Sister species within the *Triops cancriformis* lineage (Crustacea, Notostraca)

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We investigated the phylogenetic relationships among the three presently recognized subspecies of the tadpole shrimp, *Triops cancriformis*, using mitochondrial 16S and 12S rDNA sequences. Our results indicate that the taxon is divided into two distinct lineages. One lineage is formed of *T. c. cancriformis* populations and samples from northern Spain that had been classified as *T. c. simplex* in the most recent literature. The second lineage comprises all populations of *T. c. mauritanicus* and northern African populations of *T. c. simplex*. These two main lineages separated 2.3 to 8.9 million years ago, based on the range of inferred molecular clocks recognized for crustacean mtDNA sequence divergence. Percentages of divergence are in the range reported for recognized species in other notostracan lineages and we therefore propose to recognize them as two species, *Triops cancriformis* and *Triops mauritanicus*. The latter would comprise two subspecies in northern Africa, one consisting of the Moroccan populations of the former *T. c. mauritanicus*, the other comprising the African populations of the former *T. c. simplex*. It also includes three as-yet unnamed lineages. A comparison of morphological characters with the molecular data revealed that the former *T. c. simplex* cannot be reliably separated from *T. c. cancriformis*, using morphological characters that have hitherto been used to distinguish among subspecies of *T. cancriformis*. Our investigation is the first to demonstrate the presence of *T. c. cancriformis* in Africa (Tunisia). The genetic haplotypes of these populations are identical with haplotypes also occurring in Central and Western Europe, as well as in Sicily. Therefore, we hypothesize that the African populations of *T. c. cancriformis* represent a result of repeated long-distance dispersal across the Mediterranean Sea.

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Introduction

The Notostraca are an ancient group of branchiopod crustaceans, their fossil record dating back to the upper Carboniferous (Dumont & Negrea 2002). They consist of two genera with worldwide distributions, *Triops* and *Lepidurus*. Both occur almost exclusively in temporary pools where they can endure prolonged dry phases via resting eggs (encysted embryos in diapause). A well-known peculiarity of the Notostraca is their high variability in most — or even all — morphological key characters, even within populations. For

example, the number of segments may vary by three or even four within a sample of specimens from one locality (Linder 1952; Longhurst 1955) and partially reduced or extended, spiral-shaped body rings can also be observed (Linder 1952). This poses great difficulties for morphological classification of the group (e.g. Linder 1952), resulting in a high number of species (more than 60) being described, sometimes on the basis of just a single specimen.

The Notostraca were revised by Linder (1952) and Longhurst (1955). The latter author reduced the number of species to nine, four of which were in the genus *Triops*. More recent studies have concentrated on North American populations, using allozyme electrophoresis and DNA sequence analysis. These studies revealed the existence of cryptic species among North American *Lepidurus* and *Triops* (Sassaman *et al*. 1997; King & Hanner 1998; Rogers 2001).

In temperate Europe and the Mediterranean region, *T. cancriformis* is the predominant species of *Triops*. Mantovani *et al*. (2004) concluded that there were no cryptic species among *T. cancriformis* but they only investigated populations of the subspecies *T. c. cancriformis*. However, at present three subspecies are recognized, all of which occur in the Mediterranean region. Originally, four species were described (Ghigi 1921) for these Mediterranean populations: *Triops mauritanicus*, *Triops simplex*, *T. cancriformis* and *Triops apulius*, of which the first three were later treated as subspecies of *T. cancriformis* (Longhurst 1955), while *T. apulius* was regarded as a synonym of either *T. c. cancriformis*, or less probably, *T. c. simplex* (Longhurst 1955).

The subspecies differ markedly in sex ratio. Typical *T. c. cancriformis* populations are either unisexual or female-biased, whereas populations of *T. c. simplex* and *T. c. mauritanicus* have equal distribution of the sexes (Eder & Hödl 2003; Scanabissi *et al*. 2005). Ignorance of the fact that sex ratios in *T. cancriformis* appear to be strictly linked to subspecies led to the simplified assumption of a geographical parthenogenesis in *T. cancriformis* (Zaffagnini & Trentini 1980) with a latitudinal gradient, as only *T. c. cancriformis* occurs in Central and northern Europe, whereas the other subspecies are restricted to more southern regions. However, if there is a geographical gradient in the distribution of reproductive modes in European populations of *T. cancriformis*, it is directed rather from west to east than from north to south, as, for example, no gonochoric populations (i.e. those that have an obligately outcrossing mode of reproduction, with separate male and female individuals) of this species have been reported from mainland Italy.

Triops cancriformis cancriformis has a wide distribution, from Europe and western Russia through the Middle East to northern India (Longhurst 1955). The range of *T. c. mauritanicus* covers north-west Africa, southern Spain and the island of Menorca (Longhurst 1955). Authors disagree in the geographical distribution of the subspecies *T. c. simplex*. Longhurst (1955) reported it only in northern Africa, from Ceuta to Egypt. Recently, the range of this subspecies has been extended to the Arabian Peninsula, where it was found at a single locality in Yemen (Thiéry 1996). According to Longhurst (1955), the populations occurring in the northern part of the Iberian Peninsular belong to *T. c. cancriformis*. However, Margalef (1953) and Alonso (1985, 1996) regarded these northern Iberian populations as belonging to *T. c. simplex*. They also reported the absence of *T. c. cancriformis* from the Iberian Peninsula. This discordance deserves further investigation, for Longhurst (1955) did stress that it might be difficult to ascribe single specimens to *T. c. cancriformis* or *T. c. simplex*. The same perhaps might be true for whole populations, given the high morphological variability inherent to this group. The problems that arise in morphological determinations within *Triops* may even be apparent at the species level. For some southern African populations, there is no consensus regarding their affiliation to *T. cancriformis* (e.g. Barnard 1929) or *Triops numidicus* (e.g. Longhurst 1955). After re-examination of the type specimens, Hamer & Rayner (1995) again classified these populations as *T. cancriformis*, but without assigning them to one of the recognized subspecies. Thus, the status of these southern African populations remains to be investigated.

In this study, we use 16S and 12S rDNA sequences to investigate the phylogenetic relationships among the subspecies of *T. cancriformis* and to clarify their distributions in the western Mediterranean region. We compare the sequence data with key morphological characters and with reproductive mode.

Materials and methods

Taxon sampling

We attempted to acquire as many different samples of *Triops cancriformis* from Europe and North Africa as possible (locality data are listed in Table 1). We used both wild-caught specimens and specimens raised in the laboratory from eggs from sediments. Most samples were conserved in absolute ethanol until extraction (a few samples were fixed in 70% ethanol). Tissue vouchers were deposited in the 'Tissue' collection of the 'Museum fuer Tierkunde' (Dresden, Germany) under the MTD-TW numbers listed in Table 1. Voucher specimens (with the exception of those loaned from a private collection, see Acknowledgements section for details) from the morphological analyses were deposited in the 'Invertebrates' collection of the same museum, under the numbers MTD Crus 2624–MTD Crus 2666. Sequences were submitted to GenBank with accession numbers AM183821–AM183917 for 16S and AM184165–AM184184 for 12S sequences. Sequences already available in GenBank were also included in the phylogenetic analyses (see Table 2).

Table 1 Geographical origin and museum specimen tissue voucher numbers (MTD-TW) of specimens of which we obtained 16S sequences in this study and short names of haplotype groups. (For definition of haplotype groups see Appendix 1).

Taxon	Voucher number	Geographical origin	Haplotype group
T. c. cancriformis	8, 9	Italy, Sicily, Gela	C. Eur
T. c. cancriformis	34	Italy, Favignana Island, pond 1	C. Eur
T. c. cancriformis	256	Italy, Favignana Island, pond 2	C. Eur
T. c. cancriformis	101, 102	Tunisia, Bou Salem	C. Eur
T. c. cancriformis	47, 48, 49	Germany, Ingolstadt	C. Eur
T. c. cancriformis	109, 110	Malta	C. Eur
T. c. cancriformis	249, 250	Serbia, Melenci, pond 1	C. Eur
T. c. cancriformis	251, 252, 253	Serbia, Melenci, pond 2	C. Eur
T. c. cancriformis	22, 23, 24	Italy, Ustica Island	Sicily
T. c. cancriformis	25, 26, 27	Italy, Sicily, Custonaci	Sicily
T. c. cancriformis	28, 29, 30	Tunisia, Jendouba	Sicily
T. c. cancriformis	74	Hungary, Kunszentmiklós	Hungary
T. c. cancriformis	254	Hungary, Poroszló	Hungary
T. c. cancriformis	255	Hungary, Tiszabercel	Hungary
T. c. cancriformis	50, 51, 52	Austria, commercial kit	Austria
T. c. cancriformis	156	United Arab Emirates, Sharjah	Austria
T. c. cancriformis	246, 247, 248	Russia, Uljanowsk	Russia
T. c. simplex	10, 11, 12	Tunisia, Tunis	Tunisia
T. c. simplex	31, 32, 64	Tunisia, Jendouba	Tunisia
T. c. simplex	15	Tunisia, Kairouan, pond 064	Interm.
T. c. simplex	146, 147	Morocco, Ain-Benimathar	T.c.s. M.
T. c. simplex	20, 21, 103, 104, 105, 106	Spain, Girona	N. Spain
T. c. mauritanicus	1, 2, 3	Morocco, El-Hajeb, pond 058	M 058
T. c. mauritanicus	4, 37	Morocco, Timahdite	Rabat
T. c. mauritanicus	5, 6	Morocco, Rabat, pond 059	Rabat
T. c. mauritanicus	39	Morocco, Rabat, pond 060	Rabat
T. c. mauritanicus	126	Morocco, Casablanca, pond 002	Casab.
T. c. mauritanicus	136, 137	Morocco, Casablanca, pond 005	Casab.
T. c. mauritanicus	134, 135	Morocco, Safi	S. W. M.
T. c. mauritanicus	138, 139	Morocco, Essaouira, pond 049	S. W. M.
T. c. mauritanicus	141	Morocco, Essaouira, pond 050	S. W. M.
T. c. mauritanicus	142, 143	Morocco, High Atlas S. of Marrakech	H. Atlas
T. c. mauritanicus	144, 145	Morocco, Mrirt, pond 056	M 056
T. c. mauritanicus	54, 55	Spain, Extremadura, pond Gitanilla	Gitanilla
T. c. mauritanicus	158–169	Spain, Extremadura, from 4 pools	S. Spain
T. c. mauritanicus	65, 66, 67	Spain, Sevilla	S. Spain
T. c. mauritanicus	68, 69, 70	Spain, Huelva	S. Spain
T. c. mauritanicus	18, 19, 76, 77, 78	Portugal, Sagres	Portugal
T. c. ssp. 'intermediate'	13, 14	Tunisia, Kairouan, pond 063	Interm.

Determination of specimens

The characters given by Longhurst (1955) were used to determine specimens of *T. cancriformis* provisionally to subspecies (see Table 3).

The northern Spanish population investigated here had already been classified as *T. c. simplex* by Margalef (1951, 1953) and Alonso (1985, 1996); thus we only determined a few specimens. These showed the typical morphological features of this subspecies (but see Results section below). Longhurst (1955) had reported the absence of *T. c. simplex* from the Iberian Peninsula, but did not investigate specimens from the same population (his classification was based on a population from Valencia in eastern Spain).

All specimens with large furcal spines were classified as *T. c. mauritanicus*. Central European and Italian samples, of which all determined specimens showed a smooth carina, were nevertheless classified as *T. c. cancriformis* because of the absence of males in these samples and the ability of individuals to reproduce in the absence of males, which we tested in all of the populations in question. Unisexual Tunisian populations (one with specimens showing carinal spines, one in which almost all specimens showed a smooth carina) were also classified as *T. c. cancriformis*. Their ability to reproduce in the absence of males was tested using the population in which most specimens lacked carinal spines. One population from Tunisia (pond 063, Kairouan; Table 1) could not be classified using the characters given in the literature, because specimens showed a mixture of characters of all subspecies. This population had an equal distribution of sexes (53% males, $n = 17$).

Table 2 Overview of the sequences retrieved from the GenBank for this study with their accession numbers. *Lepidurus cryptus* (Rogers 2001) refers to the cryptic lineage found by King & Hanner (1998).

Taxon	Accession numbers	Gene
T. cancriformis	AB084514	16S + 12S
T. cancriformis	AY115613, AY159571–579	16S
T. numidicus	AF200963–971, AY115612	16S
T. longicaudatus	AY639934, AY115605, AY115609, AY115610–611, AY159580–581	16S
L. a. apus	AY159584	16S
L. a. lubbocki	AY159582–583	16S
L. arcticus	AY159585	16S
L. lemmoni	AY115614	16S
T. cancriformis	AY115603, AY159563–65, AF494482	12S
T. numidicus	AY115602	12S
T. australiensis	AY050646	12S
T. longicaudatus	AY115595–600, AY159566, TLAJ0817, AY639934, AY115601	12S
L. a. apus	AF494483, AY159568	12S
L. a. lubbocki	AY159567	12S
L. arcticus	AY159569	12S
L. lemmoni	AY115604, LLAJ0823–824, LLAJ0830	12S
L. packardi	LPAJ0820–822	12S
L. couesii	LCAJ0827	12S
L. cryptus	LCAJ0819, LCAJ0825–826, LCAJ0829	12S
L. bilobatus	LBAJ0818, LBAJ0828, LBAJ0831	12S

DNA extraction, PCR amplification and sequencing

For total DNA extraction, a piece of the thoracopod (or the whole specimen if it was very small) was washed twice with 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and then placed into 500 µL DTAB buffer (65 °C; 37.5 mM EDTA, 1.125 mM NaCl, 75 mM Tris-HCl, pH 8.0) in an Eppendorf cup. The material was crushed with a small pestle or cut into small pieces with scissors and incubated for 30 min at 65 °C. After addition of Proteinase K (0.5 mg/sample), the extractions were incubated overnight at 55 °C. To remove surplus RNA, the samples were incubated with 0.1 mg RNAase A for 30 min at 37 °C, after which the extracts were cleaned with chloroform/isoamyl-alcohol (24 : 1) twice. After addition of 1/5 volume 4 M LiCl and 1 volume isopropanol, the DNA was precipitated for 30 min at –20 °C and pelleted by centrifugation at 4 °C. The DNA pellet was washed twice with ice-cold 70% ethanol, dried and dissolved in 50 µL TE buffer.

Part of the 16S rDNA gene was amplified using the universal primers 16Sar and 16Sbr (Palumbi *et al*. 1991; corresponding to primers 16SA and 16SB from Simon *et al*. 1994) and the 12S fragment with the universal primers published in Colbourne & Hebert (1996) and Murugan *et al*. (2002). Each PCR was performed with about 500 ng template DNA in a 50-µL volume (5 pmol of each primer, 1.25 nmol of each dNTP, and 0.2 u of *Taq* polymerase (Bioron) buffered with 10 mM Tris-HCl, 50 mM KCl, 0.5% Triton X-100, 1.5 mM $MgCl_2$). Following the initial 4-min denaturation at 94 °C, the program consisted of 35–50 cycles (depending on DNA quantity) of 45 s at 94 °C, 60 s at 55 °C, 90 s at 72 °C, and 5 min at 72 °C for final elongation. For sequencing, the PCR products were sent to the DNA Sequencing Facility of the Max Planck Institute of Molecular Cell Biology and Genetics (Dresden, Germany). The forward primers were used for direct sequencing of the PCR product, and if the sequence was not of sufficient quality the complement/reverse sequence was obtained additionally.

Sequence alignment, nucleotide composition, and substitution patterns

Alignment was performed by hand using the program BioEdit (Hall 1999). In the 16S dataset one *Lepidurus* and one *Triops longicaudatus* sequence were included as outgroups. In the ingroup we encountered only one insertion in the 16S gene (an additional A at the position 272 in the unequivocal alignment); additional gaps had to be introduced at three positions with respect to the outgroups. Two deletions and two insertions had to be introduced within the ingroup in the 12S dataset (alignment available upon request). Nucleotide composition, substitution frequencies, pairwise transition/transversion frequencies, and pairwise distances were calculated with PAUP* 4.0b10 (Swofford 1998). To enable an assessment of the overall range of intrageneric sequence divergence found among recognized species of Notostraca, we compared the mean genetic distances between congeneric

Table 3 Literature data (Longhurst 1955) used for the classification of subspecies of *Triops cancriformis*. Longhurst (1955) notes that it may be difficult to ascribe single specimens (with a smooth carina) to the nominate race or *T. c. simplex*, but he found no large samples of *T. c. cancriformis* without specimens showing carina spines. To date, it still is not clear if the maleless 'hermaphrodite' populations reproduce by selfing or by parthenogenesis. There are also populations known that are strongly female/hermaphrodite dominated.

Character	*T. c. cancriformis*	*T. c. simplex*	*T. c. mauritanicus*
Carina spines	0–10, generally 2–3	complete absence, carina 'quite' smooth	more numerous and much stronger than in *T. c. cancriformis*
Size of furcal spines	small	small	very large
No. of apodous segments of female	4–6	5–7	5–7
Reproductive mode	hermaphrodite and bisexual	bisexual	bisexual

species pairs (calculated with MS Excel). MEGA version 2.1 was used to illustrate parsimony-informative characters and singletons (Kumar *et al.* 2001). The program ForCon 1.0 (Raes & Van de Peer 1998) was used to interconvert input files between formats. To assess saturation effects in this data set, pairwise comparisons of transitional and transversional changes were plotted against pairwise distances in DAMBE version 4.2.13 (Xia & Xie 2001; whereby the result that the data were not saturated was stable with all distance correction methods implemented).

Phylogenetic analyses

To investigate relationships among the subspecies, several data sets were used for calculations of phylogeny reconstruction. First, a 16S dataset comprising 107 *T. cancriformis* sequences and two outgroup sequences from *Lepidurus a. apus* and *T. longicaudatus* (GenBank accession numbers in Table 2 and Fig. 4) was analysed using maximum parsimony (MP; settings gapmode = new; add = cl) as implemented in PAUP* 4.0b10 (Swofford 1998) and maximum likelihood (ML) using PHYML (Guindon & Gascuel 2003; via the online Web interface http://atgc.lirmm.fr/phyml/). As a measure of branch support, bootstrap values were calculated with MP and neighbour-joining (ML-corrected distances) in PAUP* (settings nreps = 1000, maxtree = 1000) and with PHYML (nreps = 500; presented in percent). The best evolutionary model for the data was established by hierarchical likelihood testing, performed with the program ModelTest (Posada & Crandall 1998). A second 16S dataset consisting of the subset of ingroup samples for which 12S sequence data were additionally available (30 *T. cancriformis*) was analysed with 10 outgroup sequences in the same manner. Similarly, the 12S sequences of this selection of samples, as well as the combined 16S and 12S sequences, were analysed (also as described above) as third and fourth datasets.

Timing of diversification events

To estimate whether rates of mtDNA molecular evolution are equivalent among the sequences of *T. cancriformis* (a condition necessary for dating cladogenetic events), we compared the 16S maximum-likelihood trees (excluding all outgroup taxa and some very close taxa of *T. cancriformis* to reduce computation time; neighbour-joining starting tree; in PAUP*) obtained without (option multrees in effect) and with (setting maxtree = 1) the assumption of a molecular clock. The latter analysis was undertaken by enforcing the molecular clock option in PAUP* (using a UPGMA starting tree rooted on a sample of *T. c. mauritanicus* from southern Spain as outgroup). We used the S–H test in PAUP* to compare alternative trees (unrooted to enable comparison). As no significant difference was detected (the best tree was the first of the two obtained without the molecular clock enforced, and the one with the clock enforced resulted in $P = 0.254 > 0.05$, i.e. it was not significantly different at the 5% level; Shimodaira & Hasegawa 1999), we assumed clock-like evolution of the sequences of *T. cancriformis*. Approximate times of diversification for selected clades were calculated by converting pairwise genetic distances into units of time, following the divergence range of inferred crustacean molecular clocks for mtDNA (16S) sequence divergence published in Schubart *et al.* (2000): 0.65–0.88% per million years.

Morphological re-analyses

For comparison with the sequence data, we investigated three key morphological characters that have been used to discriminate among subspecies of *T. cancriformis* (Longhurst 1955; Table 3): (1) size of furcal spines located postero-laterally on the telson; (2) dorsal carina spines; and (3) number of apodous abdominal segments.

Furcal spines. Furcal spines are part of the telson armature, which comprises four sets of spines (see Longhurst 1955 for details). They are positioned around the bases of the furcal rami and are few and large in *T. cancriformis* (Longhurst 1955). The two most prominent furcal spines are typically situated dorso-laterally, to each side of the telson, followed ventrally by several smaller spines (in some specimens, a smaller spine may also be positioned dorsally from one of the prominent spines). For practical reasons, only the most prominent spines (one from each side of the telson) were taken into account and are referred to below as the furcal spines.

We used the ratio of furcal spine length to the distance between furcal spine tip and the anterior-lateral edge of the telson (henceforth called telson length ratio) to characterize the size of the furcal spines. Because the spines show no clearly identifiable starting point at their base in most specimens, we used subsidiary lines to define the anterior starting point. One subsidiary line was drawn from the foremost anterior edge-point of one furcal ramus to the corresponding edge-point of the other furcal ramus in dorsal view (see Fig. 1). Further subsidiary lines were directed along the distal sides of each furcal ramus. The distance from the spine tip to the point where the subsidiary lines meet was defined as the furcal spine length. Telson length ratio was measured for both sides of the telson to form a mean value, except for specimens with a damaged spine on one side.

Measurements were made on digital photographs of the telson (taken in dorsal view) using Scion Image for Windows (Release Alpha 4.0.3.2 available at www.scioncorp.com). Subsidiary lines were drawn in Adobe Photoshop Elements 2.0.

Dorsal carina spines. In all specimens, carina spines were counted using a stereomicroscope at ×50 magnification. Very small spines were included in the counts. Only bulges with increased

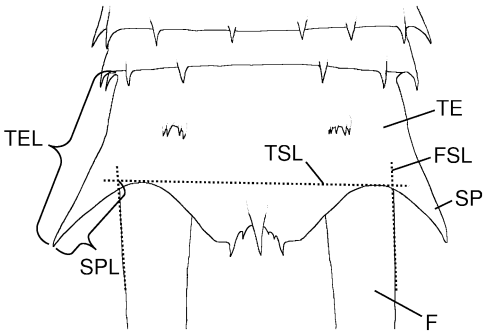

Fig. 1 Schematic drawing of the posterior part of the abdomen of a *Triops cancriformis mauritanicus* specimen in dorsal view, showing the position of subsidiary lines used for furcal spine measurements (dotted lines). Telson length ratio is defined as the ratio of furcal spine length to telson length. Abbreviations: TSL, telson subsidiary line; FSL, furcal subsidiary line; TE, telson; SP, furcal spine; F, furcal ramus; SPL, furcal spine length; TEL, telson length.

sclerotization were recognized as spines. In *T. c. mauritanicus*, small spines situated on top of larger ones were often observed. These were regarded as the remnants of once separate spines, as comparisons among specimens suggested that in this subspecies, spines often fuse to different degrees. They were therefore included in the counts.

Number of apodous abdominal segments. The number of apodous abdominal segments was counted using a stereomicroscope at ×6.5–50 magnification. Linder (1952) and Longhurst (1955) concluded that legs grow independently of the segments and thus may end at any point of the abdomen. It is therefore not appropriate to simply count the number of segments without any legs. For segments that were only partially covered by legs, the legless portion was estimated and was added to the number of apodous segments as one decimal unit. Consistent measurements of this character are impossible in fixed specimens because of variable degrees of body contraction during fixation (Longhurst 1955). Contrary to Longhurst (1955), we did not include incomplete segments in the counts, as they generally do not appear to increase the length of the abdomen. Often, they form only a small scale that partially covers the base of the telson. Thus, we consider that neglecting these incomplete segments provides a better impression of the size of the apodous part of the abdomen. Whether segments were incomplete or not was assessed in ventral view of the abdomen.

Statistical analyses of morphological data. For each morphological dataset, the null hypothesis that there were no significant differences between means of statistical populations was tested with a single-factor analysis of variance (ANOVA). The genetic haplotype group was considered as the fixed factor for each analysis, where haplotype groups are defined as subgroups of sequences sharing autapomorphic sites, without consideration of singletons (Appendix 1). There was one exception from grouping specimens by haplotype group: the northern Spanish population was treated like a separate haplotype group to evaluate its unusual position in the present classification in which it is assigned to a subspecies (i.e. *T. c. simplex*) different to the other members of its haplotype group (*T. c. cancriformis*). The dependent variables were telson length ratio, number of apodous abdominal segments and number of dorsal carina spines, respectively. To test for homogeneity of variance, Levene's test was used and normality was checked by plotting expected normal values against observed values. When the null hypothesis was rejected, differences among single statistical populations were investigated using a Tukey post-hoc test.

The number of specimens available for morphological analysis varied among populations and ANOVA is much less robust to violations of assumptions, particularly homogeneity of variances, when sample sizes differ (Quinn & Keough 2003). Thus, to avoid an unbalanced design, we randomly excluded 'excess' data points. For telson length ratio and number of dorsal carina spines, all statistical populations (i.e. haplotype groups) were set to 10 specimens, while for the apodous abdominal segment counts in females, the number was set to five specimens per haplotype group. Populations for which these numbers were not attained were excluded (i.e. haplotype groups 'Russia' and 'Hungary'; Appendix 1).

The dorsal carina spine counts showed positive skewness, resulting in nonhomogeneous variances. Thus, a square root transformation was used, which greatly improved the approximation to a normal distribution and homogeneity of variances within this dataset. However, the assumption of homogeneity of variances was still clearly violated. Therefore, only a data subset that met all the assumptions of ANOVA was used for calculating statistics by eliminating some populations with unusual low variability. In this data subset, we retained all populations that were most important in evaluating the usefulness of this morphological character for discriminating among subspecies, e.g. populations of *T. c. mauritanicus* with an important overlap in this character with other subspecies (such overlap is not recognized in the present literature).

Following data analysis, a sequential Bonferroni test (Rice 1989) was carried out for the three ANOVAs performed. All statistics on morphological data were undertaken with STATISTICA 6.0 (StatSoft, Inc.).

Biogeography

For a better understanding of present patterns of genetic diversity and the geographical distribution of populations with differing reproductive modes, we reconstructed the possible maximum distribution range of *T. cancriformis* in Europe

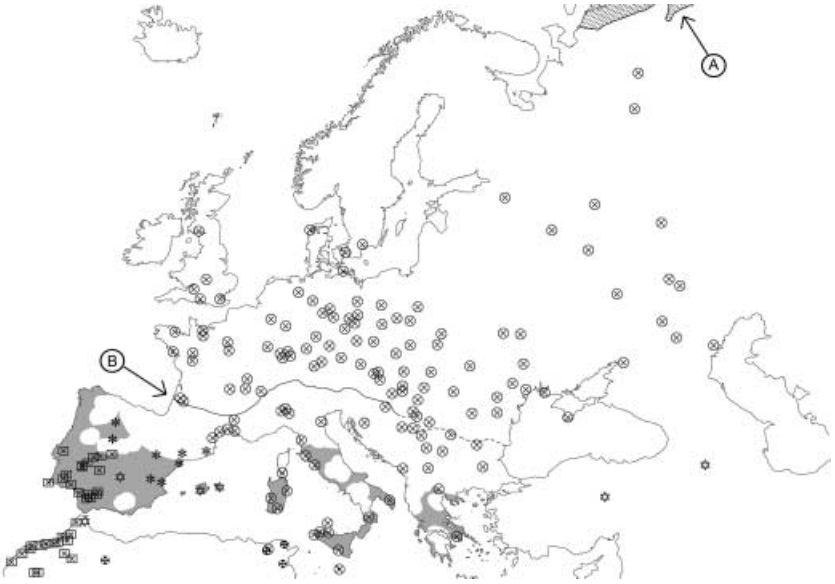

Fig. 2 The present distribution of *Triops cancriformis* subspecies in Europe (literature data dating back one century and own records), the reconstructed maximum distribution of *Triops* in Europe during the last Ice Ages (shaded area; for details see Materials and methods section), the present extent of massive ice wedges (ice-filled ground frost cracks) and the main distribution of ice-wedge-casts (fossil ice wedges; indicative of former permafrost: see Murton & Kolstrup 2003). Symbols: ⊗, *Triops cancriformis cancriformis*; ⊠, *T. c. mauritanicus*; ✠, North African *T. c. simplex*; ✻, Spanish *T. c. simplex* (including population from Girona, northern Spain); ✡, *T. cancriformis*, without specification of subspecies or with doubtful classification; A and dotted area, the present extent of massive ice wedges (redrawn from a map available at http://nsidc.org/data/ggd600.html); B, southern borderline of the main region in which ice-wedge casts occur (redrawn from Flint 1971).

during the last Ice Ages. The reconstruction is based on the assumption that the minimum distances of present *Triops* sites to permafrost regions or high alpine glaciers are indicative of the general potential maximum distribution extent of this taxon towards such low temperature regions. We thus used indications of the extent of permafrost and glaciers during the Ice Ages to infer the possible distribution of *Triops* during this period. The palaeodistribution of permafrost can be withdrawn from the distribution of fossil ground frost cracks, so-called ice-wedge-casts (Murton & Kolstrup 2003). For our reconstruction, we used distribution maps of ice-wedge-casts (redrawn from Flint 1971; see Fig. 2), present-day massive ice wedges (ground frost cracks filled with ice; map available at the Frozen Ground Data Center homepage, see http://nsidc.org/data/ggd600.html) and Ice Age glaciers (redrawn from Flint 1971). The present distribution of *T. cancriformis* was referred from literature data and own observations (not all literature data could be checked for correctness). The minimum geographical distance of a reported *Triops* site to regions in which massive ice wedges occur (refer to site 86 in Vekhoff 1993; see northernmost locality in Fig. 2) is approximately 400 km and the minimum geographical distance to an alpine glacier is approximately 50 km (refer to the site indicated for Haute Savoie region in Defaye *et al*. 1998). Consequently, the reconstructed area of maximum palaeodistribution of *Triops* in Europe is the area situated at least 400 km south of the area in which ice-wedge-casts occur and which at the same time is situated outside a range of 50 km to any montane regions that were covered by glaciers during the Ice Ages.

Results

Nucleotide composition, substitution patterns and sequence variability

The nucleotide composition in the 16S rDNA gene segment sequenced showed a pronounced AT bias (33.1% T, 31.8% A, 12.8% C, 22.3% G) within *T. cancriformis*. Higher AT than GC levels are not unusual for mitochondrial DNA (Simon *et al*. 1994); however, it should be noted that the assumption of equal base frequencies in certain substitution models used in phylogenetic analyses is violated.

The alignment consisted of 433 sites, of which 393 sites were conserved within *T. cancriformis*, constituting 90.8%. Within this lineage 37 sites were variable and 31 of these (7.2% of the total sequence) were parsimony informative. The mean 16S sequence divergences between the *Triops* taxa analysed are presented in Table 4a. Table 4b shows 16S distances between taxa of *Lepidurus* for comparison. Within *T. cancriformis* transitions had not reached saturation (Fig. 3), whereas transversions exhibited a slight tendency to saturation, indicated by the cessation of linear correlation.

Table 4 Mean genetic divergences calculated from all 16S sequences analysed between taxa of (a) *Triops* and (b) *Lepidurus* and from all 12S sequences available of (c) *Triops* and (d) *Lepidurus* in percent, based on the uncorrected p-distances (below diagonal) and the ML-distance (above diagonal). The ML-parameters were estimated by hierarchical likelihood ratio tests in Modeltest 3.06 (Posada & Crandall 1998; parameter values can be obtained from A.K.H. upon request). Comparisons with *T. cancriformis* are also broken down by the two lineages observed in the study at hand (see text and Fig. 4), whereby the samples from Girona (northern Spain) were included in *T. c. cancriformis* and excluded from *T. c. simplex*. *Lepidurus cryptus* (Rogers 2001) refers to the cryptic lineage found by King & Hanner (1998). Abbreviations: *T.c.c.*, *T. c. cancriformis*; *T.c.m.*, *T. c. mauritanicus*; *T.c.s.*, *T. c. simplex*; *T. cancrif.*, *T. cancriformis*; *T. longic.*, *T. longicaudatus*; *T. numid.*, *T. numidicus*; *L. a. lub.*, *L. a. lubbocki*; *T. austral.*, *T. australiensis*. * marks single values (i.e. not mean values).

(a)

	T.c.c.	*T.c.m. + T.c.s.*	*T. cancrif.*	*T. longic.*	*T. numid.*
T.c.c.	—	3.3	—	16.3	14.3
T.c.m. + T.c.s.	2.9	—	—	15.5	13.8
T. cancrif.	—	—	—	15.9	14.0
T. longic.	9.4	9.0	9.2	—	8.2
T. numid.	8.8	8.6	8.7	6.0	—

(b)

	L. a. apus	*L. a. lub.*	*L. arcticus*	*L. lemmoni*
L. a. apus	—	6.7	3.2*	4.2*
L. a. lub.	5.1	—	7.5	7.5
L. arcticus	2.8*	5.6	—	3.8*
L. lemmoni	3.3*	5.5	3.1*	—

(c)

	T.c.c.	*T.c.m. + T.c.s.*	*T. cancrif.*	*T. longic.*	*T. numid.*	*T. austral.*
T.c.c.	—	4.3	—	12.2	14.5	13.6
T.c.m. + T.c.s.	4.1	—	—	12.7	14.1	12.8
T. cancrif.	—	—	—	12.4	14.3	13.2
T. longic.	10.9	11.3	11.1	—	10.1	6.5
T. numid.	12.7	12.4	12.6	9.2	—	9.4*
T. austral.	12.1	11.4	11.8	6.1	8.6*	—

(d)

	L. a. apus	*L. a. lub.*	*L. arcticus*	*L. lemmoni*	*L. packardi*	*L. couesii*	*L. cryptus*	*L. bilobatus*
L. a. apus	—	7.2	4.8	5.1	7.3	8.7	8.8	7.0
L. a. lub.	6.7	—	9.6*	8.5	10.7	11.7*	12.4	10.6
L. arcticus	4.6	8.8*	—	6.8	7.2	6.4*	10.0	8.9
L. lemmoni	4.8	7.9	6.4	—	7.1	9.6	7.2	5.2
L. packardi	6.8	9.7	6.7	6.6	—	10.4	8.3	7.2
L. couesii	8.1	10.7*	6.1*	8.9	9.6	—	13.6	10.1
L. cryptus	8.2	11.2	9.3	6.8	7.8	12.4	—	6.6
L. bilobatus	6.6	9.7	8.2	4.9	6.8	9.4	6.4	—

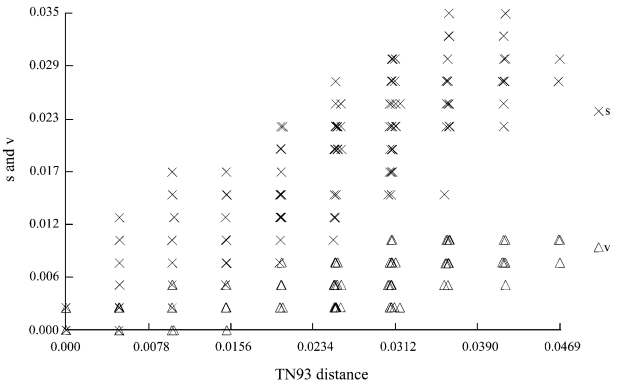

Fig. 3 Abundances of transitions (S; crosses) and transversions (V; triangles) as a function of model-corrected ML-distances for each pairwise comparison of *Triops* taxa, plotted in DAMBE (Xia & Xie 2001).

The dataset of 12S sequences of *T. cancriformis* (n = 30) also showed the AT bias (31.5% T, 38.0% A, 19.3% C, 11.2% G) and consisted of 353 sites, of which 307 were conserved, 41 were variable and 26 were parsimony informative (outgroups not included). Although the corresponding subset of 16S sequences selected from the large dataset for the combined dataset (12S and 16S sequences) did not show much fewer variable sites (34 compared to 37 in the large dataset), only 21 sites remained parsimony informative.

Phylogenetic analyses and timing of cladogenetic events

Both phylogeny reconstructions based on the first dataset (107 *T. cancriformis* 16S sequences) resulted in the topology presented in Fig. 4A. The first of the two most parsimonious trees (MPT) is presented, and the second MPT and the tree of the PHYML calculation (not shown) do not differ from this one except for details irrelevant for the phylogenetic interpretation. The dataset with 16S and 12S sequences combined also resulted in the same topology (Fig. 4B) within *T. cancriformis*, indicating a robust result: The samples of *T. cancriformis* cluster into two groups — *T. c. cancriformis* plus *T. c. simplex* from Girona (northern Spain) and *T. c. mauritanicus* together with North African *T. c. simplex*. The analyses of the corresponding datasets of the gene sequence fragments separately (the 12S dataset and the corresponding subset of 16S sequences) resulted in topologies with less resolution (especially the MP calculations), but without major conflict to the topologies presented in Fig. 4. The only noteworthy difference is that the separate 12S PHYML reconstruction resulted in sister group formation of the Portuguese sample with the samples of North African *T. c. simplex* instead of with the sample from the Spanish pond Gitanilla (Extremadura).

The intrageneric genetic divergences between recognized notostracan species in the 12S and 16S genes are presented in

Table 5 Splits of divergence of selected clades, subclades or haplotype groups of *Triops cancriformis*, estimated from applying the slowest and the fastest (Schubart *et al*. 2000) inferred molecular clock for Crustacea to pairwise 16S rDNA genetic distances.

	Myr BP at 0.65% per Myr						Myr BP at 0.9% per Myr					
	p-distance			ML-distance			p-distance			ML-distance		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
T.c.c. to T.c.m. + T.c.s.	4.5	3.2	7.2	5.1	3.5	8.9	3.2	2.3	5.2	3.7	2.6	6.4
within T.c.m. + T.c.s.:												
'S. Spain' to 'Gitanilla'	3.8	3.6	4.0	4.1	3.9	4.5	2.7	2.6	2.9	3.0	2.8	3.2
'S. Spain' to 'Portugal'	2.5	2.5	2.6	2.7	2.7	2.8	1.8	1.8	1.9	1.9	1.9	2.0
'S. Spain' to T.c.s.	2.2	2.2	2.2	2.2	2.2	2.3	1.6	1.6	1.6	1.6	1.6	1.7
'S. Spain' to T.c.m. Morocco	1.9	1.4	2.6	2.0	1.5	2.7	1.4	1.0	1.9	1.4	1.1	1.9
T.c.s. to T.c.m. Morocco	2.4	1.4	3.2	2.5	1.5	3.5	1.7	1.0	2.3	1.8	1.1	2.5
'Gitanilla' to 'Portugal'	4.1	3.9	4.3	4.7	4.5	4.9	3.0	2.9	3.1	3.4	3.2	3.5
within T.c.c.:												
'C. Eur.' to 'Russia'	0.7	0.4	1.1	0.7	0.4	1.1	0.5	0.3	0.8	0.5	0.3	0.8
'C. Eur.' to 'Austria'	0.4	0.0	1.1	0.4	0.0	1.1	0.3	0.0	0.8	0.3	0.0	0.8
'C. Eur.' to 'Sicily'	0.4	0.4	1.1	0.4	0.4	1.1	0.3	0.3	0.8	0.3	0.3	0.8
'C. Eur.' to 'Hungary'	0.4	0.4	0.7	0.4	0.4	0.7	0.3	0.3	0.5	0.3	0.3	0.5

Table 4. Within *Triops* the lowest value of divergence between recognized species was observed between *T. longicaudatus* (New World and Japan) and *Triops australiensis* (Australia): 6.1% p-distance (12S; Table 4c). For comparison, the p-distance between the two lineages found within *T. cancriformis* (Fig. 4), i.e. *T. c. cancriformis* and *T. c. mauritanicus* + *T. c. simplex*, is 4.1% (12S; Table 4c). Within *Lepidurus* the lowest values of divergence (p-distance) are observed between *L. a. apus* and *Lepidurus arcticus*: 2.8% (16S; Table 4b) and 4.6% (12S; Table 4d). These values are in the range of the 2.9% (16S; Table 4a) and 4.1% (12S; Table 4c) observed among the two lineages within *T. cancriformis*.

The estimates of divergence times of selected clades within *T. cancriformis* are presented in Table 5. Splits within the *T. c. cancriformis* lineage are clearly more recent than those within the lineage of *T. c. mauritanicus* plus *T. c. simplex*. Although the two haplotype groups 'Portugal' and 'Gitanilla' form a monophyletic clade in the reconstructions with sufficient resolution, the split among them is nearly as high as between the two main lineages within *T. cancriformis*.

The Triops cancriformis cancriformis *lineage*

The Russian samples branch off first within this lineage, illustrating the 'Russian' haplotype group. Next to branch off is the 'Austrian' haplotype group, including the sample from the United Arab Emirates (and one sample from Italy, interpreted as an artefactual cluster formation, details in Discussion section). The sequences of *Triops c. simplex* from Girona (northern Spain) genetically belong to the same haplotype group as those of *T. c. cancriformis* samples from Malta, Serbia, Germany as well as certain samples from Tunisia (#101, 102) and Italy (#34; Fig. 4A). For purposes of morphological and taxonomic comparison, however, the former are assigned to a different haplotype group ('Northern Spain') than the latter ('Central European'). Nevertheless, they are not a separate lineage and the group consisting of '*T. c. simplex*' from Girona (northern Spain) and *T. c. cancriformis* is referred to below simply as the *T. c. cancriformis* clade. Genetic data also demonstrate that the Japanese specimen belongs to the *T. c. cancriformis* lineage (Fig. 4, labelled T.c. AB084514). Certain samples from Sicily (but not all from this island), Ustica and Tunisia (but not all from this country) form a separate subclade, which is named the 'Sicilian' haplotype group. Similarly, the samples from different localities in Hungary cluster together, illustrating the existence of the haplotype group 'Hungary'.

The Triops cancriformis simplex *group*

Triops cancriformis simplex consist of two genetically very distinct lineages, one forming part of the *T. c. cancriformis* lineage (see above), the other grouping within *T. c. mauritanicus*. Thus, genetic data indicate that a separate *T. c. simplex* lineage as indicated by morphological similarities (see below) and most recent classification as a separate subspecies (with populations occurring in northern Africa as well as in Iberia) does not exist.

The Triops cancriformis mauritanicus *lineage*

Triops cancriformis mauritanicus is paraphyletic in both the MP and ML reconstructions as it includes those North African samples assigned to *T. c. simplex* (Fig. 4). The *T. c. mauritanicus* samples form three separate monophyletic clusters (Fig. 4A):

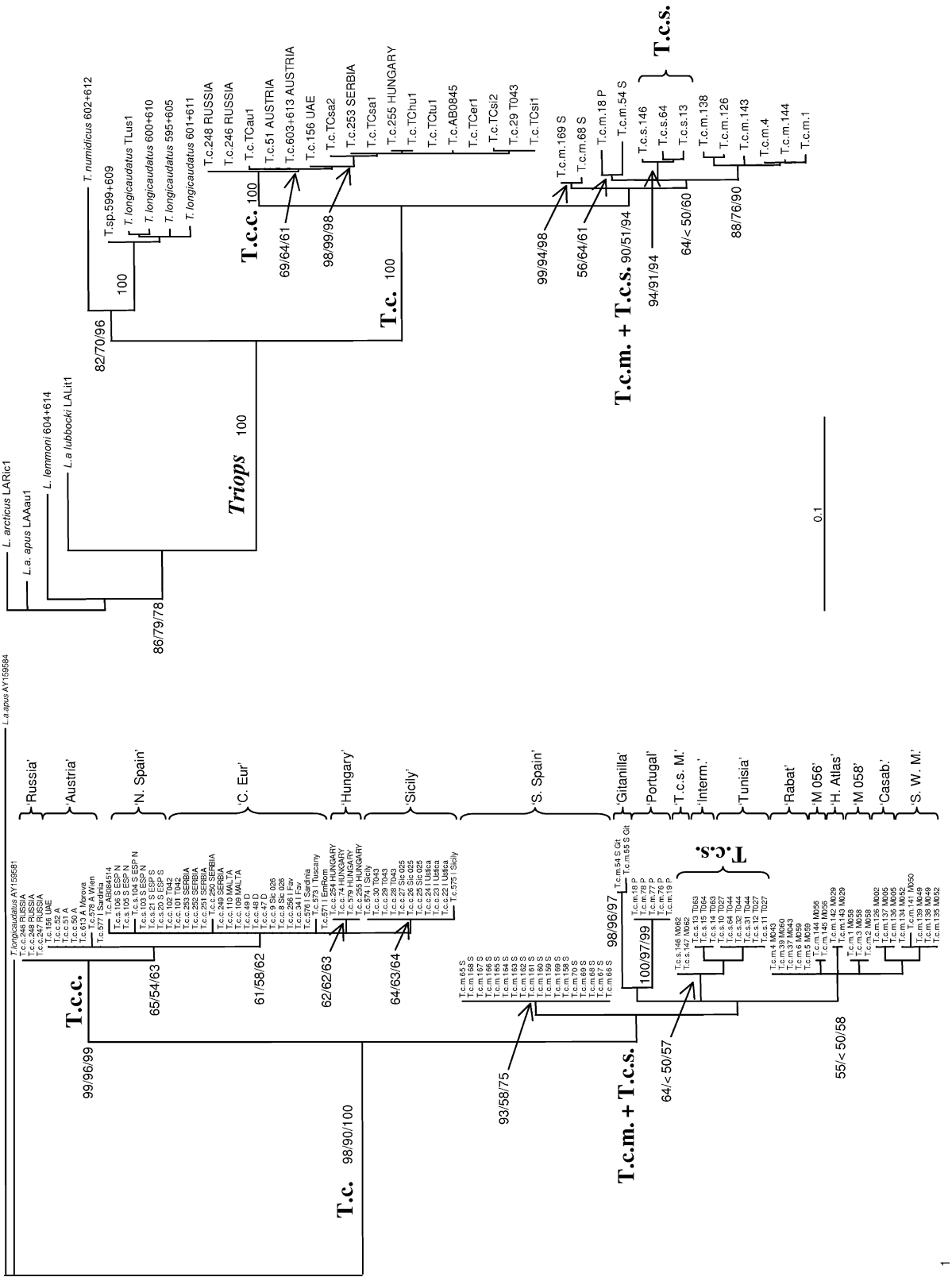
A
B
L.a.apus AY159584
'Russia'
'Austria'
'N. Spain'
'C. Eur'
'Hungary'
'Sicily'
'S. Spain'
'Gitanilla'
'Portugal'
'T.c.s. M.'
'Interm.'
'Tunisia'
'Rabat'
'M 056'
'H. Atlas'
'M 058'
'Casab.'
'S. W. M.'
T.c.c.
T.c.
T.c.m. + T.c.s.
T.c.s.
99/96/99
98/90/100
65/54/63
61/58/62
62/62/63
64/63/64
93/58/75
98/96/97
100/97/99
64/< 50/57
55/< 50/58
T.longicaudatus AY159581
T.c.248 RUSSIA
T.c.248 RUSSIA
T.c.247 RUSSIA
T.c.156 UAE
T.c.51 A
T.c.50 A
T.c.613 A Morova
T.c.AB084514 Wien
T.c.577 I Sardinia
T.c.s.106 S ESP N
T.c.s.106 S ESP N
T.c.s.104 S ESP N
T.c.s.103 S ESP N
T.c.s.21 S ESP S
T.c.s.20 S ESP S
T.c.101 T042
T.c.100 T042
T.c.253 SERBIA
T.c.252 SERBIA
T.c.251 SERBIA
T.c.250 SERBIA
T.c.249 SERBIA
T.c.110 MALTA
T.c.109 MALTA
T.c.48 D
T.c.47 D
T.c.9 Sic 026
T.c.8 Sic 026
T.c.586 I Fav
T.c.544 I Fav
T.c.576 I Sardinia
T.c.573 I Tuscany
T.c.572 I Tuscany
T.c.254 HUNGARY
T.c.74 HUNGARY
T.c.579 HUNGARY
T.c.255 HUNGARY
T.c.574 Sicily
T.c.30 T043
T.c.29 T043
T.c.28 T043
T.c.27 Sic 025
T.c.26 Sic 025
T.c.25 Sic 025
T.c.24 I Ustica
T.c.23 I Ustica
T.c.22 I Ustica
T.c.575 I Sicily
T.c.m.65 S
T.c.m.168 S
T.c.m.167 S
T.c.m.166 S
T.c.m.165 S
T.c.m.164 S
T.c.m.163 S
T.c.m.162 S
T.c.m.161 S
T.c.m.160 S
T.c.m.159 S
T.c.m.158 S
T.c.m.70 S
T.c.m.69 S
T.c.m.67 S
T.c.m.66 S
T.c.m.54 S Gt
T.c.m.55 S Gt
T.c.m.18 P
T.c.m.78 P
T.c.m.77 P
T.c.m.76 P
T.c.m.19 P
T.c.s.146 M062
T.c.s.147 M062
T.c.s.13 T063
T.c.s.15 T064
T.c.s.14 T063
T.c.s.10 T027
T.c.s.64 T044
T.c.s.32 T044
T.c.s.31 T044
T.c.s.12 T027
T.c.s.11 T027
T.c.m.4 M043
T.c.m.139 M060
T.c.m.137 M043
T.c.m.6 M059
T.c.m.5 M059
T.c.m.144 M056
T.c.m.145 M056
T.c.m.142 M029
T.c.m.143 M029
T.c.m.1 M058
T.c.m.3 M058
T.c.m.2 M058
T.c.m.126 M002
T.c.m.137 M095
T.c.m.136 M095
T.c.m.134 M052
T.c.m.141 M050
T.c.m.139 M049
T.c.m.138 M049
T.c.m.135 M052
1
T.numidicus 602+612
T.sp.599+609
T.longicaudatus TLus1
T.longicaudatus 600+610
T.longicaudatus 595+605
T.longicaudatus 601+611
T.c.248 RUSSIA
T.c.246 RUSSIA
T.c.TCau1
T.c.51 AUSTRIA
T.c.603+613 AUSTRIA
T.c.156 UAE
T.c.TCsa2
T.c.253 SERBIA
T.c.TCsa1
T.c.255 HUNGARY
T.c.TChu1
T.c.TChu1
T.c.AB0845
T.c.TCer1
T.c.TCsi2
T.c.29 T043
T.c.TCsi1
T.c.m.169 S
T.c.m.68 S
T.c.m.18 P
T.c.m.54 S
T.c.s.146
T.c.s.64
T.c.s.13
T.c.m.138
T.c.m.126
T.c.m.143
T.c.m.4
T.c.m.144
T.c.m.1
L.arcticus LARic1
L.a.apus LAAau1
L.lemmoni 604+614
L.a lubbocki LALit1
82/70/96
100
T.c.c. 100
69/64/61
98/99/98
T.c. 100
Triops 100
86/79/78
99/94/98
56/64/61
T.c.m. + T.c.s. 90/51/94
94/91/94
64/< 50/60
88/76/90
T.c.s.
0.1

Table 6 Results from the single factor analysis of variance (ANOVA) for morphological characters. The genetic haplotype group was considered as the fixed factor for each analysis (haplotype groups were defined as subsets of sequences sharing diagnostic sites, without consideration of singletons). The northern Spanish population was treated as a separate haplotype group due to clear differences in morphological characters to specimens of the same haplotype but with a different mode of reproduction. *P*-levels shown are corrected values, after application of sequential Bonferroni procedure.

Dependent variable for ANOVA model	d.f.	F	*P*
Telson length ratio	15	116.1	< 0.001
Apodous abdominal segments in females	15	6.2	< 0.001
No. of dorsal carina spines	9	33.2	< 0.001

first a clade consisting of the Moroccan samples, and second, a clade formed of the Portuguese samples and the Spanish samples from Gitanilla. The North African *T. c. simplex* samples cluster as a monophylum in an unresolved trichotomous relationship with these two *T. c. mauritanicus* subclades. The southern Spanish samples of *T. c. mauritanicus* from Extremadura, Sevilla and Huelva group into a third clade that is the sister group of the rest of the lineage.

Morphological re-analyses

All three ANOVA models yielded significant results ($P < 0.001$; Table 6). Thus, a Tukey post-hoc test was used to compute all pairwise comparisons among populations included in each model.

Telson length ratio. The ratio of furcal spine length to the distance between the tip of the furcal spine and the anterior-lateral edge of the telson. High values of this ratio are indicative of long furcal spines. Telson length ratio divides the populations into two significantly different groups (ANOVA, $P < 0.001$; see Appendix 2B; Fig. 5A). It separates all *T. c. mauritanicus* (haplotype group means ranging from 0.38 to 0.50) from the remaining populations, which show much lower values (haplotype group means: 0.21–0.27). Within *T. c. mauritanicus*, specimens of the Moroccan haplotype groups exhibit the longest furcal spines, separating them from the 'Portuguese' (with the single exception of the 'M 056' haplotype group; Appendix 2) and 'Gitanilla' (Extremadura) populations, the latter having the shortest spines in this subspecies. The southern Spanish haplotype has an intermediate position, being clearly separated only from the 'Gitanilla' (and from the 'M 058') haplotype group (Fig. 5A; Appendix 2B). North African *T. c. simplex* (haplotype groups 'Tunisia' and 'T.c.s. M.') and the northern Spanish population previously assigned to this subspecies are almost identical in respect to this character. The intermediate form has a mean value that is even lower than that of North African *T. c. simplex*. The latter is reported to show a frequent tendency to a general weakness in the strength of the armature (Longhurst 1955, 1958; in Notostraca, the armature consists of posterior carapace spines, abdominal spines and telson spines, the latter including the furcal spines).

Dorsal carina spines. Most *T. c. mauritanicus* investigated had numerous dorsal carina spines, often exceeding 50. However, three of the investigated populations of this subspecies included specimens that had less than 20 spines, and counts as low as eight spines were observed, which is less than the 10 reported in the literature as being the maximum value for *T. c. cancriformis* (Table 3). In our samples, most specimens of *T. c. cancriformis* showed 0–4 spines. However, the number of spines ranged from 0 to 30 in the Austrian commercial kit population, although most of these spines were extremely small. This clearly exceeds the maximum value reported hitherto and demonstrates that there is clear overlap in this character among these subspecies. The discrepancy between our spine counts and the number of spines reported in the literature was possibly caused by the fact that other authors did not include the smallest spines in their counts. However, we found these spines to show a size gradient in most specimens, and their classification into 'large' and 'small' spines would be arbitrary. In addition, Moroccan *T. c. mauritanicus* often showed a strong reduction in the size of carina spines, attaining a condition more typical for *T. c. cancriformis* in one

Fig. 4 A, B. The hypotheses of *Triops cancriformis* phylogeny as reflected by our mitochondrial sequence data. —A. The first of two most parsimonious trees (score = 129, CI = 0.8682, RI = 0.9742) based on the large 16S dataset and calculated with PAUP* (version 4.0b10, Swofford 1998; settings gapmode = new, add = cl). Bootstrap support is given above (or to the left of) selected branches calculated with PHYML (nreps = 500; presented in percent)/ML-NJ (PAUP*; nreps = 1000)/MP (PAUP*; gapmode = new, maxtree = 1000, nreps = 1000). The 16S haplotype groups (defined by diagnostic sites, see Appendix 1) are indicated to the right of each clade. —B. The PHYML reconstruction based on the combined 12S and 16S sequences of a selection of samples and calculated with parameters corresponding to the TVM + G model (evaluated by Modeltest 3.06; Posada & Crandall 1998): Base = (0.3553 0.1519 0.1515); Nst = 6; Rmat = (1.4243 9.7012 3.4980 0.0000001 9.7012); Rates = gamma; Shape = 0.2194; Pinvar = 0.0. Bootstrap support is presented in the same manner as in A. In both A and B the scale enables comparison of evolutionary changes with the branch lengths, which are proportional to the evolutionary difference between taxa. Selected nodes are labelled for convenient comparison of the two topologies. Abbreviations and symbols: T.c., *Triops cancriformis*; T.c.c., *T. c. cancriformis* incl. *T. c. simplex* from Girona (northern Spain); T.c.m., *T. c. mauritanicus*; T.c.s., North African *T. c. simplex*.

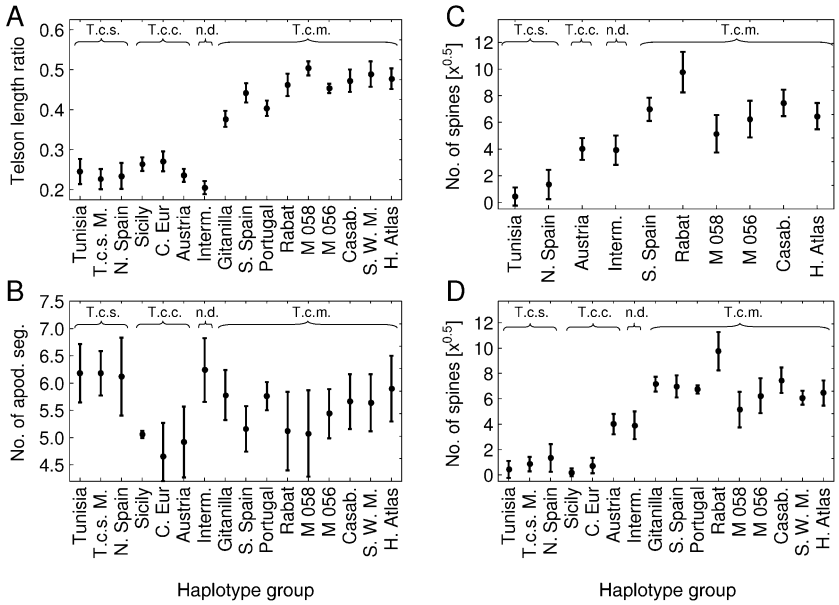

Fig. 5 A–D. Quantification of a selection of the morphological variability of *Triops cancriformis* haplotype groups, whereby these were defined as subgroups of sequences sharing diagnostic sites, without consideration of singletons (the population from Girona, northern Spain, is treated as a separate haplotype group here to investigate a nonconcordance in the present classification of this population as *T. c. simplex* and genetic data, which suggests its affiliation to *T. c. cancriformis*). For details on haplotype groups see Table 1 and Appendix 1. The present nomenclature is indicated in the graphs, above the data points. Note the different scales. Error bars: 95% confidence intervals. —A. The ratio of furcal spine length to total telson length (including the spine). —B. The number of apodous segments in females. —C, D. The square root transformed numbers ($x^{0.5}$) of dorsal carina spines of —C. A subset of haplotype groups used for statistics —D. All haplotype groups included. Abbreviations: T.c.s, *Triops c. simplex*; T.c.c, *T. c. cancriformis*; T.c.m, *T. c. mauritanicus*; n.d., not defined (unusual combination of key morphological characters).

of the populations studied (such a reduction was never observed in Iberian *T. c. mauritanicus* specimens). Thus, care should be taken that the counts of dorsal carina spines will not simply be a function of quality or magnification of the microscope used.

In Tunisia, Sicily and on Ustica Island we found three apparently male-less populations of *T. cancriformis* ('Sicilian' haplotype) that almost completely lack carina spines. Only 5% of these specimens (n = 57) had dorsal carina spines and in one case, the single spine present was almost completely reduced. Most surprisingly, the morphological reinvestigation of northern Spanish material revealed that there is a rather high percentage of specimens with spines in this population (50%, n = 22), although the spines are mostly very small. Spines are arranged at irregular distances, all close to the terminal spine. According to descriptions given in the literature, *T. c. simplex* is diagnosed as lacking these carinal spines completely (Ghigi 1921; Longhurst 1955). In the northern Spanish population, the number of spines was found to range from 0 to 14, which is well within the range we found for typical *T. c. cancriformis*.

We even found specimens with up to seven very small carina spines in a sample of typical *T. c. simplex* from Tunisia, and up to four spines in Moroccan *T. c. simplex*, resulting in mean values not markedly different to that of the northern Spanish population (Fig. 5C,D). The intermediate population from Tunisia was found to have 0–28 carinal spines, a number also within the range we found for typical *T. c. cancriformis*. In this population, spines are arranged in a regular row in front of the terminal spine, which is typical for *T. c. mauritanicus*, but most of the spines are very small, even smaller than the most reduced forms found among Moroccan *T. c. mauritanicus*.

Number of apodous abdominal segments in females. This character clearly separates typical *T. c. cancriformis* from *T. c. simplex*, the intermediate form and the northern Spanish population ($P < 0.05$, Tukey post-hoc test, see Appendix 2A), the latter showing the highest values (Fig. 5B). Most of the *T. c. mauritanicus* haplotype groups have intermediate mean values. There are no clear differences in the number of apodous segments among populations of *T. c. mauritanicus* (Fig. 5B), but in Moroccan haplotype groups, there seems to be a tendency towards a higher number of apodous segments towards haplotype groups found in the south. The values for *T. c. mauritanicus* also almost completely encompass those of the other two subspecies.

Discussion

Identification of haplotype groups

Within *Triops cancriformis* 17 haplotype groups were identified by characterizing diagnostic sites in the 16S sequences [Appendix 1; the haplotype groups 'Central European' (*Triops c. cancriformis*) and 'Northern Spain' (*T. c. simplex*) are genetically identical, but are listed and treated separately in the morphological analyses to quantify phenotypic differences for the assessment of possible taxonomic implications]. The comparison of the 16S sequence of *T. cancriformis* from Japan included in our analyses (Fig. 4, labelled T.c. AB084514) to the one published in Suno-Uchi *et al.* (1997; this sequence was not included in our analyses due to the high amount of indels in otherwise conserved positions) indicates that the Adenin in position 25 (Appendix 1) represents a diagnostic site and Japanese specimen appear to represent a separate haplotype group within *T. c. cancriformis*. The identified 16S haplotype groups are corroborated by 12S sequences (data not shown), which were obtained (or were available in GenBank) for a subset of samples. However, the 16S GenBank sequences AY159576 and AY159577 from specimen collected in Oristano, Sardinia, split to two of our 16S haplotype groups ('Central European' and 'Austrian', respectively). The 12S sequences of these specimen clearly show that both specimen belong to the 'Central European' haplotype group and that the Adenin in position 253 of the 16S sequence AY159577 (Appendix 1; indicative of the 'Austrian' haplotype) is a misleading concurrence, and possibly erroneous. Nevertheless, for the assignment of single *T. c. cancriformis* specimen to one of our haplotype groups, additional 12S data should be consulted. For the single sample from the United Arab Emirates, 12S data confirm its affiliation to the 'Austrian' haplotype group.

The Triops cancriformis cancriformis *lineage*

Our genetic data show that the specimens from Girona (northern Spain) belong to *T. c. cancriformis*, and not to *T. c. simplex* as stated by Margalef (1951, 1953) and Alonso (1985, 1996). Furthermore, they do not form a monophyletic subgroup within *T. c. cancriformis* but are unresolved within the clade (Fig. 4A). However, northern Spanish females do reach similar high values in the number of apodous abdominal segments to females of *T. c. simplex*, and in this character, both differ significantly ($P < 0.05$, Tukey post-hoc Test) from all typical *T. c. cancriformis*, which show much lower values. This is the only morphological character investigated that allows separation of all typical *T. c. cancriformis* from *T. c. simplex*. Thus the northern Spanish population, which is morphologically identical to North African *T. c. simplex*, is morphologically clearly separated from typical *T. c. cancriformis*, revealing it to be a cryptic lineage of this subspecies. Only the presence of dorsal carina spines in this population could have suggested its morphological affiliation to *T. c. cancriformis* (Ghigi 1921; Longhurst 1955; Alonso 1996). However, our results clearly demonstrate that numerous dorsal carina spines may also be found in *T. c. simplex* (see below), which seems to render a morphological separation of both taxa impossible.

The same is also true for reproductive mode. Typical *T. c. cancriformis* are either unisexual or, if males are present, they occur only in low numbers, typically not reaching 30% of the population (see References in Scanabissi *et al.* 2005). All documented cases of southern bisexual populations with equal sex ratio refer to populations of Spain and northern Africa (Scanabissi *et al.* 2005; for more information see also Thiéry 1987 and Machado *et al.* 1999). However, table 2 and fig. 4 in the same paper (Scanabissi *et al.* 2005) indicated the occurrence of a population with equal sex ratio in the Corbières, southern France. We expect this most probably to be a mistake, as Thiéry (1988) only reported one population of *Triops* (*T. c. cancriformis*) in this region, populating the Opoul temporary pool, from which Knoepffler (1978) reported the complete absence of males in a sample of 300 specimens. Thus, we can conclude that typical *T. c. cancriformis* have never been observed to reach an equal sex ratio [a sample of three males and two females of *T. c. cancriformis* reported for Favignana Island, Sicily (Cottarelli & Mura 1995) was too small to give an accurate estimate of this population's sex ratio, and it also appears reasonable to speculate that it was actually a sample of *T. c. simplex*, considering that no *T. cancriformis* populations with equal sex ratio have been reported for the islands of Sardinia and Sicily or for mainland Italy]. There is no consensus about the reproductive mode of these populations, which are either thought to reproduce by parthenogenesis or are regarded as selfing hermaphrodites (reviewed in table 1 in Scanabissi *et al.* 2005). Recent genetic studies (Cesari *et al.* 2004) indicate the occurrence of both hermaphroditic and parthenogenetic populations. Parthenogenesis was suggested for a unisexual population from Oristano, Italy, while hermaphroditism was reported for a population with male occurrence from the Morava floodplain, Austria (Cesari *et al.* 2004). Interestingly, in typical *T. c. cancriformis*, reproductive modes do not appear to correlate to haplotype groups, as populations with males occur at least in three different haplotype groups that also form apparently unisexual populations (M.K. pers. obs.).

The cryptic *T. c. cancriformis* population from Girona (northern Spain) did not show a significant deviation from an equal sex ratio in 37 of 52 samples where adult specimens were present, including those made at both early and late phases of the inundation events (Boix *et al.* 2002). Thus, this population typically has an equal sex ratio. Additionally, single females that were raised separately did lay some eggs in the absence of males, but no larvae hatched (M.K. pers. obs.). Therefore, this is a typical gonochoric population. Thus, the *T. c. cancriformis* lineage clearly shows high plasticity in

reproductive modes, with at least two different modes of reproduction occurring even within a single haplotype. These different reproductive modes may be associated with significant morphological differences, which in turn led to the assignment of populations that belong to the same haplotype to two different subspecies (*T. c. cancriformis* and *T. c. simplex*). A similar pattern has been reported in North American *Triops*: the number of apodous abdominal segments in *T. longicaudatus* was found to be 10.31 (SD = 0.755, n = 53) in females, but only 5.75 (SD = 0.483, n = 60) in unisexuals (see table 5 in Sassaman *et al*. 1997). These findings, together with our data, suggest that in nongonochoric *Triops*, a reduced number of apodous abdominal segments is linked to reproductive mode rather than reflecting the specimens phylogenetic position.

The* Triops cancriformis simplex *group: no separate lineage

The only support for a separate *T. c. simplex* lineage comes from the number of apodous segments in females, a character that appears to be linked to reproductive mode in *Triops* species and is thus of little value for phylogenetic classification of lineages that include nongonochoric populations (see above). The key diagnostic character used hitherto to discriminate the former *T. c. simplex* from other subspecies, i.e. the complete loss of dorsal carina spines, proved to be erroneous: all gonochoric populations from which we were able to investigate a sufficient amount (more than three) of specimens and that were found in the distribution range of the former *T. c. simplex* would have been determined as *T. c. cancriformis* using this character.

Our genetic data demonstrate that 'Spanish *T. c. simplex*' form part of the *T. c. cancriformis* lineage while 'North African *T. c. simplex*' are part of the *T. c. mauritanicus* lineage. This is supported by the obvious geographical isolation of both lineages (Fig. 2). Furthermore, geographical distribution of both lineages suggests that ecological requirements are basically different for these lineages: within Spain, the former *T. c. simplex* appears to be refined to northern localities (Fig. 2), whereas the distribution of African populations of the former *T. c. simplex* suggests that they must be well adapted to desert conditions: they have, for instance, repeatedly been reported in central Algeria (Gauthier 1934; Longhurst 1958).

Thus, we conclude that the *T. c. simplex* lineage as defined in the most recent literature does not exist.

The* Triops mauritanicus *lineage

As currently constituted, *T. c. mauritanicus* is paraphyletic (Fig. 4), because the North African *T. c. simplex* samples nest within it. The southern Spanish samples of *T. c. mauritanicus* form the sister group of the remainder of the lineage.

For the intermediate population from pond 063 (Kairouan) in Tunisia, comparison of genetic and morphological characters suggests that it is a *T. c. simplex* population with one of the key morphological characters inverted. Its classification as *T. c. simplex* is supported by the genetic data, very small telson length ratio (indicative of very short furcal spines), high number of apodous abdominal segments, general appearance, geographical locality, and apparently equal sex ratio (53% males, n = 17). Only the occurrence of numerous dorsal carina spines contradicts this classification, because *T. c. simplex* is diagnosed by complete lack of these spines (Ghigi 1921, 1924; Longhurst 1955). However, Gauthier (1934) described a specimen of *Apus* (= *Triops*) *cancriformis* ssp. *simplex* with five small spines, arranged in a regular row anteriorly, next to the terminal spine. This specimen was one of three females in a sample from Ghardaïa (Mzab, Algeria) that also contained one male. This was the only specimen of 73 from seven localities reported by Gauthier (1934) as having dorsal carina spines.

Our investigation shows that the occurrence of dorsal carina spines in this taxon is much more frequent than previously thought, even though the spines are usually very small. Longhurst (1955) may have accounted for this by calling its carina 'rather smooth'. In our intermediate population, the spines appear to be of similar size to the spines of *T. c. cancriformis* in which similar high numbers of spines have been observed. However, some Moroccan *T. c. mauritanicus* specimens with reduced spines also have a rather similar appearance, although the furcal spines of these Moroccan populations are much larger. Thus, morphologically the population in question would be regarded as a *T. c. cancriformis* population with extremely short furcal spines, and with an unusually high number of apodous segments. Since genetically it is clearly *T. c. simplex*, it could also be regarded as a cryptic lineage.

Taxonomic implications

Our sequence data indicate a clear differentiation of *T. cancriformis* into two main lineages (Fig. 4): *T. c. cancriformis* (including '*T. c. simplex*' from Girona, northern Spain) and *T. c. mauritanicus* (including 'true' *T. c. simplex*).

This result provides new insight into the relationships of the subspecies of *T. cancriformis*. Mantovani *et al*. (2004) found the species to be very homogenous and thus ruled out the possibility of cryptic species within *T. cancriformis*. However, they did not include samples from within the geographical range of the subspecies *T. c. mauritanicus* and *T. c. simplex*. They did include data from Japanese *T. cancriformis*, but these appear to come from a unisexual population (Suno-Uchi *et al*. 1997) and this characteristic should have indicated the specimen to belong to the taxon *T. c. cancriformis* (referring to the data given by Longhurst 1955; on the reproductive mode of subspecies; see also Fig. 4). We therefore conclude that they only examined one subspecies and could therefore not make any conclusions regarding the entire species. Our study also reveals the *T. c. cancriformis* lineage as rather homogeneous. The diversity of haplotypes is low with only four

parsimony-informative sites and five singletons observed (for sequences from only European specimens, i.e. excluding the Japanese sequence: four singletons; if more sequences from Japanese specimen were included the alignment position 25 would be parsimony informative, see section 'Identification of haplotype groups'). In contrast, the *T. c. mauritanicus* lineage is very diverse. The sequences reveal 23 parsimony-informative sites and one singleton. Within the subclade *T. c. simplex* there are already three parsimony-informative sites.

The two main lineages based on *T. c. cancriformis* and *T. c. mauritanicus* are reciprocally monophyletic sister groups (Fig. 4). The two lineages have diverged by an average of 2.9–3.3% in the 16S gene (Table 4a) and 4.1–4.3% in the 12S gene (Table 4c). This clear division of the *T. cancriformis* samples into two main lineages is in strong contrast to the current taxonomy, which classifies *T. cancriformis* into three subspecies of equal rank. Rather, our genetic data support a classification into two main lineages of subspecific or even specific rank, in which the *mauritanicus* lineage also contains the clearly distinguished subclade of North African *simplex* samples. This subclade (including the atypical population from pool 063, Kairouan) is morphologically clearly separated from the remaining *mauritanicus* lineage by the much smaller size of the furcal spines and was described as a separate species by Ghigi (1921, 1924; telson morphology, including furcal spines, was the most important source of characters used by Longhurst 1955 to separate *Triops* species). Its distinct position is also reflected by two autapomorphic substitutions in our 16S rDNA dataset (alignment positions #115: A instead of G, #281: A instead of T; Appendix 1).

We therefore reinstate the *mauritanicus* lineage to full species status as *Triops mauritanicus* Ghigi, 1921, **stat. rev.**, with two subspecies in North Africa. *Triops mauritanicus mauritanicus* (described by Ghigi 1921; from specimens collected in Morocco and now held in the MNHN, Paris) is restricted to western Morocco north of the High Atlas, including the western ridges and mountain slopes of the latter. The Moroccan and Tunisian populations of the former *T. c. simplex* are here treated as *T. m. simplex* Ghigi, 1921, **syn. and stat. nov.**, on the basis of page priority and the Principle of First Reviser (Algerian, Libyan, Egyptian, Sudanese and Arabian populations of the former *T. c. simplex* possibly also belong to this subspecies)**.** The clade *T. m. mauritanicus* (*T. c. mauritanicus* from Morocco in Fig. 4) can be distinguished from the Iberian populations of the former *T. c. mauritanicus* by the strong reduction in size of dorsal carina spines in most populations, the very long furcal spines (Fig. 5A) and the extremely high variability in the number of dorsal carina spines within most populations (Fig. 4D). Its monophyletic status is genetically reflected by two autapomorphic substitutions in our 16S rDNA dataset (alignment positions #377: G instead of A, #422: T instead of C; Appendix 1).

We also include the Portuguese and Spanish populations of the former *T. c. mauritanicus* in *T. mauritanicus*. However, further studies are needed to validate their substructure, position and status, and thus we refrain here from assigning them formal subspecific names. We do not have access to sufficient samples for genetic studies from the Iberian Peninsula to undertake this investigation yet [most samples available in collections for morphology are conserved in (only 70%) denatured alcohol or formalin, which degrades DNA, or are too old]. Although the sister clade formation of the haplotype groups 'Portugal' and 'Gitanilla' appears in most reconstructions (Fig. 4 and other topologies not shown), it may be an artefact of long branch attraction. One reconstruction (PHYML tree of the separate subset of 16S sequences) did show a different clade formation ('Portugal' sister to *T. m. simplex*), indicating the need for further study. Since the divergence among these Iberian samples is of the same magnitude as their differentiation from *T. m. mauritanicus* (Table 5), we expect that further morphological and genetic analyses may differentiate further subspecies in Iberia.

As mentioned above, *T. cancriformis* and *T. mauritanicus* lineages have diverged by an average of 2.9–3.3% in the 16S gene (Table 4a). Unfortunately, no sequence data were available from *Triops newberryi*, the cryptic adelphotaxon of *T. longicaudatus* (Sassaman *et al.* 1997), and no 16S sequences were available from *T. australiensis*. The latter represents the closest relative of the adelphotaxa *T. longicaudatus* and *T. newberryi* and thus the 16S distances among these three taxa are expected to be much lower than the smallest value (6.0%) indicated among recognized *Triops* species in Table 4a. This is in line with the distance range observed with 12S sequences, whereby the value of the distance between *T. longicaudatus* and *T. australiensis* is the lowest (6.1%; Table 4c). These two species are morphologically similar (both species always have a completely reduced second maxilla, which is unique in Notostraca), but are nevertheless recognized by all authors as distinct species. Occurring on different continents, it is not surprising that their divergence is slightly higher than the one observed between *T. cancriformis* and *T. mauritanicus* (4.1%; Table 4c), which both occur in the western Palaearctic.

Further comparative data are available for *Lepidurus* species from North America and Europe for the same DNA fragment as used in the present study. Mantovani *et al.* (2004) indicated that genetic distances between *L. a. apus* and *L. a. lubbocki* are of the same order of magnitude as those observed between American *Lepidurus* species and furthermore do not represent a monophyletic clade (the closest relative of *L. a. apus* is *L. arcticus*, not *L. a. lubbocki*), which is why we perform distance comparisons with the two taxa separately. Among well-recognized taxa of *Lepidurus*, 16S sequence divergences (Table 4b; p-distance) may be as low as 2.8% (between *L. arcticus* and *L. a. apus*; 4.6% in 12S), 3.1% (between *Lepidurus lemmoni*

and *L. arcticus*; 6.4% in 12S) or 3.3% (between *L. lemmoni* and *L. a. apus*; 4.8% in 12S). It should be noted that despite low genetic divergences, two of these comparisons are among morphologically most distinct species. *Lepidurus lemmoni* has much higher numbers of body segments and legs than the other two taxa (e.g. there is no overlap in these characters among *L. lemmoni* and *L. arcticus* referring to Rogers 2001). Thus, the divergence into two taxa within the former *T. cancriformis* by 2.9–3.3% (16S; and 4.1–4.3% 12S) as revealed by our results is similar to that observed among other notostracan species. It is supported morphologically by the formation of extraordinary long furcal spines in the *T. mauritanicus* lineage (with the single exception of the *T. m. simplex* clade), a morphological state unique among Notostraca in a character shown to have low variability within the haplotype groups (Fig. 5A).

In addition, the distance of 2.9–3.3% between the adelphotaxa *T. cancriformis* and *T. mauritanicus* is higher than that observed in two other pairs of crustacean sister species in the genus *Perisesarma* (Sesarmidae; Gillikin & Schubart 2004). We estimate the latter to be 1.5–2.5% from the distance tree presented in their Fig. 3. A second pair of crustacean sister species, *Aegla occidentalis* and *Aegla bahamondei* (Aeglidae), also has lower average divergences of 1.4–1.6% (Jara *et al.* 2003). However, higher sequence divergences can also occur between sister species of Crustacea, e.g. *Euchaeta marina* and *Euchaeta rimana* (Copepoda; Braga *et al.* 1999), which diverged by 5.4% (recalculated from the divergence times presented and the molecular clock used). Therefore, compared to the variability observed among other taxa, the reclassification of the former *T. cancriformis* into two species appears justified.

Validation of morphological characters

As a high number of carina spines can be found in all of the former *T. c. mauritanicus*, including the southern Spanish samples that are the sister group of the clade that includes *T. m. simplex*, this character state appears to be plesiomorphic, at least within the *T. mauritanicus* lineage. Typical specimens of *T. m. simplex* show an autapomorphic complete (or very strong) reduction of carina spines. Thus, for the intermediate population from pond 063 (Kairouan) in Tunisia, this character state seems to have reversed. The haplotype groups of *T. cancriformis* display different degrees of reduction of spines, including complete loss. The same is true for the reduction in the size of these spines within Moroccan *T. m. mauritanicus*. Also, statistics indicate that even population means may lack significant differences among representatives of all three of the former subspecies (see Appendix 2C). This leads to the conclusion that the morphological character 'size and number of carina spines' is very plastic within the *T. cancriformis* group and cannot be used to distinguish between *T. cancriformis* and *T. m. simplex*, and sometimes even between *T. m. mauritanicus*, *T. m. simplex* and *T. cancriformis*. Also, the number of apodous segments may vary greatly, even within the same haplotype, as seen in the examples of northern Spanish and typical *T. cancriformis*. We suggest that telson morphology, including the size of furcal spines, represents the most useful source of morphological characters for determining specimens of the *T. cancriformis* group. However, we do not know of a morphological character that clearly separates *T. cancriformis* and *T. m. simplex*.

***Biogeography of* Triops cancriformis *and* T. mauritanicus**

Dispersal abilities and distribution. To understand present distribution patterns in both species, it is important to take into account the consequences that may arise from different reproductive modes. Nongonochoric modes of reproduction have properties inherently more effective for fast distribution. One resting 'egg' (embryo in diapause) from a parthenogenetic female transported into a new habitat can rapidly form a new population, whereas a minimum of two of these 'eggs', a male and a female (that of course also would have to hatch during the same flooding event), are necessary for a gonochoric population to colonize new habitats, which is much less probable (e.g. Dumont & Negrea 2002). In addition, a gonochoric population increases at a slower rate.

We present the first record of *T. c. cancriformis* in northern Africa (north-west Tunisia), where we found two unisexual populations. The populations belong to two different haplotype groups that also occur in Europe. One of these haplotype groups is shared only with northern Sicily and Ustica Island, the other ranges throughout Western and Central Europe. The discovery of these two European haplotype groups in northern Africa might be evidence for repeated long-distance passive dispersal events across the Mediterranean Sea. The haplotypes have not yet diverged, so they must have reached northern Africa long after the salinity crisis formed a land bridge between the two continents 5.6–5.3 million years ago (Mya) (Blondel & Aronson 1999). Similar evidence comes from the occurrence of 'Central European' haplotypes on Sardinia and Malta. Furthermore, single haplotype groups may have a vast distribution. For example, the 'Central European' haplotype group occurs at least from eastern Spain to Serbia (and from Germany to Tunisia) and populations belonging to the 'Austrian' haplotype group occur in Austria as well as in the United Arab Emirates. This is in accordance with our hypothesis that nongonochoric populations might disperse with a high probability.

For gonochoric populations, we could not find indications of long-distance dispersal in the present investigation. No North African haplotypes of *T. mauritanicus* were found in the Iberian Peninsula and vice versa (Fig. 4 and Appendix 1), indicating that dispersal of this lineage between Europe and Africa may be limited. However, the lack of evidence for passive dispersal across the Strait of Gibraltar in *T. mauritanicus*

could also be due to low establishment success of dispersed haplotypes: in southern Spain, as well as in Morocco, suitable ecological niches may already have been occupied by locally adapted populations of native *T. mauritanicus* subspecies with large resting propagule banks. Thus, new invaders may have to cope with a numerical as well as with a fitness effect, keeping effective gene flow among populations low (Monopolization Hypothesis; De Meester *et al.* 2002). Although effective long-distance dispersal across the sea may be a scarce event in gonochoric populations, there is evidence for its occurrence in the present range of *Triops* species. For example, the distribution of two closely related species, *T. longicaudatus* and *T. australiensis*, on different continents, may only be explained by long-distance dispersal. However, for *T. cancriformis*, we could not find any clear indication of gonochoric populations occurring outside of a rather restricted range in northern Spain (distribution of 'Spanish *T. c. simplex*' in Fig. 2). For this species, we hypothesize that a single nongonochoric lineage descended from a gonochoric ancestor located in Spain (or possibly Western Europe) that possibly formed parthenogenetic as well as androdioecious (with a mating system involving outcrossing and facultative selfing) or hermaphroditic populations. This nongonochoric lineage may then have further diverged into several haplotype groups while spreading eastwards. Evidence for this hypothesis comes from the first genetic data on reproductive mode (Cesari *et al.* 2004), the geographical distribution of sex ratios (Scanabissi *et al.* 2005: Fig. 4, Table 2), the distribution of haplotypes in Europe as well as male occurrence in at least three of the nongonochoric lineages (see above). This scenario, with only nongonochoric and thus easily dispersing populations spreading eastwards, might best explain how this rather recently divergent taxon could expand its range to vast areas of Asia, including India (Tiwari 1952; Longhurst 1955) and Japan (Suno-Uchi *et al.* 1997). Most samples described from India (Tiwari 1952), as well as samples from Japan (Suno-Uchi *et al.* 1997), do not contain males. Further evidence comes from the apparent absence of gonochoric populations from mainland Italy (Scanabissi *et al.* 2005) and Israel (Kuller & Gasith 1996), two areas that should not have been depopulated during the Ice Ages, and thus could have harboured ancient lineages.

Possible triggers for diversification. *Triops cancriformis* and *T. mauritanicus* lineages are indicated to have separated 2.3–8.9 Mya (Table 5). The inferred fluctuations of surface water temperature in the Mediterranean, based on calcareous nanoplankton records (fig. 8 in Veith *et al.* 2003; modified after Müller 1985), suggests that after a long time of constant climatic conditions, two periods of strong climatic oscillations may have occurred in the range of this period. The first occurred during 6.5–5.1 Mya followed by a second one during 3.9–1.5 Mya. We hypothesize that the onset of these fluctuations, 6.5 Mya, was the trigger for the split of the *T. cancriformis* and *T. mauritanicus* lineages. The second phase of strong climatic changes coincides with most splits among clades within *T. mauritanicus* suggesting that these climatic changes might have been the trigger for the divergence of these subspecific lineages, possibly caused by successive range extensions during phases with favourable conditions followed by range fragmentations and geographical isolation of populations during phases with less favourable conditions (e.g. Veith *et al.* 2003). Calculations with different reconstruction algorithms based on separate 16S and 12S sequence datasets, as well as on the combined dataset all result in a polytomy between three of the subclades of *T. mauritanicus* (Fig. 4 and other reconstructions, data not shown). This consistent pattern indicates that the observed polytomy may not be an artefact caused by insufficient data, but rather suggests that a divergence into three different clades may have occurred within a very short time. This appears to have been after the first *T. mauritanicus* lineage (represented by the recent 'Southern Spanish' haplotype group) had evolved from proto-*T. mauritanicus*. The fact that gonochoric populations of both species as well as the 'Southern Spanish' lineage of *T. mauritanicus* all occur within the Iberian Peninsula suggests that the biogeographical origin of both *T. cancriformis* and *T. mauritanicus* may be situated in the Iberian Peninsula. Thus, we hypothesize that a common ancestor of *T. m. mauritanicus*, *T. m. simplex* as well as of the lineages represented by the haplotype groups 'Portugal' and 'Gitanilla' was located in the Iberian Peninsula and may have diverged during a massive range expansion into northern Africa (to both sides of the Atlas Mountains) and other parts of Iberia during a phase of especially favourable conditions. For *Triops*, such conditions might have been a degradation of formerly dense forests. Actually, a drying trend led to the development and expansion of steppe faunal associations around 2.3 Mya (Blondel & Aronson 1999), possibly linked to a phase of unusual cold conditions 2.5 Mya (nanoplankton data). The strong genetic diversification especially within the lineage represented by 'Portuguese' and 'Gitanilla' haplotype groups indicates that the three clades in polytomy evolve faster than their sister clade, the 'Southern Spanish' haplotype group. Molecular data from more populations are needed to corroborate this possible asynchronous evolution pattern and the biogeographical scenario formulated above.

Within *T. cancriformis* the different haplotype groups may have diverged during a third period of strong climatic oscillations that occurred since 0.6 Mya and lasts until present times (as suggested by nanoplankton records, see above). Our genetic data suggest that they have diverged around 1.08–0.26 Mya, indicating that they were most probably in existence well before the last Ice Age. They might thus have formed during repeated range extensions and fragmentations caused by one or several of the preceding glacial cycles.

Patterns of genetic diversity and postglacial recolonization. Within the *T. mauritanicus* lineage, we observed several genetically highly divergent subclades indicating the occurrence of several subspecies (see above and Fig. 2). Our data indicate that diversity is much lower among the haplotype groups of the *T. c. cancriformis* lineage, such that in this species, all populations investigated (i.e. gonochoric as well as nongonochoric populations) appear to have diverged rather recently from a common ancestor. There is no indication of a differentiation into subspecies. The latter species clearly has a more northerly distribution (Fig. 2), which may be linked to its lower genetic diversity (see below).

Our reconstruction of the possible maximum distribution of *Triops* during the Ice Ages suggests that suitable refuges for *T. cancriformis* within Europe may have existed in southern Iberia, Sardinia, Sicily as well as parts of mainland Italy and Greece. The reported distribution of the former subspecies with their differing reproductive modes (see above; Fig. 2) demonstrates that only nongonochoric populations have been found to inhabit areas that had been depopulated during the Ice Ages. Their high dispersal ability must have led to a rather fast recolonization of formerly depleted areas. This may explain the low genetic diversity found among populations inhabiting this area as compared to the high levels of divergence found within North African lineages of *T. mauritanicus.* Populations of this species are expected not to have been depleted by the Ice Ages, enabling ancient polymorphisms to be retained. This pattern of a latitudinal gradient in diversity agrees with that found in other animals (e.g. grasshopper, brown bear and hedgehog, Hewitt 1999, 2000; and a hawkmoth, Hundsdoerfer *et al.* 2005). Parts of southern Europe (and northern Africa) appear to be more diverse in species' haplotypes than Central and northern Europe. When the latter areas were depopulated by the Ice Ages, the former acted as refugia. Central and northern Europe appear to have been recolonized from these southern gene pools by leptokurtic dispersal (leading edge dispersal) resulting in low diversity. Thus, we suppose that the present haplotype groups in *T. cancriformis* might reflect different refuge areas during the last Ice Age. The present distribution of reproductive modes and haplotype groups suggests that there might have been two separate refuges for gonochoric and nongonochoric populations of the 'Central European/Northern Spanish' haplotype group (these are identical, see Appendix 1). Thus, we hypothesize that Spain provided refuges for the gonochoric populations of the 'Central European/Northern Spanish' haplotype group and southern Italy gave refuge to nongonochoric 'Central European' as well as 'Sicilian' haplotype groups. For this study, no material was available from possible refuge areas in Greece or Turkey, and notostracans of both areas have not been well studied yet. Thus, further diversified gonochoric populations might exist in these typical refuge areas. However, the lack of high diversification observed within *T. cancriformis* in the present study (see above) suggests that, if such populations exist, they most likely did not have an important contribution to the recolonization of Central and northern Europe after the last Ice Ages. Also, the single literature report on a southern Greek *T. cancriformis* population rather indicates the presence of a nongonochoric than of a gonochoric population (the sample included seven females and a single male; Colosi 1923). Furthermore, we have to consider that even within these main refuges, most populations may have become extinct due to repeated fast climatic oscillations surely associated with changes in available habitats.

Gonochoric populations often may have failed to follow fast climatic fluctuations and habitat changes since this mode of reproduction has properties inherently less effective for fast passive distribution (see above). They might thus have survived only in a few separated localities, promoting allopatric diversification (Hewitt 1999). This might explain why three highly divergent clades of *T. mauritanicus* could be found within a rather small area in the southern Iberian Peninsula. The lack of diversity among the populations of the 'Southern Spanish' haplotype group compared to the high diversity among Moroccan populations of *T. mauritanicus* may be explained by recent recolonization events of formerly depopulated areas within the Iberian Peninsula. This may suggest that the only suitable refuges for *T. mauritanicus* may have been located in lowland plains at the southernmost edge of the Iberian Peninsula.

Future investigations should include further sources of data, most importantly nuclear gene sequences, and specimens from the Mediterranean islands, the Arabian Peninsula, Algeria, Libya, Egypt and Sudan, as well as more specimens from the Iberian Peninsula, to gain broader insight into the biogeographical scenario and dispersal abilities of *T. mauritanicus*. Also, future studies should investigate the status of southern African material.

Conclusion

Within a group that is as difficult to handle morphologically as the Notostraca as a whole, molecular methods greatly improve the understanding of phylogenetic relationships. In this study, we suggest that the species *Triops cancriformis*, which in the most recent classification comprises three subspecies, actually divides into two distinct species, *T. cancriformis* and *T. mauritanicus*. The latter shows high substructuring and may include at least five subspecies. Thus, the geographical distribution of some lineages of the former *T. cancriformis* is much more restricted than previously thought.

Since we found only one locality for the *T. mauritanicus* haplotype 'Gitanilla' in Spain, in the pond Laguna de la Gitanilla (Extremadura), this haplotype may be highly endangered. This pond deserves formal protection. In south-western

Portugal (Sagres), a part of the known populations of *T. mauritanicus* occur in a natural park, the 'Parque Natural do Sudoeste Alentejano e Costa Vicentina' (PNSACV). This study shows that these populations deserve a higher conservation status within this park, since the population investigated represents a distinct subclade of *T. mauritanicus*. Similarly, the current conservation status of *Triops* in Catalunya (north-eastern Spain) is important due to their unique combination of morphology and reproductive mode within the *T. cancriformis* lineage. The northern Spanish populations may be the only gonochoric representatives of this lineage. We therefore also encourage the protection of their habitats.

High substructuring may also occur in other notostracan taxa. More comparative studies, including both morphological and genetic data, are needed to clarify the phylogenetic relationships among Eurasian and African populations of this group. Such studies could also improve knowledge of passive dispersal abilities, as cryptic taxa may be responsible for the currently assumed huge geographical ranges of some gonochoric notostracan taxa like *T. numidicus* or *Lepidurus couesii*.

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Appendix 1 Diagnostic sites in the alignment of 16S sequences of *Triops cancriformis* for the definition of the haplotype groups considered as fixed factor for the statistical analyses of the morphological data. Haplotype groups were defined as subgroups of sequences sharing diagnostic sites, without consideration of singletons. The population from Girona, northern Spain, was treated as a separate haplotype group ('Northern Spain') to investigate a nonconcordance in the present classification of this population as *T. c. simplex* and genetic data, which suggests its affiliation to *T. c. cancriformis*. For details on haplotype group specimens see Table 1.

	0	0	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4
Haplotype	2	9	0	1	4	7	8	0	1	1	1	4	5	5	6	7	8	8	8	8	9	9	0	1	2	6	7	1	2	3	3	3
group	5	8	5	5	1	9	0	9	1	2	8	4	3	6	5	5	1	4	5	6	0	2	2	9	0	8	7	6	2	1	2	3
Russia	G	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	A	T	T	A	T	T	T	A	A	**T**	C	A	C	A	A	G	C	**C**	**G**	**G**
Austria	G	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	A	T	T	A	T	T	T	A	A	**T**	C	***G***	C	A	A	G	C	**C**	**G**	**G**
Japan	**A**	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	**G**	T	T	A	T	T	T	A	A	**T**	C	***G***	C	A	A	G	C	**C**	**G**	**G**
Hungary	G	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	**G**	T	T	A	T	T	T	A	A	**T**	C	***G***	C	**T**	A	G	C	**C**	**G**	**G**
Sicily	G	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	**G**	T	T	A	T	T	**C**	A	A	**T**	C	***G***	C	A	A	G	C	**C**	**G**	**G**
C. Eur	G	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	**G**	T	T	A	T	T	T	A	A	**T**	C	***G***	C	A	A	G	C	**C**	**G**	**G**
N. Spain	G	T	T	G	**A**	**T**	**G**	**A**	C	T	C	G	**G**	T	T	A	T	T	T	A	A	**T**	C	***G***	C	A	A	G	C	**C**	**G**	**G**
Tunisia	G	T	T	**A**	**A**	**T**	A	G	C	T	C	G	A	T	T	A	**A**	T	T	A	**G**	A	C	A	C	A	A	G	C	A	A	A
Interm.	G	T	T	**A**	G	**T**	A	G	C	T	C	G	A	**C**	T	A	**A**	T	T	A	**G**	A	C	A	C	A	A	G	C	A	A	A
T.c.s. M.	G	T	T	**A**	G	C	A	G	C	T	C	G	A	T	T	A	**A**	T	T	A	**G**	A	C	A	C	A	A	G	C	A	A	A
S. Spain	G	T	T	G	G	C	A	G	C	T	C	G	A	T	T	A	T	T	T	**G**	A	A	C	A	C	A	A	G	C	A	A	A
Gitanilla	G	**G**	T	G	G	**T**	A	G	**T**	**C**	**T**	G	A	T	T	A	T	T	T	A	**G**	A	**T**	A	**T**	A	A	**A**	C	A	A	A
Portugal	G	T	T	G	**A**	C	A	G	C	T	C	**A**	A	T	T	**G**	T	T	T	A	**G**	A	C	**C**	**T**	A	A	G	C	A	A	A
Rabat	G	T	T	G	G	C	A	G	C	T	C	G	A	T	T	A	T	T	T	A	**G**	A	C	A	C	A	**G**	G	**T**	A	A	A
M 058	G	T	T	G	G	C	A	G	C	T	C	G	A	T	**C**	A	T	T	T	A	**G**	A	C	A	C	A	**G**	G	**T**	A	A	A
M 056	G	T	T	G	G	C	A	G	C	T	C	**A**	A	T	T	A	T	T	T	A	**G**	A	C	A	C	A	**G**	G	**T**	A	A	A
Casab.	G	T	T	G	G	**T**	A	G	C	T	C	G	A	T	T	A	T	T	T	A	**G**	A	C	A	C	A	**G**	G	**T**	A	**G**	A
S. W. M.	G	T	T	G	G	C	A	G	C	T	C	G	A	T	T	A	T	**C**	T	A	**G**	A	C	A	C	A	**G**	G	**T**	A	**G**	A
H. Atlas	G	T	**G**	G	G	C	A	G	C	T	C	G	A	T	T	A	T	T	T	A	**G**	A	C	A	C	A	**G**	**A**	**T**	A	A	A

Appendix 2 Results from Tukey post-hoc comparisons of morphological data. —A. Apodous segments —B. Telson length ratio —C. No. of carina spines. Significant results ($P < 0.05$) are given in bold letters. (For details on haplotype specimens see Table 1).

A	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}	{13}	{14}	{15}	{16}
1 Tunisia		1.000	1.000	**0.014**	**0.000**	**0.003**	1.000	0.986	**0.039**	0.978	**0.026**	**0.017**	0.384	0.883	0.850	1.000
2 T.c.s. M.	1.000		1.000	**0.014**	**0.000**	**0.003**	1.000	0.986	**0.039**	0.978	**0.026**	**0.017**	0.384	0.883	0.850	1.000
3 N. Spain	1.000	1.000		**0.026**	**0.000**	**0.006**	1.000	0.997	0.070	0.995	**0.048**	**0.032**	0.530	0.953	0.934	1.000
4 Sicily	**0.014**	**0.014**	**0.026**		0.986	1.000	**0.007**	0.431	1.000	0.480	1.000	1.000	0.991	0.727	0.772	0.195
5 C. Eur	**0.000**	**0.000**	**0.000**	0.986		1.000	**0.000**	**0.014**	0.911	**0.017**	0.953	0.978	0.299	**0.048**	0.058	**0.004**
6 Austria	**0.003**	**0.003**	**0.006**	1.000	1.000		**0.001**	0.167	1.000	0.195	1.000	1.000	0.883	0.384	0.431	0.058
7 Interm.	1.000	1.000	1.000	**0.007**	**0.000**	**0.001**		0.953	**0.021**	0.934	**0.014**	**0.009**	0.261	0.772	0.727	0.997
8 Gitanilla	0.986	0.986	0.997	0.431	**0.014**	0.167	0.953		0.680	1.000	0.580	0.480	0.997	1.000	1.000	1.000
9 S. Spain	**0.039**	**0.039**	0.070	1.000	0.911	1.000	**0.021**	0.680		0.727	1.000	1.000	1.000	0.911	0.934	0.384
10 Portugal	0.978	0.978	0.995	0.480	**0.017**	0.195	0.934	1.000	0.727		0.630	0.530	0.999	1.000	1.000	1.000
11 Rabat	**0.026**	**0.026**	**0.048**	1.000	0.953	1.000	**0.014**	0.580	1.000	0.630		1.000	0.999	0.850	0.883	0.299
12 M 058	**0.017**	**0.017**	**0.032**	1.000	0.978	1.000	**0.009**	0.480	1.000	0.530	1.000		0.995	0.772	0.813	0.226
13 M 056	0.384	0.384	0.530	0.991	0.299	0.883	0.261	0.997	1.000	0.999	0.999	0.995		1.000	1.000	0.953
14 Casab.	0.883	0.883	0.953	0.727	**0.048**	0.384	0.772	1.000	0.911	1.000	0.850	0.772	1.000		1.000	1.000
15 S. W. M.	0.850	0.850	0.934	0.772	0.058	0.431	0.727	1.000	0.934	1.000	0.883	0.813	1.000	1.000		1.000
16 H. Atlas	1.000	1.000	1.000	0.195	**0.004**	0.058	0.997	1.000	0.384	1.000	0.299	0.226	0.953	1.000	1.000	

B	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}	{13}	{14}	{15}	{16}
1 Tunisia		0.997	1.000	0.998	0.947	1.000	0.341	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
2 T.c.s. M.	0.997		1.000	0.480	0.181	1.000	0.991	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
3 N. Spain	1.000	1.000		0.849	0.517	1.000	0.856	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
4 Sicily	0.998	0.480	0.849		1.000	0.902	**0.009**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
5 C. Eur	0.947	0.181	0.517	1.000		0.606	**0.001**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
6 Austria	1.000	1.000	1.000	0.902	0.606		0.790	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
7 Interm.	0.341	0.991	0.856	**0.009**	**0.001**	0.790		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
8 Gitanilla	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**		**0.001**	0.924	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
9 S. Spain	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.001		0.413	0.996	**0.005**	1.000	0.819	0.129	0.592
10 Portugal	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.924	0.413		**0.010**	**0.000**	0.070	**0.001**	**0.000**	**0.000**
11 Rabat	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.996	0.010		0.280	1.000	1.000	0.916	1.000
12 M 058	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.000	**0.005**	**0.000**	0.280		0.064	0.785	1.000	0.936
13 M 056	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.000	1.000	0.070	1.000	0.064		0.997	0.573	0.968
14 Casab.	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.000	0.819	**0.001**	1.000	0.785	0.997		0.999	1.000
15 S. W. M.	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.000	0.129	**0.000**	0.916	1.000	0.573	0.999		1.000
16 H. Atlas	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.000	0.592	**0.000**	1.000	0.936	0.968	1.000	1.000	

C	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}
1 Tunisia		0.951	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
2 N. Spain	0.951		**0.009**	**0.013**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
3 Austria	**0.000**	**0.009**		1.000	**0.002**	**0.000**	0.825	0.054	**0.000**	**0.022**
4 Interm.	**0.000**	**0.013**	1.000		**0.001**	**0.000**	0.755	**0.038**	**0.000**	**0.015**
5 S. Spain	**0.000**	**0.000**	**0.002**	**0.001**		**0.005**	0.219	0.988	1.000	0.999
6 Rabat	**0.000**	**0.000**	**0.000**	**0.000**	**0.005**		**0.000**	**0.000**	**0.043**	**0.000**
7 M 058	**0.000**	**0.000**	0.825	0.755	0.219	**0.000**		0.854	**0.040**	0.673
8 M 056	**0.000**	**0.000**	0.054	**0.038**	0.988	**0.000**	0.854		0.768	1.000
9 Casab.	**0.000**	**0.000**	**0.000**	**0.000**	1.000	**0.043**	**0.040**	0.768		0.915
10 H. Atlas	**0.000**	**0.000**	**0.022**	**0.015**	0.999	**0.000**	0.673	1.000	0.915	