Population Structure in the Roundtail Chub (Gila robusta Complex) of the Gila River Basin as Determined by Microsatellites: Evolutionary and Conservation Implications

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Abstract

Ten microsatellite loci were characterized for 34 locations from roundtail chub (Gila robusta complex) to better resolve patterns of genetic variation among local populations in the lower Colorado River basin. This group has had a complex taxonomic history and previous molecular analyses failed to identify species diagnostic molecular markers. Our results supported previous molecular studies based on allozymes and DNA sequences, which found that most genetic variance was explained by differences among local populations. Samples from most localities were so divergent species-level diagnostic markers were not found. Some geographic samples were discordant with current taxonomy due to admixture or misidentification; therefore, additional morphological studies are necessary. Differences in spatial genetic structure were consistent with differences in connectivity of stream habitats, with the typically mainstem species, G. robusta, exhibiting greater genetic connectedness within the Gila River drainage. No species exhibited strong isolation by distance over the entire stream network, but the two species typically found in headwaters, G. nigra and G. intermedia, exhibited greater than expected genetic similarity between geographically proximate populations, and usually clustered with individuals from the same geographic location and/or sub-basin. These results highlight the significance of microevolutionary processes and importance of maintaining local populations to maximize evolutionary potential for this complex. Augmentation stocking as a conservation management strategy should only occur under extreme circumstances, and potential source populations should be geographically proximate stocks of the same species, especially for the headwater forms.
Introduction

In deserts of western North America, long periods of aridity have been punctuated by occasional wet interludes [1], leading to fluctuating levels of habitat connectivity within terrestrial and aquatic environments over the last 2–3 million years. Glacial cycles (at approximately 100 kyr intervals) are correlated at middle latitudes with pluvial cycles, causing relatively regular patterns of isolation and connectedness [2]. For some taxa, fluctuating levels of isolation combined with ecological opportunity in arid environments are thought to have resulted in elevated rates of lineage diversification [3–6]. However, in North American freshwater fishes, species richness is lower west of the continental divide, as only about 150 of 750 species reside there [7]. This pattern has been influenced, in part, by tectonic activity and severity of the environment, leading to elevated extinction rates [8]. More recently, human actions have exacerbated this situation, and freshwater and diadromous fishes of North America are declining at an alarming rate [9]. Approximately 39% of described taxa are considered imperiled, representing a 92% increase since 1989. The situation is especially dire as 89% of imperiled taxa listed in 1989 exhibit the same status or worse, indicating that little has been achieved in the past quarter century to improve the status of most endangered fishes.

An interesting case involves the roundtail chub: a complex of closely related species of the cyprinid genus Gila (G. robusta, G. intermedia, and G. nigra [the last formerly “grahami”]) that are endemic to the Gila River basin in the southwestern United States. Like most other fishes of the region, the geographic distribution of populations has been reduced dramatically by human impacts; numbers are dwindling throughout their ranges, and remaining populations face myriad threats to their persistence [10, 11]. This has resulted in listing G. intermedia as endangered [12], petitions to similarly list G. nigra and G. robusta [13, 14], and inclusion of one or all species in regional conservation plans [15, 16].

The three species of the G. robusta complex provided an excellent opportunity for examining the distribution of genetic variation in threatened and endangered species. These species have had a complex and confused taxonomic history, and several detailed studies of variation of meristic and morphological traits have been completed [17–19]. In addition, DeMarais [20] examined genetic variation at 25 presumptive allozyme loci within and among populations of this complex. Analysis of the distribution of genetic variation identified significant differences among locations ($F_{ST} = 0.410$), however, this analysis did not identify significant structure associated with hydrogeography or species. Given observed distributional patterns and levels of genetic variation, DeMarais [20] hypothesized that the form “grahami” arose through past introgression between G. intermedia and G. robusta.

Minckley and DeMarais [21] summarized available distributional, morphological, and molecular data and examined the taxonomic status of all three species of the complex. Because each morphologically discrete form was consistently collected at the same locations and was always allopatric, they concluded that G. intermedia, G. robusta, and “grahami” represented three distinct taxonomic species. They also noted that some type specimens of “grahami” were actually G. robusta, invalidating this nomen; the earliest available replacement name was Gila nigra. Minckley and DeMarais [21] further discuss origins of G. nigra, hypothesizing that it may have multiple, independent origins through discrete hybridization events between G. intermedia and G. robusta.

Schwemm [22] found similar results to those of DeMarais [20] when he characterized sequence variation of mtDNA and two nuclear loci (introns of S7 and TPI). He found limited divergence among alleles/haplotypes; however, many locations exhibited unique variants, and were frequently monomorphic for these private alleles/haplotypes. Hierarchical analysis failed to associate patterns of sequence variation with species or hydrogeographic connection, and
patterns of variation were best explained by fragmentation and independent evolution of local subpopulations.

The present study extends the existing population genetic literature on the *G. robusta* complex by providing an analysis of microsatellite DNA loci for the same samples used by Schwemm [22]. Our results demonstrate differences in spatial genetic structure between the mainstem form (*G. robusta*) and the headwater forms (*G. intermedia* and *G. nigra*) that have important implications for managing local populations associated with different types of desert stream habitats. The patterns we observed are consistent with patterns of gene flow reported for many desert fishes, where genetic connectivity is a function of hydrogeographic connectivity, and often varies between populations occupying different desert stream environments [23]. Our findings have important implications for conservation management the *G. robusta* complex and other aquatic organisms that inhabit desert stream environments.

**Materials and Methods**

**Ethics Statement**

Permission to undertake field work and collect specimens was obtained under permits from the states of Arizona and New Mexico, and the U. S. Fish and Wildlife Service (FWS Federal Fish and Wildlife Service Native Endangered Species Recovery Permit Number TE0-39716-1). Specimens were obtained under Arizona State University Institutional Animal Care and Use Committee (IACUC) approval 05-768R.

**Study organisms**

The three species studied here were once common inhabitants of lower Colorado River basin streams and rivers. *Gila robusta* historically was found in moderate size and larger mainstem waters including Bill Williams, Gila, Salt, San Pedro and Verde rivers in the lower Colorado River basin and in Colorado, Gunnison, San Juan, and Green rivers in the upper basin where it occupied the largest, deepest pools and attained more than 45 cm total length. Smaller individuals inhabited smaller habitats, as with many western fishes [24], where they prefer shaded, deeper pools with cover such as undercut banks, boulders, or debris. Best described as a creek fish, *Gila intermedia* is a Gila River basin endemic that occupies well-developed pools with abundant cover in small to middle-size headwater creeks [25]; it is most common in marshy areas and ciénegas. *Gila intermedia* and *G. robusta* have never been taken syntopically despite spatial proximity of some populations. *Gila nigra*, also a Gila River basin endemic, inhabits smaller, first-order to middle reaches of medium sized streams, where it is strongly associated with cover such as undercut banks, boulders, and debris. It does not occur in ciénegas and does not co-occur with either of its congeners.

**Sampling and DNA extraction**

Sampling of Gila and Bill Williams river drainages encompassed 34 sites in seven sub-basins in Arizona and New Mexico (Bill Williams, Agua Fria, Verde, Salt, Santa Cruz, San Pedro, and Gila River mainstem--Fig 1), representing most known extant and some extirpated populations of these taxa. Minckley and DeMarais [21] summarized information meristic, morphometric and pigmentation characters of samples from locations examined here and identified them to species; therefore, we follow their taxonomic designations.

Efforts were made to sample up to 25–30 individuals/locality; however, the rarity of these species sometimes made it difficult to achieve this goal. Fourteen to 30 individuals were collected from each locality (Table 1). Some of these locations were represented by frozen tissues
from whole specimens collected in the 1980s for an allozyme study by DeMarais [20]. Specimens obtained for DNA studies (Schwemm [22] and here) were captured using standard fisheries methods (i.e., electrofishing, seining, or trapping). Tissues were obtained by removing a piece of right pectoral fin (< 3 mm square) from larger individuals with ethanol sanitized surgical scissors; after which fish were immediately released unharmed. This process was fast (a
Table 1. Locality data and groupings of samples for hierarchical analyses of samples from the *Gila robusta* complex, Arizona-New Mexico. All sub-basins are within the Gila River basin except for the Bill Williams River, which is a direct tributary to the lower Colorado River. Taxonomic identity of sampled individuals follows Minckley and DeMarais [21]. Coordinates are provided in UTM, elevation is in meters, and the column “N” provides number of individuals analyzed, with the superscript identifying the original source of material.

| Location Acronym | Acronym | Coordinates (UTM) | Elevation | Species | N |
|------------------|---------|------------------|-----------|---------|---|
| **Gila River basin** | | | | | |
| Agua Fria River basin | | | | | |
| Silver Creek, Yavapai Co., AZ | SIL | 12S 414762E 3793564N | 1301m | *intermedia* | 29 |
| **Gila River sub-basin** | | | | | |
| Blue River, Gila Co., AZ | BLU | 12S 566895E 3708078N | 1261m | *intermedia* | 19 |
| Bonita Creek, Graham Co., AZ | BON | 12S 637253E 3647293N | 1077m | *intermedia* | 20 |
| Dix Creek, Greenlee Co., AZ | DIX | 12S 671832E 3675085N | 1200m | *intermedia* | 22 |
| East Fork Eagle Creek, Greenlee Co., AZ | EFE | 12S 643185E 3707050N | 1716m | *intermedia* | 20 |
| Harden-Cienega Creek, Greenlee Co., AZ | HCN | 12S 673853E 3674850N | 1210m | *intermedia* | 22 |
| Eagle Creek—lower, Greenlee Co., AZ | LEG | 12S 648752E 3651019N | 1020m | *robusta* | 20 |
| East, Middle and West Forks Gila River, Catron Co., NM | NMFKS | 12S 758619E 3677745N | 1713m | *nigra* | 19 |
| Turkey Creek, Grant Co., NM | TURNM | 12S 734293E 3662829N | 1462m | *nigra* | 18 |
| Eagle Creek—upper, Greenlee Co., AZ | UEG | 12S 641212E 3704936N | 1653m | *intermedia* | 18 |
| **Salt River sub-basin** | | | | | |
| Black River, Greenlee Co., AZ | BLK | 12S 639590E 3724720N | 2003m | *robusta* | 21 |
| Cherry Creek, Gila Co., AZ | CHR | 12S 515036E 3740368N | 917m | *robusta* | 21 |
| Marsh Creek, Gila Co., AZ | MAR | 12S 497689E 3780336N | 1519m | *nigra* | 27 |
| **Salt River sub-basin** | | | | | |
| Rock Creek, Gila Co., AZ | ROC | 12S 489588E 3759443N | 1586m | *nigra* | 20 |
| Spring Creek, Gila Co., AZ | SPRSA | 12S 495954E 3765606N | 1428m | *nigra* | 20 |
| Tonto Creek, Gila Co., AZ | TON | 12S 491190E 3786183N | 1229m | *nigra* | 16 |
| **Santa Cruz River sub-basin** | | | | | |
| Cienega Creek, Pima Co., AZ | CC | 12S 540260E 3524841N | 1280m | *intermedia* | 20 |
| Sabino Canyon, Pima Co., AZ | SAB | 12S 519663E 3577319N | 940m | *intermedia* | 14 |
| Sheehy Spring, Santa Cruz Co., AZ | SHY | 12S 540028E 3470448N | 1433m | *intermedia* | 25 |
| **San Pedro River sub-basin** | | | | | |
| Aravaipa Creek, Pinal Co., AZ | ARA | 12S 551774E 3640104N | 922m | *robusta* | 24 |
| Bass Canyon, Cochise Co., AZ | BAS | 12S 571383E 3579679N | 1234m | *intermedia* | 20 |
| O’Donnell Canyon, Santa Cruz Co., AZ | ODN | 12S 544969E 3491596N | 1505m | *intermedia* | 20 |
| Redfield Canyon, Pima Co., AZ | RDF | 12S 562895E 3588722N | 1111m | *intermedia* | 20 |
| Turkey Creek, Santa Cruz Co., AZ | TURAZ | 12S 546154E 3489828N | 1516m | *intermedia* | 18 |
| **Verde River sub-basin** | | | | | |
| East Verde River, Gila Co., AZ | EVR | 12S 465504E 3794077N | 1355m | *nigra* | 20 |
| Fossil Creek, Yavapai Co., AZ | FOS | 12S 447363E 3861552N | 1328m | *nigra* | 26 |
| Canal downstream from confluence Salt and Verde rivers, Maricopa Co., AZ | LSALT | 12S 438441E 3712009N | 404m | *robusta* | 29 |
| Spring Creek, Yavapai Co, AZ | SPRVE | 12S 415889E 3853475N | 1170m | *intermedia* | 20 |
| Verde River, Perkinsville, Yavapai Co, AZ | VDP | 12S 391039E 3862667N | 1163m | *robusta* | 20 |
| Walker Creek, Yavapai Co, AZ | WAK | 12S 435967E 3833635N | 1227m | *intermedia* | 24 |
| West Clear Creek, Yavapai Co, AZ | WCL | 12S 436195E 3822226N | 1102m | *robusta* | 29 |
| Williamson Valley Wash, Yavapai Co, AZ | WVW | 12S 364924E 3822226N | 1102m | *intermedia* | 20 |
| **Bill Williams River basin** | | | | | |
| Boulder Creek, Yavapai Co., AZ | BOL | 12S 302702E 3834064N | 1156m | *robusta* | 30 |

(Continued)
few seconds), required minimal handling, and caused no harm to the fish so no anesthesia was applied. For larvae/young-of-year, specimens were euthanized in 500 mg/L MS-222. All material was transferred immediately after acquisition to 95% ethanol for storage. Genomic DNA was extracted from tissues by standard proteinase K/phenol/chloroform protocol as modified by Tibbets and Dowling [26].

**Microsatellite loci**

Primers for ten microsatellite loci used here were derived from several sources. Six loci (36, 222, 223, 225, 227, 300) were developed by Keeler-Foster et al. [27] from a *G. elegans* library. One locus (G294) was developed by Meredith and May [28] from a *G. bicolor obesa* library. The remaining three loci, C2 (repeat unit, (GACA)$_4$GTCA (GACA)$_3$ (GATA)$_3$; primers 5' - GACAAAGCGGTAGACAAAACCA - 3' and 5' - AATCTGAACTGGCTAACCTT - 3'), D17 (repeat unit, (GT)$_{15}$; primers 5' - TGGGCCAGGAAAAGAGAAACT - 3' and 5' - ATAAAGAGACGGTAAAGAActC - 3'), and D42 (repeat unit, (TCTA)$_5$; primers 5' - TTGCCTGTATAGGGTTGTGA - 3' and 5' - GTTGCTCATTGTTAGTTTGTG - 3'), were obtained from a library generated from *G. robusta* using enrichment methods provided by Glenn and Schable [29]. Amplifications used GoTaq (Promega) and the buffer supplied, dNTPs (200 mM final concentration of each dNTP), and IRD labeled primers (0.5 μM final concentration). Reactions were started with a long denature step (95°C, 5 min) followed by a series of touchdown steps where annealing temperature was decreased 1°C each cycle (94°C, 30 sec; 65–50°C, 30 sec; 72°C, 30 sec) to a final temp of 50°C. These same steps were repeated for additional cycles until 25 or 30 total cycles were completed, and the run finished with a long extension step (72°C, 7 min). Products were separated by electrophoresis through 6.5% denaturing gels (KBPlus, LI-COR Biotechnology) for 90–105 mins at 40 W with a minimum of four ladder lanes (50 bp—350 bp size standard, LI-COR Biotechnology) included on each gel. Fragments were visualized on a LI-COR 4300 DNA Analysis system and analyzed using SAGA GT (version 3.3, LI-COR Biotechnology).

**Statistical analyses**

Deviations from Hardy-Weinberg equilibrium ($F_{IS}$) and multilocus equilibrium were examined using FSTAT version 2.9.3.2 [30]. Significance level (0.05) for single and multilocus tests was adjusted using the B-Y correction [31]; adjusted critical values of 0.00797 and 0.00653, respectively). The unbiased estimate of gene diversity [32] and allelic richness ($A_R$—corrected for sample size by rarefaction) were calculated using FSTAT and HP-Rare [33], respectively. Basic statistical analyses (e.g., ANOVA, Kruskal-Wallis) were performed using PASW Statistics (formerly SPSS), release 18.

To examine distribution of variation among sample populations we also used FSTAT to generate Weir and Cockerham [34] $F$-statistics. Significance of values for $f$ ($\approx F_{ST}$), $\Theta$ ($\approx F_{IT}$), and $F$ ($\approx F_{TT}$) were obtained by jackknifing (over individuals and all loci) and bootstrapping (loci only). Comparison of the levels of $\Theta$ among the three species was obtained by
bootstrapping across samples (2500 permutations) using the comparison among groups of samples function in FSTAT. $F_{ST}$ was further partitioned by species or drainage using AMOVA with Arlequin version 3.11 [35]. Sample populations were clustered by neighbor-joining using POPTREE2, using corrected $F_{ST}$ as the estimate of genetic distance with confidence of nodes assessed by bootstrapping (1000 replicates) [36].

Stream distance were estimated from stream data from the Digital Chart of the World [37] using the network extension in the GIS software ARC/INFO. To test for isolation by (stream) distance, PASSaGE 2 [38] was used to perform Mantel tests and build Mantel correlograms [39–41]. The standardized Mantel statistic ($r_M$) was used to measure the correlation between pairwise $F_{ST}$ and stream distance over all sample populations. Mantel correlograms were constructed with five distance classes with an approximately equal number of pairs in each class. For each distance class, sites belonging to the same distance class received a value of 1 and the other pairs received value of 0 and design matrices were compared to a resemblance matrix based on pairwise $F_{ST}$. In this context, the standardized Mantel coefficient ($r_M$) was used as a measure of spatial autocorrelation for distance data, and can be used in the same way in a correlogram. For both the Mantel test and Mantel correlograms, the statistical significance of the standardized Mantel statistic ($r_M$) was tested by randomly permuting the rows and columns of one matrix in tandem (= 9999 permutations) and then counting the number of cases that yielded a Mantel coefficient greater than or equal to the observed value.

We also used Bayesian Assignment Tests to determine whether individuals (or groups of individuals) could be sorted into discrete gene pools. Assignment of individuals to gene pools was generated using STRUCTURE version 2.2 [42, 43] and assignment of groups of individuals to demes was examined using BAPS 5.1 [44]. For BAPS 5.1 analyses, separate runs were completed for each species, treating each sample location as an "informed prior." We then combined all sample locations (over all species) and repeated the analysis. For all runs, we entered a vector of replicate $K$ values (10 replicates per $K$, from $K = 2$ to $K = n$, where $n$ is the number of sample populations); BAPS 5.1 reports the set of estimates with the "best" partition and probability associated with different a priori assumptions.

For STRUCTURE, the default assumption (admixture among samples, correlated allele frequencies across loci) was employed. For each $a$ priori assumed number of populations ($K$), 10 independent runs of 110,000 replicates each (burn-in = 10,000) were performed. Optimal number of groups ($K$) was determined using the method of Evanno et al. [45] as implemented by the web-based program STRUCTURE HARVESTER [46]. The distribution of Q values across runs for each $K$ was summarized using CLUMPP [47] and the statistic $H'$ calculated to provide assessment of similarity across replicates; results were visualized using DISTRUCT [48].

Results

Variation within populations

Genetic variation in *Gila* was characterized using 744 individuals from 34 locations and 10 microsatellite loci. Genotypes for each individual are provided in S1 Table. Most samples had complete data, with an average amplification failure rate of 5.0 individuals/locus or 0.5% of all samples. Locus 300 had the highest failure rate where 15 individuals (or 2% of all individuals) failed to amplify. Failed amplifications were scattered across populations, reducing concerns over potential impact of null alleles.

Average allelic richness per locus was variable across loci (S2 Table), ranging from 1.3 to 7.4 (for loci C2 and 227, respectively). Average allelic richness per sample ranged from 1.7 (SAB) to 8.9 (NMFKS), with the majority of lower values reported for *G. intermedia* and *G. nigra* (Table 2). Populations of *G. robusta* exhibited higher levels of variation ($A_R = 6.0$) than those of...
G. intermedia and G. nigra (A<sub>u</sub> of 4.7 and 5.0, respectively), with these values significantly different among species (Kruskal-Wallis, P = 0.006).

Average gene diversity per locus ranged from 0.073 to 0.752 (for loci C2 and 300, respectively) while average gene diversity per sample ranged from 0.221 to 0.754 (FOS and UEG, respectively) (S3 Table). Fit to Hardy-Weinberg expectations (as indicated by variation in average F<sub>IS</sub> values across loci and populations) did not vary significantly among species as indicated by variation in average F<sub>IS</sub> values (across loci and populations) for G. robusta, G. nigra, and G. intermedia (F<sub>IS</sub> = -0.008, -0.025, and 0.055, respectively; Kruskal-Wallis, P = 0.077). Of 340 individual tests conducted (10 loci, 34 locations), 13 showed deviations from Hardy-Weinberg equilibrium after B-Y correction, with more significant tests identifying heterozygote

Table 2. Population genetic statistics for each sample from the Gila robusta complex, Arizona-New Mexico. *Species* follows designations in Minckley and DeMarais [21], "N" is sample size, "A<sub>u</sub>" is allelic richness averaged across loci, "#M" is the number of monomorphic loci, and "HWE" provides the number of significant deficiencies/excesses of heterozygotes per locus for each sample.

| Species        | Location | Drainage | Acronym | N  | A<sub>u</sub> | #M | HWE  |
|----------------|----------|----------|---------|----|---------------|----|------|
| intermedia     | Silver   | Agua Fria| SIL     | 29 | 2.9           | 1  | 1/0  |
| intermedia     | Blue     | Gila     | BLU     | 19 | 3.5           | 2  | 0/0  |
| intermedia     | Bonita   | Gila     | BON     | 20 | 6.0           | 1  | 0/0  |
| intermedia     | Dix      | Gila     | DIX     | 22 | 4.2           | 0  | 1/1  |
| intermedia     | E Fork Eagle | Gila   | EFE     | 20 | 7.2           | 0  | 0/0  |
| intermedia     | Harden-Cienaga | Gila | HCN     | 22 | 4.1           | 1  | 1/0  |
| intermedia     | Upper Eagle | Gila  | UEG     | 18 | 6.8           | 0  | 0/0  |
| intermedia     | Bass     | San Pedro| BAS     | 20 | 5.0           | 1  | 0/0  |
| intermedia     | O’Donell | San Pedro| ODN    | 20 | 6.2           | 1  | 0/0  |
| intermedia     | Redfield | San Pedro| RDF    | 20 | 4.3           | 1  | 0/0  |
| intermedia     | Turkey, AZ | San Pedro| TURAZ | 20 | 5.5           | 0  | 0/0  |
| intermedia     | Cienaga  | Santa Cruz| CC     | 20 | 2.1           | 5  | 0/0  |
| intermedia     | Sabino   | Santa Cruz| SAB    | 14 | 1.7           | 5  | 0/0  |
| intermedia     | Sheehy   | Santa Cruz| SHY    | 25 | 2.7           | 0  | 0/0  |
| intermedia     | Spring   | Verde    | SPRVE  | 20 | 7.1           | 1  | 0/0  |
| intermedia     | Walker   | Verde    | WAK    | 24 | 3.5           | 2  | 2/0  |
| intermedia     | Williamson Valley | Verde | WWV  | 20 | 6.4           | 1  | 2/0  |
| nigra          | Gila Forks, NM | Gila | NMFKS | 19 | 8.9           | 0  | 0/0  |
| nigra          | Turkey, NM | Gila     | TURNM  | 18 | 3.6           | 1  | 0/2  |
| nigra          | Marsh    | Salt     | MAR    | 27 | 5.8           | 0  | 1/0  |
| nigra          | Rock     | Salt     | ROC    | 20 | 5.4           | 0  | 0/1  |
| nigra          | Spring   | Salt     | SPRSA  | 20 | 6.4           | 0  | 0/0  |
| nigra          | Tonto    | Salt     | TON    | 20 | 6.5           | 1  | 1/0  |
| nigra          | East Verde | Verde | EVR    | 20 | 1.8           | 5  | 0/0  |
| nigra          | Fossil Spring | Verde | FOS   | 26 | 1.8           | 4  | 0/0  |
| robusta        | Boulder  | Bill Williams| BOL | 30 | 2.5           | 3  | 0/0  |
| robusta        | Trout    | Bill Williams| TRT | 30 | 4.0           | 1  | 0/0  |
| robusta        | Aravaipa | Gila     | ARA    | 25 | 6.8           | 1  | 0/0  |
| robusta        | Lower Eagle | Gila     | LEG    | 19 | 7.1           | 1  | 0/0  |
| robusta        | Black    | Salt     | BLK    | 18 | 8.3           | 1  | 0/0  |
| robusta        | Cherry   | Salt     | CHR    | 21 | 5.7           | 0  | 0/0  |
| robusta        | Lower Salt | Verde     | LSALT | 29 | 6.4           | 1  | 0/0  |
| robusta        | Verde, Perkinsville | Verde | VDP | 20 | 6.7           | 1  | 0/0  |
| robusta        | West Clear | Verde | WCL   | 29 | 6.7           | 1  | 0/0  |

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deficiency than excess (9 and 4, respectively, Table 2). Given the rarity of deviations (< 4% of comparisons) and their scatter across loci (seven loci exhibit deviations), impact from null alleles would be minimal, so all samples and loci were included in remaining analyses.

Remaining deviant samples exhibited significant heterozygote deficiencies. Most locations exhibited only a slight deficiency of heterozygotes, while deviations for G. intermedia from SAB and WAK were larger (overall $F_{IS} = 0.268, P = 0.0096$ and overall $F_{IS} = 0.140, P = 0.0015$, respectively). Samples from these locations yielded smaller numbers of alleles ($A_R = 1.7$ and 3.5, respectively). At SAB, five loci were monomorphic, while four of five polymorphic loci exhibited a deficiency of heterozygotes that was not statistically significant.

All pairs of polymorphic loci were tested for genotypic linkage disequilibrium within each population, with 21 of 1184 pairwise tests (1.8%) significant after B-Y correction, with nearly half of the significant tests coming from two sample populations: ROC and TURNM (4 and 6 significant pairs, respectively). TURNM was unusual in that eight of the nine polymorphic loci exhibited an excess of heterozygotes, with two of those values significant (overall $F_{IS} = -0.274$, $P < 0.0001$), potentially indicating close relatedness among these individuals. Locus pairs exhibiting significant disequilibrium were not consistent from sample population to sample population, indicating that loci are assorting independently.

Variation among populations

Partitioning of genetic variation into within and among population components identified significant population structure. Jackknife estimates of total genetic variation ($F \approx F_{IT}$) for each locus ranged from 0.211–0.407 (loci 222 and 36, respectively), with a jackknife average $F$ across loci of 0.293 (95% confidence interval 0.249–0.342). The within population component ($f \approx F_{IS}$) was small and not significantly different from 0 (range -0.083 [locus C2] to 0.075 [locus 36]), consistent with Hardy-Weinberg equilibrium results discussed above (jackknife average $f = 0.02$, 95% confidence interval -0.01 to 0.048). Therefore, the majority of variation was partitioned among populations: $\Theta (\approx F_{ST})$ ranged from 0.227 (locus 300) to 0.384 (locus C2) with a significant jackknife average of 0.278 (95% confidence interval 0.249–0.314).

To further examine the role of historical factors and geography, among population variation ($F_{ST}$) was partitioned by either taxonomy (three species) or river drainage (seven drainages, Fig 1) to see how these factors explain the distribution of genetic variation (calculated as weighted average across loci). When taxonomy was used to define partitions, the majority of the variation was found among local population within species ($F_{SC} = 0.271$) instead of among species ($F_{CT} = 0.016$). A similar result was obtained when samples were partitioned by drainage, with considerably more variation attributable to samples within drainages ($F_{SC} = 0.245$) than among drainages ($F_{CT} = 0.052$).

When all sample populations from the Gila robusta complex were pooled, the spatial correlation between pairwise $F_{ST}$ and stream distance was weak and not statistically significant ($r_M = 0.165, P = 0.109$; Fig 2A). However, when sample pairs were binned into distance classes (Fig 3A), the Mantel correlogram indicated statistically significant standardized Mantel coefficient in the first distance class ($r_M = 0.174; P = 0.007$).

Analysis of population structure independently for each species provides a different picture. Estimates of $F_{ST}$ for G. robusta, G. nigra, and G. intermedia were comparable, and not significantly different among species ($F_{ST} = 0.191, 0.338$, and 0.287, respectively; $P = 0.263$). However, when samples from the Bill Williams River drainage (BOL, TRT) were excluded, the average for G. robusta dropped dramatically ($F_{ST} = 0.071$) and there were significant differences among the three species ($P = 0.009$). This is reflected in the neighbor joining network of pairwise $F_{ST}$ values (Fig 4), where many samples of G. nigra and G. intermedia exhibited long
terminal branches while samples of G. robusta (except for those from the Bill Williams drainage) were shorter. Most nodes were not supported by bootstrap analysis with the exception of some pairs of samples in relatively close proximity.

For G. robusta, pairwise $F_{ST}$ ranged from 0.03–0.42. When pairwise $F_{ST}$ was plotted against stream distance, there were three main clusters of points and one outlier in the scatter diagram (Fig 2B). The main cluster of points corresponded to comparisons between sample locations within the Gila River drainage. The remaining two clusters of points (766 to 1094 km) corresponded to comparisons between Bill Williams River samples (i.e., BOL and TRT) and Gila River samples. The outlier point was the pairwise estimate for BOL and TRT. Over all sample locations of G. robusta, we found a moderate correlation ($r_M = 0.61$) between pairwise $F_{ST}$ and stream distance that was statistically significant ($P = 0.027$). When BOL and TRT (from the Bill Williams River drainage) were excluded, the correlation became negative and was not statistically significant ($r_M = -0.24$, $P = 0.237$). Differences in connectivity between major drainages are also supported by the Mantel correlogram (Fig 3b), where the standardized Mantel coefficient decreased precipitously for comparisons between sample populations in the Gila and Bill Williams drainages.

For G. intermedia, pairwise $F_{ST}$ ranged from 0.034–0.638. Over all sample locations, the correlation between pairwise $F_{ST}$ and stream distance ($r_M = 0.20$) was weak and not statistically significant. The Mantel correlogram (Fig 3C) displayed a decreasing trend in the value of $r_M$ with increasing stream distance. For G. nigra, pairwise $F_{ST}$ ranged from 0.066–0.702. Although
Fig 3. Mantel correlograms for samples of the *Gila robusta* complex from Arizona and New Mexico. (A) all species, (B) *G. robusta* (C) *G. intermedia*, and (D) *G. nigra*. Distance classes with statistically significant ($\alpha = 0.05$) standardized Mantel coefficients are indicated by a filled circle.

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Fig 4. Neighbor-joining network for sample locations of the *Gila robusta* complex constructed using pairwise estimates of $F_{ST}$. Location acronyms are provided in Table 1. Red, blue, and black labels and symbols identify samples from *G. intermedia*, *G. nigra*, and *G. robusta*, respectively. Numbers on branches reflect the proportion of 1000 bootstrap replicates in which the defined node was found.

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sparse, scatterplot shape (Fig 2D) resembled the pattern for G. intermedia (Fig 2C). The correlation between pairwise $F_{ST}$ and stream distance was weak ($r_M = 0.20$) over all sample populations and not statistically significant. In the Mantel correlogram, the standardized Mantel statistic was significant only for the first distance class (Fig 3D; $r_M = 0.680$, $P = 0.001$).

Assignment testing

BAPS and STRUCTURE were used to estimate the number of groups encompassed by the 34 samples. BAPS determined that $K = 28$ with each identified group represented by single samples except for two, one containing samples EFE and UEG from G. intermedia and the other comprised of most G. robusta samples (ARA, BLK, LEG, WCL, and VDP) and NMFKS from G. nigra. STRUCTURE was used to characterize assignment probability for all $K$ from 2–34. There was inconsistency across replicates for more divergent samples (e.g., BOL and TRT), as indicated by their consistent assignment to different groups for each value of $K$ and reduced $h'$ values for these replicates (Fig 5). The method of Evanno et al. [45] indicated $K = 20$ ($\Delta K = 16.0$), and In likelihood values also reached a plateau at $K = 20$, supporting that conclusion [42].

Evaluation of assignment probability plots from STRUCTURE is difficult due to variation among replicates and the large number of distinct samples. Analyses from $K = 20$ and $K = 28$ (as predicted by STRUCTURE and BAPS, respectively, Fig 5) yielded similar results, with an increase in number of samples that were distinct for the latter $K$. Even at higher $K$ certain sets of geographically proximate locations are consistently grouped together: G. robusta from the Verde River (WCL and VDP); G. nigra from Tonto Creek (ROC and SPRSA); and three separate groups of G. intermedia samples (EFE-UEG from Eagle Creek, ODN-TURAZ from the San Pedro River, and CC-SHY from the Santa Cruz River).

While it is difficult to obtain much information from examination of assignment plots for each $K$ (Fig 5), several samples are notable. Individuals from BLK (G. robusta, Salt River), BON and SPRVE (G. intermedia, Gila and Verde rivers, respectively), and NMFKS and TON (G. nigra, Tonto Creek drainage) are routinely difficult to assign to specific groups, especially at low values of $K$ ($\leq 10$). In addition, individuals from three of these locations (BON, SPRVE, TON) exhibit signs of admixture at lower levels of $K$ ($\leq 10$) as there is considerable probability of assignment to a group that includes most samples of G. robusta. Similar perspective of two sites assigned to G. nigra (NMFKS, TURNM) indicates that individuals from these locations may actually belong to G. robusta.

Discussion

Results of the present study were consistent with previous molecular genetic studies of the G. robusta complex [20, 22]. Most of the genetic variation was attributable to differences among local populations within species, with minimal differentiation due to the presence of multiple drainages or species in the analysis. However, our results provided new insight into spatial genetic structure among local populations associated with different stream habitats, with evidence of widespread gene flow among local populations of the mainstem form within the Gila River basin, as well as evidence of more recent, maybe even ongoing, gene flow between proximate populations than distant populations within each headwater form.

Characterization of microsatellite variation for 10 loci failed to group samples by recognized species, a result consistent with the allozyme study of DeMarais [20] and Schwemm’s [22] characterization of mtDNA and introns from two single copy nuclear genes. Levels of divergence among populations in this complex were high, and clustering of pairwise $F_{ST}$ yields a topology with short internodal and long terminal branches, with limited support for grouping
of pairs of most populations, let alone those in the same taxonomic group (Fig 4). High divergence also affects Bayesian group assignment, with assignment of sites to a particular group inconsistent across replicates, yielding a distinctive stacked bar pattern for divergent samples, especially at lower values of $K$ (Fig 5). These studies illustrate the importance of local isolation in the evolution of this complex, producing a large number of diagnostically distinct, local populations ($K = 20$ and 28 for STRUCTURE and BAPS, respectively). It is important to emphasize here that these patterns are probably not an artifact of Bayesian methods [49] as indicated by high $F_{ST}$ values among populations; nor are they an artifact of microsatellite markers, as Schwemm [22] also noted that many local populations are distinct enough to be diagnosable with unique mtDNA haplotypes and/or nuclear alleles.

Despite the high level of divergence among populations for mtDNA and nuclear sequence data and microsatellites, diagnostic markers were not identified for species. There are a few potential explanations for our inability to identify diagnostic molecular markers. When genetic divergence among subpopulations is large, it is possible that differences among individual subpopulations can obscure differences at deeper hierarchical levels (e.g., species, drainages), reducing the effectiveness of such markers for identifying these higher categories. For example, Hedrick [50] noted that hypervariable markers like microsatellites are less effective at estimating $F_{ST}$ due to high levels of variation within populations. Extending this logic further, high levels of divergence among local subpopulations would further reduce the amount of variation between populations.
available to discriminate among higher order groups (e.g., species). Resolving hierarchy may require a substantially larger data set, with additional markers capable of resolving deeper evolutionary events.

The observed pattern could also reflect how different evolutionary forces have shaped this complex. Genetic structure within the *Gila robusta* complex may represent insularization of a historically panmictic population due to natural and artificial habitat fragmentation, with observed patterns of morphological variation due to convergent selection for a common habitat-based morphotype (e.g., ciénegas, headwater reaches), analogous to convergent selection observed in sticklebacks [51]. Assessment of this hypothesis would require identification of specific genes that are the unit of selection for different habitats as well as additional morphological analyses.

Past introgressive hybridization could also be partly responsible for the observed pattern. These species have never been reported to be sympatric naturally despite occurring in close proximity (e.g., Eagle Creek) [52]. DeMarais [20] and Minckley and DeMarais [21] hypothesized that *Gila nigra* was a taxon of hybrid origin, resulting from introgression between *G. intermedia* and *G. robusta*. During dry periods, *G. intermedia* and *G. robusta* are expected to be geographically isolated in headwater and mainstem reaches, respectively; however, during wetter times these species could co-occur and interbreed, producing local hybrid swarms. As streams again became desiccated, these hybrid populations would become isolated in headwater reaches, allowing for divergence through local adaptation. It is such populations that Minckley and DeMarais [21] hypothesized might be recognized as *Gila nigra*, which is morphologically intermediate to *G. intermedia* and *G. robusta*. Because the present analysis of microsatellite DNA loci did not identify diagnostic markers for each species, it is impossible to specifically test the introgression hypothesis here. Regardless of the reason, the lack of diagnostic molecular characters to date does not inform the status of *G. intermedia*, *G. nigra*, and *G. robusta* relative to their recognition as distinct species. Instead these results highlight the role that local evolution has played in shaping patterns of variation in these taxa and the importance of accounting for this variation when managing the complex.

**Genetic structure**

The high level of genetic subdivision detected in the present study indicates that forces acting on location populations (e.g., mutation, drift, selection) are driving patterns of genetic variation within the *Gila robusta* complex. Similar patterns were identified with allozymes [20] and nuclear and mtDNA sequences [22], indicating that this result does not solely reflect the rapid rate of microsatellite evolution. These patterns more likely reflect varying levels of hydrogeographic connectivity among stream habitats over the last 2–3 million years [2], with samples of *G. robusta* (the mainstem species) from the Gila River basin exhibiting increased variability and lower levels of divergence and hierarchical structure than *G. nigra* and *G. intermedia*, which are typically found in smaller, more isolated streams.

Although the results of the present study support substantial divergence among local populations [20, 22], spatial genetic and clustering analyses performed here indicate that relative impact of evolutionary processes on genetic variation depends on distance between localities, as well as potential barriers to dispersal. For example, F-statistic analyses of *G. robusta* identified considerable variation in allele frequencies among samples (*F_{ST} = 0.191*); however, removal of samples from the Bill Williams River reduced structure considerably (*F_{ST} = 0.071*). This inference was also supported by scatterplots of pairwise *F_{ST} versus stream distance* (Fig 2B) and a Mantel correlogram (Fig 3B), demonstrating high rates of gene flow relative to drift within the Gila River basin and differentiation between populations from Gila and Bill.
Williams basins (as well as between sample locations within the Bill Williams basin). While long stream distances separate populations from the Gila and Bill Williams basins, observed differentiation may be best explained by inhospitable habitat in the lowermost Colorado and Gila rivers, which may be acting as an isolating mechanism.

*Gila intermedia* is found in headwater reaches throughout the Gila River drainage and exhibits lower levels of variation within and more differentiation among populations than *G. robusta*, as expected. Scatterplots of pairwise $F_{ST}$ versus stream distance (Fig 2C) and the Mantel correlogram (Fig 3C) indicate isolation by distance up to some threshold level, beyond which effects of drift predominate [53]. Presence of significant divergence among distant locations likely reflects historical processes attributable to the strongly fluctuating environment [54]. Frequent dry periods would have led to divergence among locations due to drift and selection; gene flow would have been possible during pluvial times. Divergence may have been exacerbated by recent anthropogenic modifications to the stream network that formed barriers to dispersal [55]. While the scatter plot, Mantel correlogram, and clustering analyses indicate that gene flow is more effective than drift at shorter distances (with the effects of drift predominating at longer distances), ongoing hydrogeographic isolation is likely to intensify the strength of drift and erode any signal of localized isolation by distance.

*Gila nigra* also occupies headwater reaches and was also expected to show substantial differentiation among populations, and results based on various types of genetic markers corroborate this inference [20, 22]. While gene flow dynamics generally mirrored those of *G. intermedia*, drift induced divergence at long distances was less extreme for *G. nigra*, consistent with the observation that genetic variability was uniformly lower for *G. intermedia* relative to *G. nigra*. There are, however, caveats associated with this interpretation. Sampling of *G. nigra* was limited, with half the samples (MAR, SPRSA, ROC, TON) coming from the same relatively small tributary network of the Salt River (Fig 1). Also, the outcome of hierarchical analysis of assignment probabilities indicated that some samples may not be discrete; the samples NMFKS and TURNM were especially problematic, as they may actually be *G. robusta* or hybrids (Fig 5).

In general, the results of the spatial genetic analyses indicate that gene flow/drift dynamics depend on stream distance and differ between the mainstem form and the headwater forms. These results support key differences in microevolutionary processes among ecological variants within the *G. robusta* complex and should be informative for conservation genetic management of local populations irrespective of species designations.

**Comparison to other fishes from western North America**

Results from our study of the *Gila robusta* complex indicate considerable evolution at the local population level but also are consistent with the “Stream Hierarchy” model of gene flow [23], which predicts varying levels of genetic connectivity (and hierarchical structure) within a stream network depending on degree of hydrogeographic connectivity among local populations. Many studies of fishes from desert regions of western North America yield valuable perspective on the role of geographic connectedness and life history on distribution of genetic variation in arid environments. Tibbets and Dowling [26] contrasted levels of divergence among three species of stream-dwelling desert cyprinids (*Agosia chrysogaster*, *Meda fulgida*, and *Tiaroga cobitis*) from the Gila River basin, noting that patterns of genetic variation reflected expectations derived from consideration of life history and predicted levels of movement among locations. Studies of genetic variation in other cyprinids yielded variable results. In their study of mtDNA variation in *Richardsonius*, Houston et al. [56] found most variation distributed among, but not within, major regions, indicating high levels of gene exchange.
within but not among regions. This contrasts with studies of other minnows (e.g., *Rhinichthys osculus*–[57, 58] and *Lepidomeda*–[59]), where there was considerable divergence among localities within drainages as well as among drainages. Johnson [60] also identified considerable divergence within and among drainage groups in the cyprinid *Gila atraria* but also noted additional divergence associated with evolved life history differences within this species.

Diversity of pattern is not restricted to cyprinids. Whiteley et al. [61] quantified allozyme and microsatellite variation within and among populations of mountain whitefish (*Prosopium williamsoni*) where variation was hierarchically arrayed into five distinct assemblages corresponding to major drainage basins, but with no differentiation within major drainage basins. This contrasts to other salmonids from the same region, which exhibit more divergence among locations than within drainages [62]. Hopken et al. [63] also examined the importance of geographic structure on patterns of genetic variation in bluehead sucker (*Catostomus discobolus*), an endemic to the Colorado River basin. They identified three evolutionarily significant units and seven management units within this species, with each group defined by a geomorphological barrier and/or isolation due to aridity. Together, these studies show that levels of genetic connectivity within drainages can vary among taxa based on hydrogeographical patterns and the life history of a species.

**Conservation implications**

Molecular and morphological variation provides critical information for management of this complex. In such situations, it is critical to understand evolutionary processes that generated the underlying genetic diversity, allowing for preservation of the evolutionary legacy and adaptive potential of the complex. To maximize preservation of evolutionary potential, we advocate an approach that preserves available genetic diversity as identified by morphological and molecular analyses. Conservation units should be defined in a hierarchical manner, with genetically distinct units identified within each morphologically recognized species and each subbasin. Importance of local adaptation, drift, and gene flow makes it advisable to consider hydrogeography as well as divergence when developing conservation plans. Note, however, that we found some discrepancies between assignments based on microsatellite data and putative taxonomic status based on morphological traits as defined by Minckley and DeMarais [21]. Because morphological identifications are based upon museum records, such conflicts could represent change in species composition. Given the significance of morphological as well as genetic variation, it is critical that remaining populations of these taxa are characterized to allow for fully informed management of this group.

In addition to maintaining discreteness associated with geographic isolation and evolutionary independence, it is possible that *G. nigra* may result from admixture of *G. robusta* and *G. intermedia*. Connectedness among populations is difficult to envision in today’s environment that includes both physical and biological barriers to exchange; however, there is no obvious resolution of those issues. Instead, we must overcome the general need of placing specific populations into categories and acknowledge that conservation should focus on preserving processes that generate observed patterns as well as the patterns themselves, thus requiring preservation of the entire complex and not just individual species.

Among members of the *Gila robusta* complex only *G. intermedia* is listed under the Endangered Species Act, and it thus is the only one to receive protection. There currently is no accommodation for integrated conservation of the complex and little likelihood this will change. Given this restriction, we advocate managing each species independently by sub-drainage, with efforts to avoid mixing stocks from different sub-basins to avert negative consequences associated with outbreeding depression. This requires genetic characterization to
match donor and recipient populations prior to translocation. Because of high levels of local differentiation, augmentation should only occur under extreme circumstances (i.e., population collapse, physical evidence of inbreeding depression), with special care to preserve local stocks. Efforts to establish new populations should utilize the nearest geographic population as a source, while avoiding transfer across different subdrainages; this is especially important for headwater forms.

Supporting Information
S1 Table. Genotypes for each locus and individual examined in this study of the *Gila robusta* complex, Arizona—New Mexico. (DOCX)

S2 Table. Allelic richness (*A*_r) for each locus and sample, including averages, for each sample of the *Gila robusta* complex, Arizona—New Mexico. (DOCX)

S3 Table. Gene diversity (unbiased estimate [32]) for each locus and sample of the *Gila robusta* complex, Arizona—New Mexico. (DOCX)

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Author Contributions
Conceived and designed the experiments: TED PCM. Performed the experiments: TED. Analyzed the data: TED CDA MSR. Contributed reagents/materials/analysis tools: TED CDA PCM MSR. Wrote the paper: TED CDA PCM MSR.

References
1. Axelrod DI. Age and origin of Sonoran desert vegetation. Occ Pap CA Acad Sci 1979; 132:1–74.
2. Morrison RB. Quaternary stratigraphic, hydrologic, and climatic history of the Great Basin, with emphasis on Lakes Lahontan, Bonneville, and Tecopa. In: Morrison RB, editor. Quaternary nonglacial geology: Contiguous US. GSA DNAG Vol. K-2; 1991. pp 283–321.
3. Douglas ME, Douglas MR, Schuett GW, Porras LW. Evolution of rattlesnakes (Viperidae; Crotalus) in the warm deserts of western North America shaped by Neogene vicariance and Quaternary climate change. Molecular Ecology 2006; 15:3353–3374. PMID: 16968275
4. Neiswenter SA, Riddle BR. Landscape and climatic effects on the evolutionary diversification of the *Perognathus fasciatus* species group. Journal of Mammology 2011; 92:982–993.
5. Bryson RW Jr, Riddle BR. Tracing the origins of widespread highland species: a case of Neogene diversification across the Mexican sierras in an endemic lizard. Biological Journal of the Linnaean Society 2012; 105:382–394.
6. Bryson RW Jr, Riddle BR, Graham MR, Smith BT, Prendini L. As old as the hills: montane scorpions in southwestern North America reveal ancient associations between biotic diversification and landscape history. PLOS One 2013; 8:e5282.
7. Minckley WL, Douglas ME. Discovery and extinction of western fishes: A blink of the eye in geologic time. In: Minckley WL, Deacon JE, editors. Battle against extinction: Native fish management in the American West. Tucson: University of Arizona Press; 1991 pp. 7–18.
8. Smith GR, Badgley C, Eiting TP, Larson PS. Species diversity gradients in relation to geological history in North American freshwater fishes. Evolutionary Ecology Research 2010; 12:693–726.

9. Jelks HL, Walsh SJ, Burkhead NM, Contreras-Balderas S, Diaz-Pardo E, Hendrickson DA, et al. Conservation status of imperiled North American freshwater and diadromous fishes. Fisheries 2008; 33:372–407. Minckley WL. Fishes of Arizona. Phoenix: Arizona Game and Fish Department; 1973.

10. Weedman DA, Girmendonk AL, Young KL. Status review of Gila chub, Gila intermedia, in the United States and Mexico. Nongame technical report 91. Phoenix: Arizona Game and Fish Department; 1996. 120 pp.

11. Voeltz JB. Roundtail chub (Gila robusta) status survey of the lower Colorado River basin. Nongame technical report 186. Phoenix: Arizona Game and Fish Department; 2002. 221 pp.

12. U.S. Fish and Wildlife Service. Endangered and threatened wildlife and plants; listing the Gila chub as endangered with critical habitat; proposed rule. Federal Register 2002; 67(154), 51948–51985.

13. U.S. Fish and Wildlife Service. Endangered and threatened species. Review of plant and animal taxa: proposed rule. Federal Register 2006; 71(89):26007–260017.

14. U.S. Fish and Wildlife Service. Endangered and threatened wildlife and plants; 12-month finding on a petition to list a distinct population segment of the roundtail chub (Gila robusta) in the lower Colorado River basin; proposed rule. Federal Register 2009; 74(128):32352–32387.

15. Arizona Game and Fish Department. Arizona statewide conservation agreement for roundtail chub (Gila robusta), headwater chub (Gila nigra), flannelmouth sucker (Catostomus latipinnis), Little Colorado River sucker (Catostomus spp. [sic.]), bluehead sucker (Catostomus discobolus) and Zuni bluehead sucker (Catostomus discobolus yarrowi). Phoenix: Arizona Game and Fish Department; 2006. 63 pp.

16. Utah Department of Natural Resources. Rangewide conservation agreement and strategy for roundtail chub (Gila robusta), bluehead sucker (Catostomus discobolus), and flannelmouth sucker (Catostomus latipinnis). Publication Number 06–18. Salt Lake City: Utah Department of Natural Resources; 2006.

17. Rinne JN. Cyprinid fishes of the Gila from the lower Colorado River. Wassman Journal of Biology 1976; 34:65–107.

18. DeMarais BD. Morphological variation in Gila (Pisces: Cyprinidae) and geologic history: lower Colorado River basin. Thesis, Arizona State University. 1986.

19. Douglas ME, Minckley WL, DeMarais BD. Did vicariance mold phenotypes of western North American Fishes? Evidence from Gila River cyprinids. Evolution 1999; 53:238–246.

20. DeMarais BD. Genetic relationships among fishes allied to the genus Gila (Teleostei: Cyprinidae) from the American Southwest. Dissertation, Arizona State University. 1992.

21. Minckley WL, DeMarais BD. Taxonomy of chubs (Teleostei: Cyprinidae, genus Gila) in the American Southwest with comments on conservation. Copeia 2000; 2000:251–256.

22. Schwemm MR. Genetic variation in the Gila robusta complex (Teleostei: Cyprinidae) in the lower Colorado River. Thesis, Arizona State University. 2006.

23. Meffe GK, Vrijenhoek RC. Conservation genetics in the management of desert fishes. Conservation Biology 1988; 2:157–169.

24. Smith GR. Effects of habitat size on species richness and adult body sizes of desert fishes. In Naiman RJ and Soltz DL, editors. Fishes in North American deserts. New York: John Wiley and Sons; 1981. pp 125–171.

25. Minckley WL. Fishes of Arizona. Phoenix: Arizona Game and Fish Department; 1973.

26. Tibbets CA, Dowling TE. Effects of intrinsic and extrinsic factors on population fragmentation in three North American minnows (Teleostei: Cyprinidae). Evolution 1996; 50:1280–1292.

27. Keeler-Foster CL, Spies IB, Bondu-Hawkins V, Bentzen P. Development of microsatellite markers in bonytail (Gila elegans) with cross-species amplification in humpback chub (Gila cypha). Molecular Ecology Notes 2004; 4:23–25.

28. Meredith EP, May B. Microsatellite loci in the Lahontan tuft chub, Gila bicolor obesa, and their utilization in other chub species. Molecular Ecology Notes 2002; 2:156–158.

29. Glenn TC, Schable NA. Isolating microsatellite DNA loci. Methods in Enzymology 2005; 395:202–222. PMID: 15865969

30. Goudet J. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). 2001. Available from http://www2.unil.ch/popgen/softwares/fstat.htm. Accessed 3 August 2009.

31. Narum SR. Beyond Bonferroni: Less conservative analyses for conservation genetics. Conservation Genetics 2006; 7:783–787.

32. Nei M. Molecular Evolutionary Genetics. New York: Columbia University Press; 1987.
33. Kalinowski ST (2005) HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. Molecular Ecology Notes 5:187–189.

34. Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.

35. Excoffier L, Laval G, Schneider S. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 2005; 1:47–50.

36. Takezaki N, Nei M, Tamura K. POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with Windows-interface. Molecular Biology and Evolution 2010; 27:747–752. doi: 10.1093/molbev/msp312 PMID: 20022889

37. ESRI [Environmental Systems Research Institute]. Digital chart of the world. Redlands: Environmental Systems Research Institute; 1993.

38. Rosenberg MS, Anderson CD. PASSaGE: Pattern Analysis, Spatial Statistics and Geographic Exegesis. Version 2. Methods in Ecology and Evolution 2011; 2:229–232.

39. Bocard D, Legendre P. Is the Mantel correlogram powerful enough to be useful in ecological analysis? A simulation study. Ecology 2012; 93:1473–1481. PMID: 22834387

40. Meirmans PG. The trouble with isolation by distance. Molecular Ecology 2012; 21:2839–2846. doi: 10.1111/j.1365-294X.2012.05578.x PMID: 22574758

41. Diniz-Filho JAF, Soares TN, Lima JS, Dobrovolski R, Landeiro VL, de Campos Telles MP, Rangel TF, Bini LM. Mantel test in population genetics. Genetics and Molecular Biology 2013; 36:475–485. doi: 10.1590/S1415-47572013000400002 PMID: 24385467

42. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics 2000; 155:344–359. PMID: 10835412

43. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. Genetics 2003; 164:1567–1587. PMID: 12930761

44. Corander J, Walmann P, Marttinen P, Sillanpaa MJ. BAPS2: enhanced possibilities for the analysis of population structure. Bioinformatics 2004; 20:2363–2369. PMID: 15073024

45. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 2005; 14:2611–2620. PMID: 15969739

46. Earl DA, vonHoldt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 2011; 4:359–361.

47. Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 2007; 23:1801–1806. PMID: 17485429

48. Rosenberg NA. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 2004; 4:137–138.

49. Frantz AC, Cellina S, Krier A, Schley L, Burke T. Using spatial Bayesian methods to determine the genetic structure of a continuously distributed population: clusters or isolation by distance? Journal of Applied Ecology 2009; 46:493–505.

50. Hedrick PW. Highly variable loci and their interpretation in evolution and conservation. Evolution 1999; 53:313–318.

51. Colosimo PF, Hossemeann KE, Balabhadra S, Villereal G Jr., Dickson M, Grimwood J, Schmutz J, Myers RM, Schluter D, Kingsley DM. Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. Science 2005; 307:1928–1933. PMID: 15790847

52. Minckley WL, Marsh PC. Inland fishes of the greater southwest: chronicle of a vanishing biota. Tucson: Univ. Ariz. Press, Tucson; 2009. 426 pp.

53. Hutchison DW, Templeton AR. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 1999; 53:1898–1914.

54. Polya VJ, Asmerom Y. Late Holocene climate and cultural changes in the southwestern United States. Science 2001; 294:148–151. PMID: 11588259

55. Clarkson RW, Marsh PC, Dowling TE. Population prioritization for conservation of imperiled warmwater fishes in an arid-region drainage. Aquatic Conservation: Marine and Freshwater Ecosystems 2012; 22:498–510.

56. Houston DD, Shiozawa DK, Riddle BR. The roles of Neogene geology and late Pleistocene lake levels in shaping the genetic structure of the Lahontan redside shiner Richardsonius egregius (Teleostei: Cyprinidae). Biological Journal of the Linnaean Society 2011; 104:163–176.
57. Ardren WR, Baumsteiger J, Allen CS. Genetic analysis and uncertain taxonomic status of threatened Foskett Spring speckled dace. Conservation Genetics 2010; 11:1299–1315.

58. Billman EJ, Lee JB, Young DO, McKell MD, Evans RP, Shiozawa DK. Phylogenetic divergence in a desert fish: Differentiation of speckled dace within the Bonneville, Lahontan, and upper Snake River basins. Western North American Naturalist 2010; 70:39–47.

59. Johnson JB, Dowling TE, Belk MC. Neglected taxonomy of rare desert fishes: Congruent evidence for two species of leatherside chub. Systematic Biology 2004; 53:841–855. PMID: 15764555

60. Johnson JB. Evolution after the flood: phylogeography of the desert fish Utah chub. Evolution 2002; 56:948–960. PMID: 12093030

61. Whiteley AR, Spruell P, Allendorf FW. Can a common species provide valuable information for conservation? Molecular Ecology 2006; 15, 2767–2786. PMID: 16911199

62. Whiteley AR, Spruell P, Allendorf FW. Ecological and life history characteristics predict population genetic divergence of two salmonids in the same landscape. Molecular Ecology 2004; 13:3675–3688. PMID: 15548282

63. Hopken MW, Douglas MR, Douglas ME. Stream hierarchy defines riverscape genetics of a North American desert fish. Molecular Ecology 2012; 22:956–971. doi: 10.1111/mec.12156 PMID: 23279045