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# Age, phylogeography and population structure of the microendemic banded spring snail, *Mexipyrgus churinceanus*

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**Abstract**

**Recent theoretical and empirical studies of phylogeography and population structure indicate that many processes influence intraspecific evolutionary history. The present study represents the first examination of various forces influencing the spatial and temporal patterns of sequence variation in the freshwater Mexican banded spring snail, *Mexipyrgus churinceanus*. This snail occurs in one of the most critically endangered centres of freshwater endemism, the desert ecosystem of Cuatro Ciénegas. From cytochrome *b* mtDNA sequence variation, there is strong evidence of long-term isolation of three regions, suggesting that these regions represent evolutionarily distinct lineages. Molecular clock estimates of clade age indicate a time to most recent common ancestor of approximately 2.5 million years ago (Ma). The three regions differ considerably in the historical and demographic forces affecting population structure. The western populations have extremely low mtDNA diversity consistent with a severe bottleneck dating to 50 000 years before present (BP). The nearby Rio Mesquites drainage is characterized by fragmentation events, restricted gene flow with isolation by distance, and higher levels of mtDNA polymorphism. These patterns are consistent with the long-term stability of this drainage along with habitat heterogeneity and brooding contributing to population isolation and restricted gene flow. Southeastern populations show evidence of range expansion and a strong influence of genetic drift. Migration rates between drainages indicate very little gene flow between drainages except for asymmetric migration from the Rio Mesquites into both western and southeastern drainages.**

*Keywords*: Cuatro Ciénegas, *Mexipyrgus*, mtDNA sequence variation, phylogeography, population structure

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## Introduction

A central concern of statistical phylogeography is to understand how historical processes mould the spatial and temporal patterns of intraspecific genetic variation (Avise 1989, 2000; Templeton 1998, 2004). Recent spatial and temporal analyses of genetic variation suggest that current patterns of genetic structure are likely the result of complex interactions among many processes (Althoff & Pellmyr 2002; Branco *et al*. 2002). Multiple techniques are required to assess how historical isolation, gene flow, and demographic processes influence current patterns of population structure and genetic diversity. Understanding the relative roles of these processes for a species' evolutionary history has implications for numerous issues. For example, spatial patterns of sequence divergence and diversity can provide important information regarding conservation and management units as well as an understanding of whether parallel evolution can explain the independent evolution of similar adaptations (Schluter & Nagel 1995; Thompson *et al*. 1997). For aquatic organisms, habitat fragmentation probably enhances population differentiation, because suitable habitat is often embedded within unsuitable terrestrial environments. Therefore, different drainages may not experience gene flow depending

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upon the organism's dispersal capabilities. For freshwater organisms, long-term isolation among drainages may lead to considerable divergence, but drainages may differ in the influence of demographic processes such as population bottlenecks and expansions (Rogers & Harpending 1992; Rogers 1995; Harpending *et al*. 1998). If certain geographic areas are subject to severe reductions in population size or there is greater dispersal between habitats in some geographic areas vs. others, the spatial scale of genetic differentiation may vary among geographic regions (Bousset *et al*. 2004).

The present study examines microgeographic variation in cytochrome *b* mtDNA sequences in an aquatic snail, the banded spring snail (*Mexipyrgus churinceanus*). This ovoviviparous, hydrobiid snail is endemic to the small, closed basin of Cuatro Ciénegas, which is located in the Chihuahuan Desert in northeastern Mexico. At present, there are no phylogeographic studies of sequence variation for any organism in this valley, which is considered a Priority Class I ecoregion because of its vulnerability to ecosystem alteration and its biological distinctiveness (Abell *et al*. 2000). From a conservation management perspective, an assessment of phylogeographic structure and genetic diversity will provide important information about evolutionarily significant units (ESUs) and levels of genetic variation. This information is especially critical because certain aquatic habitats are threatened by anthropogenic disturbances such as canal diversions and development (Minckley 1969; Johnson, personal observations).

Temporal and spatial patterns of sequence variation can also provide important insights into the unusual shell morphology of this freshwater snail. Compared to most freshwater snails, *M. churinceanus* has ribbing, sculpturing, and thick shells that are more characteristic of marine snails (Vermeij & Covich 1978; Bertness & Cunningham 1981). *Mexipyrgus churinceanus* also exhibits microgeographic variation in shell structures and load strength (Taylor 1966; Hershler 1983; Hershler & Minckley 1986; Tang & Roopnarine 2003; Hulsey & Johnson, submitted). Vermeij & Covich (1978) proposed that this is an ancient basin in which elaborate shell architecture of *M. churinceanus* is the result of co-evolutionary interactions between endemic snails and the molluscivorous morph of an endemic cichlid, *Hericthys minckleyi*. Other authors have also suggested an ancient origin given the high levels of endemism in numerous taxa (Taylor 1966). A goal of the present study is to provide the first age estimates for an organism from this unique ecosystem.

Another focus is to provide a historical framework for understanding spatial variation in phenotypic characters such as shell architecture and resistance to predation. The approach used in this study was to examine an independent character set (mtDNA sequence variation) in order to assess whether divergent genetic lineages are associated with drainage isolation. Phylogenetic evidence of independent lineages can then be used to assess whether prey defensive traits have evolved multiple times (Brodie *et al*. 2002; Janzen *et al*. 2002). An intriguing possibility is that similar patterns of phenotypic diversification in shell architecture and load strength have been generated within divergent lineages, perhaps driven by interactions with the molluscivorous cichlid, *H. minckleyi.* Given that suitable aquatic habitats are embedded within the inhospitable matrix of the Chihuahuan Desert, isolated drainages represent islands, perhaps promoting local adaptation.

The physiogeographic features of the basin may structure genetic divergence in *M. churinceanus*. Spring-fed pools and stream outflows occur primarily around the base of the central mountain range (Sierra de San Marcos; see Fig. 1). Genetic divergence of *M. churinceanus* populations may occur at various scales. The two most obvious physiographic features that may play a role in isolation are (i) the northward-jutting mountain range, the Sierra de San Marcos, which bisects the basin into eastern and western lobes, and around which most pools and streams are clustered, and (ii) the microgeographic isolation of pools and riverine systems (Fig. 1). *Mexipyrgus churinceanus* is most common in the following three areas: two western drainages consisting of spring-fed pools and stream outflows (Churince and the Becerra/Garabatal systems); the Rio Mesquites near the northern tip of Sierra de San Marcos and draining to the southeast; and isolated pools of the southeastern basin (Tio Candido, Pozas Azules, and Santa Tecla). These three regions appear isolated at present, and gene flow may be restricted between the major drainages. Even though there are no direct surface connections at present, the proximity of the upper headwater of the Rio Mesquites and the downstream areas of Rio Garabatal may have facilitated exchange historically, especially prior to canal diversions (Minckley 1969).

Spatial differentiation in *M. churinceanus* may also be enhanced by habitat specialization and brooding. This snail occurs only where there are ample and invariable sources of water, gentle or negligible water currents, warm and constant temperature, and soft substrates (Hershler 1983). Hard travertine sediment exposed by strong water currents may effectively isolate *M. churinceanus* populations within drainages. Because female snails brood their larvae, dispersal may be restricted. Therefore, important genetic structuring may occur among drainages and perhaps within drainages due to limited chance for dispersal.

The following questions were addressed regarding the spatial and temporal distribution of mtDNA sequence variation in *M. churinceanus*. (i) Does phylogenetic reconstruction of mtDNA sequence variation indicate deep divergences consistent with long-term isolation caused by physiographic features? (ii) What is the time to most recent

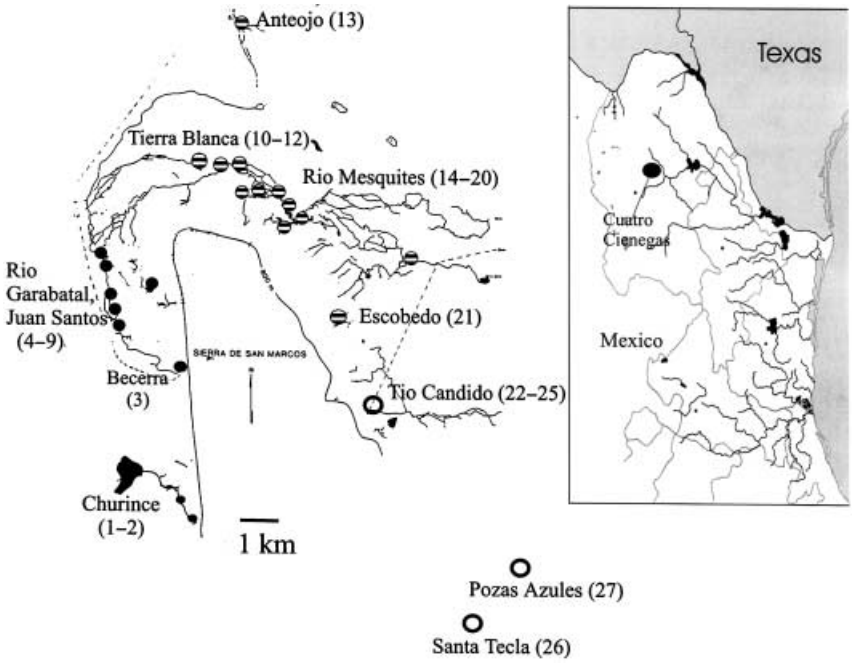


**Fig. 1** Map of geographical locality of Cuatro Ciénegas and the major drainages and locations of sampled sites within the basin. Symbols for the three regions are solid circles (western), horizontal-line circles (Rio Mesquites), and unfilled circles (southeastern). See Appendix for sample size and more detailed locality information.

common ancestor (tMRCA) employing molecular clock estimates? (iii) How do historical and contemporary processes influence the current patterns of mtDNA sequence variation within and among drainages?

## Materials and methods

### *Geographic sampling and molecular methods*

Twenty-seven sites containing *Mexipyrgus churinceanus* were sampled from throughout the entire Cuatro Ciénegas basin (Fig. 1; Appendix). All specimens were frozen in liquid nitrogen and then stored at –80 °C. Total DNA was extracted from foot tissue using the QIAGEN DNeasy Plant Mini Kit™. A portion of *M. churinceanus* cytochrome *b* was amplified with primers designed by Collins *et al*. (1996). For each population, a 710-base-pair fragment of mitochondrial cytochrome *b* was amplified and sequenced. Mitochondrial cytochrome *b* amplifications were performed in 50 μL solutions containing 10 mM Tris (pH 8.3), 50 mM KCl, 5.5 mM $MgCl_2$, each dNTP at 200 mM, 30 pmol of each primer, 1–2 μL of undiluted template DNA, and 2 units of *Taq* polymerase. Polymerase chain reaction (PCR) cycling parameters were 45 s at 92 °C, 50 s at 47 °C and 75 s at 72 °C, for 30 cycles followed by 72 °C for 5 min. Amplification products were purified with Wizard PCR Preps™ (Promega) or GeneClean™ (QBIOgene), and sequenced on either an ABI 377 or 3100 automated sequencer (Applied Biosystems). A 697-bp fragment was aligned using CLUSTAL W (Thompson *et al*. 1994).

### *mtDNA gene tree and clade ages*

To examine temporal divergences in the gene tree, phylogenetic relationships were reconstructed among cytochrome *b* haplotypes using maximum-likelihood and Bayesian approaches. The best-fitting, least-parameter rich model of sequence evolution was based on hierarchical likelihood-ratio tests performed in MODELTEST 3.06 (Posada & Crandall 1998). This method identified the GTR + I + G as the optimal model. This model of sequence evolution and its parameter estimates were used to perform a heuristic maximum-likelihood search with 10 replications of stepwise addition and tree-bisection–reconnection (TBR) branch swapping in PAUP* (Swofford 2001). MRBAYES (Huelsenbeck 2000) was used to estimate the clade support by running the Markov chain for 5 000 000 generations and using a burn-in of 500 000. The remaining trees were imported into PAUP*, and the numbers at interior branches of a majority-rule consensus tree were used as the probability of that clade existing.

To provide an estimate of tMRCA for two very divergent mtDNA lineages in the eastern and western drainages (see Results), divergence dates were estimated using BEAST 1.0, which employs a Bayesian Markov chain Monte Carlo (MCMC) algorithm (Drummond & Rambaut 2003). An HKY model of sequence evolution was employed with the substitution rate based on gastropod-specific 4.0% sequence divergence per million years (Myr) for third-base-pair transitions (Collins *et al*. 1996). Only third-base-pair transitions were used. Five million chains were conducted with

a burn-in of 500 000, and stability of estimates was achieved by examining ESS values. Because BEAST can only determine tMRCA for two sequences, haplotypes were randomly chosen from the western drainage and the eastern drainages. Seven BEAST runs were conducted with eastern haplotypes randomly drawn from the following locations: Tierra Blanca, Los Remojos, Mojarral West, Laguna Escobedo, Tio Candido, Pozas Azules, and Rio Mesquites. There were three independent MCMC runs for each of seven comparisons.

### *Population structure, diversity measures, gene flow, and demography*

To test the hypothesis that drainage isolation plays the primary role in generating genetic subdivision, analysis of molecular variance (AMOVA) was used to examine genetic structuring among three regions: (i) western populations; (ii) Rio Mesquites including Escobedae and Anteojo; and (iii) southeastern pools. The total sequence variation was partitioned into differences among the three regions ($\Phi_{CT}$), among localities within the three regions ($\Phi_{SC}$), and among localities ($\Phi_{ST}$). Significance of each estimator was based on 1000 permutations and all analyses were run in ARLEQUIN 2.000 (Schneider *et al*. 2000). To test the hypothesis that drainages differ in genetic diversity, gene and nucleotide diversities were calculated for all 27 populations, and then the hypothesis that these two measures do not differ among regions was tested using a Mann–Whitney *U*-test.

MIGRATE was used to estimate gene flow between the three geographic regions (Beerli & Felsenstein 2001; Beerli 2002). This method gives a maximum-likelihood approximation based on coalescence theory and employs a Metropolis–Hastings MCMC algorithm to estimate migration rates. Starting theta and *M* values were generated from $F_{ST}$ calculations, and a migration matrix model with variable theta was employed under the following conditions: 10 short chains with 100 000 trees sampled, three long chains with 1 000 000 trees sampled with a burn-in of 100 000 trees, and three replicates of these Markov chain settings.

Nested clade analysis (NCA) was used to examine causes of geographic associations of haplotypes due to either historical processes such as fragmentation, colonization or range expansions or present-day processes such as restricted gene flow (Templeton 1998, 2004). Haplotype networks were constructed using TCS 1.13 (Clement *et al*. 2000) and nesting categories followed Templeton & Sing (1993). Closed loops were resolved in the cytochrome *b* network based on coalescence theory (Crandall *et al*. 1994). The GEODIS 2.0 program (Posada *et al*. 2000) was employed for statistical analyses of geographic associations of haplotypes and clades and all analyses used 10 000 Monte Carlo simulations. Surface distances from GPS longitude and latitude were calculated (USDA website: http://www.wcrl.ars.usda.gov/cec/java/lat-long.html). The revised inference key (Templeton 2004) was used to infer whether genetic structuring was due to restricted gene flow, various forms of fragmentation, range expansion, long-distance colonization or combinations of these factors.

Two techniques were used to examine whether the three drainages differed in demographic events that may lead to nonequilibrium patterns. Following the approach of Hutchison & Templeton (1999), the relationship between $F_{ST}$ and geographic distance was examined in pairwise population comparisons for the three regions. Assuming a regional equilibrium between gene flow and drift, there should be a positive, monotonic relationship between $F_{ST}$ and geographic distance, which is the case I pattern indicating isolation by distance. Where regional populations are not in equilibrium, no relationship between $F_{ST}$ and geographic distance can arise under two scenarios. First, case II where there is little variance in estimates in divergence either due to recent bottlenecks and/or founding of populations from homogenous source population. Second, case III where, subsequent to a founding event, populations are fragmented into small, isolated populations, and drift is stronger than gene flow. Under two possible scenarios, there would be high variance in divergence estimates and a wide degree of scatter of point estimates. Population pairwise $F_{ST}$ estimates were determined using Kimura 2-parameter (K2P) distances with no gamma correction in ARLEQUIN 2.000 (Schneider *et al*. 2000). Mantel tests (1000 permutations) were used to assess the significance of the relationship between pairwise $F_{ST}$ estimates and surface distances for each of the three regions.

A mismatch distribution analysis (MDA) of mtDNA sequences tested for evidence of population expansion (Schneider & Excoffier 1999). The sum of squared deviations was used as a test of the goodness of fit (1000 bootstrap replicates) to the sudden expansion model. Initial and final population size ($\theta_0$ and $\theta_1$, respectively) and tau ($\tau$) were estimated from a sudden expansion model. Unimodal distributions usually characterize population expansions, and the MDA age expansion parameter ($\tau$) was used to date the initiation of the range expansion assuming a generation time of 1 year (Johnson, unpublished) and a 2% sequence divergence per Myr (see Rogers & Harpending 1992 for equations).

## Results

### *Sequence variation*

There were 59 unique mitochondrial haplotypes among the 193 *Mexipyrgus churinceanus* individuals. Unique mtDNA haplotypes have been deposited in GenBank (Accession nos AY851303–AF851361). Amino acid translation using the *Drosophila* genetic code in DNASP (Rozas *et al*. 2003) of

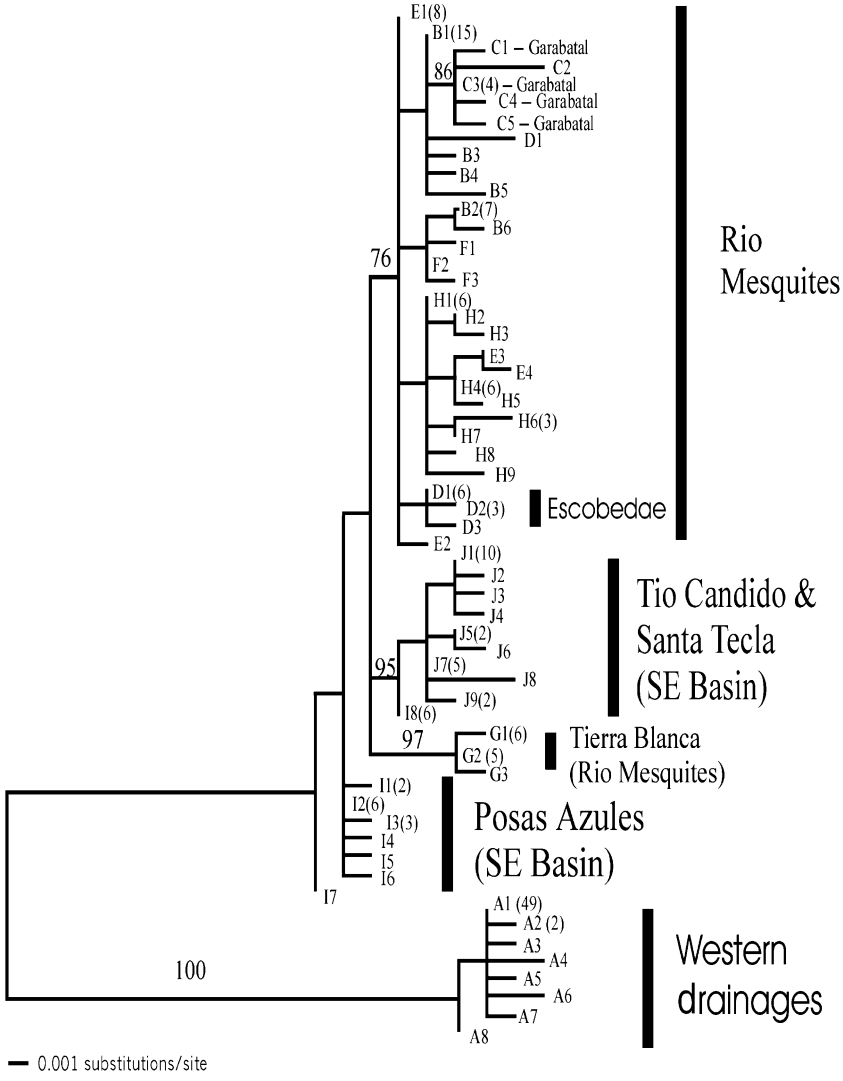


**Fig. 2** Maximum-likelihood phylogram for 49 unique cytochrome *b* haplotypes with midpoint rooting. The model of sequence evolution was GTR + I + G. See text for parameter estimates. Numbers on branches represent Bayesian support for nodes. Samples sizes for each unique haplotype are one except where noted. The scale bar at the bottom left is proportional to branch length, measured as the number of DNA substitutions per site.

all unique haplotypes indicated no stop codons. The maximum K2P distance among these haplotypes was 4.18% and ranged from 0.14% to 4.18%. Of the 65 variable nucleotide positions, 38 were parsimony informative, and there were 50 synonymous and 15 nonsynonymous changes. The average nucleotide base frequencies among haplotypes were A = 0.28, T = 0.41, C = 0.15, and G = 0.16.

### *Phylogenetic relationships and clade ages*

Maximum-likelihood analysis of the 59 unique mitochondrial haplotypes under GTR + I + G (I = 0.67, G = 0.92) model of sequence evolution resulted in a single tree with a likelihood score of –ln L = 1499.88 (Fig. 2). The most obvious feature of the midpoint-rooted tree is considerable divergence of mtDNA haplotypes from western drainages from haplotypes from the other two drainages with 100% Bayesian support for the western clade. The average pairwise divergence between these two groups is 3.8%. Within this western clade, there is a single, common haplotype (A1) that is geographically widespread among the Churince and Garabatal drainages, and seven haplotypes with limited sequence divergence from common haplotype. In the other major grouping, there are well-supported groups for Rio Mesquites haplotypes, peripheral populations within the Rio Mesquites drainage (Escobedo and Tierra Blanca), and southeastern haplotypes from Tio Candido and Santa Tecla. Haplotypes restricted to Pozas Azules also show strong bootstrap support (77%), although the midpoint-rooted tree places them as the basal group to this clade.

The mean Bayesian estimate of tMRCA for the seven comparisons of western and eastern haplotypes was 2.55 Ma with upper and lower confidence limits of 3.94 and 1.37 Myr. For the seven analyses, tMRCA ranged from 2.51 to 2.57 Myr.

*Population structure, diversity measures, gene flow, and demography*

The AMOVA for mtDNA sequence variation indicated that most of the total variation among haplotypes (74.8%) was explained by differences among the three regions ($\Phi_{CT} = 0.748$, $P < 0.005$). Differences among populations within regions accounted for just 9.0% of the total variation ($\Phi_{SC} = 0.358$, $P < 0.001$) and differences among populations accounted for 16.2% of the total variation ($\Phi_{ST} = 0.838$, $P < 0.001$). Gene and haplotype diversity for each population are presented in the Appendix. Populations in lower reaches of the Rio Garabatal (G4 and G5) have elevated nucleotide diversities because of the presence of two divergent mtDNA haplotypes (approximately 4% sequence divergence). Neither gene nor nucleotide diversity differed significantly among regions ($P > 0.10$ for both tests). When divergent western haplotypes, probably derived from the Rio Mesquites (see Fig. 1), were excluded, western drainages had significantly lower nucleotide diversity (Kruskal-Wallace = 5.97, $P = 0.05$). Results of the MIGRATE analysis indicated there were highly asymmetrical patterns of historical migration: there is no gene flow between drainages except for very moderate levels of gene flow from the Rio Mesquites into both the western and southeastern drainages (Table 1).

Using the statistical parsimony algorithm in TCS, mtDNA haplotypes differing by nine or fewer substitutions could be connected with 95% probability. Cladogram estimation resulted in two independent networks corresponding to the two major groups present in the maximum-likelihood phylogram. For the western network, the null hypothesis of no association between clades and geographic location could not be rejected ($P = 0.673$), indicating lack of genetic structure among western populations. There is a common, widespread interior haplotype with rare tip haplotypes restricted to specific habitats. For the network presented in Fig. 3, the null hypothesis of no association between clades and geographic locations was rejected for eight clades (Table 2). Four processes leading to geographic associations of haplotypes were inferred: range expansion, allopatric fragmentation, restricted gene flow with isolation by distance, and long-distance colonization followed by recent fragmentation.

Range expansion was inferred for clades from the southeastern (clade 2-1) and western (clade 3-3) regions (see Table 1 and Fig. 3 for all significant associations). For the southeastern clade 2-1, tip clade 1-1 has a significantly large clade distance with individuals from Tio Candido and the distant Santa Tecla pools, while interior clade 1-4 has a significantly small clade distance with individuals only from Tio Candido. The I–T clade distance is also significantly small. A contiguous range expansion was inferred for clade 3-3: the interior clade 2-7 has a significantly small clade distances. This range expansion represents the dispersal of Rio Mesquites' individuals into the Rio Garabatal in the western drainage.

Various forms of fragmentation were inferred with one case from the southeastern region (clade 2-2) and three cases from the Rio Mesquites (clades 2-3, 3-2, and 3-4). For the southeastern clade 2-2, clade 1-5 contained individuals from Tio Candido and clade 1-6 contained individuals from Pozas Azules. Clade distances are significantly small and there is a significantly large $D_n$ for clade 1-5. The low level of sequence divergence between these two populations suggests recent fragmentation. For Rio Mesquites clade 2-3, long-distance colonization with recent fragmentation was inferred: tip clade 1-11 has a significantly large nested clade distance and includes mostly individuals from the isolated Anteojo population (Fig. 1). For clade 3-2, allopatric fragmentation was inferred: clade 2-5 has a significantly small clade distance with all individuals restricted to Escobedo, which is an isolated pool some distance from the other clades that are restricted to the Rio Mesquites. Because there are extremely short branch lengths separating the Escobedo clade from clade 2-4, this restricted distribution is probably of recent origin. Allopatric fragmentation was also inferred for Rio Mesquites clade 3-4: clade 2-8 is restricted to Mojarral east pool and clade 2-9 is restricted to the upper headwaters of the Rio Mesquites drainage

**Table 1** Maximum-likelihood estimates of gene flow between *Mexipyrgus churinceanus* populations from western, Rio Mesquites, and southeastern regions. The analysis used an unrestricted migration matrix with variable subpopulation size. The ML estimate (bold) and 95% profile confidence intervals (in parentheses, below) are shown for the number of immigrant females per generation ($2N_f m$), where $N_f$ is the female effective population size and u is the mutation rate per generation per site

| Population → *i* | Western → *i* | Rio Mesquites → *i* | Southeastern → *i* |
|---|---|---|---|
| Western | — | **0.4328**<br>(0.0972, 1.2107) | **0.0000**<br>(0.0000, 0.3718) |
| Rio Mesquites | **0.0000**<br>(0.0000, 0.2660) | — | 0.0000<br>(0.0000, 0.2635) |
| Southeastern | **0.0000**<br>(0.0000, 0.4199) | **0.6253**<br>(0.1482, 1.6617) | — |

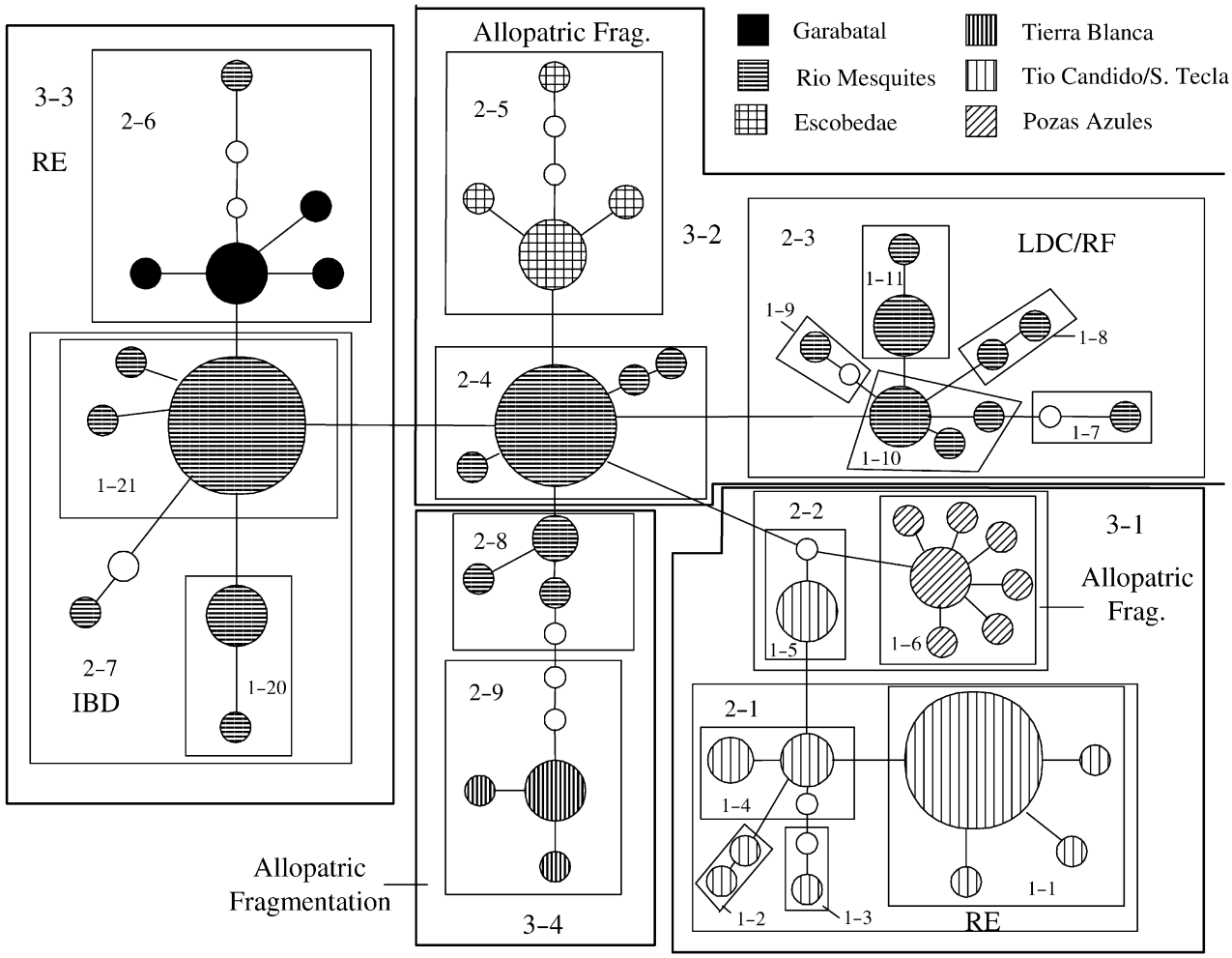


**Fig. 3** TCS haplotype network estimated from the cytochrome *b* mtDNA sequences. Haplotypes frequencies are proportional to circle area. Small open circles are inferred missing haplotypes that were not observed in the data. Clade nesting levels are used to infer the underlying population processes: RE, range expansion; IBD, restricted gene flow with isolation by distance; LDC/RF, long-distance colonization with recent fragmentation.

(Tierra Blanca). For the overall cladogram, the southeastern clade 3-1 had a significantly small clade distance and a significantly large nested clade distance, a pattern consistent with long-distance colonization and/or past fragmentation. A long-distance colonization event and recent fragmentation was inferred because of the short branch lengths separating the southeastern clade from the Rio Mesquites 3-step clades. Restricted gene flow with isolation by distance was inferred for Rio Mesquites clade 2-7: tip clade 1-20 has a significantly small clade distance being restricted to Mojarral East and nearby site in the Rio Mesquites.

Based on the limited sequence divergence among haplotypes in the western drainages and evidence for range expansion for the western and southeastern drainages, demographic events leading to nonequilibrium patterns were examined. The relationship between pairwise $F_{ST}$ and geographic distance measures for each of the three regions is presented in Fig. 4. The only significant relationship between pairwise $F_{ST}$ values and geographic distance occurs in the Rio Mesquites drainage ($r = 0.45$, $P = 0.015$), consistent with isolation by distance and the case I pattern. There are high values of pairwise $F_{ST}$ estimates for Rio Mesquites populations. Consistent with a bottleneck, a case II pattern is seen in the western populations where there is limited divergence among populations and no significant relationship between pairwise $F_{ST}$ values and geographic distance ($r = 0.07$, $P = 0.24$). For southeastern populations, there is also no significant relationship between pairwise $F_{ST}$ values and geographic distance ($r = -0.11$, $P = 0.65$). There is also a wide degree of scatter around point estimates, consistent with a case III pattern.

For the western and southeastern drainages, the hypothesis of a sudden range expansion could not be rejected ($P = 0.58$ and 0.39 for western and southeastern populations, respectively). However, the timing and severity of

**Table 2** Results of the nested clade analysis of cytochrome *b* for *Mexipyrgus churinceanus* populations. Only nested clades with significant geographical associations and associated probability values in parentheses are reported along with the clade dispersion ($D_c$), displacement from clades at higher nesting levels ($D_n$) and tip-interior contrasts when available. Significantly small or large $D_c$ or $D_n$ ($P < 0.05$) are indicated by S or L superscripts and are based on 10 000 permutations of the data under the null hypothesis of no geographical association. The inference is based on Templeton's revised key (2004)

| | Position | $D_c$ | $D_n$ | Inference |
|---|---|---|---|---|
| Clade 2-1 ($P < 0.001$) | | | | |
| Clade 1-1 | tip | $5.46^{L}$ | 4.78 | Contiguous Range Expansion |
| Clade 1-2 | tip | 0.04 | 1.96 | |
| Clade 1-3 | tip | 0.00 | 10.38 | |
| Clade 1-4 | interior | $0.28^{S}$ | 2.67 | |
| I-T Clades | | $-3.83^{S}$ | –1.93 | |
| Clade 2-2($P < 0.001$) | | | | |
| Clade 1-5 | interior | $0.00^{S}$ | $4.95^{L}$ | Allopatric Fragmentation |
| Clade 1-6 | tip | $0.37^{S}$ | $2.48^{S}$ | |
| I-T Clades | | –0.37 | $2.48^{L}$ | |
| Clade 2-3 ($P = 0.011$) | | | | |
| Clade 1-7 | tip | 0.47 | 3.13 | Long-Distance Colonization |
| Clade 1-8 | tip | 0.00 | 1.66 | with Recent Fragmentation |
| Clade 1-9 | tip | 0.00 | 1.66 | |
| Clade 1-10 | interior | $0.91^{S}$ | $2.07^{S}$ | |
| Clade 1-11 | tip | $0.46^{S}$ | $3.33^{L}$ | |
| I-T Clades | | 0.56 | $-0.83^{S}$ | |
| Clade 2-7 ($P = 0.027$) | | | | |
| Clade 1-20 | tip | $0.27^{S}$ | 0.73 | Restricted Gene Flow with |
| Clade 1-21 | interior | 0.83 | 0.83 | Isolation by Distance |
| I-T Clades | | $0.56^{L}$ | 0.10 | |
| Clade 3-1 ($P < 0.001$) | | | | |
| Clade 2-1 | tip | 3.74 | 4.33 | Inconclusive Outcome |
| Clade 2-2 | interior | 3.30 | 3.84 | |
| I-T Clades | | –0.43 | –0.49 | |
| Clade 3-2 ($P < 0.001$) | | | | |
| Clade 2-3 | tip | 2.75 | 3.49 | Allopatric Fragmentation |
| Clade 2-4 | interior | 3.45 | 3.46 | |
| Clade 2-5 | tip | $0.00^{S}$ | 2.87 | |
| I-T Clades | | 1.64 | 0.18 | |
| Clade 3-3 ($P < 0.001$) | | | | |
| Clade 2-6 | tip | 0.76 | 2.94 | Range Expansion |
| Clade 2-7 | interior | $0.81^{S}$ | 2.86 | |
| I-T Clades | | 0.05 | –0.05 | |
| Clade 3-4 ($P = 0.001$) | | | | |
| Clade 2-8 | interior | $0.00^{S}$. | $1.59^{L}$ | Allopatric Fragmentation |
| Clade 2-9 | tip | 0.54 | $0.56^{S}$ | |
| I-T Clades | | $-0.54^{S}$ | $1.02^{L}$ | |
| Overall Cladogram ($P < 0.001$) | | | | |
| Clade 3-1 | tip | $4.14^{S}$ | $6.09^{L}$ | |
| Clade 3-2 | interior | $3.38^{S}$ | $4.22^{S}$ | |
| Clade 3-3 | tip | $2.91^{S}$ | 6.63 | |
| Clade 3-4 | tip | $0.62^{S}$ | 4.83 | |
| I-T Clades | | $0.27^{\cdot}$ | $-1.14^{S}$ | |

the initial bottleneck varied considerably between these two regions. For the western populations, there is evidence for a severe bottleneck ($N_0 \sim 0$) dating to approximately 50 000 BP with evidence for a limited recovery of population size ($N_1 = 14\,535$). The observed mean number of differences was 0.38. The southeastern expansion occurred approximately 94 000 BP with $N_0 = 22\,000$ and $N_1 = 450\,000$. The observed mean number of differences was 2.74.

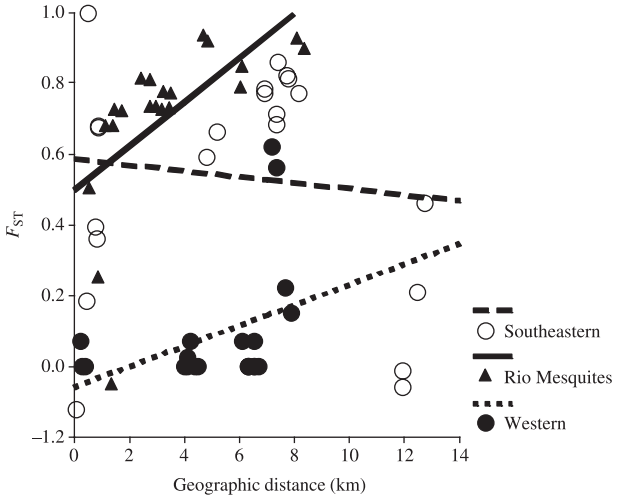


**Fig. 4** Scatter plots of $F_{ST}$ estimates against geographical distance separating each pairwise combination of populations within each region.

## Discussion

The results of the current study provide the first examination of sequence variation for an organism inhabiting the unique desert ecosystem of Cuatro Ciénegas. Through a combination of multiple analytical techniques to examine spatial and temporal patterns of mtDNA sequence variation, a complex pattern of historical and demographic influences on sequence variation was uncovered. There are two important findings from the present study: (i) a Pliocene origin of this endemic snail and pronounced sequence differentiation at small spatial scales and; (ii) complex historical and demographic influences on sequence divergence and diversity within and among drainages.

Previous authors postulated an ancient origin of the Cuatro Ciénegas fauna because of the high local diversity and endemism of the hydrobiid snails and the remarkable diversification in *Mexipyrgus* shell morphology (Taylor 1966; Vermeij & Covich 1978; Hershler 1983). Recent studies of other marine-like freshwater gastropods from Lake Tanganyika also indicate ancient origins of an incredibly diverse fauna (Wilson *et al*. 2004). Taylor (1966) suggested *Mexipyrgus churinceanus* arose 2–3 Ma although other authors have suggested the potential for a much older age for the fish fauna with evidence that the *Herichthys* clade in northeastern Mexico originated approximately 5 Ma (Hulsey *et al*. 2004). The Bayesian estimate, using a gastropod-specific molecular clock, is approximately 2.5 Ma, suggesting a late Pliocene origin of this endemic snail. Preliminary analysis of mtDNA sequence divergence among major clades of the other two endemic snail genera (*Nymphophilus* and *Mexithauma*) and the endemic cichlid (*Herichthys minckleyi*) indicate origins of these taxa of 1–4 Ma (Hulsey & Johnson, in prep).

Based on the Bayesian estimate of clade age and the presence of two cytochrome *b* networks, an allopatric fragmentation event occurred around 2.5 Ma. The most obvious mechanism for this deep split of eastern and western clades is the isolation of eastern and western drainages by the Sierra de San Marcos. There is a strong signal of historical isolation and divergence among the major drainages in the basin at an extremely small spatial scale. As opposed to organisms with higher dispersal capabilities (Fordyce & Nice 2003), small-scale genetic differentiation is also seen in other snails, primarily associated with habitat fragmentation associated with river drainages and mountain ranges (Ross 1999; Watanabe & Chiba 2001; Holland & Hadfield 2002). We are currently examining whether the other two snail genera and cichlids show congruent phylogeographic patterns (Hulsey & Johnson, in prep).

The divergence among drainages suggests that these are independent lineages in which one can examine the role of predator–prey interactions in shaping snail morphological diversification. The age of cichlid and snail lineages also suggest that there is the potential for long-term interactions. Pronounced geographic differences in the strength of interspecific interactions, such as occur between predators and prey, may be common (Benkman 1999; Brodie *et al*. 2002). Certain habitats such as the Tierra Blanca and Tio Candido have exaggerated shell strength (Hulsey & Johnson, submitted). These geographic differences in snail shell strength may be the result of habitat variation in the strength of natural selection and reciprocal interactions (Thompson & Cunningham 2002). Certain habitats may be hotspots of co-evolution in which strong selection exerted by cichlid molariforms may favour higher crushing resistance by snails. Spatial variation in selection pressure on snail shell strength may be driven by variation among habitats in the abundance, distribution and density of the mollusc-ivorous cichlid.

While there is evidence of genetic divergence among drainages, different historical and contemporary processes have influenced levels of genetic variation within drainages and geographic association of haplotypes. Demographic processes can influence current patterns of genetic variation, and there is evidence that the timing and severity of bottlenecks and demographic expansions vary among drainages. In the western drainages, mtDNA sequence variation is extremely low and there is evidence of strong bottleneck. The widespread distribution of the common mtDNA haplotype in clade A across the Churince and Becerra/Garabatal drainages is consistent with a severe Pleistocene bottleneck and limited recovery of population size. However, these drainages also suffer from the greatest anthropogenic disturbances: canals built in the 1900s have dramatically reduced the water flow from the Becerra system, and there has been a considerable drop of water levels in the Rio Garabatal within the last 10 years (Johnson,

personal observation). Given the unique haplotypes found in this drainage, considerable attention must be given to alleviate habitat destruction.

Another important finding in the western drainages was the presence of divergent mtDNA haplotypes and unidirectional migration from the Rio Mesquites. Many phylogeographic studies have documented similar patterns of genetic divergence through vicariance followed by subsequent range expansion and secondary contact resulting in hybridization (Watanabe & Chiba 2001; Zamudio & Savage 2003). An alternative hypothesis for the presence of divergent mtDNA haplotypes in western drainages is retention of ancestral polymorphism, which is plausible given the large population sizes of *Mexipyrgus*. An argument against retention of ancestral polymorphism is based on the inference of range expansion from the NCA and the evidence of unidirectional migration from the Rio Mesquites into western drainages. Nuclear sequence variation also indicates that there is elevated heterozygosity of individuals from the western drainages, consistent with hybridization (Johnson, in prep). There are a couple plausible mechanisms of interdrainage dispersal. First, even though there is no surface connection between the Rio Garabatal and the Rio Mesquites, these drainages are in near proximity in the upper reaches of the Rio Mesquites. Perhaps during wetter periods, these drainages may have coalesced. Drainage transfer could also occur through passive dispersal from birds, horses, and humans.

In contrast to the above results, there are strong haplotype associations with geography in the Rio Mesquites drainage. The evolutionary processes inferred were fragmentation, isolation by distance, and long-distance colonization followed by fragmentation. Well-supported mtDNA clades are restricted to Laguna Escobedo and the upper headwaters of the Rio Mesquites (Tierra Blanca). Recent isolation of Escobedae is likely because mtDNA haplotypes show limited sequence divergence from their nearest sister clade in the Rio Mesquites. An older event isolated Tierra Blanca haplotypes from their nearest sister clade in the Rio Mesquites. The persistent isolation of the Tierra Blanca haplotypes is probably attributable to a downstream, subterranean channel that isolates Tierra Blanca from the Rio Mesquites (Minckley 1969). Because *Mexipyrgus* prefer soft, flocculent substrates, the hard substrates of the underground channels probably represent a strong barrier to dispersal. Additionally, *M. churinceanus* females brood their larvae leading to limited dispersal. Additionally, large stable populations persist in the pools and river channels of the Rio Mesquites, in contrast to high rates of extinction in the Garabatal system.

In the southeastern part of the Cuatro Ciénegas basin, range expansion was inferred from the NCA, and the mismatch distribution indicates that a moderate bottleneck and demographic expansion dates to approximately 100 000 BP. There was also no relationship between pairwise $F_{ST}$ and geographic distances with a high variance. This case III pattern suggests that, subsequent to a founding event, populations are fragmented into small, isolated populations, and drift is stronger than gene flow. All analyses suggest a common pattern of fragmentation of small isolated populations with strong effects of drift. Even more striking is that the range expansion inferred from the cytochrome *b* network involves large tip clade distances with identical haplotypes found in Tio Candido and Santa Tecla. The most likely explanation for these patterns is that the southeastern lobe of the basin has extreme karst topography (Minckley 1969). New pits are scattered throughout these regions and these sinkholes often occur in a linear series, perhaps marking routes of subterranean channels. The colonization of newly foundered pits may involve strong founder effects, and long-distance colonization through subterranean channels may account for the observed patterns of range expansion and high variance in pairwise $F_{ST}$ estimates.

## Conclusions

The present study represents the first examination of historical and demographic forces influencing the spatial and temporal patterns of sequence variation in an organism inhabiting one of the most important centres of freshwater biodiversity. Through the use of multiple analytical techniques, a complex evolutionary history of genetic diversification was uncovered in the banded spring snail, *Mexipyrgus churinceanus*. There is strong evidence of long-term isolation of the major drainages in the basin, suggesting that these drainages represent evolutionarily distinct lineages. Molecular clock estimates of clade age indicate a tMRCA of approximately 2.5 Ma. Whether predator–prey co-evolution has caused the independent evolution of exaggerated shell strength in these drainages is the focus of current studies. While historical vicariance has promoted genetic divergence among drainages, the three drainages differ considerably in the forces affecting population structure. The western populations have extremely low mtDNA diversity consistent with a severe bottleneck dating to 50 000 years. In contrast, the nearby Rio Mesquites drainage is characterized by fragmentation events, restricted gene flow with isolation by distance, and higher levels of mtDNA polymorphism. These patterns are consistent with the long-term stability of this drainage along with habitat heterogeneity and brooding contributing to population isolation and restricted gene flow. Lastly, the southeastern populations show evidence of range expansion and strong influence of genetic drift in these small, isolated pools. The final conclusion is that recent gene flow from the Rio Mesquites into the western drainage has resulted in hybridization between divergent lineages.

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## Appendix

Collection data for *Mexipyrgus churinceanus* included in this study. For each population, I list the drainage and population, number of cytochrome *b* haplotypes, haplotypic (*H*) and nucleotide diversity ($\pi$) and standard errors and GPS coordinates

| Drainage/population | Haplotypes | *H* (± SE) | $\pi$ (± SE) | GPS coordinates |
|---|---|---|---|---|
| Western | | | | |
| 1. Laguna Churince | 5 | 0.00 | 0.00 | 26°50.53′N, 102°08.20′W |
| 2. Churince Stream | 12 | 0.1667 (0.1343) | 0.0002 (0.0004) | 26°50.61′N, 102°08.30′W |
| 3. Becerra | 11 | 0.4909 (0.1754) | 0.0013 (0.0011) | 26°52.82′N, 102°08.44′W |
| 4. Rio Garabatal 1 | 5 | 0.7000 (0.2184) | 0.0154 (0.0099) | 26°53.68′N, 102°09.66′W |
| 5. Rio Garabatal 2 | 6 | 0.0000 | 0.0000 | 26°53.76′N, 102°09.72′W |
| 6. Rio Garabatal 3 | 5 | 0.7000 (0.2184) | 0.0011 (0.0011) | 26°53.88′N, 102°09.73′W |
| 7. Rio Garabatal 4 | 6 | 0.7333 (0.1552) | 0.0211 (0.0128) | 26°54.23′N, 102°09.80′W |
| 8. Rio Garabatal 5 | 6 | 0.8000 (0.1721) | 0.0216 (0.013) | 26°54.48′N, 102°09.90′W |
| 9. Juan Santos | 8 | 0.0000 | 0.0000 | 26°53.97′N, 102°08.96′W |
| Rio Mesquites | | | | |
| 10. Tierra Blanca 1 | 4 | 0.6667 (0.2041) | 0.0010 (0.0011) | 26°55.74′N, 102°08.10′W |
| 11. Tierra Blanca 2 | 5 | 0.7000 (0.2184) | 0.0012 (0.0011) | 26°55.65′N, 102°08.31′W |
| 12. Tierra Blanca 4 | 3 | 0.00 | 0.00 | 26°55.39′N, 102°08.43′W |
| 13. Anteojo | 6 | 0.3333 (0.2152) | 0.0005 (0.0006) | 26°58.24′N, 102°07.80′W |
| 14. Mojarral West | 10 | 0.9778 (0.0540) | 0.0037 (0.0025) | 26°55.47′N, 102°07.50′W |
| 15. Mojarral East | 20 | 0.8684 (0.0504) | 0.0028 (0.0018) | 26°55.48′N, 102°07.28′W |
| 16. Rio Mesquites 1 | 5 | 0.9000 (0.1610) | 0.0023 (0.0019) | 26°55.47′N, 102°06.67′W |
| 17. Rio Mesquites 2 | 5 | 0.7000 (0.2184) | 0.0038 (0.0028) | 26°55.19′N, 102°06.28′W |
| 18. Rio Mesquites 3 | 5 | 0.4000 (0.2373) | 0.0023 (0.0019) | 26°55.24′N, 102°06.24′W |
| 19. Rio Mesquites 4 | 4 | 0.00 | 0.00 | 26°54.33′N, 102°03.48′W |
| 20. Los Remojos | 8 | 0.4643 (0.2000) | 0.0033 (0.0023) | 26°55.01′N, 102°06.67′W |
| 21. Laguna Escobedo | 12 | 0.7424 (0.1158) | 0.0022 (0.0016) | 26°53.59′N, 102°05.34′W |
| Southeastern | | | | |
| 22. Tio Candido 1 | 4 | 0.8333 (0.2224) | 0.0029 (0.0024) | 26°52.33′N, 102°04.85′W |
| 23. Tio Candido 2 | 6 | 0.7333 (0.1552) | 0.0020 (0.0016) | 26°52.31′N, 102°04.87′W |
| 24. Tio Candido 3 | 6 | 0.00 | 0.00 | 26°52.73′N, 102°05.09′W |
| 25. Tio Candido 5 | 5 | 0.00 | 0.00 | 26°52.73′N, 102°04.80′W |
| 26. Santa Tecla | 7 | 0.7143 (0.1809) | 0.0036 (0.0025) | 26°47.40′N, 102°00.24′W |
| 27. Pozas Azules | 14 | 0.7912 (0.0894) | 0.0015 (0.0012) | 26°49.83′N, 102°01.76′W |