FINAL REPORT

TO: U. S. Fish and Wildlife Service

PROJECT: Cooperative Agreement No. 201814J826
Gila River Basin Native Fish Conservation Program, Tasks 3-65, 3-68, 3-70
Project CA-3-65

TITLE: Pupfish Genetics:
Genetic Structure of Wild and Refuge Stocks of Desert Pupfish

BY: A. A. Echelle, D. Loftis, H. Koike, and R. A. Van Den Bussche
Oklahoma State University

DATE: 18 December 2007

OBJECTIVES

1. Assess genetic status of desert pupfish refuge populations, using wild populations as a reference
2. Develop management protocols for exchange of genetic material

SUMMARY

This report on the genetic structure of wild and refuge stocks of the federally endangered Desert Pupfish complex (*C. macularius* and *C. eremus* is in three sections: Part I, genetic structure of wild populations, Part II, genetic status of refuge populations, and Part III, management protocols for refuge populations.

Stocks of the Desert Pupfish complex have been maintained in semi-natural refuges in a variety of private and public facilities since the 1970s. In the 30+ years of the refuge program for the Desert Pupfish complex, the lineages have been managed independently of one another and of the wild populations. Some refuge stocks have been supplemented by fish from other populations of the same refuge lineage and one refuge population (Boyce Thompson Arboretum = BT) was initiated with fish from two separate lineages. There has been only one recorded inoculation of a refuge population with wild-caught fish, and, except for BT, there have been no mixtures of lineages.

For wild populations (Part I), we assayed microsatellite DNA loci (7 for each species) in each of 11 populations in California, Sonora, and Baja California del Norte, and compared the results with a previous mtDNA analysis on the same populations. For refuge populations (Part II), we assayed for variation at four microsatellite DNA loci in each of the two species of the complex. This included 30 of the 45 or so extant refuge populations, including nine lineages (24 populations; one sampled in two separate years) of *C. macularius* and four lineages (6

populations) of *C. eremus*, where a lineage is a group of population descended from a single translocation from the wild. Part III presents some generalizations from population genetics that we used for insights into a program of genetic exchange among refuge populations.

Management recommendations include establishment of at least four large, well-managed "primary-refuge" populations each representing one of four groups of wild populations, two for *C. macularius* (lower Colorado River delta and Salton Sea area) and two for *C. eremus* (Río Sonoyta and Quitobaquito Springs). These primary populations would be continually maintained at large sizes, preferably 2000 adults or more, and (as long as wild stocks are available) they would periodically receive "migrants" via artificial inoculation with fish from the wild-source regions. The diversity in each of these core populations would then be dispersed into a second set of "secondary" populations via a program of artificial gene flow at an average rate of about 1-2 immigrants per generation for each population. Collectively, these secondary populations serve two important roles: (1) they function as a safeguard against severe losses of diversity in the global refuge population should catastrophic loss occur in the primary refuge, and (2) they function to inflate the effective population size of the global refuge population to levels that should preserve evolutionary adaptability.

Results: Part I—Wild Populations

- The results from microsatellite DNA variation (7 loci each for species) for wild populations were consistent with a previous mtDNA survey in supporting management of the complex as two separate species.
- For *C. macularius*, small, but statistically significant proportions of diversity was attributable to differences between populations in the Salton Sea area and those on the lower Colorado River delta: 0.8% for microsatellites and 3.7% for mtDNA.
- For *C. eremus*, 10% of the microsatellite variation and 2.9% of the mtDNA variation was attributable to differences between the Quitobaquito Springs and Río Sonoyta populations.

Results: Part II—Refuge Populations

- There have been large declines in genetic diversity in local refuge populations compared with the wild populations.
- However, global diversity of the refuge programs for the two species was within the range of variation in wild populations.
- The global effective population size (N_e) of the refuge program between the present and the time of the original translocations from the wild was 742 (95% *CI* = 477-1156) for *C. eremus* and 1059 (*CI* = 777-1404) for *C. macularius*.
- The point-estimates of global N_e were moderately greater than the minimum ($N_e = 500$) recommended for long-term preservation of evolutionary adaptability.

Results: Part III—A Suggested Protocol Of Genetic Exchange

The following is a condensation of the recommended protocol for managing refuge populations.

- Establish primary-refuge populations or identify such populations among existing refuges.
    - One for each of the following wild populations of *C. eremus*:

        - Río Sonoyta
        - Quitobaquito Springs
    - One for each of the following groups of wild populations of *C. macularius*.
        - Colorado River Delta (El Doctor/Santa Clara Slough/Laguna Salada/Cerro Prieto)
        - San Felipe Creek/Salton Sea area
    - Manage to maintain populations at more than 2000 adults
    - Assure diversity in the primary populations
- Monitor population sizes and genetic diversity in the primary-refuge populations
    - Census adult population size annually
    - Assay genetic diversity
- Periodically inoculate primary refuges with fish from the wild
    - Aim for an average of 2-8 adult "immigrants" per generation per primary refuge
    - Use larger numbers if transplantations occur at longer intervals
    - Bias immigrant sex ratio toward females, say 2:1 or greater.
    - Use fish from the same region (not necessarily the same locality--see 3.4) that served as the original wild-source for the refuge population
    - Avoid repeated inoculation with fish from the same wild locality
    - Assure high diversity in the transplanted fish.
    - Avoid unwanted transplants, particularly non-native species and disease organisms.
- Establish and/or maintain a set of 10 or more secondary-refuge populations representing the source-region for each primary-refuge population.
    - Maintain populations at 100 adults or more.
    - For new refuges, use as founders, a minimum of 100 fish from the primary-refuge population or from the wild source of that population.
    - Periodically inoculate the secondary populations with fish from the corresponding primary-refuge population.
        - Aim for an average of 1-2 migrants per generation (2-4/year) for each refuge.
        - For extremely small populations (<100) that have not been inoculated for several years, remove a moderate proportion of the population, say 10% or so, and replace with an equivalent number of migrants.
        - For existing secondary populations with low diversity, destroy and re-establish, or remove a large proportion (>50%) and replace with an equivalent number of migrants.
        - For guidance with respect to the existing refuges, "Low diversity" is arbitrarily defined in this report as <70% of the allele richness of the wild populations (12.5 alleles/locus/population in this study).
    - Avoid unwanted transplants, particularly non-native species and disease organisms

CONTENTS

.

PART I: GENETIC STRUCTURE OF WILD POPULATIONS

INTRODUCTION

Conservation of native, non-game fishes has been said to have begun with Carl Hubbs' campaign in the 1940s to protect the Devil's Hole Pupfish, *Cyprinodon diabolis*, and its habitat (Deacon and Williams 1991). Since then, and despite extensive legislation and management action aimed at conservation, native fish in the American Southwest, including pupfishes (Minckley et al. 1991; Echelle et al. 2003), continue to decline as a result of anthropogenic habitat alteration and introductions of nonnative fishes (Minckley and Deacon 1991; Contreras and Lozano 1994). One of the most dramatic declines has been the status of the Desert Pupfish complex, a once wide-ranging group that has disappeared from perhaps 95% of its historical range in the past several decades (Miller and Fuiman 1987; Hendrickson and Varela 1989). This complex has arguably received more attention from conservation managers than any other non-game species of fish (Minckley et al. 1991).

In this paper, we use variation at microsatellite loci to assess genetic structure of the remaining wild populations of the Desert Pupfish complex. The complex comprises two federally endangered, allopatric species, the Desert Pupfish, *C. macularius*, and the Sonoyta Pupfish, *C. eremus*. *Cyprinodon macularius* historically ranged from Gila River tributaries in southeastern Arizona and northern Sonora, Mexico, westward to the Salton Sea area of southern California and southward into the Colorado River Delta region of Sonora and Baja California (Miller 1943). *Cyprinodon eremus* is restricted to Quitobaquito Springs in Organ Pipe Cactus National Monument, southern Arizona (Miller and Fuiman 1987) and a short reach of the Río Sonoyta, northern Sonora (Hendrickson and Varela-Romero 1989; Varela-Romero et al. 2002). Because of dramatic population declines, *C. macularius* and *C. eremus* are listed (under the name *C. macularius*) as endangered by United States (Federal Register 51(61):10842) and Mexico (http://www.conabio.gob.mx/conocimiento/ise/fichasnom/).

The prognosis for the existing wild populations of the Desert Pupfish complex is poor (Dunham and Minckley 1998). Consequently, state and federal agencies have established more than 40 populations in quasi-natural refuges ranging in surface area from ~3 m^2 to ~6000 m^2 in facilities as diverse as a national fish hatchery, national wildlife refuges, public schools and universities, state parks, and private zoos and arboretums in California, Arizona, New Mexico, and Sonora (Dunham and Minckley 1998; Koike, 2007). Two important purposes of such stocks are to protect genetic resources against catastrophic loss of natural populations, and to provide stocks that might eventually be used for reintroductions into the historical ranges of the two species. To serve these purposes, captive populations should be managed to preserve, as closely as possible, the original genetic diversity of the species. Thus, there is a need for documentation of the pattern of diversity in wild populations.

Previous studies of genetic variation in natural populations of the Desert Pupfish complex consist of allozyme surveys of populations in the United States (Turner 1983; Dunham and Minckley 1998) and a mitochondrial DNA (mtDNA) survey throughout the range of the complex,

including the known populations in Mexico (Echelle et al. 2000). In this paper, we provide a survey of microsatellite DNA variation in the samples assayed by Echelle et al. (2000).

Our purposes are to (1) compare the pattern of microsatellite DNA variation with results from previous studies of genetic variation in the Desert Pupfish complex, (2) use the results as a basis of recommendations for genetic management of the complex, and (3) revaluate the species-level taxonomy of the complex. Miller and Fuiman (1987) described *C. m. eremus* as a subspecies endemic to Quitobaquito Springs, southern Arizona, with the nearby Río Sonoyta population (Sonora, Mexico) being somewhat intermediate between that subspecies and the remainder of *C. macularius*. Subsequently, on the basis of mtDNA variation and color pattern in breeding males, Echelle et al. (2000) elevated the subspecies names to species status, with *C. eremus* comprising populations in the Río Sonoyta and Quitobaquito Springs and *C. macularius* comprising all other populations of the complex. The pattern of microsatellite DNA variation presented herein provides a nuclear DNA perspective on this taxonomy.

RECENT HISTORY OF THE COMPLEX

Construction of upstream dams and water diversions on the Colorado and Gila rivers has eliminated most of the flow that once supported an expansive system of shallow-water pupfish habitats on river floodplains and the Colorado River delta at the head of the Gulf of California (Hendrickson and Varela-Romero 1989; Mueller and Marsh 2002). Elsewhere, including the Río Sonoyta in Sonora, and tributaries of the Gila River in Arizona, overgrazing by livestock and other anthropogenic factors caused extensive erosion, entrenching channels and drying the shallow marshy habitats occupied by the species (Minckley 1973; Miller and Fuiman 1987).

Unfortunately, historical records of the distribution and abundance of *C. macularius* are sparse, making it difficult to compare past and present distributions (Hendrickson and Varela-Romero 1989). Regardless, collecting records starting in the 1940's (Miller 1943) indicate a declining trend in distribution and abundance (Varela-Romero et al. 2002), with *C. macularius* extirpated from as much as 95% of its historical range (Hendrickson and Varela-Romero 1989, Varela-Romero et al. 2002).

The history of the Salton Sea includes an irregular cycle of inundation by overflow from the Colorado River, followed by gradual desiccation when the river flow returned to the Gulf of California. According to Carpelan (1961), the river overflowed into the Salton Sink seven times between 1849 and 1907. The present Salton Sea originated in 1905-1907 when, during a period of high flow, the Colorado River emptied into the Salton Sink, an event that would have inundated springs and marshes supporting *C. macularius* (Miller and Fuiman 1987). In the early 1960s the pupfish was abundant in shoreline pools of the Salton Sea (Barlow, 1961). Now, largely because of competition with non-native fishes (Moyle 2002), populations are sparse, but they still occur along the shoreline of the Salton Sea and in San Felipe and Salt creeks, various irrigation canals and drains, and San Sebastian marsh at the southwestern corner of Salton Sea (Martin and Saiki 2005).

The population of *C. eremus* at Quitobaquito Springs consists of several thousand adults (Douglas et al. 2001) in a remote, springfed habitat in the federally protected Organ Pipe Cactus National Monument. The flow from the springs has been declining since the 1970s, and, in September and October of 2007, apparently because of a leak in the dam or elsewhere, the water depth in the receiving pond declined dramatically from 75 cm and then stabilized at about 10 cm (T. Tidditts, pers. comm.). Persistence of the nearby (~2 km) Rio Sonoyta population is tenuous because the short reach of stream presently occupied is subject to desiccation during drought (C. O. Minckley, pers. comm.). Miller and Fuiman (1987:603) concluded from fossil spring deposits that the two populations probably had been in permanent contact at "some time in the Holocene and certainly during Pleistocene and earlier times." They wrote that "field reconnaissance" contradicted Miller's original assumption that Quitobaquito Springs and the the Río Sonoyta shared a direct water connection sometime within the past 100 yr.

MATERIALS AND METHODS (Part 1)

Sample acquisition.—DNA samples used in this study were those used by Echelle et al. (2000) from collections made in 1997 and 1998 at 11 sites (n = 18-25, avg. = 20.7) representing all known wild populations of *C. macularius* and *C. eremus* (Fig. 1; Appendix A).

PCR amplification.—We assayed 10 microsatellite loci in this analysis, nine (listed below) from Burg et al. (2002; listed below), and one (WSP-02) from Stockwell et al. (1998). Microsatellites were amplified via polymerase chain reaction (PCR) using a standard protocol (15-µl reactions consisting each of 9.0 µl True Allele PCR Premix (Applied Biosystems Inc.), 3.8 µl ddH_2O, 1.0 µl each of 5.0 µM primer pairs, and 1.2 µl template DNA). All PCR reactions were carried out using either an MJ Research PTC 100 Thermal Cycler, or a Perkin Elmer 9600 Thermal Cycler using a two-step annealing process. Annealing temperatures (T_{A1} and T_{A2}) are given in Appendix B. Reaction conditions for the PTC 100 consisted of one cycle at 95°C for 12 minutes; five cycles of 94°C for 30 s, T_{A1} for 30 s, 72°C for 30 s; 32 cycles of 94°C for 30 s, T_{A2} for 30 s, 72°C for 20 s; and one cycle at 72°C for 2 minutes. Reaction conditions for the PE-9600 consisted of one cycle at 95°C for 12 minutes; five cycles of 94°C for 30 s, T_{A1} for 30 s, 72°C for 30 s; 35 cycles of 94°C for 45 s, T_{A2} for 45 s, 72°C for 1 minute; and one cycle at 72°C for 2 minutes. Each primer pair was end-labeled with a fluorescent dye as follows: GATA2, 6-FAM; GATA5, HEX; GATA9, NED; GATA10, 6-FAM; GATA26, HEX; GATA39, NED; GATA73, HEX (reverse primer); CmD1, NED; CmD16, 6-FAM; WSP-02; HEX).

Genotyping and data analysis. —Samples were genotyped on an Applied Biosystems Inc. Prism 377 or 3130 automated DNA sequencer. Electropherogram processing was performed with Genescan (version 3.1) and alleles were manually assigned using Gentotyper (version 2.5). At least one previously genotyped sample was included with nearly every set of samples to identify machine drift in output of allele sizes.

MICRO-CHECKER (version 2.2.0.3; Van Oosterhout et al. 2004) was used to identify genotyping errors due to null alleles, short allele dominance, and the scoring of stutter peaks. Brookfield's (1996) Method I was used to estimate null allele frequencies. We used GENEPOP (version 3.4; http://genepop.curtin.edu.au) to test for linkage disequilibrium and deviation from

Hardy-Weinberg Equilibrium (exact test; Guo and Thompson 1992). We used GDA (Lewis and Zaykin 2001), GENEPOP, and CONVERT (Glaubitz 2004) to compute observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles (A), allele richness (A_R), and number of private alleles (A_P). The sequential Bonferroni correction (Rice 1989) was used to reduce Type I errors for multiple tests applied to the same hypothesis.

We used ARLEQUIN (version 2.001; Schneider et al. 2000) for analysis of molecular variance (AMOVA; Excoffier et al. 1992) and to compute pairwise F_{ST} and R_{ST} values among subpopulations (Raymond and Rousett, 1995). We also ran AMOVAs on mtDNA sequence data from Echelle et al. (2000).

We used SPAGeDi (Hardy and Vekemans 2002) for an allele-size permutation test (5000 iterations) to indicate whether differences in microsatellite allele size (mutation) contributed to genetic divergence ($R_{ST} > F_{ST}$) or whether divergence can be attributed to genetic drift alone. For microsatellites, R_{ST} is the appropriate measure of divergence when $R_{ST} > F_{ST}$; the appropriate measure is F_{ST} when $R_{ST} \leq F_{ST}$ (Hardy and Vekemans 2002).

We used GENEPOP to perform a Mantel test of correlation between mtDNA and microsatellite divergence among populations (Echelle et al. 2000). Pairwise Φ_{ST} (mtDNA) and R_{ST} (microsatellites) values were used in the test across both species. In the test for *C. macularius* alone, we used F_{ST} for both mtDNA and microsatellites. To test for isolation-by-distance in *C. macularius* we used the ISOLDE program in GENEPOP (10,000 permutations) and tested for association between Slatkin's linearized F_{ST} estimates ($F_{ST}/(1-F_{ST})$) and the natural logarithm of geographic distances. .

The neighbor-joining algorithm in MEGA 3.1 (Kumar et al. 2004) was used to summarize pairwise genetic divergences based on microsatellites (5 loci; R_{ST}) and mtDNA (Φ_{ST}) across all populations of the two species. We used multidimensional scaling in SYSTAT (version 10, SPSS Inc.) to visualize among-population divergences (F_{ST}) for the seven loci assayed in *C. macularius*.

RESULTS (Part I)

The ranges in allele sizes are given in Appendix B. One locus (GATA73) was removed from the analysis because of consistent evidence of null alleles at frequencies >0.15. It is worth noting, however, that GATA73 amplified in all except two specimens of *C. eremus* (both in Quitobaquito Springs), but did not amplify in *C. macularius* (185 specimens from 9 localities were tested). Similar results were obtained in a subsequent analysis of captive populations (H. Koike, pers. comm.). Therefore GATA73 amplification appears effectively diagnostic of the two species.

Nine loci were retained in the analysis (GATA2, GATA5, GATA9, GATA10, GATA26, GATA39, CmD1, CmD16, and WSP-02). Two, GATA10 and GATA26, were not used for *C. macularius* because of consistent null-allele frequencies ≥ 10%, and two other loci, GATA2 and GATA5, were not used for *C. eremus* because of, respectively, inconsistent scoring and large

heterozygote deficiencies. Removing two loci for each species left seven loci for intraspecific analyses and five for analyses that included both species.

There was no evidence of linkage disequilibrium (i.e., nonrandom association among alleles of different loci) and with the Bonferroni correction (2, 9, or 11 populations per locus; initial P for significance, respectively, 0.025, 0.006, and 0.005) no evidence of HWE deviation, except for GATA5 at locality 2 (heterozygote deficiency, $P < 0.001$). Without the correction, there were six deviations ($P = 0.012$ to 0.048) in *C. macularius* (all were heterozygote deficiencies). There was no statistically significant frequency of null alleles. Expected heterozygosity and other measures of diversity for each locus in individual populations are in Appendixes C and D. The measures of diversity across loci are in Table 1.

The allele-size permutation test indicated that R_{ST} was larger than F_{ST} when all populations of both species were included in the analysis (5 loci; $P < 0.0001$). Comparisons of the two species were the major contribution to this effect (see Table 2). The test was also significant for the comparison of the two populations of *C. eremus* (7 loci; $P = 0.02$); it was not significant for *C. macularius* (7 loci; $P = 0.14$).

All interspecific, pairwise estimates of F_{ST} and R_{ST} were significant, as were those between the two populations of *C. eremus* (Table 2). Within *C. macularius*, none of the R_{ST} values were significant, but, based on F_{ST}, the three populations from the Salton Sea were significantly divergent from each other and from most populations on the lower Colorado River delta. The latter group showed few significant differences except for comparisons involving the Laguna Salada population (locality 5).

Neighbor-joining trees for mtDNA and the five shared microsatellite loci grouped *C. macularius* and *C. eremus* separately (Fig. 2). Most microsatellite alleles were shared between the two species, as indicated by the number of private alleles (Table 1). The locus most nearly diagnostic of the two species was WSP-02. Allele 204 occurred only in a single heterozygote in *C. macularius,* whereas all *C. eremus* individuals were either homozygous or heterozygous for this allele.

For the seven loci assayed in *C. macularius*, multiple dimensional scaling separated the three populations in the Salton Sea area from the six in the lower Colorado River Delta, although the geographically intermediate population at locality 4 could have been placed in either group (Fig. 3). The Mantel test for isolation-by-distance in *C. macularius* was significant for microsatellites ($P = 0.0001$), but not for mtDNA ($P = 0.26$). Nonetheless, a tendency for parallel geographic variation in the two sets of data is indicated by the Mantel test of correlation between mtDNA and microsatellite genetic distances, which was significant, both across all populations of both species (5 microsatellite loci, $P = 0.003$) and for *C. macularius* alone (7 loci, $P = 0.048$).

Diversity indexes for each locus-by-population sample are in Appendixes C and D. AMOVA attributed 23% of the microsatellite diversity (R_{ST}) to differences between *C. macularius* and *C. eremus*, 2% to differences among populations within species, and 75% to variation within populations (Table 3). For *C. macularius* alone, a small (0.8%), but statistically significant portion of diversity (F_{ST}) was attributed to variation among groups (Salton Sea area vs lower

Colorado River delta), 1.9% was attributed to variation among populations within groups, and 97.3% to variation within populations. For *C. eremus*, 10.0% and 90% of the diversity (R_{ST}) was attributed to, respectively, differences between the two populations and variation within populations.

The AMOVA results for mtDNA generally corresponded closely with those for microsatellites (Table 3). The major discrepancy was with *C. eremus*, where, for mtDNA, variation reflecting divergence between the two populations was a statistically non-significant 2.9% ($P = 0.19$), whereas for microsatellites it accounted for 10% of the variation ($P < 0.00001$).

DISCUSSION (Part I)

Taxonomy and interspecific divergence.—The species-level taxonomy of the Desert Pupfish complex is consistent with results from three sets of data on genetic structure: (1) Populations of the two species, *C. macularius* and *C. eremus*, clustered separately in the neighbor-joining tree from microsatellites; (2) in a previous allozyme analysis, *C. eremus* from Quitobaquito Springs clustered outside of a group of five populations of *C. macularius* (four from the Salton Sea area and a captive stock derived from Santa Clara Slough; Turner 1983); and (3) the two species are reciprocally monophyletic for mtDNA (Echelle et al. 2000), with lineages that have been diverging for about 0.9 myr, based on a Bayesian estimate with the upper limit of divergence time set at 2 myr (Echelle *in press*). These results, together with subtle morphometic differences (Miller and Fuiman 1987) and a difference in intensity of yellow pigmentation on the caudal fin and peduncle in breeding males, support recognition of two species.

The proportion of diversity attributable to between-species differences in microsatellites (23%) was markedly less than that for mtDNA (86%). This potentially reflects two factors: (1) the maternal, haploid inheritance of mtDNA gives it a roughly four-fold smaller effective population size, hence greater genetic drift, in comparison with biparental, diploid markers like microsatellites, (2) microsatellite allele-sizes are prone to exhibit convergence (Orti et al. 1997), particularly among closely related species (Culver et al. 2001). It is worth noting, however, that the rather weak microsatellite divergence between *C. macularius* and *C. eremus* is paralleled by similarly weak allozyme divergence (Turner 1983, Echelle and Echelle 1993), indicating that the nuclear genomes are recently divergent. Nonetheless, the finding that allele size contributes to divergence of the two species ($R_{ST} > F_{ST}$) demonstrates that divergence is not explainable on the basis of genetic drift alone and that the two species have been isolated sufficiently long that accumulation of new mutations has played a role (Hardy et al. 2003).

Overall genetic diversity.—Both species of the Desert Pupfish complex show notably high levels of microsatellite diversity. Number of alleles per locus/population was 15.4 for *C. macularius* and 15.1 for *C. eremus*, and heterozygosity (H_e) per locus/population was, respectively, 0.91 and 0.84. These values are markedly higher than the averages (7.5 alleles/locus, $H_e = 0.46$) reported in a review of freshwater fishes (DeWoody and Avise 2000). Although comparisons involving different suites of loci must be tentative, the estimates for the Desert Pupfish complex are higher (*C. macularius*) or similar to (H_e in *C. eremus*) those reported for six microsatellite loci in the

four most diverse populations of *C. nevadensis* (11.6 alleles/locus, $H_e = 0.83$), a species characterized as having abundant variation in both microsatellites and mtDNA (Duvernell and Turner 1998, Martin and Wilcox 2004). The estimates are markedly higher than reported for two microsatellite loci in native populations of *C. tularosa* (4.0 alleles/locus; $H_e = 0.48$; Stockwell et al. 1998), a species having low allozyme variation in comparison with several other pupfishes (Echelle et al. 1987, Stockwell et al. 1998).

Intraspecific divergence.—As suggested by Miller and Fuiman (1987), the two populations of *C. eremus* (in Quitobaquito Springs and Río Sonoyta) might have a long history of isolation, despite being separated by only about 2 km. This is consistent with (1) morphometric divergence of the two populations (Miller and Fuiman 1987), (2) the rather large proportion (10%) of microsatellite diversity attributable to differences between the two populations, and (3) the indication that, unlike *C. macularius*, mutation subsequent to isolation contributes significantly to the divergence in microsatellites ($R_{ST} > F_{ST}$). In contrast, a statistically non-significant 2.9% of the mtDNA diversity in *C. eremus* reflected differences between the two populations. Failure to detect significant mtDNA divergence despite divergence in microsatellites might reflect, in part, the smaller sample of mtDNA markers (n = number of fish) compared with microsatellites (n = 2 x number of fish).

The genetic structure of *C. macularius* shows rather weak, albeit statistically significant, geographic structure. About 1% of the microsatellite diversity and 4% of the mtDNA diversity was attributable to differences between populations in the Salton Sea area and those from the lower Colorado River Delta, with 97% and 94% of, respectively, microsatellites and mtDNA diversity occurring within the average local population. This probably reflects gene flow across the Colorado River delta in historical times prior to the presently depleted river flow and the construction of levees that control the course of the river in Mexico.

The test for isolation-by-distance in *C. macularius* was statistically significant for microsatellites, but not for mtDNA, possibly reflecting the above-mentioned difference in sample size for the two types of markers. Presently, most of the populations are isolated by uninhabitable stretches of dry desert, and the Salton Sea populations have been isolated from those on the Colorado River delta for more than a century. But, historically, there was much greater connectedness among populations, including more frequent connections between the lower delta and the Salton Sea (Sykes 1926; Carpelan 1961). In those times, distance, and not geographical isolation per se, could have played a role in restricting gene flow among populations.

The population in Laguna Salada (Locality 5, Fig. 1) harbors the most isolated population of *C. macularius*. This population had no unique alleles and its microsatellite diversity, although high, was the lowest observed for the species. Follett (1960) and Miller and Fuiman (1987) cited information from Carl Hubbs in reporting that the species had been extirpated from the basin by the 1960s or 1970s. In 1983-84, the species would have gained access to Laguna Salada when flows from the Colorado River temporarily filled the basin, the first such connection in more than 20 years (Hendrickson and Varela-Romero 1989). By 1987, the resulting lake was reduced in size and desiccating (Hendrickson and Varela-Romero 1989). When the collection for our

study was made in 1998, the lake was dry except for a small, spring-fed area (Pozo del Tules) near the northwest end of the basin that has persisted to the present and still supports a pupfish population (C. O. Minckley, pers. comm.).

The largest populations of *C. macularius* now occur in springfed marshes and artificial pools and canals in the vicinity of the Cerro Prieto geothermal field, the site of a large geothermal power plant (Varela-Romero et al. 2002). This population is in the Rio Hardy watershed, which drains directly to the Gulf of California. Historically, however, the low divide separating Cerro Prieto from the Salton Sink was occasionally breached during high flows in the Colorado River, when the river emptied into the Salton Sea via "Volcano Lake," a shallow wetland encompassing the Cerro Prieto area (MacDougal 1906; Sykes 1926).

Conservation implications.—The remnant wild populations of both *C. macularius* and *C. eremus* retain large amounts of genetic variation. This diversity in the face of severely reduced inter-population connectedness and numbers and sizes of populations probably is explained by life history traits. Most pupfishes are hardy, small-bodied omnivores capable of maintaining extremely dense populations, particularly in harsh, marginal environments where there is little competition from other species (Echelle et al. 1972; Naiman 1976). Consequently, recent surveys found the densest populations of *C. macularius* in harsh, highly saline habitats where they are least exposed to non-native fishes (Hendrickson and Varela-Romero, 1989; Varela-Romero et al. 2002). Dunham and Minckley (1998:13) commented that, "Fortunately, most non-native species . . . cannot survive the severe conditions pupfish accommodate readily." They suggested that this aspect of pupfish biology should be incorporated into conservation plans for *C. macularius*.

Results from both microsatellite DNA and mtDNA, together with a difference in coloration of breeding males, support management of the Desert Pupfish complex as two separate species. Within species, there is no genetic evidence of separate evolutionarily significant units (ESUs), neither as defined by Waples (1991) on the basis of reproductive isolation and ecological or adaptive uniqueness, nor as defined by Moritz (1994) on the basis of reciprocal mtDNA monophyly.

Conservation biologists often use measures of genetic divergence (e.g., F_{ST}) to identify management units (MUs), which can be defined as demographically independent (isolated or relatively so) populations or groups of populations important for the long-term persistence of the species. This effort is confounded by two factors when based on highly variable loci like microsatellites (Hedrick 1999): (1) Because of high statistical power, detected divergence might not be biologically significant, and (2) on the other hand, when heterozygosity is high, as in the Desert Pupfish complex (~0.90), the among-population component of diversity will be small, even when populations share no alleles and clearly have experienced little or no gene flow for sufficient time to allow adaptive divergence. In addition, because of reduced genetic drift in large populations, they can be demographically independent while showing little divergence for neutral markers (Allendorf and Luikart 2007). In the following, we identify an MU as a population or network of populations that occupies a critical portion of the geographic range. In

our judgment, the loss of any one of these MUs would be a significant step toward extinction of the species in the wild.

We recommend recognition of two management units (MUs) in *C. eremus* (Quitobaquito Springs and Río Sonoyta) and five in *C. macularius*, three in the lower Colorado River delta (Laguna Salada, Cerro Prieto, and the Santa Clara Slough/El Doctor area) and two in the Salton Sea region (the San Felipe Creek/San Sebastian Marsh system and the remaining Salton Sea system).

There is some danger of misinterpreting the implications of MU recognition. Recognition of a set of populations (for example, shoreline pool and canal populations connected to the Salton Sea) as an MU does not mean that all except one of those populations are expendable or that we should preserve the most distinctive member of the MU. Instead, conservation management should aim to preserve the natural network of genetic connections between populations, thereby preserving the processes that maintain diversity and evolutionary potential (Crandall et al. 2000).

For *C. macularius*, management activities over the past three decades, primarily establishment of captive stocks in artificial refuges (Hendrickson and Varela-Romero 1989, Dunham and Minckley 1998), have treated populations in the Salton Sea and Santa Clara Slough separately. This seems generally appropriate, given the statistically significant, although rather small, amount of diversity (~2%) attributable to differences between these two groups of populations.

Similarly, the Quitobaquito and Río Sonoyta populations of *C. eremus* have been managed as separate units, with no mixing of the two in the refuge program for *C. eremus* (this report, Part II). Management has concentrated primarily on the Quitobaquito Springs population, which has been transplanted into several captive refuges (Hendrickson and Varela-Romero 1989, Dunham and Minckley 1998, Part II of this report). Recently, the Río Sonoyta population has received increased attention, with a refuge stock established in 2005 at Cabeza Prieta National Wildlife Refuge, Arizona, and establishment of two refuge stocks in 2007 in Sonora (D. Duncan, pers. comm.). A third captive stock of *C. eremus* has been maintained for several years at Hermosillo, Sonora (A. Varela-Romero, pers. comm.).

PART II: REFUGE POPULATIONS

INTRODUCTION

Since the 1970s, translocations of fishes into semi-natural or artificial refuges have played an increasingly important role in the conservation management of imperiled fishes in the American Southwest (Williams 1991; Minckley 1995). The primary purposes of such stocks are to protect genetic resources against catastrophic loss of natural populations and to provide fish for release into the wild to augment existing populations or to re-establish populations within the historical range of the species. Ultimately, the success of such a program depends heavily on maintenance of genetic variability in the refuge populations (Quattro and Vrijenhoek 1989, Hedrick and Miller 1992). As noted by Allendorf and Phelps (1980:537), "genetic variability is the primary biological resource" in the successful management of artificially propagated populations.

Captive threatened fishes in the American Southwest fall into two general groups with reference to how they are managed for propagation (Echelle 1991): "spontaneous breeders," such as cyprinodontoids and smaller cyprinids, and "artificial breeders," such as salmonids, catostomids, and larger cyprinids. Spontaneous breeders spawn and propagate with little or no human intervention, even in relatively small holding facilities, whereas artificial breeders require more handling and manipulation (hormone application, stripping, etc.). Losses of variability in captive stocks of artificial breeders are well documented (Allendorf and Ryman 1987; Dowling et al. 1996) and this has heightened awareness of the potential for unwanted genetic effects when captive stocks are managed without attention to preserving diversity. In general, however, spontaneous breeders have received less attention, in part because of their biology (Echelle 1991). They typically are smaller, shorter-lived fishes that, in hatchery ponds and other artificial situations, can quickly form large populations requiring little or no management effort, and this has allowed a rather laissez faire approach to management of such stocks. In this paper, we assess levels and patterns of genetic diversity in refuge populations of the Desert Pupfish complex (Cyprinodontidae: *Cyprinodon macularius* complex), some of which have been maintained for more than 30 years.

In many respects, pupfishes are ideal for low-maintenance refuge programs dependent on spontaneous reproduction. They are small bodied (usually <40 mm SL) omnivores with high reproductive potential. Members of the Desert Pupfish complex have extended spawning seasons and can breed at sizes as small as about 15 mm SL and only two months post-hatching (Cox 1966; Kinne 1962; Constantz 1981). These features resemble those of other pupfishes, such as *C. nevadensis*, which can reach densities as high as 89 fish/m^2 (Naiman 1976) in harsh situations that, like most of the refuges, have few other fish species. These life history attributes allow quick rebound from founder events and other population bottlenecks and promote large populations in small refuges.

On the other hand, the observed number of animals in a refuge (N) undoubtedly overestimates the evolutionarily effective size (N_e) of the population. The N_e/N ratio in animals often is <0.10 and generally 0.25-0.50 (Frankham 1995). In the Desert Pupfish complex, the breeding males are intensely territorial (Barlow 1961; Cox 1966), and this, together with other life-history

variables, can greatly reduce N_e/N (Frankham 1995). Thus, the rate of loss of genetic diversity should be similar to that of an "ideal" population (e.g., 1:1 sex ratio, equal individual contributions to reproduction, and constant N_e) considerably smaller than the actual number of pupfish supported by a refuge.

In an allozyme survey of refuge populations of *C. macularius*, Turner (1984) found no evidence of marked change in genetic diversity compared with wild populations and, with caveats regarding management, commented (p. 368) that this was "grounds for cautious optimism about the ability of artificial refugia to preserve the natural genetic variation of fish populations." Edds and Echelle (1989) obtained similar results, but with some evidence of loss of rare alleles, in an allozyme survey of six- to eight-year old captive stocks of two pupfish species (*Cyprinodon bovinus* and *C. elegans*) and a third cyprinodontoid, *Gambusia nobilis*. Dunham and Minckley (1998) found some evidence of reduced allozyme variability after about 10 years in captivity for two populations (one lineage) of nine refuge stocks of *C. macularius*, although the authors did not have the benefit of comparison with the wild-source population in Mexico. In contrast to the studies just described, marked genetic change, including reduced diversity was observed in four refuge populations of *Gambusia affinis* established in western United States in the 1920s to 1940s (Stockwell et al. 1996).

Our purpose was to use variation at microsatellite DNA loci to evaluate the effectiveness of the refuge programs for *C. macularius* and *C. eremus*. The analysis covers 30 of the 45 or so existing, non-aquarium, refuge populations of the Desert Pupfish complex and includes a comparison of diversity with most of the existing wild populations, estimates of local and global effective population sizes of refuge populations since translocation from the wild, and an assessment of factors contributing to declines in genetic diversity in the refuge populations. We also present recommendations for management of the refuge program for the Desert Pupfish complex.

HISTORY OF REFUGE POPULATIONS

The first refuge stock of Desert Pupfish was established in June 1970, when 50 Salton Sea individuals were translocated to Anza Borrego State Park, San Diego County, California. Subsequent translocations from the wild took place in the 1970s and 1980s with founding population sizes varying from 10 to 280 (Figs. 4-6; Tables 4 and 5). Some captive stocks were renovated to eliminate unwanted species, such as *Gambusia affinis* and *Notemigonus crysoleucas* (Dunham and Minckley 1998). These renovations involved eliminating the pupfish population and replacing it with pupfish from another captive stock or from the salvaged original population.

Refuge stocks of *C. macularius* comprise seven lineages representing original translocations from wild populations (Figs. 4 and 5), two from the lower Colorado River delta in Sonora, Mexico (Terrace Springs and a pool near the terminus of Canal Sanchez Taboada) and five from the Salton Sink region in California, USA. Refuge stocks of *C. eremus* (excluding three in Sonora not included in this analysis) comprise three lineages from Quitobaquito Springs and one (Finley Tank = FT) that, prior to this study, was considered by the Arizona Department of Game

and Fish (AZGF) to be of unknown, potentially mixed, origin. Our results and personal correspondence with B. Kynard (7 August 2007) indicate that this stock came from Río Sonoyta. Kynard collected the fish from the river in 1976, kept them at the University of Arizona, and released them at FT in the spring of 1978. Although there were some between-refuge transfers of pupfish, only one refuge population is known to contain genetic material from two or more lineages. This exception is Boyce Thompson Arboretum (BT, Fig 2), which was established with fish from the DNFH and WLM lineages. One of the two ponds at Living Desert Zoo and Gardens (LD1) is the only known instance of supplementation of a refuge stock with wild-caught fish (Fig. 5).

For *C. macularius*, there are about 25 refuge populations in Arizona, 15 in California, and 1 in New Mexico. The refuge program for *C. eremus* includes about six refuges established in Arizona from wild stocks obtained from Quitobaquito Springs. At least one refuge population from Río Sonoyta is being maintained in Hermosillo, Sonora, Mexico (A. Varela-Romero, pers. comm.; not included in this study).

MATERIALS AND METHODS (Part II)

Sampling and molecular techniques.--Data from wild populations included in this analysis (Fig. 1) are from Part I. For the refuge populations, fish were collected from 30 sites (N = 25 each) in 2005 and 2006 and stored in 100% ethanol (Tables 4 and 5, Appendix E), and we included DNA samples prepared by Echelle et al. (2000) from a collection made at one of those sites (DNFH) in 1998.

Habitat size was estimated at the time of collection as the product of length and width of water-surface area of the refuge. History of each captive stock was obtained from Dunham and Minckley (1998), managers of individual sites, and records provided by AZGF and the California Department of Fish and Game. Differences in history from these sources were trivial. The major discrepancy among sources had to do with the refuge at Boyce Thompson Arboretum (BT). We followed Dunham and Minckley (1998) who reported four steps from the wild for the fish descended from the Terrace Springs population (Fig. 4); AZGF records indicate only two steps.

DNA from each specimen was extracted using the DNeasy kit (Quiagen). Six microsatellite loci with tetranucleotide repeats (Table 6) were amplified using primers from Burg et al. (2002). Different combinations of four of these were used with the two species: GATA2, GATA5, GATA9, and GATA39 for *C. macularius* and GATA9, GATA10, GATA26, and GATA39 for *C. eremus*. GATA10 and GATA26 were not used for *C. macularius* because of high frequencies of null alleles and, for the same reason, GATA2 and GATA5 were not used for *C. eremus* (this report, Part I). Loci were amplified using the polymerase chain reaction with15-ul reactions (9.0 ul Applied Biosystems True Allele premix, 3.8 ul ddH2O, 1.0 ul of 5.0 uM primer pairs, and 1.2 ul template DNA). Table 3 shows the characteristics for the six loci. All PCR reactions were carried out using either an MJ Research PTC 100 Thermal Cycler, or a Perkin Elmer 9600 Thermal Cycler using a two-step annealing process. Reaction conditions for the PTC 100 consisted of one cycle at 95°C for 12 minutes; five cycles of 94°C for 30 s, T_{A1} for 30 s, 72°C for

30 s; 32 cycles of 94°C for 30 s, T_{A2} for 30 s, 72°C for 20 s; and one cycle at 72°C for 2 minutes. Reaction conditions for the PE-9600 consisted of one cycle at 95°C for 12 minutes; five cycles of 94°C for 30 s, T_{A1} for 30 s, 72°C for 30 s; 35 cycles of 94°C for 45 s, T_{A2} for 45 s, 72°C for 1 minute; and one cycle at 72°C for 2 minutes. Primers were end-labeled with a fluorescent dye as follows: GATA2 and GATA10, 6-FAM; GATA 5 and GATA26, HEX; GATA9 and GATA39, NED. In an analysis of the origin of the Finley Tank (FT) population, each FT individual was assayed for three additional loci (CmD1, CmD16, and WSP02; methods as in Part I).

Each individual was genotyped using the Applied Biosystems Inc. (ABI) 377 or Prism 3130 automated sequencer and the ABI Genescan 3.1 Software. The PCR products for the various loci were multiplexed in the automated sequencer in two groups: (1) GATA2, GATA5, and GATA9; (2) GATA10, GATA26, and GATA39. Allele sizes were scored with ABI Genotyper 2.5 software and GeneMapper v. 3.5. To minimize scoring errors, we performed blind re-genotyping of 5% of samples for each locus, with samples and loci randomly assigned by random number generator. The error rate was calculated and used for identifying loci prone to error.

Data analyses.--Genetic diversity indices, including number of observed alleles (A), observed heterozygosity (H_o) and expected heterozygosity (H_e), were calculated using GENEPOP (Raymond and Rousett 1995). Allele richness (A_R) was calculated with FSTAT 2.9.3.2 (Goudet 1995). GENEPOP was also used for exact tests of deviations from Hardy-Weinberg (H-W) equilibrium and linkage disequilibrium. When deviations were detected at a locus, we used MICRO-CHECKER (van Oosterhout et al. 2004) to check for large-allele drop out, null alleles, and scoring of stutter peaks. Brookfield's (1996) Method I was used to estimate null allele frequencies.

We used STRUCTURE v. 2.0 (Pritchard et al., 2000) to assess the origin of the Finley Tank (FT) population. First, we used the dataset for five loci in both *C. macularius* and *C. eremus* from Part I of this report, added the FT genotypes for those loci, assumed two groups, and left FT unassigned. STRUCTURE used Markov Chain Monte Carlo (MCMC) iterations to compute the Bayesian probability of the unassigned individuals. Based on the results, FT individuals were assigned to *C. eremus,* and the analysis was rerun with the same parameters as before. This gave the probability of assignment to each of the two species and the probability that individuals had hybridization in their ancestry. In a third analysis, we used the dataset for seven loci in wild populations of *C. eremus* (see Part I), added the FT genotypes for those loci, assumed two groups (Quitobaquito Springs and Río Sonoyta) and left FT unassigned. All analyses used the population assignment for the reference individuals (those assigned to *C. macularius* or *C. eremus*), assumed correlated alleles among populations, and computed probabilities from 520,000 MCMC iterations with the first 20,000 discarded as burnin.

Arlequin (Schneider et al. 2000) was used to compute overall and pairwise F_{ST} values among collections and to conduct analyses of molecular variance (AMOVA). For each species, we used a simple AMOVA to test for among-population divergence in wild populations and hierarchical AMOVA to estimate partitioning of diversity among groups: one analysis with two groups,

refuge vs wild, and another with groups identified as refuge populations descended from an original translocation from the wild.

The partial Mantel test (Douglas and Endler 1982) in the zt software package (Bonnet and Van de Peer, 2002) was used to test for association between change in genetic diversity (H_e and A_R) and number of founders, habitat size, and time since founding for the refuge populations of *C. macularius*. In these tests, the input data comprised three half-matrices of pairwise population differences: a matrix of differences in the response variable (H_e or A_R), a matrix of differences in an independent variable of interest, and a matrix of differences in a covariate. To correct for heterozygosity differences between different parent populations, we followed Dunham and Minckley (1998) and expressed the difference in heteozygosity between parent and daughter population (H_{eDIFF}) as the absolute difference divided by H_e of the parent population. Mantel tests of association were not performed for *C. eremus* because of the small number of refuge populations. The two species were not combined in a single analysis because the suites of loci differed for the two species.

We used the difference in H_e between wild-source and refuge population as the response variable ($H_{e(SOURCE)}$) in partial Mantel tests of association between number of founding steps and heterozygosity in *C. macularius*. Following Dunham and Minckley (1998), $H_{e(SOURCE)}$ is the ratio of the absolute difference in H_e divided by the wild-source H_e. Groups of populations served as the wild sources because the exact source locality generally was not known for most refuge populations. For example, the founding stock from Terrace Springs was taken from a complex of springs that could include sites 7-9 in Figure 1 (C. Minckley, pers. comm.), the locality within San Felipe Creek is not known, and the Salt Creek population was extirpated by the time collections were made. To represent the wild source for refuge populations derived from the Salton Sea area, we used the collections from sites 1-3 (Fig. 1), and for those derived from the lower Colorado River delta, we used populations from sites 6 to 9. A given composite of wild source-populations was treated as a single, large sample with heterozygosity equal to the average for the individual populations. In computing wild-source A_R, we included this sample with the samples from the associated descendant populations. The resulting estimate for each population is based on the expectation for a sample the size of that of the smallest sample in the group.

We used the temporal-sampling method (Waples 1989) available in NeEstimator (The State of Queensland, Department of Primary Industries and Fisheries 2004) to calculate effective population sizes (N_e). The method assumes discrete, non-overlapping generations, but the bias caused by violating this assumption is minor when the number of elapsed generations is greater than about five (Waples and Yokota 2007). Four kinds of estimates were made: (1) N_e for DNFH05 since establishment from DNFH98, (2) long-term N_e for each refuge population since the original translocation from the wild, (3) N_e for each lineage, where a lineage is defined as a group of populations descended from an original refuge population established from the wild, and (4) global N_e for the refuge programs of the two species since inception of the programs in the 1970s. Global N_e was computed in two ways, one with all refuge samples lumped as a single population (Global-1), and the other with one randomly chosen refuge from each lineage lumped as a single population (Global-2).

With one exception, we used Quitobaquito Springs as the wild source in estimating long-term N_e for each refuge population of *C. eremus*. The exception was refuge population FT, for which we made two estimates, one using Río Sonoyta as the wild source and the other using Quitobaquito Springs. AZGF records indicated that the wild source was unknown for FT. For *C. macularius*, N_e of each refuge population was computed based on deviation from the corresponding wild-source group (described above); for 95% confidence intervals, we used the lowest of the 95% minima and the highest of the 95% maxima among the individual analyses (= CI_{max}).

To estimate N_e for each lineage of refuge populations, we treated all refuge populations of that lineage as a single collection and we treated all populations of the corresponding wild source-group as a second collection. We used these as two temporally separated collections from the same population. To estimate global N_e of the refuge programs, all wild and all refuge populations were combined into two separate collections, with separate analyses for *C. macularius* and *C. eremus*. For the analyses involving lumped refuge populations, the initial estimate of N_e was divided by 1-F_{ST} of the captive populations to remove bias associated with geographic structure (Waples 2002).

For N_e computations, we used twice the number of years since original founding from the wild as the number of generations separating refuge and wild populations. This is based on the extended breeding season of *C. eremus* and *C. macularius* (Cox, 1966; Constantz 1981), and the observation that individuals begin breeding at 15 mm standard length (Constantz 1981), a size attainable within two to three months post-hatching (Kinne 1962). For global N_e with all populations included in the analysis, we used the weighted mean of number of generations since the original founding from the wild (50.5 for *C. macularius*: 46.4 for *C. eremus*). This was straight-forward except for BT, which was founded from two original refuges, WLM and DNFH, established, respectively, 29 and 22 years before our collections. For BT, we used the average to represent time since the original founding from the wild (25.5 years = 51 generations). For global N_e with each lineage represented by a single refuge population we used the average age across lineages (50.0 generations for *C. macularius*; 49.3 for *C. eremus*).

RESULTS (Part II)

Accuracy for blind re-genotyping was 100% except for GATA 26 (97%). Genotypes for each individual are available from A. A. Echelle. There was no evidence of linkage disequilibrium among loci, and, with the Bonferroni correction (*C. macularius*, 34 populations per locus, critical $P = 0.05/34 = 0.001$; *C. eremus*, 8 populations per locus, critical $P = 0.006$), no instances of deviation from Hardy-Weinberg equilibrium (HWE; Appendices D and E). Without the correction, there were four heterozygote excesses and nine deficiencies among the 136 tests (P = 0.003 to 0.040). MICROCHECKER indicated the following instances of significant null-allele frequencies: 13% for GATA2 at AHS, 14% for GATA2 at FT, 11% for GATA5 at DP2, and 9% for GATA39 at DNFH05.

Measures of genetic diversity for each locus in individual refuge populations are given in Appendixes F and G. Numbers of alleles per locus ranged from 23 to 35 for the four loci assayed in *C. macularius* and 19 to 29 for the four assayed in *C. eremus* (Table 7). As a group,

the refuge populations of *C. macularius* had 89% of the alleles detected in wild populations. The refuge populations of *C. eremus* had more alleles (103%) than the wild populations, and the percentage remained high (95%) even when we excluded OPC, which was founded from Quitobaquito Springs only two months before we made our collection. The measures of diversity for refuge populations of both species were consistently lower than those for wild populations (Tables 8 and 9; Fig. 7).

The STRUCTURE analysis of assignment of FT individuals in *C. macularius* or *C. eremus* (5 loci) placed all individuals in *C. eremus* at probabilities of 0.905 to 0.991, with only one individual showing 90% CI overlap for membership in the two groups. When the analysis was redone with all FT fish assigned to *C. eremus*, that individual classified as *C. eremus* at P = 0.954, and probability of membership in *C. macularius* or having a hybrid ancestry were, respectively, 0.004 and 0.042. In the analysis of membership in the Quitobaquito Springs versus Río Sonoyta populations of *C. eremus* (7 loci), 17 of the 19 FT individuals had their highest (albeit low) probabilities of membership in the Río Sonoyta population (P = 0.530-0.678); the remaining two were assigned to the Quitobaquito population (P = 0.501-0.549). Confidence intervals for membership in the two groups were broadly overlapping for all FT individuals. The indicated tendency for FT fish to group with the Río Sonoyta population is also reflected in the results of the neighbor-joining analysis of F_{ST}-values among populations (Fig. 8).

The matrices of pairwise divergence (F_{ST}) among populations (available from Echelle) are summarized in Figure 6. Nearly all comparisons involving captive stocks were statistically significant. With 589 comparisons across all wild and captive stocks, the initial P-value for significance with the sequential Bonferroni correction was 0.0001. With this correction, all except two of the 312 comparisons of wild vs captive stocks were significant. The only exceptions were the comparisons of the two most recently established captive lineages of *C. eremus* (OPC and SCC) with the ancestral wild stock at Quitobaquito Springs.

Nearly all pairwise comparisons of refuge populations were statistically significant: 13 of 15 (87%) for *C. eremus* and 296 of 300 (99%) for *C. macularius*. The two exceptions for *C. eremus* involved SCC, which was not significantly different from its parent refuge population (ASU2) or from the recently established OPC. The four exceptions for refuge populations of *C. macularius* involved comparisons of highly similar populations within the DNFH lineage (Fig. 6). The one comparison involving the same refuge sampled in two separate years, DNFH in 1998 and 2005, was not significant (F_{ST} = 0.009; P = 0.054).

Statistically significant divergence was notably less common in pairwise comparisons of wild populations. Comparison of the two wild populations (QS and RS) of *C. eremus* was marginally significant with the Bonferroni correction (F_{ST} = 0.02, P = 0.0001 = critical P). For *C. macularius*, 17 of 36 (47%) comparisons of wild populations were significant; F_{ST} for those 17 ranged from 0.04 to 0.05.

The AMOVA results indicate that 11.5% and 16.5% of total diversity in refuge populations of, respectively, *C. eremus* and *C. macularius*, is attributable to differences among populations, with 88.5% and 83.5% attributable to within-population variation (Table 10). For the same loci in wild populations of both species, only about 2% of total diversity reflected among-population

variation. A significant portion of the among-population variation in refuges of both species was attributable to differences among lineages, where lineages were defined as refuge populations descended from an original translocation from the wild: 7.5% and 7.6%, respectively, for *C. eremus* and *C. macularius*.

For the one refuge population sampled in separate years (DNFH, 1998 and 2005), the estimate of effective population size (N_e) was 529 (*CI* = 143.1-infinity). The long-term N_e for individual refuge populations of *C. macularius* (between the original translocation from the wild and the time collections were made for this study) ranged from a low of 151 for DNFH98 (CI_{max} = 114-468) to a high of 817 for LD1 (CI_{max} = 327-2166). For *C. eremus*, the lowest N_e was 275 for SDM (*CI* = 190-506) and the highest was 470 for ASU2 (*CI* = 317-1932). The average across all refuge populations was 299 for *C. macularius* and 396 for *C. eremus* (Tables 8 and 9).

The weighted mean number of generations since the original translocation from the wild was 50.5 for the refuge program of *C. macularius*. The weighted average for *C. eremus* was 46.4 when the recently founded OPC population was not considered (38.7 with OPC included). Using these ages, the global estimate of N_e computed over all refuge populations (global-1) was 1059 (*CI* = 777-1404) for *C. macularius* and 797 (*CI* = 501-1291) for *C. eremus*. Using only a single population from each lineage (global-2), gave similar results, with a moderate increase in N_e for *C. macularius* (1273, *CI* = 903-1755), and little change for *C. eremus* (746; *CI* = 470-1198). Deleting the recently established OPC refuge had little effect on the estimate for *C. eremus* (741; *CI* = 477-1156). The lowest N_e for individual lineages was 297 (*CI* = 212-402) for the DNFH lineage and the highest was 783 (*CI* = 541-1107) for the PC lineage (Tables 8 and 9; Fig. 9).

Mantel tests were marginally significant for association between number of founding steps and differences among refuge populations in heterozygosity ($H_{e(SOURCE)}$; r = 0.13; P = 0.06), but not for differences in allele richness ($A_{R(SOURCE)}$; r = 0.10; P = 0.12; Table 11). The tests for association between lineage age (time since founding from the wild) and differences in both heterozygosity (r = 0.11-0.12; P = 0.07-0.09) and allele richness (r = 0.16-0.17; P = 0.05-0.06) were marginally significant. Using number of founders as a covariate in the test with lineage age, and vice versa, effectively had no effect on the results, and neither did other covariates, including number of founders of the immediate population, refuge size, and number of supplements to the refuge.

Mantel tests for association between differences in heterozygosity (H_{eDIFF}) and number of founders, refuge size, number of supplements, and number of years since establishment of the refuge were not significant (r = -0.00 to +0.07; P = 0.21-0.65). However, a highly significant association was found between refuge size and number of founders for the population (r = 0.88; P = 0.0002)

DISCUSSION (Part II)

Estimates of global N_e over the 30+ years of the refuge programs for *C. macularius* and *C. eremus* were over 500, which is the minimum size mentioned in the literature on the size necessary to maintain historical diversity and the long-term, adaptive potential of populations

(Franklin 1980; Nelson and Soulé 1987). This number has been debated on theoretical grounds, with Franklin and Frankham (1998) arguing that the minimum size is between 500 and 1000 and Lynch and Lande (1998) arguing that it is between 1000 and 5000. Regardless, our global N_e estimates, and, for *C. macularius*, the lower 95% *CI*, for refuge populations are at least within the range of sizes recommended in the literature. This is reflected by the global diversity of the refuge programs, which is similar to that in individual wild populations and only moderately lower than in global wild populations of the two species. This was achieved without inoculation with individuals from the wild, and with essentially no interchange among refuge lineages, both of which are recommended for avoiding unwanted change in managed stocks (Allendorf 1986).

The success of the refuge program benefited from maintaining multiple stocks representing several wild-source populations. Microsatellite and mtDNA diversity in the wild populations of both *C. macularius* and *C. eremus* show very little geographic structure, with less than 3% attributable to differences among populations (Echelle et al. 2000; Part I, this report). Still, maintenance of multiple stocks from the extremes of the extant ranges of both species undoubtedly captured more diversity than would have been captured from single wild-source populations.

In contrast with the global refuge programs, the long-term effective sizes of local refuge populations and the global estimates for individual lineages were, with few exceptions, below 500, reflecting their generally low levels of genetic diversity compared with the wild populations. Reduced microsatellite diversity signals reduced diversity, particularly of rare alleles, throughout the genome, including variation for the quantitative traits that often are the targets of natural selection (Lande 1980). Such losses potentially detract from long-term success by compromising the health and adaptability of local populations (Lesica and Allendorf, 1995; Frankham et al. 2002), and, ultimately, the refuge program. Wild populations of both species of the Desert Pupfish complex still exist and the long-term goals of refuge management would benefit from implementing artificial immigration from the wild. Minimal amounts of immigration, on the order of several individuals per generation, can essentially counteract the deleterious effects of genetic drift in small populations (Mills and Allendorf 1996; Vucetich and Waite 2001).

The history and attributes of the various refuges provide several case studies of management consequences. For example, the DNFH lineage formed a cluster of highly similar populations with low diversity. Dunham and Minckley (1998) attributed low allozyme diversity in the parent DNFH refuge and one of its descendant refuges (DBG herein) to founder effect during establishment of the lineage in 1983 with 280 fish, all from a single, recently dug pool alongside the terminus of Canal Sanchez Taboada that might have been colonized by a small number of founders. Since translocation to DNFH, the stock has consistently remained above 500 adults (M. Ulibarri, pers. comm.), conforming to our N_e estimate of 529 between 1998 and 2005. Although the size of the founding stock and the N_e in captivity were fairly large, the lineage apparently originated with low diversity that was passed on to descendant refuge populations. This illustrates the perils of choosing a single, local population with unknown genetic diversity as the wild source for a lineage of refuges.

Reduced variability in several lineages probably reflects, to a large extent, genetic drift in the parent refuge population subsequent to founding. The WLM lineage, for example, originated in 1976 with 64 fish from five separate springs and was kept in a small backyard pool (<4 m^2 in surface area) until 2002, when about 75 fish of mixed gender and age were transplanted to an aquarium at Arizona State University (P. Marsh, pers. comm.). Although the population was estimated to have "persisted in the low hundreds" (Dunham and Minckley 1998:10), the long-term N_e undoubtedly was considerably lower because of breeding-male territoriality and fluctuations in population size, which varied from perhaps "a few 10s to several hundreds" (P. Marsh, pers. comm.). The consistently low diversity among populations of this lineage illustrates the importance of ensuring that parent refuges are sufficiently large to avoid passing low diversity on to descendant refuges.

The population at Boyce Thompson Arboretum (BT) shows the effect of lineage mixing, together with population size. This large refuge (~6000 m^2), which was established in 1984-1985, was stocked with pupfish from both DNFH and WLM. Consequently, the estimates of allele richness and effective population size were higher for BT than for any other population in the DNFH or WLM lineages (A_R = 9.3 vs 4.4-6.9; N_e = 435 vs 151-234), including the global populations of those lineages (A_R = 6.9 and 7.0; N_e = 297 and 383). Additionally, branch lengths in the neighbor-joining tree (Fig. 6) indicate that BT is less divergent from the wild populations than are the unmixed WLM and DNFH populations.

One population, LD1, is noteworthy because (except for the recently established OPC) it is the only refuge population that retains genetic diversity within the range for the wild populations (A = 14.8 vs 12.0-16.5; A_R = 11.5 vs 10.8-13.6). This refuge was stocked only with wild fish, 10 in1985 and 250 in 1987 (S. Keeney, pers. comm.), and it is one of the largest refuges (470 m^2) in our study. The success in retaining diversity after two decades probably is a result of the size of the refuge, together with the inoculation from the wild, two years after it was founded with an inadequate number of fish.

The general lack of association between diversity and the history of management for individual refuges of *C. macularius*, except for marginal significance for number of founding steps and lineage age, probably is the result of pupfish biology. The amount of genetic variation remaining after a bottleneck is a function of not just the severity of the bottleneck, but how rapidly the population size rebounds (Nei et al.1975). As mentioned earlier, various aspects of pupfish biology (small body size, omnivory, extended breeding season, early age at maturity, and extreme hardiness) would promote rapid rebound from population bottlenecks, thereby moderating losses of diversity. The majority of refuges for *C. macularius* are in the DNFH and WLM lineages, which experienced reduced diversity as results of either severe founder effect in the local, wild population used as the source for the lineage (DNFH) or an extended bottleneck resulting from the small size of the parent refuge (WLM). Other factors in the management of these lineages appear to have had relatively little effect on the overall pattern of diversity.

In conclusion, the global refuge program has been reasonably successful at maintaining the original diversity in wild populations. However, there have been declines in local populations, which, if continued unchecked, will result in unacceptable levels of overall diversity. Fortunately, wild populations still exist and managers have a number of available options. At

one extreme, some of the existing refuge populations could be destroyed and replaced with stock from the wild. On the other hand, all existing stocks could be retained and managed in a way to increase levels of diversity. Removal of a portion of the population in individual refuges and replacement with individuals from the wild can have an immediately large effect on genetic diversity. This is logistically simple, particularly for the smaller refuges where a single seine haul can remove a large proportion of the adults. Part III of this report provides more detailed recommendations for refuge management.

The prognosis for wild populations of the Desert Pupfish complex is not good (Dunham and Minckley, 1998). The river segment supporting one of the two existing wild populations of *C. eremus* (Rio Sonoyta) could disappear rather soon as a result of an ongoing drought and habitat desiccation (C. Minckley, pers. comm.). Most populations of *C. macularius* in the lower Colorado River delta and the Salton Sea area are sparse and severely threatened from interactions with non-native fishes (Hendrickson and Varela-Romero 1989; Varela-Romero et al. 2002; Martin and Saiki 2005).

Fortunately, pupfish require "little more than water to survive as long as non-native species are excluded" (Dunham and Minckley 1998). They can, therefore, be maintained in a diversity of refuge situations with relatively little management beyond monitoring for non-natives and amount of habitat. With minor, relatively simple alterations in management, the refuge programs for *C. macularius* and *C. eremus* appear adequate to preserve a large proportion of the wild genetic diversity.

PART III: PROTOCOL FOR EXCHANGE OF GENETIC MATERIAL

INTRODUCTION & RATIONALE

The Desert Pupfish Recovery Plan (USFWS 1993) recommends a protocol for genetic exchange among refuges that uses "quantitative modeling techniques to determine the frequency and number of individuals to be exchanged between populations and to ensure that each desert pupfish stock maintains its genetic integrity." Quantitative modeling is beyond the scope of this report. Instead, we present general guidelines based on genetic theory, pupfish biology, and, to some extent, logistic feasibility in the face of limited resources.

Many recommendations for conservation genetics management depend on the ratio (N_e/N) between the observed number of adults (N) and the effective population size (N_e). Referring to Frankham's (1995) average of 0.10 (10%) in a review of N_e/N in natural populations, Allendorf and Luikart (2006) state that "20% of the adult population size is perhaps a better general value to use for N_e." Actual values vary depending on social structure, sex ratio, and other factors (Allendorf and Luikart 2006).

Presently, the only available pupfish data relevant to N_e/N is from the refuge population of *C. macularius* at DNFH, which suggests that N_e is a large proportion of N. Our point estimate of N_e (529) between 1998 and 2005 is consistent with the report (M. Ulibarri, pers. comm.) that the population has been continually maintained in excess of 500 fish. The latter value is based on estimated numbers of fish recovered in the fall, brought into aquaria for the winter, and returned to the hatchery pond in the spring. If $N = 500$ is as much as 100% less than the consistently maintained population size, then N_e is about 50% of N. For purposes of gaining rough insight into a protocol of exchange, we will assume $N_e/N = 0.50$.

The minimum effective population size (N_e) required to prevent extinction in the long-term (i.e., over hundreds or thousands of generations) is between 500 and 1000 according to Franklin and Frankham (1998) and between 1,000 and 5,000 according to Lynch and Lande (1998). If we accept the common ground of extremes between those two alternatives ($N_e = 1{,}000$) and if N_e/N is 0.50, then 2,000 adults would be required to maintain the long-term evolutionary success of a single, isolated population in the Desert Pupfish complex. At such a size, two primary factors operate in preserving long-term evolutionary adaptability: (1) genetic drift is less likely to cause losses of rare alleles contributing to additive genetic variation for evolutionarily important quantitative characters, and (2) natural selection prevents the buildup in frequency of mildly deleterious mutations that, over evolutionary time, can result in "mutational meltdown" and extinction (Lynch and Lande 1998).

Avoidance of unwanted short-term effects (e.g., during founding of a population) of inbreeding depression and loss of alleles requires relatively small effective population sizes. A commonly mentioned rule of thumb is that this requires a minimum N_e of about 50 (Franklin 1980), although this should only be applied as a rough approximation (see Allendorf and Luikart 2007). If N_e is 50% of N, then achieving $N_e = 50$ requires 100 adults.

The average number of migrants per generation required to preserve genetic diversity in a subpopulation without compromising local adaptation (e.g., allele frequencies for quantitative traits) is about 1-8 migrants over a wide range of values for N_e/N and size (N) of the recipient population (Mills and Allendorf 1996; Vucetich and Waite 2001). Only two migrants per generation are required when $N > 50$ and N_e/N is near the 0.50 value used in the above paragraphs; this increases to only about 8 migrants if N_e/N is as low as 0.10 (Vucetich and Waite 2001). In addition, these numbers represent an *average* number per generation, such that immigration can occur in pulses with larger numbers of migrants. Such estimates are based only on genetics, and other factors can affect the optimum number of migrants.

Mills and Allendorf (1996), while "hesitating to give 'cookbook' prescriptions," made the following suggestions: "an increase in migration above the mean of one [or two] migrant[s] per generation may be desirable under the following conditions [our comments in brackets]:

(1) inbreeding depression is thought to be a problem in the local population [negligible with a program of gene flow and $N_e > 50$];
(2) migrants are closely related to each other or to the local population;
(3) social, behavioral, or logistical factors prevent single individuals from immigrating, so that movement is in pulses of several animals every several generations [for the Desert Pupfish complex, this is probably dictated by logistical factors alone];
(4) N_e is much less than total population size [e.g., 10% or less; Vucetich and Waite 2001];
(5) migrants are likely to be at a disadvantage in terms of survival and breeding success [because of male territoriality in pupfish, this likely applies primarily to male migrants];
(6) the receiving population has been isolated for many generations [like many of the existing refuge populations of the Desert Pupfish complex]; and
(7) demographic or environmental variation indicates a high danger of extinction without aggressive supplementation."

In theory, there are reasons why too much gene flow might be a problem for the management of genetic resources in a network of refuge populations. These include outbreeding depression, negating local adaptation, and disruption of social structure. Mills and Allendorf (1996) ended their discussion of such concerns with the suggestion that, "up to 10 migrants per generation is not likely to tip the balance too far by causing uniformity of allele frequencies across subpopulations." In the protocol below, we recommend minimal levels (1-2 migrants/generation) to keep from homogenizing allele frequencies in a given set of primary/secondary populations.

A HIERARCHY OF REFUGES: PRIMARY & SECONDARY POPULATIONS

Most refuges in Part II of this report qualify as Tier 3 populations in the Desert Pupfish Recovery Plan (USFWS 1993); that is, they are artificially established populations in natural or quasi-natural (non-aquarium) refuges that may be considered suboptimal habitat. Tier 1 populations are extant wild populations, and Tier 2 populations are replicates (of wild populations) artificially established in the best available wild habitats within the probable historical range.

The plan recommended exchanges from Tier 2 to Tier 3 and among Tier 3 populations, but not from Tier 3 to Tier 2. To our knowledge, attempts to establish Tier 2 populations generally have been unsuccessful.

To facilitate management of Tier 3 populations, we recommend establishing four sets of refuge populations, minimally two for each species. Each set would include two types of refuges (1) a large, well-managed "primary-refuge" population, and (2) a group of "secondary-refuge" populations. The primary-refuge populations would be continually maintained at large sizes, preferably 2000 adults or more, and (as long as wild stocks are available) they would periodically receive "migrants" via artificial inoculation with fish from the wild-source regions.

The secondary-refuge populations would be continually maintained at 100 adults or more. These populations would receive migrants from the primary population at an average rate of about 1-2 immigrants per generation for each population, which is low enough to prevent severe inbreeding and maintain adequate diversity, but not so high as to create among-refuge homogeneity in allele frequencies.

The population sizes required to serve short-term and long-term goals can be relaxed by implementation of a program of artificial gene flow among refuge populations (Lacy 1987; Mills and Allendorf 1996; Vucetich and Waite 2001). However, given our crude approximation of N_e/N and uncertainties regarding factors such as the effects of refuge size on social structure, effective sex ratios, and other variables, we recommend that refuge managers aim for minimum, continually maintained sizes of $N > 2000$ for primary refuge populations. For the secondary refuges, population sizes of $N > 100$, together with a program of artificial immigration, should be adequate to avoid severe inbreeding and loss of diversity via genetic drift. The population sizes recommended for primary and secondary refuges generally are relatively easily achieved with pupfish and, with a program of genetic exchange, should be adequate to preserve the global long-term evolutionary adaptability of the species.

The primary-refuge/secondary-refuge design represents an effort to strike a balance between what is ideal (generally, larger and greater numbers of populations) and what seems logistically feasible. For example, designating primary-refuge populations is an attempt to have a few, relatively large, especially well-managed populations, as opposed to having management distributed more diffusely over a larger number of refuges. These primary refuges can be monitored and managed for high genetic diversity, which then is parceled out to a second set of refuges that, collectively, serve two important roles: (1) they guard against severe losses of diversity in the global refuge population should catastrophic loss occur in the primary-refuge, and (2) they inflate the effective population size of the global refuge population to levels that should preserve long-term evolutionary adaptability.

The suggested protocol rests in part on inadequate information, particularly N/N_e and effects of refuge variables (e.g., size and structure) on N/N_e. For example, if N/N_e is considerably lower than 0.50, then larger population sizes are required to serve the goals of genetic conservation. On the other hand, if N/N_e is larger than 0.50, then those numbers are reduced. So long as diverse wild populations are available, adjustments to the refuge program are rather easily achieved based on future knowledge without much loss of diversity.

A SUGGESTED PROTOCOL OF EXCHANGE

1. Establish primary-refuge populations or identify such populations among existing refuges:

1.1. Two for *C. eremus*, one for each of the following wild populations:
- 1.1.1. Río Sonoyta
- 1.1.2. Quitobaquito

1.2. Two for *C. macularius*, one for each of the following groups of wild populations:
- 1.2.1. Colorado River Delta (El Doctor/Santa Clara Slough/Laguna Salada/Cerro Prieto)
- 1.2.2. San Felipe Creek/Salton Sea area

1.3. Each primary refuge should provide sufficient habitat, or be managed (e.g., supplemental feeding) to continually maintain populations at more than 2000 adults. That is, the number should not drop below 2000.

1.4. Assure diversity in the primary populations
- 1.4.1. Use a large number of founders; 250 fish or more.
- 1.4.2. Introduce fish early in breeding season to allow rapid increase in population size.
- 1.4.3. Genetically assay the potential source-population prior to establishment OR . . .
- 1.4.4. Use a mixture of fish from several areas within the region represented by the primary refuge--for example:
  - 1.4.4.1. Lower Colorado River: Laguna Salada, El Doctor, Cerro Prieto
  - 1.4.4.2. Salton Sea: San Felipe Creek, Sebastián Marsh, shoreline pools
- 1.4.5. If primary populations identified among existing refuges have low diversity (below the range for wild populations) remove a large number of adults (say 10% or more) and replace with an equivalent number from the wild.

2. Monitor population size and genetic diversity in the primary-refuge populations

2.1. Census adult population size annually

2.2. Assay genetic diversity
- 2.2.1. Minimally once every five years
- 2.2.2. Assay a minimum of 50 individuals for at least seven microsatellite loci
- 2.2.3. Compute genetic diversity indexes and effective population size for each interval between assays

3. Periodically inoculate primary refuges with fish from the wild

3.1. Aim for an average of 1-4 adult "immigrants" per generation for each primary refuge
- 3.1.1. Translates to 2-8 fish per year (assuming 2 generations/yr)
- 3.1.2. Use larger numbers if transplantations occur at longer intervals

3.2. Bias immigrant sex ratio toward females, say 2:1 or greater.

3.3. Use fish from the same region (not necessarily the same locality--see 3.4) that served as the original wild-source for the refuge population

3.4. Avoid repeated inoculation with fish from the same wild locality
- 3.4.1. alternate among localities within a wild-source region, OR . . .
- 3.4.2. use fish from more than one locality in a given inoculation

3.5. Assure high diversity in the transplanted fish.
- 3.5.1. Avoid using fish from small or potentially just-colonized situations where the population might have undergone severe bottlenecking.
- 3.5.2. Inoculate with fish from several widely separated populations (different spring systems, etc.) OR . . .
- 3.5.3. Take fish from a locality where the population (a) is known from previous genetic surveys to have high diversity and (b) there is some assurance that it has not undergone subsequent bottlenecking, OR . . . .
- 3.5.4. Assay genetic diversity prior to releasing individuals into the primary population.
  - 3.5.4.1. Assay at least 7 microsatellite loci (see Part I of this report).
  - 3.5.4.2. Assay a minimum of 30 fish sampled non-lethally--e.g., pelvic fin clips
  - 3.5.4.3. Compute diversity indexes, compare with results for wild populations in Part I of this report.

3.6. Avoid unwanted transplants, particularly non-native species and disease organisms.
- 3.6.1. Examine each potential immigrant for identification to species
- 3.6.2. If other pupfish species are suspected, discard the entire collection (not just suspect individuals) because the collection is likely to contain hybrids--OR . . .
- 3.6.3. Genetically assay a sample of 30 or so at a minimum of one informative locus--one such strategy is as follows:
  - 3.6.3.1. Sequence the mitochondrial ND2 gene (this has has been done for most species of *Cyprinodon* and the sequences are deposited in GenBank.
  - 3.6.3.2. Use the BLAST option in GenBank to search for the species of origin for the sequence.
- 3.6.4. Use accepted procedures to guard against transferring parasites and diseases into the population.

4. Establish and/or maintain a set of 10 or more secondary-refuge populations representing the source-region for each primary-refuge population.

4.1. Each refuge should provide sufficient habitat to continually maintain populations at 100 adults or more.

4.2. For new refuges, use as founders, a minimum of 100 fish from the primary-refuge population or from the wild source of that population.

4.3. Periodically inoculate the secondary populations with fish from the corresponding primary-refuge population.
    4.3.1. Aim for an average of 1-2 migrants per generation (2-4/year) for each refuge.
    4.3.2. For extremely small refuge populations (<100) that have not been inoculated for several years, remove a moderate proportion of the population, say 10% or so, and replace with an equivalent number of migrants.
    4.3.3. For existing secondary populations with low diversity, destroy and re-establish, or remove a large proportion and replace with an equivalent number of migrants. See Figure 10 for recommendations for existing refuges.
    4.3.4. For guidance with respect to the existing refuges, "Low diversity" is arbitrarily defined in this report as <70% of the allele richness of the wild populations (12.5 alleles/locus/population in this study).
4.4. Avoid unwanted transplants, particularly non-native species and disease organisms (see item 3.6)

ACKNOWLEDGMENTS

We thank C. Minckley, G. Knowles, and B. Bagley for providing the Mexican collections and numerous people, but especially P. Barrett, A. F. Echelle, A. Hervatin, S. Keeney, J. Schooley, and R. Vu-Loftis for assistance in the field, S. Keeney, B. Kynard, P. Martin, P. Siminski, and J. Voeltz for information on refuge histories, and D. Duncan and R. Clarkson provided helpful comments on an earlier draft of the report. The Oklahoma Cooperative Fish and Wildlife Research Unit provided administrative assistance.

LITERATURE CITED

Allendorf, F. W. 1986. Genetic drift and the loss of alleles versus heterozygosity. Zoo Biol. 5:181-190.

Allendorf, F. W. 1988. Conservation biology of fishes. Conservation Biology 2:145-148

Allendorf, F. W., and G. Luikart. 2007. Conservation and the genetics of populations. Blackwell Publishing, Malden, MA.

Allendorf, F.W., and S.R. Phelps. 1980. Loss of genetic variation in a hatchery stock of cutthroat trout. Trans. Am. Fish. Soc. 109:537–543.

Allendorf, F.W., and N. Ryman. 1987. Genetic management of hatchery stocks, p. 141-159. *In*: Population Genetics and Fisheries Management (N. Ryman and F. M. Utter., eds.). Univ. Washington Press, Seattle.

Barlow, G. W. 1961. Social behavior of the desert pupfish, *Cyprinodon macularius* in the field and in the aquarium. Amer. Midl. Nat. 65:339-359.

Bonnet E, and Y. Van de Peer. 2002. ZT: a software tool for simple and partial Mantel tests. J. Stat. Software 7:10.

Brookfield, J. F. Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Molecular Ecology 5:453-455.

Burg, T. M., J. L. Wilcox, and A. Martin. 2002. Isolation and characterization of polymorphic microsatellite loci in pupfish (genus *Cyprinodon*). Conserv. Genetics 3:197-204.

Carpelan, L. H. 1961. History of the Salton Sea, p. 9-15. *In*: The ecology of the Salton Sea, California, in relation to the sportfishery. B. W. Walker (ed.). California Department Fish Game, Fish. Bull. 113.

Constantz, G. D. 1981. Life history patterns of desert fishes, p. 237-290. *In*: Fishes in North American Deserts. (R. J. Naiman and D. L. Soltz, eds.). John Wiley and Sons, NY.

Contreras-B., S., M. L. Lozano-V. 1994. Water, endangered fishes, and development perspectives in arid lands of Mexico. Conservation Biology 8:379-387.

Cox, T. J. 1966. A behavioral and ecological study of the desert pupfish (*Cyprinodon macularius*) in Quitobaquito Springs, Organ Pipe National Monument, Arizona. Unpubl. Ph.D. diss., Univ. Arizona, Tucson.

Crandall, K. A., O. R. P. Bininda-Emonds, G. M. Mace, and R. K. Wayne. 2000. Considering evolutionary processes in conservation biology. Trends Ecol. Evol. 15:290-295.

Culver, M., M. A. Menotti-Raymond, and S. J. O'Brien. 2001. Patterns of size homoplasy at 10 microsatellite loci in pumas (*Puma concolor*). Mol. Biol. Evol. 18:1151-1156.

Deacon, J. E., and C. D. Williams. 1991. Ash Meadows and the legacy of the Devils Hole pupfish, p. 69-92. *In*: Battle against extinction: Native fish management in the American West. W. L. Minckley and J. E. Deacon (eds.). Univ. of Arizona Press, Tucson.

DeWoody, J.A., and J. C. Avise. 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. Journal of Fish Biology 56:461–473.

Douglas, M. E., and J. A. Endler. 1982. Quantitative matrix comparisons in ecological and evolutionary investigations. J. Theoret. Biol. 99: 777-795.

Douglas, M. R., M. E. Douglas, and P. C. Brunner. 2001. Population estimates, movements, and size structure of the endangered Quitobaquito pupfish, *Cyprinodon macularius eremus*. Southwest. Nat. 46:141-150.

Dowling, T. E., W. L. Minckley, P.C. Marsh, and E. Goldstein. 1996. Mitochondrial DNA diversity in the endangered razorback sucker (*Xyrauchen texanus*): analysis of hatchery stocks and implications for captive propagation. Conserv. Biol. 10, 120–127.

Dunham, J. B., & W. L. Minckley. 1998. Allozymic variation in desert pupfish from natural and artificial habitats: genetic conservation in fluctuating populations. Biol. Conserv. 84:7-15.

Duvernell, D. D., and B. J. Turner. 1998. Evolutionary genetics of Death Valley pupfish populations: mitochondrial DNA sequence variation and population structure. Molecular Ecology 7:279-288.

Echelle, A. A. 1991. Conservation genetics and genic diversity in freshwater fishes of western North America, p. 141-154. *In*: Battle Against Extinction: Native Fish Management in the American West (W. L. Minckley and J. E. Deacon, eds.). Univ. Arizona Press, Tucson.

Echelle, A. A. *In Press*. The western pupfish clade (Cyprinodontidae: *Cyprinodon*): mtDNA divergence times and drainage history. *In*: Late Cenozoic Drainage History of the Southwestern Great Basin and Lower Colorado River Region: Geologic and Biotic Perspective (M. Reheis and R. Hershler, eds.). Special Paper, Geological Society of America.

Echelle, A. A., and A. F. Echelle. 1993. Allozyme perspective on mitochondrial DNA variation and evolution of the Death Valley pupfishes (Cyprinodontidae: *Cyprinodon*). Copeia 1993:275-287.

Echelle, A. A., and A. F. Echelle. In press. Cyprinodontidae: The Pupfishes. In R. L. Mayden and B. M. Burr, editors. North American Freshwater Fishes: Evolution, Ecology, and Behavior. Academic Press, NY.

Echelle, A. A., A. F. Echelle, and D. R. Edds. 1987. Population structure of four pupfish species (Cyprinodontidae: *Cyprinodon*) from the Chihuahuan desert region of New Mexico and Texas: allozymic variation. Copeia 1987:668-681.

Echelle, A. A., A. F. Echelle, S. Contreras-B., M. L. Lozano-V. 2003. Pupfishes of the northern Chihuahuan Desert: Status and conservation, p. 111-126. *In*: Aquatic fauna of the northern Chihuahuan Desert (N. Allan and G. P. Garrett, eds.). Special Publ. Mus. Texas Tech Univ., Lubbock.

Echelle, A. A., A. F. Echelle, and L. G. Hill. 1972. Interspecific interactions and limiting factors of abundance and distribution in the Red River pupfish, *Cyprinodon rubrofluviatilis*. American Midland Naturalist 88:109-130.

Echelle A.A., E.W. Carson, A.F. Echelle, R.A. Van Den Bussche, T.E. Dowling, A. Meyer. 2005. Historical biogeography of the New World genus *Cyprinodon* (Teleostei: Cyprinodontidae). Copeia 2005:320–339.

Echelle, A. A., R. A. Van Den Bussche, T. P. Malloy, Jr., M. L. Haynie, & C. O. Minckley. 2000. Mitochondrial DNA variation in pupfishes assigned to the species *Cyprinodon macularius* (Atherinomorpha: Cyprinodontidae): taxonomic implications and conservation genetics. Copeia 2000:353-364.

Edds, D. R., & A. A. Echelle. 1989. Genetic comparisons of hatchery and natural stocks of small endangered fishes: Leon Springs pupfish, Comanche Springs Pupfish, and Pecos Gambusia. Trans. Am. Fish. Soc. 118:441-446.

Excoffier, L., P. Smouse, and J. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131:479-491.

Follett, W.I. 1960. The freshwater fishes: Their origins and affinities. Symposium on biogeography of Baja California and adjacent seas. Syst. Zool. 9:212-232.

Frankham, R. 1995. Effective population-size adult population size ratios in wildlife: a review. Genet. Res. 66:95–107.

Frankham, R., J. D. Ballou, and D. A. Briscoe. 2002. Introduction to Conservation Genetics. Cambridge Univ. Press, New York.

Franklin, I. R. 1980. Evolutionary change in small populations, p. 135-150. *In*: Conservation Biology: An Evolutionary-Ecological Perspective (M. Soulé and B. A. Wilcox, eds.). Sinauer Associates, Sunderland, MA.

Franklin, I. R., and R. Frankham. 1998. How large must populations be in order to retain evolutionary potential. Anim. Conserv. 1:69-73.

Girard, C. 1859. Ichthyological Notices. XLI-LIX. Proceedings of the Academy of Natural Sciences, Philadelphia 11:157-161.

Glaubitz, J.C. 2004. CONVERT: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Molecular Ecology Notes 4:309-310.

Goudet, J. 1999. PCA-GEN Version 1.2. Institute of Ecology, Biology Building, UNIL, Lausanne, Switzerland.

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. J. Hered. 86: 485-486.

Goudet, J. 1999. PCA-GEN Version 1.2. Institute of Ecology, Biology Building, UNIL, Lausanne, Switzerland.
Hardy, O. J., and X. Vekemans (2002). SPAGeDi : a versatile computer program to analyse spatial genetic structure at the individual or population levels. Molecular Ecology Notes 2:618-620.
Hardy, O. J., N. Charbonnel, H. Fréville and M. Heuertz, 2003. Microsatellite allele sizes: a simple test to assess their significance on genetic differentiation. Genetics 163:1467-1482.
Hendrickson, D. A., & A. Varela-R. (1989) Consevation status of desert pupfish, *Cyprinodon macularius* in Mexico and Arizona. Copeia 1995:478-483.
Hedrick, P. W. 1999. Perspective: Highly variable loci and their interpretation in evolution and conservation. Evolution 53:313-318.
Hedrick, P. W., and P. Miller. 1992. Conservation genetics: techniques and fundamentals. Ecological Applications 2:30-46.
Hendrickson, D. A., and A. Varela-Romero. 1989. Conservation status of desert pupfish, *Cyprinodon macularius*, in Mexico and Arizona. Copeia 1989:478–483.
Higgins, K., and M. Lynch. 2001. Metapopulation extinction caused by mutation accumulation. Proc. Natl. Acad. Sci. 98:2928-2933.
Jones, A. G., G. A. Stockwell, D. Walker, and J. C. Avise. 1998. The molecular basis of a microsatellite null allele from the White Sands pupfish. J. Hered. 89:339-342.
Jordan, D. S., and B. W. Evermann. 1896. The fishes of North and Middle America: a descriptive catalogue of the species of fish-like vertebrates found in the waters of North America, north of the Isthmus of Panama, Part 1. Bulletin of the United States National Museum 47:1-1240.
Kinne, O. 1962. Irreversible non-genetic adaptation. Comp. Biochem. Physiol. 5:265-282.
Koike, H. 2007. Genetic structure of refuge populations of the desert pupfish complex. Unpubl. M.S. thesis, Oklahoma State University, Stillwater.
Kumar, S, K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-163.
Lacy, R. 1987. Loss of genetic diversity from managed populations: interacting effects of drift, mutation, selection, and population subdivision. Conserv. Biol. 1: 143-157.
Lande, R. 1980. Genetic variation and phenotypic evolution during allopatric speciation. Am. Nat. 116:463–479.
Lesica, P., and F. W. Allendorf. 1995. When are peripheral populations valuable for conservation? Conserv. Biol. 9:753–760.
Lewis P. O., and D. Zaykin. 2001. Genetic Data Analysis Software, version 1. http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php.
Longmire, J. L., M. Maltbie, and R. J. Baker. 1997. Use of "Lysis Buffer" in DNA isolation and its implication for museum collections. Occasional Papers, Museum Texas Tech University 163:1–3.
Lynch, M., and R. Lande. 1998. The critical effective size for a genetically secure population. Anim. Conserv. 1:70-72.
MacDougal, D. T. 1906. The delta of the Rio Colorado. Bulletin of the American Geographic Society 38:1-16.

Martin, A. P., and J. L. Wilcox. 2004. Evolutionary history of Ash Meadows pupfish (genus *Cyprinodon*) populatons inferred using microsatellite markers. Conservation Genetics 5:769-782.

Martin, B. A., and M. K. Saiki. 2005. Relation of desert pupfish abundance to selected environmental variables in natural and manmade habitats in the Salton Sea basin. Environ. Fish Biol. 73:97-107.

Michalakis, Y., and L. Excoffier. 1996. A generic estimation of population subdivision using distances between alleles with special reference to microsatellite loci. Genetics 142:1061-1064.

Miller, R. R. 1943. The status of *Cyprinodon macularius* and *Cyprinodon nevadensis*, two desert fishes of western North America. Occ. Pap. Mus. Zool., Univ. Mich. 473:1-25.

Miller, R. R., and L. A. Fuiman. 1987. Description and conservation status of *Cyprinodon macularius eremus*, a new subspecies of pupfish from Organ Pipe Cactus National Monument Arizona. Copeia 1987:593-609.

Mills, L. S., and F. W. Allendorf. 1996. The one-migrant-per-generation rule in conservation and management. Conservation Biology 10:1509-1518.

Minckley, W. L. 1973. Fishes of Arizona. Arizona Game Fish Department, Phoenix, Arizona.

Minckley, W. L. 1995. Translocation as a tool for conserving imperiled fishes: Experiences in western United States. Biol. Conserv. 72, 297-309.

Minckley, W. L., and J. E. Deacon (eds.). 1991. Battle against extinction: Native fish management in the American West. Univ. of Arizona Press, Tucson.

Minckley, W. L., G. K. Meffe, and D. L. Soltz. 1991. Conservation and management of short-lived fishes: the cyprinodontoids, p. 247-282. *In*: Battle Against Extinction: Native Fish Management in the American West (W. L. Minckley and J. E. Deacon, eds.). Univ. Arizona Press, Tucson.

Moritz, C. 1994. Defining "evolutionarily significant units" for conservation. Trends Ecol. Evol. 9:373-375.

Moyle, P. B. 1976. Inland fishes of California. University of California Press, Berkeley.

Mueller, G. A., and P. C. Marsh. 2002. Lost, a desert river and its native fishes: A historical perspective of the lower Colorado River. Fort Collins, CO: U.S. Geological Survey. Information and Technology Report USGS/BRD/ITR 2002-0010. 69 pp.

Naiman, R. J. 1976. Productivity of a herbivorous pupfish population (*Cyprinodon nevadensis*) in a warm desert stream. J. Fish. Biol. 9:125-137.

Nei, M., T. Maruyama, and R. Chakraborty. 1975. The bottleneck effect and genetic variability in populations. Evolution 29:1–10.

Nelson, K, and M. Soulé. 1987. Genetical conservation of exploited fishes, p. 345-368. *In*: Population Genetics and Fisheries Management (N. Ryman and F. Utter, eds.). Univ. of Washington Press, Seattle.

O'Connell, M., and J. M. Wright. 1997. Microsatellite DNA in fishes. Rev. Fish Biol. Fisheries 7:331-363.

Orti, G., D. E. Pearse, and J. C. Avise. 1997. Phylogenetic assessment of length variation at a microsatellite locus. Proceedings of the National Academy of Science, USA 94:10745-10749.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using mulitlocus genotype data. Genetics 155:945–959.

Quattro, J. M., and R. C. Vrijenhoek. 1989. Fitness differences among remnant populations of the Sonoran topminnow, *Poeciliopsis occidentalis*. Science 245:976-978.
Raymond, M. and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. J. Heredity. 86:248-249.
Rolston, H., III. 1991. Fishes in the desert. Paradox and responsibility, p. 93-108. *In*: Battle against extinction: Native fish management in the American West. W. L. Minckley and J. E. Deacon (eds.). Univ. of Arizona Press, Tucson.
Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223-225.
Schneider, S., D. Roessli, & L. Excoffier. 2000. Arlequin: A software for population genetic data. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
Sheldon, A. L. 1988. Stream fishes: Patterns of diversity, rarity and risk. Conservation Biology 2:149-156.
Stockwell, C. A., M. Mulvey, and A. G. Jones. 1998. Genetic evidence for two evolutionarily significant units of the White Sands pupfish. Anim. Conserv. 1:213-225.
Stockwell, C. A., M. Mulvey, and G. L. Vinyard. 1996. Translocations and the preservation of allelic diversity. Conserv. Biol. 10:1133-1141.
Sykes, G. 1926. The delta and estuary of the Colorado River. Geographical Review 16:232-255.
Turner, B. J. 1983. Genetic variation and differentiation of remnant natural populations of the desert pupfish, *Cyprinodon macularius*. Evolution 37:690–700.
Turner, B. J. 1984. Evolutionary genetics of artificial refugium populations of an endangered species, the desert pupfish. Copeia 1984:364-369.
U. S. Department of the Interior. 1986. Endangered and threatened wildlife and plants; determination of endangered status and critical habitat for the desert pupfish. Federal Register 51:10843-10851.
U. S. Fish and Wildlife Service. 1993. Desert Pupfish Recovery Plan. Phoenix, Arizona. 93 pp.
van Oosterhout, C. V., W. F. Hutchinson, D. M. Willis, and P. Shipley. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535-538.
Varela-Romero, A., G. Ruiz-Campos, L. M. Yepiz-Velazquez, and J. Alaniz-Garcia. 2002. Distribution, habitat and conservation status of desert pupfish (*Cyprinodon macularius*) in the lower Colorado River basin, Mexico. Rev. Fish Biol. Fisheries 12:157-165.
Vucetich, J. A., and T. A. Waite. 2001. Migration and inbreeding: the importance of recipient population size for genetic management. Conservation Genetics 2:167-171.
Waples, R. S. 1989. A generalized approach for estimating effective population size from temporal changes in allele frequency. Genetics 121:379-91.
Waples, R. S. 1991. Pacific salmon, *Oncorhynchus* spp., and the definition of "species" under the Endangered Species Act. Mar. Fish. Rev. 53:11-22.
Waples R.S., 2002. Definition and estimation of effective population size in the conservation of endangered species, p. 147-168 *In*: Population Viability Analysis (Beissinger, S. R., and D. R. McCullough, eds.). University of Chicago Press.
Waples, R. S., and M.Yokota. 2007. Temporal estimates of effective population size in species with overlapping generations. Genetics 175:219-233.
Williams, J. E. 1991. Preserves and refuges for native western fishes: History and management, p. 171-189. *In*: Battle Against Extinction: Native Fish Management in the American West (W. L. Minckley and J. E. Deacon, eds.). Univ. of Arizona Press, Tucson.

Table 1. Population genetic statistics averaged over 7 microsatellite loci in *Cyprinodon macularius* and a separate suite of 7 loci in *Cyprinodon eremus*. Locality numbers are as in Figure 1. N = sample size, H_o = mean observed heterozygosity, H_e = mean expected heterozygosity, A = mean number of alleles, A_R = mean allele richness. A_P = number of private alleles in each species considered separately and (after the slash) when all populations of both species are considered together for the five shared loci.

	C. macularius									*C. eremus*	
	Salton Sea			Lower Colorado River Delta						Río Sonoyta Basin	
	1	2	3	4	5	6	7	8	9	10	11
N	19-20	18-19	24-25	19-20	20	20	19-20	20	20	19-23	20
H_o	0.878	0.882	0.897	0.883	0.879	0.871	0.892	0.950	0.843	0.830	0.850
H_e	0.870	0.922	0.914	0.914	0.892	0.900	0.926	0.923	0.925	0.840	0.842
A	13.3	15.6	17.1	14.6	13.0	15.6	16.9	16.6	16.0	14.3	11.9
A_R	11.5	13.0	14.1	14.0	11.7	13.4	14.8	14.2	13.5	10.6	8.8
A_P	4/4	8/5	8/5	4/1	0/0	5/4	6/4	2/1	4/1	46/4	29/1

Table 2. Pairwise estimates of R_{ST} (above diagonal) and F_{ST} (below diagonal) among 11 wild populations (numbered as in Fig. 1). Within species values are based on 7 loci (different suite for each species), while among species values are based on 5 loci in common between the two species. Population numbers are as in Figure 1. Bold values indicate statistical significance with the sequential Bonferroni correction (55 tests each half-matrix; <0.001 = initial *P* for significance at table-wide $\alpha = 0.05$).

	C. macularius									*C. eremus*	
	1	2	3	4	5	6	7	8	9	10	11
1		0.032	0.038	0.000	0.022	0.029	0.004	0.002	0.013	**0.216**	**0.283**
2	**0.033**		0.041	0.036	0.082	0.058	0.032	0.066	0.081	**0.322**	**0.371**
3	**0.029**	**0.007**		0.013	0.068	0.045	0.001	0.029	0.066	**0.290**	**0.364**
4	**0.020**	**0.016**	**0.017**		0.000	0.052	0.000	0.002	0.011	**0.250**	**0.309**
5	**0.050**	**0.039**	**0.037**	**0.028**		0.069	0.039	0.011	0.027	**0.267**	**0.291**
6	**0.040**	**0.030**	**0.030**	**0.017**	**0.029**		0.048	0.006	0.067	**0.177**	**0.231**
7	**0.030**	**0.019**	0.015	0.008	**0.022**	0.015		0.013	0.021	**0.245**	**0.335**
8	**0.030**	0.007	0.013	0.008	0.016	0.014	0.004		0.021	**0.205**	**0.268**
9	**0.053**	**0.021**	**0.025**	**0.019**	**0.028**	**0.027**	0.009	0.008		**0.103**	**0.168**
10	**0.128**	**0.119**	**0.118**	**0.097**	**0.108**	**0.103**	**0.093**	**0.099**	**0.104**		**0.100**
11	**0.118**	**0.103**	**0.094**	**0.094**	**0.109**	**0.103**	**0.093**	**0.101**	**0.103**	**0.025**	

Table 3. Analyses of molecular variance in wild populations of the Desert Pupfish complex. Based on allele size diversity (R_{ST}) for microsatellites and sequence diversity (Φ_{ST}) for mtDNA. 1, 2, and 3 asterisks signify significance at, respectively, $P < 0.05$, $P < 0.005$, and $P < 0.00001$.

	Percentage of total diversity attributable to differences:		
Groupings	Among groups	Among populations within groups	Within populations
C. macularius and *C. eremus*			
Microsatellites (5 loci), F_{ST}	8.0***	1.9***	90.0
Microsatellites (5 loci), R_{ST}	22.6***	2.0**	75.3
mtDNA, Φ_{ST}	86.1***	0.6*	13.3
C. macularius sites 1-3 & 4-9			
Microsatellites (7 loci), F_{ST}	0.8***	1.9***	97.3
Microsatellites (7 loci), R_{ST}	0.7	2.9***	96.4
mtDNA, Φ_{ST}	3.7*	2.1	94.2
C. eremus sites 10 & 11			
Microsatellites (7 loci), F_{ST}	2.5***	-	97.5
Microsatellites (7 loci), R_{ST}	10.0***	-	90.0
mtDNA, Φ_{ST}	2.9[a]	-	97.1

[a] $P = 0.19$

Table 4. Attributes of refuge populations of *C. macularius*. Population abbreviations are described in Appendix E.

Source/ Refuge population	Number of founders	Surface area of refuge (log m^2)	Years since founding	Number of supplements	Number of founding steps from the wild
Lower Delta					
DNFH98	280	2.30	22	0	1
DNFH05	280	2.30	22	0	1
DBG	250	2.95	18	0	2
AHS	100	1.02	1	1	2
DVH	300	2.90	8	0	2
IWM	150	1.60	2	2	3
LE	820	3.04	8	2	3
FWJ	90	1.32	19	0	3
BT	1450	3.78	20	0	3[a]
PZ	400	0.69	19	1	2
ASU1	50	2.48	17	0	3
CNWR	37	1.70	6	0	3
PVH	25	1.48	6	0	3
INWR	23	1.40	5	0	4
BWR	200	1.70	0.6	0	3
Salton Sea					
LD1	10[b]	2.67	20	1	1
LD2	40	1.67	33	0	2
AZBC	375	2.02	24	1	1
OS1	77	1.26	28	0	1
OS2	20	1.15	26	0	2
AZBP	45	1.18	24	1	2
AZBV	20	1.88	26	1	2
SS	203	1.45	23	0	2
DP1	395	3.00	15	0	4
DP2	198	2.70	15	1	4

[a] This is the average for the two parental lineages contributing to the founding of this population (see text).
[b] An additional 250 wild fish were added, two years after establishment of the population.

Table 5. Attributes of refuge populations of *C. eremus*. Population abbreviations are described in Appendix E. Unk = unknown.

Source/ Refuge population	Number of founders	Surface area of refuge (log m^2)	Years since founding	Number of supplements	Number of founding steps from the wild
Quitobaquito Springs					
SDM	Unk	1.16	28	0	2
TCP	Unk	0.70	18	0	3
ASU2	80	1.18	16	0	1
SCC	50	1.78	5	0	2
OPC	235	1.10	0.2	0	1
Río Sonoyta					
FT	150	2.30	3	0	2

Table 6. GenBank accession numbers and attributes for the six microsatellite loci (Burg et al. 2002) used in this study. T_{A1} and T_{A2} = PCR annealing temperatures.

Locus	Accession numbers	Marker size range	Repeat motif	T_{A1}/T_{A2}
GATA2	AF398010	207-351	$(GATA)_{30}$	45/48
GATA5	AF398011	181-281	$(GATA)_{39}$	50/53
GATA9	AF398012	213-361	$(GATA)_{29}$	50/53
GATA10	AF398013	140-340	$(GATA)_{33}$	50/53
GATA26	AF398018	200-288	$(GATA)_{39}(GACA)_3$	50/53
GATA39	AF398019	228-356	$(GATA)_{28}$	50/53

Table 7. Total numbers of alleles detected, numbers found only in refuge or wild population, and ratio of total number detected in refuges to the number detected in wild populations.

Species Locus	Number detected Refuge and wild	Only in refuges	Only in wild	Ratio Refuge:wild
C.macularius				
GATA2	35	0	5	0.86
GATA5	23	3	4	0.95
GATA9	33	3	5	0.93
GATA39	30	2	6	0.86
All loci	121	8	20	0.89
Percent of total	--	6.6%	16.5%	--
C.eremus				
GATA9	29	6	2	1.17
GATA10	28	2	4	0.92
GATA26	22	1	1	1.00
GATA39	19	2	1	1.06
All loci	98	11	8	1.03
Percent of total	--	11.2%	8.2%	--
C.eremus minus OPC				
GATA9	29	6	4	1.09
GATA10	27	1	6	0.81
GATA26	21	0	1	0.95
GATA39	18	1	1	1.00
All loci	95	8	12	0.95
Percent of total	--	8.2%	12.6%	--

Table 8. Population genetic statistics averaged over four microsatellite loci in wild and refuge populations of *Cyprinodon macularius*. Source (Lower Delta or Salton Sea area) is indicated for the refuge populations. Locality abbreviations are as in Appendix E for refuge populations and as in Figure 1 for wild populations. Global-1 = all refuge populations lumped as a single sample; Global-2 = one population from each refuge lineage lumped as a single sample. N = sample size, H_o = mean observed heterozygosity, H_e = mean expected heterozygosity, A = mean number of alleles, A_R = mean allele richness. For N_e, of individual refuges (i.e., not the global or lineage estimates) the values shown are the mean and maximum range of 95% confidence interval from multiple analyses, each with a different wild population as the potential source (See Methodology).

Populations	N	H_e	H_o	A	A_R	N_e (Refuge)	
						Mean	*CI*
Wild Populations							
Global Population	183-186	0.92	0.88	28.3	13.7	--	--
CLD	19-20	0.87	0.86	12.8	10.8	--	--
SFC	21-22	0.93	0.88	15.0	12.4	--	--
SPSS	25	0.91	0.90	16.3	12.4	--	--
CP	18-19	0.90	0.85	12.0	11.3	--	--
LS	20	0.88	0.93	13.3	11.3	--	--
CST	20	0.88	0.85	15.3	12.6	--	--
FDD	20	0.92	0.88	15.5	12.8	--	--
ED1	20	0.91	0.94	16.5	13.6	--	--
ED2	20	0.93	0.83	15.8	13.2	--	--
LCRD region (lumped)	118-119	0.92	0.87	24.5	13.8	--	--
Salton Sea area (lumped)	65-67	0.91	0.88	22.0	12.9	--	--
All wild (Avg.)	20.5	0.90	0.88	14.7	12.3	--	--
Refuge Populations							
Global-1	577-581	0.90	0.75	25.3	11.1	1059	777-1404
Global-2	199	0.90	0.79	21.5	11.4	1273	903-1755
Source: Lower Colorado River Delta (LCRD)							
DNFH lineage	140-142	0.81	0.77	12.0	7.0	297	212-402
DNFH98	32-33	0.76	0.81	8.3	6.2	151	114-468
DNFH05	24-25	0.78	0.80	7.3	6.4	236	80-277
DBG	25	0.82	0.90	8.3	6.9	239	123-468
AHS	21-22	0.80	0.67	7.5	6.7	231	114-433
DVH	24-25	0.77	0.71	6.8	5.8	205	109-362
IWM	19-20	0.77	0.72	7.3	6.6	228	112-436

LE	25	0.77	0.79	6.8	6.0	209	106-374
WLM lineage	163-165	0.78	0.69	11.8	6.9	383	274-518
FWJ	24-25	0.74	0.67	7.5	6.4	263	140-477
PZ	25	0.72	0.75	6.0	5.4	229	103-444
ASU1	24-25	0.60	0.59	5.0	4.4	199	94-344
CNWR	24	0.67	0.72	6.5	5.5	219	105-414
PVH	25	0.66	0.59	6.0	5.4	216	101-395
INWR	20	0.72	0.73	4.8	4.5	208	99-384
BWR	20-21	0.68	0.78	5.3	5.0	234	131-388
BT mixed lineage	24-25	0.84	0.82	11.3	9.3	435	215-961
LCRD (Avg.)	24	0.74	0.74	7.0	6.0	234	--
Source: Salton Sea area							
PC Lineage	100	0.87	0.77	17.8	9.9	783	541-1107
LD2	25	0.78	0.80	8.0	6.9	193	84-426
AZBP	25	0.70	0.64	6.5	5.6	317	179-565
AZBV	25	0.87	0.87	11.0	8.9	493	232-1044
SS	25	0.79	0.75	7.8	6.7	342	182-651
CVP Lineage	47	0.82	0.75	13	8.8	367	242-546
DP1	25	0.82	0.78	10.8	8.9	277	116-659
DP2	22-25	0.76	0.71	9.5	7.4	223	106-484
OS Lineage	25	0.8	0.72	12.3	8.3	665	436-1000
OS1	25	0.88	0.84	11.5	9.8	645	281-1847
OS2	25	0.61	0.59	4.5	3.9	212	104-385
Single population lineages							
AZBC	25	0.87	0.86	11.0	9.2	454	219-1227
LD1	25	0.88	0.81	14.8	11.5	817	327-2166
Salton Sea (Avg.)	24.9	0.80	0.77	9.5	7.9	397	--
All refuges (Avg.)	24.4	0.76	0.75	8.0	6.8	299	--

Table 9. Population genetic statistics averaged over four microsatellite loci in wild and refuge populations of *Cyprinodon eremus*. Locality abbreviations are as in Appendix E for refuge populations and as in Figure 1 for wild populations. Except for Finley Tank (FT), estimates of N_e used Quitobaquito Springs as the wild source. For FT, N_e estimated with the Río Sonoyta population as the wild source is also shown.[a] The recently established OPC refuge was not included in the global estimate or the average. Remainder of legend as in Table 5.

Populations	*N*	H_e	H_o	*A*	A_R	N_e (Refuge)	
						N_e	*CI*
Wild							
Global	42-43	0.94	0.84	21.8	14.4	--	--
QS	22-23	0.93	0.90	15.3	13.5	--	--
RS	20	0.94	0.93	16.8	13.1	--	--
All wild (Avg.)	21.25	0.94	0.92	16.1	--	--	--
Refuge							
Global-1	123-131	0.90	0.81	22.5	11.5	742	477-1156
Global-2	68-70	0.89	0.77	18.5	11.0	746	470-1198
SDM lineage	44-45	0.77	0.70	8.5	6.5	360	239-529
SDM	24-25	0.72	0.67	6.5	5.6	275	190-506
TCP	20	0.75	0.75	5.5	5.2	290	192-551
ASU2 lineage	42-50	0.88	0.86	16.5	10.6	493	302-828
ASU2	24-25	0.86	0.86	13.5	10.3	470	317-1932
SCC	17-25	0.87	0.87	12.3	10.1	383	265-1574
Single population lineages							
OPC	16	0.94	0.92	14.8	13.5	∞	169-∞
FT	19-20	0.87	0.79	11.3	10.0	456 561	267-808 304-1149
All refuges (Avg.)	20.92	0.84	0.81	10.7	9.1	396[b]	--

[a] Finley Tank was the only population of *C. eremus* showing a larger N_e when Río Sonoyta was treated as the wild source rather than Quitobaquito Springs.
[b] Computed with the larger of the two estimates for FT.

Table 10. Distribution of diversity in refuge and wild populations of the Desert Pupfish Complex. Asterisks signify probability: * < 0.01, ** < 0.00001.

	Percentage of total diversity attributable to differences:		
Species: Populations	Among groups	Among populations	Within populations
C. macularius			
Wild	--	1.8**	98.2
Refuge[a]	7.6**	8.9**	83.5
Wild vs Refuge	1.9*	12.2**	85.9
C. eremus			
Wild	--	2.1**	97.9
Refuge[b]	7.5**	4.0**	88.5
Wild vs Refuge	0.0	7.5**	92.6

[a] Groups = the eight lineages identified in Table 5, including the mixed BT lineage as a single-population lineage.
[b] Groups = SDM, ASU2 and FT lineages; the recently founded OPC lineage was excluded.

Table 11. Results of Mantel tests of correlation between genetic diversity ($A_{R(SOURCE)}$, H_{eDIFF} and $H_{e(SOURCE)}$) and various independent variables. P = probability; r = correlation coefficient.

Comparison	Covariate	P	r
Number of founding steps vs:			
$A_{R(SOURCE)}$	---	0.12	0.10
$H_{eSOURCE}$	---	**0.06**	0.13
$A_{R(SOURCE)}$	Number of founders	0.13	0.10
$H_{eSOURCE}$	Number of founders	**0.05**	0.13
$A_{R(SOURCE)}$	Refuge size	0.15	0.09
$H_{eSOURCE}$	Refuge size	**0.06**	0.14
$A_{R(SOURCE)}$	Number of supplements	0.12	0.10
$H_{eSOURCE}$	Number of supplements	**0.06**	0.13
$A_{R(SOURCE)}$	Age of lineage	0.10	0.11
$H_{eSOURCE}$	Age of lineage	**0.05**	0.14
Age of lineage	---	0.15	-0.07
Age of lineage vs:			
$A_{R(SOURCE)}$	---	**0.06**	0.16
$H_{eSOURCE}$	---	**0.09**	0.11
$A_{R((SOURCE)}$	Number of founding steps	**0.05**	0.17
$H_{eSOURCE}$	Number of founding steps	**0.07**	0.12
Local Refuges			
Number of founders vs H_{eDIFF}	---	0.26	0.04
Refuge size vs H_{eDIFF}	---	0.21	0.07
Number of supplements vs H_{eDIFF}	---	0.65	-0.00
Year Since Establishment vs H_{eDIFF}	---	0.29	0.04
Number of founders vs refuge size	---	**0.0002**	0.88

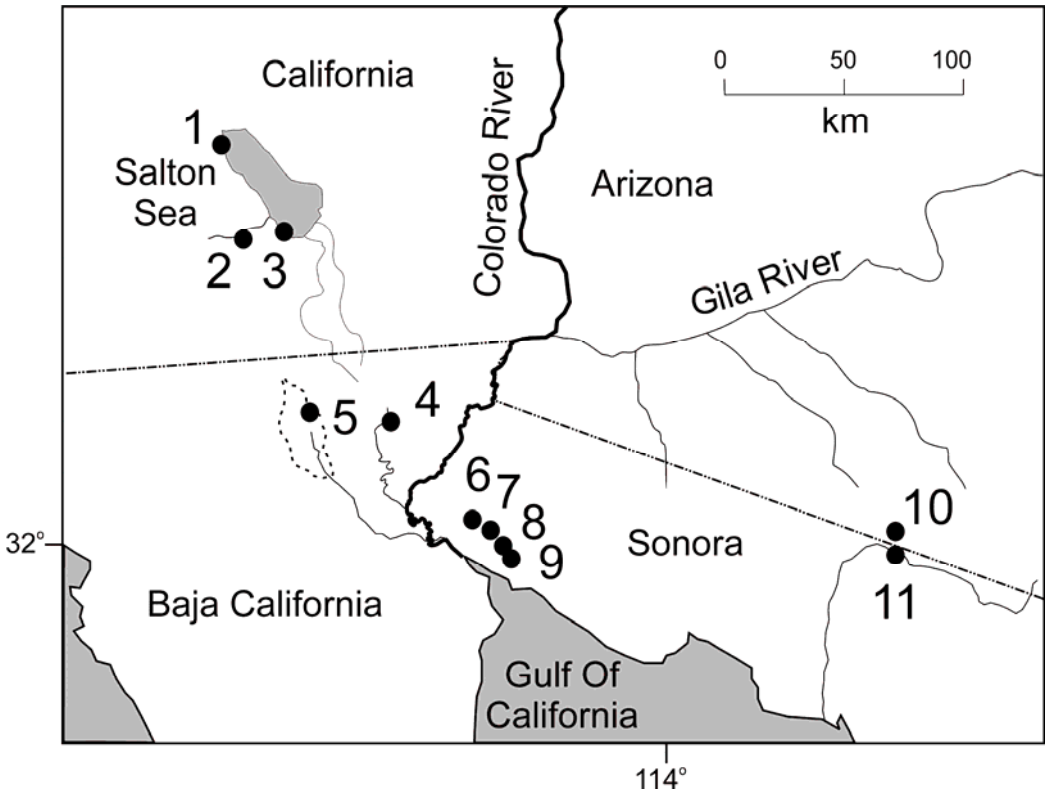

Figure 1. Collection localities for wild populations of the Desert Pupfish complex. Localities 1-9 represent the remaining populations of *C. macularius*, which historically occurred in the Gila River of Arizona and the lower Colorado River of California and Arizona. Localities 10 and 11 represent the two populations of *C. eremus*. Locality abbreviations used in the text are as follows: 1 = County Line Drain (CLD), 2 = San Felipe Creek (SFC), 3 = shoreline pool of Salton Sea (SPSS), 4 = Cerro Prieto (CP), 5 = Pozo del Tules in Laguna Salada (LS), 6 = Canal Sanchez Taboada (CST), 7 = Flor del Desierto (FDD), 8 = El Doctor 1 (ED1), 9 = El Doctor 2 (ED2), 10 = Quitobaquito Springs (QS), 11 = Río Sonoyta (RS).

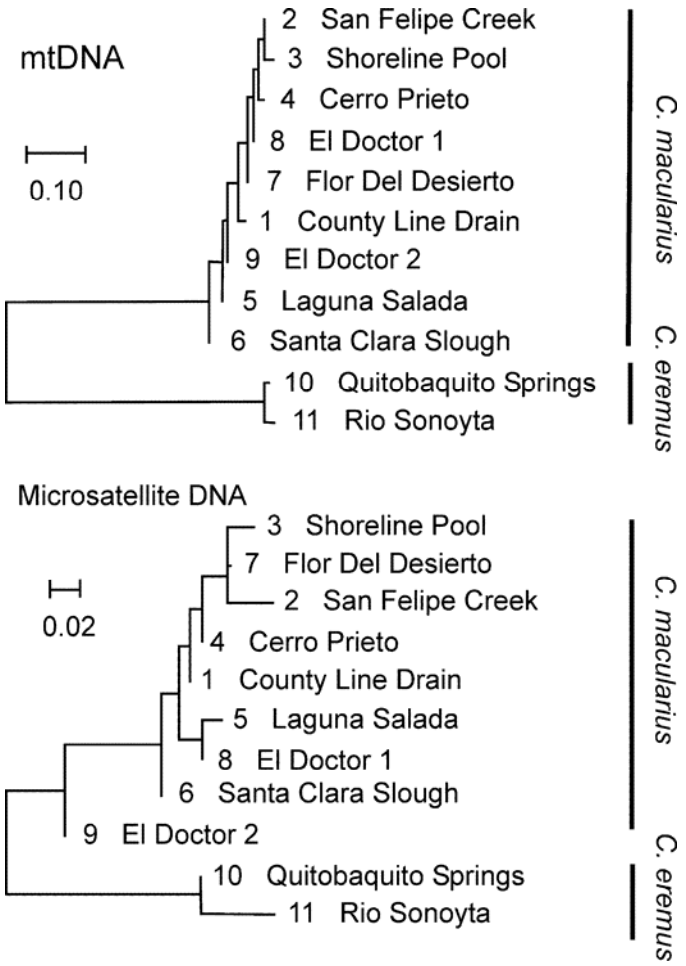

Figure 2. Neighbor-joining trees based on pairwise Φ_{ST} (top) and R_{ST} (bottom) values. Numbers on terminal nodes are from Figure 1. The microsatellite tree is based on the five loci in common between the two species.

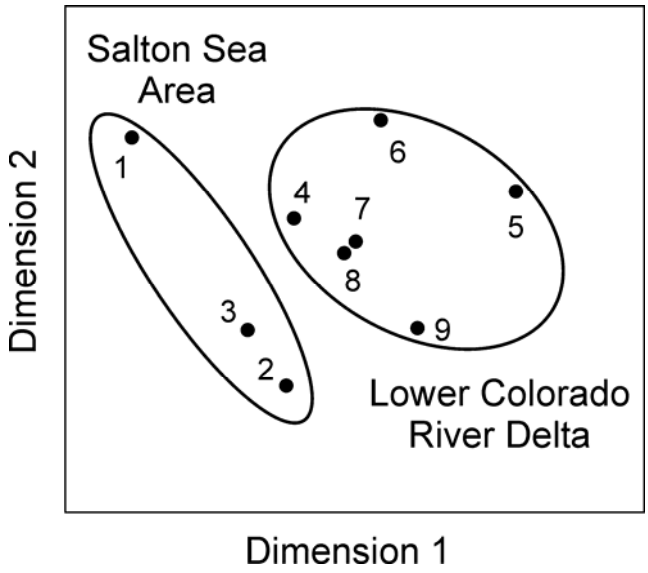

Figure 3. Multidimensional scaling of pairwise F_{ST} values from microsatellite DNA variation in *C. macularius*. Based on the seven loci scored for this species. Population numbers are from Figure 1.

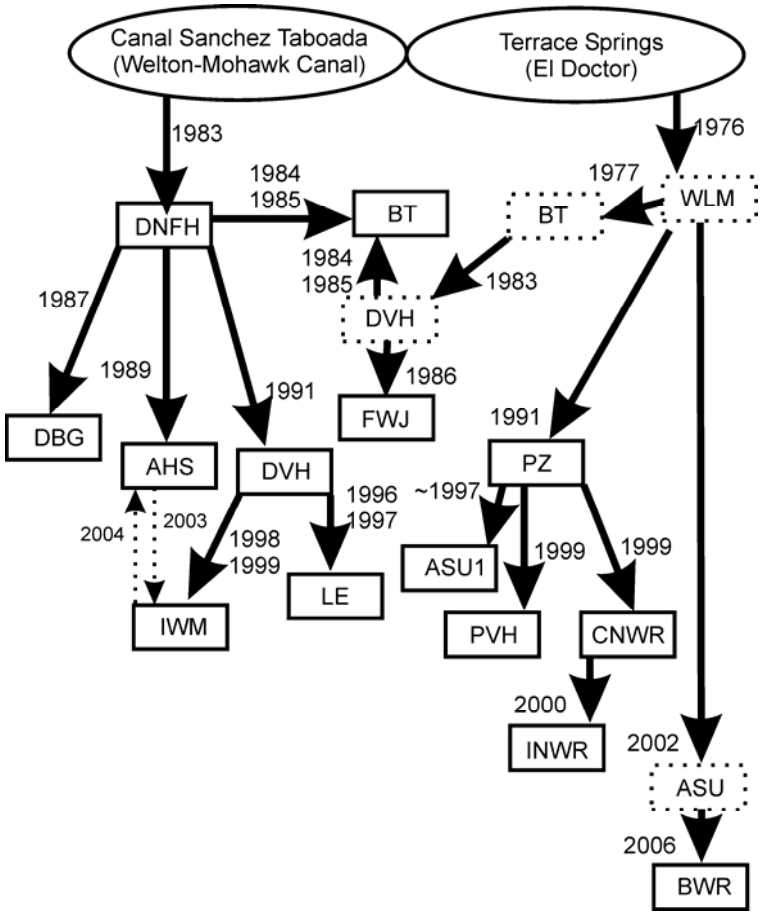

Figure 4. History of refuge populations of *C. macularius* from the lower Colorado River Delta. Boxes drawn with solid lines represent populations assayed in this study (abbreviations as described in Appendix E), boxes drawn with dotted lines have been extirpated. Large, solid arrows and associated years indicate initial founding events; small, dotted arrows and years indicate subsequent supplementations.

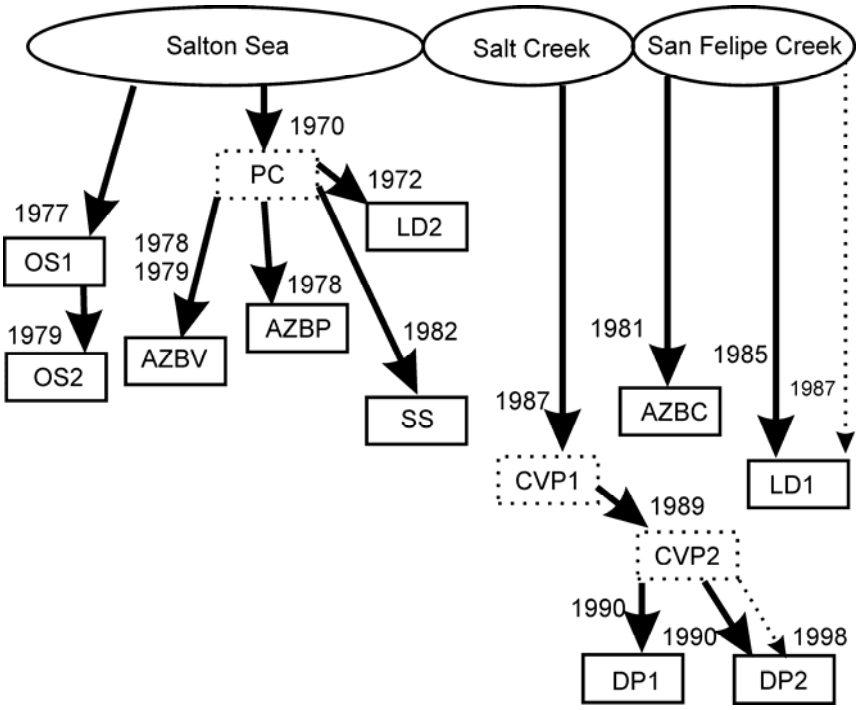

Figure 5. History of refuge populations of *C. macularius* from the Salton Sea area. PC is an extirpated population at Anza-Borrego State Park, California that is referred to as the Palm Canyon refuge in records of the history of the refuges. Boxes drawn in dotted lines are populations not examined in this study; CVP1 = a population at Coachella Valley Preserve, CVP2 = a population at the visitor center at the preserve. Other abbreviations are described in Appendix E.

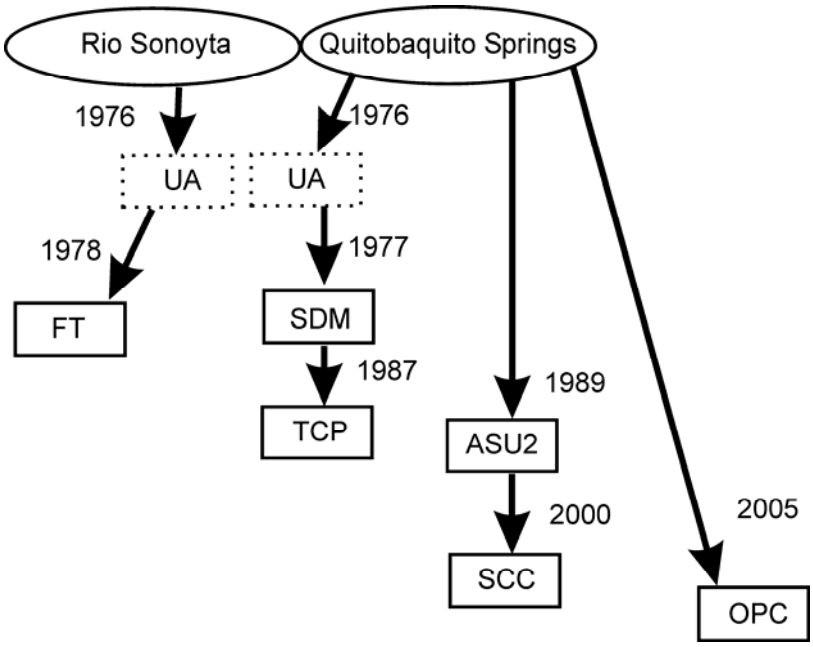

Figure 6. History of refuge populations of *C. eremus*. UA = one or more extirpated stocks previously maintained at the University of Arizona. Year of translocation from the wild to UA is not known; 1976 is used as an approximation for purposes of the analysis. The wild source for FT (Finley Tank) was questionable, but results herein confirm comments from B. Kynard (pers. comm.) that it was Río Sonoyta. Remainder of abbreviations are described in Appendix E.

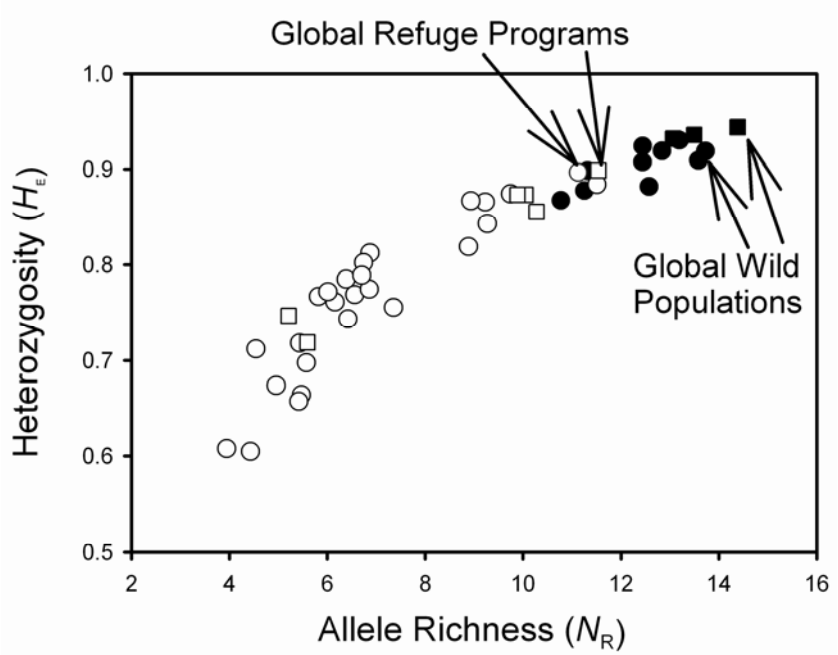

Figure 7. Heterozygosity versus allele richness in refuge and wild populations of the Desert Pupfish Complex. Circles = *C. macularius*, squares = *C. eremus*; solid = wild populations, open = refuge populations. The recently established refuge (OPC) for *C. eremus* is not included. It had essentially the same genetic diversity as the two wild populations of the species.

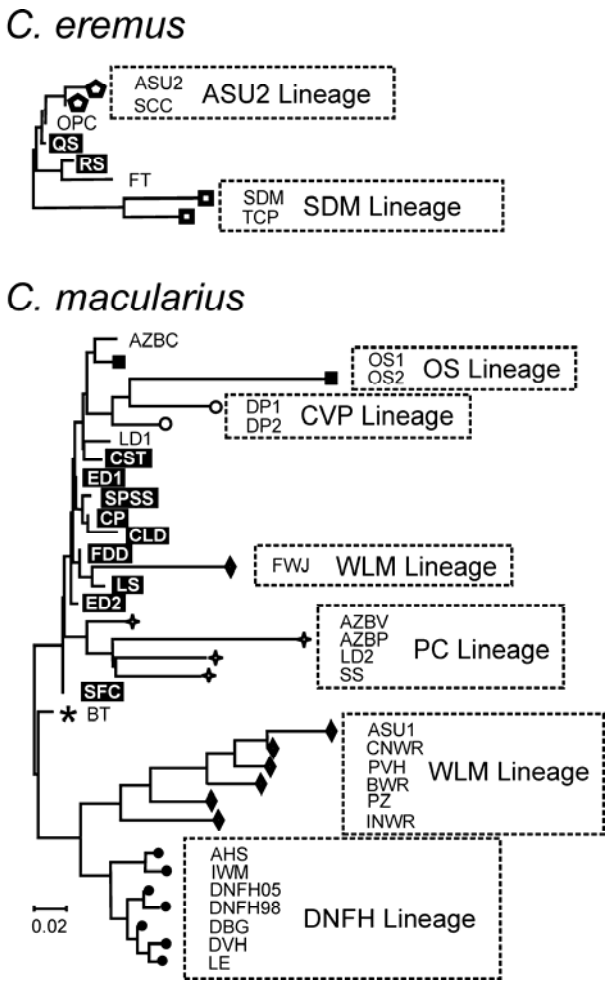

Figure 8. Neighbor-joining dendrograms summarizing pairwise F_{ST}-values among populations of *C. eremus* and *C. macularius*. Abbreviations inside black rectangles = wild populations. Terminal nodes with the same symbol = populations from the same lineage established from the wild; nodes with no symbol = refuge populations established as independent translocations. BT (asterisk) was established as a mixture of WLM and DNFH stocks.

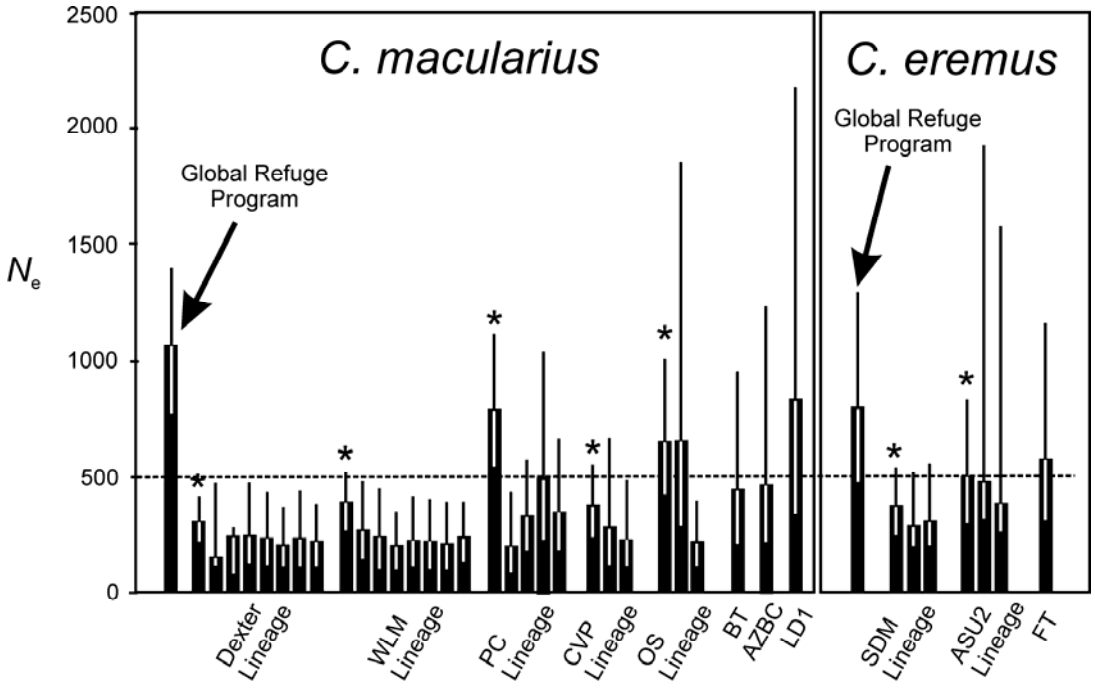

Figure 9. Long-term effective population sizes (N_e and 95% *CI*) for individual refuge populations, lumped populations within lineages of multiple refuge populations (asterisks), and the global refuge programs for *C. macularius* and *C. eremus*. Effective population sizes were computed over the number of generations since the original translocation from the wild.

Number	Population	A_R	%
1	OS2	3.9	31.2
2	ASU1	4.4	35.2
3	INWR	4.5	36.0
4	BWR	5.0	40.0
5	TCP	5.2	41.6
6	PZ	5.4	43.2
7	PVH	5.4	43.2
8	CNWR	5.5	44.0
9	AZBP	5.6	44.8
10	SDM	5.6	44.8
11	DVH	5.8	46.4
12	LE	6.0	48.0
13	DNFH98	6.2	49.6
14	DNFH05	6.4	51.2
15	FWJ	6.4	51.2
16	IWM	6.6	52.8
17	AHS	6.7	53.6
18	SS	6.7	53.6
19	DBG	6.9	55.2
20	LD2	6.9	55.2
21	DP2	7.4	59.2
22	AZBV	8.9	71.2
23	DP1	8.9	71.2
24	AZBC	9.2	73.6
25	BT	9.3	74.4
26	OS1	9.8	78.4
27	FT	10.0	80.0
28	SCC	10.1	80.8
29	ASU2	10.3	82.4
30	CLD	10.8	86.4
31	CP	11.3	90.4
32	LS	11.3	90.4
33	LD1	11.5	92.0
34	SFC	12.4	99.2
35	SPSS	12.4	99.2
36	CST	12.6	100.8
37	FDD	12.8	102.4
38	RS	13.1	104.8
39	ED2	13.2	105.6
40	OPC	13.5	108.0
41	QS	13.5	108.0
42	ED1	13.6	108.8

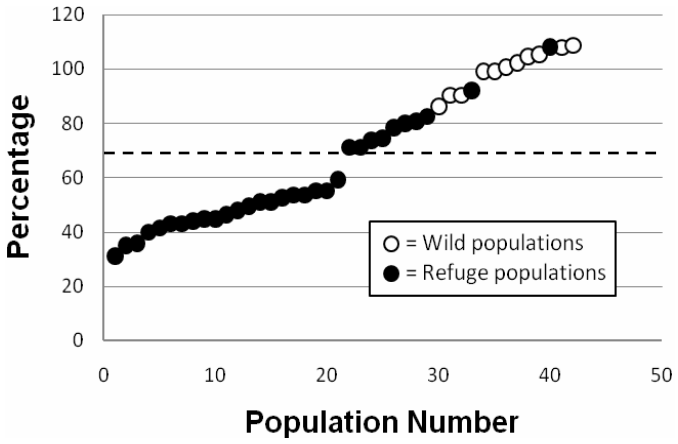

Figure 10. Allele richness (A_R) plotted in ascending order as percentage of the average (12.4 alleles/locus) for wild populations of the Desert Pupfish complex. Shaded portions of the legend of populations indicate refuge populations. It is suggested herein that >70% (dashed line) of the average for wild populations might be used as the level where management might not need to replace all, or a large portion, of the existing refuge population prior to inoculating (or re-establishing) with fish from the wild.

APPENDIX A

Collection localities for 11wild populations of the Desert Pupfish Complex. Locality numbers correspond with those in Figure 1 and Table 1.

Salton Sea area. (1) Shoreline pool of Salton Sea near Trifolium 20A drain, about 15 km northwest of Westmorland, Imperial County, California.; (2) San Felipe Creek at Highway 86 bridge, 18 km southeast of Salton City, Imperial County, California; (3) Irrigation drain, 100 m from shore of Salton Sea near boundary between Riverside and Imperial Counties, 3 km north of Desert Shores, Imperial County, California

Colorado River Delta. (4) A slough near Cerro Prieto and 0.8 km north of a geothermal power plant, Baja California; (5) a spring-fed area, Pozo del Tules, at east edge of Laguna Salada, 20.6 km south of Highway 2, Baja California; (6) Santa Clara Slough at terminus of Wellton-Mohawk canal, Sonora, Mexico; (7) a canal at Flor del Desierto, Sonora on Highway 003 (Highway 40 on some maps; (8) a spring at El Doctor, Sonora; (9) a spring about 150 m northwest of locality 8.

Río Sonoyta/Quitobaquito area. (10) Quitobaquito Springs/Pond, Organ Pipe Cactus National Monument, Pima County, Arizona; (11) Río Sonoyta 11.5 km west, 1.5 km south of Sonoyta, Sonora, Mexico.

APPENDIX B

Characteristics of primers used in this study. GATA and CmD primers are from Burg et al. (2002); WSP-02 is from Stockwell et al. (1998). WSP-02 has dinucleotide repeats, the GATA loci have tetranucleotide repeats.

Locus	T_{A1}/T_{A2}[1]	Marker size range
GATA2	45/48	207-351
GATA5	50/53	181-269
GATA9	50/53	237-357
GATA10	50/53	212-332
GATA26	50/53	200-280
GATA39	50/53	228-348
GATA73	40/43	276-324
CmD1	58	218-370
CmD16	58	244-424
WSP-02	55	204-294

[1] T_{A1} and T_{A2} are the first and second annealing temperatures (see text)

APPENDIX C

Population genetics statistics summarizing variation at 7 microsatellite loci in wild populations of *C. macularius*. Locality numbers are as in Figure 1. N = sample size, H_O = observed heterozygosity, H_E = expected heterozygosity, A = number of alleles, A_R = allele richness, A_P = number of private alleles. Bold H_o values with asterisks signify $P < 0.05$ for the individual test. None was significant with the correction.

	Locus						
Population	GATA2	GATA5	GATA9	GATA39	CmD1	CmD16	WSP02
Salton Sea							
Locality 1							
N	20	20	20	19	20	20	20
H_o	1.000	0.700	0.850	0.895	0.950	0.950	0.800
H_e	0.928	0.740	0.915	0.885	0.912	0.906	0.805
A	15	8	14	14	15	15	12
A_R	12.1	6.9	11.4	11.7	12.5	12.0	9.7
A_P	0	0	1	0	0	2	1
Locality 2							
N	22	22	22	21	19	19	19
H_o	0.910	**0.700***	0.955	1.000	0.842	0.947	0.895
H_e	0.945	0.882	0.938	0.928	0.953	0.940	0.868
A	20	12	14	14	18	17	14
A_R	15.8	10.7	11.9	12.3	15.6	13.7	11.8
A_P	1	3	0	1	0	4	0
Locality 3							
N	25	25	25	25	24	25	25
H_o	1.000	0.800	0.880	0.920	0.875	0.920	0.880
H_e	0.925	0.834	0.932	0.938	0.942	0.931	0.895
A	17	10	18	20	20	19	16
A_R	12.9	8.5	14.3	14.8	14.9	14.1	12.4
A_P	2	1	1	1	1	2	0
LCRD							
Locality 4							
N	16	17	15	13	20	20	19
H_o	0.750	0.765	**0.867***	1.000	0.950	1.000	0.842
H_e	0.910	0.831	0.910	0.935	0.960	0.935	0.913
A	12	9	13	14	21	16	17
A_R	12.0	9.0	12.0	14.0	17.0	14.0	13.0
A_P	0	1	0	0	0	0	1
Locality 5							
N	20	20	20	20	20	20	20

H_o	1.000	0.800	1.000	0.900	0.900	0.750	0.800
H_e	0.935	0.754	0.899	0.928	0.908	0.881	0.940
A	16	8	14	15	12	10	16
A_R	13.4	7.0	11.6	13.0	11.0	9.1	13.6
A_P	0	0	0	0	0	0	0
Locality 6							
N	20	20	20	20	20	20	20
H_o	0.950	0.650	0.950	**0.850***	0.950	0.850	0.900
H_e	0.956	0.681	0.941	0.945	0.940	0.931	0.904
A	21	8	15	17	17	16	15
A_R	16.3	6.5	13.3	14.2	14.0	13.3	12.2
A_P	1	0	1	1	0	0	2
Locality 7							
N	20	20	20	20	19	20	20
H_o	0.950	0.700	0.950	0.900	0.947	0.950	0.850
H_e	0.945	0.837	0.938	0.950	0.967	0.924	0.922
A	17	9	18	18	22	14	20
A_R	14.3	7.3	14.1	14.9	17.5	12.3	15.1
A_P	1	0	1	2	1	0	1
Locality 8							
N	20	20	20	20	20	20	20
H_o	1.000	0.800	0.950	1.000	0.950	1.000	0.950
H_e	0.960	0.798	0.950	0.941	0.944	0.941	0.933
A	21	9	18	18	19	15	16
A_R	16.8	8.1	14.9	14.6	15.0	13.2	13.2
A_P	0	0	2	0	0	0	0
Locality 9							
N	20	20	20	20	20	20	20
H_o	0.950	**0.750***	0.850	**0.750***	0.850	0.850	0.900
H_e	0.946	0.881	0.930	0.951	0.960	0.920	0.891
A	19	12	15	17	21	13	15
A_R	15.2	10.4	12.7	14.5	16.7	11.3	12.1
A_P	0	2	0	1	1	0	0

APPENDIX D

Population genetic statistics summarizing variation at 7 microsatellite loci in *Cyprinodon eremus*. Remainder of legend as in Appendix C.

	Locus						
Population	GATA9	GATA10	GATA26	GATA39	CmD1	CmD16	WSP02
Río Sonoyta							
Locality 10							
N	23	23	22	23	22	23	23
H_o	0.870	1.000	0.955	0.913	0.955	**0.783***	0.348
H_e	0.930	0.940	0.951	0.920	0.922	0.912	0.312
A	17	18	17	15	16	13	4
A_R	16.7	17.7	16.8	12.9	14.8	12.9	4.0
A_P	7	10	3	6	11	6	3
Locality 11							
N	20	20	20	20	20	20	20
H_o	0.800	1.000	0.900	0.900	1.000	0.850	0.500
H_e	0.938	0.943	0.958	0.883	0.900	0.860	0.412
A	16	16	18	11	9	9	3
A_R	16.0	16.0	18.0	11.0	10.0	9.0	3.0
A_P	6	8	4	2	5	2	2

APPENDIX E

Description of refuge localities.

Abbreviation	Locality Name	Locality
AHS	Arizona Historical Society	Tucson, AZ
ASU1	ASU Desert Arboretum	Tempe, AZ
ASU2	Arizona State University	Tempe, AZ
AZBC	Anza Borrego Desert State Park: Camp Ground Pool, Inyo County, CA	Borrego Springs, San Diego Co., CA
AZBP	Anza Borrego Desert State Park: Palm Spring	San Diego Co., CA
AZBV	Anza Borrego Desert State Park: Visitor Center, Inyo County, CA	Borrego Springs, San Diego Co., CA
BT	Boyce Thompson Arboretum State Park	Pinal Co., AZ
BWR	Bill Williams National Wildlife Refuge	La Paz Co., AZ
CNWR	Cibola National Wildlife Refuge	La Paz Co., AZ
DBG	Desert Botanical Garden	Phoenix, AZ
DNFH	Dexter National Fish Hatchery and Technology Center	Dexter, Chaves Co., NM
DP1	Dos Palmas (Large), CA	Riverside Co., CA
DP2	Dos Palmas (Small), CA	Riverside Co., CA
DVH	Deer Valley High School	Glendale, AZ
FT	Finley Tank	Appleton-Whittell Research Ranch, Elgin, Santa Cruz Co., AZ
FWJ	Flowing Wells Junior High School	Tucson, AZ
IWM	International Wildlife Museum	Tucson, AZ
INWR	Imperial National Wildlife Refuge	Yuma Co., AZ
LD1	The Living Desert Zoo and Gardens; Sonoran Pond	Indio, Riverside Co., CA
LD2	The Living Desert Zoo and Gardens; Oasis Pond	Indio, Riverside Co., CA
LE	Scott L. Libby Elementary School	Litchfield Park, AZ
OPC	Organ Pipe Cactus National Monument	Pima Co., AZ
OS1	Oasis Spring Ecological Reserve (Tamarisk Palm)	Riverside Co., CA
OS2	Oasis Spring Ecological Reserve (Date Palm)	Riverside Co., CA
PVH	Palo Verde High School	Tucson, AZ
PZ	Phoenix Zoo	Phoenix, AZ
SDM	Sonoran Desert Museum	Tucson, AZ
SCC	Scottsdale Community College	Scottsdale, Maricopa Co., AZ
SS	Salton Sea State Recreation Area	Riverside Co., CA
TCP	Tohono Chul Park	Tucson, AZ

APPENDIX F

Genetic statistics for refuge populations of *C. macularius*. (p<0.05 bolded)

		Loci			
		GATA2	GATA5	GATA9	GATA39
DNFH98	N	33	33	33	32
	H_e	0.71	0.74	0.81	0.78
	H_o	0.91	0.61	0.85	0.88
	A	5	9	11	8
	A_R	4.7	8.0	9.9	7.2
	F_{IS}	**-0.279**	**0.183**	-0.042	-0.124
DNFH05	N	25	25	25	24
	H_e	0.74	0.68	0.86	0.85
	H_o	0.96	0.76	0.80	*0.67*
	A	7	4	9	9
	A_R	5.8	4.0	7.8	8.0
	F_{IS}	-0.312	-0.112	0.076	**0.224**
DBG	N	25	25	25	25
	H_e	0.73	0.84	0.86	0.84
	H_o	0.96	0.84	0.96	0.84
	A	6	9	10	8
	A_R	4.8	7.4	8.2	7.0
	F_{IS}	-0.324	-0.006	-0.125	-0.005
AHS	N	21	22	22	22
	H_e	0.72	0.80	0.84	0.84
	H_o	0.48	0.73	0.77	0.68
	A	5	6	11	8
	A_R	4.9	5.9	9.0	7.2
	F_{IS}	0.343	0.092	0.078	0.193
DVH	N	25	25	25	24
	H_e	0.71	0.68	0.86	0.81
	H_o	0.60	0.68	0.72	0.83
	A	6	4	10	7
	A_R	4.6	3.9	8.7	6.1
	F_{IS}	0.156	0.005	0.167	-0.028
IWM	N	19	20	20	20
	H_e	0.62	0.76	0.85	0.83
	H_o	0.53	0.75	0.70	0.90
	A	5	5	10	9
	A_R	4.7	5.0	8.8	7.8
	F_{IS}	0.159	0.016	0.18	-0.081
LE	N	25	25	25	25

	H_e	0.73	0.73	0.82	0.81
	H_o	0.80	0.72	0.80	0.84
	A	5	5	10	7
	A_R	4.5	4.5	8.7	6.3
	F_{IS}	-0.1	0.014	0.023	-0.036
FWJ	N	25	25	25	24
	H_e	0.80	0.61	0.72	0.83
	H_o	0.68	0.60	0.56	0.83
	A	11	3	5	11
	A_R	8.7	3.0	4.8	9.2
	F_{IS}	0.153	0.016	0.231	0.001
BT	N	25	25	25	24
	H_e	0.84	0.74	0.92	0.86
	H_o	0.84	0.72	0.92	0.79
	A	11	10	14	10
	A_R	8.8	8.1	11.6	8.5
	F_{IS}	0.002	0.034	0.003	0.081
PZ	N	25	25	25	25
	H_e	0.59	0.65	0.89	0.74
	H_o	0.60	*0.72*	0.92	0.76
	A	5	3	9	7
	A_R	4.3	3.0	8.7	5.8
	F_{IS}	-0.017	**-0.106**	-0.03	-0.028
ASU1	N	25	25	24	25
	H_e	0.15	0.64	0.81	0.81
	H_o	0.16	0.52	0.83	0.84
	A	3	3	7	7
	A_R	2.4	3.0	6.3	6.0
	F_{IS}	-0.049	0.194	-0.027	**-0.038**
CNWR	N	24	24	24	24
	H_e	0.37	0.67	0.83	0.79
	H_o	0.42	0.83	0.79	0.83
	A	6	4	9	7
	A_R	4.4	3.5	7.7	6.3
	F_{IS}	-0.139	-0.247	0.05	-0.055
PVH	N	25	25	25	25
	H_e	0.49	0.57	0.87	0.69
	H_o	0.44	0.52	0.88	*0.52*
	A	5	4	9	6
	A_R	4.4	3.5	8.5	5.2
	F_{IS}	0.11	0.096	-0.012	**0.25**
INWR	N	20	20	20	20
	H_e	0.66	0.74	0.77	0.69

	H_o	0.70	0.80	0.80	0.60
	A	5	4	5	5
	A_R	4.6	4.0	5.0	4.6
	F_{IS}	-0.062	-0.088	-0.046	0.13
BWR	N	21	21	21	20
	H_e	0.75	0.46	0.86	0.64
	H_o	0.81	0.62	1.00	0.70
	A	5	3	9	4
	A	4.9	2.9	8.2	3.9
	F_{IS}	-0.086	-0.347	-0.173	-0.095
LD2	N	25	25	25	25
	H_e	0.83	0.60	0.84	0.84
	H_o	0.92	0.64	0.84	*0.80*
	A	9	5	10	8
	A_R	7.8	4.5	7.9	7.2
	F_{IS}	-0.114	-0.077	0.001	**0.046**
AZBC	N	25	25	25	25
	H_e	0.87	0.76	0.92	0.92
	H_o	0.72	0.76	1.00	0.96
	A	9	6	14	15
	A_R	7.8	5.5	11.4	12.2
	F_{IS}	0.171	-0.006	-0.09	-0.043
OS1	N	25	25	25	25
	H_e	0.88	0.78	0.93	0.91
	H_o	0.92	0.76	0.84	0.84
	A	12	7	14	13
	A_R	9.8	6.4	11.9	10.9
	F_{IS}	-0.046	0.024	0.097	0.077
OS2	N	25	25	25	25
	H_e	0.64	0.53	0.56	0.70
	H_o	0.52	0.64	0.52	0.68
	A	3	3	6	6
	A_R	3.0	2.5	4.8	5.4
	F_{IS}	0.185	-0.213	0.071	0.035
AZBP	N	25	25	25	25
	H_e	0.72	0.45	0.81	0.81
	H_o	0.76	0.44	0.72	0.64
	A	7	3	8	8
	A_R	5.7	3.0	6.8	6.8
	F_{IS}	-0.059	0.019	0.113	0.211
AZBV	N	25	25	25	25
	H_e	0.86	0.83	0.88	0.89
	H_o	0.96	0.72	0.84	0.96

	A	10	8	13	13
	A_R	8.5	6.8	10.3	10.1
	F_{IS}	-0.115	0.137	0.049	-0.08
SS	N	25	25	25	25
	H_e	0.82	0.78	0.71	0.84
	H_o	*0.76*	0.64	0.84	0.76
	A	9	6	7	9
	A_R	7.7	5.8	5.7	7.7
	F_{IS}	**0.08**	0.183	-0.189	0.096
LD1	N	25	25	25	25
	H_e	0.94	0.78	0.93	0.87
	H_o	0.92	0.68	0.88	*0.76*
	A	20	10	17	12
	A_R	14.7	7.9	13.3	10.1
	F_{IS}	0.024	0.135	0.057	**0.131**
DP1	N	25	25	25	25
	H_e	0.82	0.62	0.80	0.78
	H_o	0.84	0.64	0.72	0.64
	A	11	4	14	9
	A_R	8.4	3.8	9.8	7.4
	F_{IS}	-0.019	-0.031	0.098	0.179
DP2	N	25	22	25	25
	H_e	0.88	0.66	0.86	0.87
	H_o	0.92	0.45	0.88	0.88
	A	12	5	15	11
	A_R	9.8	4.5	11.7	9.5
	F_{IS}	-0.045	0.314	-0.019	-0.011
SPSS	N	25	25	25	25
	H_e	0.92	0.83	0.93	0.94
	H_o	1.00	0.80	0.88	0.92
	A	17	10	18	20
	A_R	12.7	8.4	14.0	14.7
	F_{IS}	-0.083	0.042	0.057	0.02
SFC	N	22	22	22	21
	H_e	0.95	0.88	0.94	0.93
	H_o	0.91	*0.64*	0.95	1.00
	A	20	12	14	14
	A_R	15.0	9.9	12.5	12.3
	F_{IS}	0.039	**0.283**	-0.018	-0.08
CLD	N	20	20	20	19
	H_e	0.93	0.74	0.92	0.88
	H_o	1.00	0.70	0.85	0.89
	A	15	8	14	14

	A_R	12.6	6.8	12.0	11.7
	F_{IS}	-0.08	0.055	0.073	-0.012
CP	N	16	17	15	13
	H_e	0.91	0.83	0.91	0.94
	H_o	0.75	0.76	*0.87*	1.00
	A	12	9	13	14
	A_R	11.2	8.0	12.0	14.0
	F_{IS}	0.18	0.082	**0.05**	-0.072
CST	N	20	20	20	20
	H_e	0.96	0.68	0.94	0.94
	H_o	0.95	0.65	0.95	*0.85*
	A	21	8	15	17
	A_R	16.3	6.5	13.3	14.2
	F_{IS}	0.007	0.046	-0.01	0.103
FDD	N	20	20	20	20
	H_e	0.94	0.84	0.94	0.95
	H_o	0.95	0.70	0.95	0.90
	A	17	9	18	18
	A_R	14.2	8.0	14.3	14.8
	F_{IS}	-0.006	0.167	-0.013	0.054
ED1	N	20	20	20	20
	H_e	0.96	0.79	0.95	0.94
	H_o	1.00	0.80	0.95	1.00
	A	21	9	18	18
	A_R	16.8	8.1	14.9	14.6
	F_{IS}	-0.043	-0.015	0	-0.064
ED2	N	20	20	20	20
	H_e	0.95	0.88	0.93	0.95
	H_o	0.95	*0.75*	0.85	*0.75*
	A	19	12	15	17
	A_R	15.2	10.4	12.7	14.5
	F_{IS}	-0.004	0.152	0.088	**0.216**
LS	N	20	20	20	20
	H_e	0.93	0.75	0.90	0.93
	H_o	1.00	0.80	1.00	0.90
	A	16	8	14	15
	A_R	13.4	7.0	11.6	13.0
	F_{IS}	-0.072	-0.063	-0.116	0.031

APPENDIX G

Genetic statistics for refuge populations of *C. eremus.*

		Loci			
		GATA9	GATA10	GATA26	GATA39
SDM	N	25	25	24	25
	H_e	0.81	0.73	0.61	0.73
	H_o	0.72	0.60	0.63	0.72
	A	10	5	7	4
	A_R	8.9	4.6	6.0	4.0
	F_{IS}	0.11	0.178	-0.027	0.011
TCP	N	20	20	20	20
	H_e	0.79	0.71	0.79	0.69
	H_o	0.95	0.55	0.90	0.60
	A	6	4	7	5
	A_R	6.0	4.0	6.8	4.8
	F_{IS}	-0.211	0.234	-0.136	0.135
	N	25	25	24	25
	H_e	0.88	0.90	0.77	0.87
	H_o	0.84	0.88	0.79	0.92
	A	14	15	13	12
	A_R	11.9	12.0	11.1	10.3
	F_{IS}	0.047	0.02	-0.026	-0.058
SCC	N	25	25	25	17
	H_e	0.88	0.86	0.88	0.87
	H_o	0.84	0.80	0.88	0.94
	A	16	11	12	10
	A_R	12.8	9.9	10.8	9.9
	F_{IS}	0.052	0.067	-0.002	-0.08
OPC	N	16	16	16	16
	H_e	0.93	0.94	0.95	0.93
	H_o	0.94	0.81	1.00	0.94
	A	14	13	18	14
	A_R	14.0	13.0	18.0	14.0
	F_{IS}	-0.009	0.137	-0.05	-0.014
FT	N	20	19	20	20
	H_e	0.84	0.89	0.93	0.83
	H_o	0.60	0.79	0.90	0.85
	A	10	12	13	10
	A_R	9.6	11.3	12.5	9.1
	F_{IS}	0.291	0.115	0.031	-0.029