Molecular taxonomy and phylogeny of the 'living fossil' lineages *Triops* and *Lepidurus* (Branchiopoda: Notostraca)

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European *Triops cancriformis* and *Lepidurus apus* were analysed for 12S and 16S mitochondrial genes and compared to North American and Japanese taxa. There are no cryptic species among European *T. cancriformis* populations, which are highly homogeneous in comparison to conspecific Japanese samples. *T. cancriformis* differs from congeneric taxa all over its range, which can be explained by its antiquity. In contrast, the parapatric subspecies *L. apus apus* and *L. apus lubbocki* are morphologically conserved and differ substantially at the mtDNA level. The genetic distance values between them are of the same order of magnitude as those observed between American *Lepidurus* species. Their subspecific status therefore requires further analysis. *L. apus apus* is more closely related to a *L. arcticus* sample from Iceland than to *L. apus lubbocki*. It is also related to a Canadian *L. couesii* population. Further analyses of populations from the whole range of *L. arcticus* and the European range of *L. couesii* are needed to understand the relationships among these notostracan taxa. When considering the two genera, it is clear that *Lepidurus* is a well supported monophyletic unit, while *Triops* is polyphyletic, embodying very divergent taxa.

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Introduction

Notostraca is an ancient order of branchiopod crustaceans dating from the Cambrian. It represents a challenging group from both evolutionary and ecological aspects. First, its two genera, *Triops* Schrank, 1803 and *Lepidurus* Leach, 1816, constitute a well-known example of 'living fossils', i.e. lineages surviving 'over a long period of time with minimal morphological change' as a result of 'unusual morphological conservatism' (Fisher 1990). This nearly 200 Myr stasis in gross morphology contrasts with extensive variation in many individual characters and reproductive biology. This leads to taxonomic and phylogenetic difficulties at the species and subspecies levels. Taxa have frequently been synonymized and redescribed (Linder 1952; Longhurst 1955; Lynch 1966, 1970). While the presence of a supra-anal plate in *Lepidurus* and its absence in *Triops* allows the immediate distinction of the two genera, a rudimentary plate can be found in some specimens of *T. cancriformis* (Bosc, 1801) (Longhurst 1955).

Triops and *Lepidurus* are present in all continents except Antarctica (Longhurst 1955; Brtek & Thiéry 1995). This world-wide distribution is due to their antiquity, but possibly also to their passive transport: geographical barriers are more effective for nonpassively distributed animals. From an ecological point of view, notostracans, like most branchiopods, are restricted to temporary pools (Kerfoot & Lynch 1987). The ephemerality of these extreme habitats may have selected for the development of resistant stages (dried eggs or cysts) and some unusual reproductive strategies. Despite histological and lab-rearing analyses (Sassaman 1991; Tommasini *et al*. 1989; Tommasini & Scanabissi 1992; Scanabissi & Mondini 2002a,b), the reproductive mechanisms of many notostracans still remain to be clarified. Furthermore, these studies demonstrated that a taxon may show quite different reproductive strategies. For example, *T. cancriformis* comprises bisexual populations — with an equal male : female sex ratio or with a female bias — and unisexual populations, either hermaphroditic or parthenogenetic. *T. granarius* (Lucas, 1864) is a bisexual and outbreeding taxon, but the sex ratio in its populations ranges from equal to a female or a male bias. *T. newberryi* (Packard, 1871) includes bisexual populations embodying males at low frequencies and self-compatible hermaphrodites (Sassaman 1991; Sassaman *et al*. 1997). A survey of the reproductive features of Italian *Lepidurus apus lubbocki* Brauer, 1873 showed that, despite the existence of male and female individuals in the population, male gametogenesis is abortive.

The role of these non-functional males remains to be elucidated (Scanabissi & Mondini 2002b). Obviously, these peculiar reproductive strategies have a bearing on population genetic structure and therefore on taxa definition.

Only recently have biochemical and molecular approaches made their appearance, making for a deeper understanding of notostracan systematics. At the generic level, the analysis of the mitochondrial 12S gene in two genera of each of the four branchiopod orders demonstrated that *Triops* and *Lepidurus* are less differentiated than other compared genera (Hanner & Fugate 1997). No other comparisons between the two notostracan genera have been performed.

In *Triops*, Sassaman *et al*. (1997) demonstrated by allozyme analyses that North-American *T. longicaudatus* Le Conte, 1846 *sensu* Linder (1952) and *T. newberryi* are genetically distinct and reproductively isolated. 12S and 16S mitochondrial gene sequencing in Mexican *Triops* populations indicates that in this area the nominal species *T. longicaudatus* is a mixture of several species. In the same paper, the analysis of an Austrian *T. cancriformis* sample suggests that this taxon may represent a group independent from the other species in the genus (Murugan *et al*. 2002). Analyses of 929 bp of the mitochondrial 16S gene in Japanese populations of *T. granarius*, *T. longicaudatus* and *T. cancriformis* indicated a higher affinity between the first and second taxa than between the first and third (Suno-Uchi *et al*. 1997).

In North-American *Lepidurus*, a study of 330 bp of the mitochondrial gene 12S and data from nine allozyme loci indicated five genetically divergent clades among the populations chosen to represent the four nominal species *L. couesii* Packard, 1875 (two distinct clades), *L. bilobatus* Packard, 1877, *L. lemmoni* Holmes, 1894 and *L. packardi* Simon, 1886 (King & Hanner 1998).

Given the obvious interest in organisms which diverged at least 200 Mya and the absence of molecular data on European taxa, the aims of this paper are to verify the taxonomic status and level of differentiation of *T. cancriformis* and the level of divergence between the two formally described European subspecies, *L. apus apus* (L.) and *L. apus lubbocki* (Longhurst 1955). In all, five subspecies have been reported, but the morphological differences among them are such that unambiguous recognition of single individuals is impossible (Fryer 1988).

As a final goal, having analysed 12S and 16S genes, we also try to relate all mitochondrial data available on the two genera, keeping in mind that, owing to maternal inheritance, this molecular approach is particularly relevant to organisms with contrasting types of sexual reproduction.

Materials and Methods

DNA was extracted either from single alcohol-preserved or frozen individuals (field caught or taken from laboratory cultures), or from cysts. Sample pertinent information is given in Table 1. At least two individuals from each collecting site were analysed.

For single adults we used the method of Preiss *et al*. (1988), while for cysts we followed Moorad *et al*. (1997). PCR amplification was performed in 50 µL reactions using the Invitrogen PCR kit with recombinant *Taq* DNA polymerase. Thermal cycling was done in a GeneAmp PCR System 2400 (Applied Biosystems) programmable cyclic reactor. Thirty cycles were scheduled as follows: denaturation at 94 °C for 30 s, annealing at 46 °C for 30 s, extension at 72 °C for 30 s. The amplified products were purified with the Nucleospin kit (Macherey-Nagel) and both strands were directly sequenced with the DNA sequencing kit (Dye terminator cycle sequencing, Applied Biosystems) in an ABI PRISM 310 Genetic Analyser. The primers for PCR amplification and sequencing were mtD-35 = SR-J-14233/mtD-36 = SR-N-14588 and mtD32 = LR-J-12887/mtD34 = LR-N-13398 obtained from the Biotechnology Laboratory (NAPS), Vancouver, University of British Columbia.

Alignments performed with the Clustal algorithm of the SEQUENCE NAVIGATOR program (ver 1.0.1, Applied Biosystems) were also checked by sight.

The nucleotide sequences of the analysed specimens have been entered into GenBank under A.C. AY159563–9 (12S) and AY159571–85 (16S). One individual *Leptestheria dahalacensis* (Rüppel, 1837) has also been analysed (12S, AY159570; 16S, AY159586) and utilized as outgroup.

Phylogenetic analyses followed standard methods. Neighbor-Joining (NJ) was performed using MEGA 2 (Kumar *et al*. 2001), Maximum Parsimony (MP) and Maximum Likelihood (ML) with PAUP (ver. 4.0, Swofford 2001). Bootstrap analysis involved 1000, 500 and 100 replicates, respectively. For ML, Modeltest (ver. 3.06; Posada & Crandall 1998) was run to determine the best substitution model (TVM + G for both genes) with the evaluation of base frequencies, R-matrix, proportion of invariable sites and value of gamma shape parameters. Bayesian analysis of the data was performed with MRBAYES 1.1 (Huelsenbeck 2000) setting the ML parameters as follows: 'lset nst = 6', 'rates = invgamma' and 'basefreq = estimate'. 500 000 generations were run, with trees being sampled every 100 generations for a total of 5000 trees in the initial sample. Examination of ML score variation indicated that 'stationarity' had occurred by the 1000th tree; therefore, the first 1000 trees were discarded and the posterior probability of the phylogeny and its branches were determined on the remaining 4000 trees.

For appropriate comparisons, sequences from GenBank were utilized (Table 2). We also used the 16S sequences of Suno-Uchi *et al*. (1997; in which they are published in the 3′ vs. 5′ direction).

Table 1 Collecting sites, acronyms and haplotypes of *Triops* and *Lepidurus* specimens analysed for 12S and 16S genes (ND: haplotype not determined).

Species	Collecting site	Country	Acronym	Haplotype 12S	Haplotype 16S
T. cancriformis					
	Jolanda di Savoia, Ferrara	Italy (Emilia-Romagna)	TCer1	H1	H1
			TCer2	H1	ND
			TCer3	H2	ND
			TCer4	H1	ND
			TCer5	H1	H2
			TCer6	ND	H1
	Principina a Mare, Grosseto	Italy (Tuscany)	TCtu1	H1	H3
			TCtu2	H1	H3
	Baglio Cofano, Trapani	Italy (Sicily)	TCsi1	H1	H4
			TCsi2	H1	H5
	Oristano	Italy (Sardinia)	TCsa1	H1	H6
			TCsa2	H1	H7
	Wien (laboratory hatched)	Austria	TCau1	H3	H8
			TCau2	H3	H8
	Apaj	Hungary	TChu1	H1	H9
			TChu2	H1	H9
	Volgograd	Russia	TCru1	H3	ND
			TCru2	H3	ND
T. longicaudatus					
	Fresno	USA	TLus1	H4	H10
			TLus2	H4	H11
L. apus apus					
	Markthof	Austria	LAAau1	H6	H14
			LAAau2	H6	H14
L. apus lubbocki					
	Castel Porziano, Roma	Italy (Lazio)	LALit1	H5	H12
			LALit2	H5	H12
			LALit3	H5	H13
L. arcticus					
	Thjorsarver	Iceland	LARic1	H7	H15
			LARic2	H7	H15

Results

We sequenced 347–348 bp of the 12S gene and 464–509 bp of the 16S gene. The first tract corresponds to the *T. longicaudatus* 12S gene (AN: AJ000817, King & Hanner 1998) with an extension of 19–21 bp at the 5′ end in comparison to the sequences drawn from GenBank. The 16S tract starts at position 12169 of the complete mitochondrial genome of *A. franciscana* Kellogg, 1906 (AN: NC_001620).

The 12S gene of *T. cancriformis* shows only three haplotypes (Table 1). Among these, haplotype 1 is found in all Italian locations and in the Hungarian sample. TCer3 shows a haplotype characterized by a point mutation. A more differentiated haplotype is the one scored in the Austrian and Russian samples: it differs from the others in five and six substitutions, respectively. The two analysed specimens of *T. longicaudatus* show the same haplotype, H4 (Table 3), which differs from *T. cancriformis* in 35–36 mutations.

In *Lepidurus*, sample-specific haplotypes have been observed, with the *L. apus apus* sequence showing almost the same number of substitutions (20–21) (Tables 1, 3) in comparison to *L. apus lubbocki* and *L. arcticus* (Pallas, 1793).

When the two genera are compared, 43–49 substitutions differentiate *Triops* and *Lepidurus* haplotypes (Table 3).

Nine haplotypes were scored in *T. cancriformis* for the 16S sequence (Table 1). While haplotypes H1, H2, H3 and H6 differ for single indels, the remaining sequences show 1–4 substitutions; 39–47 substitutions diagnose haplotypes H10 and H11 found in the two analysed specimens of *T. longicaudatus* (Table 4).

In *Lepidurus*, sample-specific haplotypes have been again observed; in this case, though, the *L. apus apus* sequence shows only 12 substitutions with respect to the *L. arcticus* haplotype and as many as 26 mutations with respect to *L. apus lubbocki* (Table 4). The haplotypes of the two genera are distinguished by 50–62 mutations. Kimura-2 parameter distances show the same ranges and trends with respect to the total number of substitutions (Tables 3, 4).

When all available sequences (Tables 1, 2) are taken into account and the transition-transversion ratio is plotted

Table 2 16S and 12S sequence source (from the literature or GenBank) and geographical origin of the *Triops* and *Lepidurus* taxa utilized for comparison.

Species	Reference — GenBank A.N.	Country	Acronym 16S	Acronym 12S
Triops cancriformis				
	Suno-Uchi *et al.*, 1997	Japan	*Tcancriformis*JAP1	
Triops longicaudatus				
	Suno-Uchi *et al.*, 1997	Japan	*Tlongicaudatus*JAP1	
	Suno-Uchi *et al.*, 1997	Japan	*Tlongicaudatus*JAP2	
	AJ000817	USA		*Tlongicaudatus*USA1
Triops granarius				
	AF200963	Japan	*Tgranarius*JAP1	
	AF200964	Japan	*Tgranarius*JAP2	
	AF200965	Japan	*Tgranarius*JAP3	
	AF200968	Japan	*Tgranarius*JAP4	
	AF200971	Japan	*Tgranarius*JAP5	
	Suno-Uchi *et al.*, 1997	Japan	*Tgranarius*JAP6	
Triops australiensis				
	AY050646	Australia	*Taustraliensis*AUS1	
Lepidurus bilobatus				
	AJ000831	USA		*Lbilobatus*USA1
	AJ000828	USA		*Lbilobatus*USA2
	AJ000818	USA		*Lbilobatus*USA3
Lepidurus lemmoni				
	AJ000830	USA		*Llemmoni*USA1
	AJ000824	USA		*Llemmoni*USA2
	AJ000823	USA		*Llemmoni*USA3
Lepidurus couesii				
	AJ000829	USA		*Lcouesii*USA1
	AJ000826	USA		*Lcouesii*USA2
	AJ000825	USA		*Lcouesii*USA3
	AJ000819	USA		*Lcouesii*USA4
	AJ000827	Canada		*Lcouesii*CAN1
Lepidurus packardi				
	AJ000822	USA		*Lpackardi*USA1
	AJ000821	USA		*Lpackardi*USA2
	AJ000820	USA		*Lpackardi*USA3

Table 3 Kimura-2-parameter distances (above diagonal) and number of substitutions (below diagonal) between haplotypes scored for the 12S gene (for haplotype numbers see Table 1).

	H1	H2	H3	H4	H5	H6	H7
H1		0.0029	0.0146	0.1094	0.1531	0.1416	0.1488
H2	1		0.0175	0.1128	0.1568	0.1452	0.1524
H3	5	6		0.1094	0.1495	0.1381	0.1452
H4	35	36	35		0.1593	0.1513	0.1549
H5	47	48	46	49		0.0606	0.0928
H6	44	45	43	47	20		0.0635
H7	46	47	45	48	30	21	

against the total number of substitutions (Fig. 1), a clear trend towards an increase in the total number of mutations from intraspecific to intergeneric comparisons emerges. Transversions prevail in transitions in intergeneric comparisons, while in the remainder, transitions are higher than transversions in all instances for the 12S gene. The same does not apply to the 16S haplotypes: in 10 intra- and interspecific comparisons only transversions are scored.

Dendrogram evaluations first involved all sequences given in Tables 1 and 2 (available from the authors). A complete overlapping topology was obtained with newly sequenced haplotypes and only a subsampling of sequences available from GenBank or from the literature (Figs 2, 3). MP and NJ dendrograms for 12S sequences (Fig. 2A) show a highly supported dichotomy between *Triops* and *Lepidurus* haplotypes. *Triops cancriformis* sequences appear well differentiated from the cluster embodying both *T. australiensis* and the American *T. longicaudatus* samples. On the other hand, the *Lepidurus* group shows a polytomy within which the poorly supported cluster (54% bootstrap value) between *L. apus* subspecies and the affinity of the presently analysed *L. arcticus* samples with *Lcouesii*CAN1 are to be noted. Topology and probability of the Bayesian strict consensus tree (not shown) completely overlap the MP and NJ ones. In the ML dendrogram (Fig. 2B), neither the monophyly of *Triops*, nor the relationships between *L. apus apus* and *L. apus lubbocki* are supported.

Table 4 Kimura-2-parameter distances (above diagonal) and number of substitutions (below diagonal) between haplotypes scored for the 16S gene (for haplotype numbers see Table 1).

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15
H1		0.0000	0.0000	0.0020	0.0041	0.0000	0.0021	0.0041	0.0020	0.0941	0.0964	0.1355	0.1354	0.1302	0.1226
H2	0		0.0000	0.0022	0.0043	0.0000	0.0022	0.0022	0.0022	0.0900	0.0900	0.1336	0.1333	0.1252	0.1175
H3	0	0		0.0020	0.0041	0.0000	0.0021	0.0041	0.0020	0.0964	0.0987	0.1355	0.1351	0.1302	0.1226
H4	1	1	1		0.0020	0.0020	0.0042	0.0060	0.0040	0.0956	0.0979	0.1377	0.1328	0.1260	0.1207
H5	2	2	2	1		0.0040	0.0063	0.0080	0.0059	0.0979	0.1001	0.1402	0.1352	0.1283	0.1231
H6	0	0	0	1	2		0.0021	0.0040	0.0020	0.0933	0.0956	0.1351	0.1304	0.1260	0.1184
H7	1	1	1	2	3	1		0.0021	0.0042	0.0935	0.0935	0.1357	0.1354	0.1304	0.1226
H8	2	1	2	3	4	2	1		0.0060	0.0941	0.0964	0.1355	0.1316	0.1271	0.1194
H9	1	1	1	2	3	1	2	3		0.0956	0.0978	0.1327	0.1281	0.1236	0.1161
H10	43	39	44	45	46	44	42	44	45		0.0020	0.1133	0.1171	0.1224	0.1147
H11	44	39	45	46	47	45	42	45	46	1		0.1157	0.1194	0.1247	0.1171
H12	59	55	59	60	61	59	58	59	58	50	51		0.0042	0.0564	0.0586
H13	60	56	60	61	62	60	59	60	59	54	55	2		0.0536	0.0555
H14	58	53	58	58	59	58	57	58	57	56	57	26	26		0.0242
H15	55	50	55	56	57	55	54	55	54	53	54	27	27	12	

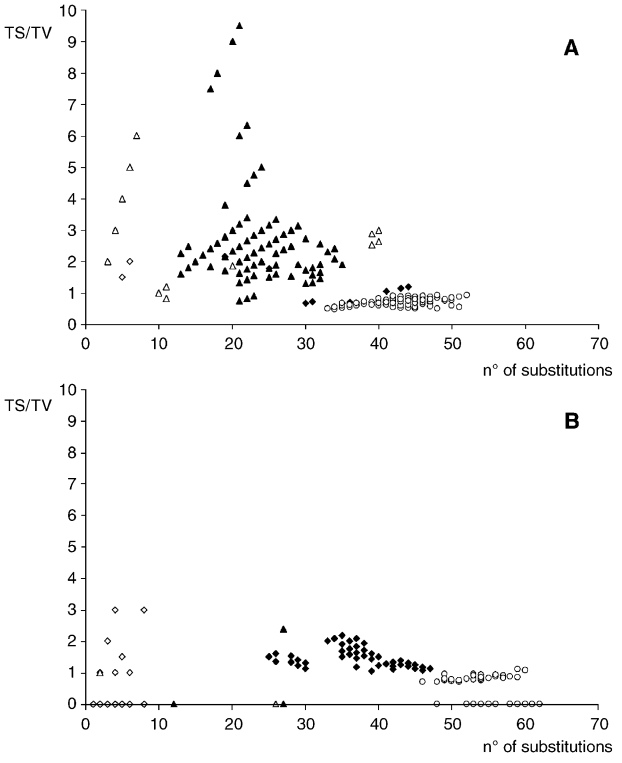

Fig. 1 Plot of transition-transversion ratio to the total number of substitutions for all available 12S (A) and 16S (B) sequences. Comparisons are as follows: (◇) within *Triops* species; (◆) between *Triops* species; (△) within *Lepidurus* species; (▲) between *Lepidurus* species; (○) between *Triops* and *Lepidurus* species.

The analysis of the 16S gene shows a picture comparable that of the ML tree on 12S gene. The MP and NJ dendrograms (Fig. 3A) agree, with high bootstrap values, in showing three well differentiated clusters for *Triops*: the first one comprises the European *T. cancriformis* haplotypes plus the conspecific Japanese sample; the second group clusters the Japanese *T. granarius* haplotypes; the third cluster is given by the two american *T. longicaudatus* haplotypes scored in the present analyses plus the conspecific Japanese sample. The *Lepidurus* group (given the absence of *Lcouesii*CAN1) shows the clustering of *L. apus apus* with the *L. arcticus* sequences. In the ML evaluation (Fig. 3B) and in the Bayesian strict consensus tree (not shown), the terminal branching pattern is maintained, although deep branch topology is modified, owing to the clustering of *T. longicaudatus* and *T. granarius* with *Lepidurus* samples.

Discussion

Italian, Austrian, Hungarian and Russian samples of *T. cancriformis* are highly homogeneous and close to conspecific Japanese samples. This seems to rule out the possibility of the existence of cryptic species within this taxon. The result should not be due to a failure of the genes utilized, since the 12S gene analysis of King & Hanner (1998) provided evidence of the presence of a cryptic species within *L. couesii* and the same molecular markers allowed the identification of at least six phylogenetic species within Mexican *T. longicaudatus* (Murugan *et al*. 2002). Furthermore, *T. cancriformis* appears highly differentiated from congeneric taxa over its whole range: in most dendrograms, *T. cancriformis* haplotypes never cluster with congeneric taxa. Further analyses are needed based on larger samples, but present data support the hypothesis that *T. cancriformis* could represent a third genus within the Notostraca (Linder 1952; Murugan *et al*. 2002). This differentiation possibly relates to its antiquity: the fossil *Triops* has been described as *T. cancriformis minor* (Trusheim 1938).

Taking the Fresno samples as reference, it emerges that both Japanese and American *T. longicaudatus* belong to the

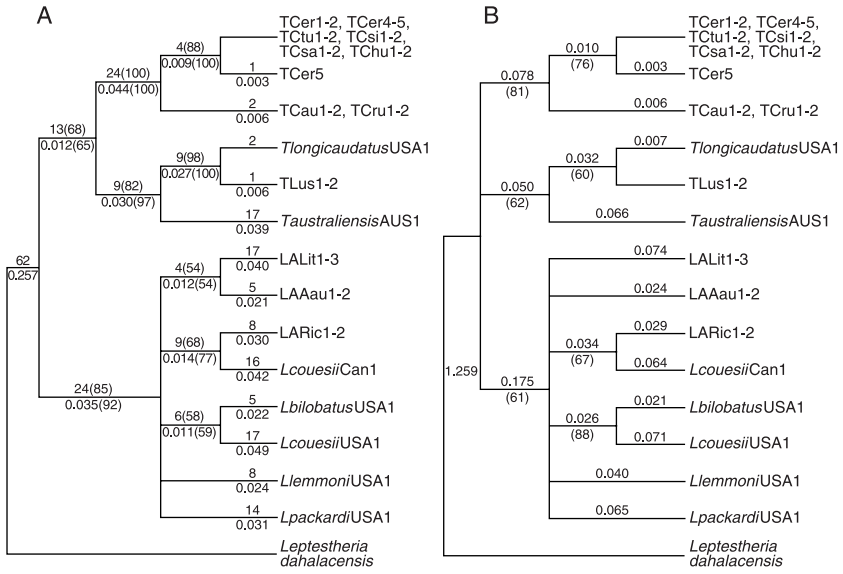

Fig. 2 A, B. Neighbor-Joining combined with —A. MP (consistency index: 0.725, homoplasy index: 0.275) and —B. ML (–Ln likelihood = 1363.623) trees obtained from 12S sequences. Acronyms as listed in Tables 1 and 2. Values in bold type indicate mutational steps (above branches) or distance values (below branches) in A and substitutions/site (above branches) in B. Numbers in parentheses represent bootstrap percentages.

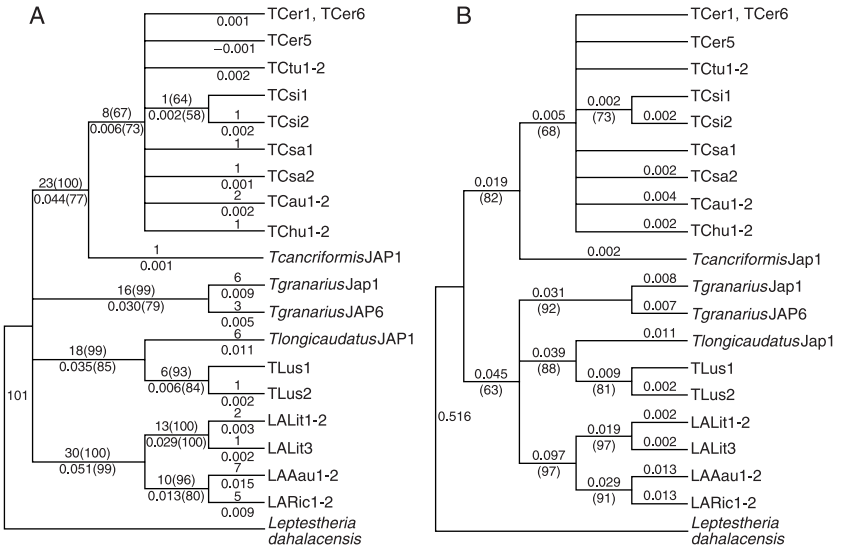

Fig. 3 A, B. Neighbor-Joining combined with —A. MP (consistency index: 0.794, homoplasy index: 0.206) and —B. ML (–Ln likelihood = 1812.401) trees obtained from 16S sequences. Acronyms as listed in Tables 1 and 2. Values in bold type indicate mutational steps (above branches) or distance values (below branches) in A and substitutions/site (above branches) in B. Numbers in parentheses represent bootstrap percentages.

same taxonomic entity which, on the whole, appears well diversified from other *Triops* species, and is possibly slightly more closely related to *T. granarius* (16S) or *T. australiensis* (12S) than to *T. cancriformis*. Owing to the very complex situation found in North-American *Triops* (Sassaman *et al.* 1997; Murugan *et al.* 2002), these results further support the divergence of *T. cancriformis*.

As for *Lepidurus*, the mitochondrial haplotypes of *L. apus apus* and *L. apus lubbocki* are very divergent: they cluster together only in the 12S MP, NJ and Bayesian trees. However, the poor bootstrap values support the clear differentiation and high level of divergence seen in all other dendrograms. Whether this differentiation occurs at the subspecific or specific level will be ascertained by further analyses. Yet, for both genes, the magnitude of all distance parameters between these two entities is clearly comparable to that observed between other *Lepidurus* species surveyed here and to the divergence among populations of different specific clades scored in King & Hanner (1998). It should be remembered that *L. apus lubbocki* inhabits temporary ponds in Italy, while *L. apus apus* is found throughout the rest of Europe (Brtek & Thiéry 1995). Bird migratory routes should explain these clearly distinct ranges, which correspond to genetically highly differentiated lineages in sharp contrast

with morphological character conservation. Further, albeit indirect, support at the specific level of differentiation between *L. apus lubbocki* and *L. apus apus* derives from the observation that our topology closely agrees with that scored in King & Hanner's (1998) dendrograms on American *Lepidurus* for 12S. In addition, our elaboration, regardless of the different number of taxa taken into account, demonstrates an equal differentiation of *L. lemmoni* and *L. packardi*, a higher affinity of *L. bilobatus* with American *L. couesii* and a consistent differentiation of the Canadian sample of *L. couesii*. This taxon, already indicated as a cryptic species by King & Hanner (1998), is in our dendrogram more closely related to *L. arcticus* in Iceland than to other nearctic *Lepidurus* taxa. On the other hand, in the 16S tree, where only taxa that have been sequenced for this work are considered, *L. arcticus* clusters with the Austrian *L. apus apus*, even if quite differentiated from it. Further sampling covering the whole range of *L. arcticus* and the European range of *L. couesii* (Brtek & Thiéry 1995) is required to highlight their natural relationships. These are of particular interest, especially for the circumpolar arctic taxon, the only notostracan living in permanent water bodies.

Owing to the antiquity of their divergence, the picture emerging from the present analysis for the two notostracan genera is quite unexpected. *Triops* and *Lepidurus* haplotypes behave as belonging to distinct genera only in MP-NJ and Bayesian trees of 12S gene (Fig. 2A; not shown). Elsewhere, *Triops* haplotypes show a basal polytomy (Figs 2B, 3A) or even cluster with those of *Lepidurus* (Fig. 3B). On the whole, while *Lepidurus* appears a well defined and monophyletic entity, *Triops* does not completely embody private mitochondrial lineages. The possibility that this is due simply to convergent homoplasies requires further analysis.

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