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Evidence for cryptic species in the tadpole shrimp *Triops granarius* (Lucas, 1864) (Crustacea: Notostraca)

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Abstract

We used three ribosomal DNA markers to investigate the genetic divergence of *Triops granarius* (Lucas, 1864) populations from Tunisia, Namibia and Japan. The comparison of the genetic distances between these samples and those found among other species of Notostraca (both *Triops* and *Lepidurus*) strongly suggests that the three *Triops granarius* populations investigated belong to different, possibly cryptic species.

Key words: *Triops numidicus*, *Lepidurus*, molecular phylogeny, 16S, 12S, 28S, ribosomal gene

Introduction

Triops granarius shows one of the largest geographical distributions among all Notostraca. It ranges from Africa through the Middle East to India, China and Japan (Longhurst 1955; Suno-Uchi *et al*. 1997) and extends north as far as Mongolia (Brtek *et al*. 1984) and Transbaikalia (Vekhoff 1993). It is thus not astonishing that the list of synonyms for this species is high, with 19 species group names (Brtek 1997). In addition, morphological variability is higher than in other congeneric species. Among *Triops* species, Longhurst (1955) reported the highest range in the number of segments and in the number of apodous segments in *T. granarius* (see Table 1). This high variability is due to the fact that populations of considerably different morphology were grouped together, such as the former species *Apus granarius* Lucas, 1864 sensu Uéno (1940) and *Apus sinensis* Uéno, 1925, both occurring in Manchuria. Uéno (1940) reported much lower numbers of apodous segments in *A. sinensis*, apparently with no overlap with *A. granarius* or intermediate forms. *A. sinensis* was synonymised with *A. granarius* on evidence of the

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original description, i.e., without reinvestigation of the type material (Longhurst 1955).

A careful reinvestigation of American *Triops* (Sassaman *et al*. 1997) demonstrated that the wide range of 5–12 apodous segments reported for females of *Triops longicaudatus* (LeConte, 1846) by Longhurst (1955) is misleading, as it actually gives the total range of three separate forms that overlap in the number of body and apodous segments. Although these forms may not be separated from each other based on morphology alone, they are reproductively isolated (Sassaman *et al*. 1997). *Triops longicaudatus* sensu Longhurst (1955) was shown to comprise gonochoric (these have a high no. of apodous segments) and unisexual populations (with a low no. of apodous segments) of one species, and androdioecious (i.e. consisting of hermaphrodites and males) as well as unisexual populations (both with an intermediate no. of apodous segments) of a second, cryptic species (Sassaman *et al*. 1997). The variability of the character "number of apodous segments" in gonochoric females of *T. longicaudatus* is thus comparatively low (SD=0.755, N=53, Table 5 in Sassaman *et al*. 1997), suggesting that this character might be of a higher discriminatory power in morphological classifications than suggested by Longhurst (1955). *Triops granarius* is reported to consist entirely of bisexual populations (e.g. Longhurst 1955), and there are no reports of nongonochoric reproduction in this species. This is also true for populations with a low number of apodous segments, for an example Japanese *T. granarius* (Akita 1976). The clear differences in the number of apodous abdominal segments reported for different populations of *T. granarius* may therefore also suggest the presence of cryptic species in *T. granarius*.

TABLE 1. The number of body segments and the number of apodous segments (Total range; amount of difference, Δ, given in parentheses) in *Triops* species reported by Longhurst (1955).

	No. of body segments		No. of apodous segments	
	male	female	Male	female
Triops cancriformis	32–35 (Δ=4)	32–35 (Δ=4)	5–9 (Δ=5)	4–7 (Δ=4)
Triops australiensis	36–44 (Δ=9)	35–43 (Δ=9)	9–13 (Δ=5)	5–12 (Δ=8)
Triops longicaudatus	35–44 (Δ=10)	35–43 (Δ=9)	10–13 (Δ =4)	5–12 (Δ=8)
Triops granarius	32–43 (Δ=12)	32–42 (Δ=11)	6–14 (Δ=9)	4–13 (Δ=10)

Recent molecular investigations of American populations reported not only the occurrence of cryptic species in *Triops* (Sassaman *et al*. 1997), but also in *Lepidurus* (King & Hanner 1998; Rogers 2001). Similarly, Mantovani *et al*. 2004 hypothesized the possible occurrence of a cryptic species in European *Lepidurus* [within *Lepidurus apus* (Linné, 1758)]. In addition, *Triops cancriformis* (Bosc, 1801) [sensu Longhurst (1955)] has been shown to consist of two separate species (Korn *et al*. 2006), giving further evidence that Longhurst´s (1955) revision of the Notostraca may have been too restrictive, rather describing species groups than species.

A reinvestigation of the phylogeny of all species sensu Longhurst (1955) is thus considered necessary. The present work may be regarded as the first step towards a detailed reinvestigation of *T. granarius*. It is the first study to investigate phylogenetic relationships among populations of *T. granarius* from different continents, based on sequence data from mitochondrial and nuclear genes. The aim of the present study was to investigate the possible occurrence of cryptic or just unrecognized species in *T. granarius*.

Note

In the recent literature, two names have been applied to the species of *Triops* under investigation here: *Triops granarius* (Lucas, 1864), and *Triops numidicus* (Grube, 1865). In his review of the Notostraca, Longhurst (1955) synonymised the two species, treating *Apus numidicus* Grube, 1865 as a junior synonym, according to the publication dates. However, Brtek (1997) subsequently suggested that *Apus granarius* Lucas, 1864 was not described before 1886 (Simon 1886) and that the synonymy thus should be inverted, with *T. numidicus* being the valid name for this species. We followed this latter interpretation in our last publication (Korn *et al*. 2006). In our present work, however, we carefully investigated the original descriptions ourselves and came to the conclusion that the name *A. granarius* was made available in a protocol of the short oral species description of Lucas given in a meeting of the "Societé Entomologique de France" in the "Bulletin Entomologique" (later "Bulletin de la Societé Entomologique de France") on 24th February 1864 (Lucas 1864). We thus conclude that *Triops granarius* (Lucas, 1864) is the valid species name under the principle of priority.

Material and methods

The determination key given by Longhurst (1955) was used to determine specimens of *Triops* collected for the present study. We used both wild caught specimens and specimens raised in the laboratory from eggs in sediments. Samples were preserved in ethanol until extraction. For total DNA extraction we followed a modified DTAB procedure (Gustincich *et al*. 1991). Fragments of the 12S and 16S ribosomal genes were amplified using universal primers and PCR programs (details in Murugan *et al*. 2002). The 28S fragment was amplified using the primers 28S_L1 (5'-AGCGGAGGAAAAGAAACTA-3') and 28S_H4lv1 (5'-ACGATCGATTTGCACGTCAG-3'; taken from Sonnenberg et al. in prep.) and a standard PCR program with 30–40 cycles at an annealing temperature of 60°C. 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 products, and if the sequence was not of good quality, the reverse sequence was also obtained. We refrained from sequencing both strands for all samples, since most sequences were of extraordinarily

good quality and we replicated the sequences by analysing 2–3 individuals per locality. The 12S alignment comprised 383 base positions, the 16S alignment of 474 and the 28S alignment of 955 base positions. The sequences have been submitted to GenBank under the accession numbers AM269416-AM269423 (12S); AM269426-AM269433 (16S) and AM269437-AM269444 (28S) and will be made available to the public together with further sequences in a more detailed study of the North African populations of *T. granarius*. Tissue vouchers were deposited in the frozen tissue collection (part MTD-TW) of the "Museum für Tierkunde" of the State Natural History Collections in Dresden (Germany) under the numbers given in Table 2. Vouchers of the whole animals were deposited in the Crustacean collection of the same museum under the accession numbers MTD Crus 2667–MTD Crus 2674. As outgroups for the 28S sequence divergence comparison we included one specimen of *Triops longicaudatus* sensu Longhurst (1955) (*Triops*, INC. commercial kit) and *Triops cancriformis* (commercial kit from Austria) each (vouchers MTD-TW 479 and 51 and accession numbers AM269435 and AM269436 respectively).

TABLE 2. The specimens of *Triops granarius* analysed in this study. The voucher number refers to the tissue voucher in the frozen tissue collection (part MTD-TW) of the "Museum für Tierkunde" of the State Natural History Collections in Dresden, Germany.

Voucher number	Country of origin	Locality
107, 108	Namibia	Aronedis
86–88	Tunisia	Pond 032, Hajib Al´Uyun
41–43	Tunisia	Pond 064, Kairouan

We used 12S, 16S and 28S mitochondrial DNA sequences to compare genetic distances (calculated with PAUP* v. 4.0b10; Swofford 1998) between *Triops granarius* populations from Tunisia and Namibia (localities in Table 2) and *T. granarius* from Japan (for Japanese populations only 12S and 16S sequence data were available). The sequences of the latter were taken from the Genbank or extracted manually from Suno-Uchi *et al*. (1997). All other notostracan 12S and 16S sequences available in Genbank were included in phylogenetic analyses to assess the relationships among them [except for four *Lepidurus arcticus* (Pallas, 1793) 12S sequences, as the relative position of the gene fragment sequenced in these samples does not overlap sufficiently with that of the other taxa so that too many N's would have had to be inserted]. *Lepidurus cryptus* Rogers, 2001 (12S sequences LCAJ0819, LCAJ0825, LCAJ0826 and LCAJ0829 databased with the taxon name *Lepidurus couesii* Packard, 1875 in GenBank) was originally observed (but not described) as a cryptic species within *L. couesii* by King & Hanner (1998), but has since been described in detail as the new species *Lepidurus cryptus* Rogers, 2001 and shown actually to conform to the *Lepidurus packardi* Simon, 1886 morphotype by Rogers

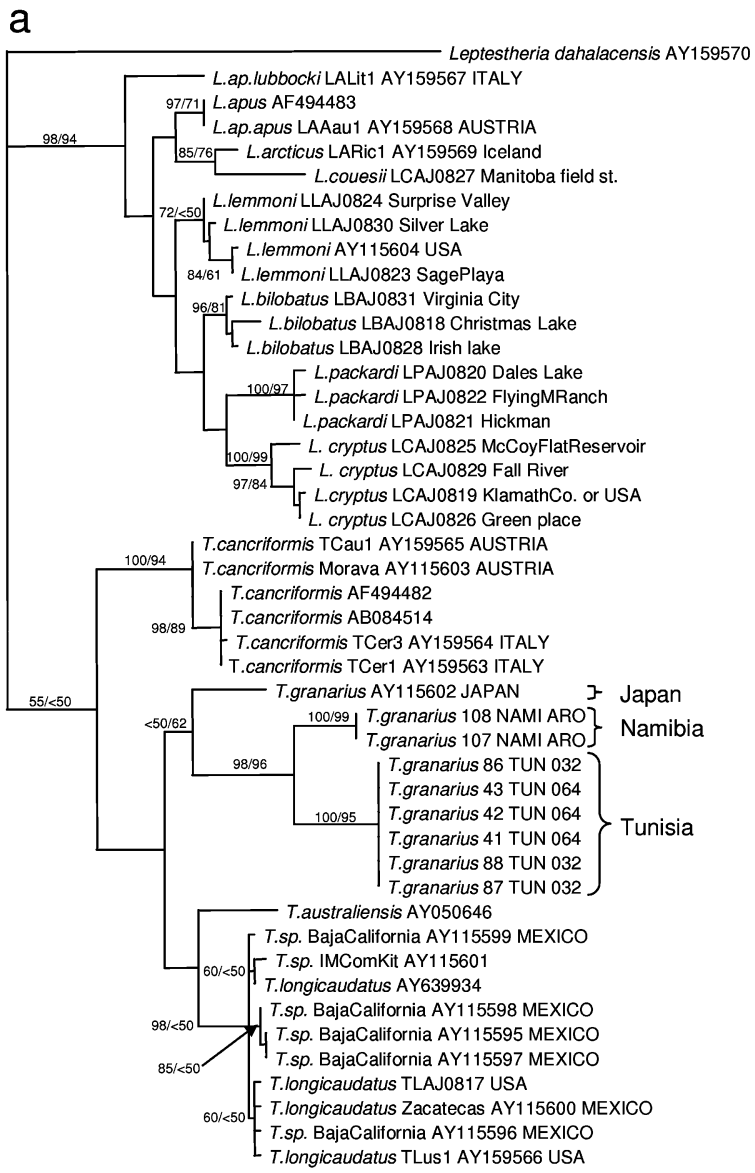
(2001). Sequences of *Leptestheria dahalacensis* (Rüppell, 1837) were included as outgroup in the phylogenetic analyses of the 12S and 16S gene fragments. The accession numbers of the sequences of *L. dahalacensis*, *Lepidurus* and *Triops* that were extracted from Genbank are presented as part of the taxon labels of the trees in Fig. 1. Taxon labels lacking accession or tissue voucher numbers indicate sequences from Suno-Uchi *et al*. (1997). The taxon sampling for the two genetic markers is very different due to the differing availability of sequences. The 16S focuses on the relationships within *Triops*, whereas the 12S provides more information about the relationships within *Lepidurus*.

Phylogenetic analyses were performed with PAUP*. We performed searches for each gene separately with the optimality criterion maximum parsimony (MP; heuristic search; gaps treated as fifth base). Support for nodes was determined by bootstrapping with MP (hs settings nreps=1000, maxtrees=1000) and Neighbour Joining (NJ). The distances for the NJ bootstraps were corrected by maximum likelihood, whereby the models of sequence evolution (12S and 16S: TVM+G) were selected by hierarchical ratio testing with the program Modeltest (v. 3.06; Posada & Crandall 1998).

For a better interpretation of the sequence divergences found within *Triops granarius*, we assessed the overall range of intra-generic sequence divergence (12S and 16S sequences) found among recognized species of Notostraca, i.e., genetic distances were calculated for congeneric species pairs to determine what level of genetic differentiation is indicative of separation on species level. For the purposes of this study, *Lepidurus a. apus* (Linné, 1758) and *L. a. lubbocki* Brauer, 1873 are treated as separate species, because Mantovani *et al*. (2004) indicated that genetic distances among these European taxa are of the same order of magnitude as those observed between recognized *Lepidurus* species from America and furthermore they do not form a monophyletic group (see also Tab. 3 and Fig. 1).

Results and discussion

Two reconstructions of the phylogenetic relationships within the Notostraca are presented in Fig. 1. Fig. 1a represents one of the most parsimonious trees (phylogram) found in the analysis based on the 12S data. Fig. 1b is based on the 16S data. Both trees indicate that the genera *Triops* and *Lepidurus* are monophyletic, although bootstrap support for the monophyly of *Triops* based on 12S sequences (Fig. 1a) is very low. Indeed, the monophyly of *Triops* has been questioned by several authors (e.g. Murugan *et al*. 2002 and Mantovani *et al*. 2004), but this problem is not the focus of the present paper. The phylogenetic analyses of both the 12S (Fig. 1a) and 16S (Fig. 1b) sequence data indicate three well supported clades among the populations of the species *Triops granarius*. The 16S data (Fig. 1b) even implies that the species is not monophyletic, but rather represents two separate lineages.



to be continued (Fig. 1)

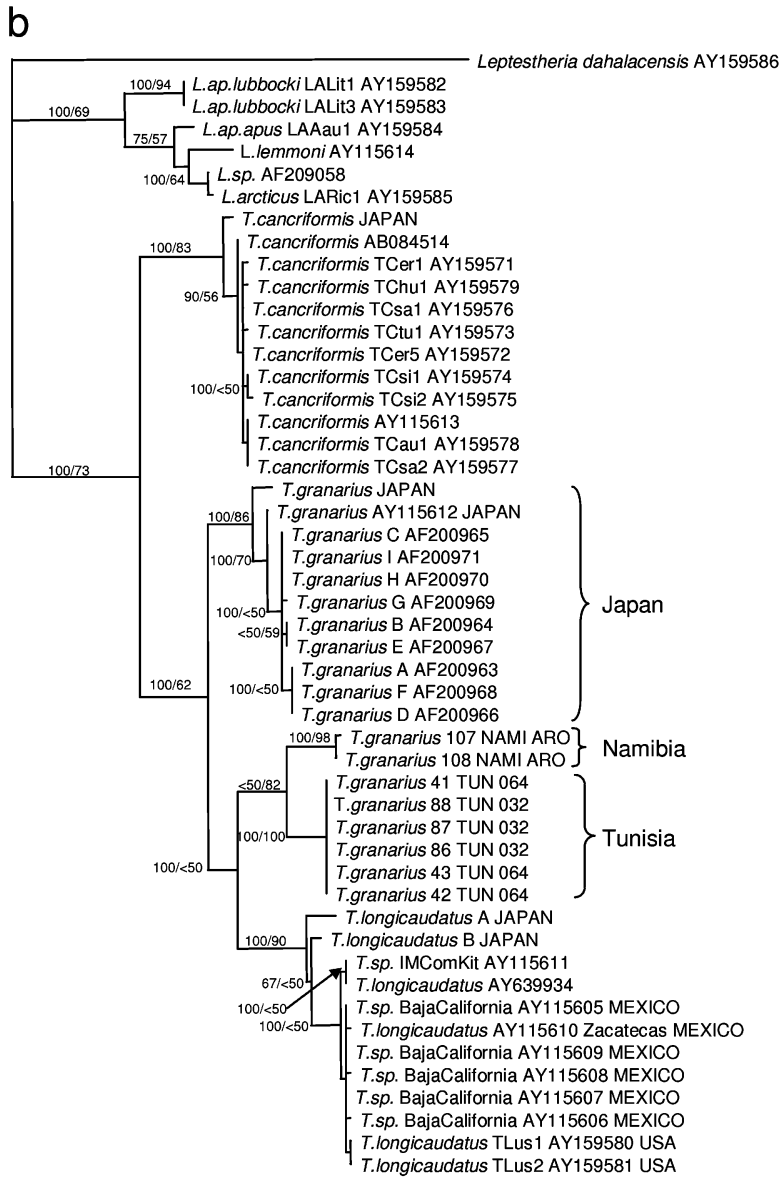


FIGURE 1. The phylogenetic relationships within the Notostraca as reconstructed from (a) 12S (one of 12 MP trees; tree length=350, CI=0.6200, RI=0.8811) and (b) 16S (one of 4 MP trees; tree length=321, CI=0.7539, RI=0.9345) gene sequences with maximum parsimony (heuristic search with the settings gapmode=new, add=cl). Branch lengths are proportional to genetic divergence. Numbers above (or to the left of) branches indicate bootstrap support calculated by maximum parsimony (settings as above and with maxtrees=1000, nreps=1000) to the left and (for an additional comparison) calculated by NJ with ML-distances (nreps=1000) to the right of the "/".

Table 3 (a–d) shows the magnitude of genetic distances within *T. granarius* compared to interspecific distances between other congeneric species of both *Triops* and *Lepidurus*. For the 12S gene, genetic distances among species pairs range from 5.9 to 15.5% in *Triops* and from 4.5 to 12.4% in *Lepidurus.* For the 16S gene, the range in *Lepidurus* is from 2.6 to 5.9% (Table 3c) and up to 9.0% in *Triops*. Unfortunately, no sequence data were available for *Triops newberryi* (Packard, 1871) (assuming that the species names in the Genbank are correct), the sister species of *T. longicaudatus* (LeConte, 1846) (Sassamann *et al.* 1997), and no 16S sequences were available for *Triops australiensis* (Spencer & Hall, 1896). The latter represents the closest relative of the sister species *T. longicaudatus* and *T. newberryi* and thus the 16S distances among these three taxa are expected to be much lower than the values indicated in Table 3d. The high divergence of *T. longicaudatus* from Japan to the new world samples indicated by the 16S sequence data (Fig. 1b) deserves further study. Based on an interpretation of the Phylogenetic Species Concept favoring a strong breakdown into species, Murugan et al. (2002) suggested the existence of six phylogenetic species within seven of the American populations studied. Following an interpretation that allows for intraspecific variation (as does one of the most important traditional species concepts, the morphospecies concept), the data suggest that all samples they analysed may simply belong to a single species (compare to Fig. 1a). We found profound genetic divergence within *T. granarius*: the genetic distances between *T. granarius* from Japan and Africa are 11.0% in the 12S gene and 6.3% in the 16S gene, and between *T. granarius* from Tunisia and Namibia, we found genetic distances of 7.0% in the 12S gene and 3.8% in the 16S gene. Thus, in contrast to the slight genetic variation found by Murugan et al. (2002) within new world *Triops* (12S: max 1.6%; 16S: max 0.4%), the divergence we detected within *T. granarius* is of the same magnitude as genetic distances found between recognized species of Notostraca.

Table 3e shows the genetic divergence of *Triops* species and the three African *T. granarius* populations studied, based on nuclear sequences (28S rDNA). The values are much lower than those observed in the mitochondrial DNA, but nuclear ribosomal genes are known to evolve more slowly than mitochondrial ribosomal genes (Hillis and Dixon 1991). The value observed between the specimens of *T. granarius* from Namibia and Tunisia (0.7%) was lower than those observed between *T. cancriformis*, *T. longicaudatus*, and *T. granarius* in pairwise comparisons (3.1–5.4%). This value (0.7%) is, however, not much lower than those observed between other species of Branchiopoda in this gene fragment: two recognized species of the genus *Parartemia* (*Parartemia contracta* Linder, 1941 AF209042 and *Parartemia longicaudata* Linder, 1941 AF209043; Anostraca) have also only diverged by 0.9%. Although this value is slightly higher than that between the two African *T. granarius* populations studied in this paper, these two *Parartemia* species are not sister species (Remigio *et al.* 2002), indicating that values between recognized sister species are probably lower. Furthermore, the different ecology of these crustaceans should be taken into account: *Parartemia* are saltwater and *T. granarius* freshwater crustaceans. It has been shown for Australian anostracans and daphniids that levels of

divergence in lineages from saline inland waters were substantially greater than levels of divergence in lineages occurring in freshwaters (Hebert *et al*. 2002; Remigio *et al*. 2001). This pattern may also hold for Notostraca (the populations we studied inhabit freshwaters). Several arguments thus suggest that the divergence of 0.7% between the two African *T. granarius* populations lies within the range typical for separation on species level.

We conclude that our results indicate that *Triops granarius* comprises at least three separate cryptic species.

TABLE 3. Genetic (p-) distances (in percent) between pairs of species, broken down by gene. Mean values are presented except for those marked with *, which indicate single values due to single comparisons of two sequences (i.e. no mean can be calculated). Due to differences in availability of the gene sequences of the species not all comparative values could be calculated for both genes. *Lepidurus a. apus* and *L. a. lubbocki* were treated as separate species (for details, see Materials and Methods section). a. 12S *Lepidurus*, b. 12S *Triops* (A.=Africa, N.=Namibia, Tu.=Tunisia, J.=Japan), c. 16S *Lepidurus* (the sequence from *L. sp.* AF209058 was excluded from comparisons), d. 16S *Triops* e. 28S *Triops*.

a	*L.a.apus*	*L.a.lubbocki*	*L.arcticus*	*L.bilobatus*	*L.couesii*	*L.cryptus*	*L.lemmoni*
L.a lubbocki	6.6	-	-	-	-	-	-
L.arcticus	4.5	8.8*	-	-	-	-	-
L.bilobatus	6.4	9.7	8.2	-	-	-	-
L.couesii	8.0	10.7*	6.1*	9.4	-	-	-
L.cryptus	8.0	11.2	9.3	6.4	12.4	-	-
L.lemmoni	4.7	7.9	6.4	4.9	8.9	6.7	-
L.packardi	6.7	9.7	6.7	6.8	9.6	7.8	6.7

b	*T. austr.*	*T. cancrif.*	*T. gran.* A.	*T. gran.* N.	*T. gran.* Tu.	*T. gran.* J.
T. cancrif.	11.8	-	-	-	-	-
T. gran. A.	10.9	15.3	-	-	-	-
T. gran. N.	10.3	15.5	n.a.	-	-	-
T. gran. Tu.	11.0	15.2	n.a.	7.0	-	-
T. gran. J.	8.2	12.2	11.0	10.3	11.3	-
T. longic.	5.9	10.5	11.4	11.6	10.9	8.4

c	*L.a.apus*	*L.a.lubbocki*	*L.arcticus*
L.a.lubbocki	5.2	-	-
L.arcticus	2.6*	5.6	-
L.lemmoni	3.3*	5.9	3.1*

to be continued.

TABLE 3 (continued).

d	*T. cancrif.*	*T. gran.* A.	*T. gran.* N.	*T. gran.* Tu.	*T. gran.* J.
T. gran. A.	8.5	-	-	-	-
T. gran. N.	8.6	n.a.	-	-	-
T. gran. Tu.	8.5	n.a.	3.8	-	-
T. gran. J.	8.7	6.3	6.0	6.3	-
T. longic.	9.0	6.9	7.4	6.8	6.2

e	*T. cancrif.*	*T. gran.* A.	*T. gran.* N.	*T. gran.* Tu.
T. gran. A.	5.2	-	-	-
T. gran. N.	5.4	n.a.	-	-
T. gran. Tu.	5.2	n.a.	0.7	-
T. longic.	3.6*	3.2	3.3	3.1

Notostracan species often do not show clear morphological differentiation which, for an example, has resulted in the classification of *Lepidurus packardi* and *L. couesii* as two subspecies of *L. apus* by Longhurst (1955). Small but consistent morphological differences suggest a possible differentiation at the species level. Thus, literature data give numerous indications on the possible occurrence of further distinct species in *T. granarius* even within rather small geographic areas. For example, Thiéry (1996) reported two different morphotypes within the Arabian Peninsular. Similar results have been reported for Moroccan (Thiéry 1987), Indian (Tiwari 1951), Southern African (Barnard 1929) and Northern Chinese (Uéno 1940) populations; for Southern Africa, India and Northern China, several species of *Triops* have even been described on the basis of minor morphological differences. These were later regarded as synonyms of *T. granarius* by Longhurst (1955). Future surveys should therefore examine genetic distances among populations of *T. granarius* on a fine geographical scale within its total range and compare them to morphological characters and ecological traits, to investigate the potential number of cryptic or presently unrecognized species within this taxon.

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