

Phylogeny, molecular ecology and taxonomy of southern Iberian lineages of *Triops mauritanicus* (Crustacea: Notostraca)

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Abstract We investigated the phylogeography of the main lineages in the tadpole shrimp *Triops mauritanicus* Ghigi in the south-western Iberian Peninsula, using mitochondrial 12S and 16S rDNA sequences. Our results indicate that a fourth, hitherto unknown main phylogenetic lineage occurs in Iberia, so that in total, the species is divided into six distinct clades, comprising *T. m. mauritanicus*, *T. m. simplex* Ghigi, and four as yet unnamed lineages that appear to be endemic to Iberia. Percentages of sequence

divergence among the main clades in *T. mauritanicus* reach the range reported for recognized species in other notostracan lineages. A thorough morphological investigation also revealed that the differentiation among these lineages is higher than previously thought, and that populations of three of the main clades within *T. mauritanicus* can be reliably separated from each other and from the remaining lineages based on the morphology of adult males. The remaining clades also show a significant level of morphological

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differentiation, but include a certain proportion of populations for which the additional application of molecular methods is needed for a reliable determination. The geographic distributions of 12S haplotypes are indicative of frequent dispersal events and gene flow among populations belonging to the same main lineage, but give no evidence of recent migration events among different main lineages, suggesting that there is no gene flow among the latter. Our data thus suggest that the six main lineages within *T. mauritanicus* represent distinct species. We therefore describe the Iberian lineages as *T. baeticus* Korn n. sp., *T. emeritensis* Korn & Pérez-Bote n. sp., *T. gadensis* Korn & García-de-Lomas n. sp., and *T. vicentinus* Korn, Machado, Cristo & Cancela da Fonseca n. sp., and reinstate *T. simplex* Ghigi to full species status. Our data confirm the general, previously recognized pattern of a lower dispersal probability in gonochoric *Triops* taxa. However, we found evidence that passive dispersal in *Triops* may be further complicated by a strong habitat dependence of dispersal probability, mediated by prevailing dispersal vectors.

Keywords Phylogeography · Gene diversity · Passive dispersal · Dispersal probability · Gene flow · Waterbird

Introduction

Low levels of morphological differentiation in many passively dispersed aquatic invertebrates had led to the assumption that these taxa had a cosmopolitan distribution and that there was little restriction to frequent long-distance dispersal (Bohonak and Jenkins 2003). The application of molecular tools has greatly changed our knowledge on these taxa, and led to the general conclusion that many zooplankton species have small geographic distributions and often show a high level of genetic substructure (e.g. Colbourne et al. 2006; Ishida and Taylor 2007), indicating that high potential for dispersal does not necessarily translate to high effective dispersal rates or gene flow (Bohonak and Jenkins 2003). Similar cryptic diversification has also been reported in large branchiopods (e.g. Korn et al. 2006; Korn and Hundsdoerfer 2006; Sassaman et al. 1997). The factors that may interact to uncouple dispersal from gene flow are summarized in the Monopolization Hypothesis formulated by De Meester et al. (2002). This hypothesis suggests that for many freshwater organisms, the impact of new immigrants was reduced by a numerical effect (high population growth rates and large resting propagule bank) and a fitness effect (local adaptation of residents), leading to the monopolization of resources by first colonizers, enhancing priority effects and reducing gene flow, so that neighbouring populations may commonly show pronounced genetic differentiation despite high dispersal capacity. Bohonak and Jenkins (2003) argue that

the Monopolization Hypothesis may not be generally applicable to the majority of freshwater invertebrates, and stress that generalizations about overland dispersal in freshwater taxa are not valid, and that specific information is needed for each taxon. The case of the Notostraca supports this argumentation, for example, because indirect evidence suggests that closely related species in this group show different dispersal probabilities (Korn et al. 2006; Sassaman et al. 1997: Fig. 3). These differences appear to be linked to reproductive modes, and gonochoric taxa (i.e. those that have an obligately outcrossing mode of reproduction, with separate male and female individuals) have lower inferred dispersal probabilities.

The Notostraca comprise two genera with worldwide distributions, *Triops* Schrank, 1803, and *Lepidurus* Leach, 1819. Both occur almost exclusively in temporary bodies of water and can even inhabit ponds that remain dry for several years, as they are capable of enduring prolonged dry phases via resting eggs (e.g. Fryer 1988; Longhurst 1955). Among the European regions inhabited by *Triops*, the Iberian Peninsula is of special interest to studies on dispersal abilities and phylogeography, since several highly divergent phylogenetic lineages of possibly subspecific status have been recorded in a rather small geographic region in south-west Iberia (Korn et al. 2006). The predominant species of *Triops* in the Iberian Peninsula is *T. mauritanicus*. Originally established as a species by Ghigi (1921) it was later treated as a subspecies of *T. cancriformis* (Longhurst 1955), but has been returned to full species status (Korn et al. 2006). The northern African populations of the former *T. c. simplex* (originally described as a separate species; Ghigi 1921) are presently recognized as a subspecies of *T. mauritanicus* (Korn et al. 2006).

In this study, we use 12S and 16S rDNA sequences to investigate the phylogeography of the main lineages within *Triops mauritanicus* in the south-western Iberian Peninsula, and to infer dispersal abilities in these gonochoric taxa. In addition, we conduct a thorough morphological investigation. The different datasets on genetic divergence, phylogeography, inferred patterns of gene flow and morphological diversification are used to re-evaluate the taxonomy of the group.

Material and methods

Taxon sampling

We attempted to acquire as many different samples of *Triops mauritanicus* from southern Iberia as possible (for locality data, see Appendix section, Table A1). We used both wild-caught specimens and specimens raised in the laboratory from eggs from sediments. Samples were

preserved in 70–99.8% ethanol until extraction. Tissue vouchers have been deposited in the tissue collection of the Museum of Zoology (Museum für Tierkunde), Senckenberg Dresden, Germany, under the MTD-TW numbers listed in Table A1. Voucher specimens from the morphological analyses have been deposited in the invertebrate collection of the same museum, under the numbers MTD Crus 3046–3436 [in addition, samples MTD Crus 2640–2645, 2647, 2676–2680, 2688–2691, 2697–2701, 2705–2709, 2713–2718, 2724–2727, 2734–2738, 2744–2749, 2754–2759, 2765–2770, 2775–2778, 2781–2787 and 2792–2801 were included in morphological analyses (these are samples from Korn et al. 2006; MTD numbers originally referred to populations but were later partially redistributed in order to store individual specimens separately)]. Sequences are available from GenBank under accession numbers FN691389–FN691428 (12S) and FN689861–FN689867 (16S). Earlier sequences of *T. mauritanicus* and *T. cancriformis* were retrieved from GenBank and also included in the phylogenetic analyses (AM183829–AM183832, AM183836–AM183840, AM183842, AM183854–AM183861, AM183867; samples listed in Tables 1, A1).

Table 1 Overview of sequences retrieved from GenBank, including short names used in present study for haplotypes of *Triops c. cancriformis*

| Taxon | Accession no. | Gene | Haplotype |
|---------------------------|---------------|------|------------------------------|
| <i>T. c. cancriformis</i> | AB084514 | 12S | <i>T.c.c.</i> 4 |
| <i>T. c. cancriformis</i> | AY159564 | 12S | <i>T.c.c.</i> 3 |
| <i>T. c. cancriformis</i> | DQ369308 | 12S | <i>T.c.c.</i> 5 |
| <i>T. c. cancriformis</i> | AY159575 | 16S | <i>T.c.c.</i> 4 ^a |
| <i>T. c. cancriformis</i> | AY159577 | 16S | <i>T.c.c.</i> 6 ^a |
| <i>T. c. cancriformis</i> | AB084514 | 16S | <i>T.c.c.</i> 8 |
| <i>T. longicaudatus</i> | AY639934 | 12S | |
| <i>T. granarius</i> | AY115602 | 12S | |
| <i>L. a. apus</i> | AF494483 | 12S | |
| <i>L. a. lubbocki</i> | AY159567 | 12S | |
| <i>L. arcticus</i> | AY159569 | 12S | |
| <i>L. lemmoni</i> | AY115604 | 12S | |
| <i>T. longicaudatus</i> | AY639934 | 16S | |
| <i>T. granarius</i> | AY115612 | 16S | |
| <i>L. a. apus</i> | AY159584 | 16S | |
| <i>L. a. lubbocki</i> | AY159583 | 16S | |
| <i>L. arcticus</i> | AY159585 | 16S | |
| <i>L. lemmoni</i> | AY115614 | 16S | |

^a Our 12S haplotype *T.c.c.* 4 corresponds to H1 in Mantovani et al. (2004), so that short names in Fig. 2b–c for the dataset with 12S and 16S sequences combined are *T.c.c.* 4 4 and *T.c.c.* 4 6 for Mantovani et al.'s (2004) specimens Tcsi2, AY159575 and Tcsa2, AY159577, respectively

Determination of specimens

The characters given by Korn et al. (2006) were used to assign samples of *Triops* to species. All specimens from southern Iberia had large furcal spines, and thus could be determined unambiguously as *T. mauritanicus*. The present study's 'S. Iberian' lineage within *T. mauritanicus* corresponds to the 'S. Spanish' haplotype group in Korn et al. (2006). Eurasian and North African populations of *T. cancriformis* are referred to as *T. c. cancriformis* here, to avoid confusion with certain populations in southern Africa (Hamer and Rayner 1995) whose actual status (subspecies of *T. cancriformis*?) and species affiliation remain to be investigated.

DNA extraction, PCR amplification and sequencing

DNA methods followed Korn et al. (2006), with the exception that PCR products were sequenced on an ABI 3130 sequencer (Applied Biosystems) at the Museum of Zoology, Senckenberg Dresden.

Sequence alignment, nucleotide composition, and substitution patterns

Computerized alignments (obtained with Clustal W in the program BioEdit; Hall 1999) were modified by hand using BioEdit (alignments available from <http://purl.org/phylo/treebase/phylovs/study/TB2:S10349>). In both, the 12S and the 16S datasets, four *Lepidurus* and two *Triops* sequences (*T. longicaudatus* and *T. granarius*) were included as outgroups (Table 1). Nucleotide composition, substitution frequencies, pairwise transition/transversion frequencies, and pairwise distances (uncorrected p-distances) were calculated with PAUP* 4.0b10 (Swofford 2003). To enable an assessment of the overall range of sequence divergence found among the sublineages of the ingroup (*T. mauritanicus* and *T. c. cancriformis*), we compared the mean genetic distances between all *T. mauritanicus* lineages and between these lineages and *T. c. cancriformis* (calculated with MS-Excel). MEGA version 3.1 (Kumar et al. 2001) was used to illustrate parsimony-informative characters and singletons. The program ForCon 1.0 (Raes and Van de Peer 1998) was used to convert input files between formats. To assess saturation effects in this dataset, pairwise comparisons of transitional and transversal changes were plotted against pairwise distances in DAMBE version 4.2.13 (Xia and Xie 2001; for all distance correction methods implemented in DAMBE, we consistently found that the data were not saturated).

Phylogenetic analysis

The 428 12S ingroup sequences obtained were collapsed to 53 haplotypes (Tables 1, A1), 48 of which were detected

within *Triops mauritanicus*. A second dataset comprised 26 ingroup 16S haplotypes (18 within *T. mauritanicus*; collapsed from 119 ingroup sequences). A third dataset used to investigate relationships among the ingroup lineages consisted of combined 12S and 16S sequences of those samples for which both gene fragments were available. This combined 12S and 16S dataset comprised 38 mitotypes (29 within *T. mauritanicus*). Data analysis for all three datasets was performed using maximum parsimony (MP; settings gapmode=new; add=cl) and maximum likelihood (ML) as implemented in PAUP* 4.0b10 (Swofford 2003). Additional ML searches performed with the program RAxML (Stamatakis et al. 2008) consistently resulted in tree topologies identical (with respect to the relationships among the main ingroup lineages) to those of ML phylograms obtained with PAUP*. The best evolutionary models for the data were selected using the program Modeltest (Posada and Crandall 1998; best-fit models were: TVM+G for 12S, HKY+I+G for 16S, and GTR+G for the combined dataset, selected by AIC; parameter values can be obtained from the first author upon request). As a measure of branch support, bootstrap values were calculated with MP in PAUP* (settings nreps=1,000, maxtree=10,000), and with ML in GARLI (version 0.95; Zwickl 2006; setting bootstrapreps=100). Bayesian analysis was additionally applied to the combined 12S and 16S mitotype dataset using MRBAYES 3.1.2 (Huelsenbeck and Ronquist 2001); the settings were four runs with six chains of 5,000,000 generations, sampling every 500, and a burn-in of 1,000. The analysis was partitioned by gene (evolutionary models as specified above, but parameter values were estimated: no priors).

Geographic distribution of Iberian lineages

The geographic distribution of the Iberian lineages within *T. mauritanicus* was derived from mitochondrial sequence data obtained from 422 specimens originating from a total of 103 populations. Different samples obtained from the marisma habitat (natural temporary marshes; see Serrano et al. 2006) of Doñana National Park were considered as belonging to different populations (Table A1).

Genetic diversity of populations and lineages

Analysis of molecular variance (AMOVA) was calculated in Arlequin 2.000 (Schneider et al. 2000) to investigate the level of differentiation in the main phylogenetic lineages of *Triops mauritanicus*. We included only populations in the analysis for which sequence data from at least six individuals were available (populations 001–039, 082–087 and 094–097; Table A1). The ‘S.Iberian’ lineage was broken down to subgroups referring to four main habitat types: (1) marismas of Doñana National Park (populations

001–009); (2) ponds surrounded by forest or shrubland (populations 010–020); (3) ponds in open habitat within 25 km distance to the marismas (populations 021–030; these ponds were situated within 2 km distance from the marismas before vast areas of marshland were transformed to farmland during the 20th century—the sampling sites are now mainly used as pastures or fields); (4) ponds in open habitat more than 75 km from the marismas of Doñana National Park. In the last category, we included only Portuguese samples (populations 033–039) in order to compare data obtained from geographical areas of similar size (i.e., populations 031 and 032 from eastern Sevilla province were excluded from the AMOVA, but were included in this habitat category to investigate gene diversity, see below). A pond was considered to be situated in open habitat when wide areas of meadows or fields were flanking the pond at least to one side. Significance tests were based on 1,023 permutations.

To further investigate genetic diversity in regard to ecological factors, the index of gene diversity H (Nei 1987) as implemented in Arlequin 2.000 (Schneider et al. 2000) was calculated for each of the 39 populations obtained from the ‘S.Iberian’ lineage for which we investigated a minimum of 6 individuals each (populations 001–039). A single-factor analysis of variance (ANOVA) was used to compare levels of gene diversity among the habitat categories as defined above. It is common knowledge that ponds situated in open habitat are more attractive to waterbirds (a major dispersal vector for branchiopod crustaceans, see e.g. Sánchez et al. 2007) than ponds surrounded by forest or shrubs (for a case study demonstrating a clear avoidance of habitats flanked by hedges, see Tourenq et al. 2001). As a result, probabilities of dispersal by waterbirds should be higher in the absence of wooded margins, and we predicted higher gene diversities in *Triops* populations situated in open habitat, including the marismas. Simple regression analysis was used to further investigate a possible correlation of gene diversity in *Triops* to waterbird abundances. As no exact data on waterbird abundances in our sampling sites were available, we estimated relative abundances of waterbirds for each of the sampling sites within Sevilla and Huelva provinces (populations 001–032), at a scale of 5 abundance levels (from 1=low to 5=high). Estimates were based on regular observations during recent years (performed by A.J.G. while blind to the genetic data; see also Rendon et al. 2008).

In addition, we tested for a possible effect of land use for pastures on the gene diversity in *Triops*, as cattle could represent an important vector for dispersal on a local scale. We used an ANOVA with two levels of land use: no pasture use vs. pasture use (populations 001–032 and 035–039; no data available for 033 and 034). Finally, a possible correlation of gene diversity in *Triops* to abiotic factors

was investigated with a multiple regression analysis. Our data on abiotic factors for marisma sites were incomplete, as the central regions within the extensive marismas of Doñana could not be accessed during the flooding phase, so that *Triops* samples from these sites were raised from sediment samples collected during the dry season. Thus, abiotic factors were only investigated for non-marisma habitats. We included pH, conductivity, surface area of the ponds, and distance to marismas as dependent variables in the multiple-regression model. Surface area was measured on satellite photographs obtained from Google Earth version 4.0.2091 beta (Google, Inc.) using PixeLINK Capture SE (Version 3.1. obtained from www.pixelink.com); distances were measured directly in Google Earth (alternatively, for Portuguese ponds, surface area was derived from GPS signals or tracks taken in the field, using UTHSCSA Image Tool software, available at <http://ddsdx.uthscsa.edu/dig/itdesc.html>).

As part of the samples were obtained from sediments, we used three populations of *T. mauritanicus* to test if diversity measurements obtained from field-collected specimens differed from those obtained from lab-raised samples. For each of these populations, we obtained 12S sequences from six field-collected specimens and from an additional six specimens that were raised from eggs from sediment samples. Haplotype diversity was identical for two of the populations and differed by only one (four vs. five haplotypes) in the third sample, indicating that results are comparable and that it is a valid procedure to merge both types of data into a single data file for statistical analysis of diversity measurements (generalization to other studies may not be valid unless the sediment sampling procedure equals that used for the present study, which implies collecting numerous subsamples from different parts of a pond; subsamples can be pooled).

Evidence for recent passive dispersal and gene flow

To test if the present geographic segregation of the main phylogenetic lineages within south-western Iberia might be the result of a general dispersal limitation in gonochoric Notostraca (Korn et al. 2006), we inferred the order of magnitude of dispersal abilities from geographic haplotype distributions. We calculated the additive minimum geographic distance between all occurrence sites of shared haplotypes. We call this the ‘accumulative minimum dispersal distance’ (AMDD) of the haplotype. For the geographic distance measurements performed in the present study, we made the simplifying assumption that shared haplotypes were always the result of dispersal (an independent evolution of identical 12S haplotypes at different sites is assumed to be a rare event, since evolutionary rates in the 12S gene are low enough to be applied to phylogenetic

studies at higher taxonomic levels, see, e.g. Ballard et al. 1992).

As the whole area covered by the marismas of Doñana is interconnected at certain flood events (Serrano et al. 2006), active dispersal may occur in addition to strictly passive dispersal among populations situated within the marismas. Consequently, the marismas were treated as a single site for our passive-dispersal distance measurements, referring to the original range and borderlines of the marismas around the year 1900 (Montes et al. 1998: Fig. 6.4), before large areas of natural marshes were transformed into farmland. In addition, we measured total dispersal distances (including obligately passive overland dispersal among strictly separated ponds as well as potentially active dispersal among marisma sites during high flood events), i.e. those obtained if all sampling localities that were originally situated (or are still situated) within the marismas were treated as separate sites.

For comparison to the results obtained for south-western Iberian lineages of *Triops mauritanicus*, we measured AMDDs for *T. c. cancriformis*. As no exact coordinates were available for some of the sites, AMDDs for this species were rounded off to the nearest multiple of 100 km.

Only co-occurrences of geographically spread haplotypes (i.e. those that were found in at least two sites) were regarded as evidence for recent gene flow between populations, because the mere existence of a single haplotype at numerous sites could simply be the result of unique dispersal events (of sufficient individuals to found a new population), and need not imply that there was an exchange of individuals between different populations. It is important to make this differentiation, because dispersal may be uncoupled from gene flow (e.g. Bohonak and Jenkins 2003).

Morphology

Material and characters

A wide variety of morphological characters was investigated systematically for a representative subset of samples (established, distinctive characters and new ones were tested in a consistent manner). Only those characters that were of sufficiently low variability and showed differences among the major phylogenetic sublineages of *Triops mauritanicus* were subsequently studied for the remaining samples. For males, additional samples of the African *T. m. mauritanicus* (comprising specimens from populations 108, 109, 112, 113 and 115–119; Table A1) and *T. m. simplex* (populations 104–107) were investigated in order to compare the morphology among all known sublineages in the species. In total, the morphology of 459 individuals was investigated. The size of the studied specimens varied

widely, so that several of the morphological characters needed to be standardized for size. We used the minimal width of the telson at its anterior margin (henceforth called telson width; Fig. 1a) as a surrogate for body size. Consistent measurements of total body length are impossible in fixed specimens because of variable degrees of body contraction during fixation (Longhurst 1955).

To validate the usefulness of telson width as a measure of body size, we compared it to the carapace length for a representative subset of samples [carapace length shows isometric growth in *Triops* (see Longhurst 1955), but in *T. mauritanicus* the long terminal carina spines are frequently broken in preserved specimens, raising the need for another character to represent body size]. Graphic presentation of the resulting data using logarithmic scales revealed a growth coefficient (k) of 1.018, which is indicative of isometric growth of carapace length and telson width (carapace length = $7.57 * \text{telson width}^{1.018}$; Hartnoll 1978) and confirms the usefulness of the latter as a measure of body size. All measurements were made on digital photographs using PixelINK Capture SE (Version 3.1, obtained from www.pixelink.com). Photographs of trunk limbs (mounted on microscope slides) were taken with a PL-B686CU PixelINK colour microscopy camera on a Nikon Eclipse E200 microscope, using 2–40 \times magnification lenses.

Telson morphology

Korn et al. (2006) established the telson length ratio (the ratio of furcal spine length to the distance between furcal spine tip and the anterior-lateral edge of the telson) to characterise the size of the furcal spines. Following Korn

et al. (2006), we used two subsidiary lines (telson subsidiary line and furcal subsidiary line; Fig. 1a) to define the anterior starting point of furcal spines as the point where both subsidiary lines meet. In the present survey, we used additional characters to describe the shape of furcal spines: (1) the furcal spine width, measured at the anterior starting point of furcal spines as defined above (to standardize for the size of investigated specimens, the ratio of furcal spine width to telson width was used to represent this character in statistical analysis); (2) the ratio of furcal spine length to furcal spine width (henceforth called furcal spine size ratio).

We call the central part of the telson (i.e., excluding furcal spines) posterior to the telson subsidiary line (Fig. 1a) the telson posterior marginal section. Its posterior margin is typically incised medially, giving it a bilobed appearance. We measured the distance from the foremost point of the margin within the medial incision to the telson subsidiary line (henceforth called minimum length of posterior marginal section). In addition, the distance from the telson subsidiary line to the posteriormost points of the lobes was determined (maximum length of posterior marginal section, expressed as the mean from measurements of both lobes), as was the distance between these two posteriormost points (lobe distance of posterior marginal section). Furthermore, we measured the area of the telson posterior marginal section. In specimens lacking a clear incision in the posterior margin, we used the distalmost points in which the maximum length of the posterior marginal section was reached as fixpoints for measuring maximum length and lobe distance of the posterior marginal section. Measurements were made on digital photographs of the telson taken in dorsal view. Subsidiary

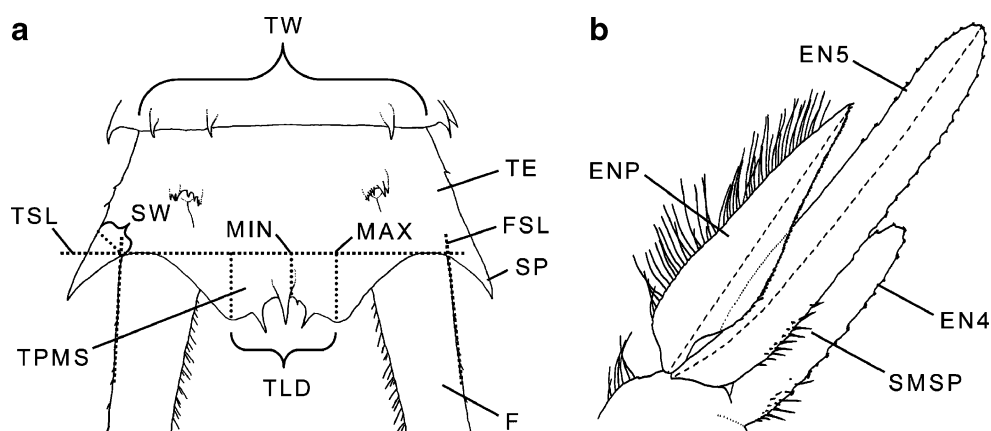


Fig. 1 *Triops mauritanicus*, schematic drawings of morphological features and measurements. **a** Posterior part of abdomen, dorsal view (modified from Korn et al. 2006), with dotted lines used in measurements concerning furcal spines and posterior marginal section of telson. **b** Distal part of second trunk limb, anterior view, with dashed lines used in length measurements of endopodite and fifth endite.

Abbreviations: ENP = endopodite; EN4, 5 = fourth, fifth endite; F = furcal ramus; FSL = furcal subsidiary line; MAX, MIN = maximum, minimum length of telson posterior marginal section; SMSP = submarginal spines; SP = furcal spine; SW = furcal spine width; TE = telson; TLD = telson lobe distance; TPMS = telson posterior marginal section; TSL = telson subsidiary line; TW = telson width

lines were drawn in PixeLINK Capture SE using the ‘Annotate’ function.

Trunk limb morphology (nomenclature following Fryer 1988)

Spine counts of the tenth trunk limb Each endite bears a row of submarginal spines on the anterior face, and one row of meshwork spines each on the anterior and posterior faces of the endite. For the present survey, we counted the number of spines of the anterior row of meshwork spines on endite three, as well as the number of submarginal spines on endite four in the tenth trunk limb. In some specimens, the smallest submarginal spines are positioned at the edge of the endite, or are even displaced to its posterior face. Thus, to count the number of submarginal spines, the fourth endite was investigated in anterior and posterior views, at 100–400× magnification.

Morphology of the second trunk limb The characters of the second trunk limb were studied in males only, since they show different levels of modification between sub-lineages, possibly linked to the functional role of the anterior trunk limbs in mating (for further modifications attributed to the role of anterior trunk limbs in mating, see Lynch 1972). The characters investigated in this study show different patterns of allometric growth. Formulas to standardize these characters for the size of investigated specimens were derived from best-fit curves as described for the following example: if the curve was indicated as $Y = a - b * X$, where X is telson width [mm], then each observed point (X_i, Y_i) was transformed into a size-standardized point ($X_{\text{standard}}, Y_i^*$). Standardized values of Y_i (i.e., Y_i^*) were thus obtained by application of the formula: $Y_i^* = Y_i + b * X_i$.

Maximum length of submarginal spines on the second trunk limb The proportional length of spines gradually decreases in size towards the anteriormost trunk limbs. On the fifth endite of the second trunk limb, they are confined to the proximal region of the endite, and do not appear to play any functional role (M.K. pers. obs.). In male specimens, we measured the proportional (percent) length of the longest submarginal spine of the fifth endite in relation to the length of the fifth endite (henceforth called proportional spine length). We used the ‘polyline’ tool implemented in PixeLINK Capture SE to measure the length of the fifth endite, as it is usually curved towards its base in *Triops mauritanicus* (see Fig. 1b). To standardize this character prior to analysis, the following formula was applied: $Y_i^* = Y_i + 0.7 * X_i$, where Y = proportional spine length, and X = telson width [mm].

Proportional length of the endopodite in relation to length of the fifth endite In male specimens, we measured the proportional (percent) length of the endopodite in relation to the length of the fifth endite on the second trunk limb (henceforth called proportional endopodite length). This character shows a non-linear correlation to body size. Thus, prior to analysis, data were transformed by applying the formula: $Y_i^* = Y_i + 30 * \text{LOG}_{10} X_i$, where Y = proportional endopodite length, and X = telson width [mm]. Measurements were made on digital microscopy photographs of the trunk limbs in anterior view. The length of the fifth endite was measured using the ‘polyline’ function as described above, whereas endopodite length was measured using the ‘caliper’ tool implemented in PixeLINK Capture SE.

Number of apodous abdominal segments

The number of apodous abdominal segments was counted using the methods described in Korn et al. (2006; nomenclature following Longhurst 1955; whether or not Notostraca have a true abdomen still awaits confirmation, see Schram and Koenemann 2004).

Size of resting eggs

The outer coating (comprising an alveolar layer, covered distally by an outer cortex) of the resting eggs is still smooth at the time when the eggs are deposited. [Thiéry (1987) states that the alveolar layer swells after the eggs are exposed to water, when they are released from the brood pouches. During this process, the thickness of the alveolar layer is reported to expand from approx. 20 μm to 55–100 μm. Clearly, this requires that the outer cortex and the alveolar layer are still flexible at that time.] Consequently, the eggs adapt their shape in response to the morphology of the sediment (M.K. pers. obs.). Very fine sediments typically result in a roughly ball-shaped resting egg, whereas coarse sediments usually result in asymmetric shapes of the outer coating. Thus, a simple measurement of the egg diameter appeared to be inappropriate to characterize the size of the eggs. Therefore, we used digital images to measure profile area, and calculated egg-diameter values by using the standard formula for diameter-area relationships in a circle, resting egg diameter = $2 * \text{square root}(\text{profile area} / \pi)$, to get a more accurate estimate of the size of the eggs. All eggs were measured in dry condition and were extracted from natural sediments or sediments obtained from lab cultures.

Analysis of morphological data

Data for morphological characters in adult *Triops* had to be tested separately for males and females due to a high level

of sexual dimorphism in several characters, so that two separate datasets were formed for males and females from the Iberian populations. A third dataset included males of all known sublineages of *T. mauritanicus*, i.e. including additional samples of the two recognized subspecies occurring in northern Africa, *T. m. mauritanicus* and *T. m. simplex*. For each of these morphological datasets, the null hypothesis that there were no significant differences between means of statistical populations was tested with discriminant function analysis. Predetermination of statistical groups was based on the molecular determination of *Triops* populations.

Our sampling was highly asymmetrical due to the fact that levels of abundance and the sizes of distribution ranges clearly differed among the phylogenetic lineages studied. In the Iberian Peninsula, the ‘S.Iberian’ lineage clearly outnumbered the other lineages (Table A1). Thus, in order to achieve a less unbalanced design, we included data from only a single randomly chosen individual from each of the populations of the ‘S.Iberian’ lineage in the set of samples used to calculate discriminant functions. The remaining samples were treated as ungrouped cases in the discriminant function analysis and were classified using the classification functions derived from the model. The set of dependent variables was chosen individually for the three datasets to meet all the assumptions of

discriminant function analysis. Some variables had to be ln-transformed or square root-transformed in order to reach homogenous variances (see Table 2). Variables that did not reach homogeneity of variances were excluded from analysis. To test for homogeneity of variance, the Hartley F-max statistic, Cochran C statistic, and the Bartlett Chi-square test were calculated, and normality was checked by plotting expected normal values against observed values. A priori classification probability was set to ‘same for all groups’.

The standard method for sampling observations for post-hoc classification, i.e. resubstitution, may result in underestimation of the classification error rate even at rather high sample sizes (Lance et al. 2000). To minimize this bias, we additionally used a Jackknife sampling procedure (e.g. Quinn and Keough 2003) to classify observations. For the ‘S.Iberian’ lineage, we had sufficient samples to perform a modified, population-level Jackknife sampling, i.e. for each of the populations all observations were classified based on a model that contained only samples of the remaining populations. The resulting classifications thus represent a realistic, unbiased estimate of the classification success for new independent observations (i.e. individuals from hitherto uninvestigated populations).

As a measure of differentiation between phylogenetic lineages, squared Mahalanobis distances (obtained by DFA)

Table 2 Morphological characters and character ratios included in discriminant function analysis for each of the three datasets investigated

| Dependent variable | Lineages: Sex: | All Male | Iberian Male | Iberian Female |
|---|-------------------|-----------------------|-----------------------|-----------------------|
| Number of anterior meshwork spines on 3rd endite of 10th trunk limb | | Included | Included | Included |
| Number of submarginal spines on 4th endite of 10th trunk limb | | Included | Included | Included |
| Proportional spine length on 5th endite of 2nd trunk limb [%] | | Included ^a | Included | Unavailable |
| Proportional endopodite length of 2nd trunk limb [%] | | – | Included | Unavailable |
| Telson length ratio | | Included | Included | Included |
| Furcal spine width / telson width | | Included ^b | – | Included |
| Furcal spine size ratio | | Included ^a | Included ^a | Included |
| Number of apodous abdominal segments | | – | Included | Included |
| Minimum length of TPMS / area of TPMS | | – | – | Included |
| Maximum length of TPMS / minimum length of TPMS | | – | Included | Included |
| Length of telson posterior incision / telson width | | Included | Included | Included |
| Length of telson posterior incision / maximum length of TPMS | | Included | Included | Included |
| Length of telson posterior incision / area of TPMS | | Included ^b | Included | Included ^a |
| Area of TPMS / telson width | | Included | Included | Included |
| Telson lobe distance / maximum length of TPMS | | Included ^a | – | – |
| Telson lobe distance / area of TPMS | | Included ^a | Included | Included |
| Telson lobe distance / telson width | | Included | Included | Included |

– excluded from analysis, as variable did not reach homogeneity of variances even after data transformations were applied

TPMS telson posterior marginal section

^a data ln-transformed

^b data square root-transformed

were calculated between the group centroids (multivariate means) of the phylogenetic lineages. For comparison with molecular phylogenetic reconstructions, a NJ tree based on squared Mahalanobis distances between the group centroids of males of all phylogenetic lineages was calculated using PAUP* (Swofford 2003).

For the size of resting eggs, the null hypothesis that there were no significant differences between means of populations was tested with a single-factor analysis of variance (ANOVA). To test for homogeneity of variance Levene's test was used, and normality was checked by plotting expected normal values against observed values. A logarithmic transformation was used, which greatly improved the approximation to a normal distribution and homogeneity of variances within this dataset. However, since the assumption of homogeneity of variances was still clearly violated, only a data subset that met all the assumptions of ANOVA was used for calculating statistics. This subset excluded some populations of *Triops c. cancriformis* with unusually low variability (populations 121–123 and 130, see Table A1), but retained all populations that were important in evaluating the usefulness of this morphological character for discriminating among phylogenetic lineages, as the mean values of excluded populations were within the range of those observed in the other populations of this species. As the null hypothesis was rejected, differences among single populations were investigated using a Tukey post-hoc test. All statistics on morphological data were undertaken with STATISTICA 6.0 (StatSoft, Inc.).

Results

Nucleotide composition, substitution patterns and sequence variability

The nucleotide composition in the 12S rDNA gene segment sequenced showed a pronounced AT-bias (33.0% T, 38.9% A, 17.9% C, 10.2% G) for the ingroup (*Triops cancriformis* + *T. mauritanicus*). The alignment consisted of 552 sites, of which 446 (80.8%) were constant within the ingroup. Within this lineage 101 sites were variable, and 78 of these (14.1% of the total sequence) were parsimony informative. The mean 12S sequence divergences between the sublineages of the ingroup, and the maximum sequence divergences within the sublineages are presented in Table 3.

The dataset of 16S sequences also showed the AT bias (33.1% T, 31.9% A, 12.7% C, 22.3% G); it consisted of 432 sites, of which 389 were constant, 40 were variable, and 24 were parsimony informative. The combined 12S and 16S dataset consisted of 984 sites, of

which 847 were constant, 130 were variable, and 97 were parsimony informative (outgroups not included, but calculated using the alignments that were used for phylogenetic reconstructions).

Phylogenetic analysis

The molecular analysis of a high number of *Triops* populations from south-western Iberia revealed the presence of a sixth, previously undiscovered main lineage within *T. mauritanicus*, occurring in western parts of Cádiz province (Fig. 2; 'Cádiz' haplotypes in Table A1). The ML calculation based on the 16S dataset (not shown; results available from <http://purl.org/phylo/treebase/phylo/phylo/TB2:S10349>) indicates the newly discovered lineage in a sister-group position to the remaining ingroup taxa, thus rendering *T. mauritanicus* paraphyletic with respect to *T. c. cancriformis*. The MP calculation (16S) could not resolve the relationships among the ingroup lineages. In contrast, all calculations based on the 12S haplotypes, as well as the dataset with 12S and 16S sequences combined, indicated *T. mauritanicus* and *T. c. cancriformis* as monophyletic sister taxa (Fig. 2). The ML calculations resulted in similar topologies for the 12S and the combined datasets (Fig. 2a, b). For the combined dataset, the first of two ML trees is presented (Fig. 2b; topologies of the second ML phylogram and the Bayesian inference majority rule tree were identical with respect to relationships among the main lineages). The *T. mauritanicus* samples form three separate monophyletic clusters (Fig. 2a, b): (1) a clade consisting of the western Cádiz samples belonging to the 'Cádiz' haplotype group; (2) a clade formed by the south central Portuguese samples and Spanish samples from Extremadura, Sevilla, Huelva and northern Cádiz provinces ('S.Iberia' haplotype group); (3) a clade including the south-west Portuguese samples ('Portugal' haplotype group), the samples from 'Gitanilla' lineage, as well as the samples of *T. m. simplex* and *T. m. mauritanicus*. Within that third cluster, south-west Portuguese samples and samples from the 'Gitanilla' lineage form a monophylum either in an unresolved trichotomy with the African subspecies *T. m. simplex* and *T. m. mauritanicus* (dataset with 12S and 16S sequences combined) or forming the sister group to the latter two (12S dataset). MP calculations could not resolve relationships among the *T. mauritanicus* clades using 12S sequences alone, but resolved all *T. mauritanicus* clades in the calculation based on the combined dataset (strict consensus tree presented in Fig. 2c): in this phylogeny reconstruction, the 'S.Iberian' haplotype group forms the sister group to the remaining samples within *T. mauritanicus*. Among these remaining samples, the 'Cádiz' haplotype group is in a sister-group relationship with a clade comprising two monophyletic

Table 3 Genetic divergences (uncorrected p-distances) within and between *Triops* ingroup lineages (*T.c.c.* = *T. cancriformis cancriformis*; *T.m.* = *T. mauritanicus*; *T.m.m.* = *T. m. mauritanicus*, *T.m.s.* = *T. m. simplex*) calculated from 12S and 16S sequences and from the combined dataset; pairwise inter-lineage distances given as means with ranges in parentheses

| Taxon | Genetic marker | | |
|--|----------------|---------------|---------------|
| | 12S | 16S | 12S+16S |
| Pairwise distances between lineages [%] | | | |
| <i>T.m.</i> to <i>T.c.c.</i> : | | | |
| ‘Cadiz’ to <i>T.c.c.</i> | 6.0 (5.5–7.0) | 3.3 (2.6–3.7) | 4.9 (4.0–5.5) |
| ‘S.Iberia’ to <i>T.c.c.</i> | 5.6 (4.1–7.2) | 2.8 (2.1–3.3) | 4.6 (3.1–5.2) |
| ‘Portugal’ to <i>T.c.c.</i> | 5.8 (5.3–6.8) | 3.2 (2.8–3.5) | 4.7 (4.0–5.3) |
| <i>T.m.m.</i> to <i>T.c.c.</i> | 5.2 (4.3–6.1) | 3.2 (2.1–4.0) | 4.4 (3.2–5.0) |
| ‘Gitanilla’ to <i>T.c.c.</i> | 5.7 (5.2–6.1) | 4.3 (3.7–4.7) | 5.0 (4.5–5.4) |
| <i>T.m.s.</i> to <i>T.c.c.</i> | 5.1 (4.6–5.5) | 3.0 (2.1–3.5) | 4.2 (3.5–4.6) |
| Within <i>T.m.</i> : | | | |
| ‘Cadiz’ to ‘S.Iberia’ | 5.0 (4.2–5.7) | 1.2 (0.9–1.4) | 3.2 (3.0–3.5) |
| ‘Cadiz’ to ‘Portugal’ | 4.3 (3.9–5.0) | 2.3 (2.1–2.3) | 3.4 (3.2–3.7) |
| ‘Cadiz’ to <i>T.m.m.</i> | 4.8 (4.2–5.2) | 1.9 (1.4–2.3) | 3.4 (3.1–3.7) |
| ‘Cadiz’ to ‘Gitanilla’ | 5.1 (4.6–5.5) | 2.7 (2.6–2.8) | 3.9 (3.7–4.1) |
| ‘Cadiz’ to <i>T.m.s.</i> | 4.2 (3.9–4.6) | 1.9 (1.4–2.1) | 3.1 (2.8–3.4) |
| ‘S.Iberia’ to ‘Portugal’ | 4.5 (3.7–5.1) | 1.8 (1.6–1.9) | 3.3 (3.0–3.5) |
| ‘S.Iberia’ to <i>T.m.m.</i> | 4.9 (4.4–5.7) | 1.4 (0.9–1.9) | 3.3 (2.9–3.6) |
| ‘S.Iberia’ to ‘Gitanilla’ | 4.7 (4.2–5.2) | 2.7 (2.6–2.8) | 3.7 (3.5–3.8) |
| ‘S.Iberia’ to <i>T.m.s.</i> | 4.3 (3.7–4.8) | 1.4 (0.9–1.6) | 2.9 (2.6–3.2) |
| ‘Portugal’ to <i>T.m.m.</i> | 3.7 (3.3–4.2) | 1.9 (1.4–2.1) | 2.9 (2.5–3.2) |
| ‘Portugal’ to ‘Gitanilla’ | 2.9 (2.6–3.5) | 2.8 (2.8–2.8) | 3.0 (2.8–3.2) |
| ‘Portugal’ to <i>T.m.s.</i> | 3.3 (2.9–3.9) | 1.8 (1.6–2.1) | 2.7 (2.4–3.1) |
| <i>T.m.m.</i> to ‘Gitanilla’ | 4.1 (3.9–4.4) | 2.8 (2.6–3.3) | 3.4 (3.3–3.6) |
| <i>T.m.m.</i> to <i>T.m.s.</i> | 3.7 (3.3–4.2) | 1.6 (0.9–2.1) | 2.7 (2.3–3.2) |
| ‘Gitanilla’ to <i>T.m.s.</i> | 3.7 (3.3–4.0) | 2.6 (2.6–2.6) | 3.2 (3.0–3.3) |
| Maximum divergence within each lineage [%] | | | |
| <i>T.c.c.</i> | 1.5 | 0.9 | 1.0 |
| <i>T.m.s.</i> | 0.9 | 0.5 | 0.7 |
| <i>T.m.m.</i> | 1.5 | 1.2 | 1.1 |
| ‘Portugal’ | 0.7 | 0.0 | 0.4 |
| ‘S.Iberia’ | 1.1 | 0.5 | 0.5 |
| ‘Cadiz’ | 1.3 | 0.5 | 0.8 |
| ‘Gitanilla’ | 0.2 | 0.0 | 0.0 |

groups: one represented by *T. m. mauritanicus*, the other including *T. m. simplex* as a sister group to samples from ‘Gitanilla’ lineage and south-west Portuguese samples, the latter two being in a sister-group relationship to each other.

The genetic divergences between ingroup lineages in the 12S and 16S genes and in the dataset with 12S and 16S sequences combined are presented in Table 3. The ‘Cádiz’ and the ‘S.Iberian’ lineage have the lowest inter-lineage sequence divergences in the 16S gene, while their levels of divergence are among the highest observed within *Triops mauritanicus* in the 12S gene. In contrast, sequence divergences are lowest between ‘Portugal’ and ‘Gitanilla’ lineages in the 12S gene, while these lineages were highly divergent in the 16 gene. Combining the 12S and 16S sequences in a single dataset buffered such effects, which

resulted in an improved estimate of total inter-lineage divergences (assuming that differing branch lengths among these closely related taxa are likely to represent an artefact of short sequences, rather than differences in evolutionary rates). In this combined dataset, uncorrected average inter-lineage *p* distances between *T. c. cancriformis* and the lineages within *T. mauritanicus* range between 4.2 and 5.0%. Uncorrected average inter-lineage *p* distances observed within *T. mauritanicus* are lower but of similar magnitude, ranging from 2.7 to 3.9%. Intra-lineage divergences are highest for *T. m. mauritanicus*, reaching a maximum of 1.1% (uncorrected p-distance). Thus, within *T. mauritanicus* uncorrected average inter-lineage *p* distances were at least 2.5 times higher than the maximum intra-lineage divergence observed, which indicates that all

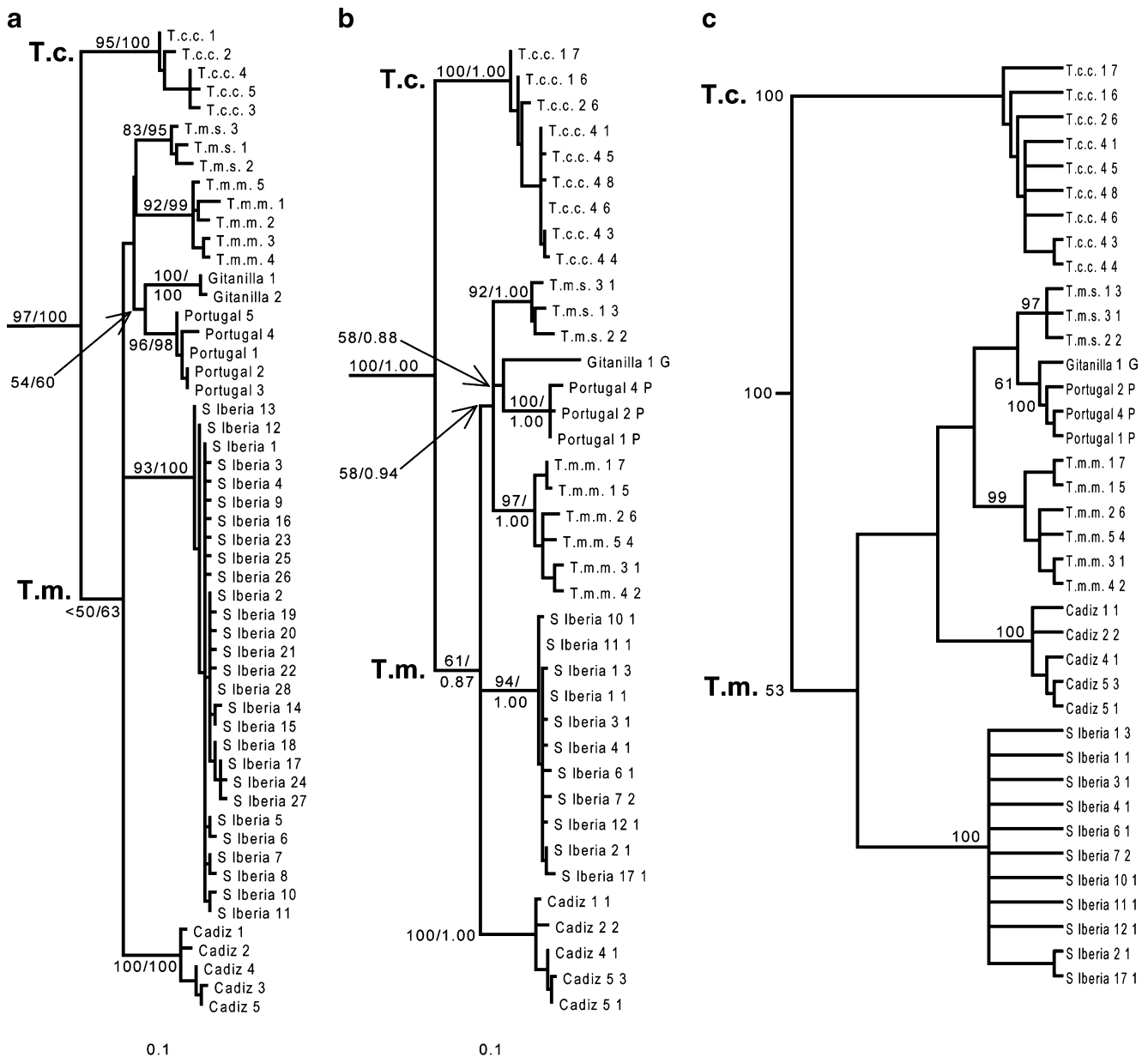


Fig. 2 Hypotheses on *Triops mauritanicus* (“T.m.”) and *T. c. cancriformis* (“T.c.”) phylogeny as reflected by our mitochondrial sequence data; outgroups (*Triops longicaudatus*, *T. granarius*, *Lepidurus a. apus*, *L. a. lubbocki*, *L. arcticus*, *L. lemmoni*) removed for clarity. **a** ML 12S tree based on large 12S dataset, using TVM+G model of evolution; ML/ MP bootstrap support values given for selected branches. **b** First of two ML trees based on combined 12S and 16S sequences from selected samples, using GTR+G model; ML

bootstrap support/ Bayesian posterior probabilities given for selected branches. **c** Strict consensus tree based on combined 12S and 16S sequences (gaps included as fifth character); MP bootstrap support values given for selected branches. All samples labelled with short haplotype names as given in Table A1; for combined datasets, 12S haplotype number given first, followed by blank space and 16S haplotype number

lineages within *T. mauritanicus* are well separated from each other.

Geographic distribution of Iberian lineages

In general, suitable habitats for Notostraca form in low-relief landscapes with impermeable surface soils or with

upwelling groundwater (if upwelling lasts for prolonged periods of time after heavy rains). Within the study area, these conditions are predominantly met within the wide valleys of the lower reaches of streams as well as in the coastal lowlands. Thus, the majority of south-west Iberian *Triops* populations obtained for this study were found at altitudes below 300 meters. However, higher altitudes were

recorded for populations 058 and 059 (Table A1) in Extremadura (see also Alonso 1985; Pérez-Bote et al. 2006). Distribution records are illustrated in Fig. 3. All four lineages show continuous distribution ranges with sharp range boundaries, and we found no evidence of a mosaic contact zone where different lineages meet.

The ‘S.Iberian’ lineage has the most extensive range, covering wide areas within the valleys of the Guadalquivir and Guadiana rivers and adjacent areas. In south central Portugal it is restricted to the area north of a complex of mountain ranges including the Serra de Monchique and Serra do Caldeirão. The northernmost records are from Cáceres province, Extremadura. The ‘Gitanilla’ lineage appears confined to a small area within the distribution range of the ‘S.Iberian’ lineage and could thus be regarded as sympatric with the latter. However, the minimum geographic distance recorded between the two lineages is 40.5 km. The distribution of ‘Cádiz’ and ‘S.Iberian’ lineages appears to be a typical parapatric one due to the comparatively low minimum distance of 25.8 km recorded between these neighbouring lineages, and to the apparent absence of a geographic barrier between their distribution ranges (if waterbirds are considered as major dispersal vectors, see, e.g., Green and Figuerola 2005). The ‘Portugal’ lineage may be geographically more isolated from the ‘S.Iberian’ lineage due to mountain ranges (Serra de Monchique and Serra do Caldeirão) forming its northern range limit, and to a possible lack of suitable habitats towards its eastern range limit, where the mountains approach the sea so that the coastal lowlands are confined

to a narrow belt along the shoreline. Within this coastal belt, the two lineages are separated by a distance of 53.2 km.

Dispersal abilities inferred from haplotype distribution

For each of 50 populations, we obtained 12S sequences from a minimum of six individuals. Among these populations, at least 52% show associations of multiple haplotypes (percentage of populations with multiple haplotypes was 44% if all populations were considered for which we obtained sequences from at least two individuals each). We identified 18 ingroup haplotypes that occurred in more than a single habitat and thus can be used to infer recent successful dispersal events, i.e. where haplotypes successfully established in new habitats. Most of these geographically spread haplotypes belong to the ‘S.Iberian’ lineage, for which also the highest accumulative minimum dispersal distances (AMDDs) were observed within *Triops mauritanicus* (Table 4), exceeding 200 km in three of the haplotypes. The common haplotype ‘S.Iberia 1’ even dispersed by an accumulative distance of more than 500 km and was detected in a total of 35 different habitats that can only have been reached via overland dispersal. Despite its much smaller range, the ‘Cádiz’ lineage also showed rather high values of AMDD, up to almost 73 km. Inferred successful dispersal was considerably lower for the ‘Portugal’ and ‘Gitanilla’ lineages, with AMDDs of 16.4 km and 1.9 km, respectively. Indeed, for the ‘Portugal’ lineage we found no evidence of recent dispersal among

Fig. 3 Distribution of *Triops mauritanicus* lineages in south-western Iberian Peninsula, limited to records from this and a preceding study (Korn et al. 2006), as literature records could not be assigned to the phylogenetic lineages. *Black lines* show political borders, *grey lines* the major rivers; *dashed* area indicates extension of marismas (natural temporary marshes) in Guadalquivir River delta around the year 1900

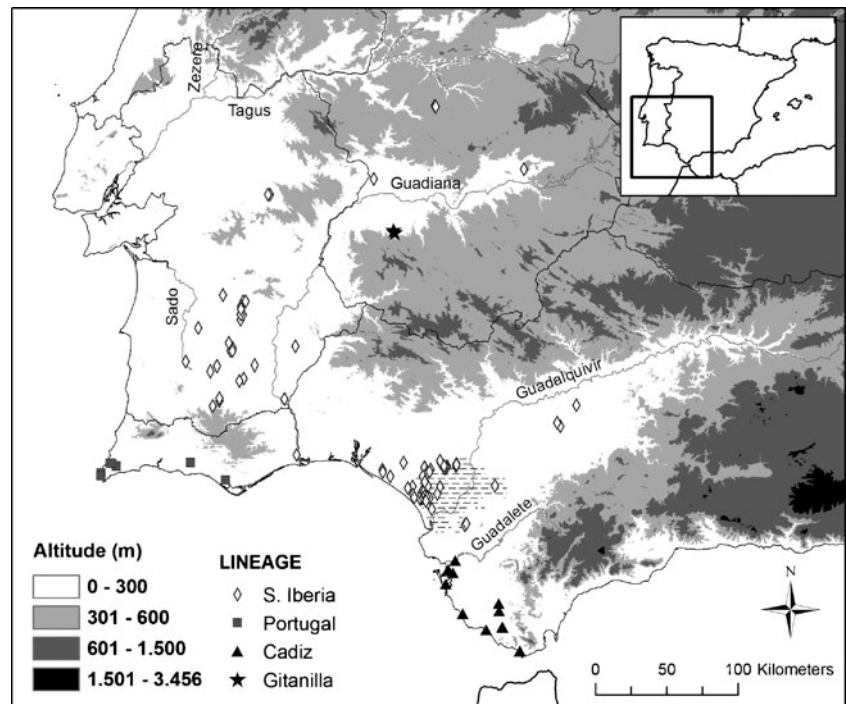


Table 4 Accumulative minimum geographic distances over which single haplotypes must have been passively dispersed to show the present geographic distribution (marismas of Doñana treated as a single site)

| 12S haplotype | Accumulative minimum dispersal distance [km] | Number of sites |
|-----------------|--|----------------------|
| S.Iberia 1 | 505.9 (613.7) ^a | 35 (41) ^a |
| S.Iberia 12 | 263.1 | 3 |
| S.Iberia 7 | 230.7 | 4 |
| S.Iberia 2 | 39.2 (69.4) ^a | 10 (16) ^a |
| S.Iberia 6 | 33.8 | 3 |
| S.Iberia 4 | 15.8 | 9 |
| S.Iberia 5 | 2.9 | 2 |
| S.Iberia 13 | 1.3 | 2 |
| S.Iberia 19 | 0.9 | 2 |
| S.Iberia 17 | 0.6 (28.9) ^a | 2 (8) ^a |
| S.Iberia 18 | 0.5 (28.3) ^a | 2 (5) ^a |
| S.Iberia 3 | 0.4 (79.1) ^a | 3 (13) ^a |
| S.Iberia 24 | 0.0 (8.6) ^a | 1 (3) ^a |
| Cádiz 5 | 72.8 | 5 |
| Cádiz 1 | 43.9 | 6 |
| Cádiz 2 | 16.7 | 2 |
| Portugal 1 | 16.4 | 6 |
| Gitanilla 1 | 1.9 | 2 |
| <i>T.c.c.</i> 4 | 11,100.0 | 5 |
| <i>T.c.c.</i> 1 | 2,300.0 | 2 |

^a Values in parentheses indicate total dispersal and total number of sites, respectively, if *Triops* collected from all sampling sites located within the former range of marismas (around year 1900) were treated as separate populations (the temporary lakes and ponds within the marismas are interconnected at certain flood events, so that additional active dispersal may occur among populations within this habitat)

populations from Costa Vicentina (SW Portugal) and the two populations from south central Algarve, as we found no haplotypes shared among these regions.

Despite the overall high level of populations with shared haplotypes, we did not observe any population with a co-occurrence of haplotypes belonging to different main phylogenetic lineages. The most divergent 12S haplotypes observed to co-occur were ‘S.Iberian’ haplotypes 3 and 24 (uncorrected p-distance 0.9, representing 82% of the maximum intra-lineage divergence observed) and ‘Cádiz’ haplotypes 2 and 5 (uncorrected p-distance 1.1, representing 85% of the maximum intra-lineage divergence).

The differentiation of populations from the south central Algarve and Costa Vicentina as observed for the ‘Portugal’ lineage was also confirmed by AMOVA (Table 5), indicated by a high proportion of the total (within-lineage) genetic variation observed among populations (80.65%). The ‘Cádiz’ lineage had a similarly high proportion of the variation observed among populations (83.59%), and for both, the ‘Portugal’ and the ‘Cádiz’ lineage, these values were considerably higher than in the ‘S.Iberian’ lineage (within the same type of habitat: 56.26% and 66.84%, see Table 5; from the ‘Gitanilla’ lineage not enough samples were available to perform AMOVA).

For comparison, AMDD values for two haplotypes common in unisexual populations of *T. c. cancriformis* are shown (Table 4), demonstrating the possible effect of reproductive mode on dispersal success (Korn et al.

2006). Clearly, despite lower sampling efforts, observed AMDD values are higher in this species than in the gonochoric *T. mauritanicus*.

Evidence for recent gene flow

Evidence for recent gene flow was found on a smaller geographical scale than evidence for dispersal. 38% of the populations for which 12S sequences were available from a minimum of six specimens each, showed associations of geographically spread haplotypes (Table A1; 32% if all populations were considered for which we investigated at least 2 individuals) and thus were indicative of gene flow between populations. However, these populations (i.e. the ones showing associations of geographically spread haplotypes) were only observed within the Guadalquivir delta and a small area in southern Cádiz province (between Tahivilla and Benalup).

Genetic diversity among habitat types

Gene diversity of *Triops* populations differed markedly among all three sampled habitat types within the Guadalquivir delta (i.e. within a distance of 25 km to the marismas of Doñana National Park; ANOVA, $p < 0.001$, Tukey post-hoc test, $p < 0.001$ for all pairwise comparisons; Fig. 4a). *Triops* populations at greater distances to the Guadalquivir delta (>75 km distance to marismas of

Table 5 Results from analysis of molecular variance (AMOVA) based on 12S haplotype frequencies of main lineages within *Triops mauritanicus*, broken down by habitat types

| Lineage | Habitat type | Source of variation | Degrees of freedom | Percent of variation | Probability | Fixation index |
|------------|--------------------------|---------------------|--------------------|----------------------|-------------|----------------|
| 'S.Iberia' | Open, far ^a | Among populations | 6 | 66.84 | <0.001 | 0.67 |
| | | Within populations | 35 | 33.16 | | |
| 'S.Iberia' | Open, close ^b | Among populations | 9 | 56.26 | <0.001 | 0.56 |
| | | Within populations | 51 | 43.74 | | |
| 'S.Iberia' | Forest/shrubs | Among populations | 10 | 95.50 | <0.001 | 0.95 |
| | | Within populations | 55 | 4.50 | | |
| 'S.Iberia' | Marismas | Among populations | 8 | 9.94 | <0.001 | 0.10 |
| | | Within populations | 57 | 90.06 | | |
| 'Cadiz' | Open | Among populations | 5 | 83.59 | <0.001 | 0.84 |
| | | Within populations | 36 | 16.41 | | |
| 'Portugal' | Open | Among populations | 3 | 80.65 | <0.001 | 0.81 |
| | | Within populations | 20 | 19.35 | | |

^a Ponds in >75 km distance to the marismas (natural temporary marshes) of Doñana

^b Ponds located ≤25 km from the marismas

Doñana) were all found in open habitat but had a lower gene diversity than populations found in open habitat within the delta region (Tukey post-hoc test, $p < 0.05$; Fig. 4a). These geographically distant populations showed similarly low gene diversity to populations in closed woodland or shrub habitat within the delta (Tukey post-hoc test, not significant). Gene diversity of *Triops* populations was positively correlated with estimates of waterbird abundances (regression analysis, $R = 0.70$, $p < 0.001$; Fig. 4b). Conversely, we found no clear evidence for a possible effect of pasture land use on gene diversity of *Triops* (ANOVA, $p = 0.096$), and a statistical model considering only abiotic factors (conductivity, pH, \log_{10}

surface area, \log_{10} distance to marismas) could also not sufficiently explain the variability observed among populations (multiple regression; with distance measurements referring to the present extension of marismas: $R = 0.49$, $p = 0.20$, $F = 1.6$; with distance measurements considering the borderlines of marisma habitat around year 1900: $R = 0.55$, $p = 0.12$, $F = 2.12$).

The differences in genetic diversity observed among habitat types were also confirmed by AMOVA. The proportion of genetic variation observed among populations differed markedly among all three habitat types, ranging from 9.9% in marisma habitat to 95.5% in enclosed forest or shrubland (Table 5), indicating a low level of gene flow

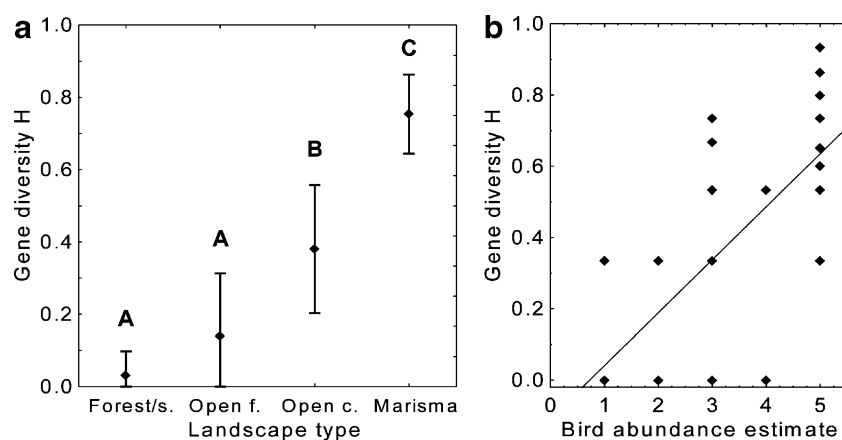


Fig. 4 Gene diversity H calculated from 12S haplotype frequencies in populations of the 'S.Iberian' lineage of *Triops mauritanicus*. **a** H in relation to main habitat types [Forest/s. = forest or shrubland; Open f. (open, far) = ponds at >75 km distance from marismas of Doñana National Park; Open c. (open, close) = ponds ≤25 km from marismas], based on populations 001–039 (see Table A1); error bars indicate

95% confidence intervals, capital letters show statistical classification from Tukey post-hoc test ($p < 0.05$; ANOVA, $p < 0.001$). **b** H in relation to estimates of waterbird abundance (1=low, 5=high), based on populations 001–032 from Sevilla and Huelva provinces; regression analysis showed strong correlation ($R = 0.70$, $p < 0.001$)

among populations surrounded by forest or shrubland and highest gene flow within marisma habitat. Populations in open habitat were indicated to have intermediate levels of gene flow and, according to the differences in gene diversity observed between the Guadalquivir delta and more distant areas, also showed a difference in the proportion of genetic variation observed among populations ('open, close', delta region: 56.26% of total variation; 'open, far', >75 km geographic distance to marismas: 66.84%).

Morphological analysis of adult specimens

All three discriminant function analysis models of morphological data yielded significant results ($p < 0.001$; Table 6). Thus, the multivariate models successfully separated the statistical groups representing phylogenetic lineages of *Triops mauritanicus*.

Morphological data of males in all presently known lineages

The overall discriminating power of the discriminant function analysis (DFA) model was high, indicated by a low value of Wilk's lambda (0.009; see Table 6) and a high classification success: 85% of all individuals were classified correctly. The latter value was only slightly lower (82% correct) when a jackknife procedure was applied to the analysis. Classification success was highest for *Triops m. simplex* and the 'Portugal' and 'Gitanilla' lineages (Table 7). In contrast, the 'S.Iberian' lineage showed the lowest levels of correct classification (Table 7), apparently due to the fact that its morphology is intermediate between that of the 'Cádiz' lineage and *T. m. mauritanicus* (Fig. 5), so that individuals of the 'S.Iberian' lineage with a less typical combination of characters may easily be misclassified to one of those morphologically similar lineages. The morphological differentiation of the 'S.Iberian' lineage from *T. m. mauritanicus* is lower than its differentiation from the 'Cádiz' lineage (squared Mahalanobis distances 4.4 and 6.1, respectively; Table 8). This morphological proximity of the 'S.Iberian' lineage to the nominotypical subspecies is also

apparent in the prevailing placement of misclassified observations into that taxon (up to 28% of all individuals of the 'S. Iberian' lineage if a jackknife procedure was applied at the population level). When the DFA model was used for identification of Iberian populations, this resulted in a high proportion of populations in the 'S.Iberian' lineage that could not clearly be assigned to that lineage (Table 9). Among the 19 populations of the 'S.Iberian' lineage classified using a population-level jackknife procedure, two populations were even misclassified as *T. m. mauritanicus*.

The NJ tree (Fig. 5) based on squared Mahalanobis distances between the group centroids of the phylogenetic lineages is in agreement with the phylogenetic reconstructions based on molecular data, with the exception of the position of *Triops m. mauritanicus*: the 'Cádiz' and 'S. Iberian' lineages group between the nominotypical subspecies and *T. m. simplex*, the 'Portuguese' and the 'Gitanilla' lineage, whereas the latter four form a monophyletic clade in all molecular phylogenetic reconstructions with sufficient resolution (see above).

Morphological data of Iberian males

The reduced dataset including only males from the south-west Iberian Peninsula caused fewer problems in retaining homogeneity of variances, so that in total, one more dependent variable could be included in the model (Table 2). The set of variables in the model resulted in better separation of all four south-west Iberian lineages, as indicated by the consistently higher squared Mahalanobis distances between group centroids of Iberian lineages as compared to distances achieved for the above-described model (Table 8). Consequently, classification success of observations was higher for the morphologically less well differentiated 'S.Iberian' and 'Cádiz' lineages. For populations, classification success was 89%, both for the 'S. Iberian' lineage and for total number of populations (Table 9). None of the populations was misclassified, as the individuals from populations which were not classified correctly could not be assigned clearly to a single lineage.

Morphological data of Iberian females

The results of discriminant function analysis obtained for Iberian females were in agreement with those for males. However, with a value of 0.084 for Wilks' lambda, discriminating power was lowest for that DFA model (Table 6). Accordingly, distances between groups tended to be lower (Table 8) and classification success of observations was clearly lower than that obtained for males (Table 7). This resulted in almost 20% lower success of population classification (70% correct classification; data not shown) compared to the classification based on males.

Table 6 Results from discriminant function analysis (DFA) for morphological characters

| Specification of DFA model | Wilks' lambda | F | p |
|----------------------------|---------------|------|--------|
| Males, all lineages | 0.009 | 16.8 | <0.001 |
| Males, Iberian lineages | 0.037 | 13.0 | <0.001 |
| Females, Iberian lineages | 0.084 | 8.9 | <0.001 |

The haplotypes were used to pre-define statistical groups tested in each analysis

Table 7 Classification success of observations (individuals) for discriminant function analysis (DFA) of morphological characters; a priori classification probability set to ‘same for all groups’

| Classification based on resubstitution sampling | | | | | | | | Classification based on jackknife sampling | | | | | | | |
|---|-----------|----|----|----|-----|----|----|--|-----------|----|----|----|-----|----|----|
| Males of all lineages | | | | | | | | | | | | | | | |
| | % correct | C | G | P | S.I | MM | MS | | % correct | C | G | P | S.I | MM | MS |
| C | 80 | 16 | 1 | 0 | 2 | 1 | 0 | C | 80 | 16 | 1 | 0 | 2 | 1 | 0 |
| G | 94 | 0 | 16 | 0 | 1 | 0 | 0 | G | 94 | 0 | 16 | 0 | 1 | 0 | 0 |
| P | 97 | 0 | 0 | 35 | 1 | 0 | 0 | P | 97 | 0 | 0 | 35 | 1 | 0 | 0 |
| S.I | 59 | 4 | 1 | 2 | 20 | 7 | 0 | S.I | 56 | 4 | 1 | 2 | 19 | 8 | 0 |
| MM | 90 | 0 | 0 | 0 | 3 | 26 | 0 | MM | 79 | 0 | 0 | 0 | 6 | 23 | 0 |
| MS | 100 | 0 | 0 | 0 | 0 | 0 | 17 | MS | 94 | 0 | 1 | 0 | 0 | 0 | 16 |
| S.I ^a | 63 | 6 | 5 | 3 | 65 | 24 | 0 | S.I ^a | 56 | 7 | 5 | 3 | 58 | 30 | 0 |
| Males of Iberian lineages | | | | | | | | | | | | | | | |
| | % correct | C | G | P | S.I | | | | % correct | C | G | P | S.I | | |
| C | 85 | 17 | 0 | 0 | 3 | | | C | 75 | 15 | 1 | 0 | 4 | | |
| G | 94 | 0 | 16 | 0 | 1 | | | G | 94 | 0 | 16 | 0 | 1 | | |
| P | 100 | 0 | 0 | 36 | 0 | | | P | 97 | 0 | 0 | 35 | 1 | | |
| S.I | 85 | 3 | 1 | 1 | 29 | | | S.I | 71 | 5 | 3 | 2 | 24 | | |
| S.I ^a | 85 | 7 | 4 | 4 | 88 | | | S.I ^a | 82 | 10 | 4 | 5 | 84 | | |
| Females of Iberian lineages | | | | | | | | | | | | | | | |
| | % correct | C | G | P | S.I | | | | % correct | C | G | P | S.I | | |
| C | 79 | 26 | 1 | 0 | 6 | | | C | 58 | 19 | 2 | 1 | 11 | | |
| G | 89 | 0 | 8 | 0 | 1 | | | G | 78 | 0 | 7 | 0 | 2 | | |
| P | 94 | 0 | 2 | 31 | 0 | | | P | 88 | 0 | 4 | 29 | 0 | | |
| S.I | 72 | 10 | 0 | 1 | 28 | | | S.I | 59 | 13 | 2 | 1 | 23 | | |
| S.I ^a | 81 | 11 | 4 | 2 | 72 | | | S.I ^a | 78 | 13 | 4 | 3 | 69 | | |

C ‘Cadiz’ lineage, G ‘Gitanilla’ lineage, P ‘Portuguese’ lineage, S.I ‘S.Iberian’ lineage, MM *Triops m. mauritanicus*, MS *T. m. simplex*

^a Additional samples not included in DFA models, but classified via classification functions derived from these models

However, 100% correct classification was achieved for populations of the ‘Portugal’ and ‘Gitanilla’ lineages also with this DFA model.

Size of resting eggs

This character divides the populations into four significantly different groups of taxonomic importance (ANOVA, $p < 0.001$; Tukey post-hoc test, $p < 0.01$). It separates all *Triops mauritanicus* lineages (population means of resting-egg diameter 412–553 μm) from *T. c. cancriformis*, which

shows consistently lower values (population means 367–390 μm). Within *T. mauritanicus*, the ‘Gitanilla’ lineage exhibits the largest eggs, separating them from all the remaining lineages (Fig. 6). The populations of the ‘Portuguese’ lineage have the largest eggs of the remaining lineages. *Triops m. mauritanicus*, *T. m. simplex* as well as the ‘S.Iberian’ and ‘Cádiz’ lineages assume intermediate positions between *T. c. cancriformis* and the ‘Gitanilla’ plus ‘Portuguese’ lineages. Within this group, *T. m. simplex* tends to have the smallest eggs (observed population means 412 and 439 μm), while the ‘S.Iberian’ lineage tends to

Fig. 5 Unrooted NJ tree of squared Mahalanobis distances between group centroids obtained from discriminant function analysis of morphological data on adult males of all known *Triops mauritanicus* lineages. Abbreviations: T.m.m. = *T. m. mauritanicus*; T.m.s = *T. m. simplex*

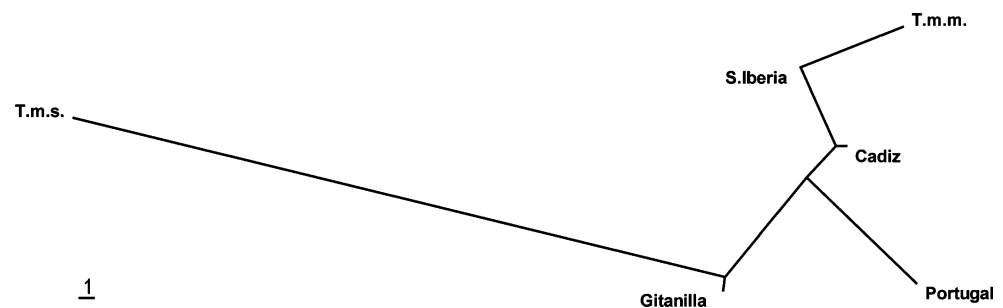


Table 8 Squared Mahalanobis distances between centroids (multivariate means) of statistical groups (main phylogenetic lineages in *Triops mauritanicus*; lineage and taxon abbreviations as in Table 7) in the discriminant function analysis (DFA) of morphological characters; probabilities $p < 0.001$, except $p = 0.004$ for 'S.I' vs. 'C' in females (*)

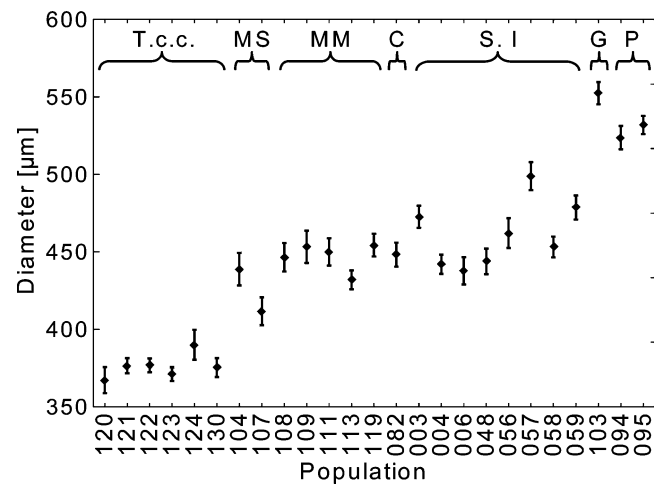
| Males of all lineages | | | | | |
|-----------------------------|------|------|------|------|------|
| | G | P | S.I | MM | MS |
| C | 11.3 | 18.6 | 6.1 | 12.0 | 59.1 |
| G | | 28.3 | 15.5 | 22.6 | 48.4 |
| P | | | 13.6 | 26.9 | 60.0 |
| S.I | | | | 4.4 | 63.1 |
| MM | | | | | 81.4 |
| Males of Iberian lineages | | | | | |
| | G | P | S.I | | |
| C | 17.8 | 22.6 | 6.5 | | |
| G | | 37.4 | 18.0 | | |
| P | | | 18.1 | | |
| Females of Iberian lineages | | | | | |
| | G | P | S.I | | |
| C | 17.8 | 20.6 | 2.3* | | |
| G | | 15.0 | 17.4 | | |
| P | | | 18.5 | | |

reach larger egg sizes, especially one of the four populations from Extremadura, which showed a population mean diameter of 499 μm (population 057, see Table A1). Nauplius sizes were exemplarily measured for two of the populations (003, *T. mauritanicus*, 'S.Iberian' lineage; 121, *T. c. cancriformis*; data not shown). Total length of naupliae was significantly higher in the *T. mauritanicus* population (ANOVA, $p < 0.001$, $F = 99.8$, $df = 1$) compared to the *T. c. cancriformis* population (and was approximately 40–50% bigger than the diameter of resting eggs).

Table 9 Success of population classification for the discriminant function analysis (DFA) based on jackknife sampling

Classification success was calculated for populations for which a minimum of four individuals were available. A population was regarded as classified correctly when $\geq 75\%$ of its individuals were classified correctly. A population was regarded as misclassified when $\geq 75\%$ of its individuals were attributed to a lineage other than that indicated by mitochondrial sequence data (lineage and taxon abbreviations as in Table 7)

| Males of all lineages | | | | |
|---------------------------|-----------|-------------|--------------------|-------------------|
| | % correct | No. correct | No. indeterminable | No. misclassified |
| C | 67 | 2 | 1 | – |
| G | 100 | 1 | – | – |
| P | 100 | 5 | – | – |
| S.I | 32 | 6 | 11 | 2 |
| MM | 60 | 3 | 2 | – |
| MS | 100 | 3 | – | – |
| Total | 56 | 20 | 14 | 2 |
| Males of Iberian lineages | | | | |
| | % correct | No. correct | No. indeterminable | No. misclassified |
| C | 67 | 2 | 1 | – |
| G | 100 | 1 | – | – |
| P | 100 | 5 | – | – |
| S.I | 89 | 17 | 2 | – |
| Total | 89 | 25 | 3 | 0 |

**Fig. 6** Resting-egg size in populations of *Triops cancriformis* and main lineages of *T. mauritanicus* (C = 'Cádiz' lineage; G = 'Gitanilla' lineage; MM = *T. m. mauritanicus*; MS = *T. m. simplex*; P = 'Portuguese' lineage; S. I = 'S.Iberian' lineage; T.c.c. = *Triops c. cancriformis*). Eggs from populations 082, 084, 058, 108–111, 119, 120–123, 130 and some eggs from population 103 obtained from lab cultures, remaining samples extracted from field-collected sediments; for details on populations see Table A1. Error bars indicate 95% confidence intervals

This demonstrates that bigger egg sizes are not merely the result of an increased thickness of the outer coating of resting eggs.

Discussion

Morphological determination of populations

Among the lineages within *Triops mauritanicus*, we observed the lowest level of morphological differentiation

in the ‘S.Iberian’ lineage (Table 7). Interestingly, its morphological differentiation from the northern African *T. m. mauritanicus* is lower than its differentiation from the ‘Cádiz’ lineage (Table 8), even though geographical separation is clear in the former case but next to none in the latter (Fig. 3). Consequently, the inclusion of samples of the nominotypical subspecies in discriminant function analysis clearly reduces classification success in the ‘S.Iberian’ lineage (Table 7) and even results in misclassifications of some of the populations (Table 9) due to overlap in morphological characters. However, since our phylogeographic data suggest a clear geographical separation between the ‘S.Iberian’ lineage and the nominotypical subspecies (with the Mediterranean Sea and the ‘Cádiz’ lineage situated in between), this overlap in morphological traits will cause fewer determination problems if geographical origin of samples is taken into account. The probability that populations of *T. m. mauritanicus*, which appears to be endemic to western Morocco (Korn et al. 2006; Table A1), might occur within the known range of the ‘S.Iberian’ lineage appears to be low. Consequently, for the determination of Iberian samples, a priori classification probabilities could be lowered for the nominotypical subspecies in order to improve classification success in Iberian populations. Alternatively, Iberian populations could primarily be classified using the DFA model including only Iberian males, as this model has a higher discriminatory power. The DFA model including males of all lineages could then be additionally applied to identify Iberian populations that might be assigned to *T. m. simplex* or *T. m. mauritanicus*. The identity of such populations should be confirmed via molecular methods.

Evidence for asynchronous morphological evolution

Within *Triops mauritanicus*, morphological differentiation is highest for *T. m. simplex*, followed by the ‘Portugal’ and ‘Gitanilla’ lineages (Table 8; Fig. 5). A comparison with molecular phylogenetic reconstructions (Fig. 2) demonstrates that these lineages have branched off last within *T. mauritanicus* (Fig. 2c) or are among the lineages that have diverged last (Fig. 2a, b). Hence, the more recently diverged lineages are the ones with the highest level of morphological differentiation within *T. mauritanicus*. This suggests that this monophyletic group within *T. mauritanicus* evolves faster morphologically than the ‘S.Iberian’ and ‘Cádiz’ lineages. The low level of morphological differentiation between *T. m. mauritanicus* and the ‘S.Iberian’ lineage might therefore be a secondary effect, assuming that the common ancestor of *T. m. mauritanicus*, the ‘Portugal’ and ‘Gitanilla’ lineages and *T. m. simplex* might have been morphologically more similar to the latter three.

Dispersal, gene flow and differentiation among populations

The marismas (natural temporary marshes) of Doñana are habitat for an outstanding number of waterbirds. With an average of more than 300,000 wintering waterbirds (Rendón et al. 2008) in the Doñana wetland complex, this is one of the most important wintering sites for migratory waterbirds in the Western Palaearctic (Rendón et al. 2008). There is increasing evidence that waterbirds may represent an important passive dispersal vector for a variety of invertebrates via internal transport (Frisch et al. 2007; Green and Figuerola 2005; Green et al. 2008). Among numerous other taxa, *Triops* have been successfully reared from material that was recovered from the lower digestive tract of domesticated ducks fed on a mixture of crustacean eggs extracted from dry natural playa sediments (Proctor 1964). Furthermore, Notostraca are known to form part of the natural diet of waterfowl (Krapu and Swanson 1977) and herons (Kazantzidis and Goutner 2005), and may even serve as the major food item during periods of high abundance (Lo 1991). As notostracan resting eggs are carried in brood pouches before they are deposited in sediments, it appears likely that at least part of the eggs from brood pouches (those which are almost ready for deposition) will survive gut passage (see, e.g., Sánchez et al. 2007 on the viability of anostracan eggs from ovisacs of ingested adults). Thus, predation by waterbirds should result in dispersal of a high number of *Triops* resting eggs, especially when temporary ponds are drying out and *Triops* are easy prey items.

Pasture use of the marismas should further increase dispersal probabilities, as external transport of *Triops* eggs on the feet of hoofed mammals has been demonstrated by Thiéry (1987). Furthermore, floating eggs could be transported over wide distances during peak floods. In addition to the presence of numerous potential dispersal vectors, we also recorded *Triops* in all parts of the marismas, and eggs reached high densities in several of the sediment samples investigated (M.K. pers. obs.). We would thus expect to observe the highest frequency of dispersal within the marismas. Indeed, AMOVA showed that among all habitat types investigated, the amount of differentiation between populations was lowest in the marismas ($F_{ST}=0.10$; Table 5). Accordingly, we found clear indirect evidence for recent gene flow among the marisma sites, indicated by commonly observed co-occurrences of geographically spread haplotypes (see above). Nevertheless, AMOVA indicated a significant level of diversification ($F_{ST}=0.10$, $p<0.001$) among the marisma populations studied.

This is supported by morphological data: variables used to characterize the size of the furcal spines (which represent an important part of the armature) showed significant differences among samples obtained from the eastern edge

and from central and western parts of the marismas (MANOVA, $p < 0.05$). This is unlikely to represent the result of local adaptation, as predation risk is expected to be of the same magnitude in these sites (waterbird abundance estimated at level 5, ‘highly abundant’, for all marisma sites). The differences in furcal spine morphology might rather be associated with relative abundances of a certain haplotype or group of haplotypes, suggesting that *Triops* from central to western and from eastern parts of the marismas originated from different source populations with differing morphology: the samples in which we observed the largest furcal spines (populations 002 and 005; Table A1) were dominated by haplotypes ‘S.Iberia 17, 18 and 24’, which together with haplotype 27 form a subclade within the ‘S.Iberian’ lineage (Fig. 2a). These haplotypes were not observed in the samples from the eastern edge of the marismas, which showed smaller furcal spines (populations 007 and 046). Korn et al. (2006) had demonstrated that the furcal spine size shows low within-lineage variability, which suggests that this character evolves slowly. In contrast, the size and number of dorsal carina spines shows high variability (Korn et al. 2006) and may quickly adapt to local predation regimes.

Additive effects of a large resting propagule bank and adaptation to local environmental conditions may reduce the impact of new immigrants and thus keep the populations distinct despite high levels of dispersal, as proposed for zooplankton by De Meester et al. (2002). Although the capacity for local adaptation is expected to be strongest for zooplankton that reproduces via cyclical parthenogenesis, obligately sexually reproducing taxa are also predicted to have a high capacity for adaptation to local conditions (De Meester et al. 2002). While competition for resources and adaptation to local predation regimes are regarded as the main driving forces for local adaptation in zooplankton (De Meester et al. 2002), the special morphological features of *Triops* appear to allow for an additional mechanism that could enforce a fast selection for local adaptation, i.e. cannibalism among early instar larvae: in *T. mauritanicus*, juveniles of approximately 5 mm total length (which is usually reached within 1 week after hatching, M.K. pers. obs.) are already capable of feeding on metanaupliae (M.K. pers. obs.). These observations on cannibalism among juveniles were made in a small outdoor mesocosm with natural sediments (population 002), as well as on freshly field-collected specimens (population 086). Cannibalistic juveniles eliminate potential competitors and at the same time may increase their growth rates so that they may reproduce with a higher probability before predation pressure increases towards later stages of succession (Moorhead et al. 1998) or before the pond dries out. We thus hypothesize that in *T. mauritanicus* the timing of hatching and the size of resting eggs (and thus naupliae)

may be among the key factors determining establishment success of new invaders. For the ‘S.Iberian’ lineage, we found a high level of shared haplotypes only in the marisma sites, and in populations assigned to the habitat type ‘open, close’, i.e. temporary ponds in open habitat situated within the Guadalquivir delta but located outside the marisma habitat. These are the habitats in which we predicted *Triops* to have the highest dispersal probabilities. This may suggest that in *T. mauritanicus*, new immigrants have a discernible effect on the local gene pool only where dispersal rates are exceptionally high.

Within the Guadalquivir delta, we found genetic diversity of *Triops mauritanicus* populations to differ among the three major habitat types: populations in the marismas showed the highest diversity, while populations in enclosed woodland or shrubs had the lowest. Populations in open habitat outside the marismas showed intermediate values of genetic diversity. This habitat type probably has been established in the Guadalquivir delta only recently, as a result of forest degradation and clearing following human settlement. Within the Doñana National Park, the natural forest vegetation was still present in 1636, but was rapidly replaced by small pioneer shrub species within a few decades, due to overgrazing, burning, charcoal making and lumbering (Granados Corona et al. 1988). Forest degradation is likely to have occurred earlier in adjacent areas, since the above-mentioned changes in vegetation resulted from a change in land use from an exclusively aristocratic hunting estate to a pasture for livestock (livestock from neighbouring towns was not admitted to the Doñana until 1628, see Granados Corona et al. 1988). The opening of formerly forested areas appears to have resulted in increased gene diversity of populations situated within these areas. It appears unlikely that the increase in gene diversity has been the result of increased population sizes due to higher primary productivity following a reduction in shading (e.g. Mokany et al. 2008), because we did not find a positive correlation of habitat size to gene diversity despite the fact that our study ponds covered a considerable range of surface areas (approx. 60–124,480 m², marisma sites not included). Instead, we attribute the rise in gene diversity to increased dispersal probabilities. The fact that ponds in open habitat more than 75 km away from the marismas had similarly low diversity to the forest/shrubland populations near the marismas supports this interpretation. Furthermore, the morphology of four of our study ponds in open habitat close to the marismas suggests that they are artificial [a comparison with aerial photographs taken in 1956 (available at <http://www.juntadeandalucia.es>) also suggests that at least two of these ponds did not exist at that time; M.K. pers. obs.] and thus were colonized only recently by two to three *Triops* haplotypes each, which supports our interpretation of high dispersal probabilities for ponds in open habitat.

The observed pattern is in line with our hypothesis that dispersal by waterbirds should result in an increased diversity in open habitats, but less likely in ponds surrounded by forests or shrub. Waterbirds may indeed have been the main dispersal vector leading to an increase in gene diversity in the open habitats, as we found a clear positive relation of waterbird abundance estimates to gene diversity (Fig. 4b). However, in these temporary ponds located within 2 km of the original (approximately year 1900) marisma borderline, wind dispersal and human activities such as ploughing could also have resulted in an increased diversity in the open habitat, but would be less likely in ponds surrounded by forest or shrubs. Thus, our data do not allow discriminating between these dispersal vectors, because all are likely to contribute to dispersal on a local scale. However, our data suggest that mammals are less effective dispersal vectors for *Triops*, because although they might represent a major dispersal vector within forest habitat (Vanschoenwinkel et al. 2008), their presence in forest and shrublands did not result in an increase in gene diversity (gene diversity was 0 in all but one of the forest/shrubland ponds studied). Consequently, waterbirds may represent the main vector for dispersal on a supra-local scale, as suggested for other large branchiopods (Hawes 2009).

The inferred lower dispersal probabilities in forested areas may have been a key factor for the evolution of Iberian lineages of *T. mauritanicus*, because regular strong fluctuations between forest and steppe vegetation have occurred in the Mediterranean region since approximately 2.5 million years before present (Thompson 2005) and the onset of these fluctuations roughly coincides with estimates of divergence times within these lineages (Korn et al. 2006). During phases with forest vegetation maxima, dispersal may have been limited, promoting diversification into sublineages. During these periods, dispersal may have occurred mainly among a few temporary marshes and associations of large temporary ponds, which might have been the most attractive to waterbirds, whereas in areas with small scattered ponds *Triops* may have been much more isolated and prone to extinction, owing to small population sizes. The low genetic divergences within the Iberian *T. mauritanicus* lineages as compared to the high divergence observed for the northern African *T. m. mauritanicus* (Table 3; see also Korn et al. 2006) is indeed indicative of phases with increased extinction rates of Iberian populations and of resulting bottlenecks. In fact, for *Triops* in the south-west Iberian peninsula it might have been the forest maxima associated with glacial cycles, rather than the glaciation maxima or Heinrich events (episodes of massive iceberg discharge into the North Atlantic) with predominance of semi-desert vegetation (Fletcher and Sánchez Goñi 2008), that may have caused

these bottlenecks. The most unfavourable conditions may have been met in periods with closed forest under a temperate, moist, continental climate, as it appears to have formed in the study area during the late glacial Allerød interstadial, prior to the Younger Dryas (Fletcher et al. 2007). Similar conditions may also have occurred during preceding glacial cycles. To our knowledge, *Triops* has never been found in woodland ponds in regions with a temperate, moist climate, which suggests that such habitat is unsuitable. Contrary, the cold steppes found during glaciation maxima or Heinrich events may have been less unfavourable, as may be suggested by the north-eastward range extension of *T. c. cancriformis* under a cold continental climate in Russia, where the species has been reported from Syktyvkar (61°40'N) and Ukhta (63°34'N; Vekhoff 1993).

For the 'S.Iberian' lineage, the high number of private haplotypes found in the Guadalquivir delta, in a small area in south central Portugal (roughly on a line connecting Mértola and Castro Verde), and in Extremadura suggests that these areas were already colonized prior to the last Ice Age. This is supported by the fact that the area in south central Portugal that hosts numerous private haplotypes in *Triops* is also characterized by a diverse fauna of other large branchiopod taxa, most importantly an endemic species of *Tanymastigites* (Cancela da Fonseca et al. 2008), a genus typical of semi-desert climates. Furthermore, the only known records of the 'Gitanilla' lineage are from Extremadura. In both areas, a combination of local climatic conditions and soil type may have supported open-ground habitats even during forest maxima. This interpretation of our data suggests that the range of the 'S.Iberian' lineage before the last Ice Age might already have been similar to the presently occupied range, and thus might be the result of an earlier range expansion.

Three natural barriers (besides the Atlantic Ocean and the Mediterranean Sea) appear to have been limiting for this postulated earlier range expansion, but also for post-glacial recolonizations: first, a group of mountain ranges towards the eastern and northern range edges and a smaller one in southern Portugal, and second, areas already occupied by other main lineages of *T. mauritanicus*. A third factor may have been the main river systems, as we found no representatives of the 'S.Iberian' lineage south of the Guadalete River, nor between the Guadiana River and the Sierra Morena mountain range (the central mountain range in Fig. 3) within Extremadura. The possible effect of rivers as barriers to successful dispersal would imply that mammals were an important dispersal vector, since they might avoid crossing large rivers (e.g. Eriksson et al. 2004; Frantz et al. 2010) or might lose a high proportion of adherent mud with resting stages from temporary ponds (Thiéry 1987; Vanschoenwinkel et al. 2008) due to wash

off (or abrasion of macerated mud by river sediments or riverine vegetation). This appears to be in conflict with our interpretation that mammals were less effective dispersal vectors within the Guadalquivir delta (see above). However, mammals may have a relatively higher impact in areas with lower abundances of waterbirds. Furthermore, it should be noted that our interpretation of dispersal and recent gene flow is based on the present situation. Mammals might have had a higher potential for dispersal in prehistoric times, when Iberia had a diverse fauna of large herbivores, including wild horse (*Equus caballus*), steppe bison (*Bison priscus*), the bovine *Leptobos stenometopon*, steppe rhinoceros (*Stephanorhinus hemitoechus*), woolly rhinoceros (*Coelodonta antiquitatis*), and straight-tusked elephant (*Palaeloxodon antiquus*; Fortelius 2003; Kurtén 1968), and man-made barriers were still lacking. Especially during dry periods with dominance of steppe vegetation, *Triops* habitats may have been among the main water reservoirs, and thus may have attracted herds of large herbivores that often migrate over longer distances, potentially carrying *Triops* eggs with them via external transport in mud that is attached to the legs when approaching the ponds for drinking (Thiéry 1987; see also Vanschoenwinkel et al. 2008).

The inter-lineage differences observed in the amount of differentiation between populations (Table 5) and in the distribution ranges of the lineages (Fig. 3) may have resulted both from priority effects (the ‘S.Iberian’ lineage might have been the first to colonize the lower Guadalquivir valley, where high waterbird abundance may have resulted in an increase in dispersal probabilities for that lineage, see Table 4) and from individual dispersal abilities. Smaller resting propagules are generally more likely to be dispersed by birds than bigger propagules (Green and Figuerola 2005). In addition, for a certain body size of females, large sizes of resting eggs should translate into small clutch sizes and vice versa. This could clearly result in differing dispersal probabilities via birds that feed on adult females, especially at the beginning of the reproductive phase of the notostracans (or in ponds with short flooding phases), when clutch size is often limited to only a few propagules. We thus hypothesize that in *Triops* there is a trade-off between dispersal probability (favouring a reduction in the size of resting eggs) and probability of survival in local populations (promoting increased egg sizes, see above). Thus, the large sizes of resting eggs in the ‘Portugal’ and ‘Gitanilla’ lineages (Fig. 6) may have resulted in low dispersal abilities in these clades (Table 4).

Biogeography of *Triops mauritanicus*

Recently, the presence of a *Triops mauritanicus* population was reported for Ares del Maestre in northern Spain

(Zierold et al. 2007), based on COI, ATP6 and ATP8 sequence data. The authors classified that population under the invalid name *T. cancriformis mauritanicus* (the corresponding taxon had never been recorded from north-east Spain before), but did not indicate how they had determined that name. In their Table 1 Zierold et al. (2007) stated that the taxonomic identity was merely “inferred”, but did not elaborate, leaving molecular data as the likely but unconfirmed basis. [It had been demonstrated before that the former *T. c. mauritanicus* is paraphyletic (Korn et al. 2006), so that only a clear morphological classification would have allowed assigning the sample to either *T. m. simplex* or a sublineage of the former *T. c. mauritanicus* (characterized by considerably longer furcal spines; see Korn et al. 2006).] Thus, the actual taxonomic identity of the sample in question cannot be inferred from that study. However, a comparison of preliminary COI sequence data obtained from a representative subset of *T. mauritanicus* samples with the sequence by Zierold et al. (2007) retrieved from GenBank (accession number EF675908) clearly allows assignment of the Ares del Maestre sample to *T. m. simplex* (see Appendix: Fig. A1). Furthermore, in the preliminary ML phylogenetic reconstruction the northern Spanish sample branches off first within *T. m. simplex*, which suggests an origin of that taxon in the Iberian Peninsula. Apparently, *T. c. cancriformis* and *T. m. simplex* co-occur (on a regional scale) in northern Spain, which may explain why different authors disagree on the taxonomic identity of *Triops* populations in that region (see review in Korn et al. 2006). Clearly, a phylogeographic study including a sound morphological reinvestigation of northern Spanish populations is needed.

Based on our data and the interpretation of new data from northern Spanish populations (see above), we conclude that out of the six known main lineages within *Triops mauritanicus* only a single one (*T. m. mauritanicus*) appears to be absent from the Iberian Peninsula, and four of those lineages appear to be Iberian endemics, among them the two lineages that are indicated by our phylogenetic analysis to have branched off first within *T. mauritanicus* (i.e. ‘Cádiz’ and ‘S.Iberian’ lineages; see Fig. 2). This strongly supports the hypothesis formulated by Korn et al. (2006) that a common ancestor of all *T. mauritanicus* lineages was located in the Iberian Peninsula. Korn et al. (2006) had hypothesized that *T. m. simplex* and *T. m. mauritanicus* may both have diverged during a range expansion into northern Africa. However, the presence of *T. m. simplex* both in northern Africa and in Spain, and the basal position of the northern Spanish sample within *T. m. simplex* (see above) rather suggest that this taxon has evolved in the Iberian Peninsula. It might have dispersed to northern Africa more recently, well after *T. m. mauritanicus* may have diverged from a common ancestor of *T. m. simplex*, *T. m.*

mauritanicus and the 'Portugal' and 'Gitanilla' lineages during a range expansion from Iberia to northern Africa (west of the Atlas Mountains). However, more molecular data from northern Spanish and northern African populations of *T. m. simplex* are needed to corroborate this modified biogeographical scenario.

Dispersal success among populations in relation to levels of genetic divergence

Our data indicate a clear-cut difference in dispersal success in relation to levels of genetic divergence. Within the main phylogenetic lineages, gene flow as indicated by shared haplotypes appears to be high, at least on an evolutionary time scale. The fact that several of the ponds for which we observed indirect evidence of gene flow appear to be artificial and thus of recent origin (see above) suggests that gene flow may be frequent at least among populations in the Guadalquivir delta.

In contrast to this high level of exchange between populations of the same lineage, our results suggest that gene flow between the main phylogenetic lineages might be very low or even completely absent: despite the high number of 422 Iberian individuals investigated with molecular tools, we did not detect a single population with a co-occurrence of haplotypes from different main lineages. Indeed, the most divergent 12S haplotypes observed to co-occur were 'S.Iberian' haplotypes 3 and 24 (0.9% divergence as uncorrected p-distance, representing 82% of the maximum intra-lineage divergence observed; data not shown), and 'Cádiz' haplotypes 2 and 5 (1.1% divergence, 85% of maximum intra-lineage divergence). Mean inter-lineage divergences are considerably higher than divergences among these co-occurring haplotypes, ranging from 2.9% to 5.1% (uncorrected p-distances; Table 3). The lack of shared haplotypes at this magnitude of differentiation suggests that the divergences among main lineages may have reached a level of differentiation where outbreeding depression (or the formation of pre- or postzygotic isolating mechanisms) may prevent ongoing gene flow. In contrast, especially for the 'S.Iberian' and 'Cádiz' lineages, low dispersal abilities per se could hardly have caused the lack of shared haplotypes among lineages, as both lineages show rather high accumulative dispersal distances (Table 4), indicating high potential for dispersal.

Furthermore, they are geographically located close to each other in a region with only weakly expressed geographic barriers (represented mainly by the Guadalete River), and with abundant suitable habitats (indicated by high abundances of populated ponds, see Fig. 3) as well as high abundances of migrating waterbirds (Rendón et al. 2008) as potential dispersal vectors. Several of the

populations of the 'Cádiz' lineage inhabit coastal temporary marshes associated with rivers and frequented by waterbirds similar to those found in Doñana (e.g. Pérez-Hurtado et al. 1993). The probability of dispersal between these small temporary marshes and the Doñana therefore appears to be high. This supports our assumption that the lack of shared haplotypes among these regions likely is a result of low establishment success of new immigrants rather than very low dispersal rates. Although the use of mitochondrial markers would have failed to indicate successful inter-lineage dispersal by males, the apparent absence of such dispersal in females suggests that there is no free gene flow among the main lineages of *Triops* in SW Iberia.

Taxonomic implications

A recently conducted molecular and morphological reinvestigation of all subspecies formerly assigned to *Triops cancriformis* has clarified the gross phylogenetic relationships within this group (Korn et al. 2006). As a consequence, *T. mauritanicus* has regained full species status, and now includes the northern African populations of the former *T. cancriformis simplex* as a subspecies, *T. mauritanicus simplex*. Korn et al. (2006) assumed subspecific status for the lineages discovered in Portugal and Spain, but had only preliminary data for these geographic areas. Little was known about the substructure, position and status of the Iberian lineages, so that no formal subspecific names were assigned to them. The present study greatly improved our knowledge on the substructure within Iberian clades, identifying 36 further haplotypes in the 12S gene and five new haplotypes in the 16S gene (previously, only four 12S and three 16S haplotypes were known). More importantly, an additional clade was discovered in the southernmost region of Iberia. The resulting comprehensive set of sequence data indicates a clear differentiation of *T. mauritanicus* into six main lineages (Fig. 2; Table 3): *T. m. mauritanicus*, *T. m. simplex*, and four additional lineages of similar order that appear to be confined to the south-western part of the Iberian Peninsula.

The six lineages have diverged by an average of 1.2–2.8% in the 16S gene and 2.9–5.1% in the 12S gene (uncorrected p-distances; Table 3). Among well recognized and morphologically differentiated species of Notostraca (Rogers 2001), 16S sequence divergences may be as low as 2.8% (between *Lepidurus arcticus* and *L. apus apus*; uncorrected p-distances, calculated for the sequences listed in Table 1), 3.0% (between *L. lemmoni* and *L. arcticus*) or 3.3% (between *L. lemmoni* and *L. a. apus*). For the 12S gene, a corresponding low value is 6.9% (between *L. lemmoni* and *L. a. apus*; we did not calculate 12S divergences for the other *Lepidurus* species due to insufficient overlap with our long 12S fragment). Thus, the divergence into six taxa

within *Triops mauritanicus* by 1.2–2.8% (16S; average uncorrected p-distances) and 2.9–5.1% (12S) reaches a magnitude reflecting separations at species level in other notostracan lineages (see also Korn and Hundsdoerfer 2006). Consequently, it appears plausible that the six main lineages within *T. mauritanicus* represent young, but separate species.

This assumption is supported by the clear morphological differentiation observed within *T. mauritanicus*: three of the main lineages, namely *T. m. simplex*, the ‘Portuguese’ lineage and the ‘Gitanilla’ lineage, can be distinguished clearly by adult morphology alone (Table 9), and the latter two are further differentiated from the remaining lineages by their significantly larger resting eggs (Fig. 6). The remaining three lineages show less pronounced morphological differentiation (Tables 7, 9). Nevertheless, the observed high level of morphological differentiation among several of the lineages within *T. mauritanicus* is remarkable, given the overall weak morphological differentiation among notostracan species. It is the stasis in gross morphology (e.g. Suno-Uchi et al. 1997), combined with typically high within-population variability in morphological key characters (Linder 1952; Longhurst 1955), that poses great difficulties for morphological classification of the group. This has resulted, for example, in the classification of *Lepidurus packardi* and *L. couesii* as two subspecies of *L. apus* by Longhurst (1955). Similarly, some southern African populations have been variously assigned to *T. cancriformis* (e.g. Barnard 1929; Hamer and Rayner 1995) or to *T. granarius* (e.g. Longhurst 1955), and the latter includes at least three separate, possibly cryptic species and may even be paraphyletic, with *T. longicaudatus* grouping within it (Korn and Hundsdoerfer 2006).

Lastly, our molecular data suggest that there is no free gene flow between the main phylogenetic lineages, as we found evidence for a high level of exchange between populations of the same lineage but failed to detect any indication of recent gene flow among the main lineages (see above). Taken together, our results demonstrate that a taxonomic revision of *Triops mauritanicus* appears justified. We therefore reinstate *T. m. simplex* to full species status, as *Triops simplex* Ghigi 1921, and describe the Iberian lineages as new species below.

Conclusions

Dispersal abilities

Our data confirm the general, previously recognized pattern of a lower dispersal probability in gonochoric lineages of *Triops*. However, we found additional evidence that dispersal in this taxon may be further complicated by a strong habitat-

dependence of dispersal probability, mediated by prevailing dispersal vectors. Similar effects are likely to occur also in other pond-dwelling invertebrates that are passively dispersed.

Morphological determination of samples

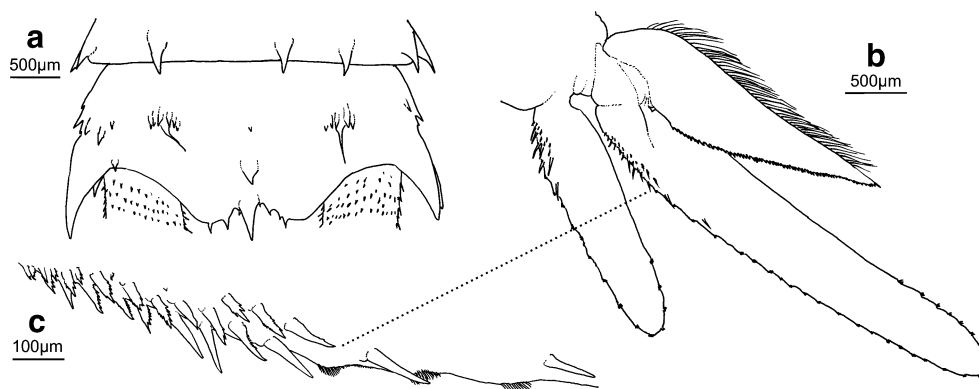
A well-known peculiarity of the Notostraca is their high variability in morphological key characters, even within populations (e.g. Longhurst 1955). Therefore, a morphology-based determination appears reasonable only at the population level, whereas reliable determination of individual specimens requires the application of genetic markers. For the morphological determination of Iberian populations we recommend a minimum of 10 male specimens per population. Although our data suggest that the ranges of the south-west Iberian lineages do not overlap, and that occurrence of *Triops simplex* or *T. m. mauritanicus* in this region is unlikely, we cannot rule out the respective alternative possibilities. Thus, we recommend the use of both discriminant function models described above. We suggest to primarily classify the Iberian populations by use of the DFA model including only Iberian males, as this model shows higher discriminatory power. For a reliable determination of morphologically indeterminable populations, we recommend the application of genetic markers. We further suggest confirming all records that are clearly outside the known range of a species via molecular methods, including south-west Iberian populations that are assigned to *T. simplex* or *T. m. mauritanicus* by the DFA model including males of all lineages. An Excel file with the morphological dataset used for DFA in the present study is available for determination purposes as “[Electronic Supplementary Material](#)” from the online version of this paper. Regarding measurements of resting-egg sizes, we recommend using eggs that were extracted from natural sediments. If lab-grown eggs are considered, great care has to be taken that not only clutches from small individuals are included, otherwise resulting mean values might underestimate actual egg sizes [preliminary results suggest that the correlation of egg size with body size varies between populations (M.K. pers. obs., data not shown)].

Taxonomy of southern Iberian lineages of the *Triops mauritanicus* species group

Abbreviations. PM = population mean(s); P.no. = population number, referring to Appendix: Table A1.

Order Notostraca Sars, 1867
Family Triopsidae Keilhack, 1909
Genus *Triops* Schrank, 1803

Fig. 7 *Triops baeticus* n. sp., adult male (holotype). **a** Telson, dorsal view. **b** Distal part of 2nd trunk limb. **c** Proximal region of 5th endite of 2nd trunk limb



Triops baeticus Korn n. sp.

(Fig. 7)

Note. This species refers to the ‘S.Iberian’ phylogenetic lineage.

Etymology. The specific epithet refers to the Roman province Baetica, named after the river Baetis (modern Guadalquivir). The delta region of that river represents the barycentre of the new species’ geographic distribution, and its diversity hotspot. The epithet is to be treated as a noun in apposition for the purposes of nomenclature.

Material examined. Holotype ♂, Museum of Zoology Dresden (MTD Crus 3434); Spain, Sevilla, Coto de Doñana National Park, Caño Travieso, P.no. 007, leg. Hugues Lefranc 6 December 2006. Paratypes: 6 ♂♂, 3 ♀♀, Museum of Zoology Dresden (MTD Crus 3299–3301, 3430–3433, 3435–3436); same data as holotype. Other material: P.no. 001, 1♂, 1♀; P.no. 002, 4♂♂, 3♀♀; P.no. 005, 4♂♂, 5♀♀; P.no. 008, 4♂♂, 3♀♀; P.no. 010, 2♂♂; P.no. 013, 3♂♂, 1♀♀; P.no. 014, 1♂; P.no. 015, 2♀♀; P.no. 019, 2♀♀; P.no. 022, 5♂♂, 7♀♀; P.no. 025, 1♂, 1♀; P.no. 026, 2♀♀; P.no. 027, 1♂; P.no. 030, 1♂; P.no. 032, 7♂♂, 4♀♀; P.no. 033, 9♂♂, 4♀♀; P.no. 034, 6♂♂, 6♀♀; P.no. 035, 1♀; P.no. 036, 5♂♂, 6♀♀; P.no. 037, 5♂♂, 3♀♀; P.no. 038, 7♂♂, 7♀♀; P.no. 039, 2♂♂, 2♀♀; P.no. 041, 5♀♀; P.no. 042, 1♂, 1♀; P.no. 045, 4♂♂, 3♀♀; P.no. 046, 6♂♂, 4♀♀; P.no. 048, 1♂, 1♀; P.no. 049, 10♂♂, 4♀♀; P.no. 053, 1♀; P.no. 054, 1♀; P.no. 056, 2♂♂, 3♀♀; P.no. 057, 4♂♂; P.no. 058, 3♀♀; P.no. 059, 2♂♂, 1♀♀; P.no. 060, 6♂♂, 9♀♀; P.no. 061, 4♀♀; P.no. 063, 3♂♂, 3♀♀; P.no. 064, 6♂♂, 6♀♀; P.no. 065, 6♂♂, 5♀♀; P.no. 066, 2♀♀; P.no. 069, 7♂♂, 6♀♀; P.no. 076, 1♀; P.no. 080, 2♂♂; P.no. 081, 2♂♂, 2♀♀.

Diagnosis. Adult male: 10th trunk limb: 7–13 (PM 10.0–11.9) anterior meshwork spines on 3rd endite, 7–15 (PM 10.4–13.5) submarginal spines on 4th endite; 2nd trunk limb: proportional endopodite length 72.9–95.5% (PM 77.3–89.6%; standardized values), proportional maximum length of spines on 5th endite 4.5–7.8% (PM 5.5–

7.0%; standardized values); telson length ratio 0.35–0.57 (PM 0.39–0.53), furcal spine width / telson width 0.11–0.20 (PM 0.12–0.16), length of telson posterior incision / telson width 0.024–0.098 (PM 0.039–0.079); apodous abdominal segments 5.4–7.7 (PM 6.2–7.2). Adult female: 10th trunk limb: 8–15 (PM 10.0–13.7) anterior meshwork spines on 3rd endite, 8–17 (PM 10.7–14.7) submarginal spines on 4th endite; telson length ratio 0.33–0.53 (PM 0.38–0.48), furcal spine width / telson width 0.11–0.18 (PM 0.12–0.16), length of telson posterior incision / telson width 0.029–0.098 (PM 0.040–0.086); apodous abdominal segments 4.8–6.7 (PM 5.1–6.4). Resting egg: diameter up to 552 µm (PM 438–499 µm).

Description of holotype. Adult male, body rings 32; total number of trunk limbs 55; 10th trunk limb: 11 anterior meshwork spines on 3rd endite, 12 submarginal spines on 4th endite; 2nd trunk limb: length of endopodite 2,558 µm, length of 5th endite 4,178 µm, longest spine on 5th endite 136 µm; length of carapace 22.52 mm, 24 dorsal carina spines, sulcus with 26 spines, length of nuchal organ 0.49 mm; 2nd maxilla well developed, with long terminal setae; telson width 2.73 mm, telson length ratio 0.42, furcal spine size ratio 2.19, posterior marginal spines of medium size, 6 median spines (the 4 anteriormost very small); approximately 7 furcal spines on both sides (not clearly separated from a row of small spines covering posterior margin of ventral side of telson), on one side, main spine not much bigger than first of the following spines; apodous abdominal segments 6.6, supernumerary spines 1.

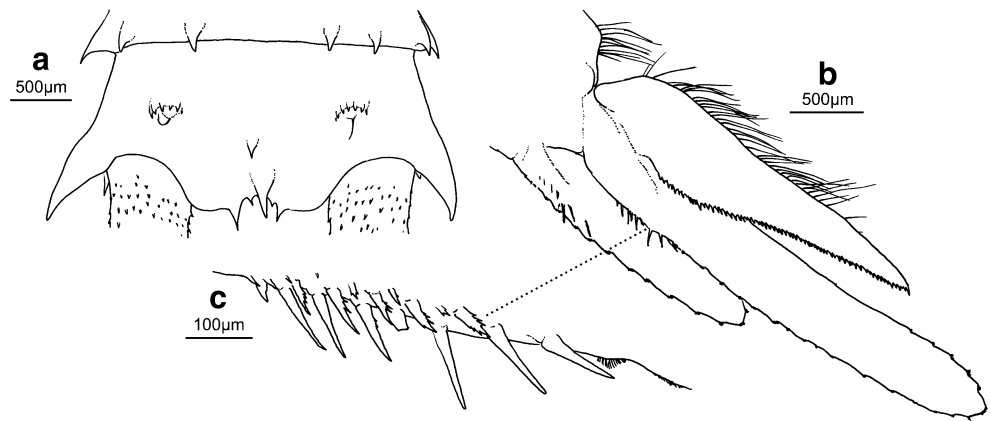
Range. Guadalquivir and Gadiana River valleys and adjacent areas. Northernmost records are from Tagus River catchment. In south central Portugal recorded from area north of Serra de Monchique and Serra do Caldeirão.

Triops gadensis Korn & García-de-Lomas n. sp.

(Fig. 8)

Note. This species refers to the ‘Cadiz’ phylogenetic lineage.

Fig. 8 *Triops gadensis* n. sp., adult male (holotype). **a** Telson, dorsal view. **b** Distal part of 2nd trunk limb. **c** Proximal region of 5th endite of 2nd trunk limb



Etymology. The specific epithet refers to Gades, the Roman name for the town of Cádiz. The epithet is to be treated as adjectival for the purposes of nomenclature.

Material examined. Holotype ♂, Museum of Zoology Dresden (MTD Crus 3089); Spain, Cádiz, Barbate, P.no. 083, leg. Juan García-de-Lomas 3 April 2006. Paratypes: 3 ♂♂, 2 ♀♀, Museum of Zoology Dresden (MTD Crus 3060–3061, 3086–3088); same data as holotype. Other material: P.no. 082, 6 ♂♂, 14 ♀♀; P.no. 084, 1 ♂, 4 ♀♀; P.no. 085, 2 ♂♂, 2 ♀♀; P.no. 086, 1 ♂; P.no. 087, 5 ♂♂, 8 ♀♀; P.no. 090, 1 ♂; P.no. 091, 3 ♀♀.

Diagnosis. Adult male: 10th trunk limb: 9–11 (PM 9.8–10.2) anterior meshwork spines on 3rd endite, 9–13 (PM 10.2–12.3) submarginal spines on 4th endite; 2nd trunk limb: proportional endopodite length 73.3–85.1% (PM 79.4–84.7%; standardized values), proportional maximum length of spines on 5th endite 4.3–6.1% (PM 4.9–5.5%; standardized values); telson length ratio 0.40–0.52 (PM 0.42–0.47), furcal spine width / telson width 0.10–0.19 (PM 0.13–0.17), length of telson posterior incision / telson width 0.034–0.099 (PM 0.055–0.068); apodous abdominal segments 5.7–7.2 (PM 6.2–6.8). Adult female: 10th trunk limb: 9–14 (PM 10.8–12.7) anterior meshwork spines on 3rd endite, 8–16 (PM 10.3–13.6) submarginal spines on 4th endite; telson length ratio 0.33–0.53 (PM 0.39–0.46), furcal spine width / telson width 0.12–0.18 (PM 0.12–0.15), length of telson posterior incision / telson width 0.033–0.082 (PM 0.058–0.064); apodous abdominal segments 4.4–6.5 (PM 5.1–5.7). Resting egg: diameter up to 479 µm (PM 448 µm).

Description of holotype. Adult male, body rings 31; total number of trunk limbs 50; 10th trunk limb: 11 anterior meshwork spines on 3rd endite, 11 submarginal spines on 4th endite; 2nd trunk limb: length of endopodite 2,674 µm, length of 5th endite 3,641 µm, longest spine on 5th endite 131 µm; length of carapace 19.05 mm, 48 dorsal carina spines, sulcus with 39 spines, length of nuchal organ 0.51 mm; 2nd maxilla

well developed, with long terminal setae; telson width 2.37 mm, telson length ratio 0.46, furcal spine size ratio 2.07, posterior marginal spines long, 2 median spines; 3+4 furcal spines, main spines considerably bigger than following spines; apodous abdominal segments 6.3.

Range. Western lowlands of Cádiz province, between El Puerto de Santa Maria and Tarifa.

Triops vicentinus Korn, Machado, Cristo & Cancela da Fonseca n. sp.

(Fig. 9)

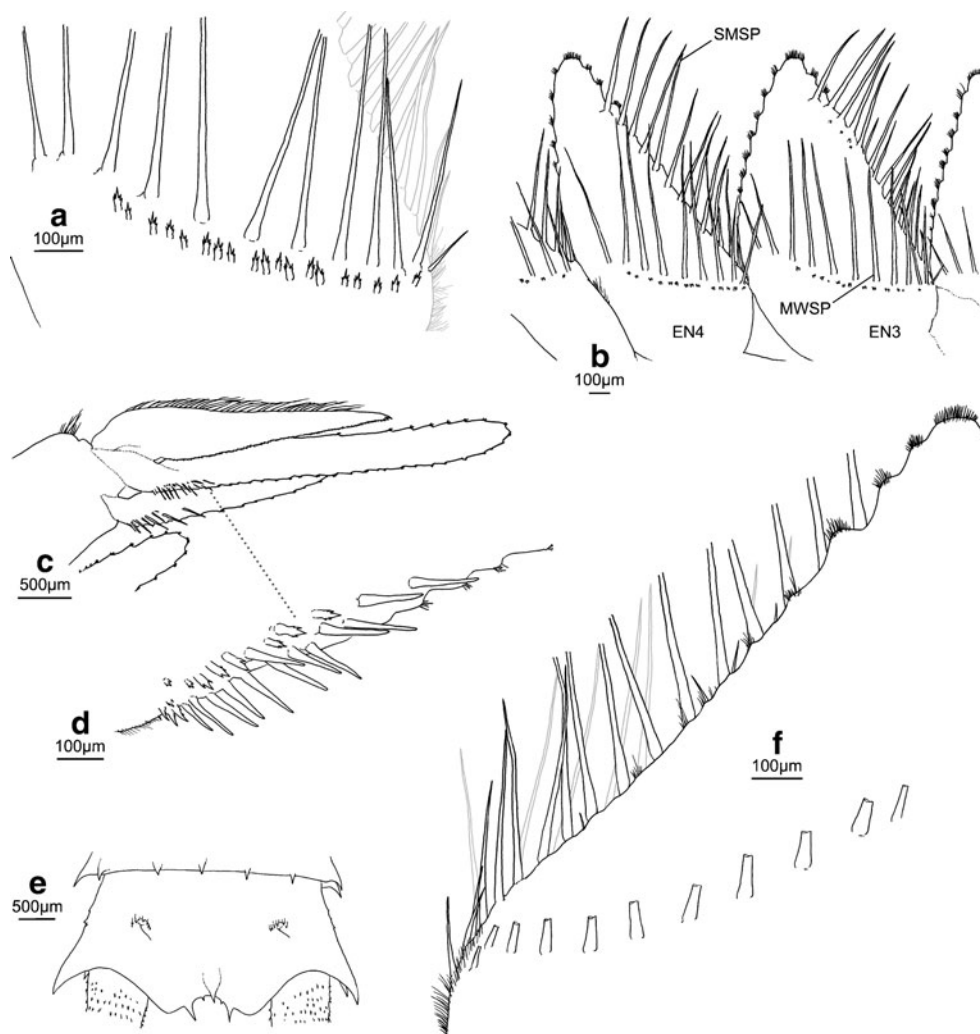
Note. This species refers to the ‘Portuguese’ phylogenetic lineage.

Etymology. The specific epithet refers to the new species’ barycentre of distribution along the Costa Vicentina in southwest Portugal. The epithet is to be treated as a noun in apposition for the purposes of nomenclature.

Material examined. Holotype ♂, Museum of Zoology Dresden (MTD Crus 3158); Portugal, Sagres, Parque Natural do Sudoeste Alentejano e Costa Vicentina PNSACV, pond G3, P.no. 095, leg. Margarida Machado, Margarida Cristo and Luís Cancela da Fonseca 3 February 2006. Paratypes: 9 ♂♂, 13 ♀♀, Museum of Zoology Dresden (MTD Crus 3124–3137, 3153–3157, 3159–3162); same data as holotype. Other material: P.no. 094, 5 ♂♂, 6 ♀♀; P.no. 096, 9 ♂♂, 3 ♀♀; P.no. 097, 6 ♂♂, 6 ♀♀; P.no. 101, 6 ♂♂, 5 ♀♀.

Diagnosis. Adult male: 10th trunk limb: 11–14 (PM 11.7–12.4) anterior meshwork spines on 3rd endite, 12–17 (PM 13.3–15.7) submarginal spines on 4th endite; 2nd trunk limb: proportional endopodite length 79.5–94.3% (PM 82.1–86.9%; standardized values), proportional maximum length of spines on 5th endite 4.3–7.0% (PM 4.6–5.9%; standardized values); telson length ratio 0.32–0.49 (PM 0.35–0.42), furcal spine width / telson width 0.11–0.16 (PM 0.12–0.14), length of telson posterior incision / telson width 0.040–0.122 (PM 0.071–0.099); apodous abdominal

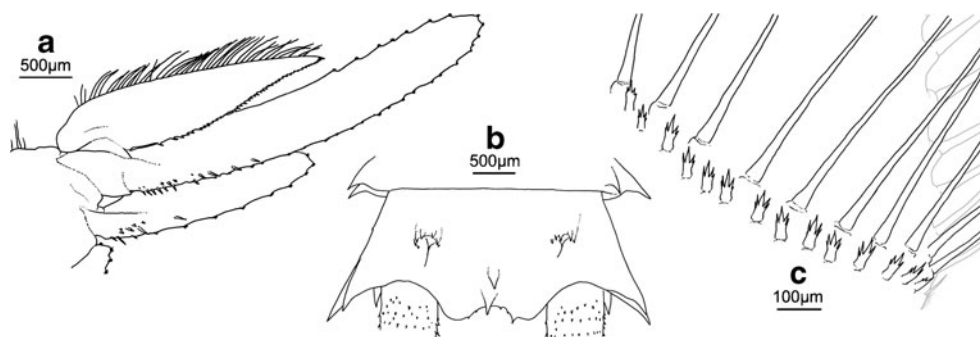
Fig. 9 *Triops vicentinus* n. sp., adult male (holotype). **a** Proximal region of 3rd endite of 10th trunk limb, anterior view; meshwork spines (and associated row of spinules) shown in *black*, submarginal spines and edge of endite in *grey*. **b** Detail of 10th trunk limb, anterior view (EN3, 4 = 3rd, 4th endite; MWSP = meshwork spines; SMSP = submarginal spines). **c** Distal part of 2nd trunk limb. **d** Proximal region of 5th endite of 2nd trunk limb. **e** Telson, dorsal view. **f** Detail of 4th endite of 10th trunk limb, posterior view; only proximalmost parts of posterior row of meshwork spines shown, anterior row of meshwork spines in *grey*



segments 5.7–7.3 (PM 6.4–7.0). Adult female: 10th trunk limb: 11–17 (PM 13.3–15.1) anterior meshwork spines on 3rd endite, 14–19 (PM 14.0–17.7) submarginal spines on 4th endite; telson length ratio 0.29–0.45 (PM 0.34–0.43), furcal spine width / telson width 0.11–0.18 (PM 0.12–0.17), length of telson posterior incision / telson width 0.048–0.134 (PM 0.080–0.099); apodous abdominal segments 5.3–6.5 (PM 5.5–6.4). Resting egg: diameter up to 583 μm (PM 524–532 μm).

Description of holotype. Adult male, body rings 33; total number of trunk limbs 57; 10th trunk limb: 12 anterior meshwork spines on 3rd endite, 17 submarginal spines on 4th endite; 2nd trunk limb: length of endopodite 2,891 μm , length of 5th endite 4,108 μm , longest spine on 5th endite 119 μm ; length of carapace 23.26 mm, 47 dorsal carina spines, sulcus with 45 spines, length of nuchal organ 0.49 mm; 2nd maxilla well developed, with long terminal setae; telson width 2.97 mm, telson length ratio 0.34, furcal

Fig. 10 *Triops emeritensis* n. sp., adult male (holotype). **a** Distal part of 2nd trunk limb. **b** Telson, dorsal view. **c** Proximal region of 3rd endite of 10th trunk limb, anterior view; meshwork spines and associated row of spinules shown in *black*, submarginal spines and edge of endite in *grey*



spine size ratio 1.72, posterior marginal spines long, 1 median spine; 7 furcal spines on both sides, main spine considerably bigger than following spines; apodous abdominal segments 6.6.

Range. Costa Vicentina to south central Algarve.

Triops emeritensis Korn & Pérez-Bote n. sp.

(Fig. 10)

Note. This species refers to the ‘Gitanilla’ phylogenetic lineage.

Etymology. The specific epithet refers to the geographic proximity of the type locality to the Roman town Emerita Augusta, the modern Mérida. The epithet is to be treated as adjectival for the purposes of nomenclature.

Material examined. Holotype ♂, Museum of Zoology Dresden (MTD Crus 3109); Spain, Badajoz, La Albuera, Laguna de la Gitanilla, P.no. 103, leg. José L. Pérez-Bote, reared from sediments). Paratypes: 16 ♂♂, 12 ♀♀, Museum of Zoology Dresden (MTD Crus 2640, 2764–2773, 3100–3102, 3104–3108, 3110–3118); same data as holotype. Other material: P.no. 102, 1♂, leg. Miguel Alonso April 2006.

Diagnosis. Adult male: 10th trunk limb: 8–10 (PM 9.2) anterior meshwork spines on 3rd endite, 9–14 (PM 10.5) submarginal spines on 4th endite; 2nd trunk limb: proportional endopodite length 69.8–80.5% (PM 74.0%; standardized values), proportional maximum length of spines on 5th endite 3.7–6.2% (PM 4.8%; standardized values); telson length ratio 0.38–0.51 (PM 0.43), furcal spine width / telson width 0.13–0.19 (PM 0.16), length of telson posterior incision / telson width 0.063–0.104 (PM 0.084); apodous abdominal segments 6.2–7.5 (PM 6.8). Adult female: 10th trunk limb: 9–12 (PM 11.4) anterior meshwork spines on 3rd endite, 10–14 (PM 12.2) submarginal spines on 4th endite; telson length ratio 0.36–0.49 (PM 0.42), furcal spine width / telson width 0.14–0.21 (PM 0.17), length of telson posterior incision / telson width 0.58–0.119 (PM 0.086); apodous abdominal segments 5.5–6.4 (PM 6.0). Resting egg: diameter up to 593 µm (PM 553 µm).

Description of holotype. Adult male, body rings 32; total number of trunk limbs 55; 10th trunk limb: 10 anterior meshwork spines on 3rd endite, 10 submarginal spines on 4th endite; 2nd trunk limb: length of endopodite 2,660 µm, length of 5th endite 4,342 µm, longest spine on 5th endite 110 µm; length of carapace 19.85 mm, 35 dorsal carina spines, sulcus with 33 spines, length of nuchal organ 0.69 mm; 2nd maxilla well developed, with long terminal setae; telson width 2.51 mm, telson length ratio 0.45, furcal spine size ratio 1.74, posterior marginal spines short, 2 median spines; 4 furcal spines on both sides, main spine only slightly bigger than following spine; apodous abdominal segments 6.6.

Range. Extremadura, Guadiana valley, recorded in two ponds near La Albuera.

Key to the southern Iberian species of *Triops*

This key uses population means of character states from both adult sexes and resting eggs; it is not designed for the identification of individual specimens. Couplet 1 includes values for the furcal spine size ratio in order to avoid possible confusion with short-spined taxa, even though these have not been reported from southern Iberia.

1. Furcal spine size ratio ≥ 0.34 ; 10th trunk limb with ≥ 11.7 (males), 13.3 (females) anterior meshwork spines on 3rd endite **and** with ≥ 13.3 (males), 14.0 (females) submarginal spines on 4th endite; length of telson posterior incision / telson width ≥ 0.074 ; diameter of resting eggs > 500 µm; geographic range: Costa Vicentina to south central Algarve *Triops vicentinus* n. sp.
– Furcal spine size ratio ≥ 0.34 ; 10th trunk limb **either** with < 11.7 (males), 13.3 (females) anterior meshwork spines on 3rd endite **or** with < 13.3 (males), 14.0 (females) submarginal spines on 4th endite, **or** counts of both types of spines below the given values 2
2. Proportional endopodite length of 2nd trunk limb in males (size standardized) $< 75\%$; length of telson posterior incision / telson width > 0.080 ; diameter of resting eggs > 500 µm; geographic range: Extremadura, Guadiana valley *Triops emeritensis* n. sp.
– Proportional endopodite length of 2nd trunk limb in males (size standardized) 77–90%; length of telson posterior incision / telson width < 0.080 ; diameter of resting eggs ≤ 500 µm 3
3. Proportional spine length of 5th endite of 2nd trunk limb in males (size standardized) $\leq 5.5\%$; geographic range: western lowlands of Cádiz province, between El Puerto de Santa Maria and Tarifa *Triops gadensis* n. sp.
– Proportional spine length of 5th endite of 2nd trunk limb in males (size standardized) $\geq 5.5\%$; geographic range: Guadalquivir and Guadiana River valleys and adjacent areas, in Cádiz province north of Guadalete River, in south central Portugal north of Serra de Monchique and Serra do Caldeirão *Triops baeticus* n. sp.

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Appendix

Table A1 Geographic origin, museum specimen tissue voucher numbers (MTD-TW) and short names of 12S and 16S haplotypes (indicating respective main phylogenetic lineage and individual haplotype) of *Triops mauritanicus* and *T. c. cancriformis* specimens used for genetic investigations and for molecular determinations of samples investigated morphologically in the present study. For samples from a preceding study (Korn et al. 2006), GenBank accession numbers (all beginning with AM18-) are given (in parentheses) instead of tissue voucher numbers. Abbreviations: Pop. no. = number code identifying population in present study; G = Gitanilla, M = Morocco, P = Portugal, S = Spain

| Pop. no. | Voucher numbers (GenBank accession numbers AM18-) | Geographic origin | Haplotypes 12S, [16S] |
|----------|---|---------------------------|------------------------------|
| 001 | 423, 796-800, 1998-2003 | S, Doñana National Park | S.Iberia 1, 2, 3, 17, 18, 22 |
| 002 | 418, 459-463, 1943-1948 | S, Doñana National Park | S.Iberia 2, 17, 18, 24, [1] |
| 003 | 3164-3169 | S, Doñana National Park | S.Iberia 1, 2, 3 |
| 004 | 3170-3175 | S, Doñana National Park | S.Iberia 3, 17, 20, 24, 26 |
| 005 | 1918-1923 | S, Doñana National Park | S.Iberia 2, 3, 17, 18 |
| 006 | 3176-3181 | S, Doñana National Park | S.Iberia 1, 2, 3, 24, 27 |
| 007 | 3185, 3227-3231 | S, Doñana National Park | S.Iberia 1, 3 |
| 008 | 1955-1960 | S, Doñana National Park | S.Iberia 1, 2, 17 |
| 009 | 557, 558, 984-987 | S, Doñana National Park | S.Iberia 17, 18, 28 |
| 010 | 419, 952-956 | S, Almonte | S.Iberia 1 |
| 011 | 801-806 | S, N. Doñana Natural Park | S.Iberia 2 |
| 012 | 778, 922-926 | S, N. Doñana Natural Park | S.Iberia 14, 15 |
| 013 | 836-841 | S, N. Doñana Natural Park | S.Iberia 9 |
| 014 | 853-858 | S, Doñana National Park | S.Iberia 1 |
| 015 | 426, 859-863 | S, Doñana National Park | S.Iberia 2, [1] |
| 016 | 427, 791-795 | S, Doñana National Park | S.Iberia 2 |
| 017 | 807-812 | S, Doñana National Park | S.Iberia 2 |
| 018 | 842-847 | S, W. Doñana Natural Park | S.Iberia 2 |
| 019 | 414, 947-951 | S, W. Doñana Natural Park | S.Iberia 1 |
| 020 | 819-824 | S, W. Doñana Natural Park | S.Iberia 2 |
| 021 | 776, 903-907 | S, Doñana National Park | S.Iberia 1, 2, 19 |
| 022 | 813-818 | S, Doñana National Park | S.Iberia 3, 19 |
| 023 | 779-784 | S, E. Doñana Natural Park | S.Iberia 1, 3 |
| 024 | 428, 962-966 | S, E. Doñana Natural Park | S.Iberia 1, 4, [1] |
| 025 | 785-790 | S, E. Doñana Natural Park | S.Iberia 1, 4 |
| 026 | 424, 912-916 | S, N. Doñana Natural Park | S.Iberia 2, 7, 21, [2] |
| 027 | 830-835 | S, N. Doñana Natural Park | S.Iberia 18 |
| 028 | 422, 957-961 | S, Villamanrique | S.Iberia 4, 7, [1] |
| 029 | 621, 848-852 | S, Villamanrique | S.Iberia 4 |
| 030 | 2004-2009 | S, El Cuervo | S.Iberia 1, 13 |
| 031 | 413, 917-921 | S, Fuentes de Andalucía | S.Iberia 1, 25 |
| 032 | 429, 932-936 | S, Cañada Rosal | S.Iberia 1 |
| 033 | 444, 967-971 | P, Castro Marim | S.Iberia 12, [1] |
| 034 | 1982-1987 | P, Serpa | S.Iberia 1 |

Table A1 (continued)

| Pop. no. | Voucher numbers (GenBank accession numbers AM18-) | Geographic origin | Haplotypes 12S, [16S] |
|----------|---|---------------------------|-----------------------|
| 035 | 431, 888-892 | P, Almodovar | S.Iberia 1 |
| 036 | 438, 883-887 | P, Castro Verde | S.Iberia 1 |
| 037 | 1976-1981 | P, Castro Verde | S.Iberia 1, 23 |
| 038 | 443, 893-897 | P, Ourique | S.Iberia 1, 8, 16 |
| 039 | 433, 1912-1916 | P, Beja | S.Iberia 1 |
| 040 | 1950, 1951 | S, Doñana National Park | S.Iberia 1 |
| 041 | 3184 | S, Doñana National Park | S.Iberia 3 |
| 042 | 1952-1954 | S, Doñana National Park | S.Iberia 2, 3 |
| 043 | 559, 992, 996 | S, Doñana National Park | S.Iberia 3, 17 |
| 044 | 416 | S, N. Doñana Natural Park | S.Iberia 2 |
| 045 | 3182 | S, Doñana National Park | S.Iberia 17 |
| 046 | 3183 | S, N. Doñana Natural Park | S.Iberia 3 |
| 047 | (3886-3888, 4183) | S, Villamanrique | S.Iberia 4, [1] |
| 048 | 475 | S, Villamanrique | S.Iberia 4 |
| 049 | 412 | S, Villamanrique | S.Iberia 4 |
| 050 | 777 | S, Villamanrique | S.Iberia 4 |
| 051 | 972 | S, Villamanrique | S.Iberia 4 |
| 052 | 973 | S, Villamanrique | S.Iberia 7 |
| 053 | 66, (3883-3885) | S, Utrera | S.Iberia 3, [1] |
| 054 | 476, 477 | S, El Cuervo | S.Iberia 13 |
| 055 | 415 | S, Fuentes de Andalucía | S.Iberia 1 |
| 056 | 165, (3913-3915) | S, Badajoz | S.Iberia 11, [1] |
| 057 | 159, 4982, 4983, (3906, 3908, 3909) | S, Navalvillar de Pela | S.Iberia 7, 10, [1] |
| 058 | 162, (3910-3912) | S, Cáceres | S.Iberia 12, [1] |
| 059 | (3907, 3916, 3917, 4184) | S, Cáceres | S.Iberia 12, [1] |
| 060 | 441 | P, Mértola | S.Iberia 6 |
| 061 | 442, 927 | P, Mértola | S.Iberia 1 |
| 062 | 435 | P, Castro Verde | S.Iberia 5 |
| 063 | 437 | P, Castro Verde | S.Iberia 5 |
| 064 | 439 | P, Castro Verde | S.Iberia 6, [1] |
| 065 | 436, 944-946 | P, Castro Verde | S.Iberia 6, [1] |
| 066 | 430 | P, Almodovar | S.Iberia 1 |
| 067 | 4986 | P, Almodovar | S.Iberia 1 |
| 068 | 4990 | P, Beja | S.Iberia 1 |
| 069 | 434 | P, Beja | S.Iberia 1 |
| 070 | 1927-1929 | P, Beja | S.Iberia 1 |
| 071 | 4989 | P, Beja | S.Iberia 1 |
| 072 | 432 | P, Beja | S.Iberia 1, [3] |
| 073 | 4988 | P, Beja | S.Iberia 1 |
| 074 | 4984 | P, Faro do Alentejo | S.Iberia 1 |
| 075 | 4985 | P, Faro do Alentejo | S.Iberia 1 |
| 076 | 1924-1926 | P, Cuba | S.Iberia 1 |
| 077 | 4991 | P, Cuba | S.Iberia 1 |
| 078 | 4987 | P, Alvito | S.Iberia 1 |
| 079 | 440 | P, Ferreira do Alentejo | S.Iberia 1 |
| 080 | 4996, 4997 | P, Estremoz | S.Iberia 1 |
| 081 | 4998-5000 | P, Estremoz | S.Iberia 1 |
| 082 | 425, 825-829, 1970-1975 | S, Tarifa | Cádiz 1, [1] |
| 083 | 488, 730-734 | S, Barbate | Cádiz 1, [1] |

Table A1 (continued)

| Pop. no. | Voucher numbers (GenBank accession numbers AM18-) | Geographic origin | Haplotypes 12S, [16S] |
|----------|---|----------------------------|------------------------|
| 084 | 485, 725-729 | S, Tahivilla | Cádiz 1, 2 |
| 085 | 486, 720-724 | S, Benalup | Cádiz 2, 5, [1, 2] |
| 086 | 421, 478, 908-911 | S, Puerto Real | Cádiz 4, [1] |
| 087 | 1988-1992 | S, El Puerto de Sta. María | Cádiz 5 |
| 088 | 420 | S, Tarifa | Cádiz 1, [1] |
| 089 | 487, 735-737 | S, Tahivilla | Cádiz 1 |
| 090 | 484, 719 | S, Benalup | Cádiz 1, 5 |
| 091 | 1934-1937 | S, Conil de la Frontera | Cádiz 5 |
| 092 | 551 | S, San Fernando | Cádiz 5, [3] |
| 093 | 1933 | S, Puerto Real | Cádiz 3 |
| 094 | 76, 1961, 1962, 1964, 1965, (3889-3893, 4181) | P, Sagres | Portugal 1, [P] |
| 095 | 447, 898-902 | P, Sagres | Portugal 1, 5, [P] |
| 096 | 446, 878-882 | P, Tunes | Portugal 2, 3, [P] |
| 097 | 445, 937-941 | P, Faro | Portugal 4, [P] |
| 098 | 1966-1969 | P, Vila do Bispo | Portugal 1 |
| 099 | 448, 1941, 1942 | P, Vila do Bispo | Portugal 1 |
| 100 | 1938-1940 | P, Vila do Bispo | Portugal 1 |
| 101 | 449, 1993, 1995 | P, Vila do Bispo | Portugal 1 |
| 102 | 634, 635, 4992-4995 | S, La Albuera | Gitanilla 1, 2 |
| 103 | (3881, 3882, 4182) | S, La Albuera | Gitanilla 1, [G] |
| 104 | (3871, 3872, 4174) | M, Ain-Benimathar | <i>T. m. s.</i> 1, [3] |
| 105 | (3868-3870, 4173) | Tunisia, Jendouba | <i>T. m. s.</i> 3, [1] |
| 106 | (3865, 3866, 4172) | Tunisia, Kairouan | <i>T. m. s.</i> 2, [2] |
| 107 | (3862-3864) | Tunisia, Tunis | <i>T. m. s.</i> [1] |
| 108 | (3873-3875, 4177) | M, El-Hajeb | <i>T. m. m.</i> 2, [6] |
| 109 | (3904, 3905, 4179) | M, Mrirt | <i>T. m. m.</i> 1, [5] |
| 110 | (3876, 3879, 4176) | M, Timahdite | <i>T. m. m.</i> 1, [7] |
| 111 | 1930-1932 | M, Timahdite | <i>T. m. m.</i> 1 |
| 112 | (3877, 3878) | M, Rabat, pond 59 | <i>T. m. m.</i> [7] |
| 113 | (3880) | M, Rabat, pond 60 | <i>T. m. m.</i> [7] |
| 114 | (3894, 4175) | M, Casablanca, pond 002 | <i>T. m. m.</i> 3, [1] |
| 115 | (3897, 3898) | M, Casablanca, pond 005 | <i>T. m. m.</i> [1] |
| 116 | (3895, 3896) | M, Safi | <i>T. m. m.</i> [2] |
| 117 | (3899, 3900, 4178) | M, Essaouira, pond 049 | <i>T. m. m.</i> 4, [2] |
| 118 | (3901) | M, Essaouira, pond 050 | <i>T. m. m.</i> [3] |
| 119 | (3902, 3903, 4180) | M, High Atlas | <i>T. m. m.</i> 5, [4] |
| 120 | 3333 | France, Crau | <i>T. c. c.</i> 4 |
| 121 | (3824-3826) | Germany, Ingolstadt | <i>T. c. c.</i> [1] |
| 122 | (3851-3853, 4171) | Tunisia, Jendouba | <i>T. c. c.</i> 4, [3] |
| 123 | (3833-3835) | Sicily, Custonaci | <i>T. c. c.</i> [3] |
| 124 | (3828) | Favignana Island, pond 1 | <i>T. c. c.</i> [1] |
| 125 | (3846, 3847) | Serbia, Melenci, pond 1 | <i>T. c. c.</i> [2] |
| 126 | (3848-3850, 4170) | Serbia, Melenci, pond 2 | <i>T. c. c.</i> 4, [1] |
| 127 | (3841, 4167) | Hungary, Tiszabercel | <i>T. c. c.</i> 4, [5] |
| 128 | (3821-3823, 4166) | Austria, commercial kit | <i>T. c. c.</i> 1, [6] |
| 129 | (3843-3845, 4168, 4169) | Russia, Uljanowsk | <i>T. c. c.</i> 1, [7] |
| 130 | (3827, 4165) | UAE, Sharjah | <i>T. c. c.</i> 2, [6] |

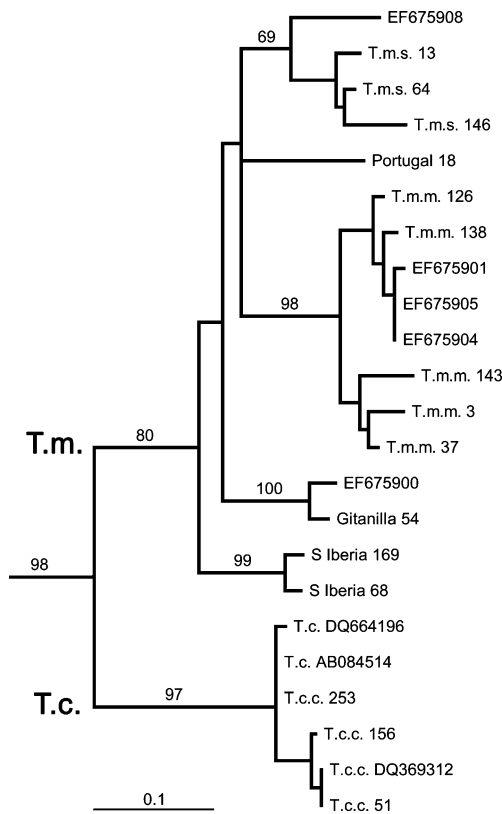


Fig. A1 ML tree based on COI sequences (RAxML program, setting 'estimate proportion of invariable sites'; best evolutionary model obtained by Modeltest was TrN+I+G, selected by AIC). ML bootstrap support (obtained with RAxML) given for selected branches. Outgroups [GenBank sequences of *Lepidurus apus* (accession number EF189669), *L. arcticus* (AF209067), *L. couesii* (DQ310622), *L. lemmonii* (GQ144447), *Triops longicaudatus* (DQ310623 and GQ144444), *T. australiensis* (DQ889135), *T. granarius* (GQ144446)] removed for clarity. Samples labelled, as applicable, with short names of main phylogenetic lineages (Table A1) followed by museum specimen tissue voucher numbers (MTD-TW; sequences submitted to GenBank, acc. nrs. FN691430–FN691444) or by GenBank accessions, or labelled with GenBank accessions containing numbers but no lineage data (samples with GenBank taxon labels apparently resulting from erroneous species identification, i.e. samples submitted to GenBank with invalid species names). Abbreviations: T.c. = *Triops cancriformis*; T.m. = *T. mauritanicus*

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