Toward a Global Phylogeny of the “Living Fossil”
Crustacean Order of the Notostraca

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Abstract

Tadpole shrimp (Crustacea, Notostraca) are iconic inhabitants of temporary aquatic habitats worldwide. Often cited as prime examples of evolutionary stasis, surviving representatives closely resemble fossils older than 200 mya, suggestive of an ancient origin. Despite significant interest in the group as ‘living fossils’ the taxonomy of surviving taxa is still under debate and both the phylogenetic relationships among different lineages and the timing of diversification remain unclear. We constructed a molecular phylogeny of the Notostraca using model based phylogenetic methods. Our analyses supported the monophyly of the two genera *Triops* and *Lepidurus*, although for *Triops* support was weak. Results also revealed high levels of cryptic diversity as well as a peculiar biogeographic link between Australia and North America presumably mediated by historic long distance dispersal. We concluded that, although some present day tadpole shrimp species closely resemble fossil specimens as old as 250 mya, no molecular support was found for an ancient (pre) Mesozoic radiation. Instead, living tadpole shrimp are most likely the result of a relatively recent radiation in the Cenozoic era and close resemblances between recent and fossil taxa are probably the result of the highly conserved general morphology in this group and of homoplasies.

Citation: Vanschoenwinkel B, Pinceel T, Vanhove MPM, Denis C, Jocque M, et al. (2012) Toward a Global Phylogeny of the “Living Fossil” Crustacean Order of the Notostraca. PLoS ONE 7(4): e34998. doi:10.1371/journal.pone.0034998

Editor: Keith A. Crandall, Brigham Young University, United States of America

Received September 14, 2011; Accepted March 8, 2012; Published April 18, 2012

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Funding: BV currently holds a postdoctoral fellowship with the Research Foundation Flanders (FWO–Vlaanderen). This research was funded by the fund for scientific research Flanders. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Tadpole shrimp (Crustacea, Notostraca) comprise one living family, the Triopsidae, including two genera: *Triops* Schrank, 1803 and *Lepidurus* Leach, 1819. Members of this group are often considered prime examples of evolutionary stasis [1–3] with the oldest confirmed notostracan fossils dating back as far as the Upper Carboniferous period [4]. Alleged to have remained virtually unchanged during an evolutionary timeframe of more than 250 million years, some surviving members of this ancient crustacean order are frequently referred to as living fossils. The contemporary *Triops cancroides* (Bosc, 1801), for instance, is regularly cited as the oldest living species because of its striking resemblance to late Permian [5] and early Triassic fossils [6–10]. Similarly, fossils from the late Cretaceous have been identified as resembling late Permian [5] and early Triassic fossils [6–10].

Consequently, various authors suggest that morphological taxonomic should be handled with utmost care, considering large numbers of individuals [13,17,18]. Further complicating systematics are the different modes of reproduction that evolved within lineages and populations making it difficult to distinguish species and subspecies [13,16]. Within *Triops*, for instance, the absence of second maxillae is a good diagnostic character to distinguish *T. australiensis* (Spencer & Hall, 1895) and *T. longicaudatus* from *T. cancroides* and *T. granarius* (Lucas, 1864). However, variation in other morphological traits such as telson armature, number of segments and shape of the dorsal organ is often less consistent. Consequently, various authors suggest that morphological taxonomic should be handled with utmost care, considering large numbers of individuals [13,17,18]. Further complicating systematics are the different modes of reproduction that evolved within the notostracans. Depending on species and population, gonochoric (separate sexes), hermaphroditic as well as androdioecious populations (containing hermaphrodites and a proportion of males) are found [19,20].

In the 1950’s, Linder [17] and Longhurst [21] revised the alpha taxonomy of the Notostraca reducing the number of accepted nominal species from more than fifty to four in *Triops* and five in...
Lepidurus. Based on molecular phylogenies, however, it was recently proposed to recognise more species, even though molecular divergence among clades is often quite low [22]. At this time there are six accepted Triops species with presumably four additional lineages deserving species status [22] and approximately 8 Lepidurus species [3,16].

Currently, molecular phylogenetic research is almost exclusively limited to representatives of Triops, but see [3,23–24], and large-scale studies considering large numbers of populations over a significant proportion of species distributions are rare [22,25]. Except for a first exploratory study by Mantovani and coworkers [26], no attempt has been made to reconstruct the phylogenetic relationships within this group at a global scale and considering most recognised species and subspecies. The main reason is that in contrast to the well studied T. cancriformis and T. mauritanicus (Ghigi, 1921) populations in Europe, material from less intensively studied continents such as Africa, South America and Australia was not available.

Here, we use DNA sequence data from two mitochondrial genes (the protein coding Cytochrome c Oxidase subunit I or COI and 12S rRNA) to elucidate the evolutionary relationships between notostracans from 60 different populations around the globe. Available sequence information is combined with a large number of newly obtained sequences, featuring several recently discovered Australian notostracan lineages. Due to the large scale of the study and the isolated nature of the considered populations, it is reasonable to assume that gene flow will be extremely low and matrilineral markers will suffice to gain insight in phylogenetic relationships at this scale.

Based on this dataset we evaluate the monophyly of both recognised genera, discuss the biogeography and phylogenetic relations among extant lineages and evaluate the potential presence of cryptic species in the light of the often controversial species delineations in notostracans. Since discussion of species status requires a taxonomic revision including morphological studies, rather than going into the taxonomic status of closely related species complexes, we focus on major evolutionary lineages. Finally, we use molecular clocks to investigate whether gene genealogies are consistent with an ancient (pre) Mesozoic radiation suggested by fossil remains.

Results

An overview of the 89 Triops and Lepidurus populations included in our analyses and their localities is provided in Table S1 and plotted in Figure 1. Detailed information about the known distribution of different Notostracan lineages can be consulted in Text S1.

Characteristics of the mitochondrial DNA sequences and alignment

41 COI and 53 12S sequences were aligned together with 123 additional COI and 74 12S sequences drawn from GenBank and trimmed to a length of 568 bp and 328 bp, respectively. Excluding the outgroup, the complete COI and 12S datasets comprised 78 and 72 unique haplotypes, respectively. The COI alignment contained 240 variable sites (42%) of which 223 (39%) were found to be parsimony informative while the 12S alignment contained 111 variable sites (34%) of which 97 (29%) were parsimony informative.

Genetic distances and mitochondrial DNA diversity

A COI maximum K2P distance of 35.7% was recorded between an Australian T. australiensis haplotype and L. couesii from Canada while the maximum 12S distance of 27.8% was calculated between South African T. granarius and T. cancriformis from Belgium. An overview of the average, minimum and maximum K2P distances within and among main notostracan lineages is provided in Table 1 and Table S2. Estimates of divergence times between main lineages are provided in Table S3. An additional genetic distance matrix calculated using uncorrected p distances is provided in Table S4.

Overall, phylogenetic analysis of both mitochondrial genes using five different methods of phylogenetic reconstruction resulted in similar topologies (Figure 2) which were confirmed in trees based on combined analysis of the two genes (Figure S1).

The monophyly of Lepidurus is confirmed in all trees, except in the 1477 tree of the COI gene and in the 1478 tree of the 12S gene. Furthermore, phylogenetic analysis of the unique amino acid sequences translated from the full COI dataset (carried out in PhyML and MrBayes under the MtMam [27] + I model (as selected with the help of ProtTest v.2.4 [28]; results not shown) supported monophyly of the genus.

Phylogenetic reconstruction yielded no statistical support for monophyly of the nominal genus Triops nor for an alternative positioning of its lineages. Only ML (52%), NJ (40%) and BI (posterior probability of 80) analyses of the COI gene provide weak support for the monophyly of Triops. In the absence of a resolved topology, we resorted to constraint analyses to formally test the hypothesis of monophyly.

Constraint analyses enforcing monophyly of all Triops representatives, were conducted for MP and ML trees. Both the Kishino-Hasegawa test and nonparametric Templeton (Wilcoxon signed-ranks) and winning-sites (sign) tests identified the constrained COI and 12S trees, with a length of 1278 and 411 respectively, as significantly more parsimonious (p<0.0001) than the unconstrained MP tree with a length of 1448 and 455 mutational events. When comparing the constrained ML COI and 12S trees (−ln 7250 and 2478 respectively) to the unconstrained ML trees (−ln 7258and 2490 respectively) in Paup*, the Kishino-Hasegawa test significantly (P<0.05) supported the constrained tree as the most likely scenario. K2P distances between the genera also largely exceed those within (Table 1). Based on the whole range of confirmed molecular clocks in invertebrates a (pre) Mesozoic radiation suggested by fossil remains is highly implausible. According to standard molecular clocks used for crustaceans (1.4–2.8% mya−1) initial diversification in the Notostraca started approximately 25.5–12.75 mya. Even according to the slowest molecular clocks, both genera presumably did not diverge before 29.75 mya (based on a COI clock of 1.2% mya−1) or before 55.6 mya (based on a 12S clock of 0.5% mya−1).

In Lepidurus, the basal position of L. apus lubbocki is supported by all phylogenetic searches in the COI tree. The Australian Lepidurus lineage, which based on morphological traits was traditionally considered a subspecies of L. apus (L. apus vivida) emerged as a sister species of a clade containing the North American L. couesii, L. australiensis and the European Lepidurus lineages previously identified as L. couesii. The monophyly of the subspecies of the presumably widespread L. apus, hence, could not be confirmed.

Analyses confirm the monophyly of five main evolutionary lineages within the genus Triops: T. granarius, T. cancriformis, T. mauritanicus and a fourth lineage containing T. longicaudatus and T. nakajimai. The fifth lineage comprised haplotypes belonging to a recently discovered Triops sp. population from the saline Lake Carey in Western Australia. The monophyly of the various Australian lineages identified as T. australiensis, however, could not be confirmed although there was weak support for this clade in the COI dataset. As a result, this taxon could be paraphyletic. Within
Triops, T. cancriformis and T. mauritanicus emerged as two sister groups. The minimum genetic distance between these two clades (11.0%) was smaller than the genetic distances to the other main Triops lineages (17.9–23.8%). The Triops population from Lake Carey in Western Australia did not cluster together with other Australian populations but instead emerged as a distinct lineage.

COI and 12S sequences diverged 12.3–17.9% and 7.4–11.1% between haplotypes from Lake Carey and T. australiensis specimens, respectively. In the 12S analysis, BI, ML, NJ and QP trees place the American Triops clade, which contains specimens morphologically identified as T. longicaudatus and T. newberryi, as an evolutionary sister of the Australian T. sp. clade from Lake Carey. K2P values further justify this position. Maximum genetic divergences between Lake Carey and T. australiensis haplotypes of 17.9% and 11.1% in the COI and 12S gene, respectively, were higher than the divergences of 16.3% and 8.6% identified between the Lake Carey species and T. longicaudatus.

**Discussion**

We reconstructed the first large-scale molecular phylogeny of the primitive crustacean order Notostraca, which is characterised by morphological stasis throughout its fossil record [1,4,8,9]. Based on results from the analysis of two mitochondrial genes (COI and 12S rDNA), we discuss the phylogenetic relationships within this enigmatic group in which morphological taxonomy is complicated by phenotypic variability within and low variability among nominal species.

A preliminary attempt to resolve phylogenetic relations in the Notostraca based on 12S and 16S rDNA markers was performed by Mantovani and coworkers [26]. Splitting the genus Triops as suggested by these authors, however, is likely to be unjustified since
in our results the monophyly of both genera is confirmed. As a result, the main morphological difference between Lepidurus and Triops, the presence of a supra-anal plate (a posteriorly directed median extension of the telson which is present in Lepidurus but never in Triops; Figure 1A, B), is supported as a systematically informative character.

In order to discuss the potential species status of the main notostracan lineages, we will focus mainly on COI, which is the standard marker for barcoding [29]. In branchiopod crustaceans average sequence divergences >7–10% at COI [30,31,32] and 4–5% at 12S [33,34] are typically considered indicative for species level differentiation, although in combination with morphological support, species status has sometimes been attributed to molecular thresholds, the species status of L. arcticus and do not form a monophyletic group. According to accepted molecular thresholds, the species status of L. arcticus and the European L. sp. clade, which was previously considered conspecific to the American L. couesii, [3] is confirmed.

Phylogenetic relations in Triops

Both analyses of a relatively rapid (COI) and a more slowly evolving mitochondrial marker (12S rDNA), consistently recovered a comb-like tree depicting hypothetical phylogenetic relations among the four main Triops lineages (T. granarius, T. australiensis, T. cancriformis-mauritanicus, T. longicaudatus-newberryi). The possibility of radiation, as suggested for other branchiopod crustaceans [40] and rapid diversification in Triops early in its evolutionary history, hence, cannot be excluded. Intercontinental dispersal and subsequent isolation followed by genetic differentiation under limited gene flow almost certainly led to speciation in the four main Triops lineages, which are largely restricted to different biogeographic regions. Divergence of the fifth lineage, T. sp., in turn, presumably results from a unique habitat shift from freshwater to saline habitats.

Based on molecular clocks, T. cancriformis and T. mauritanicus most likely diverged between 2.6–12.4 mya confirming the estimate by Korn and coworkers [41] based on 16S rDNA suggesting a potential link with the Messinian Salinity Crisis at the end of the Miocene (5–6 mya). Tectonic activity around the Gibraltar straight, isolating the Mediterranean from the Atlantic Ocean, and low rainfall resulted in strong variation in sea level including near complete drying of the basin [42]. Climate fluctuations, due to loss of the buffering capacity of the Mediterranean, may have led to contraction of suitable Triops habitat and a split between T. mauritanicus and T. cancriformis through vicariance. The clade formed by T. cancriformis, which, apart from its mostly European origin, also encompasses a Japanese population, is characterised by a large number of closely related haplotypes. As a result, Mantovani and coworkers [43] concluded that this taxon did not contain cryptic species. Low nucleotide and haplotype diversity over a wide geographical range (Europe and Asia) suggests a relatively recent postglacial colonisation of its current distribution area [25]. A growing number of studies show that postglacial colonised regions are characterised by lower genetic diversity [44,45]. From the beginning of the Quaternary (2.4 mya) until 10 kya ice sheets cyclically expanded and receded [43]. During cold periods, European T. cancriformis populations were most likely restricted to refugia southwards of the ice shelf. In contrast, cryptic diversity was demonstrated in its sister species T. mauritanicus, found in Iberia and North Africa [22]. The more southern distribution of this species can explain why it appears to have been less affected by the Pleistocene glaciations than T. cancriformis in terms of surviving lineages. Korn and coworkers [22] recognised six morphologically distinguishable lineages (five of which occur in Iberia). As argued by these authors, climate fluctuations in southern Europe associated with the Pleistocene glaciations may have contributed to fragmentation of species ranges facilitating the
emergence of different lineages in the Iberian Peninsula through founder effects and genetic drift [46].

Compared to the relatively modest genetic distances in T. cancroidiformis and T. mauritanicus, the T. granarius clade was shown to harbour more divergent haplotypes. T. granarius has a highly scattered distribution including Japan, China [47] and both northern- and southern Africa [48]. Given the vast size of its range it is not surprising that the most distant populations (Japan, southern Africa) are substantially differentiated, with a minimum genetic distance between them of 21.1%. Both COI and 12S datasets suggest that African-Eurasian T. granarius consists of different lineages including a South African, Namibian, North African and Japanese clade. Unexpectedly, the two southern African lineages did not cluster together. Instead the South African population was shown to be more closely related to lineages from Tunisia than to Namibian populations (min. K2P distance at 12S of 4.1% vs. 11.3%, respectively). Expanding on the findings by Korn and Hundsdoerfer [48], the South African haplotypes represent a fourth monophyletic lineage in T. granarius. Although this is subject to further morphological investigation, genetic distances suggest that the Japanese, the Tunisian, the South African and the Namibian clades probably represent four different species.

The T. australiensis clade, in turn, comprises several monophyletic groups and endemic haplotypes exclusive to specific localities. Four clades are restricted to rock pools on granite inselbergs, while the remaining lineages inhabit clay pans. Australian Triops are currently grouped into a single species, T. australiensis [18,49] but this may be unjustified since the monophyly of this nominal species is not strongly supported in our analyses. What is more, K2P genetic distances up to 14.2% at the COI gene are well in range of those used by other researchers to distinguish between species in other Triops lineages [22,31,35]. For example, the clade comprising rock pool populations from Walga Rock, Balan Rock and Bullamanya Rock in Western Australia, minimally diverged 9.4–11.8% at the COI gene from a clay pan population in the same area and 12.0–14.0% from the clade that inhabits the rock pools on the sandstone monolith Uluru in the Northern Territory.
relatively large genetic distances between rock pool and clay pan _Triops_ populations in Western Australia contrast with the geographic proximity of these populations providing a firm indication of habitat specialization. Overall, it is clear that _T. australiensis_ contains a lot of cryptic diversity. A detailed morphological revision of _T. australiensis_ including a discussion of the potential species status of different lineages is currently under preparation (B.V. Timms, unpublished data).

 Unexpectedly, the _Triops_ sp. population from the saline Lake Carey in Western Australia did not cluster together with other Australian populations, but instead emerged as a distinct lineage. K2P distances between _T_. sp. and its closest relatives _T. australiensis_ (min.: 12.3–17.9%) and _T. longicaudatus_ (min.: 15.8–16.3%) indicate that this lineage represents a species new to science awaiting formal description (B.V. Timms, in prep.). Tree topologies suggest that the species may have evolved during the initial radiation that gave rise to all present-day lineages coinciding with a unique habitat shift from freshwater to saline systems. Currently, it is the only notostracan population known from saline habitats (105 g L\(^{-1}\)). Finally, according to 12S tree topologies _T_. sp. could have closer affinities to American than to other Australian _Triops_ lineages and may reflect a biogeographic link mediated by historic long-distance dispersal. Considering the Cenozoic origin of living Notostraca, this biogeographic link between Australian and American lineages most likely reflects historic long-distance dispersal. Migratory birds and particularly waders, which often feed on branchiopod crustaceans and have been shown to carry propagules, are prime candidate vectors [30]. The bar-tailed godwit subspecies _Limosa lapponica baueri_, for instance, migrates back and forth from Alaska to Australia each year, often in a 11000 km nonstop flight [50] illustrating the potential of long-distance dispersal between North America and Australia.

The monophyletic _T. longicaudatus-necherryi_ clade is largely endemic to the Americas, while presumed _T. longicaudatus_ populations on Pacific islands such as the Galápagos, Hawaii and New Caledonia [21,36] may reflect efficient long-distance dispersal, presumably by avian vectors, as discussed above. Japanese records of _T. longicaudatus_, on the other hand, are attributed to recent anthropogenic introductions as a biological control agent in rice fields [48]. Based on our analyses we confirm the monophyly of North American _Triops_ populations but not the monophyly of the species _T. necherryi_ and _T. longicaudatus_. _T. necherryi_ differed only by 0.0–5.2% at COI and 1.0% at 12S from _T. longicaudatus_. The sequenced specimens, hence, should probably be considered conspecific. These findings support the need for a morphological taxonomic revision of _Triops_ across North America [51].

**Cryptic diversity and conservation implications**

Present-day Triopsidae consist of a limited number of core evolutionary lineages with generally large distributions corresponding to nominal species. However, a complex genetic substructure was shown in certain lineages, such as _T. granarius_ and _T. australiensis_, with monophyletic lineages inhabiting different parts of species ranges or contrasting habitat types (e.g. large clay pans versus small ephemeral rock pools). From a conservation point of view, these lineages can be considered evolutionary significant units [52]: appropriate conservation units of which preservation can be recommended. Whether these clades should be raised to species level, despite sometimes modest levels of genetic divergence, is open to discussion and will likely depend on whether reliable diagnostic morphological features can be formally identified.

**Evidence for an ancient radiation?**

Although fossils suggest that some living tadpole shrimp species closely resemble fossils as old as 250 million years, both standard and extreme molecular clocks for mitochondrial genes in invertebrates consistently date the most recent common ancestor of all living Triopsidae in the Cenozoic era, with estimates of divergence times among the basal lineages ranging between 29.75 and 53.6 mya (Paleogene period). An ancient (pre-) Mesozoic radiation as suggested by fossil remains, on the other hand, would explain today’s distribution of lineages by vicariance rather than long distance dispersal of several lineages. If we would assume that _Lepidurus_ and _Triops_ indeed existed as separate lineages in the middle Triassic (220 mya) then this would imply a mutation rate at the COI gene of about 0.16% per mya which is highly unrealistic since the lowest rate of evolution observed at this gene in invertebrates is 1.2% per mya [53]. Contemporary tadpole shrimp species thus almost certainly are the result of a more recent radiation from a single ancestral lineage surviving into the Tertiary rather than a group of relict lineages from an earlier (pre-) Mesozoic radiation that presumably gave rise to a number of extinct ancient lineages known from the fossil record [4]. The scenario of a recent radiation, dispersal and speciation in isolation adequately explains why, despite a (pre-) Pangaea origin of the Triopsidae, a number of lineages are linked to biogeographic regions (e.g. _T. australiensis_ and the Australian _Lepidurus_ sp. in Australia, _T. longicaudatus-necherryi_ in the Americas, and _T. cancarmiformis_ in the Palearctic).

The supra-anal plate, which is the key diagnostic character to distinguish _Lepidurus_ from _Triops_, is a trait which modern _Lepidurus_ species share with a number of Triassic and Cretaceous fossils [11,12,36]. Given the recent origin of _Lepidurus_, Mesozoic tadpole shrimp with supra-anal plates probably should not be classified in the same genus. The supra-anal plate, as such, can be a primitive character which has been lost both in a number of fossil Triopsidae as well as in the extant _Triops_ representatives. On the other hand, considering the fact that the oldest known triopsid fossils lack a supra-anal plate [5], it is also possible that it is a derived trait which has evolved multiple times both in Mesozoic triopsids and, again, in the common ancestor of modern _Lepidurus_ lineages. Evidently, current tadpole shrimp species having evolved quite recently are not living fossils and the myth that _T. cancarmiformis_ would be the oldest species on the planet must be firmly discredited. “Living fossil” is undoubtedly an attractive tag to draw attention to peculiar taxa exhibiting primitive traits. Yet, this term can be misleading and the intrinsic scientific value of such a label is not uncontested. Different definitions are in use and particularly in popular scientific literature “living fossil” is often used over-simplistically as a term to designate an ancient species which has presumably survived relatively unchanged until present day. Not surprisingly, creationist lobbyists eagerly enumerate examples of morphological stasis [54] although these by no means provide evidence against evolution by natural selection. Nonetheless, the “living fossil” concept, which was originally coined by Darwin [55], can also be more stringently and realistically defined as a taxon which belongs to a group with a long evolutionary history, has retained a number of primitive characters and has few living relatives. According to this definition the members of the order Notostraca, in general, can be considered living fossils. At least two main factors are likely to have contributed to morphological stasis in tadpole shrimp: the simple body plan consisting of a dorsal armor and serially repeated structures; traits which are also present in other “living fossils” such as horseshoe crabs [56] and chitons [57], and the very specific habitat type in which these organisms have persisted during their evolutionary
history. Since the appearance of planktivorous fish in the Devonian and Carboniferous, large predation sensitive branchiopod crustaceans such as Notostraca are restricted to extreme aquatic systems that lack fish such as temporary ponds and saline lakes: a very specific niche [4,58] in which they still persist today.

Conclusions

Although some present day tadpole shrimp species closely resemble fossil specimens as old as 250 mya, no molecular support was found for an ancient (pre) Mesozoic radiation. Instead, living tadpole shrimp are most likely the result of a relatively recent radiation in Cenozoic and close resemblances between recent and fossil taxa are probably the result of the highly conserved general morphology in this group and of homoplasies. It is clear that more and more evidence is accumulating indicating that a lack of readily observable phenotypic change (morphological stasis) during the evolutionary history of a certain lineage does not necessarily imply evolutionary stasis [59]. As shown in this study, recent species which are virtually identical to fossils in terms of their morphology may represent very different evolutionary lineages.

Methods

COI and 12S rRNA genes were sequenced for up to six tadpole shrimp specimens per population. DNA extraction, polymerase chain reaction and sequencing protocols are provided in Text S2. All new samples were collected by the authors in the field between 2008 and 2010, using a simple dipnet (5 mm mesh). Exceptionally, T. newberryi specimens from a population in Kansas, USA were laboratory-hatched from sediment in distilled water at 20°C.

Ethics statement

Collected animals were anaesthetized in carbonized water before transfer to ethanol. Collection and export permits were granted by the Free State Province Department of Tourism, Environmental and Economic affairs (South Africa): permit no.: HK/P1/07375/001 and by the Australian government: permit no. SF007548 and SF005789.

Genetic data analyses

Sequences were aligned (ClustalW multiple alignment: [60]) and trimmed in BioEdit Sequence Alignment Editor v.7.0.0 [61]. 120 additional COI and 74 12S sequences were drawn from GenBank and aligned to the newly obtained DNA fragments (Table S1 provides additional details and GenBank accession codes). The cyclosterid conchostracan Cyclasterhisoptes hislopae (Baird, 1859) was selected as outgroup. Finally the alignment was inspected by eye for any anomalies and found to be straightforward. All new sequences were deposited in GenBank under accession codes [JN175223–267; JN190396–398].

For the COI and 12S datasets, jModeltest v.0.1.1 [62] respectively selected the TIM+H+G (with a proportion of invariable sites of 0.496 and a gamma-shape parameter of 0.775) model, with nucleotide frequencies $A = 0.32, C = 0.18$, $G = 0.10$ and $T = 0.38$ and rate matrix $(1.00, 23.28, 0.06, 0.06, 14.10, 1.00)$ and the TIP2uf+G (with a gamma-shape parameter of 0.33) model, with nucleotide frequencies $A = 0.37, C = 0.17$, $G = 0.11$ and $T = 0.35$ and rate matrix $(11.35, 46.47, 11.35, 1.00, 0.46, 0.17)$ as best fitting models of evolution. Model averaged phylogeny analyses were performed in the same software, indicating that all 88 tested models rendered nearly identical trees for both the COI and 12S data.

Dating splits between passively dispersed aquatic invertebrates is problematic since long distance and even intercontinental dispersal mediated by vectors such as water birds is a realistic possibility [63,64]. In addition, the highly conserved general morphology in Notostraca throughout their evolutionary history impedes the use of fossils to calibrate molecular clocks. A likelihood ratio test [65] performed in TREE-PUZZLE [66] rejected clock-like evolution for both the COI and 12S datasets.

Even though this means that we cannot linearly calculate divergence times for individual splits in the phylogenetic trees based on genetic distance, we can broadly estimate the timing of diversification and the likelihood of an ancient radiation by using the range of molecular clocks known for invertebrates. Although this approach which is used due to the impossibility of fossil calibration is relatively coarse, at the very least it allows distinguishing between an ancient (pre) Mesozoic radiation suggested by fossil remains and a more recent Tertiary or Quaternary radiation. A prerequisite, however, is that sequences are not oversaturated in terms of accumulated mutations. As a result, substitution saturation for the third codon position was tested for both the COI data in DAMBE [64] as well as the 12S to estimate if substitution saturation is significant [64]. In addition, the highly conserved general morphology in Notostraca throughout their evolutionary history impedes the use of fossils to calibrate molecular clocks. A likelihood ratio test [65] performed in TREE-PUZZLE [66] rejected clock-like evolution for both the COI and 12S datasets.

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Phylogenetic reconstruction was performed for both mitochondrial DNA datasets independently, using neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML), quartet puzzling (QP) methods and Bayesian inference (BI). MP analyses were conducted in Paup* v.4.0b10 [75] using the PaupUp graphical interface [76]. For the ML analyses PhyML [77] was used. ML analyses in PhyML (1000 bootstrap replicates, NNI) were run according to the evolutionary model and parameters as selected by jModeltest. NJ analyses were performed in MEGA using the following settings: maximum composite likelihood, Tamura-Nei substitution model, defined G and 1000 bootstrap replicates. Quartet puzzling maximum likelihood analyses were performed in TREE-PUZZLE according to the model and parameters selected by jModeltest. Bayesian analyses were conducted in MrBayes v.3.1.2 [78] according to the evolutionary model and parameters suggested by jModeltest. MrBayes ran for 5×106 generations (let number of substitution types $= 6$, rates = invgamma, number of rate categories for the gamma distribution $= 4$, sampling frequency $= 100$ generations) until a standard deviation of split frequencies of 0.0078 was attained. An outgroup ($C. hislopi$) was defined and in order to only include trees in which divergence of the Markov chain had been reached, we chose a burn-in of 25%. The remaining trees were used to construct a 50% majority consensus tree.

Finally, in order to integrate the information provided by both genes, phylogenetic analyses were also conducted on a combined dataset containing both COI and 12S sequences. Parameters for both genes were estimated independently in MrBayes using the ‘unlink’ command (partition topologies $= 2$: 12S, COI, let applyto $= 1$, nst $= 6$, rates = invgamma, ngammacat $= 4$, let applyto $= 2$, nst $= 6$, rates = invgamma, ngammacat $= 4$, unlink
shape = all). MrBayes ran for 8×10^6 generations with a sampling frequency of 100 and a defined outgroup (C. hirsuta).

In case phylogenetic analyses did not unequivocally support monophyly of the two Notostracan genera, constraint analyses using Kishino-Hasegawa- [79] and Shimodaira-Hasegawa [80] tests for the ML tree and Kishino-Hasegawa as well as Templeton - and winning site tests for the MP tree were conducted in Paup* to test whether enforcing monophyly of genera led to a statistically significant increase in tree likelihood.

Supporting Information

Figure S1 Bayesian inference phylogram based on combined COI and 12s rRNA sequences. Numbers at nodes represent bootstrap values of maximum likelihood (ML), maximum parsimony (MP) and posterior probability values of Bayesian inference (BI). Unsupported groupings are indicated using a ‘*’. No value is provided if this method of phylogenetic inference would suggest an alternative placement of the corresponding clade in the phylogeny.

Table S1 Overview of investigated Notostraca samples.

Table S2 Kimura 2-parameter distance matrix (min.-max.) between investigated notostracan lineages based on COI (below diagonal) and 12S rRNA (above diagonal) genes. Empty cells indicate that sequence information was unavailable.

Table S3 Divergence times between main Triops clades (minimum-maximum) based on the standardly used average COI molecular clock (1.40% mya^-1; below diagonal) and 12S molecular clock (0.5% mya^-1; above diagonal) for crustaceans.

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