérgal	Favignana	Favignana
Sampling date	11/05/2018	11/05/2018	11/05/2018	11/05/2018	30/04/2018	8/04/2018	8/04/2018
Included in a Nature 2000 site	SAC ES6110008	SAC ES6110008	SAC ES6110008	SAC ES6110008	-	SAC ITA010004	SAC ITA010004
Altitude (m a.s.l.)	1551	1601	1830	1713	1050	3	30
Maximum dimensions (m)	13 × 14	67 × 30	85 × 70	74 × 50	17 × 14	95 × 16	8 × 3
Hydroperiod	Temporary	Temporary	Temporary <sup>1</sup>	Temporary <sup>1</sup>	Temporary	Temporary	Temporary
Conductivity (µS cm <sup>-1</sup> )	170	200	120	100	150	1510	316
Turbidity (NTU)	56	228	238	155	52	466	0.14
pH	8.90	8.50	8.95	9.43	9.59	n.a.	n.a.
Alkalinity (mg L <sup>-1</sup> )	74	94	55	49	59	180	60
Nitrate (N-NO <sub>3</sub> <sup>-</sup> mg L <sup>-1</sup> )	0.016	<0.002	<0.002	<0.002	0.51	1.07	0.41
Ammonia (N-NH <sub>4</sub> <sup>+</sup> mg L <sup>-1</sup> )	<0.008	<0.008	<0.008	<0.008	0.031	1.41	n.a.
Phosphates (P-PO <sub>4</sub> <sup>3-</sup> mg L <sup>-1</sup> )	<0.003	<0.003	<0.003	0.026	<0.003	0.23	0.1
Total Nitrogen (mg L <sup>-1</sup> )	1.2	1.8	1.7	1.8	2.3	8.3	1.7
Total Phosphorus (mg L <sup>-1</sup> )	0.1	0.2	0.3	0.3	<0.1	0.6	0.1
Vegetation	I, II, III	I, II, III, IV	I, II, III, IV	I, II, III, IV	I*, II, III	-	V, VI
Vertebrate Fauna	VII	VII	VII	VII	VII	-	VIII

<sup>1</sup> In exceptionally rainy years, they can hold water all the year round.



**Figure 1.** Location of the ponds referred to in this study. 1. Balsa Blanca; 2. Balsa de Caparidán; 3. Balsa del Sabinar; 4. Balsa de Barjalí; 5. Charca de Filabres; 6. Pozze dell’Ucceria; 7. Pozza di Grotta del Piano; 8. Marina de Lluçmajor; 9. Mondragó Natural Park.



**Figure 2.** (A) Balsa Blanca; (B) Balsa de Caparidán; (C) Balsa del Sabinar; (D) Balsa de Barjalí; (E) Charca de Filabres; (F) Pozze dell’Ucceria (TP072); (G) Pozza di Grotta del Piano (TP185).

Following the Köppen climate classification, the ponds included in this study are located in the Mediterranean climate, characterized by hot-dry summers and cold-wet winters [26]. On a regional scale, Andalusia is divided in five climatic regions [27], and the studied ponds are included in two of

them, i.e., in the “medium mountain Mediterranean climate” (Charca de Filabres) and in the “high mountain Mediterranean climate” (the other four ponds). These regions are characterized by an annual range of mean temperature of 11–18 °C for the medium mountain Mediterranean climate, and of 2–13 °C for the high mountain Mediterranean climate [27]. Data from meteorological stations located close to the studied ponds indicate mean temperature values between 10–15 °C in Charca de Filabres and 9–13 °C in Gádor’s ponds. The values of annual rainfall are in the range of 100–375 mm for Charca de Filabres and between 90–460 mm for Sierra de Gádor’s ponds, with mean values of 218.5 mm and 250.5 mm, respectively [28]. In all cases, summer is a dry period, with temperatures and evapotranspiration rates that favor the temporality of these ponds.

### 2.1.2. Sicily

In order to collect comparative data from the Sicilian occurrence localities of the species, a sampling campaign was carried out, leading to the confirmation of the only Sicilian site previously reported [29], and to the finding of another, to date unpublished, site inhabited by the species. Both Sicilian localities of *M. chevreuxi* are located on Favignana island, and are temporary water bodies occurring on carbonatic Meso-Cenozoic outcrops [30] (Figures 1 and 2; Table 1). “Pozze dell’Ucceria” (coded TP072, [29]) is a system of two neighboring ground pools close to the seashore, which are interconnected when at their full ponding phase. “Pozza di Grotta del Piano” (coded TP185) is a rock pool belonging to a system of kamenitzas in the inner part of the island, at the western slope of Monte Santa Caterina. The pools, located about 1.75 km apart, are subject to a typical Mediterranean climate; in Trapani meteorological station, located on Sicilian mainland 15 km east of Favignana Island, the average annual rainfall is 483 mm and the average temperature is 18 °C [31].

### 2.1.3. Majorca

No novel data could be obtained for Majorca, the other European region where *M. chevreuxi* is known to occur. However, some information about the characteristics of the inhabited water bodies was provided in previous publications for the Lluçmajor plateau [32,33] and the Mondragó Natural Park [34], both corresponding to temporary ponds. The last reference did not provide information on the exact location or characteristics of the ponds where *M. chevreuxi* was collected. Both Marina de Lluçmajor and the Mondragó Natural Park have annual precipitation values lower than 500 mm [32,34].

## 2.2. Sampling Methods and Crustacean Identification

### 2.2.1. Sampling

At each sampling site (Table 1), electric conductivity was measured in situ with a portable conductivity meter Hanna HI 98312 (Hanna Instruments, Villafranca Padovana, Italy). pH and turbidity were also measured in situ with a Hanna Checker plus and a portable turbidity meter Hanna HI 93703 (Hanna Instruments, Villafranca Padovana, Italy). Water samples for nutrient analyses were taken in sterile polyethylene vials and stored in dark and cold conditions (4 °C). Samples were brought back to the laboratory for further analysis within the following 24 h. Nitrates ( $\text{NO}_3^-$ ), ammonia ( $\text{NH}_4^+$ ), phosphates ( $\text{PO}_4^{3-}$ ) and alkalinity were measured in filtrated samples (Whatman GF/F), by using the ultraviolet spectrophotometric screening method [35] for nitrates, and colorimetric methods for alkalinity (Hanna HI775, (Hanna Instruments, Villafranca Padovana, Italy), ammonia (Hanna HI733), (Hanna Instruments, Villafranca Padovana, Italy) and phosphate (Hanna HI717), Hanna Instruments, Villafranca Padovana, Italy). Total nitrogen (TN) and total phosphorus (TP) were further analyzed in unfiltered water samples with Nanocolor 985 083 and Nanocolor 985 076 analysis kits (Macherey-Nagel GmbH & Co.KG, Düren, Germany), respectively.

A characterization of the aquatic vegetation in the studied ponds was also made. Samples were collected in each pond along two longitudinal transects, one crossing the pond, from the shoreline to the deepest point and therefrom to the opposite shoreline, and the other one along the shoreline.

This sampling strategy allowed us to evaluate the aquatic vegetation considering the potential spatial heterogeneity. Once in the laboratory, all species were identified according to [36,37].

Contextually, the amphibian larvae were censused along two longitudinal transects through each water body and identified in situ, according to [38,39]

Crustacean samples were collected by means of 63- $\mu\text{m}$  (Andalusia, Spain) or 125- $\mu\text{m}$  (Sicily, Italy) mesh-sized hand nets, paying attention to sample in each microhabitat present in each site, and fixed in situ in 95% ethanol.

### 2.2.2. Crustacean Identification

Crustacean samples collected in Andalusia and Sicily were processed in the laboratory. All crustacean individuals were identified to species level according to specific identification keys for branchiopods [40], copepods [41,42] and ostracods [43].

Samples are now stored in the crustacean collection of Federico Marrone at the University of Palermo, Italy (Branchiopoda and Copepoda) and in the crustacean collection of Francesc Mesquita-Joanes at the University of València, Spain (Ostracoda), and are available for loan on request. Voucher specimens of *M. chevreuxi* from the five Andalusian and the two Sicilian sites were deposited at the Museo di Storia Naturale, Sezione di Zoologia “La Specola”, Università di Firenze, Italy (Catalogue numbers: MZUF640-MZUF646).

In the light of the high incidence of cryptic species observed among diaptomid copepods, and in order to explore the affinities among the investigated populations, a molecular characterization of collected diaptomid specimens was carried out in parallel to their morphology-based identification. Accordingly, specimens from each of the five Andalusian and the two Sicilian populations were analysed genetically by amplifying a 308-bp long fragment of the mitochondrial gene encoding for the cytochrome b (Cyt-b), following the standard procedures for these analysis [44]. One specimen of *Metadiaptomus asiaticus* from Mongolia (site MNG010 [45]) and one specimen of *Neolovenula alluaudi* from Crete (site GR084 [44]) were processed to be included as comparative material. An additional Cyt-b sequence from a Tunisian specimen of *M. chevreuxi* (from Oued El Mekta, Kairouan [9]) was downloaded from GenBank, to be included in the analyses.

When PCR products showed a clear and single band of the expected length, they were purified using the Exo-SAP-IT<sup>®</sup> kit (Affymetrix USB, Kandel, Germany) and sequenced by MacroGen SPAIN with an ABI 3130xl (Applied Biosystems, Kandel, Germany) sequencer. Chromatograms were imported and edited with Chromas Lite 2.01 (Technelysium Pty. Ltd., South Brisbane, Australia), and the resulting sequences were aligned with BioEdit (Ibis Biosciences, Carlsbad, CA, USA) [46]. MEGA7 [47] was used to translate the Cyt-b sequences to amino acids, in order to check for the possible presence of frameshifts or stop codons, which would indicate the presence of sequencing errors or pseudogenes. Novel sequences were deposited in GenBank under Accession Numbers MT232803 to MT232810, and MT383113.

Bayesian inference (BI) of phylogeny analysis was performed, as implemented in MrBayes 3.2 [48], using a generalized time-reversible (GTR) model of sequence, as selected by the Akaike Information Criterion in JModeltest2 [49,50]. Two independent runs of 2,000,000 generations and four Markov chains with default heating values were performed; trees and parameter values were sampled every 100 generations, resulting in 20,000 saved trees per analysis. An initial fraction of 5000 trees (20%) was discarded as burn-in. The average standard deviation of split frequencies reached a value of 0.001833, and PSFR (Potential Scale Reduction Factor) were between 1.0 and 1.001 for all parameters, indicating convergence of the runs. Node supports were evaluated by their posterior probabilities.

### 3. Results

#### 3.1. The Aquatic Environment

The results obtained (see Table 1) showed that the five investigated Andalusian sites are temporary freshwater ponds ( $<500 \mu\text{S cm}^{-1}$ ), characterized by low alkalinity and low-medium turbidity. The highest turbidity values were registered in Balsa de Caparidán, Balsa de Barjalí and Balsa del Sabinar, with a range between 155 and 238 NTU (Nephelometric Turbidity Units). However, own unpublished data collected in spring 2017 showed turbidity values of 61 NTU for Balsa de Barjalí and 23.27 NTU for Balsa del Sabinar, indicating that these ponds show high turbidity variability as a consequence of wind or bioturbation caused by cattle or wild fauna.

The Sicilian sites are small fresh-subsaline temporary ponds ( $316$  and  $1510 \mu\text{S cm}^{-1}$ ), located near the coast. The alkalinity values are slightly higher than those registered in the Andalusian sites. Turbidity shows a wide range of values ( $0.14$  NTU in Pozza di Grotta del Piano and  $466$  NTU in Pozze dell'Ucceria), indicating that *M. chevreuxi* may be present both in turbid and crystal-clear waters, at least temporarily.

The ponds hosting *M. chevreuxi* in Majorca [32,33] are also temporary small freshwater-subsaline ponds (with a range of  $364$ – $1826 \mu\text{S cm}^{-1}$  for the ponds of Marina de Lluçmajor), located near the coast. Although no data on the turbidity of these ponds are provided [32], the author indicates that the waters are clear, but may be moderately muddy at the beginning of the rainy season. The alkalinity values [32] are similar to those registered in the Andalusian and Sicilian ponds, with a wide range of values (see Table 1). Following a hydrochemical classification of watertypes [51], all ponds (from Andalusia, Sicily and Majorca) are characterized by moderately high to low alkaline waters.

Generally, TN concentration was high in all Andalusian study ponds ranging from  $1.2$  (Balsa Blanca) to  $2.3 \text{ mg L}^{-1}$  (Charca de Filabres). Dissolved N fractions (nitrate and ammonia) represent a low percentage of TN and the highest proportion was found in Charca de Filabres (23%). In relation to TP concentration, it ranged from values below detection limit (Charca de Filabres) up to  $0.3 \text{ mg L}^{-1}$  (Balsa del Sabinar and Balsa de Barjalí). It is relevant to note that phosphates ( $\text{PO}_4^{3-}$ ) represent a minor fraction of the whole P pool evidencing the potential high proportion of particulate P.

In the Sicilian ponds, it is remarkable to mention the high values of TN, but also the high variability observed in its concentration between ponds, which was extremely high in Pozze dell'Ucceria ( $8.3 \text{ mg L}^{-1}$ ), likely due to the droppings of the numerous yellow-legged gulls *Larus michahellis* regularly frequenting the site, but much lower in Pozza di Grotta del Piano ( $1.7 \text{ mg L}^{-1}$ ), where no yellow-legged gulls nor their droppings were observed. Dissolved N fractions (nitrate and ammonia) represent a relatively important percentage of TN ( $>24\%$ ). In relation to TP concentration, it also differs between both study systems ( $0.6 \text{ mg L}^{-1}$  in Pozze dell'Ucceria and  $0.1 \text{ mg L}^{-1}$  in Pozza di Grotta del Piano). As in Iberian ponds, phosphates ( $\text{PO}_4^{3-}$ ) also represent a major fraction of the whole P pool, evidencing a secondary role of particulate P.

Unfortunately, no nutrient data are available for *M. chevreuxi* sites in Majorca. However, the areas where these ponds are located have been extensively modified by livestock activities in the Lluçmajor area [52], and by agriculture, livestock activities and tourism in Mondragó Natural Park [53], so that eutrophic conditions similar to those characteristics of the Andalusian ponds can be expected.

#### 3.2. Co-Occurring Flora and Vertebrate Fauna

All studied Andalusian ponds present the same aquatic vegetation (see Table 1), with the presence of the charophyte *Tolypella prolifera* and the vascular plants *Ranunculus peltatus* subsp. *saniculifolius* and *Eleocharis palustris*. In three of these ponds the Iberian endemic amphibian plant *Coronopus navasii* is also present. The vegetation in Sicilian ponds is poorer, with the presence of the amphibian plants *Callitriche* sp. and *Elatine macropoda* in one of the two ponds (Pozza di Grotta del Piano), whereas no aquatic macrophytes were observed in Pozze dell'Ucceria by [29], nor in the frame of the present survey. However, it is worth mentioning that in other rock-pools in Favignana, *Ranunculus trichophyllus* and

*Chara cf. aspera* occur (unpublished data). These species might be present also in Pozze dell'Ucceria, but were not yet grown at the sampling date. In the Majorca ponds, the aquatic vegetation species list is more extensive, with the presence of eight species in the Lluçmajor ponds [32]: one pteridophyte (*Marsilea strigosa*) and seven spermatophytes (*Ranunculus baudotii*—actually included in *Ranunculus peltatus* subsp. *peltatus*-, *Crassula vaillantii*, *Damasonium alisma*, *Zannichellia peltata*, *Callitriche brutia*, *Elatine macropoda* and *Lythrum hyssopifolia*). A more detailed study of the aquatic vegetation of these ponds was carried out by Rita and Bibiloni [52]. Some of these species, including *R. peltatus*, *C. vaillantii*, *D. alisma* and *E. macropoda*, are also found in the ponds of the Mondragó Natural Park [34].

In all the studied Andalusian ponds, natterjack toad *Epidalea calamita* larvae were found. Analogously, one of the Sicilian ponds is a breeding site for the Sicilian green toad *Bufoles boulengeri siculus* (Pozza di Grotta del Piano), and the Balearic green toad *Bufoles balearicus* breeds in the Lluçmajor [54] and Mondragó ponds [34].

### 3.3. Crustacean Assemblages

Overall, 11 crustacean taxa were identified in the studied Andalusian samples, i.e., two copepods, five branchiopods and four ostracods (Table 2). All of them could be confidently identified to species level, with the exception of *Ceriodaphnia* sp. 1, for which only a few immature specimens were collected, and *Leucocythere cf. mirabilis*, which deserve further studies, taking into account the fact that the species has been traditionally considered typical of deep oligohaline permanent lakes in central Europe [43], but it has been found recently around the Mediterranean only in temporary ponds [4,55,56].

Based on both the species list previously provided [29] and the samples collected in 2018, 14 crustacean taxa were identified in Sicilian ponds, i.e., two copepods, six branchiopods and six ostracods (Table 2). Unidentified immature cyclopoids were collected in Pozze dell'Ucceria only in 2005. In this pond, the occurrence of the ostracod *Heterocypris incongruens* has been reported [29], but in the 2018 samples, we found *Heterocypris cf. barbara*; both species are common in temporary ponds, and can sometimes co-inhabit the same water body [43].

The crustacean checklist produced for the Lluçmajor Majorcan sites [32] is based on pooled data from 22 ponds inhabited by *M. chevreuxi*, and do not include ostracods or cyclopoid copepods. Accordingly, it is not directly comparable with the other checklists presented here. Overall, 18 crustaceans are listed for the area of Marina de Lluçmajor, i.e., 14 branchiopod and four calanoid copepod taxa (see Table 4 of [32]). In the temporary ponds of the Mondragó Natural Park, the presence of the branchiopods *Triops cancriformis*, *Branchipus schaefferi* and *Coronatella (Ephemeralona) elegans* (as *Alona elegans*), has been recorded [38], but the authors do not specify if they were found together with *M. chevreuxi* in any of the four sites analyzed.

Both male and female diaptomids from the Andalusian and Sicilian sampled ponds presented a morphology in perfect agreement with [29,41,42], and were thus identified as *M. chevreuxi*. The atypical number of setae on the caudal rami represented in Figure 5A of [32], which was already stressed by [29], is surely to be ascribed to an abnormal feature of the depicted specimen, and thus of no taxonomical value. No inter-population morphological variability was observed. Moreover, the Cyt-b phylogenetic tree rooted on *Neolovenula alluaudi* showed an arrangement of the ingroup populations in good accordance to their morphology-based identification, i.e., with two well-supported and reciprocally-monophyletic *M. asiaticus* and *M. chevreuxi* clades (Figure 3). Within the *M. chevreuxi* clade, the eastern (Sicilian and Tunisian) and western (Andalusian) populations investigated here form two well-defined subclades. Two different haplotypes were observed in Andalusian populations, one limited to Charca de Filabres, the other one shared by the four populations from Sierra de Gádor. Conversely, the specimens from the two Sicilian pools and the Tunisian one downloaded from GenBank shared the same haplotype.

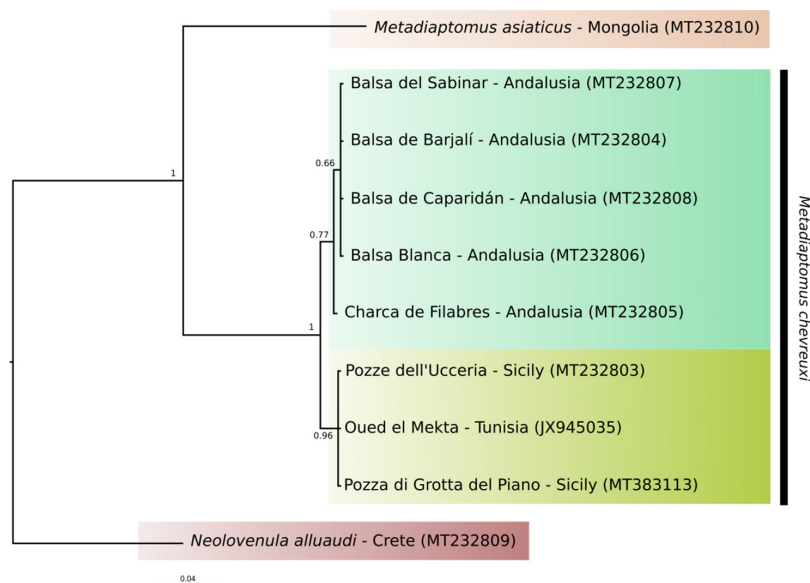
The diaptomid copepods collected in the surveyed ponds could thus be positively identified as the Paradiaptominae *M. chevreuxi*, based on both morphological and molecular evidences.



**Table 2.** Crustacean species list in Andalusian and Sicilian ponds at the sampling date. Sites 1: Blanca; 2: Caparidán; 3: Sabinar; 4: Barjalí; 5: Filabres; 6: Pozze dell’Ucceria; 7: Pozza di Grotta del Piano (see Figure 1).

Taxa	1	2	3	4	5	6	7
<b>COPEPODA</b>							
<b>Calanoida</b>							
<i>Metadiaptomus chevreuxi</i> (Guerne & Richard, 1894)	X	X	X	X	X	X	X
<b>Cyclopoida</b>							
<i>Metacyclops minutus</i> (Claus, 1863)	X	X	X	X			
Cyclopoida n.i.						X <sup>4</sup>	
<b>BRANCHIOPODA</b>							
<b>Anostraca</b>							
<i>Branchipus schaefferi</i> Fischer, 1834	X				X	X	X
<b>Notostraca</b>							
<i>Triops cancriformis</i> (Bosc, 1801)						X	
<b>Anomopoda</b>							
<i>Daphnia (Ctenodaphnia) atkinsoni</i> Baird, 1859	X	X	X	X	X	X	X
<i>Ceriodaphnia</i> sp. 1				X <sup>1</sup>	X <sup>1</sup>		
<i>Ceriodaphnia</i> sp. 2							X <sup>6</sup>
<i>Coronatella (Ephemeralona) orellanai</i> (Alonso, 1996)	X	X	X	X	X		
<i>Coronatella (Ephemeralona) elegans</i> (Kurz, 1875)						X	X
<i>Pleuroxus letourneuxi</i> (Richard, 1888)							X
<i>Macrothrix hirsuticornis</i> Norman & Brady, 1867				X			
<b>OSTRACODA</b>							
<b>Podocopa</b>							
<i>Eucypris virens</i> (Jurine, 1820)	X	X	X <sup>1</sup>	X <sup>2</sup>	X <sup>3</sup>	X	X
<i>Tonnacypris lutaria</i> (Koch, 1838)						X	
<i>Heterocypris incongruens</i> (Ramdohr, 1808)						X <sup>5</sup>	
<i>Heterocypris</i> cf. <i>barbara</i> (Gauthier & Brehm, 1928)						X	X
<i>Plesiocypridopsis newtoni</i> (Brady & Robertson, 1870)							X
<i>Cypridopsis elongata</i> (Kaufmann, 1900)	X	X	X	X			
<i>Potamocypris arcuata</i> (Sars, 1903)		X		X	X	X	X
Cyprididae n.i.						X <sup>4</sup>	
<i>Leucocythere</i> cf. <i>mirabilis</i> Kaufmann, 1892				X			

<sup>1</sup> Immature specimen(s); <sup>2</sup> Two different morphotypes; <sup>3</sup> Only a single dead valve; <sup>4</sup> Juveniles, not identified, possibly *Eucypris virens*; <sup>5</sup> Cited in [33]; <sup>6</sup> “*C. quadrangula*” sensu [39]. In fact, this is a new species pending a formal description (M. Alonso, *in litteris*).



**Figure 3.** Bayesian phylogram based on a 308-bp long fragment of the mitochondrial gene for the cytochrome b. Node support is reported as nodal posterior probabilities. Accession numbers of both novel and GenBank sequences shown in brackets. The “Andalusian” and the “Sicilian-Tunisian” subclades are reported in different shades of green.

#### 4. Discussion

Within the primarily Afrotropical subfamily Paradiaptominae, two species are, to date, reported to occur in the Ibero-Balearic region and Macaronesia: *N. alluaudi*, which is widespread in mainland Spain, Portugal, Balears and the Canary Islands [14,57]; and *M. chevreuxi*, to date reported for Majorca only [21,32] (Figure 3). This last species is characterized by a fragmented distribution, including some Sahara oases [7], the coastal countries of the western Mediterranean basin [21], and some countries of the Middle East (Jordan, Iraq and Iran, see [58–60], thus being absent in the eastern Mediterranean area. In all the inhabited areas, *M. chevreuxi* is limited to steppic and substeppic areas characterized by low precipitation values ( $<500 \text{ mm y}^{-1}$ ) (see [29,42] and references therein). To date, the alleged absence of *M. chevreuxi* from mainland Iberia, in spite of the existence of suitable habitats and climatic conditions, puzzled the researchers (cf. [32]) and, in fact, the present findings prove that its alleged absence was actually to be ascribed to an inadequate knowledge of diaptomid diversity and distribution in the Iberian Peninsula. Interestingly, another “African” crustacean species reported by Jaume [32] from Majorca and allegedly absent from the Iberian mainland, i.e., the spinicaudatan *Leptestheria mayeti*, was also recently found in eastern Spain [61] and southern Italy [62], further stressing the existence of a significant “Wallacean shortfall” affecting our understanding of inland water crustacean distribution patterns.

In accordance to the known ecological preferences of the species, all the European *M. chevreuxi* localities share a low annual mean precipitation (always lower than 500 mm per year), i.e., they can be considered substeppic zones, according to [63]. Conversely, they are quite distinct with regards to thermal regimes and elevation. The Andalusian localities of *M. chevreuxi* occur at rather high altitudes (1050–1830 m a.s.l., see Table 1), in contrast with the low altitudes at which most of the other currently known sites are located (e.g., [58] sub “*Metadiaptomus asiaticus lindbergi*”; [29,32,34,59,64,65] and present data), but in agreement with the known occurrence of the species at 1000–1300 m a.s.l. on the Algerian *Hautes Plaines* [60,63], up to 1990 m a.s.l. on the Ahaggar Mountains [60] and at about 1000 m a.s.l. in Jordan [60]. Accordingly, precipitation, rather than temperature, seems to be one of the major driving environmental features influencing the distribution of the species. However, the small size and the isolation of the inhabited water bodies might also play a role in determining the limited number of European populations of this species, since such ponds are usually characterized by the presence of a lower number of species, including fewer competitors and predators (cf. [4,66]), conditions that would have to be assessed in future studies to see its influence on the presence of this species.

The landscapes of the study areas are characterized by a pronounced incidence of endemic taxa, and by a remarkable North African or Saharo-Sindian influence [67–69]. The aquatic plant communities present in the European ponds where *M. chevreuxi* is found (see Table 1) are very similar, in relation to their autecology. All the species are typical of temporary ponds with low or moderate alkalinity values and typical from fresh-subsaline waters, with many of them being indifferent to the trophic state or typically occurring in meso-eutrophic waters [70,71]. A similar scenario can be observed for the amphibian communities: *E. calamita* (Andalusia), *B. balearicus* (Llucmajor and Mondragó, Majorca [54]) and *B. boulengeri siculus* (Sicily; unpublished data) are species which have a rapid growth and development, in order to cope with the temporality and unpredictability of these ecosystems [72,73].

The crustacean fauna co-occurring with *M. chevreuxi* in Andalusian, Majorcan and Sicilian ponds is relatively paucispecific and dominated by primarily steppic or substeppic taxa adapted to short-lasting and unpredictable temporary water bodies (Table 2, [29,32,34]). Among them, some taxa, such as the notostracan *T. cancriformis* (not collected in Andalusian ponds), the anostracan *B. schaefferi*, the anomopods *Daphnia atkinsoni* (reported sub *D. bolivari* by [32]) and *Coronatella* spp., and the ostracod *Eucypris virens*, were found in the majority of the ponds inhabited by *M. chevreuxi*. It is also remarkable the common occurrence of ostracod species considered to have mostly circum-Mediterranean distributions, particularly *P. arcuata* in both areas, *C. elongata* in the Andalusian ponds and *H. cf. barbara* in the Sicilian ones [43]. The latter is also recognized as a typical steppic species in Spain and

elsewhere [2,74], so as is the presence of males of *E. virens* in one of the Sicilian ponds, confirming their unpredictable hydroperiod [4]. In this framework, the Iberian endemic *Coronatella (Ephemeralona) orellanai* observed in Andalusia is a vicariant species of the closely related *Coronatella (Ephemeralona) elegans*, co-occurring with *M. chevreuxi* in Sicily (present work) and in Majorca [32,34]. Interestingly, no other calanoid species co-occurred with *M. chevreuxi* in Andalusia and Sicily, whereas up to three calanoid species might occur with *M. chevreuxi* in Majorca [32]. It is, however, to be considered that the species lists provided here are based on occasional samplings, and therefore likely not exhaustive of the actual crustacean assemblages inhabiting the studied sites.

The recent versus ancient origin of the crustacean communities inhabiting the arid areas of the Iberian Peninsula has long been questioned (e.g., [75,76]). Analogously, two major hypotheses can be drawn to explain the current occurrence of *M. chevreuxi* in Andalusia. The species might have recently arrived through a long-range, post-glacial dispersal event from North Africa, as proposed for the Sicilian *M. chevreuxi* populations by [77], and for some Corsican copepod species by [78], or it might rather be a Neogene relict of a period with more generalized (sub)steppic conditions [1,79].

Our exploratory molecular analysis (Figure 3) shows the presence of a single, shared haplotype in the investigated Tunisian and Sicilian populations, which is in accordance with the hypothesis of a recent immigration of the species to Sicily from Tunisia; conversely, two private and different haplotypes were found in the Andalusian populations, one limited to the four ponds located on Sierra de Gádor, the other to the single site located on Sierra de los Filabres. Even though the limited number of investigated populations and individuals prevent from drawing sound conclusions, this pattern is congruent with the existence of short-range dispersal events mediated by the cattle among the ponds occurring in Sierra de Gádor, and with the reciprocal isolation of the populations inhabiting the two Andalusian mountain complexes, i.e., with a likely relict origin of *M. chevreuxi* Betic populations. Moreover, the contrasting pattern in genetic diversity between Sicilian and Andalusian populations seems in accordance with their geographical locations. The high-altitude locations of the Andalusian ponds, away from significant bird migratory routes, is sharply different from the location of the Sicilian sites, which occur on a Mediterranean island located along one of the most important trans-Mediterranean bird migratory pathways, which might act as a “dispersal corridor” for circum-Mediterranean crustaceans passively-dispersing between Africa and Europe. In order to support this hypothesis, more specimens from the whole distribution range of the species should be investigated and sequenced, paying particular attention to the *M. chevreuxi* populations occurring in western Maghreb.

Finally, we would like to stress that more attention should be paid to the conservation of small and isolated ponds in light of their importance for ecological processes [66,80] and as considerable biodiversity hot-spots, both for vertebrates, invertebrates and plants [81–84]. Including their drainage basins under a pond-network approach [85] in the management plans is pivotal for their effective conservation, since meta-ecosystems allow for gene flow and species dispersal between the ponds through metapopulation dynamics, thus granting higher chances for a long-term survival of their biota [86]. This is the case of the Andalusian temporary ponds discussed here, which host rare animal and plant species, such as the copepod *M. chevreuxi*, the ostracod *Leucocythere* cf. *mirabilis* and the charophyte *T. prolifera*, a rare species that had not been cited in Spain since 1985 [87,88]; also being the only currently known location for two endemic diatom species: *Craticula gadorensis* and *Hantzschia gadorensis* [89,90]. Conveniently, the majority of these ponds are currently protected under EU Natura 2000 network (see Table 1). However, and although the protection measures should guarantee the conservation of these unique diaptomid copepod populations and their accompanying biota in the European context, all these ponds are actually suffering from anthropogenic impacts, such as those derived from livestock activities with the re-excavation of the pond basins in Majorcan and Andalusian ponds [52,91,92]. Moreover, impacts derived from human activities (e.g., urban development, tourism) on ponds located near the coast (as in Sicily and Majorca), and others derived from the increase in agricultural activity (in Majorca and Andalusia), could occur as well. For these reasons, we think it is

an opportune time to carry out periodic surveys to verify the persistence of *M. chevreuxi* populations in its few currently known European occurrence localities, and to carry out further sampling efforts to locate new populations of the species, both in nearby regions and in other geographical areas where ponds with similar ecological characteristics are present.

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