

Phylogeography, genetic structure, and gene flow in the endemic freshwater shrimp *Palaemonetes suttkusi* from Cuatro Ciénegas, Mexico

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Abstract The valley of Cuatro Ciénegas in Mexico has the highest degree of local endemism of any habitat in North America. A large number of endemic aquatic species occur both in the Cuatro Ciénegas basin and in the upper parts of the Río Salado de los Nadadores drainage, located immediately to the east of the valley. No natural surface flow occurs between these basins but artificial canals connect both areas. It is not clear whether the sharing of endemics between the Cuatro Ciénegas basin and the Río Salado is due to migration through canals. We conducted a phylogeographic study of mitochondrial haplotypes of the endemic shrimp *Palaemonetes suttkusi* to determine the evolutionary distinctiveness of the population found in Río Salado. We discovered that *P. suttkusi* likely has occurred naturally in both basins well into the distant past. Based on molecular clock analyses of the COI gene, the populations in the Río Salado and much of the Cuatro Ciénegas basin likely began diverging from each other between 1.9 and 11.2 Myr ago. The general levels of divergence are substantial but our results suggest there has also likely been recent gene flow between these basins. This is consistent

with migration through human-made canals, but also consistent with the occurrence of natural gene flow during intermittent wet periods in the past million years. We also found significant differentiation of the Pozas Azules area from the rest of the Cuatro Ciénegas basin, a finding that is concordant with several phylogeographic studies on other aquatic endemics in Cuatro Ciénegas. We recommend that the upper parts of the Río Salado, the Pozas Azules area, and the rest of the Cuatro Ciénegas basin should each be considered independent evolutionarily significant units for conservation, and that migration of species through human-made canals should be monitored and controlled.

Keywords Phylogeography · mtDNA variation · Gene flow · Conservation genetics · Evolutionary significant units · Shrimp

Introduction

The small ($\sim 30 \times 40$ km, 1500 km²) intermontane valley of Cuatro Ciénegas, located in the Chihuahuan desert in northern Mexico, has the highest local endemism of any area in continental North America with more than 70 endemic species (Souza et al. 2006). For comparison, Ash Meadows in the Mojave Desert has the second highest local endemism in continental North America with about 30 endemic species (Stein et al. 2000). The valley of Cuatro Ciénegas is an extremely arid site that is considered a high priority conservation ecoregion. This valley has been declared a National Protected Area by the Mexican Government, an international RAMSAR site, and a UNESCO World Heritage Biosphere Reserve (Stein et al. 2000; Hendrickson et al. 2008; Souza et al. 2008). Most of the endemic species in Cuatro Ciénegas are associated with the

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numerous aquatic habitats within the basin. There are more than 200 springs, spring-fed pools, rivers and playa lakes in the Cuatro Ciénegas basin that are physically separated into several drainages. Gene flow could therefore be currently restricted among drainages within Cuatro Ciénegas. Similarly, there is no natural surface flow outside of the Cuatro Ciénegas basin, suggesting aquatic organisms in the basin should be isolated from the Río Salado de los Nadadores basin (hereafter Río Salado) that is the physically closest drainage to the Cuatro Ciénegas basin (Johnson 2005; Hendrickson et al. 2008). The Río Salado does share several species with Cuatro Ciénegas especially in the Pozas Azules region of the valley and is currently connected via canals to the Cuatro Ciénegas basin (Hendrickson et al. 2008). Restricted gene flow within Cuatro Ciénegas and between this valley and the Río Salado should result in the origination of evolutionary significant units (ESU) for conservation, and these should be recognized and protected to maintain the full array of differently adapted geographic variants within species (Moritz 1994). Identifying ESUs for conservation of endemic species would aid in defining conservation priorities and setting long-term management policies for this unique region.

Population subdivision is present in several species within the Cuatro Ciénegas basin although not all of the

patterns are consistent across species. For instance, the two endemic pupfish species (genus *Cyprinodon*), the native variety of the largemouth bass (*Micropterus salmoides*) and the endemic aquatic snail *Mexipyrghus churinceanus* show substantial DNA sequence divergence between the west and east side of the Sierra de San Marcos (Rodríguez-Martínez 2004; Johnson 2005; Carson and Dowling 2006). The Sierra de San Marcos (hereafter Sierra) bisects the basin into eastern and western lobes and most pools and streams in the valley are clustered around this mountain (Fig. 1). Conversely, the endemic aquatic box turtle (*Terrapene coahuila*) that is one of the flagship species in the valley, exhibits minimal levels of population differentiation in Cuatro Ciénegas (Howeth et al. 2008). The differences in population genetic structure between the turtle, fish and snail could reflect differences in dispersal. Populations of species like *T. coahuila* that can move easily between subdivided drainages tend to show little genetic structure whereas fully aquatic species with lower dispersal capabilities, such as fish and spring snails, should tend to develop genetic structuring (Carini and Hughes 2004). The differences in genetic differentiation found previously for species with different dispersal abilities within Cuatro Ciénegas show the importance of studying different taxa before designing conservation strategies for entire faunas.

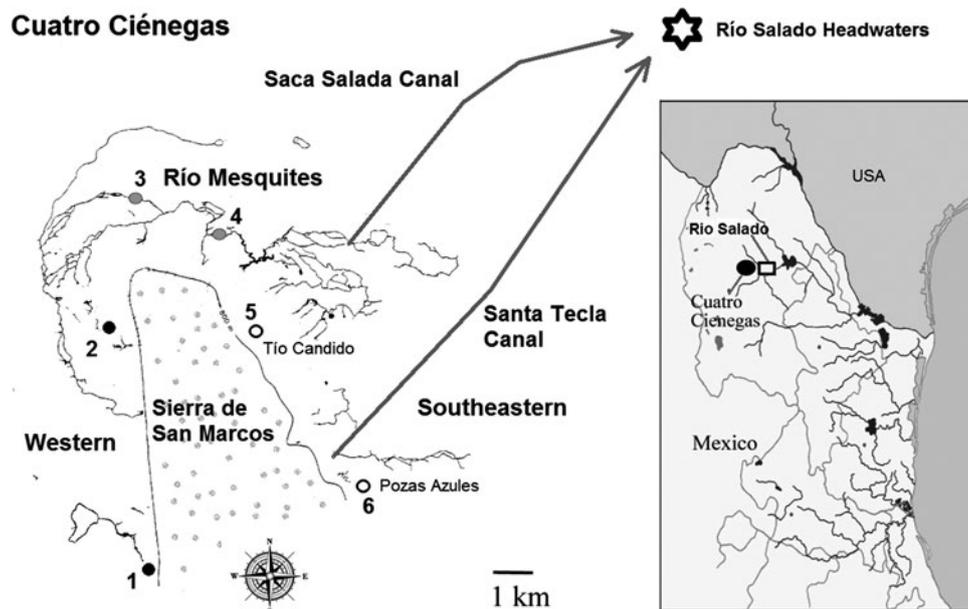


Fig. 1 The Cuatro Ciénegas basin, Río Salado de los Nadadores, and their location in Northeastern Mexico. The *inset* shows the location of Cuatro Ciénegas (*large black solid circle*) in relation to the Río Salado (*open square*). Collection sites and regions within Cuatro Ciénegas: the Western region (*small black solid circles*) in relation to the Sierra de San Marcos, includes Laguna Churince (1) and Juan Santos (2). The Río Mesquites region (*small gray solid circles*) includes Tierra Blanca (3), and Mojarral East (4). The Southeastern

region (*open circles*) includes Tío Cándido (5) and Pozas Azules (6). The headwaters of the Río Salado de los Nadadores are represented by a *star*. See [Appendix](#) for more detailed locality information. The two artificial canal systems that drain water from the Cuatro Ciénegas basin to the Río Salado headwaters (Saca Salada and Santa Tecla) are shown with *gray arrows* indicating the direction of water flow in the canals

In order to expand our limited understanding of the phylogeographic patterns in the community of endemic species found in Cuatro Ciénegas and the Río Salado, we analyzed the historical and current geographic distribution of mitochondrial haplotypes of the endemic shrimp *Palaemonetes suttkusi*. This is the only *Palaemonetes* shrimp and the only crustacean in the order Decapoda that occurs in the valley of Cuatro Ciénegas and surrounding drainages (Strenth 1976, 1994; Cole 1984; Rodríguez-Almaraz et al. 1997). Reports on the presence of *P. suttkusi* in Cuatro Ciénegas and surrounding areas span several decades (Minckley 1969; Cole 1984; Rodríguez-Almaraz et al. 1997). This can be taken as an indication that populations of this species are stable in the area. However, it is currently unknown whether there is population subdivision within the range of this species. Although freshwater shrimp are capable of swimming several kilometers per day, they are obligately aquatic and may still show population structure (Lee and Fielder 1984; Elliott 2003; Sharma and Hughes 2009). For this reason, we predict that levels of population structure in *P. suttkusi* within the Cuatro Ciénegas basin should be similar to those found in the fish and snail species examined previously.

Similar to several other Cuatro Ciénegas species, *P. suttkusi* is found not only in the Cuatro Ciénegas basin but also in the upper parts of the Río Salado (Strenth 1976; Rodríguez-Almaraz et al. 1997). The Río Salado originates just outside the eastern side of the Cuatro Ciénegas valley, 35 km away from the Sierra (Fig. 1) and ultimately is linked to rivers that flow into the Río Grande that forms the border between Mexico and the United States. Despite the lack of natural surface flow between the Cuatro Ciénegas valley and the Río Salado, there are a number of endemic taxa found both in Cuatro Ciénegas and in the upper parts of the Río Salado that are not extant anywhere else (Hendrickson et al. 2008). No study has examined whether Cuatro Ciénegas taxa found in the Río Salado are recent invaders or long diverged sister groups. Examples of endemic taxa found in both basins include darters (genus *Etheostoma*), shiners (genus *Cyprinella*), and platyfish (*Xiphophorus gordonii*). The Cuatro Ciénegas native varieties of the longear sunfish (*Lepomis megalotis*), largemouth bass (*Micropterus salmoides*), headwater catfish (*Ictalurus lupus*), roundnose minnow (*Dionda episcopa*), and the hydrobiidae snail *Cochliopina riograndensis* are also found in both basins (Hershler 1984; Hendrickson et al. 2008). No natural surface water flow occurs between these two basins currently; therefore, gene flow should not occur naturally. If migration between Cuatro Ciénegas and the Río Salado has occurred through recent aquatic connections and the basins are genetically indistinct, then the populations in Río Salado should be managed as a

sub-component of the Cuatro Ciénegas bioregion. Alternatively, both areas could be considered ESUs for conservation if the populations from both basins are genetically differentiated from each other.

There are several scenarios that could have allowed past gene flow between these two basins. North American deserts have gone through several wet cycles that increased water levels in the past (Smith 1984; Metcalfe et al. 2002; Butzer et al. 2008). A major xeric period occurred in these deserts 2.6 Myr ago, followed by a major wet period 1.3 Myr ago that preceded the major xeric period that currently characterizes the area (Smith 1984). There is also evidence that minor wet periods in the Chihuahuan desert occurred between about 11,000 and also 4,000 years ago (Castiglia and Fawcett 2006; Minckley and Jackson 2008). Hence, migration between Cuatro Ciénegas and the Río Salado could have occurred naturally several times in the past. However, it is also possible that the presence of Cuatro Ciénegas taxa in the Río Salado could be due to recent dispersal through two canal systems. The Santa Tecla and Saca Salada canals for approximately the last 100 years have carried surface water from Cuatro Ciénegas into the headwaters of the Río Salado. These canals now bridge the 30 km of desert that historically separated these two aquatic regions (Fig. 1) (Hendrickson et al. 2008). Although it would be difficult to discriminate using genetic data between the hypotheses of natural gene flow in the last 11,000 years and canal-mediated gene flow in the last 100 years, evidence for relatively recent migration events (<12,000 years) should at least be detectable using mitochondrial DNA (Lack et al. 2010).

To determine whether *P. suttkusi* naturally occurs in the headwaters of Río Salado, we asked whether haplotypes from this area are highly divergent from haplotypes found in the Cuatro Ciénegas basin. We also estimated historical and current patterns of migration between these drainages to determine two possibilities. First, whether gene flow existed between the Cuatro Ciénegas and the Río Salado drainages during the wet periods that occurred in the distant past and if it ceased after the end of those wet periods, i.e. 2.6–1.3 Myr ago and/or 11,000–4,000 years ago. Second, whether gene flow is likely to currently occur between the drainages. Investigating whether the population of *P. suttkusi* found in the Río Salado is natural, isolated, and distinct from Cuatro Ciénegas is important to determine whether the Río Salado should be considered an ESU for conservation. This would be particularly critical for the Río Salado as it lacks the federally protected status that much of the Cuatro Ciénegas basin now has. Determining whether gene flow might be occurring through human-made canals is also important in order to effectively implement management and conservation strategies in this area.

Materials and methods

Sampling and laboratory procedures

Samples of *P. suttkusi* were collected in June 2009 from the upper parts of the Río Salado basin and from six sites in the Cuatro Ciénegas basin (Fig. 1, Appendix). Within the Cuatro Ciénegas basin, we sampled two drainages consisting of spring-fed pools and stream outflows located on the western side of the Sierra (Laguna Churince and Poza Juan Santos), and the Río Mesquites near the northern tip of the Sierra (Tierra Blanca, Mojarral East). We also chose two sets of isolated pools located in the southeast part of Cuatro Ciénegas, Tío Cándido and Pozas Azules to sample. The Río Salado sampling site (San José del Águila) was located 15 km downstream from where the canals reach the Río Salado headwaters; i.e. the site is located about 45 km downstream from where the canals originate within the Cuatro Ciénegas valley. These sampling sites span the entire known geographic range of *P. suttkusi* (Rodríguez-Almaraz et al. 1997).

Samples were preserved in ethanol and DNA was extracted in the laboratory from muscle tissue. We amplified and sequenced a 623 bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) with primers LCO1490 5-GGTCAACAAATCATAAAGATATTGG-3 and HCO2198 5-TAAACTTCAGGGTGACCAAAAATCA-3 (Folmer et al. 1994). Amplifications were carried out in an Eppendorf EP Gradient thermocycler and conditions consisted of an initial denaturation step of 94°C (2.0 min) followed by cycles alternating between 54°C (1.0 min), and 72°C (1.5 min). A final incubation of 72°C for 4 min was added to ensure complete extension of products. Subsequently, the PCR products were separated from unincorporated primers and dNTPs using electrophoresis in Tris–acetate buffered agarose gels with ethidium bromide (1.5 mg/μl) added for visualization. Positively amplified DNA was then purified using an enzymatic combination of 1 μl of Exonuclease I (10.0 U/μl) and 1 μl shrimp alkaline phosphatase (2.0 U/μl) per 10 μl of PCR product. Treated PCR products were sequenced by the High Throughput DNA Sequencing Facility at the University of Washington using the same primers utilized for amplification. Complete gene sequences were assembled from individual reactions using the program Sequencher version 4.6 (Gene Codes, Ann Arbor, MI) and deposited in GenBank (accession numbers HQ324432–HQ324538).

Genetic structure analysis

To evaluate the potential for spatial genetic structure, we performed an Analysis of Molecular Variance (AMOVA) in ARLEQUIN 3.5 (Excoffier and Lischer 2010).

Specifically, we analyzed whether sequence variation among regions was greater than variation within regions. We initially defined four regions based on geographic boundaries. The Río Salado site was considered a separate region because it is located outside the Cuatro Ciénegas valley. We defined three regions within Cuatro Ciénegas based on the position of the sampling sites relative to the Sierra following previous studies (Johnson 2005; Carson and Dowling 2006). The Western region included the sites located on the west side of the Sierra, the Southeast region included the sites located on the east and south side, and the Río Mesquites region included the sites located around the tip of the Sierra (Fig. 1, Appendix). We estimated percent sequence divergence among haplotypes and regions using the Kimura 2-parameter distance model (Kimura 1980; Jin and Nei 1990). We calculated pair-wise fixation indices (F_{ST}) to estimate the level of population differentiation between populations.

Phylogeographic analysis

For the phylogeographic analysis, we first constructed a haplotype network using the median-joining method implemented in the program Network 4.5.1.6. (Bandelt et al. 1999; Fluxus technology Ltd. 2009). Once the haplotype network was built, we color coded the unique haplotypes by the four regions listed above. Then, we used jMODELTEST 1.01 to choose the best fitting, least-parameter rich model of sequence evolution based on both Bayesian and Akaike Information Criterion (Posada 2008). We simultaneously estimated gene trees and the divergence of COI haplotypes in the region using the program BEAST v 1.5.4 (Drummond and Rambaut 2007). We used a strict clock rate of 0.007 (per lineage) based on 1.4% sequence divergence per million years, which is the most commonly used rate for shrimp COI divergence (Knowlton and Weigt 1998; Zaksek et al. 2009). We chose a strict molecular clock model after confirming that *P. suttkusi* COI behaves in a clock-like fashion, by means of model comparison in BEAST using the Bayes Factor. The Bayes Factor is the ratio of the marginal likelihoods (marginal with respect to the prior) of a model that assumes a fixed rate of nucleotide change (molecular clock) and a model in which the rate of change was allowed to vary (Suchard et al. 2001). The analysis was repeated three times to confirm the topology and divergence times within the tree.

The BEAST output was inspected to examine the posterior distributions, to check for convergence, and to confirm that the effective sample size for each parameter exceeded 200 using Tracer 1.5 (Rambaut and Drummond 2009). Posterior probabilities and the “maximum clade credibility tree” were calculated using TreeAnnotator 1.5.4 (Drummond and Rambaut 2007). We ran the analysis for

10,000,000 generations starting with either UPGMA or random trees and a burn-in of at least 1,000,000 to confirm the robustness of the result. We ran the analysis with different priors (Yule and constant size coalescent) and with no data (prior only option) to confirm that the posterior was primarily driven by the data.

Gene flow analysis

We also estimated levels of gene flow between regions and divergence times between lineages using an isolation with migration model in the Bayesian program IMA2 2.0 (Hey 2010). IMA2 uses an ordered phylogenetic tree that for this study we obtained from the BEAST analysis. The method employs Markov chain Monte Carlo simulations of gene genealogies to estimate the effective number of migrant gene copies per generation. It also performs likelihood ratio tests to evaluate whether migration rates are significantly different from zero. We set our initial priors in relation to the population mutation rates (i.e. $4N_e\mu$). ARLEQUIN 3.5 was used to estimate θ , the ancestral genetic diversity, using the Watterson method (Watterson 1975). We then estimated the ancestral N_e from the relationship $\theta = 2N_e\mu$ using a mutation rate for the 623 bp COI locus calculated from the 1.4% sequence divergence per million years rate mentioned above, and a generation time of 1 year. This has been suggested to be the generation time of closely related Palaemonidae shrimps (Carini and Hughes 2004). We used 20 heated chains with 9,000,000 trees sampled with a burn-in of 90,000 trees. The effective sample sizes for each parameter estimate exceeded 200 in all runs. The procedure was repeated at least three times with different random seeds in order to confirm the robustness of the results.

Results

Genetic diversity and population differentiation

COI haplotypes were very divergent among populations of *P. suttkusi*. We found a total of 27 unique haplotypes in a sample of 107 individuals. The haplotype network is presented in Fig. 2a. The distance between unique haplotypes ranged from 0.2 to 9.6%. Based on the large divergence of Pozas Azules haplotypes from the rest of the Cuatro Ciénegas haplotypes, we split the Southeastern region into Pozas Azules and Tío Candido before conducting the AMOVA, for a total of five regions. The AMOVA showed that the haplotypes were not homogeneously distributed in the study area: 87% of sequence variation is due to differences among regions, while the remaining 13% is due to differences within regions ($F_{ST} = 0.92$, $P < 0.0001$). Most of the variation among regions is due to the large

divergence between Río Salado + Poza Azules and the rest of Cuatro Ciénegas (Table 1). Pairwise F_{ST} values between regions are very high (i.e. ≥ 0.84) and significantly different from zero, except for the comparisons among Río Mesquites, Tío Cándido and Western regions. Pairwise F_{ST} values for the last three regions are much lower (i.e. ≤ 0.33) and non significant in the case of the comparison between Río Mesquites and the Western region. However, each region contained at least one unique haplotype.

Phylogeographic analysis

The BEAST analysis (conducted using HKY + G as the model of sequence evolution according to MODELTEST) clearly identified the separation of two major lineages (posterior probability near 1.0; Fig. 2b). The first major lineage includes two well-differentiated clades, one that groups the Río Salado haplotypes and another one that groups the Pozas Azules haplotypes (posterior probabilities for each node >0.94). The second major lineage includes the rest of the sequences (posterior probability for the main node near 1.0) but does not show differentiation according to regions. Divergence between the two major clades was estimated to have started 3.4 Myr ago (95% Confidence Interval 2.4–4.7) when a Yule prior was used, and 7.9 Myr ago (95% CI 5.2–11.2) when a constant size coalescent prior was used. The divergence between the Pozas Azules clade and the Río Salado clade was estimated to have occurred 1.0 Myr ago (95% CI 0–3.2) with a Yule prior, and 1.4 Myr ago (95% CI 0–7.3) with a constant coalescence prior. We found an average distance of 2.6% between Río Salado and Pozas Azules haplotypes, 7.7% between Río Salado and the rest of Cuatro Ciénegas, and 8.1% between Pozas Azules and the rest of Cuatro Ciénegas. Despite the clear separation of Río Salado and Pozas Azules haplotypes from the rest of the Cuatro Ciénegas sequences, one individual caught in the Río Mesquites area appears within the Río Salado clade, and one individual caught in the Río Mesquites areas shows up in the Pozas Azules clade (Fig. 2b). The same pattern was recovered in the haplotype network (Fig. 2a).

Gene flow

We used the grouping pattern and splitting order of divergent populations recovered in the gene tree obtained from BEAST as input to estimate gene flow between the three divergent populations (Pozas Azules, Río Salado, and rest of Cuatro Ciénegas) and their corresponding ancestral populations using IMA2. The tree recovered, represented as a Newick string, was ((Río Salado, Pozas Azules), rest of Cuatro Ciénegas). Using the HKY model of substitution as in the BEAST analysis, IMA2 detected moderate migration

Fig. 2 Haplotype network (a) and Bayesian gene tree (b) estimated from 107 COI haplotypes from the same number of *Palaemonetes suttkusi* individuals collected from five different regions (color coded). Bayesian posterior probabilities are shown for basal nodes in the gene tree (b) to provide an indication of the support for the genetic differentiation among regions. Network (a): circle area is proportional to haplotype frequencies. Branch lengths are roughly proportional to the number of mutation steps between haplotypes and nodes; the true number of steps is shown near the corresponding branch section

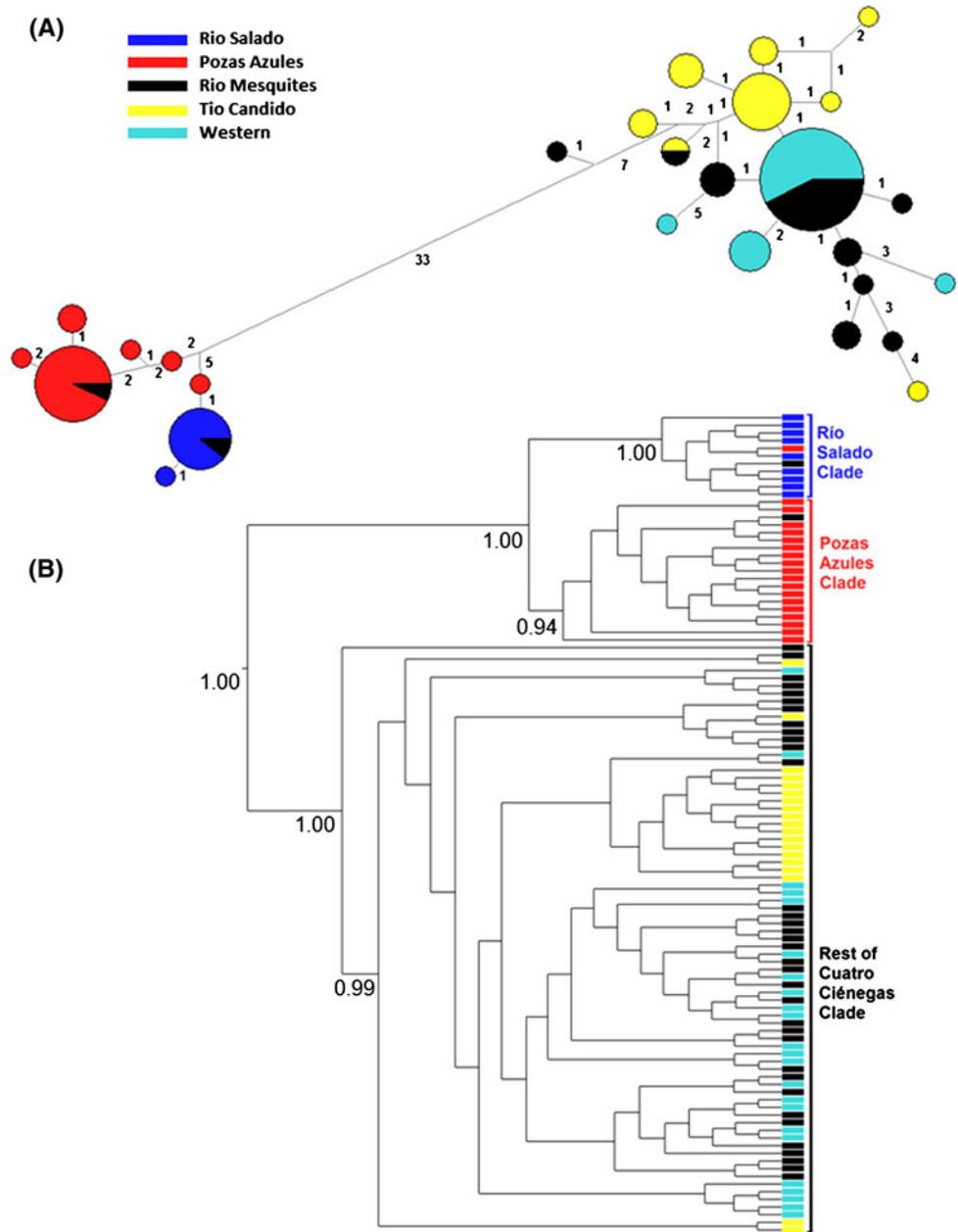


Table 1 Summary of genetic differentiation by region of *Palaemonetes suttkusi* COI haplotypes

	PA	RS	RM	TC	W
PA		<0.00001	<0.00001	<0.00001	<0.00001
RS	0.84		<0.00001	<0.00001	<0.00001
RM	0.89	0.88		<0.00001	0.14
TC	0.95	0.96	0.12		<0.00001
W	0.97	0.98	0.03	0.33	

Regions: PA Pozas Azules, RS Río Salado, RM Río Mesquites, TC Tío Cándido, W Western. Pairwise F_{ST} values are presented below the diagonal. The corresponding P -values of significance from zero are presented above the diagonal

rates, significantly different from zero, only between Río Salado and the rest of Cuatro Ciénegas, and between Pozas Azules and the rest of Cuatro Ciénegas (Table 2). However, no significant migration was detected between the rest of Cuatro Ciénegas and the ancestral population that must have split into Río Salado and Pozas Azules. This suggests that gene flow from Pozas Azules and Río Salado into the rest of Cuatro Ciénegas (0.5 migrants per generation; Table 2) occurred after Pozas Azules and Río Salado diverged from each other. The isolation with migration model implemented in IMA2 estimated that an ancestral population that occupied the entire study area split into the rest of Cuatro Ciénegas lineage and the Río Salado-Pozas

Table 2 Estimates of gene flow between populations for *Palaemonetes suttkusi* from Río Salado (RS), Pozas Azules (PA), the rest of Cuatro Ciénegas (CC), and the ancestral population (A) that split into Pozas Azules and Río Salado (see Fig. 2b)

	Donor → recipient							
	PA → RS	CC → RS	RS → PA	CC → PA	RS → CC	PA → CC	A → CC	CC → A
Estimate	0.38	0.00	0.00	0.00	0.50	0.50	0.00	0.00
95% Lo	0.06	0.01	0.00	0.00	0.11	0.12	0.05	0.03
95% Hi	5.31	3.37	1.68	1.15	4.93	7.37	9.25	8.99
LLR	1.11	0.00	0.03	0.00	3.62*	4.48*	0.00	0.00

The estimates and 95% confidence intervals are shown for the number of immigrant females per generation as estimated from IMA2. The result of a likelihood ratio test for significance from zero is also shown. Populations showing significant migration rates ($P < 0.05$) are indicated with an asterisk

Azules lineage between 1.9 (value with the highest posterior probability) and 2.5 Myr ago (mean value). It also estimated that the Río Salado population split from the Pozas Azules population between 11,000 years ago (value with the highest probability) and 1.1 Myr ago (mean value).

Discussion

Natural differentiation between Río Salado and Cuatro Ciénegas

This study demonstrates that the Río Salado populations of *P. suttkusi* are evolutionary distinct from populations found in the Cuatro Ciénegas basin. This strongly suggests that *P. suttkusi* colonized both the Río Salado and the Cuatro Ciénegas drainages in the distant past. Substantial divergence was also revealed between the Pozas Azules area and the rest of the Cuatro Ciénegas basin. However, we did not detect the same degree of divergence in *P. suttkusi* that has been found in other species between Western and Río Mesquites populations, or between these two areas and Tío Cándido (Rodríguez-Martínez 2004; Johnson 2005; Carson and Dowling 2006). Low levels of differentiation in *P. suttkusi* populations were revealed between Western, Río Mesquites and Tío Cándido (Table 1), but not enough to suggest they are distinct lineages (Fig. 2b).

Overall, *P. suttkusi* populations exhibit substantial structuring consistent with a long occupation of both the Río Salado and Cuatro Ciénegas. The BEAST and IMA2 analyses placed the initial time of divergence between the two major lineages (Río Salado + Pozas Azules and the rest of Cuatro Ciénegas) between 1.9 and 11.2 Myr ago. This estimate is large but does overlap with a major xeric period that occurred in North American deserts approximately 2.6 Myr ago. This dry period was preceded by a major wet period 3.2 Myr ago and followed by the last major wet period in the region that occurred about 1.3 Myr

ago. This scenario suggests that extensive gene flow between the Río Salado + Pozas Azules and the rest of the Cuatro Ciénegas basin could have occurred during the major wet period that occurred around 3.2 Myr ago, in which all of the drainages were presumably connected. However, the lack of putative gene flow between Río Salado + Pozas Azules and the rest of Cuatro Ciénegas until recently, along with the date of the split of these two major lineages (no more recent than 1.9 Myr ago), suggests that gene flow between these two major lineages was drastically reduced during and/or shortly after the major xeric period that started about 2.6 Myr ago.

Recent gene flow between Río Salado + Pozas Azules and the rest of Cuatro Ciénegas

Despite the long period of isolation between the Río Salado and Pozas Azules and the rest of Cuatro Ciénegas, the analysis of isolation with migration suggests that limited gene flow has recently occurred between these two major regions. No gene flow was detected between the ancestral population of Pozas Azules + Río Salado and the rest of Cuatro Ciénegas. However, moderate levels of gene flow were detected from Pozas Azules and Río Salado into the rest of Cuatro Ciénegas after the Pozas Azules and Río Salado populations diverged from each other. The BEAST and IMA2 analyses suggest that the initial time of divergence between Río Salado and Pozas Azules most likely occurred at some point between 1.4 Myr ago and 11,000 years ago. This time range includes the timeframe for the relatively major wet period that occurred 1.3 Myr ago and the short and minor wet periods that occurred during the Holocene. Two scenarios could explain this result: (1) natural gene flow that occurred during these recent wet periods or (2) recent migration through human-built canals.

Natural gene flow could have occurred several times since the last significant aquatic connection between the Río Salado and Pozas Azules was severed. Although

North American deserts are currently in a significantly drier period, a major wet period occurred 1.3 Myr ago as noted above (Smith 1984). Furthermore, the Chihuahuan desert has gone through several relatively wet cycles during the Holocene that increased water levels of lakes in this desert and caused vegetation changes (Metcalfe et al. 2002; Butzer et al. 2008). In the specific case of the Cuatro Ciénegas valley, woodlands occurred near the valley floor until ca. 11,000 years ago, when they migrated upslope as the climate warmed and the valley became arid (Minckley and Jackson 2008). There is also evidence that conditions became wet enough to support marshes in a currently dry basin in another area of the Chihuahuan desert about 4000 years ago (Castiglia and Fawcett 2006; Minckley and Jackson 2008). Water levels could have increased to the point of allowing gene flow between the rest of Cuatro Ciénegas and Pozas Azules as well as the Río Salado in the past 1.3 Myr. The lack of strong divergence among the Río Mesquites, Tío Candido, and the Western regions is consistent with the hypothesis that at least some areas in Cuatro Ciénegas were sufficiently connected to allow the movement of obligate aquatic organisms in recent times. Flooding caused by large storms such as hurricanes could also have allowed limited gene flow for short periods. Hurricane Alex flooded the area in July 2010 connecting many pools and river systems around the Sierra for about 2 weeks (A. Zertuche pers. com). Similar hurricanes hit the area in 1967 and 1988 (Furlong et al. 2007). According to Cuatro Ciénegas locals, these hurricanes also resulted in brief aquatic connections of many pools and river systems.

Despite the possibility of natural gene flow among the Río Salado, Pozas Azules, and the rest of Cuatro Ciénegas in the past million years, some pieces of information suggest that migration may be currently occurring through artificial canals. Two artificial canal systems currently connect the headwaters of the Río Salado with pools and streams near the Río Mesquites in Cuatro Ciénegas (Saca Salada canal) and the Pozas Azules region (Santa Tecla Canal) (Miller and Minckley 1963; Hendrickson et al. 2008). These two canals come in contact with each other at the eastern edge of the Cuatro Ciénegas valley as they reach the headwaters of the Río Salado. Consistent with the possibility of migration through canals is our finding that one individual carrying a Pozas Azules haplotype and one individual carrying a Río Salado haplotype were caught in the Río Mesquites area (Fig. 2). This suggests that these individuals, or recent relatives, could have reached the Río Mesquites from Pozas Azules and Río Salado using the canals. Specifically, it is reasonable to infer that one individual reached the Río Mesquites area using the Saca Salada canal system, which starts just a few km from Río Mesquites and flows uninterrupted about 45 km to the

headwaters of Río Salado. This scenario would require upstream migration, but this is not uncommon in freshwater shrimp (Elliott 2003). The other individual possibly reached the Río Mesquites area from the Pozas Azules area via the Santa Tecla canal (i.e. 30 km in the direction of the flow) and/or the Saca Salada canal (i.e. 30 km upstream). The Santa Tecla canal starts in the Santa Tecla pool, which is located near Pozas Azules (Fig. 1). The Santa Tecla pool and Pozas Azules are not currently connected but shared haplotypes are found within other Cuatro Ciénegas species inhabiting both areas (Johnson 2005), and this could presumably be what has occurred in populations of *P. suttkusi* as well.

Comparison with other phylogeographic studies of Cuatro Ciénegas endemics

The results from this study show similarities and differences with phylogeographic studies of other taxa within Cuatro Ciénegas. Previous studies on the endemic aquatic snail *M. churinceanus* and a few fish species have revealed strong population differentiation within Cuatro Ciénegas. Studies of all of these organisms recovered major phylogeographic splits within lineages that correspond to the western and eastern sides of the Sierra, and differentiation between those populations and populations located in the southeast part of the basin, i.e. Tío Cándido and Pozas Azules (Rodríguez-Martínez 2004; Johnson 2005; Carson and Dowling 2006). In the case of *P. suttkusi*, we found strong differentiation between the Pozas Azules area and the rest of the basin, but little differentiation between the western and eastern sides of the Sierra. Interestingly, we found a similar pattern of sequence divergence in *P. suttkusi* that was found in a recent phylogeographic study on the endemic hydrobiidae spring snail *Nymphophilus minckleyi* (unpublished data). Generally, gene flow in the shrimp could be limited by mobility as it likely is in many hydrobiid snails and pupfish (Barlow 1958; Lee and Fielder 1984; Schneider and Lyons 1993; Sutton 2002). The dispersal abilities of *P. suttkusi* and other fully aquatic species are likely reduced compared to the endemic turtle *Terrapene coahuila* that exhibits little population subdivision in Cuatro Ciénegas (Howeth et al. 2008). The mechanistic factors influencing gene flow among populations of the endemic species in this region are not consistent across taxa.

Conclusion and management recommendations

We provide evidence that *P. suttkusi* occurs naturally in the Río Salado de Los Nadadores drainage, just outside

the Cuatro Ciénegas valley. Before this study, it was unclear whether taxa present in both areas moved to the Río Salado from Cuatro Ciénegas through human-built canals or whether they occurred there naturally. The large levels of sequence divergence that exist between the Río Salado and most of Cuatro Ciénegas, and the long time that has passed since both areas were isolated from each other suggest that both basins should be considered distinct areas for conservation but not part of the same bioregion. The absence of complete lineage sorting between Cuatro Ciénegas and Río Salado may have been retarded by recent gene flow occurring during the last million yrs, and/or obscured by more recent gene flow occurring through human-built canals. Nevertheless, the presence of unique haplotypes in both areas, the low gene flow estimates (less than one migrant per generation), and the phylogenetic divergence concordant with a geographic barrier (the edge of a valley) should warrant considering the populations within Río Salado as an important conservation ESU (Crandall et al. 2000). The Pozas Azules area is also physically isolated from the rest of the basin (Fig. 1) and it is well differentiated genetically from both Río Salado and the rest of Cuatro Ciénegas. Hence, the populations from Pozas Azules and the rest of Cuatro Ciénegas, which are now largely managed as a single unit, should be considered two different ESUs for conservation as well. The Pozas Azules area in Cuatro Ciénegas is already well protected because it is owned by PRONATURA, a Mexican non-profit conservation organization. Much of the rest of Cuatro Ciénegas has some protection because the valley of Cuatro Ciénegas is a national protected area. Nevertheless, this protected status does not preclude regulated farming activities and most pools and rivers are located in private farms, which raises some concerns about sustainability (Hendrickson et al. 2008). Conversely, the upper parts of the Río Salado area where

P. suttkusi is found (see Appendix) are located completely outside the protected areas and this area is now very degraded due to human disturbance (Hendrickson et al. 2008). For this reason, we recommend expanding the protected area of Cuatro Ciénegas to include the upper parts of the Río Salado. This would help preserve distinct variants of *P. suttkusi* and likely other native/endemic taxa that are currently at risk in the Río Salado area due to severe human alteration.

Our study also points to the possibility of human-induced exchange of organisms between Cuatro Ciénegas and the Río Salado, and between Pozas Azules and the rest of Cuatro Ciénegas via artificial canals. These canals could be allowing the migration of individuals between otherwise naturally isolated areas. Additional studies should be conducted soon to confirm this possibility. Immediate management actions should be implemented if migration between isolated areas is confirmed to prevent the risk of losing differently adapted geographic variants in the numerous species endemic to this entire high priority conservation region.

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Appendix

See Table 3.

Table 3 Collection sites, sample size (*N*) and GPS co-ordinates for *Palaemonetes suttkusi* in the study area

Drainage	Locality	<i>N</i>	Coordinates
Western	1. Laguna Churince	5	26°50.53N, 102°08.20W
	2. Juan Santos	18	26°53.97N, 102°08.96W
Río Mesquites	3. Tierra Blanca	18	26°55.65N, 102°08.31W
	4. Mojarral East	19	26°55.48N, 102°07.28W
Southeastern	5. Tío Cándido	19	26°52.33N, 102°04.85W
	6. Pozas Azules	19	26°49.83N, 102°01.76W
Río Salado	7. San José del Águila	9	27°02.06N, 101°43.30W

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