

The use of 18S rRNA for identification of the first record of Tadpole Shrimp *Lepidurus apus* (Linnaeus, 1758) from Jordan

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Abstract. Abushattal S, Alnasarat H, Alnaimat SM, Eid E. 2024. The use of 18S rRNA for identification of the first record of Tadpole Shrimp *Lepidurus apus* (Linnaeus, 1758) from Jordan. *Biodiversitas* 25: 1223-1229. The tadpole shrimps are living fossils that survive in temporary water pools and are considered benthic organisms that swim and feed on tiny organisms. Two genera of tadpole shrimps exist worldwide: *Triops* and *Lepidurus*, which share similar ecological niches and habitats but never coincide. The occurrence of the tadpole shrimp *Lepidurus apus* in Jordan was confirmed for the first time using the 18S rRNA Gene-Based PCR Assay. The experimental approach encompassed the amplification of biological specimens utilizing a universal amplicon measuring approximately 1,500 base pairs (bp), specifically targeting the 18S ribosomal RNA (rRNA) gene. Subsequently, the acquired sequences underwent scrutiny through BlastN search against the GenBank database for validation and comparative analysis. Genetic analysis was needed to define the species accurately, especially with tadpole shrimp's generally high morphological variability. This species was recorded in a temporary freshwater pool in the desert of Al-Jafr in Ma'an governorate in southern Jordan. The DNA analysis showed a 100% identical sequence of the 18S rRNA gene with *Lepidurus apus* sequences. This record is important due to the ecological role of the tadpole shrimps as filter feeders, consuming algae, bacteria, and other small particles of organic matter, and as a food source for various fish, birds, and other aquatic animals. In addition, it will pave the way for future research to identify its economic importance, such as its role in mosquito control and its potential role in aquaculture. Finally, tadpole shrimp are sensitive to environmental changes and could be used to indicate climatic changes and habitat degradation. This is mainly due to their sensitivity to changes in water quality, including temperature, pH, and chemical composition, and their niche requirements for survival and reproduction. In addition, monitoring tadpole shrimp distribution and comparing it with historical records can reveal patterns of climate-induced range shifts.

Keywords: Crustaceans, desert, DNA barcoding, Jordan, *Lepidurus apus*, phylogenetic analysis, temporary waterpools

INTRODUCTION

The tadpole shrimps or large branchiopods belong to the order *Notostraca* and are solely aquatic life (Belk and Brtek 2015). They are living fossils (Atashbar et al. 2013), and their name is derived from the large horseshoe-shaped dorsal carapace and their superficial resemblances to frog larvae (Martin and Boyce 2005). They survive in temporary water pools (Maeda-Martinez et al. 1997; Belk et al. 2002; Boix et al. 2002; Grygier et al. 2002; Olesen and Møller 2014; Belk and Fenton 2019), where their ecology is closely connected with periodic drying off (Rogers 2009). They are considered benthic organisms that swim and feed on ventral surfaces, burrowing into the bottom sediments in search of tiny organisms (Scholnick and Snyder 1996). Tadpole shrimps are omnivorous organisms that feed on detritus, scavenge for food, and prey on other organisms (Cvetkovic-Milicic and Petrov 1999). Scholnick and Snyder (1996) stated that the wide fluctuations in population density characterize tadpole shrimps as they become more active in searching for food when their density increases. They also added that any shortage in food will reduce their development and

decrease fecundity, while mortality increases due to cannibalistic behavior. The tadpole shrimps contain two genera: *Triops* and *Lepidurus* (Rogers et al. 2019). These genera share similar ecologic niches and habitats but never co-occur (Rogacki and Brysiewicz 2021). The individuals from the two genera demonstrate different preferences related to ambient temperature, where cold water in early spring encourages *Lepidurus* species, and *Triops* species require warmer water in the spring and summer seasons. (Cáceres and Roger 2015; Subramoniam 2017).

The tadpole shrimp *Lepidurus apus* (Order: *Notostraca*) is a branchiopod freshwater crustacean recognized by its large, horseshoe-shaped dorsal carapace (Weeks and Sanderson 2016). *Lepidurus* has a supra-anal plate, distinguishing it from other *Triops* species (Sorek et al. 2016). As a species that lives in temporary water pools that dry up within weeks, it has developed a unique adaptation to survive by producing "resting eggs," or cysts, that reside in the top layers of temporary water body sediments and can withstand droughts, physical damage, lack of oxygen, extreme temperatures, UV radiation, and even survive passage through the digestive system of predators (Rogacki and Brysiewicz 2021). The eggs will hatch in batches under

favorable conditions (Savojarado et al. 2019) and can be spread passively by several means, such as animals, wind, and floods (Sorek et al. 2016). The *Lepidurus* can complete its life cycle within a week after rainfall (Chen et al. 2023; Aloufi and Obuid-Allah 2014).

Lepidurus apus is known to have a wide distribution range that exceeds other *Triops* species, as it was recorded in various continents, including Europe, Northern Africa, India, Asia, and Australia (Padhye 2015; Padhye 2016; Borgstrøm et al. 2018). The *Triops* have been recorded in Europe, Northern and Southern Africa, Southern Asia, and Japan (Zierold 2006). In the Middle East, two genera of Notostracans are present, including three species belonging to *Triops* genera; the *T. granarius*, which was found in Iraq and the temporary ponds of Qatar (Merza 2017; Chen et al. 2023), the *T. longicaudatus*, which was reported in Qatar in 2017 (Kardousha 2016; Kardousha 2017) and in northwestern Saudi Arabia (Aloufi and Obuid-Allah 2014), with very recent records from Azraq Wetland Reserve in Jordan (Ghlelat and Al-Hreisha 2023) and the *Triops cancriformis* reported from Yemen and Iran (Kangarloe et al. 2013). The *Lepidurus* is represented in two species, the *L. couesii*, confirmed from Syria and Saudi Arabia, and the *Lepidurus apus*, which have been recorded in Iran (Kangarloe et al. 2013).

This study reveals for the first time the presence of tadpole shrimp, *Lepidurus apus*, from Jordan using genetic identification and partial sequence of the 18S RNA gene. This study will shed light on important organisms for ecosystem balance, and potentially economic perspectives, especially as a food source, and it will inspire scientists working in this field to expand the available knowledge through investigations of the species distribution, ecology, taxonomy, conservation, and potential economic benefits that are poorly known to date in Jordan and neighboring countries.

MATERIALS AND METHODS

Study area

The specimens were collected from a temporary freshwater pool (30°34'30.9"N 35°52'06.3"E) situated in the desert of Al-Jafr in Ma'an governorate located in southern Jordan (Figure 1). This pool was reported by locals who noticed the presence of tadpole shrimps and promptly sent photos to the authors at Al-Hussein bin Talal University, which led to the researcher's mobilization to collect samples and investigate further. These pools tend to occur after winter and evaporate in early summer. This area is part of the Saharo-Sindian-Nubo-Sindian phytogeographical region and specifically within the gravel hamma vegetation type, which is characterized by the growth of desert annuals mainly from Asteraceae and Chenopodiaceae families. Additional species found include *Vachellia gerrardii*, *Artemisia judaica*, and *Ziziphium nummularia* (Taifour et al. 2022).

Procedures

Sample collection

Samples were obtained from the water pool and transferred to the laboratory of the biology department at Al-Hussein Bin Talal University. The specimens were frozen at -20°C.

DNA extraction

The G-spin DNA isolation kit from iNTRON Biotechnology (Korea) was used to extract DNA from the Tadpole shrimp specimens following the manufacturer's instructions. The frozen specimens underwent a process of fine grinding followed by mixing with specialized reagents from the kit. Subsequent steps involved sequential incubation at distinct temperatures of 56 and 70°C, followed by centrifugation at 13,000 revolutions per minute (rpm) for one minute to separate DNA. The elution of DNA from the Spin Column was achieved using Buffer CE. Finally, the extracted DNA was stored for preservation at -20°C.

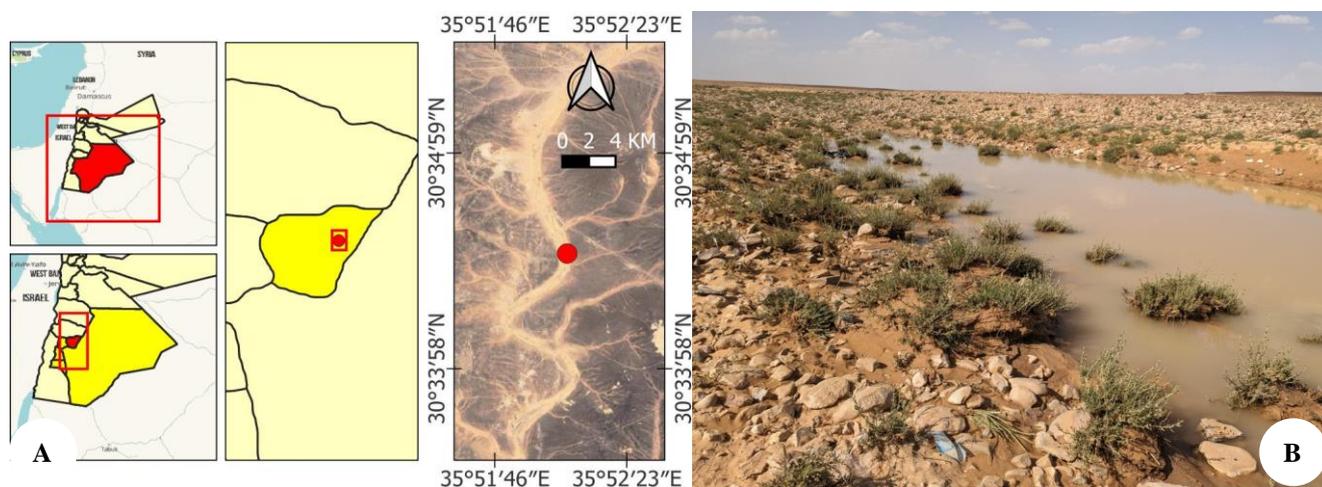


Figure 1. A. Map of the study site in Al-Jafr desert, Husseiniyyeh, Ma'an, Jordan. B. Temporary freshwater pool in Al-Jafr desert, Ma'an, Jordan

Polymerase Chain Reaction (PCR) amplification of 18S rRNA

We performed PCR amplification targeting a 700 bp segment of the DNA extracted from the specimens, explicitly focusing on the 18S rRNA gene using custom-designed primers (Ribosomal 18S marker, with 18S_F (5'-TTCACCTACGGAAACCTTGTTACG-3') and 18S_R (5'-GATACCGTCCTAGTTCTGACCA-3') primers). These primers are recognized markers for this species. PCR was conducted using a (LILIF 2x Master mix, Korea) in a 50 μ L reaction. The process involved denaturation at 94°C and annealing at 58°C, facilitating successful amplification of the 18S rRNA region. This provided the necessary DNA fragments for subsequent species identification and analysis.

Identification Using the National Center for Biotechnology Information (NCBI) GenBank

After successfully amplifying the tadpole shrimp 18S rRNA gene, species identification was performed using the NCBI GenBank database. The Basic Local Alignment Search Tool (BLAST) analysis assessed similarities between the amplified sequence and existing sequences in the database. We utilized fifteen 18S rRNA sequences sourced from NCBI, which exhibited significant identity and closer proximity to our isolate sequence, in order to construct the phylogenetic tree. Comprehensive analysis of the results involved evaluating sequence similarity scores, alignment matches, and additional parameters like sequence identity and query coverage.

Phylogenetic analysis and haplotype study

For a more comprehensive understanding of the species, a phylogenetic analysis was undertaken by comparing the yielded 18S rRNA gene sequence with those of other identified and available species globally. A phylogenetic tree was constructed using MEGA11 software and the maximum likelihood method to elucidate the evolutionary relationship between our specimens and the known *Lepidurus apus* identifications. Additionally, employing the Pearson correlation measures the strength of the linear relationship between two variables. This scale ranges from -1 to 1, where -1 represents a complete negative linear correlation, 0 indicates no correlation, and +1 denotes a total positive correlation. Moreover, a thorough genetic variation analysis was conducted using DnaSP6. This involved examining haplotypes and their frequencies within the population. A visualization of haplotype connections was achieved through a haplotype network analysis, offering insights into evolutionary relationships and potential population dynamics. This approach allowed for a deeper understanding of genetic structure and diversity, including parameters such as haplotype count (h) and nucleotide diversity (π) for all sequences, irrespective of their source. The University of Otago Popart 2023 tool was employed with the genome formatted in Nexus to create a reticulate tree of haplotypes.

RESULTS AND DISCUSSION

Results

Figure 2 shows the developed phylogenetic tree constructed using maximum likelihood analysis. It elucidates our samples' evolutionary relationships compared to 19 selected species from the NCBI. Strikingly, our results demonstrate a robust and undeniable connection between our sample, which takes the accession number OQ976891 and isolate name HSS2, and the crustacean species *Lepidurus apus* (Figure 3). Despite carefully selecting the 15 species that exhibited the highest degrees of relatedness to OQ976891 based on NCBI data, it is evident that *Lepidurus apus* shares the strongest common ancestry with our investigated specimen. The discovery of this unexpected potential link between OQ976891 and the crustacean lineage highlights the necessity for additional investigations to unravel the evolutionary significance of this compelling ecological relationship.

We present our findings on the relationship between different haplotypes and the variation of sequences observed in the target gene among compared species of tadpoles. Our study, depicted in the Figure 2.C, meticulously illustrates the number of mutations generated in the target gene across diverse species. The correlation observed in the data indicates the relationship between haplotypes (groups of genetic variants inherited together) and observed sequence differences within tadpole populations. To determine the correlation, different statistical methods were used, including the Pearson correlation coefficient, to measure the correlation within the tadpole groups. Linkage assessment involved analyzing the frequencies of different haplotypes within tadpole populations and comparing these frequencies to observed sequence variations. This comparison was performed using statistical tests to determine the degree of association between haplotypes and sequence variations.

Figure 2.B presents a detailed analysis of haplotypes that were identified through the construction of a phylogenetic tree depicting species relationships. The figure illustrates the number of distinct haplotypes identified within the species, offering valuable insights into the genetic diversity present within and among populations. Additionally, the figure visually represents the locations of specific mutations within the haplotypes, shedding light on the genomic changes that have shaped the evolutionary history of these organisms.

The results presented in Figure 4 reveal the identities between various species of tadpole shrimp, focusing on OQ976891. Notably, OQ976891 exhibited an outcome, displaying a 100% identical sequence of the 18S rRNA gene with *Lepidurus apus* sequences. The observed level of sequence similarity strongly indicates a close phylogenetic relationship between OQ976891 and *L. apus*. The 18S rRNA gene, known for its high conservation and slow evolutionary rate, serves as a reliable molecular marker in phylogenetic analyses. The identical sequence shared by OQ976891 and *L. apus* provides robust evidence of a common ancestry, suggesting that OQ976891 likely belongs to the same taxonomic group as *L. apus*. This

discovery contributes significantly to our understanding of tadpole shrimp diversity and evolution, providing insights into the genetic relationships within this group of crustaceans.

Further investigation into the biological implications of this relatedness could illuminate the ecological roles and historical biogeography of these organisms.

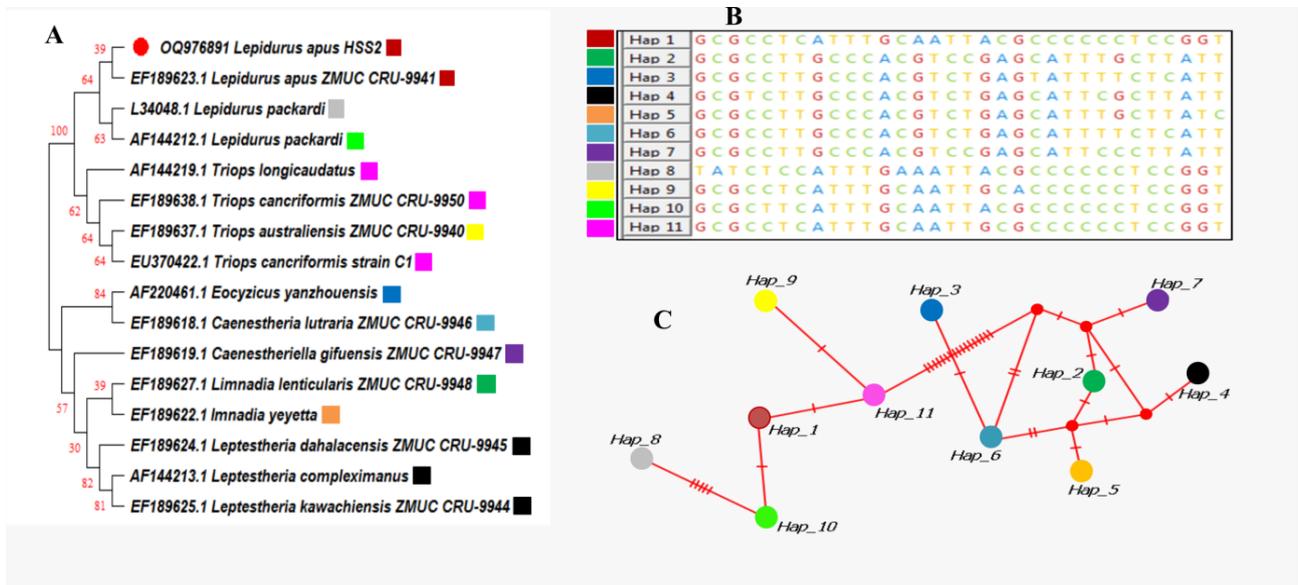


Figure 2. A phylogenetic tree was constructed using MEGA 11 software. Subsequently, haplotype analysis (B) and genetic landscape analysis for haplotypes (C) were performed. The isolate under investigation, represented by OQ976891, is highlighted proximate to the red circle in Figure 2A



Figure 3. Tadpole Shrimp *Lepidurus apus* from Al-Jafr in Ma'an governorate in Jordan

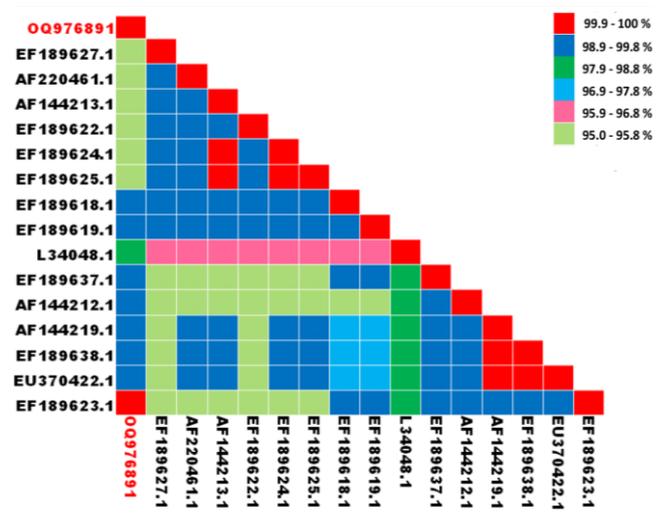


Figure 4. The Fasta sequences of all isolates developed a color contrast matrix showing sequence identity states

Discussion

This article identifies the presence of the tadpole shrimp, *Lepidurus apus*, for the first time in Jordan by sequencing the DNA barcode using the 18S rRNA Gene-Based PCR Assay and comparing the results with other relevant genetic markers to assess genetic distinctiveness. The genetic analysis was helpful in accurately defining the species, especially with the high morphological variability of tadpole shrimp, which makes it challenging to identify the species level (Rogacki and Brysiewicz 2021). By examining the distribution of haplotypes and identifying the positions of mutations, researchers can gain a deeper understanding of how these species have diverged over time and adapted to their respective environments. This comprehensive approach is crucial for identifying specific sets of alleles passed down from one generation to another, thereby tracking genetic diversity within and among populations. Studying the distribution of individual patterns provides insights into population genetic structure, offering a cumulative perspective on their evolutionary journey. Simultaneously, identifying mutations within these patterns unveils molecular signatures of evolutionary processes, serving as markers for genetic variations over time akin to a molecular clock and aiding in estimating species' divergence from their common ancestors. The phylogenetic tree represents the evolutionary connections among the species, and the accompanying data on haplotype diversity and mutation hotspots provide crucial information for unraveling the genetic mechanisms that have driven speciation and adaptation in this group. This article sheds light on the ecological significance, genetic diversity, and conservation imperatives of tadpole shrimp within the Jordanian context.

Knowledge of branchiopods in the inland waters of Jordan is inferior, with only a few observations by Nelson (1973), who reported *Chirocephalus* sp. from Qa Kanna, near Azraq. Eid (2007) has described three species for the first time in Jordan after investigating some temporary water pools within the forested region of Ajloun and Dibeen forest reserves of northern Jordan. His records included the species of *Cyzicus tetracerus*, *Chirocephalus bairdi*, and *Branchinecta ferox*. Recently, a new record of the *Triops longicaudatus* was reported by from the mudflat areas in Azraq Wetland Reserve in Jordan (Ghlelat and Al-Hreisha 2023). Our results are consistent with those reported by Subramoniam 2017, indicating that the *Lepidurus* species thrive in cold water environments. In this habitat, our specimens survive in Ma'an. At the same time, *Triops* species identified by Ghlelat and Al-Hreisha 2023 in Azraq require warmer water similar to the water found there. This research attempts underscore the dynamic nature of Jordan's aquatic ecosystems.

Despite the limited knowledge, these animals hold significant ecological importance since they are considered filter feeders, consuming algae, bacteria, and other small particles of organic matter (West 2016). This helps to keep water bodies clean and prevents the growth of harmful algae blooms. They are also a food source for various fish, birds, and other aquatic animals. Moreover, tadpole shrimp species have several economic benefits to humans, as they

often prey on the larvae of mosquitoes (Waterkeyn et al. 2016; Dambach et al. 2020; Naganawa 2020). The tadpole shrimps can play a role in aquaculture (Van Damme 2018), and they are often used in astrobiological experiments (Rogers 2011). This highlights the importance of temporary water pools as they provide a home for animals sensitive to environmental changes, which can be used to indicate the health of temporary freshwater ecosystems.

Despite the importance of this group of organisms to the environment and human economy, there is minimal research in Jordan on their diversity, population, distribution, and threats. Brendonck et al. (2008) stated that large branchiopod crustaceans are believed to be threatened globally, and other scholars confirmed that some branchiopod crustaceans have become extinct in several European countries (Eder and Hödl 2002). Cáceres and Rogers (2015) mentioned that species of this group have become progressively rare in many regions of the world. In addition, several species of Notostracan are protected in various countries and assessed as threatened according to the IUCN Red Lists (Maier 1998; Eder and Hödl 2002). Threats to these species are mainly due to the irreversible disappearance of their habitats (Brendonck et al. 2008), climate change (Jankowiak 2011; Angeler et al. 2008), intensive farming (Feber 2011; Zierold 2006) and human interference with the shape of river valleys (Feber 2011).

The issue is not limited to the lack of data about tadpole shrimps, but there is a shortage of information about aquatic crustaceans surviving in the dry areas of Jordan. This research is a starting point for studying how these aquatic crustaceans adapt to the arid environment, understanding speciation, their roles in food webs, specific ecological niches, threats, and status. In addition, studying aquatic crustaceans will play a role in understanding bird migration, especially since Ma'an is considered a route for migratory soaring birds (Khoury 2017). The availability of large numbers of these tadpole shrimps will undoubtedly provide a stopping point for many migratory birds, such as egrets, herons, ducks, coots, and grebes. In addition, adapting tadpole shrimps to the dry environment is an interesting matter, especially in light of several studies that have confirmed the adaptation of many plant and animal species to the desert and arid environment of Jordan (Al-Eisawi 2007; Saif and Al-Eisawi 2012).

Rogacki and Brysiewicz 2021 provided a complete revision of the threats facing tadpole shrimps, especially with the status of most of these rare or endangered species in many regions of the world (Cáceres and Roger 2015). Among these threats are the temporary water habitats, which are facing various challenges in Jordan due to weak rainfall and climate change (Ministry of Environment 2023). Despite the lack of studies on the impact of agriculture on tadpole shrimps, several scholars have indicated that agricultural expansion in the southern desert destroyed desert vegetation, eventually clearing the original vegetation and leveling the ground, causing the loss of the original habitat (Disi et al. 2014). Disi et al. 2014 stated that the deterioration of surface water quality due to urbanization, industrialization, agricultural practices, and effluents of wastewater treatment plants affected the

ecosystems in major surface water basins in Jordan. Since tadpole shrimps are water-dependent species, studying pollution potential in Ma'an and identifying its sources is recommended. This must include management measures to ensure that no pollutants are released into the natural ecosystems in Ma'an.

This article provides background on a newly discovered species and expands the knowledge on species composition in Jordan and their distribution. However, we have highlighted the importance of tadpole shrimp and Notostracan as flagship species for temporary waters with high environmental and potential economic benefits. Further investigations in Jordan are highly recommended to identify new localities and to understand species richness and composition in the identified areas.

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