Population structure in the roundtail chub (*Gila robusta* complex) of the Gila River basin as determined by microsatellites

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Executive Summary

Ten microsatellite loci were characterized for 33 populations from the *Gila robusta* complex of the lower Colorado River basin to quantify the distribution of genetic variation within and among samples of *G. intermedia*, *G. nigra*, and *G. robusta*. Different populations exhibited different levels of variation, with the headwater species *intermedia* tending to possess fewer alleles than the other two forms. Patterns of variation were consistent with significant structure that supported independent evolution of most samples; however, the mainstem species *robusta* exhibited smaller F_{ST}s, indicating greater connectedness among regions relative to the other taxa. Assignment testing also indicated that individuals from most localities are distinct and populations are evolving independently, and hierarchical analysis indicated that geography plays an important role in patterns of distinctiveness. While these markers did not identify any species-specific variants they are generally consistent with continued recognition of each as a taxonomic entity. Placement of some populations seemed to conflict with morphological assignment, and these should be re-examined to insure proper identification. Likewise, variation is consistent with multiple hybrid origins of *nigra*, making it important that the potential influence of introgressive hybridization be considered as management plans are developed for conserving this complex. The significance of isolation and local processes in shaping each of these populations highlights the importance of maintaining each independently to preserve unique genetic variation and maximum evolutionary potential. As programs are initiated to replace extirpated populations or establish new ones, it is important that species and geography are considered when identifying sources for translocation.

Introduction

Members of the roundtail chub complex (*Gila robusta*, *G. intermedia*, and *G. nigra*) were once common inhabitants of streams and rivers of the lower Colorado River basin. However, like most other fishes of the region, their populations have been reduced dramatically by human impacts and numbers are dwindling throughout their range (Weedman et al. 1996, Voeltz 2002), resulting in listing *intermedia* as endangered (U.S. Fish and Wildlife Service 2002). Concern for *nigra* and *robusta* is reflected in their petitions for listing as endangered (see U.S. Fish and Wildlife Service 2006) and their inclusion in regional conservation plans (e.g., Arizona Game and Fish Department 2006, Utah Department of Natural Resources 2006). To enhance management of these species, we examined patterns of microsatellite variation within and among populations and species, allowing for assessment of patterns of gene exchange and identification of units for management.

The *robusta* complex has been intensively studied and has a complicated history. Previous morphological studies (e.g., Minckley 1973, Rinne 1976, DeMarais 1986) identified intricate patterns of variation, indicating that the species may actually represent a complex of independent taxa, including *G. intermedia* and *G. robusta* and problematic forms that were referred to "*grahami*." Douglas et al. (1999) examined ecological and geological correlates with patterns of morphological variation and concluded that observed patterns were best explained by vicariant events of Pliocene age.

Analysis of molecular characters has also identified complex patterns of variation. DeMarais (1992) examined variation at 25 presumptive allozyme loci within and among populations of this complex. While this analysis identified considerable variation among populations (F_{ST} = 0.410), essentially none was partitioned by species (F_{CT} = -0.013). Likewise,

analysis of variation by drainage also failed to identify a significant geographic component (F_{CT} = -0.032), indicating that genetic variation is partitioned by sampling locality and not drainages. Examination of variation within each species independently indicated there was considerably more divergence among headwater forms (e.g., *G. intermedia*, *G.* "*grahami*") than the mainstream form (*G. robusta*) (F_{ST} = 0.431, 0.458, and 0.080, respectively). Given observed distributional patterns and levels of genetic variation, DeMarais (1992) hypothesized that the form "*grahami*" arose through past introgression between *G. intermedia* and *G. robusta*.

Minckley and DeMarais (2000) summarized available distributional, morphological, and molecular data and examined the taxonomic status of members of the complex. Because each morphologically discrete form was consistently collected at the same locations and never taken together, they concluded that *G. intermedia*, *G. robusta*, and "*grahami*" represented three distinct taxonomic species. Because some type specimens of "*grahami*" were actually *G. robusta*, this nomen was invalid, and the earliest available replacement name was *Gila nigra*. Minckley and DeMarais (2000) further discuss origins of *G. nigra*, concluding that it may have multiple, independent origins through discrete hybridization events between *G. intermedia* and *G. robusta.*

Schwemm (2006) characterized molecular variation within and among members of the *G. robusta* complex using sequences from mitochondrial (subunit 2 of NADH dehyrdrogenase) and nuclear DNA (introns from S7 and Tpi-B). While levels of divergence among alleles/haplotypes were low, there were many variants that were unique to specific subpopulations. Hierarchical analysis indicated that patterns of sequence variation were not associated with morphology or hydrographic connection, but were best explained by fragmentation and independent evolution of many subpopulations. Tests of association of mtDNA and nuclear variation failed to provide

evidence for recent admixture among forms; however, patterns of variation were still consistent with the past introgression hypothesis proposed by DeMarais (1992) and Minckley and DeMarais (2000).

Understanding patterns of variation is critical for informed management of this group of fishes because identification of independent units is essential for conservation of existing genetic and taxonomic diversity. Here we report results of microsatellite analyses designed to quantify patterns of variation within and among members of the *Gila robusta* complex. Microsatellites are small, tandemly repeated sequences (e.g., oligonucleotides AC, GATA) that are randomly distributed throughout the genome. Variant alleles at a locus result from change in the number of repeats (e.g., AC 10 times in a row versus 11), allowing easy visualization through observation of length differences. High microsatellite mutation rates (typically 10^{-3}, Jarne and Lagoda 1996, Garcia de Leon et al. 1997) result in increased levels of variation, making them extremely useful for population level studies (e.g., Dowling et al 1996, Ruzzante et al. 1996, Goldstein and Schlotterer 1999).

Materials and Methods

Sampling and DNA extraction.— Sampling of the Gila and Bill Williams river drainages encompassed 33 sites in seven sub-basins in Arizona and New Mexico (i.e., Verde River, Salt River, San Pedro River, Santa Cruz River, Agua Fria River, Gila River mainstem and Bill Williams River - Figure 1). Morphological classification and species status follow designations presented in Minckley and DeMarais (2000). Fourteen to thirty individuals were collected from each locality (Appendix 1). Fish were captured using a combination of seining, hoop nets and electrofishing. Muscle or fin clips were removed and immediately preserved in 95% ethanol, or

frozen in the field with dry ice. Frozen samples were either retained frozen (-20°C or -80°C) or transferred to 95% ethanol in the laboratory. Genomic DNA was extracted from muscle or fin clips by standard proteinase K/phenol/chloroform protocol as modified by Tibbets and Dowling (1996) and resuspended in 20-100 µL sterile-distilled water.

Microsatellite loci.— Primers for the ten microsatellite loci used in this analysis were derived from a variety of sources. Six loci (36, 222, 223, 225, 227, 300) were developed by Keeler-Foster et al. (2004) from a *G. elegans* library. One locus (G294) was developed by Meredith and May (2002) from a *G. bicolor obesa* library. The remaining three loci (C2, D17, D42) were obtained from a library generated from *G. robusta* using the enrichment methods provided by Glenn and Schable (2005). Primer sequences are provided in Table 1. Amplifications were performed using 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 the annealing temperature was decreased by 1 C each cycle (94 C, 30 sec, 65-50 C, 30 sec, 72 C, 30 sec) to final temp of 50 C. These same three steps were repeated for additional cycles until 25 or 30 total cycles were completed, and the entire run finished with a long extension step (72 C, 7 min). Products were separated by electrophoresis through 6.5% denaturing gels (KB^{Plus}, LiCor) for 90-105 mins at 40 W with a minimum of four ladder lanes (50 bp -350 bp size standard, LiCor) included on each gel. Fragments were visualized on a LiCor 4300 DNA Analysis system and analyzed using the software SAGA GT.

Statistical analyses.— Deviations from Hardy-Weinberg equilibrium (F_{IS}), allelic richness (A_R), average heterozygosity per locus (h), and multilocus equilibrium were examined using FSTAT version 2.9.3.2 (Goudet 2001). Significance values from single locus tests for equilibrium were

adjusted using the B-Y correction as described by Narum (2006) (adjusted critical value of 0.01767, 10 loci) whereas the correction provided by FSTAT was used to correct probabilities when considering tests of multilocus equilibrium. To examine the distribution of genetic variation among sample populations we also used FSTAT to generate Weir and Cockerham (1984) *F*-statistics. Significance of values was tested using jackknifing (individual and all loci) and bootstrapping (all loci). For comparisons of *F*-statistics among species, 2500 permutations were used. Genetic variation was further partitioned by species and drainage using the program Arlequin version 3.11 (Excoffier et al. 2005).

Number of groups and assignment of individuals was examined using STRUCTURE version 2.2 (Pritchard et al. 2000, Falush et al. 2003) and BAPS 5.1 (Corander at al. 2004). For STRUCTURE, the default assumption allowing for admixture among samples and correlated allele frequencies across loci was employed. This setting is recommended by the authors as it maximizes the ability to identify differences among groups (Falush et al. 2003). For each *a priori* assumed number of populations (*K)*, 50 independent runs of 55,000 replicates each (first 5000 discarded as burn-in) were performed. The output from each run is a series of probabilities of assignment (Q values) of each individual to each group. For example, if $K = 4$, then each individual will have a Q value for each of the four possible groups. Assignment of an individual to a specific group is reflected by high probability of assignment (e.g., $Q > 0.90$) whereas admixture would be indicated by low to moderate assignment probabilities to several groups.

Distribution of Q values across runs for each *K* was summarized using the program CLUMPP (Jakobsson and Rosenberg 2007) and the statistic h' ("h-prime"), which measures similarity across replicates (closer to 1 indicates greater similarity among runs). Results were visualized as assignment probabilities (Q values) for each individual across replicates and sorted

by sample, drainage, and species using the program DISTRUCT (Rosenberg 2004) as exemplified in Figure 2. In this example, we show a plot based on a subset of *robusta* samples (West Clear, Boulder, and Trout creeks) where STRUCTURE was run for three groups ($K = 3$) for 50 replicates. Q values (assignment probabilities) are plotted on the y-axis (summed across replicates) while individuals are plotted on the x-axis. Probability of assignment to different groups is identified by different colors, in this case blue, brown, and orange. In this example, individuals from West Clear and Boulder creeks are generally assigned to the blue and brown groups, respectively, across the 50 replicates. The pattern for Trout Creek reflects variation in assignment across the 50 replicates, as all individuals are assigned to the brown group in approximately 1/3 of the replicates and were placed in the orange group in the remaining 2/3 of the replicates. Evidence for variation within replicates is depicted by the West Clear Creek sample, with levels of assignment to blue and orange groups varying among individuals.

As *K* increases, population structure becomes increasingly evident as groups split off in a hierarchical manner until optimal *K* is achieved. Assignment probability plots can become complex quickly, making it difficult to draw inferences about the hierarchical structure of variation. Therefore, each set of Q values across all *K* were treated as variables for the construction of overall assignment distances among individuals (simply the multivariate Euclidean distance measured across all Q values for each pair of individuals). The hierarchical structure of the assignment distances was visualized using a neighbor-joining network constructed with MEGA 4 (Tamura et al. 2007). This tree is not meant to imply any evolutionary relationship but rather is a summary of the hierarchical structure present when examining the results from the full set of *a priori* determined cluster sizes.

BAPS 5.1 runs (Corander et al. 2004) were conducted in the "clustering of groups of

individuals" mode. In this mode, groups of individuals (pre-defined by the user) are clustered rather than individuals. First, separate runs were completed for each species, treating each sample population as a "group" within the species. Next, we treated each sample population as a group, but combined all species. 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). The software reports the set of estimates with the "best" partition and the probability associated with different *a priori* assumptions.

Results and Discussion

Variation within populations.— Genetic variation in *Gila* was characterized using 726 individuals from 33 locations and 10 microsatellite loci. Most samples have complete data as amplification rarely failed (average failure rate – 5.8 individuals/locus [0.8%]; highest failure rate - 15 individuals [2%] at locus 300, Table 1). Failed amplifications were scattered across populations, reducing concerns over the potential impact of null alleles.

There was considerable variation in numbers of alleles and allele size ranges across loci (Table 1). Allelic richness (A_R – number of alleles corrected for sample size) was calculated for each locus in each population. Average A_R values ranged from 1.70 (Sabino Canyon) to 8.86 (Forks Region of the upper Gila River), with the majority of lower values reported for populations of *intermedia* and *nigra* (Table 2). Statistical analysis indicated that these values varied significantly among loci (ANOVA, $F=24.214$, 9 df, $P<0.001$) and species ($F=5.152$, 2 df, $P=0.006$) but there was no interaction among these factors ($F=0.776$, 18 df, $P=0.728$). Post hoc tests indicated that populations of *robusta* exhibited significantly greater levels of variation than those of *intermedia* (A_R of 5.74 and 4.66, respectively) while populations of *nigra* exhibited an

intermediate value (A_R=5.08) that was not significantly different from either of the other two species. This contrasts with results from allozymes reported by DeMarais (1992) where *intermedia* (12 populations, h=0.040) and *robusta* (5 populations, h=0.041) had comparable levels of heterozygosity/locus while *nigra* exhibited considerably lower levels (4 populations, h=0.020). This difference may reflect differences in the number of samples characterized and/or differences in levels of variation between allozymes and microsatellites.

Distribution of genotypic variation within each population was examined for fit to Hardy-Weinberg expectations (HWE) using the statistic F_{IS}. Average F_{IS} values across loci and populations identified significant differences between species (*robusta* = -0.002; *nigra* = -0.039; *intermedia* = 0.055; P = 0.038), largely resulting from the excess of heterozygotes at most loci in the Turkey Creek, NM sample of *nigra*. Of the 330 individual tests conducted (10 loci, 33 locations), 17 showed deviations from HWE even when significance values were adjusted for multiple tests (Table 3). More of the 17 significant tests identified deficiency of heterozygous individuals than an excess (11 and 6, respectively). These deviations were scattered across loci with six of the ten loci exhibiting significant values. At the extremes, three of 33 samples showed significant deficiencies of heterozygotes at locus D42 (*intermedia* from Silver Creek and Dix Creek; *robusta* from Lower Eagle Creek) while three samples had significantly more heterozygotes than expected at locus 222 (*nigra* from Turkey Creek, NM and Spring Creek, Salt River drainage; *robusta* from Trout Creek). This random scatter of deviations among loci and populations also indicates that null alleles are not likely affecting results.

Examination of patterns of deviation provides useful information about processes influencing patterns of variation. Of 33 samples examined, eight exhibited significant overall deviations from HWE (two excesses and six deficiencies). *Gila nigra* from the East Verde River

(overall F_{IS} = -0.239) exhibits fewer alleles (five monomorphic loci, A_R = 1.77) while all five polymorphic loci exhibited an excess of heterozygotes (Table 3). *Gila nigra* from Turkey Creek, NM (overall F_{IS} = -0.274) exhibits higher levels of variation (A_R = 3.58), with eight of the nine polymorphic loci exhibiting excess heterozygosity and two of those values highly significant (Table 3). Boulder Creek (*G. robusta*) and Cienega Creek (*G. intermedia*) samples also tend to have an excess of heterozygotes (at 6 of 7 and 4 of 5 polymorphic loci, respectively). These sorts of patterns are consistent with small breeding populations and relatedness of individuals.

The remaining deviant samples exhibited significant heterozygote deficiencies. Several of the deviations are small; however, samples of *G. intermedia* from Sabino Canyon and Walker Creek are larger (F_{IS} = 0.268 and 0.140, respectively) and exhibit smaller numbers of alleles (AR = 1.70 and 3.47, respectively). At Sabino Canyon, four of the five polymorphic loci exhibited a deficiency of heterozygotes. These patterns are also indicative of small population sizes at each of these locations.

There were 1485 pairwise tests of multilocus equilibrium within populations (33 populations, 45 pairs of loci per population). Of these, 91 (6%) were significant at the 0.05 level with no obvious deviations associated with specific loci. Deviations were clustered by population, with Turkey Creek, NM exhibiting the most deviations (11 significant tests). These results are consistent with geographic effects; however, the loci do not appear to be physically linked.

Variation among populations.— Partitioning of genetic variation into within and among population components identified significant population structure (Table 4). Jackknife estimates of total genetic variation (*F*) for each locus ranged from 0.214 to 0.416 (loci 222 and 36, respectively), with a bootstrap average across loci of 0.297 (95% confidence interval 0.254-

0.346). The within population component (*f*) was small and not significantly different from 0 (range -0.083 [locus C2] to 0.076 [locus 36]), consistent with HWE results discussed above (bootstrap average = 0.022, 95% confidence interval -0.008-0.051). Therefore, the majority of variation was partitioned among populations ($\Theta \approx F_{ST}$) and ranged from 0.231 (locus 300) to 0.383 (locus C2) with a significant bootstrap average of 0.280 (95% confidence interval 0.251-0.318). These among population values are somewhat lower than those estimated by DeMarais (1992) from allozymic variation ($F_{ST} = 0.410$); however, this may reflect reduction in F_{ST} values associated with increased numbers of alleles at microsatellite loci relative to allozymes (Hedrick 1999).

To further examine the role of historical and geographic factors, among population variation was specifically partitioned by either taxonomy (three species) or river drainage structure (seven drainages) to see how these factors explain the distribution of genetic variation (calculated as the weighted average across loci). When taxonomy was used to define partitions, the majority of among population variation was found within species ($F_{SC} = 0.273$) instead of among them ($F_{CT} = 0.016$). A similar result was obtained when samples were partitioned by drainage, although a slightly higher fraction of the variation was explained by geographic structure (among samples within drainages [F_{SC}] = 0.246; among drainages [F_{CT}] = 0.055). This result indicates that drainage structure is slightly better at explaining patterns of variation than taxonomy. Note, however, the vast majority of the variation is partitioned at the individual population level, indicating the gene flow has been limited and local processes are driving evolution in this group of taxa.

Analysis of species separately provides a somewhat different picture. Estimates of F_{ST} among species also failed to identify differences among them (*robusta* = 0.204; *nigra* = 0.322;

intermedia = 0.287; $P = 0.386$). However, if samples from the Bill Williams river drainage are excluded, the average value for *robusta* drops dramatically ($F_{ST} = 0.076$) and there are significant differences among species in levels of population structure ($P = 0.014$). This latter pattern of differences is consistent with those reported by DeMarais (1992), with more structure in the headwater forms (*intermedia*, *nigra*) than the mainstream species (*robusta*) of the Gila River drainage. This pattern presumably results from more interconnection (and less structure) among mainstream than geographically isolated headwater forms.

Assignment testing.— A more fine-scaled perspective of genetic structure was provided by assignment testing, allowing for assessment of the number of groups represented in our samples and level of admixture among groups. Initial assessments were performed on samples from each species independently, followed by an analysis with all samples included.

For *robusta*, fit to 2 through 8 groups was assessed for 203 individuals from eight samples (Figures 3 and 4). Consistency across replicates (Figure 3) was highest for $K = 6$ (h' = 0.983); however, the fit was also very high for $K = 7$ (h' = 0.947). Each of the seven groups represents a distinct geographic sample with a high probability of including most or all of its component individuals, with only samples from West Clear Creek and Verde River at Perkinsville not distinct. For $K = 8$ (h' = 0.910), group structure is very similar to $K = 7$, with the new group scattered across samples from the entire Gila River drainage with generally low assignment probabilities. Given the solution for $K = 8$ does not appear to allow further diagnosis of samples, the optimal result appears to be that seven of eight samples form distinct groups. This is further supported by the program BAPS which also identified $K = 7$ as the optimal solution, with West Clear and Perkinsville samples clustered together.

The hierarchical nature of variation among samples of *robusta* is evident when

comparing across Q plots for various levels of *K* (Figure 3) and is reflected in the neighbor-joining network constructed from these probabilities generated by STRUCTURE (Figure 4). As *K* increases, samples are identified as distinct following a geographic pattern (Figure 3). At the lowest level (k = 2), samples from Bill Williams and Gila rivers are placed in two separate groups, consistent with greater dissimilarity between than within drainages. As *K* is increased to 3, the other Bill Williams sample from Trout Creek is often identified as distinct; however, there is variation in assignment of this group across the 50 replicates as indicated by its frequent inclusion in the Boulder group. As *K* is increased to 4, the sample from Trout Creek becomes more distinct from all others. The samples from Cherry Creek and the lower Salt River follow a similar trajectory, becoming distinct for many replicates at $K = 4$ and completely unique when group size is set at 5. The lower Salt sample also becomes distinct at $K = 5$, leaving the remaining sample from the Gila and Verde rivers in a single group. This pattern is evident in the neighbor-joining network (Figure 4), with each of each of these samples forming distinct groups that include all individuals and similarity of Q probabilities across different values of *K* that reflect the hierarchical nature of this structure.

The remaining samples are further divided as *K* increases; however, levels of distinctiveness vary (Figure 3). At $K = 6$, Gila and remaining Verde River samples split into two distinct groups; however, assignment probabilities for samples from Lower Eagle Creek are variable and not as robust. Increasing *K* to 7 dramatically improves assignment probabilities of Lower Eagle Creek samples and places these individuals in their own distinct group; however, variable assignment probabilities result in scattering of these samples throughout parts of the neighbor-joining network (Figure 4).

At $K = 7$, only two samples from the Verde River drainage (Perkinsville and West Clear

Creek) do not form discrete groups. The majority of these individuals form a single, unique group; however, some individuals have low assignment probabilities to any one group or appear to be more similar to those from Aravaipa Creek (Gila River drainage). Such individuals from the Verde River sample (also some found in other groups) are not likely the results of admixture but instead indicate that levels of variation make it difficult for the algorithm to accurately assign such individuals to any one group.

Gila nigra was represented by 170 individuals from 8 samples which were used in runs where group size was varied from 2 to 8 (Figure 5). Some replicates in some runs (especially for higher *K* values) moved towards local optima with considerably lower likelihood scores. Such runs were excluded from subsequent analyses. Consistency across replicates was highest for $K = 7$ ($h' = 0.990$). Therefore, this analysis allows for placement of individuals into seven unique samples, with only the samples from Spring Creek and its tributary Rock Creek (Salt River drainage) indistinguishable. Analysis with BAPS provides a slightly different picture, as it indicates that the optimal solution splits all eight samples into distinct groups.

The hierarchical nature of variation is evident when examining the change in group structure with increasing *K* (Figure 5). For $K = 2$, the samples from the Verde (East Verde and Fossil Creek) and Turkey Creek, NM generally are distinct from remaining populations. When $K = 3$, plot of Q values are more complex. Samples from East Verde and Turkey Creek, NM are assigned to the same group as Fossil in some replicates and to a unique group that is occasionally seen in Rock-Spring Creek samples in other replicates. The remaining samples are generally distinct from those discussed above. This trend of increasing distinctiveness of samples continues for $K = 4$ and becomes complete when $K = 5$, with similarity among groups mostly geographically aligned (Fossil Creek, East Verde River, Gila River+Turkey Creek, NM,

Tonto+Marsh creeks, and Rock+Spring creeks) and some admixture evident in the Gila River sample. Consideration of 6 groups results in recognition of separate Gila River and Turkey Creek, NM samples while $K = 7$ separates the Tonto Creek and Marsh Creek samples.

The neighbor joining network is generally consistent with these results (Figure 6), as many samples are generally cohesive (i.e., include all individuals) and levels of similarity also reflect change in group structure with increasing *K*. Samples from East Verde River and Fossil Creek are cohesive and connected by long branches near the base of the topology as expected from their distinctiveness and difficulty of assignment among replicates for lower values of *K* discussed above. In addition, samples from the upper Gila River and Salt River basins tend to be more similar to each other This analysis also identifies distinctiveness of samples from Rock+Spring creeks, Marsh+Tonto creeks, and Turkey Creek, NM. The major exception to this latter statement is due to the scattered similarity of individuals from Marsh, Rock, and Tonto creeks with some individuals from the Gila River, NM sample.

Gila intermedia was represented by 353 individuals from 17 populations. As for *nigra*, replicates in some runs (especially for higher *K* values) moved towards local optima with considerably lower likelihood scores (Figure 7). Such runs were excluded from subsequent analyses. Consistency among replicates was high for $K = 15$ (h' = 0.813); however, highest consistency across replicates was identified for 16 groups (h' = 0.836). There were similar levels of consistency when $K = 17$ (h' = 0.833); however, in these latter cases one or two of the groups (for 16 and 17, respectively) have low assignment probabilities and are scattered among locations. Therefore, inclusion of these two groups does not provide any additional information over the conclusion that group size was 15. For $K = 15$, there is considerable geographic structure, as virtually all samples from geographic collections form distinct groups (Figure 7).

The only exceptions are the two samples from Eagle Creek (upper and East Fork) which form a single group, and the combined unit of Cienega Creek, Sheehy Spring, and Sabino Canyon, with Cienega Creek being linked with either of Sheehy Springs or Sabino Canyon (depending upon the replicate). Analysis with BAPS again provides increased resolution as it recognizes 16 distinct groups under the optimal solution, with the two Eagle Creek samples included in a single unit.

The hierarchical structure is much more complex and difficult to interpret from examination across various values of *K* (Figure 7); however, the neighbor-joining network is more straight forward (Figure 8). In that analysis, all individuals from the same location are most similar to one another with the exception of a few individuals from O'Donnell Creek and Turkey Creek, AZ and the two samples from Eagle Creek (which are intermixed but form a single group). Samples from the same subdrainage often group together and are arrayed hierarchically according to geographic distance. There are a few exceptions. Walker Creek (Verde River drainage) falls as the most distant member of a distinct group that includes samples from the Agua Fria and Santa Cruz River drainages. Bonita Creek is most similar to the remaining Verde River samples; however, the branch forming this link is short, implying a similar level of difference from other Gila River samples. Samples from Blue River (a tributary to the Gila River) exhibited more similarity to a group of San Pedro River samples instead of other Gila samples. In these conflicting situations, samples are genetically quite distinct and this placement may reflect large differences between samples. Alternatively, admixture with representatives from *nigra* or *robusta* could be confounding levels of similarity among samples.

While the above analyses indicate that there has been significant fragmentation and structure within each of these species, they do not address the potential for admixture among

these species. To address this issue, all 33 populations were included in a single analysis. The size and complexity of this data set required a two step approach. Because BAPS runs do not take as much time, this program was used to determine the number of groups (k = 29). Each of the identified groups was represented by single populations except for two, one containing both East Fork and Upper Eagle Creek samples and the other comprised of Lower Eagle, Gila Forks, West Clear, and Verde River, Perkinsville.

STRUCTURE was then used characterize a subset of *K* values 3 through 10, even number runs from 12 through 28, and 29. As with previous analyses, some replicates in all runs achieved local optima with considerably lower likelihood scores. Such optima were generally more frequent than for individual species analyses, especially at larger values of k. As before, these anomalous replicates were excluded from subsequent analyses.

Consistency among replicates gradually increased until $K = 28$ (h' = 0.763), with a slight decline for $K = 29$ (h' = 0.753)(Figure 9). Like previous analyses, increasing *K* generates groups with low assignment probabilities starting at approximately $K = 24$ (h' = 0.679). These low assignment probability groups are scattered among locations, but are especially common within samples of *robusta*. Therefore, the complexity of this data set makes it difficult to definitively assess the number of groups; however, it is clear that there is considerable structure with many samples easily assignable to distinct groups. In addition certain sets of populations are consistently grouped together, even for values of *K* as high as 29. These are samples of *robusta* from West Clear Creek and Verde River at Perkinsville, *nigra* from Rock and Spring creeks, and three sets of *intermedia* samples from East Fork+Upper Eagle creeks, O'Donnell - Turkey (AZ) creeks, and Cienega Creek - Sheehy Spring. Results from *robusta* and *nigra* are consistent with species level analyses, but additional groups of populations were identified in *intermedia* that

were not found when it was analyzed without the other species.

Because of the complexity of results, it is difficult to obtain much information from examination of the Q plots for each *K* (Figure 9); however, there are a couple of samples that are notable from this perspective. Individuals from Bonita Creek, Forks region of the Gila River, Spring Creek – Verde River drainage, and Tonto Creek are routinely difficult to assign to specific groups, even at low values of *K*. In addition, individuals from four samples (Bonita Creek, Forks region of the Gila River, Spring Creek – Verde drainage, and Turkey Creek, NM) are regularly assigned to a group with samples of *robusta* at lower levels of *K* (< 10). These patterns may reflect admixture between different groups and/or species or incorrect assignment to *intermedia* or *nigra*. Morphological analyses of these populations are necessary to discriminate between these alternatives.

It is easier to examine variation across STRUCTURE runs and the hierarchical nature of similarity by looking at the results by neighbor-joining clustering (Figure 10). Because the optimal *K* is difficult to determine for this complex data set, we chose to enter data through *K* = 28 (maximum consistency across replicates) into the hierarchical analysis. Use of Q probabilities for higher values of *K* likely adds noise to the analysis; therefore, this approach is conservative. Several samples exhibited long branches that are found near the midpoint root of the network (*robusta*, Boulder and Trout; *nigra*, East Verde and Fossil; *intermedia*, Silver, Walker and Williamson Valley). These samples are often identified as unique in species level analyses, separating from others at lower hierarchical levels and values of k. The remaining *robusta* samples from the Gila River drainage exhibit high levels of similarity, clustering together; however, results are somewhat different from the single species analysis. Here, samples from Verde at Perkinsville and West Clear form separate, identifiable samples. Some individuals from

Aravaipa Creek, lower Salt River, and especially Lower Eagle Creek are scattered across the topology, consistent with admixture expected from this mainstem species. Samples of *nigra* also show high degrees of similarity among them with the majority of the Salt River samples forming groups consistent with the single species analysis; however, individuals from the Tonto Creek sample show considerable scatter, possibly reflecting some admixture with *robusta*. Most samples of *intermedia* form discrete groups of individuals, with some sets of samples clustering by drainage (e.g., Santa Cruz River, San Pedro River). This result indicates that there is some geographic relationship with similarity and the distribution of microsatellite variants.

Note, however, such relationships do not always hold, as samples for different Verde River localities are scattered across the network as are some from the Gila River drainage. There are several reasons why similarity among populations would not follow a strict geographic pattern. Microsatellites evolve very rapidly; therefore, evolution in historically disjunct (e.g., Bill Williams River samples) and isolated populations (e.g., Fossil Creek, Williamson Valley Wash, etc.) may have been extensive enough to eliminate historical similarities.

Alternatively, evolutionary processes may be responsible for breakdown of pattern. For example, the sample of *nigra* from Turkey Creek, NM requires special discussion. This sample was collected near the confluence of the creek and the Gila River, below the barrier isolating the population of *intermedia* in the headwaters from the rest of the drainage. We did not sample the population of *intermedia* above the barrier and therefore cannot address it here. Individuals from our sample near the mouth were cohesive, with all individuals similar to *nigra* from the Forks region and *robusta*. This sample was comprised of all young of the year (ca 40-50 mm SL) and exhibited significant excesses of heterozygotes at several loci. The sample was likely produced by a small number of parents; consistent with knowledge that *nigra* is becoming increasingly

rare in the Gila River in New Mexico (Paroz et al. 2006, Propst et al. 2008). Because the program STRUCTURE assumes Hardy-Weinberg equilibrium, similarity of this sample to *robusta* may result from failure to meet conditions of the algorithm and not biological factors. Note, however, the Turkey Creek, NM sample is geographically proximate to and behaves like the problematic sample of *nigra* from the Forks region. The Forks sample is also often assigned to *robusta* and appears to show some admixture with *intermedia* (especially at lower levels of k), therefore, this pattern is likely real and not an artifact of the method.

There are other additional samples that are cohesive but appear to be misplaced, being assigned to other species at lower values of *K*. Samples from Spring Creek, Verde drainage (*intermedia*) are most similar to samples of *robusta* and Turkey Creek and Gila River, NM as discussed above. Likewise, the sample of *intermedia* from Bonita Creek shows greater similarity to samples of *robusta* than with *intermedia* from their neighboring drainage, Eagle Creek. These results and examination of Q plots suggests that there may have been considerable *robusta* influence in these samples of *intermedia*. Therefore, these populations warrant closer examination, especially for morphological traits.

Other samples also require closer examination. While many samples are cohesive, there are some unusual individuals. In some samples, single individuals are misplaced (e.g., roc8, marsa22) likely reflecting unusual patterns of microsatellite divergence and not worthy of further consideration here. Other samples, however, have several individuals interspersed throughout the topology, often with other species (e.g., Lower Eagle Creek samples with *intermedia*; Tonto Creek samples with *robusta*), potentially indicating some admixture between species. The *nigra* sample from the Forks region of the Gila River is especially noteworthy in this regard, with its individuals scattered across the network, with many individuals more similar to *robusta* or

intermedia but not *nigra*.

Significance of evolutionary processes for patterns of variation.— Microsatellite results are consistent with geographic distributions and expected population sizes. The headwater forms of *intermedia* and *nigra* were expected to exhibit lower levels of variation within populations than the mainstem inhabitant *robusta* due to their isolation and greater potential for reduced population sizes, and this is precisely what was found. Patterns of population structure are also consistent with reduced isolation in *robusta* relative to the other taxa, results consistent with previous analyses based on studies of allozymes (DeMarais 1992) and nuclear DNA and mitochondrial DNA (Schwemm 2006).

Assignment testing using microsatellite data was unable to group samples by species; however, this reflects high levels of divergence among samples, especially those from *intermedia* and *nigra*. This result is consistent with F-statistic analyses from this and other data sets (DeMarais 1992, Schwemm 2006), which indicated that most of the variation is partitioned within species and drainages instead of among them. Therefore, the inability to diagnose these species with molecular data does not contradict their recognition as distinct, but highlights the role that local evolution has played in shaping the variation in these species.

Role of introgressive hybridization in this complex.— Previous studies have identified a significant role for introgressive hybridization in diversification of the genus *Gila* (Dowling and DeMarais 1993). Molecular and morphological studies identified *G. seminuda* as a taxon that originated through introgressive hybridization between *G. elegans* and *G. robusta* (DeMarais et al. 1992). There are distinct forms of *G. seminuda* in the Moapa and Virgin rivers, with the Moapa form more heavily influenced by *robusta*. In a study of molecular variation in *G. robusta*, *G. cypha*, and *G. elegans* from the upper Colorado River basin, Gerber et al. (2001) identified *G.*

jordani, a population of *Gila* isolated in the Pluvial White River that was the product of hybridization between *G. cypha* and *G. robusta*. They further noted that introgressive hybridization had been critically important in the genus as a whole as all *G. robusta* above Lake Mead exhibited mtDNA from *G. cypha*. Local introgression was found to be more important for patterns of variation than was gene exchange among locations as mtDNA from *G. cypha* and *G. robusta* from the same locality was more similar than it was to mtDNA from conspecifics at other locations.

Building upon this information, DeMarais (1992) and Minckley and DeMarais (2000) suggested that *Gila nigra* was a taxon of hybrid origin, resulting from introgression between *intermedia* and *robusta*. *Gila robusta* and *G. intermedia* occupy different habitats, with *robusta* typically found in mainstem rivers and larger tributaries while *intermedia* occupies headwater reaches and cienegas. During dry periods, these species are expected to be geographically isolated, however, during wetter times *intermedia* and *robusta* could co-occur and hybridization could result, producing local hybrid swarms. As streams again became desiccated, these hybrid populations would become isolated in headwater reaches, allowing for adaptation to the local habitat. It is these populations that are recognized as *Gila nigra*, exhibiting morphological intermediacy between *intermedia* and *robusta*.

There are several implications from the hypothesized hybrid origin for *Gila nigra*, and some data in this report can be used to address this issue. Identifying hybrid taxa is difficult because evidence of the event is ultimately erased by continuing evolution (Dowling and Secor 1997). Initially, hybrid populations would be morphologically intermediate to *intermedia* and *robusta*; however, levels of intermediacy would vary, determined by relative abundance of the parental taxa at the onset and local selection pressures (as discussed for *G. seminuda* above).

Likewise, hybrid swarms would exhibit a combination of local genetic variants from both species, and would initially have inflated levels of variation reflecting the diversity contributed by both parental species. With the passage of time, levels of variation would be reduced due to stochastic processes and selection, and the equilibrium level of variation at neutral markers (like these microsatellites) would be determined by effective population size. At this point, levels of variation would no longer provide a good indicator of hybrid origin. Since most populations of *nigra* occupy headwaters, they would be expected to ultimately attain similar levels of variation to the other headwater species, *intermedia*, which they have. Note, however, the sample from the Forks regions of the Gila River exhibits an average allelic richness (number of alleles corrected for sample size) of 8.86 per locus, which is nearly two more alleles than any other population, consistent with a recent hybrid origin for this population.

This hypothesis also has implications relative to genetic similarity and evolutionary relationships. Hybrid taxa are expected to exhibit intermediate levels of similarity and phylogenetic position relative to contributing parental forms for neutral nuclear markers like microsatellites, but they may be more similar to one or the other taxon dependent upon relative frequency of contribution. Since hybrid taxa may evolve multiple times from local pairs of progenitor species, they have the potential to be more similar to local populations of parental taxa than other phenotypically similar forms (e.g., *G. seminuda*, DeMarais et al. 1992). This pattern, however, would also depend upon age of origin. Recently formed populations would share more characteristics with each of their parental taxa; however, this signal would decay as these populations evolve new variants in geographic isolation, and they would ultimately develop their own distinctiveness, erasing evidence of their hybrid origin.

This system may provide an excellent example of temporally distinct origins of hybrid

taxa. Since *nigra* would have originated independently in each of the different drainages, populations of this species would be more similar to local populations of *intermedia* and *robusta* from which they were derived than to other populations of *nigra*. In a phylogenetic analysis, populations of *nigra* would be scattered at intermediate positions across the tree instead of forming a single cohesive entity like one would expect for *intermedia* and *robusta*. The position of each sample of *nigra* would be determined by the relative contribution of each parent, with equal contributions leading to intermediacy. Phylogenetic analyses using other markers (allozyme, DeMarais 1992; mtDNA and nuclear gene sequences, Schwemm 2006) failed to identify cohesiveness of the species; however, levels of variation were not sufficient to obtain phylogenetic resolution. Microsatellites evolve too quickly for use in phylogenetic reconstruction, but STRUCTURE analyses indicate that populations of *nigra* from different drainages exhibit varying levels of distinctiveness from each other and do not form a cohesive group. For example, samples from East Verde and Fossil Creek are highly differentiated from all other populations, identifying the significance of local evolution in these populations. Of special note, however, are samples from the upper Gila River, NM (Turkey Creek and Forks area), with some individuals showing affinities to *intermedia* and others to *robusta*, a result consistent with the hypothesized hybrid origin.

This process could be further complicated by the cyclical nature of climatic change, with introgressive hybridization possibly affecting the same populations many times, leading to overlying patterns of genetic variation. For example, samples of *nigra* from Tonto Creek are generally distinctive from all other samples from outside the basin, however, a small number of individuals appear to exhibit some influence from *robusta*. Such a pattern could have resulted from an ancient hybrid origin of the Tonto Creek populations followed by recent influences from

robusta that once occupied the Salt River.

In conclusion, there is evidence consistent with hybrid origin for *Gila nigra*, but it is not as clear cut as other members of the genus (e.g., *G. jordani* and *G. seminuda*). This is a typical problem in such cases because the origin of hybrid taxa represents one extreme of a continuum where hybridizing populations become isolated and develop there own unique evolutionary legacy. In addition, this is a continual process such that populations that may actually reflect hybridization between *intermedia* and *robusta* (e.g., *intermedia* from Spring Creek, Verde River drainage and Bonita Creek, Gila River drainage) could ultimately become isolated and evolve into admixed forms, in other words, become *nigra*. Given this information is consistent with a role for introgression and the importance for diversification in this genus, it needs to be considered as management strategies are developed and implemented.

Management implications.— The potential role of introgressive hybridization in evolution of this group complicates the process of conservation and management. Because each of the species appear to exchange genes on occasion, it is imperative that all species in the complex are afforded protection. Loss of distinct populations from any of these species may impact the evolutionary potential of significant portions of the complex. *Gila robusta*, the only species that is not currently under formal consideration for listing, may well be the most important of the three as it occupies mainstem habitats and is the potential conduit for occasional gene exchange among isolated headwater populations. Therefore, actions should be taken to insure that this species persists.

Regardless of the role of introgression, this and previous analyses have identified specific distinct groups that should be protected. In his study of nuclear and mtDNA sequence variability, Schwemm (2006) provided a specific protocol for identifying units for conservation that we also

follow here. Because data are congruent with three distinct taxonomic entities, conservation units are defined in a hierarchical manner, with genetically distinct units identified within each of the species. In this study we found some potential discrepancies between assignments based on microsatellite data and putative taxonomic status based on morphological traits (e.g., upper Gila River, NM; Spring Creek, Verde River). It is important to note that identifications based on Minckley and DeMarais (2000) are based upon museum records; therefore, such conflicts could represent change in species composition and need to be investigated further.

Previous molecular studies by DeMarais (1992) and Schwemm (2006) identified a significant role for isolation and independent evolution of populations in this complex. Schwemm (2006) used information based on F-statistics, presence of unique alleles, and geographic isolation to identify 21 distinct units across the three species for consideration in management efforts (Table 5). This study confirms and extends that work, identifying limited recent gene exchange among populations and general cohesiveness of populations based on assignment testing. Results from assignment testing are generally consistent with and supportive of Schwemm's groupings; however, because microsatellites are more sensitive, they provide increased resolution within some units. In addition, hierarchical analyses of assignment probabilities identify a significant geographic component to variation, consistent with intermittent contact among geographically proximate populations. We discuss these results below for each of the three species separately. Note, however, consideration in this manner does not lessen the burden towards conservation of the complex as a whole.

F-statistic analyses of *robusta* identified considerable variation in allele frequencies among samples ($F_{ST} = 0.204$); however, removal of the two samples from the Bill Williams River reduces structure considerably ($F_{ST} = 0.076$). Assignment testing provided consistent

results, identifying seven distinct groups out of eight samples examined (Figure 3). Most samples of *robusta* are similar, clustered together based upon hierarchical analysis of assignment probabilities (Figure 10). Samples from the Bill Williams River (Boulder and Trout creeks) are especially unique and clearly have been isolated from each other and from Gila River populations for a considerable period as members of this complex have never been observed from intervening lower Colorado River locations. Additional sampling from this drainage is necessary to better understand patterns of isolation and identify management units. The remaining samples from throughout the Gila River drainage show various levels of cohesiveness (Figures 3, 4). In general, Cherry Creek, lower Salt River, and West Clear Creek + Verde River samples are mostly discrete, with individuals from Aravaipa Creek and especially lower Eagle Creek exhibiting more variable levels of assignment probability and cohesiveness.

These results are consistent with those of Schwemm (2006) with two exceptions. We were unable to use samples from Black River, a highly variable population with many unique nuclear and mtDNA alleles. Schwemm (2006) included those as a unique management unit and noted that analysis of additional samples from the Salt River drainage is essential to understand structure in that region. The second difference involves the sample from the lower Salt River canals which Schwemm (2006) found to group with the Verde River, consistent with their source from the lower Verde River. Analysis of microsatellites allowed for discrimination of samples from the lower and remaining Verde samples (West Clear and mainstem at Perkinsville), identifying further potential subdivision.

Given these results, we advocate management on a subdrainage basis (Table 5), making attempts to maintain isolation by distance generated by evolutionary processes. If establishment of refuge populations or captive propagation are implemented as conservation strategies, this

would imply that separate stocks should be generated for use in the Gila and Verde rivers. The Salt River is more problematic, as access into many tributaries is limited and patterns of genetic variation are poorly understood. The closest and most readily available population was found in the canal system downstream from the confluence of the Salt and Verde rivers; however, that population now is depressed and too few individuals have been encountered in recent years to support stocking into lower Salt River tributaries. Finally, given that both samples of *robusta* from the upper Salt River that have been examined are diverse and contain unique genetic variants it is essential to further characterize that part of the system before proceeding with management actions. Stocking into this region with *robusta* from external sources (e.g., Verde River) could dilute or extirpate these unique populations.

Gila intermedia occupies headwater reaches throughout the Gila River drainage and was thus expected to exhibit lower levels of variation within and more differentiation among populations than *robusta*. Previous analyses of *intermedia* were consistent with these expectations as were results based on microsatellites. Assignment testing identified 16 groups from the 17 samples examined (Figure 7). This differs from Schwemm (2006) where 10 groups were identified from 16 samples. The major difference between these outcomes was the increased resolution provided by microsatellites in recognizing discrete samples from the San Pedro and Gila rivers. Examination of hierarchical patterns of assignment values was consistent with results from nuclear and mtDNA sequences, as samples within these and other rivers were generally geographically arrayed, with proximal samples exhibiting more similarity based upon hierarchical analysis of assignment probabilities (Figures 8, 10). Only three sets of samples were not discretely defined in hierarchical analyses (East Fork Eagle and Upper Eagle creeks, O'Donnell and Turkey creeks, AZ, and Sabino Canyon and two Santa Cruz populations), with

these pairs of samples each geographically proximate to one another in the same drainage. In addition samples of *intermedia* from the Verde River drainage are quite distinct and do not cluster together in hierarchical analyses. This level of difference was also found by Schwemm (2006) for Spring Creek and Williamson Valley Wash but not Walker.

As for *robusta*, we advocate conservation management of *intermedia* by subbasin (Table 5), and levels of distinctiveness among populations make it critically important that admixture be avoided. In addition, given the significance of drainage patterns to hierarchical analyses of assignment probabilities, efforts to establish new populations should utilize the nearest geographic population as a source, avoiding transfer across different subdrainages.

Gila nigra also occupies headwater reaches and was also expected to show substantial differentiation among populations, and results from all markers (DeMarais 1992, Schwemm 2006, this study) were consistent with that expectation; however, levels of variation were not as low as those found in the other headwater form, *intermedia*. Assignment testing identified seven groups from eight samples (Figure 5) with samples from East Verde River and Fossil Creek especially distinct. This result differs from the five groups recognized by Schwemm (2006) due to increased resolution, with microsatellites allowing for discrimination of Marsh-Tonto creeks and Turkey Creek, NM and the Gila Forks samples.

There are, however, caveats associated with this interpretation. Sampling of *nigra* was limited, with many of the samples (50%) coming from the Tonto subbasin of the Salt River. Also, the outcome of hierarchical analysis of assignment probabilities indicated that some of these samples may not be as discrete as indicated by examination of the probability plots. Especially problematic was the sample from the Gila Forks region, NM which was intermixed with several samples in the hierarchical network. As discussed previously, this may reflect

misassignment of this sample as *nigra* or its recent hybrid origin.

The potential hybrid origins of *Gila nigra* make development of a management plan even more difficult; recommended management units based on available data are in Table 5. Given the distinctiveness of most populations of the complex, it is important that we maintain as many of these populations as possible. Translocation efforts should utilize nearest neighboring populations of the same species. Augmentation should be avoided as mixture with other forms could result in loss of local variation.

In addition to maintaining discreteness associated with geographic isolation and evolutionary independence, we must be ready to recognize that admixing populations of *robusta* and *intermedia* have the potential to become *nigra*. Connectedness among populations is difficult to envision in today's environment; however, there is no obvious solution to that issue. Instead, we must overcome the general need of placing specific populations into categories with different values and acknowledge that conservation should be focused on preserving the processes generating observed patterns as well as the patterns themselves, requiring preservation of the complex and not individual units.

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Table 1. Locus-specific information in microsatellite variation, including number and percentage of samples (# missing and failure rate, respectively) that failed to amplify.

Locus	Repeat	Primers	# alleles	size range (bp)	# missing	failure rate (%)
C2	$(GACA)_4GTCA(GACA)_3(GATA)_3$	5' GAC AAA GCG GTA GAC AAA ACC A 3'	4	241-281	2	0.28
		5' AAT CTG AAC TGG CTA ACC TT 3'				
D17	$(GT)_{13}$	5' TGG GCA GGA AAA GAG AAA CT 3'	38	210-298	9	1.24
		5' ATA AAG AGA CGG TAA AGA ACT C 3'				
D42	$(TCTA)_5$	5' TTG CCT GTA TAG GGT TGA 3'	10	180-216	1	0.14
		5' GTT GCT CAT TGT TAG TTT GT 3'				
36	$(TG)_8TATG$	5' CTG TTC GCT AAG GCT AAA GG 3'	12	194-216	1	0.14
		5' GCT CTC GTG TTT GTG CG 3'				
222	$(CAGA)_{11}(TAGA)_4(CAGA)_2(TAGA)$ $(CAGA)_3(TAGA)(CAGA)_4(TAGA)_4$	5' AGA CTG CTC TTC AAC GAT GTC 3'	28	182-374	5	0.69
		5' TCA CAC ACT CTG GCT GTA GC 3'				
223	$(TATC)_{18}$	5' CAT AAC TGA TTT TTT TAA TTA AGC TTG 3'	29	210-322	0	0.00
		5' GTT ACT GTA GTG GTT GAG GGA AC 3'				
225	$(GATT)_3(GATA)_{28}$	5' CCT GTG GAT CAA AAA GTA GAT G 3'	28	67-215	12	1.65
		5' CGA TTC CCA CAC AGT AAG AAC 3'				
227	$(TATC)_{18}$	5' TTG CAC ATG AAC TTA CAT AGA GG 3'	37	117-265	4	0.55
		5' ACC GTA GAT AAA AAC AAT ACA ACG 3'				
300	$(GATA)_{18}TA(GATA)_4$	5' GTT ACA GAG GCC ATA GTC CG 3'	35	175-347	15	2.07
		5' AGT TCA AGA AGA CAA AAT ATG TGT G 3'				
G294	$(GATA)_7$	5' TGT TCC CCT CAT CAT CAT AG 3'	11	195-243	9	1.24
		5' AGA ACA ATA GAA CAA TAC ACA GA 3'				

Table 2. Allelic richness for each locus of each sample, including the averages across loci for each location and across locations for each locus. Information on samples is provided in the left hand columns, where N is sample size.

Species	Location	Drainage	Acronym	N	C2	D17	D42	36	222	223	225	227	300	G294	Average
intermedia	Bass Canyon	San Pedro	bas	20	1.00	5.70	3.00	3.00	6.55	8.09	5.67	5.93	6.80	4.40	5.01
intermedia	Blue	Gila	blu	19	1.00	1.74	2.00	1.00	5.92	4.67	6.34	5.65	4.67	1.74	3.47
intermedia	Bonita	Gila	bon	20	1.00	6.67	2.98	3.00	7.01	8.77	8.28	6.94	11.51	4.91	6.11
intermedia	Cienaga	Santa Cruz	cc	20	1.00	2.00	1.00	1.00	1.00	3.70	2.00	5.40	2.99	1.00	2.11
intermedia	Dix	Gila	dix	22	1.64	5.12	3.92	3.64	7.62	3.00	4.27	5.23	5.47	2.00	4.19
intermedia	E Fork Eagle	Gila	efe	20	1.70	7.65	5.89	3.89	7.33	7.29	11.19	9.95	11.04	5.87	7.18
intermedia	Harden Cienaga	Gila	hcn	22	1.00	7.25	3.59	2.87	4.87	5.54	3.99	3.98	5.51	2.64	4.12
intermedia	O'Donnell	San Pedro	odn	20	1.00	8.21	3.70	4.68	6.37	9.42	7.29	10.41	8.19	2.96	6.22
intermedia	Redfield	San Pedro	rdf	20	1.00	3.00	3.00	2.00	4.97	5.10	5.87	7.31	7.97	2.99	4.32
intermedia	Sabino	Santa Cruz	sab	14	1.00	2.00	2.00	2.00	1.00	1.00	1.00	1.00	3.00	3.00	1.70
intermedia	Sheehy	Santa Cruz	shy	25	2.00	2.00	2.55	1.81	3.61	2.97	2.00	4.04	3.00	3.00	2.70
intermedia	Silver	Agua Fria	sil	29	1.00	2.99	2.00	1.48	3.00	2.00	4.48	3.86	4.42	3.48	2.87
intermedia	Spring	Verde	sprve	20	1.00	8.84	5.59	4.91	8.95	10.37	8.14	8.20	10.98	3.98	7.10
intermedia	Turkey, AZ	San Pedro	turaz	20	1.92	7.97	3.98	2.00	6.60	9.74	6.31	8.51	4.89	2.89	5.48
intermedia	Upper Eagle	Gila	ueg	18	2.00	6.69	5.99	3.95	7.33	8.75	10.01	7.86	8.85	6.69	6.81
intermedia	Walker	Verde	wak	24	1.00	4.51	1.00	3.17	3.93	5.72	3.85	4.97	2.58	4.00	3.47
intermedia	Williamson Valley	Verde	wvw	20	1.00	6.55	7.35	2.00	6.99	9.41	8.71	6.52	12.09	3.61	6.42
nigra	East Verde	Verde	evr	20	1.00	2.00	1.00	1.00	2.00	3.00	1.00	2.00	3.70	1.00	1.77
nigra	Fossil Spring	Verde	fos	26	1.00	1.00	2.00	2.79	2.00	2.00	6.47	3.48	1.96	1.00	2.37
nigra	Marsh	Salt	marsa	27	1.52	2.00	1.52	2.89	7.60	8.57	8.32	13.02	8.32	3.89	5.76
nigra	Gila Forks, NM	Gila	nmfks	19	1.74	13.20	2.99	5.20	11.95	13.89	10.00	11.93	12.96	4.74	8.86
nigra	Rock	Salt	roc	20	2.00	5.40	2.00	3.62	4.67	10.07	6.39	7.79	7.88	3.86	5.37
nigra	Spring	Salt	sprsa	20	2.00	6.67	2.00	2.00	5.40	10.43	8.71	13.46	10.03	3.68	6.44
nigra	Tonto	Salt	ton	20	1.92	3.70	1.00	2.62	10.09	8.63	10.25	14.07	9.48	2.94	6.47
nigra	Turkey, NM	Gila	turnm	18	1.00	3.00	3.77	3.00	2.99	3.56	3.78	4.00	6.72	3.96	3.58
robusta	Aravaipa	Gila	ara	25	1.00	7.41	4.47	4.56	9.29	8.21	12.08	8.82	7.63	4.97	6.84
robusta	Boulder	Bill Williams	bol	30	1.00	2.00	1.99	1.00	3.00	2.39	6.67	2.99	3.30	1.00	2.53
robusta	Cherry	Salt	chr	21	2.99	3.67	3.00	4.94	6.65	7.18	7.49	8.95	8.52	3.89	5.73
robusta	Lower Eagle	Gila	leg	19	1.00	8.91	2.94	4.87	8.34	10.99	7.43	12.15	9.56	4.72	7.09
robusta	Lower Salt	Verde	lsalt	29	1.00	9.04	3.84	4.70	7.40	9.67	7.30	8.69	7.18	4.73	6.35
robusta	Trout	Bill Williams	trout	30	1.00	3.00	2.00	2.00	3.97	5.53	5.17	6.02	7.37	3.96	4.00
robusta	Verde, Perkinsville	Verde	vdp	20	1.00	8.06	2.97	5.51	9.43	10.13	8.51	7.55	9.98	4.00	6.71
robusta	West Clear	Verde	wcl	29	1.00	7.20	2.74	4.73	10.07	7.49	11.62	9.29	8.53	3.97	6.66
average					1.32	5.31	3.02	3.09	6.00	6.89	6.68	7.27	7.18	3.50	5.03

Table 3. Tests of Hardy-Weinberg equilibrium (F_{IS}) for each locus in each sample. Positive and negative values identify deficiency and excess of heterozygotes, respectively. An asterisk (*) identifies significant values after correction for multiple tests.

Species	Location	Drainage	Acronym	C2	D17	D42	36	222	223	225	227	300	G294	All
intermedia	Bass Canyon	San Pedro	bas	NA	-0.170	0.138	0.273	-0.200	-0.175	0.167	-0.129	-0.010	0.057	-0.016
intermedia	Blue	Gila	blu	NA	0.000	0.131	NA	0.016	0.175	-0.029	0.102	0.130	0.000	0.081
intermedia	Bonita	Gila	bon	NA	-0.017	-0.143	0.242	0.044	0.135	0.085	0.035	0.175	-0.131	0.051
intermedia	Cienaga	Santa Cruz	cc	NA	-0.229	NA	NA	NA	-0.133	0.463	-0.123	-0.115	NA	-0.098
intermedia	Dix	Gila	dix	0.000	0.241	0.442*	0.051	0.010	-0.005	0.161	-0.120	-0.309*	0.191	0.056
intermedia	E Fork Eagle	Gila	efe	0.000	0.006	0.050	0.168	0.014	-0.084	0.158	0.226	0.018	-0.045	0.054
intermedia	Harden Cienaga	Gila	hcn	NA	0.257*	-0.206	0.042	-0.089	-0.024	0.214	-0.096	-0.029	0.545*	0.061
intermedia	O'Donnell	San Pedro	odn	NA	-0.045	-0.107	0.066	0.075	0.033	0.089	0.013	0.035	-0.101	0.016
intermedia	Redfield	San Pedro	rdf	NA	-0.154	-0.049	0.240	-0.122	0.191	-0.090	0.102	0.063	0.038	0.010
intermedia	Sabino	Santa Cruz	sab	NA	0.085	0.576	0.000	NA	NA	NA	NA	0.319	0.119	0.268*
intermedia	Sheehy	Santa Cruz	shy	-0.083	-0.100	-0.095	-0.021	0.003	0.313	0.280	-0.039	-0.106	0.434*	0.075
intermedia	Silver	Agua Fria	sil	NA	0.211	0.554*	0.000	0.060	0.022	0.048	-0.161	0.144	-0.107	0.085
intermedia	Spring	Verde	sprve	NA	-0.133	0.015	0.094	0.038	0.042	-0.054	-0.049	0.065	0.082	0.010
intermedia	Turkey, AZ	San Pedro	turaz	-0.027	0.115	0.105	0.191	0.158	0.093	0.062	0.167	-0.279	-0.080	0.069
intermedia	Upper Eagle	Gila	ueg	-0.172	-0.067	0.068	-0.107	-0.079	0.066	0.063	0.059	-0.078	-0.136	-0.024
intermedia	Walker	Verde	wak	NA	-0.125	NA	0.247	-0.162	0.110	0.626*	-0.136	0.648*	0.110	0.140*
intermedia	Williamson Valley	Verde	wvw	NA	-0.110	0.135	0.297	0.139	-0.041	0.265*	-0.196	0.306*	0.208	0.097*
nigra	East Verde	Verde	evr	NA	-0.188	NA	NA	-0.462	-0.159	NA	-0.520	-0.008	NA	-0.239*
nigra	Fossil Spring	Verde	fos	NA	NA	0.096	-0.161	0.212	0.387	0.136	-0.139	-0.064	NA	0.090
nigra	Marsh	Salt	marsa	0.000	0.198	0.000	0.578	-0.071	0.100	0.208	-0.057	0.142	0.008	0.093*
nigra	Gila Forks, NM	Gila	nmfks	0.000	0.152	0.426	0.239	0.095	0.100	-0.069	-0.029	0.146	-0.071	0.085
nigra	Rock	Salt	roc	-0.438	0.075	-0.016	0.148	-0.357*	0.086	-0.361*	-0.094	0.114	0.371	-0.057
nigra	Spring	Salt	sprsa	0.123	0.229	-0.041	-0.118	-0.014	0.084	-0.054	0.099	-0.111	-0.161	0.021
nigra	Tonto	Salt	ton	-0.027	0.687*	NA	0.315	-0.094	0.158	0.057	0.099	-0.032	-0.125	0.099*
nigra	Turkey, NM	Gila	turnm	NA	-0.482*	-0.268	-0.281	-0.734*	-0.316	0.092	-0.246	-0.177	-0.115	-0.274*
robusta	Aravaipa	Gila	ara	NA	-0.057	0.051	0.157	-0.086	0.024	-0.009	-0.097	0.070	0.021	0.004
robusta	Boulder	Bill Williams	bol	NA	-0.229	-0.115	NA	-0.179	-0.058	-0.077	0.267	-0.068	NA	-0.068
robusta	Cherry	Salt	chr	0.097	0.214	-0.307	-0.024	0.107	0.211	-0.086	0.099	-0.018	0.091	0.036
robusta	Lower Eagle	Gila	leg	NA	0.054	0.469*	0.227	-0.157	0.010	0.018	-0.013	0.121	0.269	0.080*
robusta	Lower Salt	Verde	lsalt	NA	-0.001	0.113	-0.230	-0.022	-0.008	0.042	0.064	0.004	0.058	-0.003
robusta	Trout	Bill Williams	trout	NA	-0.112	0.085	-0.160	-0.254*	0.107	-0.148	0.021	-0.134	0.039	-0.065
robusta	Verde, Perkinsville	Verde	vdp	NA	0.060	-0.127	0.052	-0.078	-0.114	0.069	-0.148	-0.001	0.121	-0.015
robusta	West Clear	Verde	wcl	NA	-0.071	-0.067	-0.092	0.043	0.015	0.081	-0.145	0.112	0.092	0.005

Table 4. Mean *F*-statistics and their standard errors (obtained by jackknifing across populations) calculated for each locus, including minimum and maximum values. The total estimate was obtained by jackknifing across loci.

	F		Θ		*f*	
Locus	Mean	SE	Mean	SE	Mean	SE
36	0.416	0.056	0.368	0.059	0.076	0.039
222	0.214	0.043	0.262	0.039	-0.066	0.027
223	0.301	0.042	0.264	0.039	0.051	0.021
225	0.308	0.041	0.257	0.036	0.068	0.029
227	0.221	0.039	0.244	0.036	-0.031	0.023
300	0.255	0.047	0.231	0.041	0.030	0.027
C2	0.326	0.088	0.383	0.098	-0.083	0.101
D17	0.383	0.051	0.374	0.049	0.014	0.034
D42	0.390	0.062	0.343	0.055	0.071	0.042
G294	0.387	0.067	0.353	0.065	0.052	0.028
total	0.297	0.025	0.280	0.018	0.022	0.016
max	0.416		0.383		0.076	
min	0.214		0.231		-0.083	

Table 5. Management units and rationale for distinctiveness of *G. robusta*, *G. intermedia* and *G. nigra* based on geographic location and allele frequency differences at one mtDNA and two nDNA loci (ND2, S7 and Tpi-B), modified and updated from Schwemm (2006). Percent values indicate haplotypes/alleles per management unit and category (i.e. shared or private). Distinctiveness of individual management units are relative to other units within species. Letters in the second column denote additional subdivisions provided by analysis of microsatellites.

management unit			river basin	rationale for distinctiveness
Gila robusta (R)				
R1)	A	Verde R. (Perkinsville)	Verde	Sites share typical widspread *G. robusta* sequences at mtDNA (AA,AG; ave. 72%) plus typical nDNA at S7 (A,B; ave. 97%) and Tpi-B (A,B,C; ave. 96%)
	A	West Clear Cr.		
	B	Low. Salt R.		
R2)	A	Aravaipa Cr.	Gila	Sites are geographically distant from *G. robusta* in the Verde R., but show widespread mtDNA (AA,AB; ave. 65%), nDNA at S7 (A,B; ave. 89%) and Tpi-B (A,B,C; 100%). Sites also share rare S7 allele J (7%).
	B	Lower Eagle Cr.		
R3)		Cherry Cr.	Salt	private mtDNA (67%): AN,GI,GK,HO,IB,IK
R4)		Black R.	Salt	private mtDNA (54%): OB, FA, AJ
R5)		Boulder Cr.	Bill Williams	private mtDNA (100%): AD,PD private nDNA (100%): E (S7)
R6)		Trout Cr.	Bill Williams	private mtDNA (100%): NA,NL private nDNA (33%): C (S7)

Table 5. continued.

management unit			river basin	rationale for distinctiveness
Gila nigra (N)				
N1)	A	Turkey Cr., NM	Gila	Sites share typical widspread *G. nigra* sequences at mtDNA (AA; ave. 56%), plus typical nDNA at S7 (A,B; ave. 93%) and Tpi-B (A; ave. 65%).
	B	Gila River, Forks region		
N2)	A	Tonto Cr.	Salt	private mtDNA (ave. 69%): AK
	B	Marsh Cr.		
N3)	A	Spring Cr. (Salt)	Salt	private mtDNA (65%): CB
	A	Rock Cr.		this population was not included in Schwemm (2006), placement based on microsatellites alone
N4)		East Verde R.	Verde	private mtDNA (100%): DB
				rare nDNA (28%): I (S7)
N5)		Fossil Cr.	Verde	private mtDNA (100%): RA
Gila intermedia (I)				
I1)	A	East Fork Eagle Cr.	Gila	Sites share typical widspread *G. intermedia* sequences at mtDNA [AB; ave. 67% and/or AA; ave. 22%] except HCN fixed for AB, plus typical nDNA at S7 (A,B; ave. 82%) and Tpi-B (A; ave. 72%). Bonita Cr. shows widspread *G. robusta* mtDNA AG (60%) not common in
	A	upper Eagle Cr.		
	B	Bonita Cr.		
	C	Harden Cienega Cr.		
	D	Dix Cr.		this population was not included in Schwemm (2006), placement based on microsatellites alone
I2)	A	O'Donnell Cr.	San Pedro	Sites share unique San Pedro mtDNA haplotype FC across sites (30%,5%,100%,20%, respectively). Redfield contains a widespread mitotype AB (80%) not found elsewhere in this basin but common in the Gila River group.
	B	Bass Can.		
	C	Turkey Cr., AZ		
	D	Redfield Can.		

Table 5. continued.

management unit		river basin	rationale for distinctiveness
I2) Blue R.		Gila	private mtDNA (100%): AP,AS
I4) Cienega Cr.		Santa Cruz	private mtDNA (100%): JB
			private nDNA (85%): D (S7)
I5) Sabino Cr.		Santa Cruz	private nDNA (82%): F,G (S7)
I6) Sheehy Spr.		Santa Cruz	private mtDNA (100%): KM
I7) Silver Cr.		Agua Fria	geographical isolation
I8) Walker Cr.		Verde	Site fixed for mtDNA (100%): AG. This haplotype is not found in *G. intermedia* but is common in *G. robusta* and *G. nigra*. Geographical isolation.
I9) Williamson Valley Wash		Verde	private mtDNA (35%): AT,EB
I10) Spring Cr. (Verde)		Verde	private mtDNA (55%): BA,BG,HB,QG

Figure 1. Locality map for samples characterized in this study. Approximate sample locations are identified by symbols with shape and shading indicating species and drainage unit, respectively (see legends for detailed information). Locality data for specific locations is provided in Appendix 1.

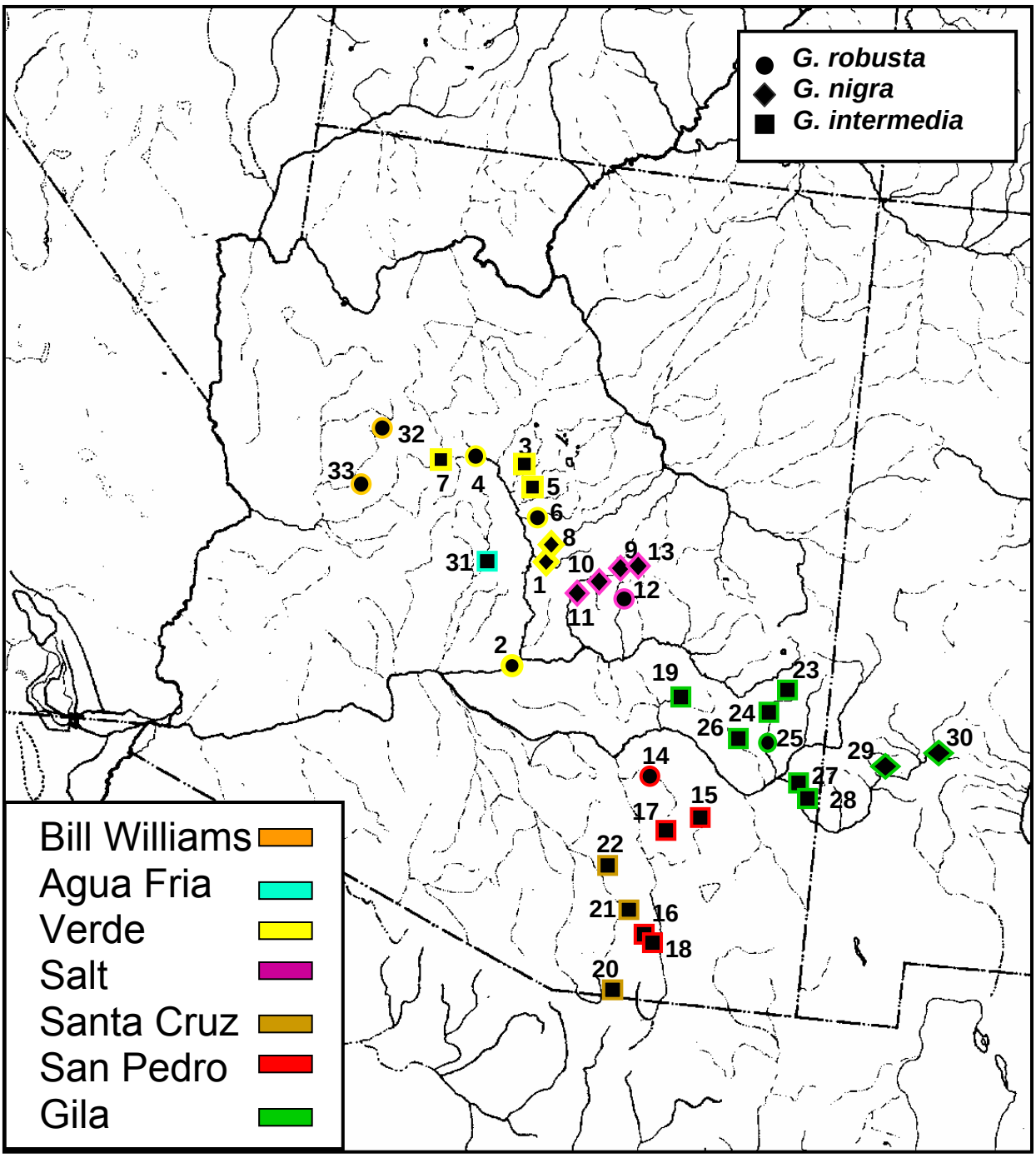


Figure 2. Example of an assignment probability plot, with probability of assignment (y-axis) to a specific group (identified by different colors) plotted for each individual (x-axis).

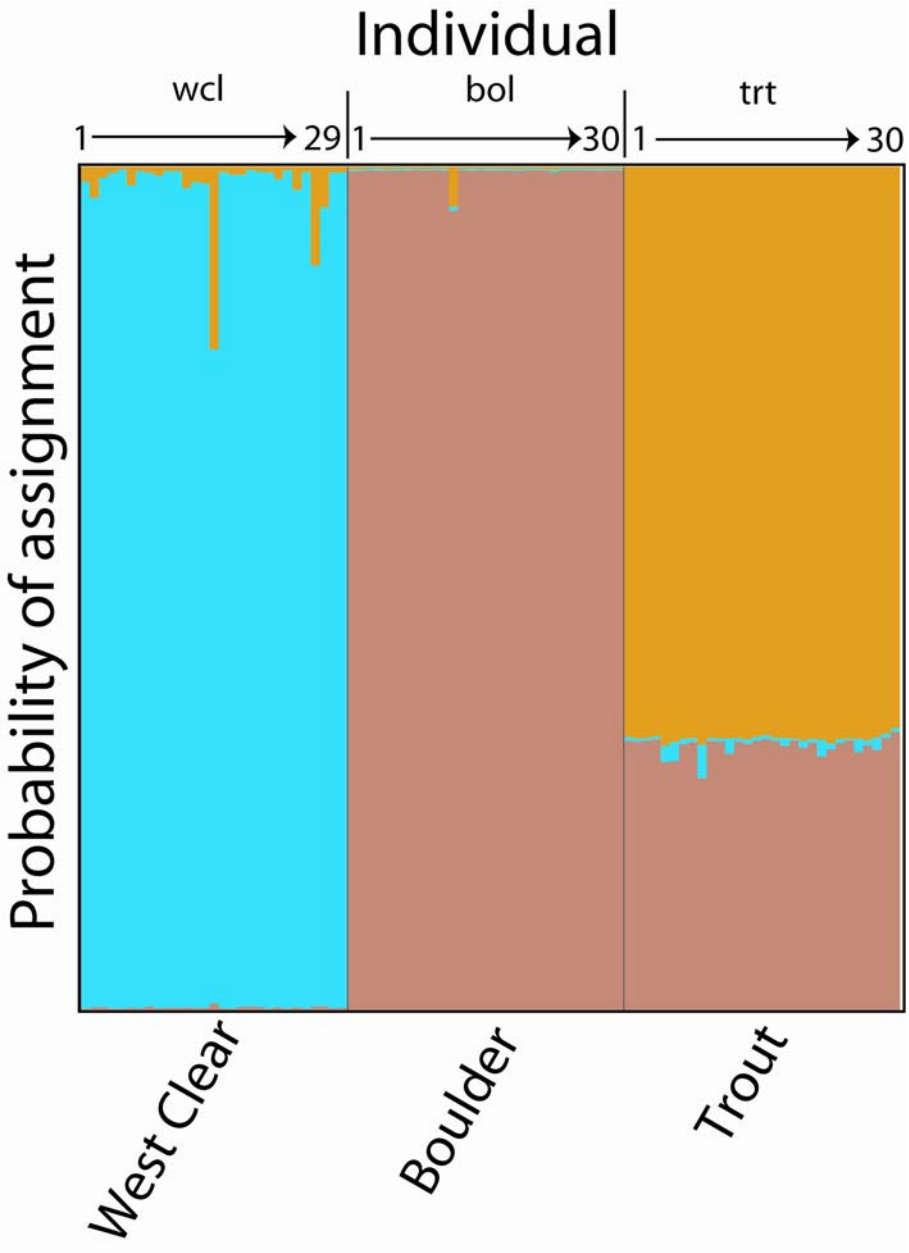


Figure 3. Assignment probability plots for all populations of *robusta*. "k#" indicates the assumed group size and "h′" the statistic measuring consistency across the 50 replicate runs at that value of *K*; see the text for additional explanation.

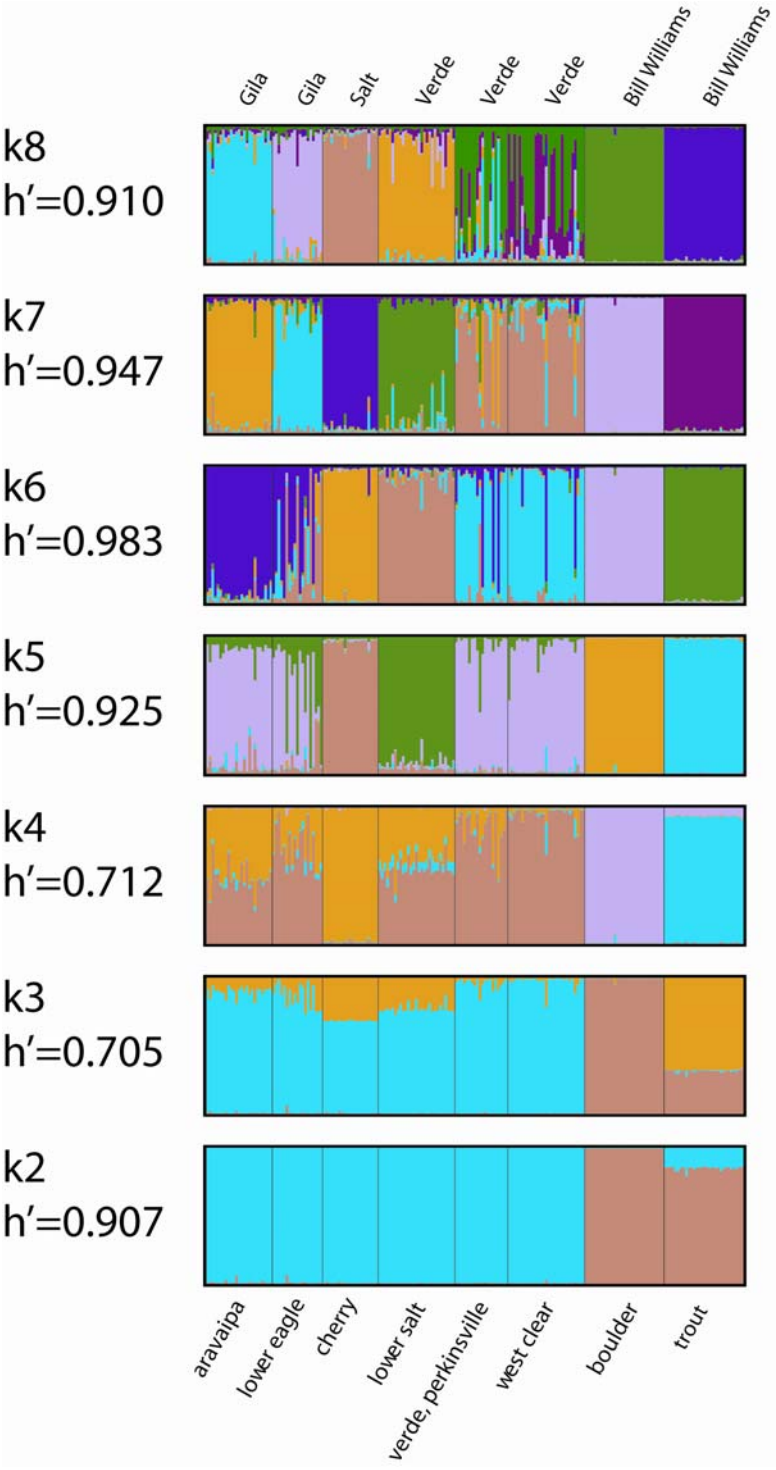


Figure 4. Hierarchical analysis of assignment probabilities for *robusta*. Scale for branch lengths is provided at the bottom. Individual samples are identified by their acronym (Table 2). To simplify the image, multiple individuals are grouped together (indicated by black triangles), with depth of triangle identifying maximum distance for the group.

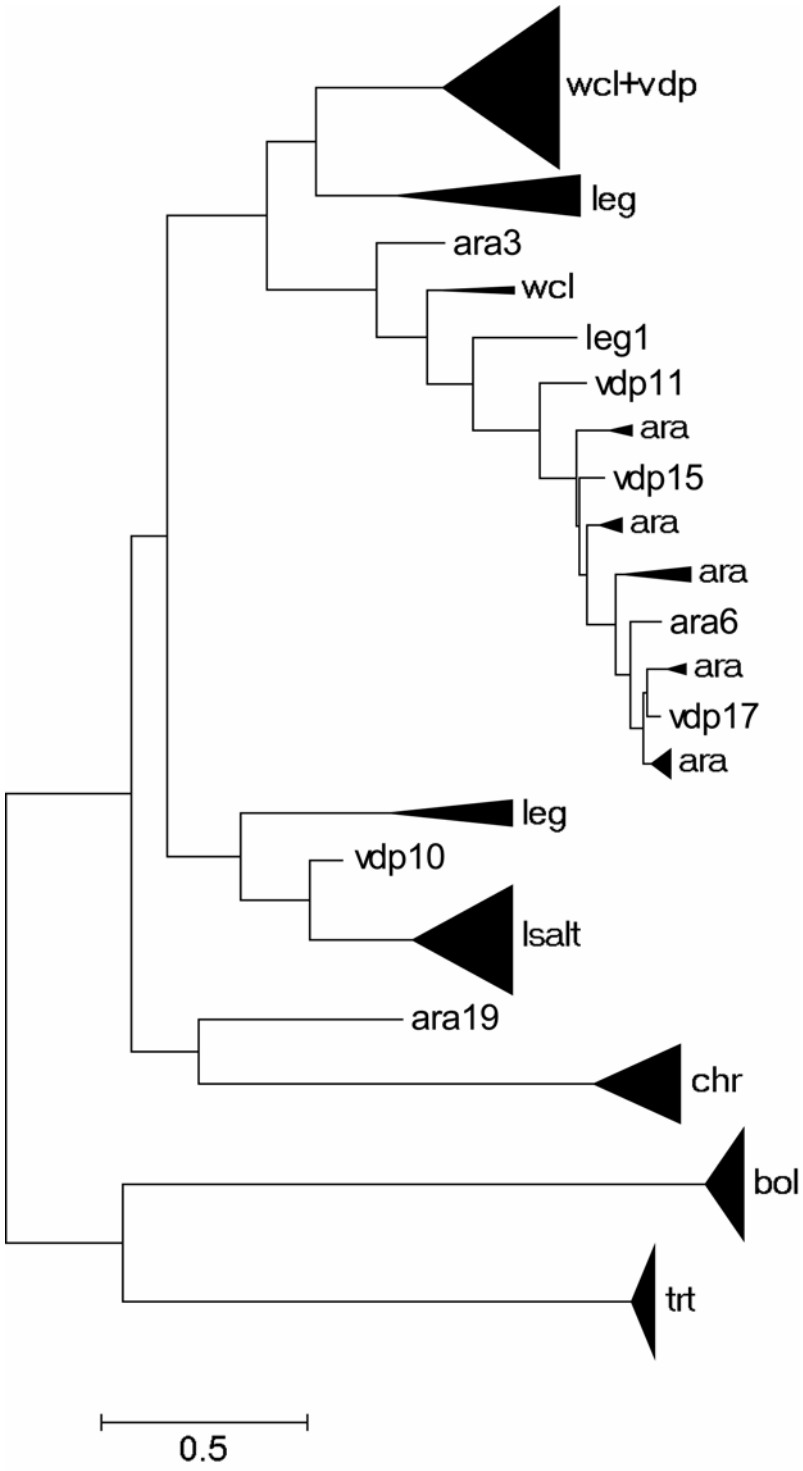


Figure 5. Assignment probability plots for all populations of *nigra*. "k#" indicates the assumed group size, the number after *K* indicates number of replicates included in the analysis, and "h′" the statistic measuring consistency across replicate runs at that value of *K*.

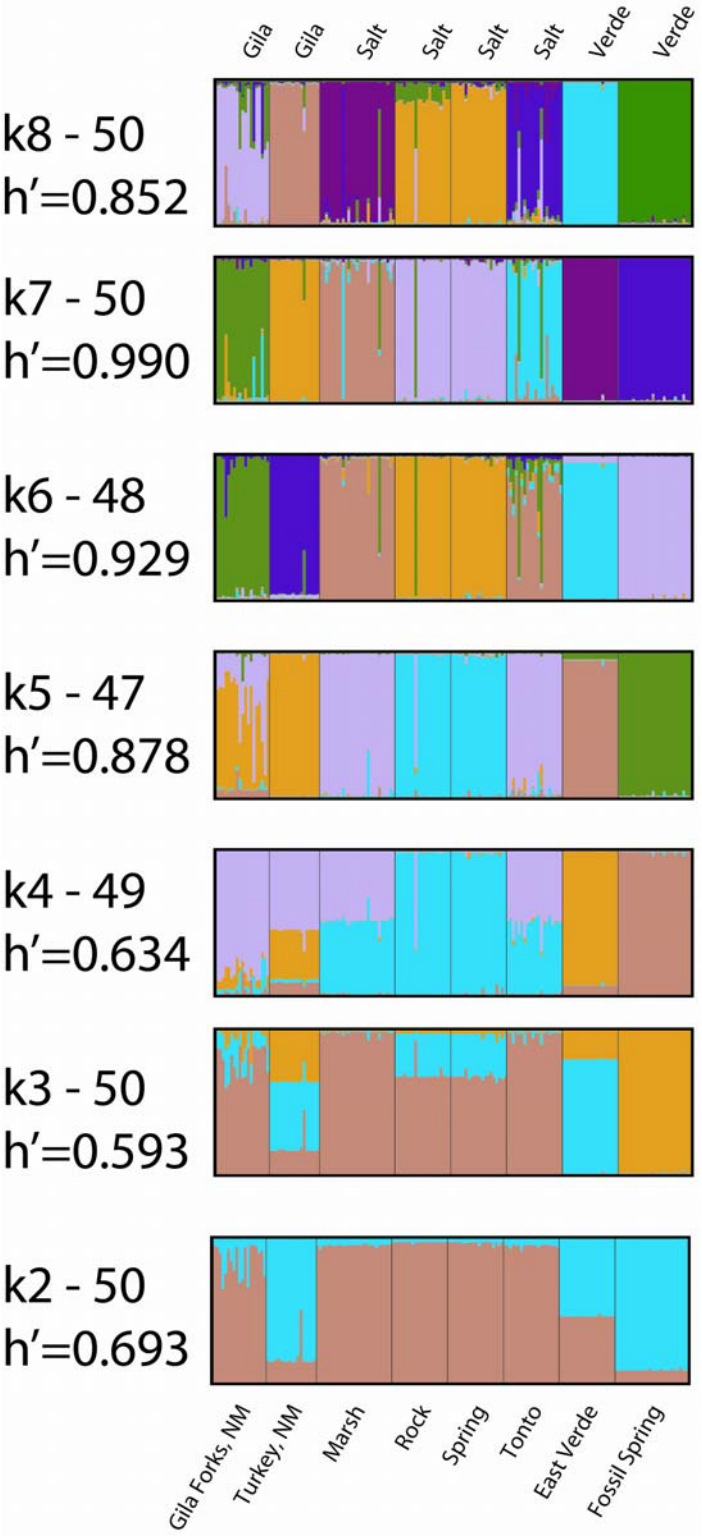


Figure 6. Hierarchical analysis of assignment probabilities for *nigra*. Scale for branch lengths is provided at the bottom. Individual samples are identified by their acronym (Table 2). To simplify the image, multiple individuals are grouped together (indicated by black triangles), with depth of triangle identifying maximum distance for the group. Samples are identified to drainage of origin with the exception of as few Salt River individuals that are interspersed in the Gila River group.

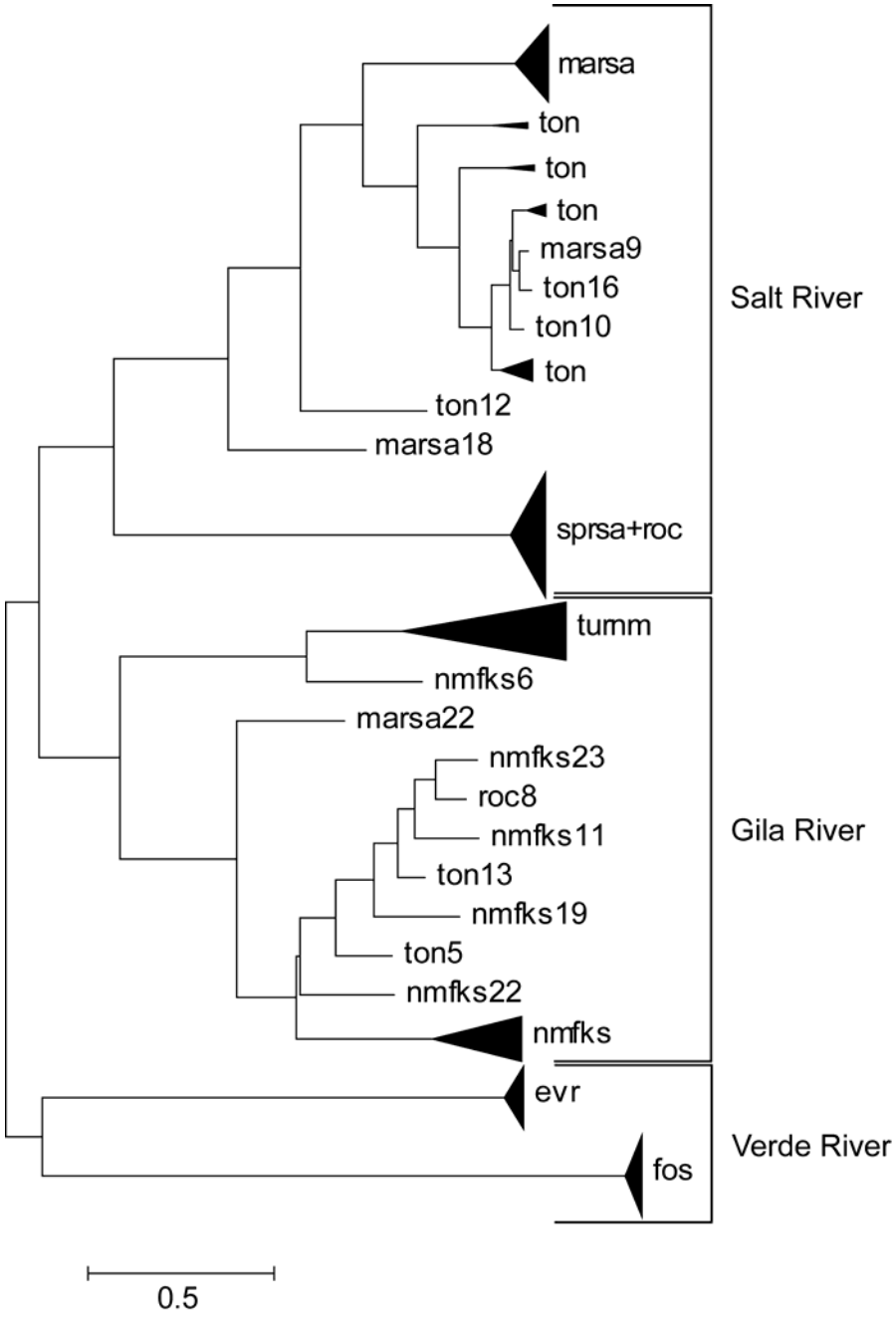


Figure 7. Assignment probability plots for all populations of *intermedia*. "k#" indicates the assumed group size, the number after *K* indicates number of replicates included in the analysis, and "h′" the statistic measuring consistency across replicate runs at that value of *K*.

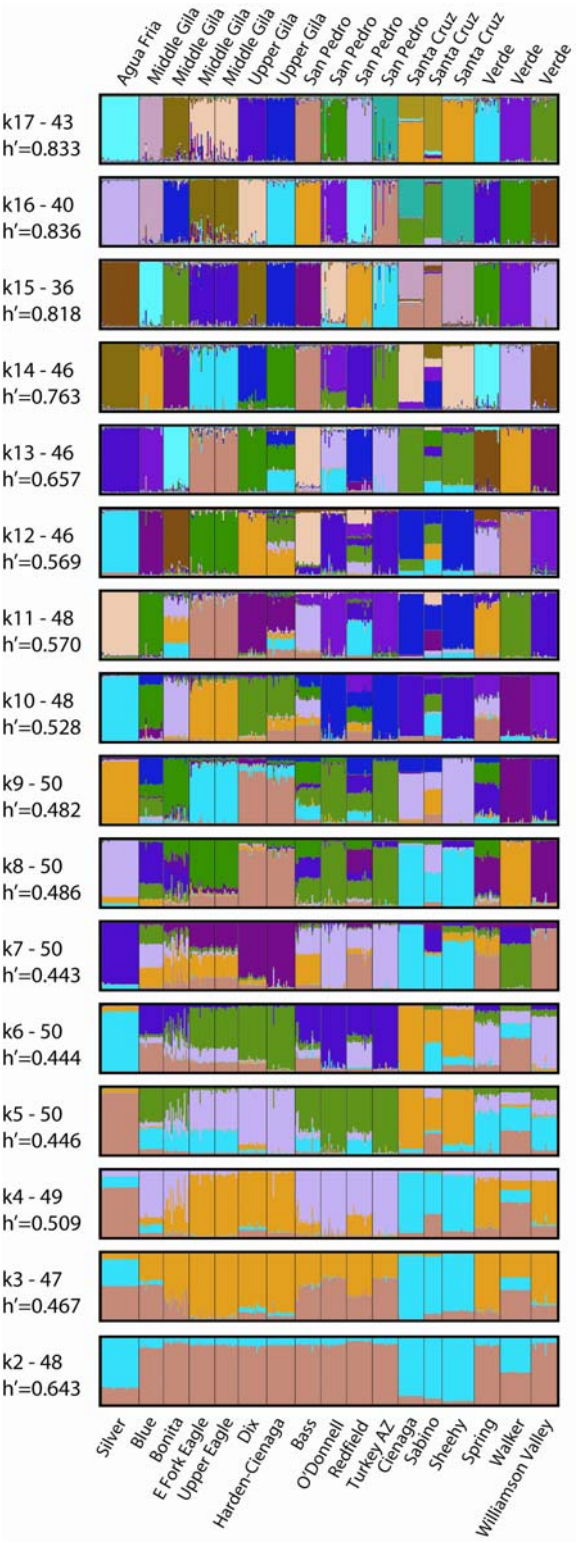


Figure 8. Hierarchical analysis of assignment probabilities for *intermedia*. Scale for branch lengths is provided at the bottom. Individual samples are identified by their acronym (Table 2). To simplify the image, multiple individuals are grouped together (indicated by black triangles), with depth of triangle identifying maximum distance for the group. Source drainage for each population is indicated at the right.

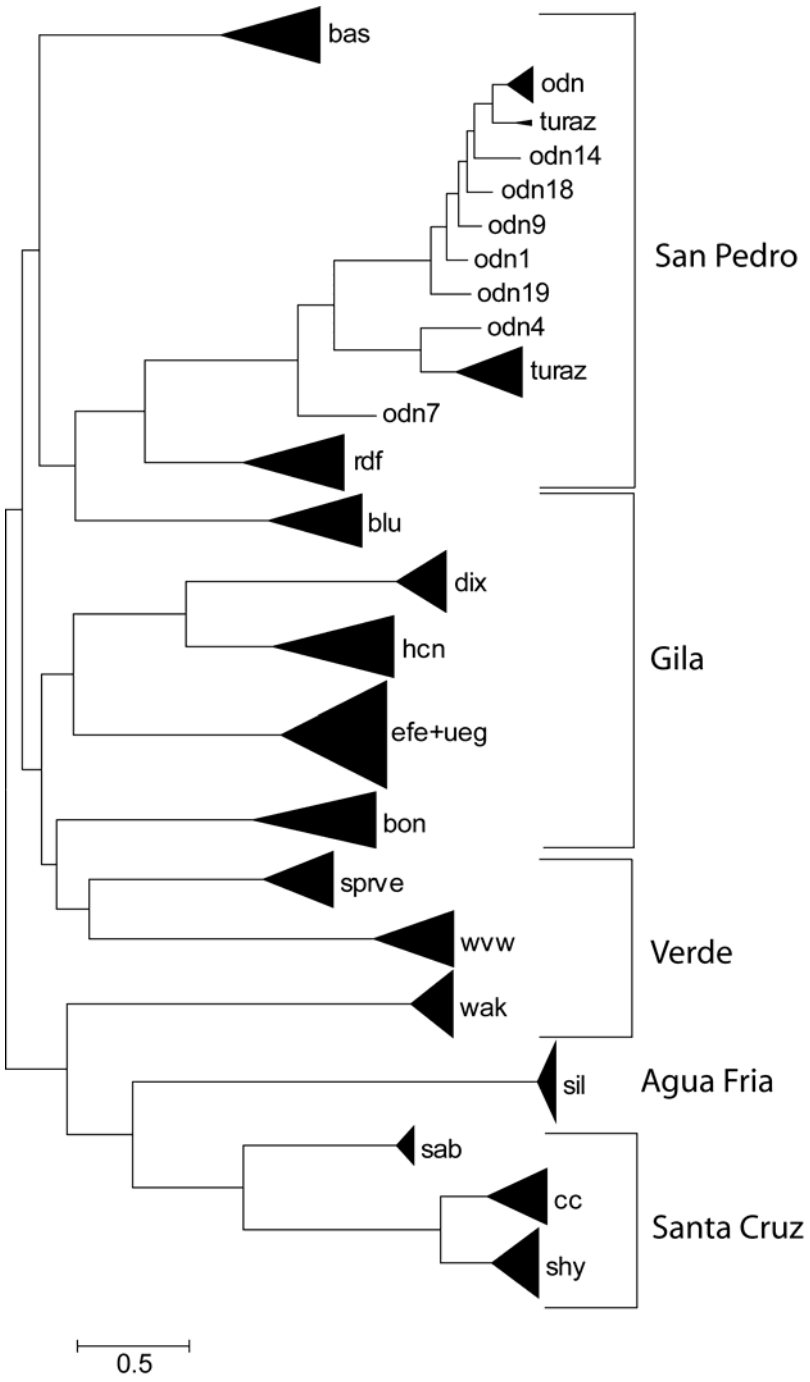


Figure 9. Assignment probability plots for all populations of all species. "k#" indicates the assumed group size, the number after *K* indicates number of replicates included in the analysis, and "h′" the statistic measuring consistency across replicate runs at that value of *K*.

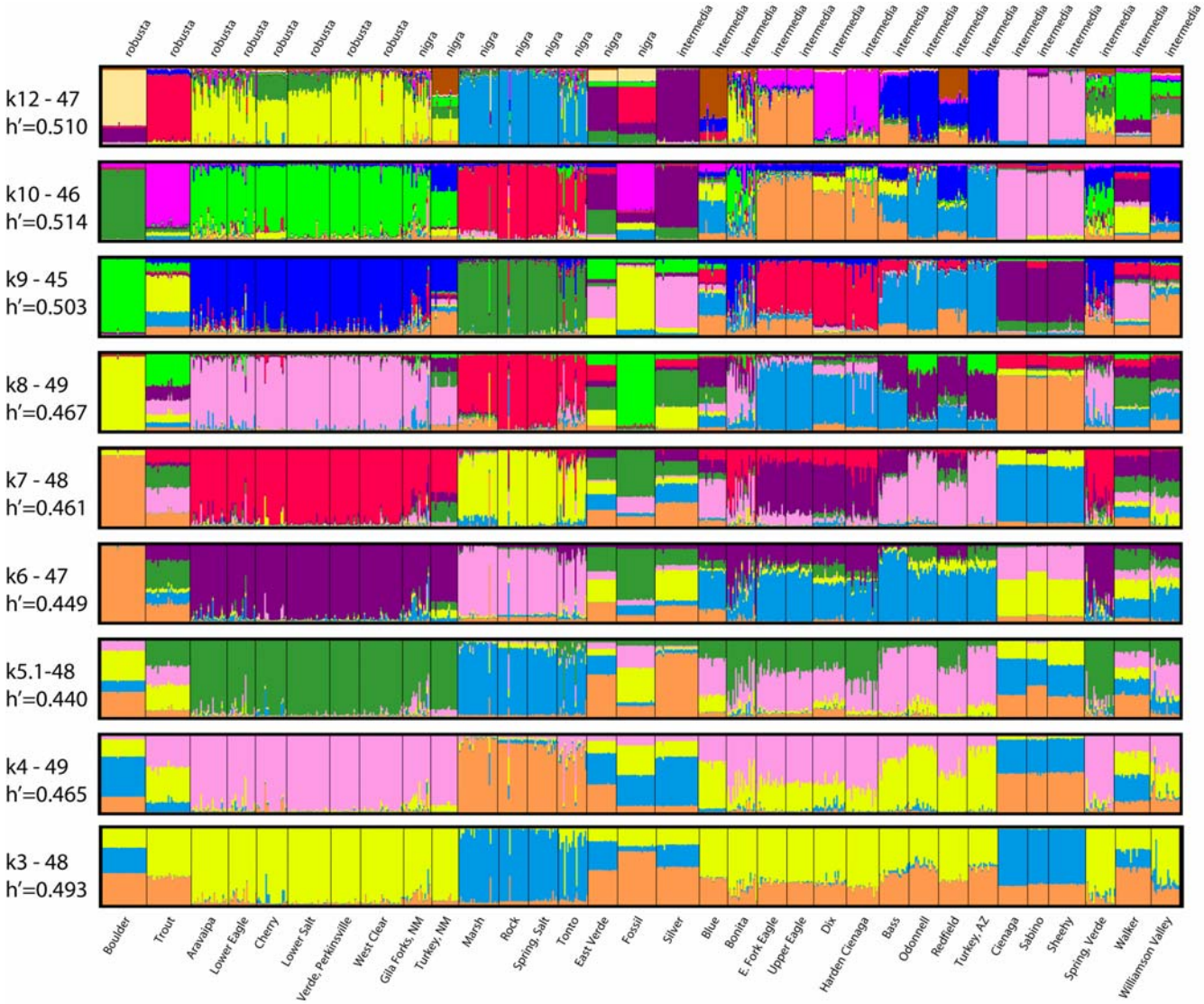


Figure 9. concluded.

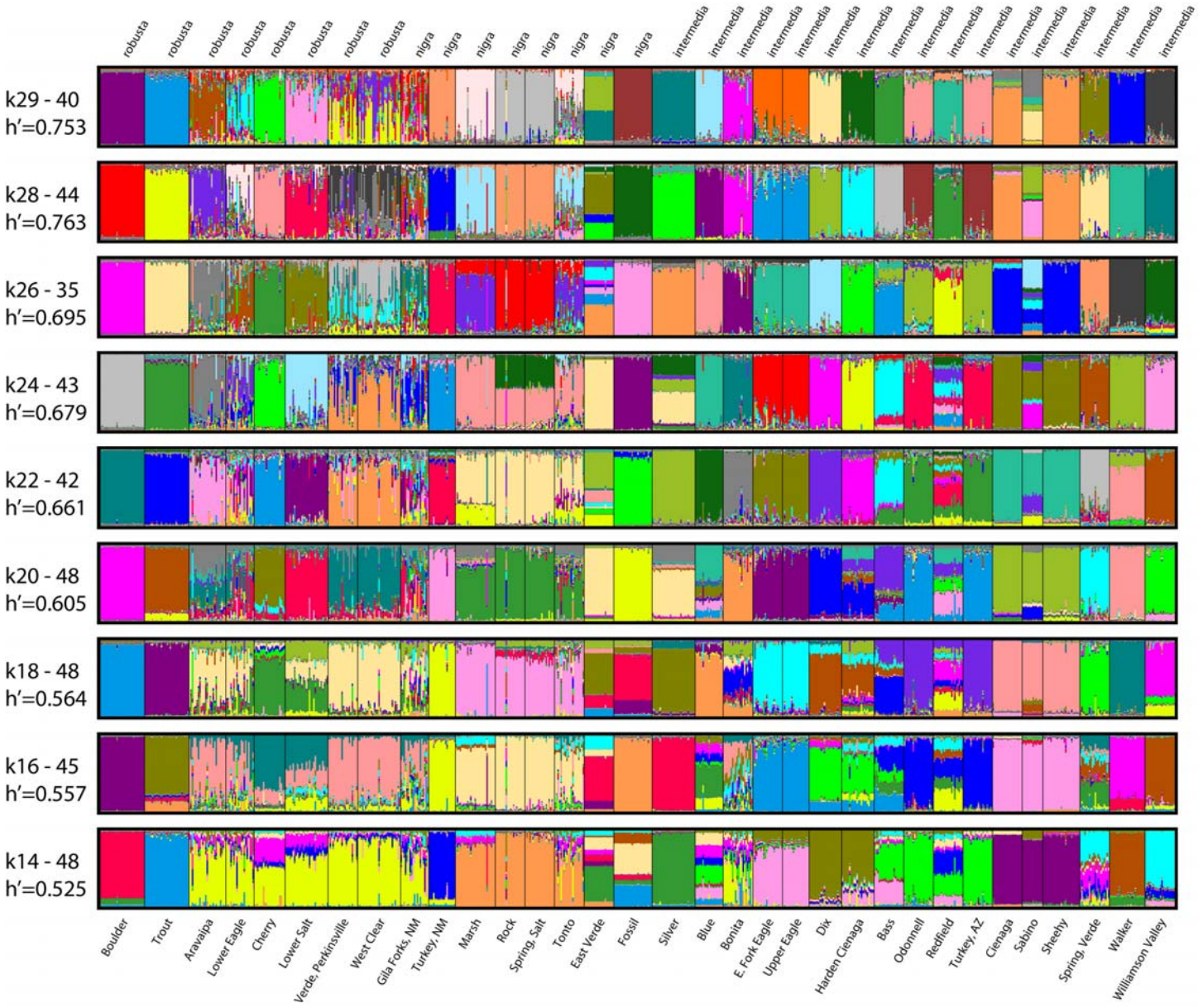
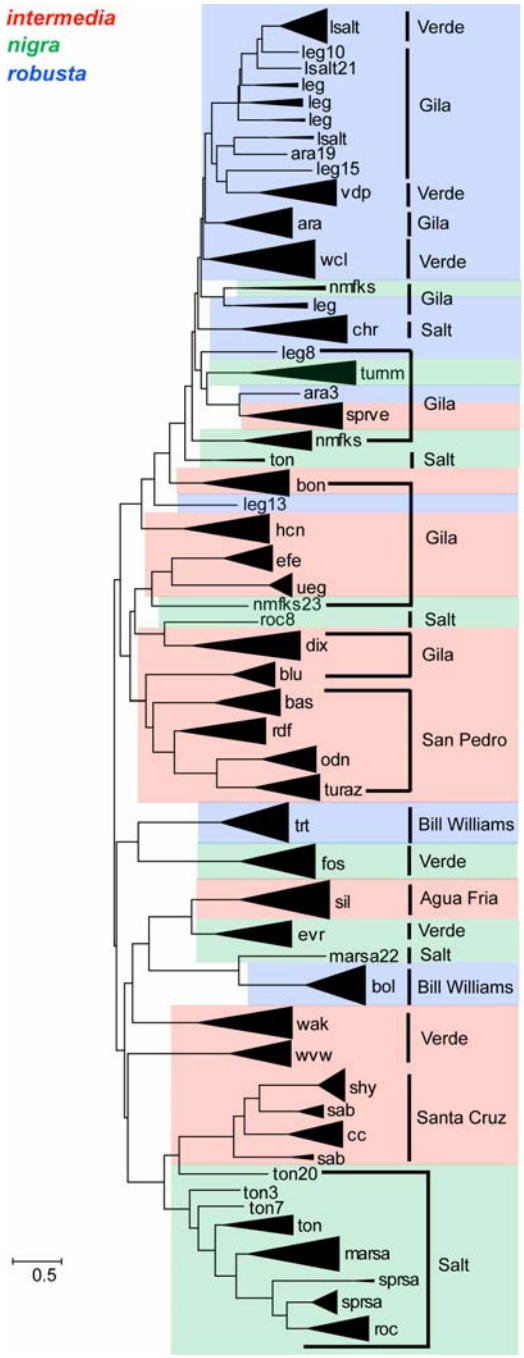


Figure 10. Hierarchical analysis of assignment probabilities for all populations. Scale for branch lengths is provided at the bottom. Individual samples are identified by their acronym (Table 2). To simplify the image, multiple individuals are grouped together (indicated by black triangles), with depth of triangle identifying maximum distance for the group. Source drainage for each population is indicated at the right; colors identify species (key in upper left corner).



Appendix 1. Locality data and groupings of samples for hierarchical analyses. Samples (abbreviations in parentheses) are arranged by drainage sub-basin of the Gila and Bill Williams rivers. All sub-basins are within the Gila River basin except for the Bill Williams River, which is a distinct tributary of the lower Colorado River. Taxonomic identity of sampled individuals follows Minckley and DeMarais (2000).

Verde River sub-basin

1. East Verde River (EVR), Gila Co., AZ [*G. nigra*; N=20]

2. Canal downstream from confluence Salt and Verde rivers (LSALT), Maricopa Co., AZ [*G. robusta*; N=29]

3. Spring Creek (SPRVE), Yavapai Co, AZ [*G. intermedia*; N=20]

4. Verde River at Perkinsville (VDP), Yavapai Co, AZ [*G. robusta*; N=20]

5. Walker Creek (WAK), Yavapai Co, AZ [*G. intermedia*; N=24]

6. West Clear Creek (WCL), Yavapai Co, AZ [*G. robusta*; N=29]

7. Williamson Valley Wash (WVW), Yavapai Co, AZ [*G. intermedia*; N=20]

8. Fossil Creek (FOS), Yavapai Co, AZ [*G. nigra*; N=26]

Salt River sub-basin

9. Marsh Creek (MAR), Gila Co., AZ [*G. nigra*; N=27]

10. Spring Creek (SPRSA), Gila Co., AZ [*G. nigra*; N=20]

11. Tonto Creek (TON), Gila Co., AZ [*G. nigra*; N=16]

12. Cherry Creek, (CHR), Gila Co., AZ [*G. robusta*; N=21]

13. Rock Creek (ROC), Gila Co., AZ [*G. nigra*; N=20]

Appendix 1. continued.

San Pedro River sub-basin

14. Aravaipa Creek (ARA), Pinal Co., AZ [*G. robusta*; N=24]

15. Bass Canyon (BAS), Cochise Co., AZ [*G. intermedia*; N=20]

16. O'Donnell Canyon (ODN), Santa Cruz Co., AZ [*G. intermedia*; N=20]

17. Redfield Canyon (RDF), Pima Co., AZ [*G. intermedia*; N=20]

18. Turkey Creek (TURAZ), Santa Cruz Co., AZ [*G. intermedia*; N=18]

San Carlos River sub-basin

19. Blue River (BLU), Gila, Co., AZ [*G. intermedia*; N=19]

Santa Cruz River sub-basin

20. Sheehy Spring (SHY), Santa Cruz Co., AZ [*G. intermedia*; N=25]

21. Cienega Creek (CC), Pima Co., AZ [*G. intermedia*; N=20]

22. Sabino Creek (SAB), Pima Co., AZ [*G. intermedia*; N=14]

Gila River sub-basin, middle section

23. East Fork Eagle Creek (EFE), Greenlee Co., AZ [*G. intermedia*; N=20]

24. Eagle Creek - upper (UEG), Greenlee Co., AZ [*G. intermedia*; N=18]

25. Eagle Creek- lower (LEG), Greenlee Co., AZ [*G. robusta*; N=20]

26. Bonita Creek (BON), Graham Co., AZ [*G. intermedia*; N=20]

Appendix 1. concludued.

Gila River sub-basin, upper section

27. Harden-Cienega Creek (HCN), Greenlee Co., AZ [*G. intermedia*; N=22]

28. Dix Creek (DIX), Greenlee Co., AZ [*G. intermedia*; N=22]

29. Turkey Creek (TURNM), Grant Co., NM [*G. nigra*; N=18]

30. East, Middle and West Forks Gila River (NMFKS), Catron Co., NM [*G. nigra*; N=19]

Agua Fria River basin

31. Silver Creek (SIL), Yavapai Co., AZ [*G. intermedia*; N=29]

Bill Williams River basin

32. Trout Creek (TRT), Mohave Co., AZ [*G. robusta*; N=30]

33. Boulder Creek (BOL), Yavapai Co., AZ [*G. robusta*; N=30]