Development of a Bio-indicator to Assess Water Quality in Ephemeral Ponds

by

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Research Problem and Objective

The overall objective for the project is to determine what environmental factors have the highest influence on the water quality of ephemeral ponds within New Mexico and how differences in water chemistry can alter the crustacean composition within ponds. Once it is known how the environment affects species distribution of a keystone crustacean species, like the tadpole shrimp, we can use this information to develop a bio-indicator for ephemeral pond water quality. The first part of my project is to characterize what effect land use practices (i.e. ranching & urban areas) can have on the water quality of ephemeral ponds located in desert scrub and desert grassland habitats. Previous research has assessed the water chemistry of ponds in a desert scrub habitat of southwestern New Mexico, near the city of Las Cruces. Next, the water chemistry of the ponds located on Otero Mesa, a desert grassland, have to be measured to facilitate comparison of the chemistry measurements across different environments.

For the second part of the project, it needs to be determined if the species of *Triops* found near Las Cruces, NM and those on the Otero Mesa are indeed different. There are slight morphological differences between the species, but to accurately assess species distinctness, a genetic analysis will be performed. By sequencing a small region of the genome, it has already been determined that there are two species of *Triops* that occur near Las Cruces, where it was previously thought all the species were the same. I will sequence the same genomic region from individuals found in ephemeral ponds from Otero Mesa and will use a phylogenetic analysis to compare *Triops* species. Combining the environmental data and the genetic data will clarify if water quality is affecting species distribution of *Triops*.

Methods

Water Chemistry

The pond water used for water chemistry analysis was reconstituted in the laboratory by adding distilled water to dry playa soil due to the infrequent filling of ephemeral ponds in the study area. The first 2 - 4 cm of soil was collected from the bed of each pond between 2011 and 2014, from randomly selected spots throughout the entire area of the playa. Soil was combined into gallon-sized bags for storage and four bags were collected from each playa lake. A previous study on playa water chemistry focused on ephemeral ponds located near Las Cruces, NM and included three natural playa lakes (PL-06, PL-07, PL-09), seven modified playa lakes referred to as stock tanks (PL-03, PL-05, PL-08, PL-11, PL-12, PL-33, PL-36), and two man-made flood retention ponds (FP-02, FP-03) (Fig. 1). The playa lakes and stock tanks (except PL-33 and PL-36) are located on the Chihuahuan Desert Rangeland Research Center (CDRRC) or the Jornada Experimental Range (PL-11 and PL-12). The PL-36 stock tank is located on public land (Bureau of Land Management) and PL-33 is on Corralito's Ranch (Las Cruces, NM). The flood retention ponds, FP-02 and FP-03, are the result of flood control dams built in the city of Las Cruces, New Mexico in 1962 and 1975, respectively. The additional playa lakes added for the current study (PL-52, PL-53, PL-54, PL-55, PL-56) are

all stock tanks and are located in the southern region of the Otero Mesa in eastern New Mexico (Fig. 1).

To approximate playa lake conditions after a rainfall, 750 g of soil were added to 2500 mL of distilled water in a plastic container referred to as a mesocosm. There were four replicate mesocosms for each playa lake. The water and soil mixture was allowed to acclimate for 48 hours prior to water chemistry analysis to simulate conditions under which *Triops* cysts hatch (Scott and Grigarick 1978; Fry et al. 1994). Dissolved oxygen (DO), pH and salinity were measured directly with a Hach model HQ 40d18 portable combination meter (Hach Company, Loveland, CO). To determine ammonia, nitrate-N (NO_3-N), nitrite-N (NO_2-N), phosphate, sulfate and sulfide content in each mesocosm, 10 mL aliquots were drawn from each mesocosm and analyzed colorimetrically with a LaMotte Smart2 colorimeter and the corresponding test kits (LaMotte Company, Chestertown, MD).

A principal components analysis (PCA) was performed in CANOCO 5 (ter Braak and Šmilauer 2012) to examine the relationship between water quality parameters and the ponds in order to assess whether presence or absence of *Triops* species was influenced by environmental parameters. The analysis was unconstrained and used the Euclidean distance between variables to separate the variation.

DNA sequencing

Live samples were collected from all playa lakes using 3mm mesh seines and immediately placed in 95% ethanol for preservation. Samples were stored at -20°C until DNA isolation. Samples from nine playa lakes (PL-03, PL-05, PL-07, PL-08, PL-09, PL-11, PL-33, PL-36, FP-03) were previously sequenced using the mitochondrial control region (mtCR; Horn et al. 2014). Therefore, only *Triops* samples from the remaining eight playa lakes (PL-06, PL-12, PL-52, PL-53, PL-54, PL-55, PL-56, FP-02) were sequenced for the current study. DNA from a total of 10 samples from each of these eight lakes was extracted using the HotShot method described by Montero-Pau et al. (2008). The protocol was modified to use 75 µl aliquots of the lysis buffer and neutralizing solution and incubation at 95°C was for 45 minutes. Amplification of the mtCR by polymerase chain reaction (PCR) was performed with primers described by Horn et al. (2014). PCR reaction volumes were a total of 25 µl and consisted of 10 µl GoTAQ Green Master Mix (Promega Corp., Madison, WI), 400 pM of each forward and reverse primer and 1 ng/µl of genomic DNA. PCR reactions were run in a Fisher thermocycler (Fisher Scientific Inc., Pittsburgh, PA) with the following conditions: 94°C for 2 minutes, followed by 35 cycles of 94°C for one minute, 50°C for one minute, 72°C for one minute and a final extension of 72°C for 15 minutes. PCR products were checked for strength of amplification on a 1% agarose gel and purification of PCR products was performed with ExoSAP-IT (USB Corporation, Clevland, OH). To further prep the samples for DNA sequencing, the purified PCR products were used in a cycle sequencing reaction using the BigDye Terminator V3.1 Kit (Life Technologies, Carlsbad, CA). Cycle sequencing products were cleaned prior to sequencing using the DyeEx 2.0 Spin Kits (Qiagen Inc., Valencia, CA). All samples were sequenced on an ABI 3130*xl* in both the forward and reverse directions (Life Technologies, Carlsbad, CA).

Sequences were aligned using the assembly function in the program Geneious Pro v5.4.6 (Drummond et al. 2011). The sequences generated from the Otero Mesa *Triops* spp., PL-06, PL-12, and FP-02 were combined with sequences from Horn et al. (2014) that included mtCR sequences from the remaining playa lakes. The program Arlequin v3.5

(Excoffier and Lischer 2010) was used to gather metrics on the sequence data including the number of transitions, transversions, indels, substitutions, polymorphic sites, the GC content, and the nucleotide and haplotype diversity. To determine the relationships between the species of *Triops* from southern New Mexico and to assess if the *Triops* spp. from Otero Mesa is a unique species, a maximum likelihood (ML) tree was assembled using the program PAUP (Swofford 2003). To assess the confidence of tree topology, the ML tree was bootstrapped using 100 replications. Evolutionary relationships between genetic haplotypes (i.e. maternal lineages) were resolved by a haplotype network using the program TCS (Clement et al. 2000) at the 95% confidence level. To further quantify genetic distinctness among *Triops* species, the genetic distance between populations was calculated using the p-distance model in the program MEGA v5.05 with uniform rates among sites and gaps treated as missing data (Tamura et al. 2011).

Results & Conclusions

Water chemistry

For each water chemical parameter, the four replications from each playa lake were averaged and is the value reported throughout the report and in Table 1. For the Otero Mesa playa lakes water chemistry, the level of ammonia ranged from 0.05 to 5.15 ppm (parts per million) and averaged across the five lakes was 2.46 ppm. Playa lakes with the most ammonia present included PL-52 (5.15 ppm) and PL-55 (3.51 ppm). PL-53 and PL-56 were comparable in ammonia levels (1.73 and 1.89 ppm). In general, the level of ammonia measured is much greater in the Otero Mesa playa lakes compared to the ammonia measured from the playa lakes previously analyzed near Las Cruces, NM, which were all below 1.00 ppm, with the exception of FP-02 (2.06 ppm; Table 1).

The nitrate values measured ranged from 12.83 ppm in PL-52 to 0.48 ppm in PL-55 with an average nitrate value across the five lakes of 6.91 ppm. Levels of nitrite ranged from 2.07 ppm in PL-53 to 0.12 ppm in PL-54 and averaged 0.78 ppm across the Otero Mesa playa lakes. Generally, these values are similar to the nitrate and nitrite values measured from the playa lakes near Las Cruces, NM (Table 1).

Measured phosphate levels were all below 1.00 ppm in the Otero Mesa lakes and averaged 0.63 ppm (Table 1). The playa lakes with the greatest phosphate levels were PL-52 (0.96 ppm) and PL-55 (0.85 ppm) and the lake with the lowest amount of phosphate was PL-53 (0.24 ppm). There were only three playa lakes from the Las Cruces, NM area that had phosphate values greater than 1.00 ppm (FP-02, PL-07, PL-33; Table 1). Values of measured sulfate ranged from 22 ppm (PL-55) to 54.75 ppm (PL-52) with an average of 32.95 ppm across all of the Otero Mesa playa lakes (Table 1). Levels of sulfide in the lakes were generally very low and ranged from 0.01 ppm (PL-55) to 0.04 ppm (PL-52, PL-54). The measures of sulfate and sulfide in the Otero Mesa playa lakes were comparable to the values measured in the lakes near Las Cruces, NM.

The measured pH of the reconstituted playa lake water averaged 8.28 for all of the Otero Mesa lakes. The range of pH was from 8.05 in PL-55 to 8.50 in PL-52 (Table 1). The pH values for the playa lakes near Las Cruces had a much larger range of pH values (7.68 to 8.38). Salinity of the lake water ranged from 0.13 ppt in PL-54 to 0.28 ppt in PL-52. The amount of dissolved oxygen ranged from 5.66 mg/L in PL-52 to 7.22 mg/L in PL-54. Both salinity and DO values were comparable between the playa lakes near Las Cruces and on the Otero Mesa.

The PCA summarized 57.6% of the variation within the water chemistry data set in the first two axes with axis one having 32.6% of the variation and axis two with 25.0% of the variation (Fig. 2). The playa lakes from Otero Mesa did not cluster together in the PCA and instead had water chemical values that were more similar to playa lakes near Las Cruces. For example, PL-36, FP-03 and PL-53 grouped together in the PCA based on their measured values of nitrate and nitrite (Fig. 1 and Table 2). The significance of each of the water chemistry measurements (i.e the response variables) in determining the grouping of playa lakes is indicated in Table 3 where a strong positive correlation is indicated by a loading value greater than 0.75. There were only two strongly significant loading values; nitrate was negatively correlated with the first axis and salinity was positive correlated with the second axis (Table 3). The other water quality parameters were not as important in explaining the variability in playa lakes.

DNA Sequencing

A total of 83 individuals were sequenced for the current study including 44 from the five Otero Mesa sites and 39 from additional playa lakes near the Las Cruces area (Table 4). For PL-54 and PL-56 located on the Otero Mesa, 10 individuals were not available so all individuals collected were sequenced. The sequence metrics calculated for each population are in Table 4. Two of the playa lakes had only one haplotype present indicating no genetic diversity (PL-12, PL-55). The most genetically diverse individuals were from FP-02, a flood pond located within the city limits of Las Cruces (Fig. 1). The nine *T. l.* "short" individuals from PL-06 all had the same haplotype and it was the same haplotype reported by Horn et al. (2014) for other playa lakes near the Las Cruces area.

The maximum likelihood tree identified three main lineages with relatively high bootstrap support (> 64), corresponding to the three *Triops* species: *T. newberryi*, *T. l.* "short" and the *Triops* sp. from Otero Mesa (Fig. 3). The tree also indicates that *T. l.* "short" and the *Triops* from Otero Mesa are more closely related than either of these species to *T. newberryi*. In addition, *T. newberryi* has much greater genetic diversity compared to the other species. The *T. l.* "short" and Otero Mesa *Triops* sequences occupy a basal position in the tree compared to *T. newberryi* indicating *T. newberryi* is more recently evolved. However, additional *Triops* lineages would be needed to accurately assign order of evolution for *Triops* in New Mexico.

The genetic network analysis displays the evolutionary relationships among the observed haplotypes by estimating how many mutational steps are present between haplotypes; the greater number of mutational steps, the more distantly related the haplotypes. In addition, because there are generally more genetic differences present among different species, a network with multiple species present will not connect allowing another way of assessing species distinctness. For the network analysis, the haplotypes present in the Horn et al. (2014) study were added to the 83 sequences from the current study. There was no network connection between the three putative *Triops* species, i.e. the Otero Mesa *Triops*, *T. l.* "short", and *T. newberryi*, indicating it is likely that these *Triops* are different species. Interestingly, there were broken network connections within the *T. newberryi* network analysis resulting in three main evolutionary lineages (Fig. 4). In both the network derived from sequenced individuals from Otero Mesa and *T. newberryi* playa lakes, there were several main haplotypes that were shared by many individuals and a large number of mutational steps between these main haplotypes. Multiple steps between haplotypes are indicative of either

haplotypes in the populations that have not yet been sampled or haplotypes that may have once been present, but are now extinct.

Genetic distance among populations of *Triops* ranged from 0.1% to 4.5% (Table 5). The populations from the Otero Mesa region were all genetically similar and had genetic distance measures all below 1.0% indicating they are all the same species of *Triops*. In contrast, when comparing the genetic distance of the Otero Mesa populations to those *T. l.* "short" or *T. newberryi* populations, the distances are much greater, from 2.8% to 4.5%. The Otero Mesa populations are genetically more similar to *T. l.* "short" than to populations with *T. newberryi*, which could imply the species of *Triops* from Otero Mesa may be a sub-species to *T. longicaudatus*.

Conclusions

The objective of the current study was to first determine if water chemistry differences exist between playa lakes found in the Otero Mesa region of New Mexico, a pristine grassland, compared to playa lakes found near Las Cruces, NM, a region dominated by desert scrubland. The second objective was to determine if the *Triops* species occurring within these playa lakes are different between regions and if the difference can be linked to a water quality parameter. The results of the water chemistry measurements indicate that there are not major differences in the water chemistry profiles between regions. Interestingly, the ammonia levels in the water from playa lakes in the Otero Mesa were, in some cases, orders of magnitude larger than that measured from lakes near Las Cruces. Cattle grazing occurs in both regions under study and waste from cattle has been shown to be a source of elevated ammonia in water (Hubbard et al. 2004). Further research is needed to determine if grazing occurs more frequently on the Otero Mesa and if increased cattle presence account for the elevated ammonia levels. The genetic analysis of the *Triops* species does indicate that there are different species of *Triops* present and the species that are present within the Otero Mesa playa lakes is not the same species that occurs near Las Cruces. With no major differences in the measured water chemistry, it is only speculation as to why a *Triops* species delineation boundary exists between Las Cruces and the Otero Mesa region. Recommendations for future research in this area would include survey of additional playa lakes found within the Otero Mesa region to identify the *Triops* species present and to determine if any playa lakes exist between the Otero Mesa and Las Cruces that might serve as a contact zone for the different *Triops* species. Further, the water should be tested for other measures not taken into account in the current study, such as metallic components, that could resolve if there is an environmental component to species differentiation. Lastly, the *Triops* that were found in the Otero Mesa region need to be morphologically identified with voucher specimens and formal species assignment needs to be made.

Beneficiaries from Research

Those who may benefit from this research project include those in the Bureau of Land Management (BLM), the Elephant Butte Irrigation District (IBID) and other academics that are interested in the species distribution of tadpole shrimp. In addition, state agencies such as the New Mexico Environment Department Surface Water Quality Bureau and the New Mexico Department of Game have interest in isolated wetlands such as playa lakes.

Presentations
A poster presentation of the project was presented at the 59th Annual New Mexico WRRI Water Conference in Santa Fe, NM on November 18-19th.

Other student/faculty assistance
Dr. Rossana Sallenave (NMSU Extension Aquatic Ecology Specialist) aided with the collection of soil and with performing the water chemistry analysis. Dr. Karen Mabry (NMSU Biology Department) allowed me use of her equipment to run gel electrophoresis and I was aided by PhD student Rebecca Kelley and Gizelle Hurtado. PhD student Andy Lawrence shadowed and assisted with the DNA extraction and PCR set-up.

Figures

Figure 1. Map of the sample area. The playa lakes sampled are denoted by black dots. The cities of Las Cruces, New Mexico and El Paso, Texas are represented by pink stars. The interstates, highways and state and county borders are also depicted.

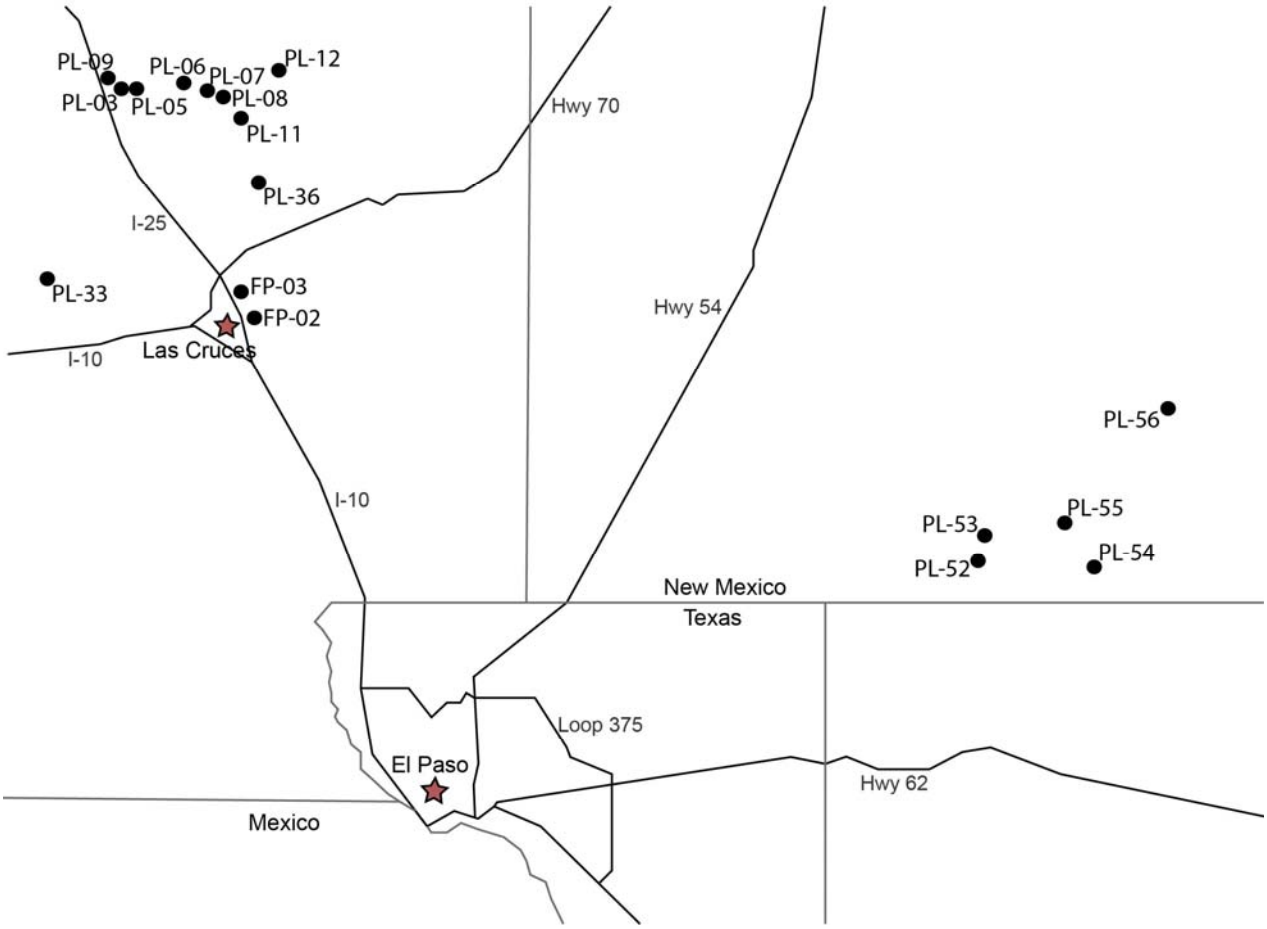


Figure 2. Graph of the principle components analysis (PCA). The first axis represents 32.6% of the variation and the second axis represents 25.0%. The black circles are those playa lakes sampled from the Otero Mesa region; the gray diamonds are those playa lakes that were previously analyzed; DO is dissolved oxygen.

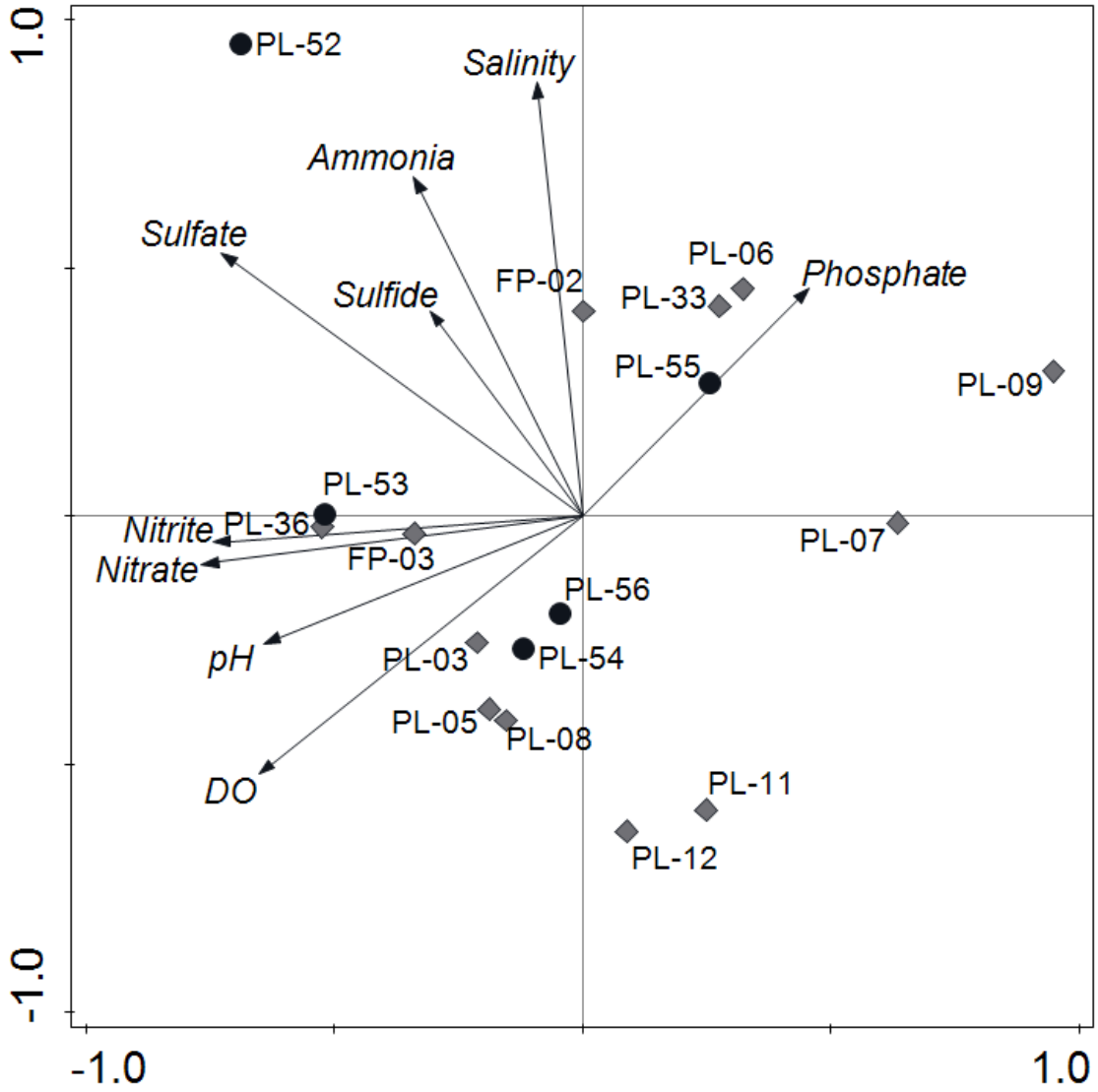


Figure 3. Maximum likelihood phylogenetic tree representing the relationships among the mitochondrial control region (mtCR) sequences. The tree is rooted with a *Triops cancriformis* mtCR sequence; haplotypes previously sequenced and are reported in Horn et al. (2014) are in black; *T. newberryi* individuals are in blue; *T. longicaudatus* "short" individuals are in orange; *Triops* sp. from Otero Mesa are in green. Numbers on each branch node correspond to the bootstrap value.

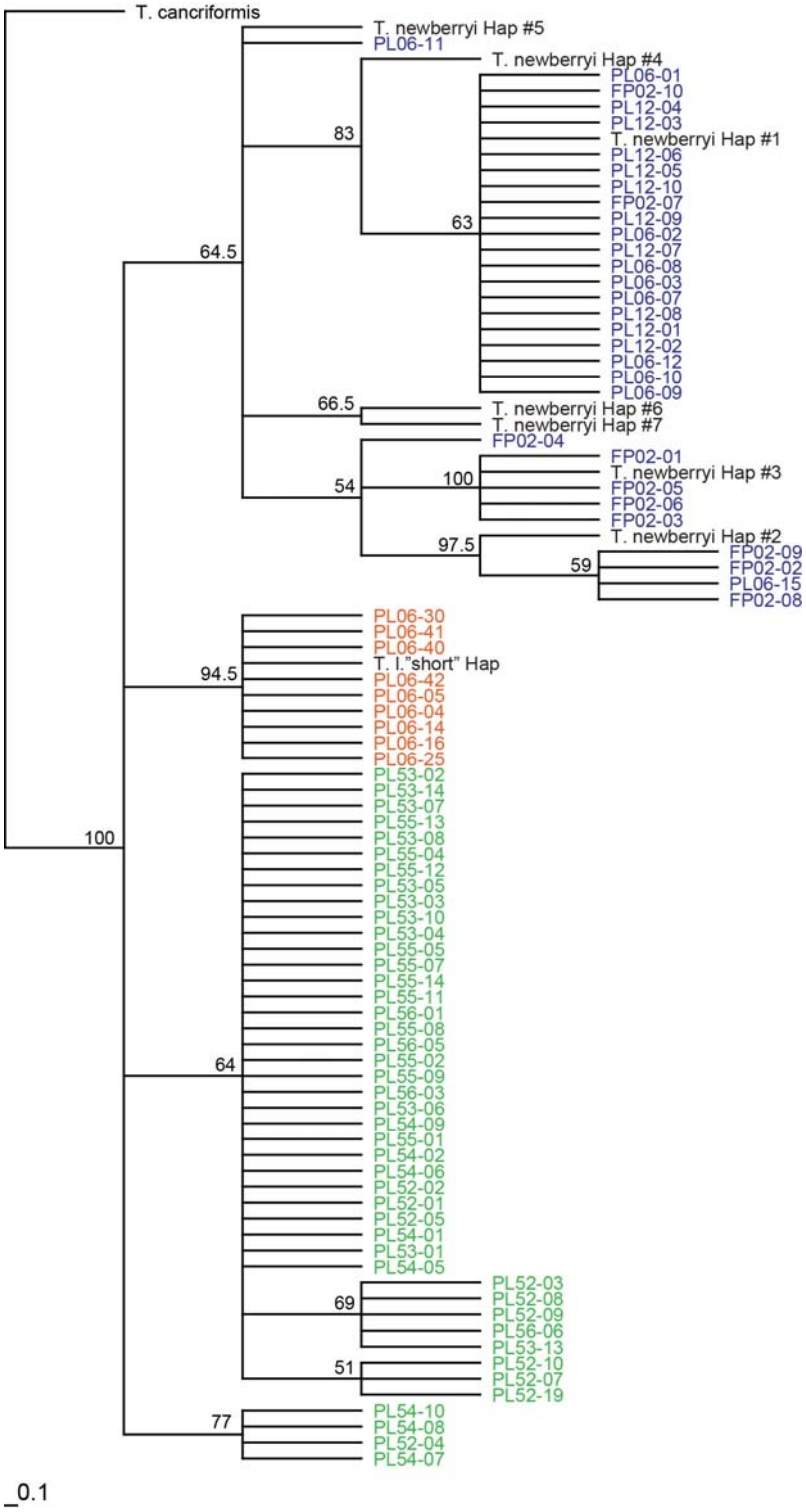
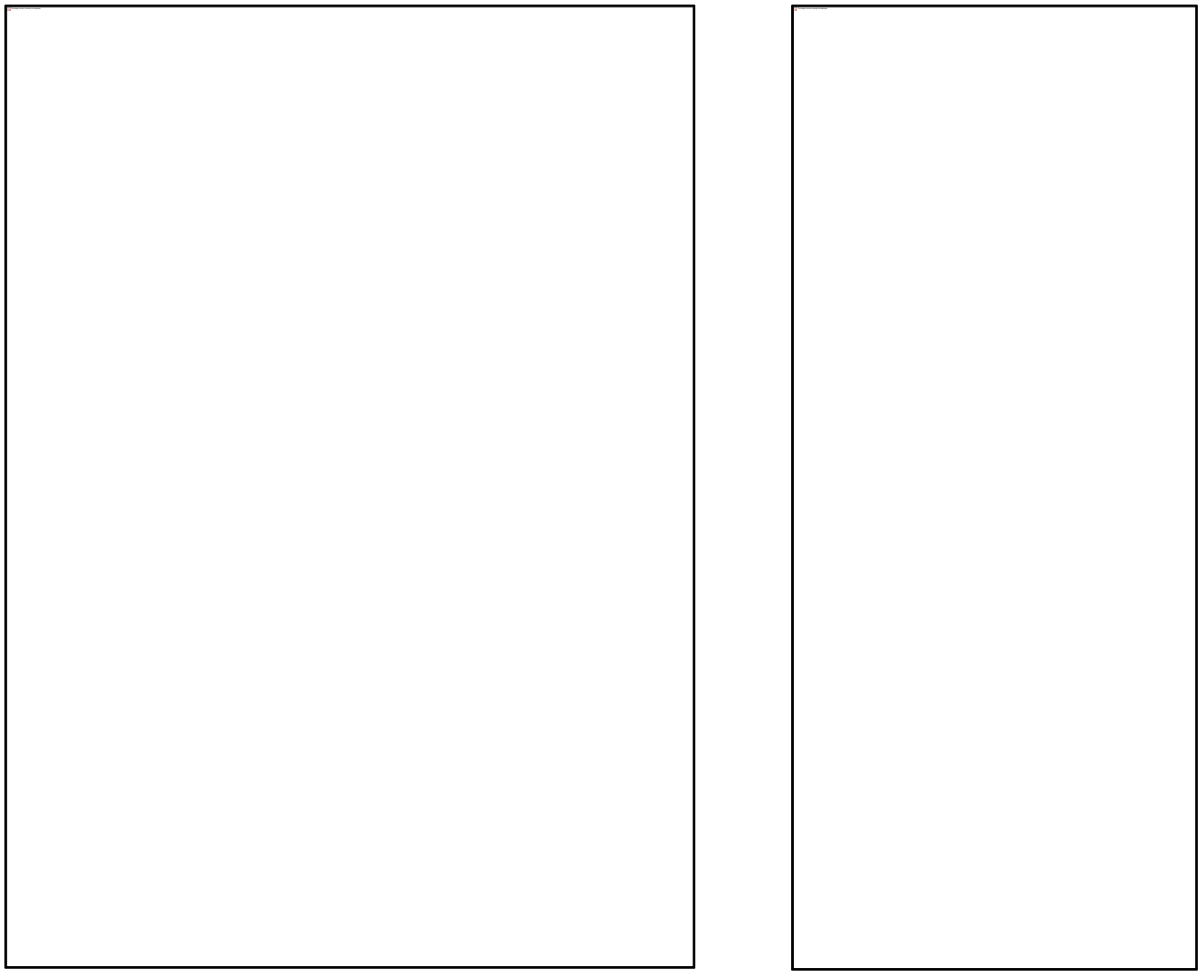


Figure 4. Network analysis for *Triops newberryi* (A) and for the Otero Mesa *Triops* (B). The numbers next to each haplotype in part A correspond to the haplotype numbering from Horn et al. (2014). The samples previously analyzed as part of Horn et al. (2014) are in black and white symbols and the newly sequenced *T. newberryi* individuals are in color. Each circle represents a playa lake population, the small black circles are hypothetical haplotypes, the lines represent one mutational step and the size of the circle is proportional to the number of individuals with that haplotype.



Tables

Table 1. The measured values for each of the water chemical parameters for the playa lakes near Las Cruces, NM and those on the Otero Mesa (italics). All measurements are in parts per million (ppm) except for salinity (parts per thousand), DO (dissolved oxygen, mg/L) and pH.

	Ammonia	Nitrate	Nitrite	Phosphate	Sulfate	Sulfide	pH	Salinity	DO
FP-02	2.06	8.20	0.71	1.43	36.00	0.00	8.19	0.27	6.10
FP-03	0.75	11.14	1.82	0.25	46.25	0.02	8.15	0.18	5.77
PL-03	0.27	8.24	1.70	0.37	18.50	0.01	8.38	0.21	6.43
PL-05	0.29	13.80	2.42	0.47	11.50	0.01	8.14	0.15	6.09
PL-06	0.69	1.43	0.25	0.63	25.00	0.03	8.03	0.27	4.11
PL-07	0.61	0.26	0.08	1.95	2.50	0.02	8.09	0.14	5.37
PL-08	0.06	19.23	0.67	0.53	17.00	0.00	8.26	0.18	6.56
PL-09	0.47	0.09	0.00	0.64	3.00	0.00	7.68	0.23	1.77
PL-11	0.01	0.55	0.02	0.53	9.50	0.01	8.32	0.09	6.65
PL-12	0.24	3.59	0.55	0.20	12.75	0.00	8.35	0.11	6.52
PL-33	0.32	2.88	0.60	1.32	40.25	0.03	7.99	0.21	4.44
PL-36	0.76	16.81	2.51	0.12	50.75	0.01	8.02	0.20	6.18
PL-52	5.15	12.83	1.26	0.96	54.75	0.04	8.50	0.28	5.66
PL-53	1.73	9.34	2.07	0.24	43.50	0.03	8.26	0.18	6.87
PL-54	0.05	10.55	0.12	0.62	21.50	0.04	8.24	0.13	7.22
PL-55	3.51	0.48	0.14	0.85	22.00	0.01	8.05	0.20	5.87
PL-56	1.89	1.33	0.34	0.50	23.00	0.02	8.36	0.14	7.06

Table 2. The principal component scores for the first two axes of the PCA.

	Axis 1	Axis 2
FP-02	0.01	1.01
FP-03	-0.82	-0.08
PL-03	-0.52	-0.62
PL-05	-0.46	-0.95
PL-06	0.79	1.12
PL-07	1.55	-0.03
PL-08	-0.37	-0.99
PL-09	2.31	0.71
PL-11	0.61	-1.44
PL-12	0.22	-1.55
PL-33	0.67	1.03
PL-36	-1.28	-0.05
PL-52	-1.67	2.31
PL-53	-1.26	0.01
PL-54	-0.28	-0.65
PL-55	0.63	0.65
PL-56	-0.11	-0.48

Table 3. PCA loading scores for the first two axes for the environmental variables. Bolded scores are significant.

	Axis 1	Axis 2
Ammonia	-0.34	0.68
Nitrate	**-0.77**	-0.10
Nitrite	-0.74	-0.05
Phosphate	0.46	0.46
Sulfate	-0.73	0.53
Sulfide	-0.31	0.42
pH	-0.64	-0.26
Salinity	-0.09	**0.88**
DO	-0.65	-0.52

Table 4. Sequence metrics for the mitochondrial control regions sequences generated from *Triops* individuals from eight playa lakes. PL-06 contains both *T. newberryi* and *T. longicaudatus* "short", therefore an * indicates that those individuals analyzed are *T. l.* "short. N, number of individuals; N_H, number of haplotypes; Transit, number of transitions; Transv, number of transversions; Subs, number of substitutions; Indels, number of indels; Poly, number of polymorphic sites; GC, the percentage of G's and C's in the sequence; *h*, haplotype diversity; π, nucleotide diversity.

	FP-02	PL-06*	PL-06	PL-12	PL-52	PL-53	PL-54	PL-55	PL-56
N	10	9	10	10	10	11	8	11	4
N_H	4	1	3	1	4	2	3	1	2
Transit	15	0	8	0	9	1	8	0	1
Transv	6	0	3	0	2	0	1	0	0
Subs	21	0	11	0	11	1	9	0	1
Indels	2	0	2	0	0	0	0	0	0
Poly	22	0	13	0	11	1	9	0	1
GC	27.9	28.3	28.3	28.3	28.5	28.5	28.4	28.5	28.6
h	0.778	0.000	0.378	0.000	0.800	0.182	0.679	0.000	0.500
π	0.022	0.000	0.007	0.000	0.006	0.0004	0.010	0.000	0.001

Table 5. The genetic distance (%) among *Triops* populations calculated using the p-distance method. The 'Hap1' to 'Hap7' are the *T. newberryi* haplotypes taken directly from Horn et al. (2014) and the 'TLS' is the *T. longicaudatus* "short" haplotype from PL-06.

	PL52	PL53	PL54	PL55	PL56	FP02	PL06	PL12	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7
PL53	0.4														
PL54	0.9	0.7													
PL55	0.3	0	0.7												
PL56	0.4	0.1	0.8	0.1											
FP02	4.5	4.4	4.3	4.4	4.4										
PL06	4.5	4.3	4.3	4.3	4.4	2									
PL12	4.5	4.4	4.4	4.4	4.4	2	0.3								
Hap1	4.5	4.4	4.4	4.4	4.4	2	0.3	0							
Hap2	4.3	4.2	4	4.2	4.2	1.7	1.8	2.1	2.1						
Hap3	4.5	4.4	4.4	4.4	4.4	1.6	2.7	2.8	2.8	2.5					
Hap4	4.1	3.9	4	3.9	4	2.1	0.9	0.7	0.7	2.3	2.5				
Hap5	3.9	3.7	3.7	3.7	3.8	1.8	0.9	0.9	0.9	1.6	2.3	1.2			
Hap6	4.3	4.2	4.2	4.2	4.2	2.3	1.4	1.4	1.4	2.1	2.8	1.6	0.5		
Hap7	4.3	4.2	4.2	4.2	4.2	2.3	1.4	1.4	1.4	2.1	2.8	1.6	0.5	0	
Tls	2.9	2.8	2.5	2.8	2.8	4.1	3.9	3.9	3.9	3.7	4.4	3.5	3.2	3.7	3.7

References

Clement M, Posada D, Crandall KA (2000) TCS: A computer program to estimate gene genealogies. Molecular Ecology, 9: 1657-1660.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2011) Geneious v5.4, Available from: http://www.geneious.com/.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources, 10: 564-567.

Fry LL, Mulla MS, Adams CW (1994) Field introductions and establishment of the tadpole shrimp, *Triops longicaudatus* (Notostraca: Triopsidae), a biological control of mosquitoes. Biological Control, 4: 113-124.

Horn RL, Kuehn R, Drechsel V, Cowley DE (2014) Discriminating between the effects of founding events and reproductive mode on the genetic structure of *Triops* populations (Branchiopoda: Notostraca). PLoS ONE, 9(5): e97473.

Hubbard RK, Newton GL, Hill GM (2004) Water quality and the grazing animal. Journal of Animal Science, 82 (E. Suppl.): E255-E263.

Montero-Pau J, Gómez A, Muñoz J (2008) Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. Limnology and Oceanography Methods, 6: 218-222.

Scott SE, Grigarick AA (1979) Laboratory studies of factors affecting egg hatch of *Triops longicaudatus* (LeConte) (Notostraca: Triopsidae). Hydrobiologia, 63: 145-152.

Swofford DL (2003) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731-2739.

ter Braak CJF, Šmilauer P (2012) Canoco reference manual and user's guide: software for ordination (version 5.0). Microcomputer Power (Ithaca, NY, USA).