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# Molecular characterization of the tadpole shrimp *Triops* (Branchiopoda: Notostraca) from the Baja California Peninsula, México: New insights on species diversity and phylogeny of the genus

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**Abstract**

Using sequence analyses of fragments of the small and large subunits of mitochondrial genes 12S and 16S rRNA, we studied the molecular identity of five *Triops* populations from the Baja California Peninsula, México. Additionally, we explored the phylogeny of the genus by comparing with sequence data from gonochoric *T. longicaudatus* (Zacatecas, México), commercial *Triops* kit (U.S.A.), *T. 'granarius'* (Japan), *T. cancriformis* (Austria), *T. australiensis* (Australia) and *Lepidurus lemmoni* (U.S.A.). The 16S fragment was not useful to discriminate the American *Triops* forms because their sequences were more than 99% similar. Molecular and phylogenetic analyses using the 12S gene fragments, in agreement with previous allozyme studies, indicate that the nominal (morphological) species *T. longicaudatus* is a mixture of several species such that, of the seven *Triops* American populations studied, six phylogenetic species can be identified and two morphologically and reproductively highly divergent forms can be grouped into a single monophyletic clade. The molecular data, rather than supporting our previous proposal that the phylogenetic relationships of *Triops* species could be deduced by similarities in the number of total and legless rings, suggest that *T. cancriformis* may represent an independent group from the rest of the species in that genus. In spite of detectable differences among American populations, our analyses indicate these represent a single monophyletic group when compared to *Triops* from outside of the New World.

## Introduction

Features like the occurrence of single-copy genes and maternal inheritance that excludes recombination make mitochondrial DNA (mtDNA) a suitable genetic marker (Avise et al., 1987). In branchiopods, the mtDNA is a molecule of less than 16 kilobases (kb). It is 15.333 kb long in the cladoceran *Daphnia pulex* (Crease, 1999) and 15.822 kb in the brine shrimp *Artemia franciscana* (Perez et al., 1994). The small and large subunits of mitochondrial ribosome RNA (rRNA) genes have been used to explore the systematics of the 'living fossil' Notostraca, which has gained attention in the last few years (Sassaman et al., 1997; Suno-Uchi et al., 1997; King & Hanner, 1998; Maeda-Martínez et al., 2000a). Suno-Uchi et al. (1997) investigated the *Triops* species from Japan using the 16S rRNA gene, and the *Lepidurus* species from North America were studied by King & Hanner (1998) using the 12S rRNA gene.

The study of Mexican notostracans started with Packard's description of two species of *Triops* using materials from México and the U.S.A. (Packard, 1871). *Triops lucasanus* (cited as *Apus lucasanus*) was described from a female labeled "Cape St. Lucas, John Xanthus, No. 4.", and from six males labeled "Kansas, No. 5". *Triops aequalis* (cited as *Apus aequalis*) was described from two males labeled "Matamoras, México, General Coach.", 13 females labeled 'Matamoras, General Coach' and "Kansas, No. 5", and one female labeled "Plains of Rocky Mts., No. 390" (Packard, 1871). Later, Packard (1883) mentioned that the material used to redescribe *T. lucasanus* (cited as *Apus lucasanus*) consisted of several

males from Museum of Chicago Academy of Sciences labeled "Cape St. Lucas, Xanthus, 4". However, this record of males from the Baja California Peninsula is most probably a mistake (see Maeda-Martínez et al., 2002). In 1895, Richard reported a new record of *T. aequalis* (cited as *A. aequalis*) from Isla Espíritu Santo (Baja California Sur). Eight decades after Packard's descriptions, Linder (1952) and Longhurst (1955) placed *T. lucasanus* and *T. aequalis* as junior synonyms of *Triops longicaudatus* (LeConte, 1846). In fact, Linder (1952) and Longhurst (1955) proposed that all *Triops* populations from the American continent were *T. longicaudatus*, and thus, very different forms of *Triops* were grouped in a single morphological species.

Maeda-Martínez (1991) recorded *Triops* sp. (cited as *Triops longicaudatus*) from arid and semiarid regions of the Mexican states of Baja California Sur, Coahuila, Chihuahua, Distrito Federal, Durango, Estado de México, Guanajuato, Hidalgo, Nuevo León, San Luis Potosí, and Zacatecas. Dodson & Silva-Briano (1996) found *Triops* sp. (cited as *Triops longicaudatus*) in the state of Aguascalientes, and Maeda-Martínez et al. (1997) reported *Triops* sp. and *Lepidurus lemmoni* Holmes from the state of Baja California (Norte). Recently, Maeda-Martínez et al. (2002) concluded that *Triops* sp. is known from 124 different localities distributed in 17 Mexican states, including new state records from Jalisco, Oaxaca, and Sonora.

According to the reproduction models of Sassaman (1989, 1991, 1995) and Sassaman & Weeks (1993), two main types of species may occur in the Notostraca: gonochoric species and androdioecious species. The gonochoric species are composed of males and obligate out-crossing females while the androdioecious species are composed of males, amphigenic hermaphrodites, and monogenic hermaphrodites. Two additional types of species may occur, the 'unisexual' species, composed only of selfing monogenic hermaphrodites, and the parthenogenetic species (Sassaman, pers. com.) (Maeda-Martínez et al., 2000a). From electrophoretic analyses made on populations from the United States, Sassaman et al. (1997) argued that the nominal species *Triops longicaudatus sensu* Linder (1952) and Longhurst (1955), is a mixture of at least two species, the androdioecious *T. 'newberryi'* (Packard, 1871), and the controversial form *T. "longicaudatus"*. The latter form is controversial because it was defined as a species composed of gonochoric (long-bodied individuals), androdioecious (long-bodied individuals), and 'unisexual' (short-bodied hermaphrodites) populations. Because of the morphological differences and uniparental reproduction, Sassaman et al. (1997) concluded that the 'unisexual' short-bodied hermaphrodites seem to be on an independent evolutionary pathway. However, given the similarity in basic genetic characteristics these authors decided to keep these short-bodied shrimps, which correspond to the nominal species *T. oryzaphagus* Rosenberg, 1947, as individuals of *T. "longicaudatus"* until further genetic studies on this form across the populations become available. Sassaman et al. (1997) reported rearing short-bodied female *T. "longicaudatus"* from Ejido Héroes de la Independencia, and female *T. "newberryi"* from near Bahía de los Ángeles, both sites located in the Mexican state of Baja California (Norte).

Sassaman et al. (1997) demonstrated the utility of the number of both the total body rings and the legless rings to identify different genetic entities of *Triops*. Using these features, we performed a meristic analysis of material collected from the Baja California Peninsula and found two short-bodied *Triops* populations with males, which represented the first record of short-bodied males from the American continent (Maeda-Martínez et al., 2000a). According to the model of Sassaman (1991), the sex ratios observed (5 males: 34 females, and 1 male: 49 females) suggest that these populations are androdioecious. In a previous paper (Maeda-Martínez et al., 2000a), we proposed that the short-bodied hermaphrodite *T. "longicaudatus"* studied by Sassaman et al. (1997), and the short-bodied androdioecious population from the Baja California Peninsula could together form a separate androdioecious species, *T. "oryzaphagus"*. Thus, we presumed that in North America there could be at least two androdioecious species (*T. "newberryi"* and *T. "oryzaphagus"*), and one gonochoric species (long-bodied *T. "longicaudatus"*). A fourth species could be represented by the androdioecious *T. "longicaudatus"* population from Arizona reported by Sassaman et al. (1997).

Based on morphological characteristics, Linder (1952) and Longhurst (1955) recognized only about 11 species of Notostraca from a group of more than 70 nominal species. However, the biochemical and molecular studies of Sassaman et al. (1997) and King & Hanner (1998) have demonstrated that Linder's and Longhurst's classifications, which caused not only misunderstandings of the species diversity in the genus *Triops* but also probably provoked a lack of

interest in studying the phylogeny and the historical biogeography of the Notostraca (Maeda-Martínez et al., 2000a), can no longer be accepted. Based on the genetic studies of Sassaman et al. (1997) and King & Hanner (1998), we (Maeda-Martínez et al., 2000a) suggested that the phylogenetic relationships among the species of *Triops* could be deduced from similarities in the number of both total body rings and legless rings. Our hypothesis was that several monophyletic species groups could be represented by the cluster formed by the short-bodied species *T. cancriformis* of Europe and *T. "oryzaphagus"* of North America, and by the cluster formed by the long-bodied species *T. australiensis* (Spencer & Hall, 1896) from Australia, *T. namaquensis* (Richters, 1886) and *T. numidicus* (Grube, 1865) from South Africa, and *T. "longicaudatus"* from North America. To test this hypothesis and define the species diversity of the genus, a research project is in process. As a part of this project, we studied the molecular identity of five *Triops* populations from the Baja California Peninsula through sequence analyses of both 12S and 16S rRNA mtDNA genes. The phylogeny of the genus was also explored by comparing to mtDNA sequences from specimens of the gonochoric *T. longicaudatus* (México), commercial *Triops* kit (U.S.A.), *T. "granarius"* (Japan), *T. cancriformis* (Austria), *Lepidurus lemmoni* Holmes, 1894 (U.S.A.), and *Triops australiensis* (Australia) (GenBank accession number AY050646).

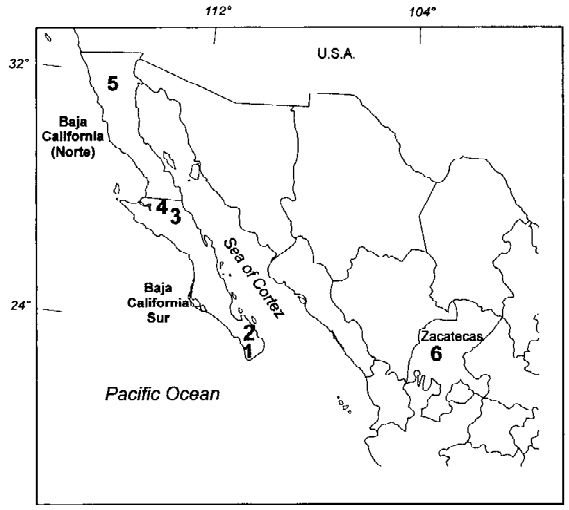


*Figure 1.* Proximate locations of the Mexican *Triops* populations used in the mtDNA characterization. 1, *Triops* sp., km 64.0, federal highway No.1, Cabo San Lucas-La Paz, Baja California Sur; 2, *Triops* sp., km 76.5, federal highway No. 1, Todos Santos-Cabo San Lucas, Baja California Sur; 3, *Triops* sp. km 0.5 W Vizcaíno, federal highway No. 1, Vizcaíno-Guerrero Negro, Baja California Sur; 4, *Triops* sp. km 28.9 S El Arco, Baja California Sur; 5, *Triops* sp. km 79.3, federal highway No. 3, Ensenada-San Felipe, Baja California (Norte); 6, *T. longicaudatus* km 27.5, federal highway No. 49, Fresnillo-Cuencamé, Zacatecas.

## Materials and methods

Considering the biochemical and molecular studies of Sassaman et al. (1997) and King & Hanner (1998) in which they demonstrated that Linder's and Longhurst's classifications can no longer be accepted, we suggest that, unless careful morphological and genetical studies are available, the temporary designation of *Triops* sp. be used. We identify one gonochoric long-bodied form from Zacatecas state as *T. longicaudatus* following Sassaman et al.'s (1997) morphological characterization for gonochoric long-bodied forms in the U.S.A.. The origin and number of notostracans analyzed are indicated in Table 1. The localities of the Mexican materials are shown in Figure 1. All analyses were made on adult specimens. Specimens from México and from the commercial kit (U.S.A.) were cultured in separate outdoor tanks and fixed in 100% ethanol. Specimens of *T. "granarius"* (Japan), *T. cancriformis* (Austria), and *Lepidurus lemmoni* (U.S.A.) were supplied by Dr. M. Grygier, Dr. S. Richter and Dr. J. King. The exoskeleton of the animals was carefully removed and only abdominal and-or thoracic muscular tissue of the body trunk was used for the analysis. Total DNA (tDNA) was isolated either by the CTAB (Hexadecyltrimethylammonium bromide) method (Doyle & Doyle, 1987) or by using a Puregene kit. The tDNA was spectrophotometrically quantified and fragments of small and large subunits of mitochondrial genes (12S and 16S rRNA mtDNA) were amplified using the primers 5′-ATG CAC TTT CCA GTA CAT CTA C and 5′-AAA TCG TGC CAG CCG TCG C (Colbourne et al., 1996), and 16Sar 5′ –CGC CTG TTT ATC AAA AAC AT and 16Sbr 5′ –CCG GTC TGA ACT CAG ATC ACG T (Palumbi et al., 1991). The PCR mixture (50 $\mu$l) consisted of 40 or 60 ng of tDNA as template, 5 $\mu$l of 10X PCR buffer, 100 $\mu$m dNTP, 100 pMol primers, 1 unit of RTS *Taq* DNA polymerase. Cycling conditions were a preliminary denaturation of DNA at 95 °C for 5 min; 40 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s), extension (72 °C for 1 min); and a final extension for 5 min at 72 °C. PCR products were purified using either QIAquick PCR purification kit or the standard Geneclean protocol described in Hillis

*Table 1.* Origin and number (*n*) of notostracans used for mtDNA analyses

| Species | *n* | Locality |
|---|---|---|
| *Triops* sp.'km 76.5' | 4 | km 76.5, federal highway No. 1, Todos Santos-Cabo San Lucas, Baja California Sur, 23° 14′ N, 110° 09′ W. |
| *Triops* sp. 'km 64.0' | 7 | km 64.0, federal highway No.1, Cabo San Lucas-La Paz, Baja California Sur, 23° 20′ 52″ N, 109° 45′ 35″ W. |
| *Triops* sp. Vizcaíno | 5 | km 0.5 W Vizcaíno, federal highway No. 1, Vizcaíno-Guerrero Negro, Baja California Sur, 27° 39′ 33″ N, 113° 23′ 23″ W. |
| *Triops* sp. El Arco | 2 | km 28.9 S El Arco, Baja California Sur, 27° 57′ 39″ N, 113°30′ 27″ W. |
| *Triops* sp. 'km 79.3' | 5 | km 79.3, federal highway No. 3, Ensenada-San Felipe, Baja California (Norte), 31° 51′ 08″ N, 116° 05′ 31″ W. |
| *T. longicaudatus* | 2 | km 27.5, federal highway No. 49, Fresnillo-Cuencamé, Zacatecas, 23° 32′ 46″ N, 102° 57′ 34″ W. |
| *Triops* sp."comm. kit" | 1 | Commercial kit: Triops Inc., Pensacola, Florida, U.S.A.. The Original batch of eggs is presumably from Utah, U.S.A. (E. Hull, pers. comm.). |
| *T. "granarius"* | 2 | Mitsukeyama 2-Chôme 8 Ibaraki, Osaka, Japan |
| *T. cancriformis* | 1 | Morava flood plains, Austria |
| *L. lemmoni* | 1 | Sage Playa, Edwards AFB, Kern, California, U.S.A. |

et al. (1996). From the purified PCR products 90 – 100 ng were labeled with Bigdye and then sequenced with either an ABI prism® 310 Genetic Analyzer or a 377 DNA Sequencer. Sequences were aligned automatically by Clustal X (Thomson et al., 1997) and checked manually by ESEE (Eye Ball Sequence Editor) (Cabot, 1998). The mtDNA sequence distances were calculated by using the Kimura 2-parameter distance method (PHYLIP 3.57c -(Phylogenetic Inference Package)) (Felsenstein, 1993), and the phylogenetic relationships were analyzed on the 12S gene sequences by using the MEGA2 (Molecular Evolutionary Genetic Analysis) (Maximum Parsimony) (Kumar et al., 2001), Treecon (Neighbour-Joining) (Van de Peer & De Wachter, 1994), and Quartet Puzzling (Neighbour-Joining) (Strimmer & von Haeseler, 1999) programs.

## Results

Fragment length, base composition, and G+C content are presented in Table 2. In the 12S fragment, all *Triops* taxa except *T. granarius* contained 570 base pairs (bp), and *Lepidurus lemmoni* contained one bp less. For the 16S fragment, we obtained 490 bases in all taxa except for *L. lemmoni*, *T. cancriformis*, and the commercial kit, where the first two taxa contained 2 extra bp and the last taxon contained one extra bp. The 12S and 16S fragments of notostracans corresponded to segments from bases 13 323 to 13 849 and 12 174 to 12 568 of the complete mitochondrial DNA of *Artemia franciscana* (GenBank accession number X69067). In both subunits, A+T content was higher than the G+C content, i.e. between 2.36 and 2.52 times higher in the small subunit and 1.88 and 2.11 times higher in the large subunit. Sequences of the two genes studied are given in Appendices 1 & 2. These sequences are deposited in GenBank (acc. num. AY115595 – AY115614).

The 16S fragment showed more than 99% similarity within the American *Triops* forms. The commercial kit population showed one insertion. The other populations presented no insertion. The populations 'km 76.5' and Vizcaíno were identical and differed from the rest of the American populations ('km 64.0', El Arco, 'km 79.3', Zacatecas, and commercial kit) in only one base, but located at different sites. Animals from 'km 64.0' differed from the 'km 79.3', El Arco, Zacatecas, and the commercial kit population at two positions. One variable site was found to be common in all populations. The other variable site was located upstream in sequences from El Arco and commercial kit, and downstream in the km 79.3 and Zacatecas population sequences. The three base differences in the 16S sequences were T or C transitions. The Kimura 2-parameter distance ranged from 0 to 0.0041 for the

*Table 2.* Fragment length, base composition (%), and G+C content (%) of mitochondrial ribosome DNA of 10 notostracan populations

| Gene | Population | No. of bases | A | C | G | T | G+C |
|---|---|---|---|---|---|---|---|
| 12S | *Triops* sp. 'km 76.5' | 570 | 36.3 | 17.7 | 10.7 | 35.3 | 28.4 |
| | *Triops* sp. 'km 64.0' | 570 | 36.1 | 18.1 | 10.9 | 34.9 | 29.0 |
| | *Triops* sp. Vizcaíno | 570 | 36.6 | 17.7 | 10.7 | 35.3 | 28.4 |
| | *Triops* sp. El Arco | 570 | 36.1 | 17.9 | 10.9 | 35.1 | 28.8 |
| | *Triops* sp. 'km 79.3' | 570 | 36.3 | 17.9 | 10.7 | 35.1 | 28.6 |
| | *T. longicaudatus* (Zacatecas) | 570 | 36.1 | 17.9 | 10.9 | 35.1 | 28.8 |
| | *Triops* sp."commercial kit" | 570 | 36.3 | 17.9 | 10.7 | 35.1 | 28.6 |
| | *T. "granarius"* (Japan) | 571 | 37.1 | 17.9 | 11.7 | 33.3 | 29.6 |
| | *T. cancriformis* (Austria) | 570 | 37.7 | 18.6 | 11.1 | 32.6 | 29.7 |
| | *L. lemmoni* (U.S.A.) | 569 | 38.3 | 17.4 | 11.2 | 33.0 | 28.6 |
| 16S | *Triops* sp. 'km 76.5' | 490 | 30.8 | 12.4 | 22.0 | 34.7 | 34.4 |
| | *Triops* sp. 'km 64.0' | 490 | 30.8 | 12.2 | 22.0 | 34.9 | 34.2 |
| | *Triops* sp. Vizcaíno | 490 | 30.8 | 12.4 | 22.0 | 34.7 | 34.4 |
| | *Triops* sp. El Arco | 490 | 30.8 | 12.2 | 22.0 | 34.9 | 34.2 |
| | *Triops* sp. 'km 79.3' | 490 | 30.8 | 12.2 | 22.0 | 34.9 | 34.2 |
| | *T. longicaudatus* (Zacatecas) | 490 | 30.8 | 12.7 | 22.0 | 34.5 | 34.7 |
| | *Triops* sp."commercial kit" | 491 | 31.0 | 12.6 | 22.0 | 34.4 | 34.6 |
| | *T. "granarius"* (Japan) | 490 | 32.0 | 12.7 | 21.0 | 34.3 | 33.7 |
| | *T. cancriformis* (Austria) | 492 | 31.5 | 12.6 | 21.7 | 34.1 | 34.3 |
| | *L. lemmoni* (U.S.A.) | 492 | 32.7 | 12.0 | 20.1 | 35.2 | 32.1 |

*Table 3.* Kimura 2-parameter distance matrix for mitochondrial 16S rRNA gene of 10 notostracan populations

| Population | | Population | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1. | *Triops* sp. 'km 76.5' | | | | | | | | | |
| 2. | *Triops* sp. 'km 64.0' | 0.002 | | | | | | | | |
| 3. | *Triops* sp. Vizcaíno | 0.000 | 0.002 | | | | | | | |
| 4. | *Triops* sp. El Arco | 0.002 | 0.004 | 0.002 | | | | | | |
| 5. | *Triops* sp. 'km 79.3' | 0.002 | 0.004 | 0.002 | 0.004 | | | | | |
| 6. | *T. longicaudatus* (Zacatecas) | 0.002 | 0.004 | 0.002 | 0.004 | 0.004 | | | | |
| 7. | *Triops* sp. "comm. kit" | 0.002 | 0.004 | 0.002 | 0.004 | 0.004 | 0.004 | | | |
| 8. | *T. "granairus"* (Japan) | 0.065 | 0.067 | 0.065 | 0.067 | 0.067 | 0.065 | 0.067 | | |
| 9. | *T. cancriformis* (Austria) | 0.095 | 0.092 | 0.095 | 0.097 | 0.095 | 0.095 | 0.097 | 0.095 | |
| 10. | *L. lemmoni* (U.S.A.) | 0.125 | 0.125 | 0.125 | 0.128 | 0.123 | 0.128 | 0.128 | 0.123 | 0.140 |

comm. kit = commercial kit.

American populations (Table 3). Within the genus *Triops*, the maximum Kimura 2-parameter distances were in the species *T. cancriformis* with a range of 0.092–0.097 (Table 3). The Kimura 2-parameter distance between American *Triops* and *Lepidurus* ranged between 0.123 and 0.128; however in the maximum genetic distance found between these genera (0.140), the non-American species *T. cancriformis* was involved (Table 3).

In the 12S fragment, out of 570 bp sequenced, a maximum of nine bp difference were observed between any two American *Triops* populations. There were 17 variable sites (Table 4) among American populations and only seven of them were informative for parsimonious analysis. Again, animals from Vizcaíno

*Table 4.* Variation in the mitochondrial 12S rRNA gene sequence of American *Triops* populations

| Population | Position of base | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 | 62 | 119 | 121 | 128 | 138 | 172 | 202 | 328 | 333 | 373 | 440 | 444 | 456 | 506 | 542 | 555 |
| *Triops* sp. 'km 76.5' | A | T | A | T | A | G | G | C | A | G | C | G | A | T | A | T | A |
| *Triops* sp. 'km 64.0' | G | C | . | C | . | A | . | . | G | . | T | A | . | C | G | . | . |
| *Triops* sp. Vizcaíno | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Triops* sp. El Arco G | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | |
| *Triops* sp. 'km 79.3' | G | C | . | . | . | A | . | T | . | . | T | A | G | C | . | C | . |
| *T. longicaudatus* (Zacatecas) | G | C | . | . | G | A | . | . | G | . | T | A | . | C | . | . | . |
| *Triops* sp. "commercial kit" | G | C | G | . | . | A | A | . | . | A | T | . | . | C | . | . | G |

*Table 5.* Kimura 2-parameter distance matrix for mitochondrial 12S rRNA gene of 10 notostracan populations

| Population | | Population | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1. | *Triops* sp. 'km 76.5' | | | | | | | | | |
| 2. | *Triops* sp. 'km 64.0' | 0.016 | | | | | | | | |
| 3. | *Triops* sp. Vizcaíno | 0.000 | 0.016 | | | | | | | |
| 4. | *Triops* sp. El Arco | 0.004 | 0.012 | 0.004 | | | | | | |
| 5. | *Triops* sp. 'km 79.3' | 0.016 | 0.011 | 0.016 | 0.012 | | | | | |
| 6. | *T. longicaudatus* (Zacatecas) | 0.014 | 0.005 | 0.014 | 0.011 | 0.009 | | | | |
| 7. | *Triops* sp. "comm. kit" | 0.016 | 0.014 | 0.016 | 0.012 | 0.012 | 0.012 | | | |
| 8. | *T. "granarius"* (Japan) | 0.126 | 0.128 | 0.126 | 0.126 | 0.124 | 0.126 | 0.132 | | |
| 9. | *T. cancriformis* (Austria) | 0.141 | 0.139 | 0.141 | 0.141 | 0.137 | 0.139 | 0.141 | 0.175 | |
| 10. | *L. lemmoni* (U.S.A.) | 0.183 | 0.187 | 0.183 | 0.183 | 0.187 | 0.187 | 0.183 | 0.209 | 0.205 |

comm. kit = commercial kit.

*Table 6.* Kimura 2-parameter distance matrix for mitochondrial 12S rRNA gene of 11 notostracan populations

| Population | | Population | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | *Triops* sp. 'km 76.5' | | | | | | | | | | |
| 2. | *Triops* sp. 'km 64.0' | 0.016 | | | | | | | | | |
| 3. | *Triops* sp. Vizcaíno | 0.000 | 0.016 | | | | | | | | |
| 4. | *Triops* sp. El Arco | 0.002 | 0.014 | 0.002 | | | | | | | |
| 5. | *Triops* sp. 'km 79.3' | 0.014 | 0.010 | 0.014 | 0.012 | | | | | | |
| 6. | *T. longicaudatus* (Zacatecas) | 0.014 | 0.006 | 0.014 | 0.012 | 0.008 | | | | | |
| 7. | *Triops* sp. comm. kit' | 0.014 | 0.014 | 0.014 | 0.012 | 0.020 | 0.012 | | | | |
| 8. | *T. "granairus"* (Japan) | 0.141 | 0.145 | 0.141 | 0.143 | 0.138 | 0.143 | 0.148 | | | |
| 9. | *T. cancriformis* (Austria) | 0.155 | 0.155 | 0.155 | 0.158 | 0.151 | 0.155 | 0.155 | 0.197 | | |
| 10. | *T. australiensis*[a] (Australia) | 0.124 | 0.121 | 0.124 | 0.124 | 0.119 | 0.119 | 0.124 | 0.180 | 0.199 | |
| 11. | *L. lemmoni* (U.S.A.) | 0.186 | 0.194 | 0.186 | 0.188 | 0.194 | 0.194 | 0.186 | 0.215 | 0.210 | 0.232 |

[a] Information of *T. australiensis* from the GenBank database contained 503 bp and according to the length sequence of other *Triops* populations was shortened.
comm. kit = commercial kit.

and 'km 76.5' showed no difference. The base differences observed in the 12S sequences were only transitional substitutions. The Japanese and European species *T. "granarius"* and *T. cancriformis* showed 77 and 83 variable sites when compared with the American populations. The Kimura 2-parameter distance

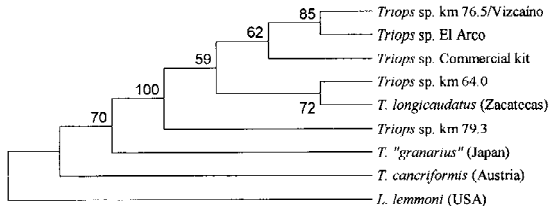


*Figure 2.* Maximum Parsimony tree of 10 notostracan populations based on 12S rRNA sequences. Sequences were bootstrapped 1000 times and the bootstrap values are given at each node as percentage.

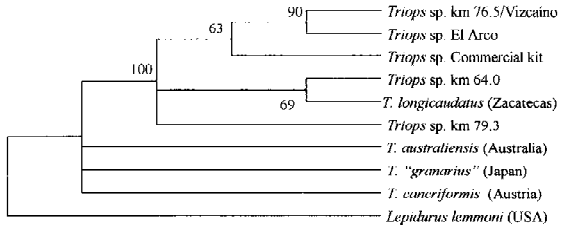


*Figure 3.* Maximum Parsimony cladogram of 11 notostracan populations (including *T. australiensis* from the GenBank) based on 12S rRNA sequences. Sequences were bootstrapped 1000 times and the bootstrap values are given at each node as percentage.

ranged from 0 to 0.016 for the American populations (Table 5). For two base differences the value was 0.004, and the highest value 0.016 was for 9 base differences. Again, within the genus *Triops* the maximum Kimura 2-parameter distances involved the species *T. cancriformis* with a range of 0.137–0.175 (Table 5). The Japanese *Triops "granarius"* and the European *T. cancriformis* showed the maximum genetic difference (0.175) within the genus *Triops*. The former species differed from the American populations by a maximum distance of 0.132 and the latter by 0.141 (Table 5). The Kimura 2-parameter distance between *Triops* and *Lepidurus* ranged between 0.183 and 0.209, where the maximum genetic distance involved the non-American species *T. "granarius"* and *T. cancriformis* (Table 5). The Kimura 2-parameter distances increased when the *Triops australiensis* 12S sequence (GenBank accession number AY050646) was added (Table 6). *Triops australiensis* showed a maximum distance with *T. cancriformis* (0.199) and a minimum distance with *T. longicaudatus* (Zacatecas) and km 79.3 populations (0.119) (Table 6).

According to the Maximum-Parsimony, Neighbour-Joining, and Quartet-Puzzling analyses, two monophyletic groups were consistently formed. One group is formed by the populations from km 76.5/ Vizcaíno and El Arco (Figs 2–7). The population from the commercial kit is genetically related to these populations,

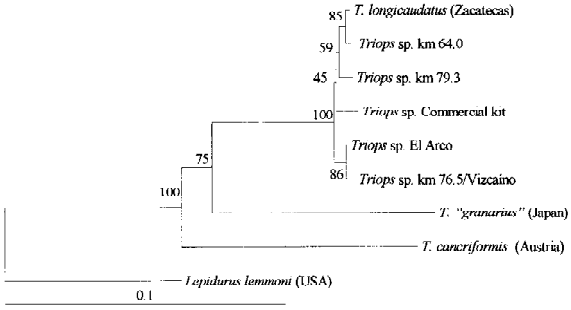


*Figure 4.* Treecon generated Neighbour-Joining tree obtained for the 12S rRNA sequences of 10 notostracan populations. Distance estimation was done by applying Kimura's (1980) method and the transition/transversion ratio was calculated from the data. Insertion and deletion were taken into account.

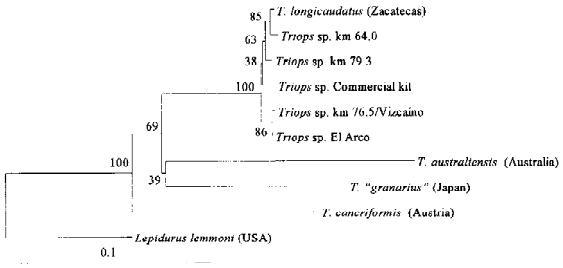


*Figure 5.* Treecon generated Neighbour-Joining tree obtained for the 12S rRNA sequences of 11 notostracan populations (including *Triops australiensis* from the GenBank). Distance estimation was done by applying Kimura's (1980) method and the transition/transversion ratio was calculated from the data. Insertion and deletion were taken into account.

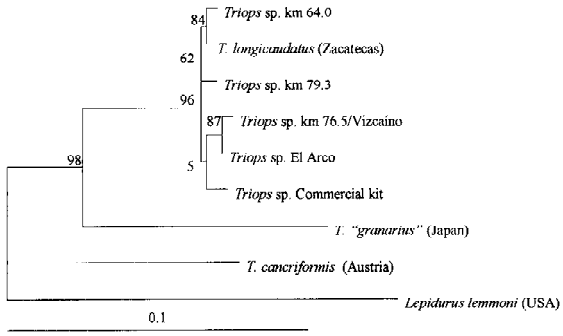


*Figure 6.* Quartet-Puzzling tree obtained for the 12S rRNA sequences of 10 notostracan populations. This is an accurate estimation of the Neighbour-Joining parameter using the HKY model of substitution. Transition/transversion estimate from data set was 1.33 ± 0.20. Out of 126 quartets, 21.4% were unresolved.

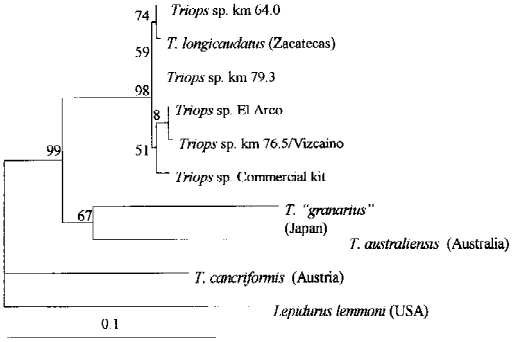


*Figure 7.* Quartet-Puzzling tree obtained for the 12S rRNA sequences of 11 notostracan populations (including *Triops australiensis* from GenBank). This is an accurate estimation of the Neighbour-Joining parameter using the HKY model of substitution. Transition/transversion estimate from data set was 1.07 ± 0.15. Out of 210 quartets, 18.6% were unresolved.

and all seem to represent a monophyletic clade (Figs 2, 3, 6 & 7). The second group is formed by the populations from km 64.0 and Zacatecas (Figs 2–7). The population from km 79.3 is genetically closer to km 64.0 and Zacatecas, and all also seem to represent a monophyletic clade (Figs 4 & 5).

## Discussion

Phylogenetic analyses grouped two forms that are highly divergent in morphology and reproduction (the uniparental short-bodied population of km 64.0, and the gonochoric long-bodied *T. longicaudatus* of Zacatecas) in a monophyletic clade (Figs 2–7). Previously, Sassaman et al. (1997) reported great similarity in genetic characteristics (allozymes) between two highly different forms from the U.S.A., the long-bodied gonochoric population *T. longicaudatus* and the 'unisexual' short-bodied population. Thus, our hypothesis that the phylogenetic relationships of *Triops* species could be deduced by similarities in both the number of total body rings and legless rings (Maeda-Martínez et al., 2000a) is not supported by molecular and biochemical data and should be abandoned. The molecular data support Linder's (1952) view that *Triops cancriformis* may represent a separate group from the rest of the species in the genus (Figs 2, 4, 5, 6 & 7). Even the Quartet-Puzzling trees indicate that *T. cancriformis* may not form a monophyletic group with the rest of the species in the genus (Figs 6 & 7). Further studies are required because these data suggest that *T. cancriformis* could represent a third genus within the Notostraca.

Phylogenetic analyses demonstrated that the American populations here studied form a monophyletic group (Figs 2–7). These analyses do not support our previous view of a possible vicariant evolution, where different species-groups could be distributed in different continents by historical processes such as continental drift (Maeda-Martínez et al., 2000a). However, to reach convincing explanations of the historical biogeography of the group, molecular studies on more species and populations of different continents are needed.

Sassaman et al. (1997) demonstrated with allozyme data that the nominal (morphological) species *Triops longicaudatus sensu* Linder (1952) and Longhurst (1955), is in fact a mixture of at least two species. Our molecular and phylogenetic analyses also indicate that this morphological species comprises several phylogenetic species. The 'phylogenetic species' was defined by Cracraft (1989) as "an irreducible (basal) cluster of organisms, diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent", and by Nixon & Wheeler (1990) as "the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals". Thus, the differences found in the base sequences of the maternally inherited mitochondrial 12S rRNA gene suggest that from the seven *Triops* American populations, six represent different phylogenetic species. One is represented by the 'male-less' populations of 'km 76.5' and Vizcaíno (geographically separated by about 550 km), the second by the 'male-less' population of El Arco (situated only about 40 km N of Vizcaíno), the third by the uniparental short-bodied population of km 64.0, the fourth by the gonochoric long-bodied *T. longicaudatus* of Zacatecas, the fifth by the 'androdioecious' short-bodied population of km 79.3, and the sixth by the population of the commercial kit (U.S.A.). The first three entities may belong to the "selfing hermaphrodite-species type", i.e. the 'unisexual' species advanced by Sassaman (pers. com.). The last entity may be of the 'androdioecious species type' given that of six individuals from the outdoor culture, only one male was obtained.

The morphology of full grown males appears as one of the keys to identify different entities of *Triops* (Maeda-Martínez et al., 2000a). Upon the presence of (morphologically distinctive) males, we consider that

the gonochoric long-bodied *T. longicaudatus* population of Zacatecas, the 'androdioecious' short-bodied population of km 79.3, and the 'androdioecious' population of the commercial kit are indeed different morphological species. The Kimura 2-parameter distance (12S mtDNA) among these three morphological species ranged from about 0.008 and 0.020 (Tables 5 & 6), which included a minimum difference of 5 bases (Table 4). These genetic distances are quite low when comparing with those obtained from other branchiopod inter-species studies. The Kimura 2-parameter genetic distances for four phylogenetic *Triops* species from Japan ranged from 0.053 to 0.150 (16S mtDNA) (Suno-Uchi et al., 1997), and between the cladocerans *Daphnia dubia* and *D. laevis* the value was 0.076, while in their inter-subspecies distances the values ranged from 0.002 to 0.006 (16S mtDNA) (Taylor et al., 1998). These values are comparable with those (0.005 and 0.002, 12S mtDNA) found between the proposed phylogenetic species gonochoric *T. longicaudatus* (Zacatecas) and the uniparental *Triops* sp. km 64, and between the 'male-less' *Triops* sp. km 76.5/Vizcaíno and *Triops* sp. El Arco, which included a sequence difference of 3 and 2 bases. Little base sequence differences between species have also been reported using other mitochondrial genetic markers. For instance, species of cichlid fishes from Lake Victoria differed from each other by an average of only 3 bases (range 1–5) in the control region (Meyer et al., 1990).

Morphological, biochemical, and reproductive characterizations of the six phylogenetic species are currently under study. Specimens of the two uniparental populations from 'km 76.5' and 'km 64.0' (found to be anatomically hermaphrodites, unpublished data) exhibited different trypsin-like enzyme patterns (Maeda-Martínez et al., 2000b) and significant differences in reproduction (cyst production) (Obregón-Barboza et al., 2001). These data support our conclusion based on molecular and phylogenetic analyses that these populations represent different entities at species level.

As mentioned above, Sassaman et al. (1997) reported great similarity in allozymic characteristics between two highly different forms, a long-bodied gonochoric population (*T. longicaudatus*, U.S.A.) and a 'unisexual' short-bodied population (U.S.A.). In line with these data, our phylogenetic analyses grouped in a monophyletic clade two forms highly differentiated in morphology and reproduction, the uniparental short-bodied of km 64.0, and the gonochoric long-bodied *T. longicaudatus* of Zacatecas (Figs 2–7). These findings suggest the possibility that, apart from the androdioecious reproductive system (Sassaman, 1989, 1991, 1995; Sassaman & Weeks, 1993), some selfing hermaphrodite populations could originate from a gonochoric reproductive system.

The 16S fragment was not informative for molecular comparisons of the American *Triops* forms because more than 99% of the sequences were similar. Absence of informative bases in the 16S gene might indicate a different rate of evolutionary change among the two subunits of mtDNA. Suno-Uchi et al. (1997) analyzed the 16S gene in Japanese *Triops*. They used a newly designed forward primer in combination with the reverse primer 16Sbr of Palumbi et al. (1991) and obtained amplified products of 929 bp. In our study, we used the forward and reverse primers (16Sar & 16Sbr) of Palumbi et al. (1991) and obtained 490 bp. However, there was no similarity between our sequences and those of Suno-Uchi et al. (1997); therefore we suggest the Japanese materials used by these authors require a molecular reexamination. Unpublished but genuine 16S sequences of Japanese *Triops* were later deposited by a different author at the GenBank (acc. num. AF200963–AF200971).

In the 12S gene, the sequence of the gonochoric *Triops longicaudatus* of Zacatecas (570 bp length) differed in 2 bp from the sequence of the gonochoric *T. longicaudatus* of Santa Rosa, New Mexico (U.S.A.) (326 bp length) reported by King & Hanner (1998) (GenBank acc.num. AJ00817). This sequence difference requires further studies. Our sequence from *Lepidurus lemmoni* did not differ from the sequence found in the GenBank database (acc.num. AJ000823). From the study of King & Hanner (1998), it is clear that the 12S sequences of the *Lepidurus* species show a higher divergence than in the *Triops* species. The sequence differences at population and species level in *Lepidurus* were mainly caused by transitions (King & Hanner, 1998). In the present study, no transversion was observed among American *Triops* indicating that the evolution at 12SrRNA gene is restrained and favors the preservation of the small subunit structure in the American notostracans.

Apart from the gonochoric phylogenetic species *Triops longicaudatus*, as here characterized, we consider it is premature to use or propose species names to the other five American identified forms. Establishing a proper nomenclature, extensive comparative molecular studies on populations of the region, and of

the type locality areas of the nominal species are still required.

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*Appendix 1.*

Appendix 1
12S rRNA

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| km 76.5 | GTTACGACTT | ATCTCTCCTT | AAG-GAAGAG | AGCGACGGGC | GATGTGTACA | CACCTCAGAG | CTTATTTCAA | ATAAGAATTT | TATTCTTAAT |
| km 64.0 | . . . . . | . . . . . | . . . - . G . . . | . . . . . | . . . . . | . . . . . | . . C . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . - . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . - . G . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . - . G . . . | . . . . . | . . . . . | . . . . . | . . C . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . - . G . . . | . . . . . | . . . . . | . . . . . | . . C . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . - . G . . . | . . . . . | . . . . . | . . . . . | . . C . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . . | . . . . . | . . . A . G . . . | . . . . . | . . . . . | . . . T . . . . . | . . C . A . . . . . | . A . . . . . | . . . . . G . . |
| *T. cancriformis* | . . . . . | . . . . . | . - . A . G . . . | . . . . . | . . . . . | . . . . . | . CA . C . . . . . | TA . . . . . | . . . . . A . . . |
| *L. lemmoni* | . . . . . | . . . . . | . . A - . G . . . | . . . . . | . . . . . | . . . T . . . . . | . . . . . T | . . . . AC . . . . | . . . . T . . . . . |
| | | | | | | | | | |
| km 76.5 | TTACTACTAA | ATCCACCTTC | ATAATTTGTA | TTACACTAAT | TAATCCGTG- | TAATTCTATA | TTATTGTAAC | CCACTACTCC | CATGTTTATA |
| km 64.0 | . . . . . | . . . . . | . . . . . | . C . . . . . | . . . . . A - | . . . . . | . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . - | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . - | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . A - | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . G . | . . . . . A - | . . . . . | . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . G | . . . . . | . . . . . A - | . . . . . | . . . . . | . . . . . | . . . A . . . . . |
| *T. "granarius"* | . . . . . | . . . . . | . . . GAA . TA . | . . . . A . T . C | C . . . . . T - | . . . A . T . . A . | . . . . . | . . . . . | . . . . C . . . . . |
| *T. cancriformis* | . . . . . | . . . . . A | . . . . . A . . . . | . - . . . TAT . C | . . . . . A - | . . . . . A . . AT | . . . . . | . . . . . T . . . . | . . . A . . . . . |
| *L. lemmoni* | . . . . . | . . . . . T | . . . . . AAT . G | . - . . . A . T . . | . . . . . AA | . . . C . TA . CT | . . . . . | . . . . . | . . . A . . . . T |
| | | | | | | | | | |
| km 76.5 | AGCTGCACCT | TGACCTGAAG | TCCCAA - TTA | ACTATTAATT | AGATTATATT | TTTTAAAAAT | AATCTGACAA | CGGCGGTATA | CAAACTGAAA |
| km 64.0 | . . . . . | . . . . . | . . . . . - . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . - . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . - . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . T . - . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . - . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . - . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . . | . . . . . | . . . T . - . . . | . . A . . . . . | . . . . . GA . | C . . . G . . . . . | . . . A . . . . . | . . . . . | . . . . . |
| *T. cancriformis* | . . . . . | . . . . . | . . AT . . A . . T | . AA . . . . . C . | . . . . . | . . C . . . . . | . . . . . | . . . . . | . . . . . T |
| *L. lemmoni* | G . . . . . | . . . . . T . | . . A . . G - . A . | . AA . C . . . . . | . . . . A . . . . . | C . . . . . | . . . . . | . . . . . | . . . . . |
| | | | | | | | | | |
| km 76.5 | ACAAAAACAT | GTACATTAAA | ACGTGGATTA | TCGATCCAAG | AGCAGGTTCC | TCTAGTAAGA | ATAAGGTACC | GCCAAATTCT | TTAGGTTTTA |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | G . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | G . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . A . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . G . A . . . . . | . . . . . | . . . . . A . |
| *T. cancriformis* | . . . . . T . . | . . . . . T . . | . . . . . A . . | . . . . . | . . . . . | . . . . . | . . G . A . . . . . | . . . . . | . . . . . A . |
| *L. lemmoni* | . . . . . | . . . A . . . T . . | T . . . . . A . . | . . . . . A . . . . | . . . . . | . . . . . | . . G . A . . . . . | . . . . . | . . . . . A . |
| | | | | | | | | | |
| km 76.5 | AAAA - - TTCT | ACTACCCCGG | CAATTAATAA | AATAAAGAAT | AATAGGGTAT | CTAATCCTAG | TTTAAATCTT | AATTTTCAAA | GATAGTTCAT |
| km 64.0 | . . . . - - . . . . | . . . . . T . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . A . . . . . |
| Vizcaíno | . . . . - - . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . - - . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . - - . . . . | . . . . . T . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . A . . G . |
| Zacatecas | . . . . - - . . . . | . . . . . T . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . A . . . . . |
| Commercial kit | . . . . - - . . . . | . . . . . T . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . - - . . T . | . . . . . A . . | . . . A . . . CTT | . . . . G . . . . | . . . . . | . . . . . | . C . . . . . C . | . . . . . T . . . | . . . TA . AT . . |
| *T. cancriformis* | . . . T - - C . . . | . . . . . T . . | . . . CA . . . TT | . . . . GG . . . . | . . . . . | . . . . . | . . . . . C . CC | T . . C . A . . . . | . T . . A . AA . . |
| *L. lemmoni* | . . . TATC . T . | . . . . . A . . | . . . . C . . - T | . . . . . | . G . . . . . | . . . . . | . . . . . G . . . | T . . . CA . . . | . . . . TAAA . A |
| | | | | | | | | | |
| km 76.5 | TCTGTTATAA | TTTTTCATTT | AAAAAAATAA | AAATTTTACC | CTTAATTTTT | TATTTAAAAA | ATTTATTTAA | AACAAATATT | TACTTCATTC |
| km 64.0 | . . . . . | C . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | G . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | C . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | C . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | C . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | C . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . T . T . . . . . | . . . . . T . C . . | . G . G . . T . . . | . . . . . T | . . . G . . CCAC | . . . . . | . . . . . C . AG . | . . T . . TA . . . | . . . . CA . . . . |
| *T. cancriformis* | . TAC . A . A . . | . . . . . A . . . . | . . . . . G . . | . G . . . . . | . C . . . . . | C . . CA . . . . . | . . CCC . . . . . | . . TT . . . . . | . . . . . A . . . . |
| *L. lemmoni* | . T . T . . . . . | . . A . . ACAA . | . C . T . TT . . . | . . . . . C . . . | . . C . . A . A . A | . . ACC . . . . . | . . . AT . . A . . | . . . G . . - . A | . . . . . A . . . . |
| | | | | | | | | | |
| km 76.5 | CACCAATCTA | TTTCATTTAA | ATCCTTCGTA | TAACC | | | | | |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | | | | | |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | | | | | |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | | | | | |
| km 79.3 | . . . . . C . . . | . . . . . | . . . . . | . . . . . | | | | | |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | | | | | |
| Commercial kit | . . . . . | . . . . . G | . . . . . | . . . . . | | | | | |
| *T. "granarius"* | . . . . . | . . . . . | . . . . . | . . . . . | | | | | |
| *T. cancriformis* | . . . . . G | . . . . . | . . . . . | . . . . . | | | | | |
| *L. lemmoni* | . . . . . AAA . | . . . T . . . . G . | CC . . A . . . . . | . . . . . | | | | | |

*Appendix 2.*

Appendix 2
12S rRNA

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| km 76.5 | ATAAATTTGA | GGTATAGCCT | GCTCTATGAA | T-TTTTTTAA | ATGGCCGCGG | TATTTTGACC | GTGCGAAGGT | AGCATAATCA | TTAGTCTTTT |
| km 64.0 | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . | . A . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | C . T . . . . A . | . . . . . | . . . . . | . . - - A . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| *T. cancriformis* | . . T . . . . . | . . . . . | . . . . . | . . - . . A . . . . . | . . . . . | . . . . C . . . . . | . . . . . | . . . . . | . . . . . |
| *L. lemmoni* | . . - . . . ACA . | . . . . . | . . . . . | . . - . G . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 76.5 | AATTGGAGGC | TGGTATGAAT | GGCTGGACGA | GAAAATTTCT | GTCTCTGTTA | TAAAATTTGA | ATTTTATTTT | TAAGTGAAAA | AGCTTAAATT |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . T . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . C . . | . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . . A . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . T . . . . . | . . . . . | . . . . . | . . . . . |
| *T. cancriformis* | . . . . . A . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . T . . . . . | . . . . G . . . . . | . . . . . | . . . . . A |
| *L. lemmoni* | . . . . . A . . . . | . . . . . | . . . . . | . . . . . | . . . . . A . . G | . . . . . | . . . . . | . . . . . | . . . . . A |
| km 76.5 | TGTTTGAGGG | ACGATAAGAC | CCTATGGATC | TTTATAATGA | TATATAGTAA | AAAGCTTAGG | T-GTGACGGC | TTACTGTTTT | AAATTATTGG |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . T . . . . . | . - . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | . . C . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . - . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . T . . . . . | . . . . . | . . . . . | . . . . . A . | . . A . . . . . | . . . . . A | . T . . A . . A . . | . . . T . A . . . . | G . . . . . |
| *T. cancriformis* | . T . . . . G . . . | . . . . . | . . . . . | . . . . . | . GAT . . A . . . | . . . . T . . . . A | A - . . T . . A . . | . . TT . AC . C . | . . G . . . . . |
| *L. lemmoni* | AT . . . . . | . . . . . | . . . . . | . . . . . TGA . | . T . . . A . . . | . . . . A . . G . T | CA . . T . . . . . | . . . T . . . AAA | T . . A . . . . T |
| km 76.5 | GTTGGGGCGA | CCGGAAGAAA | AAAATATCTC | TTCTTTAATA | - ATATAATTT | TT - TTAGAAA | TAAAA - TGAC | CCTGAATTTT | GGTTTAAAAG |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | - . . . . . | . . - . . . . . | . . . . . - . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | - . . . . . | . . - . . . . . | . . . . . - . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | - . . . . . | . . - . . . . . | . . . . . - . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . . . . . | - . . . . . | . . - . . . . . | . . . . . - . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | - . . . . . | . . - . . . . . | . . . . . - . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . | . . . . . | - . . . . . | . . - . . . . . | . . . . . - . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . . | . . A . G . . . . . | . . . G . . . . . | . . . . . A . . . . | - . A . . T . A . | . A - G . . . . . | . . . . T - . . . . | . . . . . | . . . . . |
| *T. cancriformis* | . . . . . | . T . . . . . | . . . . . | . . . . . A . . A . | T . A . . . T . A . | . . - A . . . . . T | . . . . . A . . . . | . . . A . . . . . | . . . . . GC . . |
| *L. lemmoni* | . . . . . | . T . A . . . . . | T . . T . . . . . | . . T . . A . TA . | T . A . . . GAA . | . CCG . . . . . | . . T . . - . . . . | . . . T . . . . . | . . . . . T . . . |
| km 76.5 | ACTAAGTTAC | CCTAGGGATA | ACAGCGTAAT | CTTTTTGGAG | AGTTCATATC | GATAAAAAGG | TTTGCGACCT | CGAATGTTGGA | TTAAGAGATC |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| km 79.3 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| Commercial kit | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
| *T. "granarius"* | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . G . . G . . | . . . . . | . . . . . | . . . . . C . |
| *T. cancriformis* | . TC . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . G . . . . . | . . . . . | . . . . . | . . . . . |
| *L. lemmoni* | . . . . . | . . . . . | . . . . . | T . . . CA . . . | . . . . . T . . . . | . . . G . . . . . | . . . . . | . . . . . | . . . . . T . . . |
| km 76.5 | CTTGCGGCGC | AGCCGTTGTA | AAGGAGAGTC | TGTTCGACTT | TTAAA | | | | |
| km 64.0 | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | | | | |
| Vizcaíno | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | | | | |
| El Arco | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | | | | |
| km 79.3 | . . . . . | . . . T . . . . . | . . . . . | . . . . . | . . . . . | | | | |
| Zacatecas | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | | | | |
| Commercial kit | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | | | | |
| *T. "granarius"* | . . . A . . . . . | . . . . . | . . . . . A . . . . | . . . . . | . . . . . | | | | |
| *T. cancriformis* | . . . . . | . . . A . . . . CG | G . . . . A . . . . | . . . . . | . . . . . | | | | |
| *L. lemmoni* | . . . AT . . . . . | . . . T . . . A . . | . . . . . A . . . . | . . . . . | . . . . . | | | | |