

# Microsatellite DNA analysis of success in conserving genetic diversity after 33 years of refuge management for the desert pupfish complex

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## Keywords

desert pupfish; *Cyprinodon*; refuge populations; microsatellites; conservation.

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## Abstract

Refuge populations of *Cyprinodon macularius* and *Cyprinodon eremus*, the extant members of the endangered desert pupfish complex, have been maintained for up to 33 years in semi-natural refuges. We examined the success of the refuge program in maintaining diversity at four microsatellite DNA loci in 24 refuge populations of *C. macularius* and six of *C. eremus* that include, respectively, seven and four lineages representing original translocations from the wild. These lineages have been maintained with essentially no inoculations of genetic material from the wild and, except for one refuge, no intermixing of lineages. Comparison with wild-source populations showed marked declines in diversity within local refuges and within lineages, but relatively minor declines for the composite of all refuge populations for each species. In genetic makeup, the refuge populations generally clustered by lineage, indicating significant genetic drift early in lineage history. The results indicate that, with relatively minor adjustments in management, the refuge program can successfully preserve a large portion of the wild genetic diversity in the desert pupfish complex.

## 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 (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 (Allendorf & Phelps, 1980; Hedrick & Miller, 1992).

Captive threatened fishes in the American Southwest fall into two general groups regarding 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 & 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. Spontaneous breeders

have received less attention, in part because they typically are smaller, shorter-lived fishes that, in hatchery ponds and other artificial situations, can quickly form large populations requiring little management (Echelle, 1991).

In this paper, we use microsatellite DNA variation to assess levels and patterns of genetic diversity in refuge populations of the desert pupfish complex (Cyprinodontidae: *Cyprinodon macularius* and *Cyprinodon eremus*), some of which have been maintained for more than 30 years. Pupfishes generally are small-bodied (usually <40 mm SL) omnivores with high reproductive potential, making them ideal for low-maintenance refuge programs. Members of the desert pupfish complex have extended spawning seasons and can breed at sizes as small as about 15 mm SL and only 2 months post-hatching (Kinne, 1962; Cox, 1966; Constantz, 1981). These features resemble those of other pupfishes, which can reach densities as high as 89 fish m<sup>-2</sup> (Naiman, 1976) in situations that, like most of the refuges, have few other fish species. Such attributes allow quick rebound from founder events and other population bottlenecks and promote large populations in small refuges.

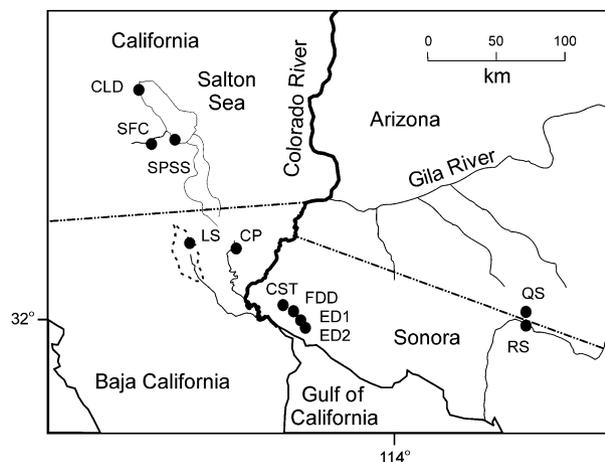
On the other hand, the observed number of animals in a refuge ( $N$ ) undoubtedly overestimates the effective size ( $N_e$ ) of the population. The  $N_e/N$  ratio in animals often is <0.10 and generally 0.25–0.50 (Frankham, 1995). In the desert pupfish complex, breeding males are intensely territorial (Barlow, 1961; Cox, 1966), suggesting high variance in male reproductive success and, therefore, greatly reduced  $N_e/N$

(Frankham, 1995). Pupfish have alternative male strategies, including sneakers and satellites (Leiser & Itzkowitz, 2002), that would moderate the effect of territoriality on  $N_e/N$ , but effects might be especially pronounced in small refuges, where a population of several hundred might include only a handful of territorial males.

Allozyme surveys of genetic diversity in refuge populations of cyprinodontoids have produced conflicting results. There was little or no evidence of change in 6- to 10-year-old refuge populations of *C. macularius* (Turner, 1984), two other *Cyprinodon* species, and the livebearer *Gambusia nobilis* (Edds & Echelle, 1989). In contrast, two of nine refuge stocks of *C. macularius* had reduced allozyme variability after about 10 years in captivity (Dunham & Minckley, 1998), and diversity showed marked declines in refuge populations of western mosquitofish *Gambusia affinis*, that were 50+ years old (Stockwell, Mulvey & Vinyard, 1996).

## The refuge program

The existing, non-aquarium refuge stocks of *C. macularius* include about 25 populations in Arizona, 15 in California and one in New Mexico. These comprise seven sets of populations in which each set ('lineage' herein) is descended from an original translocation from the wild, two lineages from the lower Colorado River delta in Sonora, Mexico and five from the Salton Sea region in California, USA (Figs 1, 2a and b). Refuge stocks of *C. eremus* (Fig. 2c; excluding three in Sonora not included in this analysis) comprise three lineages and about six populations from Quitobaquito Springs and one (Finley Tank = FT) that, before this study,



**Figure 1** Collection localities for wild populations of the desert pupfish complex. Localities QS and RS represent the two populations of *Cyprinodon eremus*. The remaining localities represent the extant populations of *Cyprinodon macularius*, which historically occurred in the Gila River of Arizona and the lower Colorado River of California and Arizona. Locality abbreviations used in the text are as follows: CLD, County Line Drain; SFC, San Felipe Creek; SPSS, shoreline pool of Salton Sea; CP, Cerro Prieto; LS, Pozo del Tules in Laguna Salada; CST, Canal Sanchez Taboada; FDD, Flor del Desierto; ED1, El Doctor 1; ED2, El Doctor 2; QS, Quitobaquito Springs; RS, Rio Sonoyta.

was considered by the Arizona Department of Game and Fish (AZGF) to be of unknown, potentially mixed, origin. Correspondence with B. Kynard (7 August 2007) indicates that the original stock was collected from the Río Sonoyta in 1976, transported to the University of Arizona, and released at FT in 1978, and this is consistent with our results.

Although there were some between-refuge transfers of pupfish, only one refuge population is known to contain genetic material from two or more refuge 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. 2).

## Material and methods

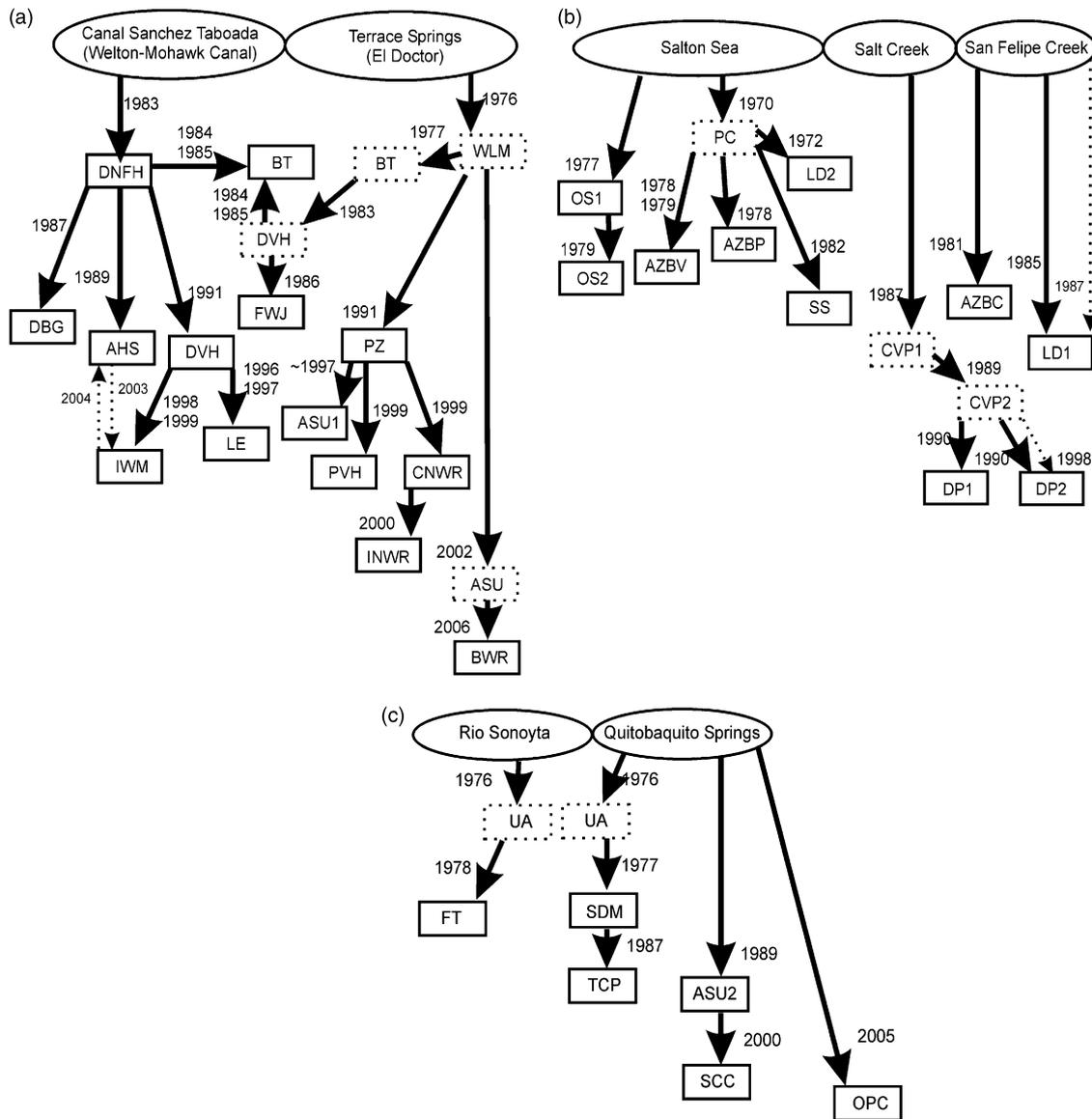
### Sampling

We used data for 11 wild populations (Fig. 1) from a survey of microsatellite DNA variation throughout the native range of the desert pupfish complex (Loftis *et al.*, 2008). That survey used the collections described by Echelle *et al.* (2000) in an earlier survey of mtDNA variation. For the refuge stocks, we collected pupfish in 2005 and 2006 from 30 sites (Table 1;  $n = 25$  each; stored in 100% ethanol). From one of those sites (DNFH) we also included genomic DNA samples used by Echelle *et al.* (2000) from a collection made in 1998.

We estimated habitat size as the product of length and width of water-surface area of the refuge and obtained the history of each captive stock (Table 2) from Dunham & 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 except for the refuge at BT. We followed Dunham & Minckley (1998) who reported four steps from the wild for the BT fish descended from the Terrace Springs population (Fig. 2); AZGF records indicate only two steps.

### Genetic assay

We used the DNeasy kit (Quiagen, Valencia, CA, USA) for DNA extractions. With primers from Burg, Wilcox & Martin (2002), we PCR amplified GATA2, GATA5, GATA9 and GATA39 for *C. macularius* and GATA9, GATA10, GATA26 and GATA39 for *C. eremus*. Based on high frequencies of null alleles in the survey of wild populations (Loftis *et al.*, 2008), GATA10 and GATA26 were not used for *C. macularius* and GATA2 and GATA5 were not used for *C. eremus*. We used fluorescent end-labeled primers in 15- $\mu$ L PCR reactions (9.0  $\mu$ L, Applied Biosystems True Allele premix, 3.8  $\mu$ L ddH<sub>2</sub>O, 1.0  $\mu$ L of 5.0  $\mu$ M primer pairs and 1.2  $\mu$ L template DNA) under the following two-step annealing conditions ( $T_{A1} = 50^\circ\text{C}$  and  $T_{A2} = 53^\circ\text{C}$  except for GATA2 where  $T_{A1} = 45^\circ\text{C}$  and  $T_{A2} = 48^\circ\text{C}$ ):  $95^\circ\text{C}$  for 12 min; five cycles of  $94^\circ\text{C}$  for 30 s,  $T_{A1}$  for 30 s,  $72^\circ\text{C}$  for 30 s; 35 cycles of  $94^\circ\text{C}$  for 45 s,  $T_{A2}$  for 45 s,  $72^\circ\text{C}$  for 1 min and one cycle at  $72^\circ\text{C}$  for 2 min. We mixed 1  $\mu$ L from each



**Figure 2** History of refuge populations of the desert pupfish complex. Parts a, b and c represent, respectively, *Cyprinodon macularius* from the lower Colorado River delta, *C. macularius* from the Salton Sea area, and *Cyprinodon eremus*. Solid-line boxes represent populations assayed in this study (abbreviations as described in Table 1); dotted-line boxes are either extant but not included in the study (CVP1 and CVP2) or they have been extirpated (CVP1 = a refuge at Coachella Valley Preserve, CVP2 = a refuge at the visitor center of the preserve). Large, solid arrows and associated years indicate initial founding events; small, dotted arrows and years indicate subsequent supplementations. 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; UA = one or more extirpated stocks previously maintained at the University of Arizona.

product with 2  $\mu$ L formamide and 0.5  $\mu$ L of ROX-labeled size standard, genotyped the product using an automated sequencer (Applied Biosystems Inc., Foster City, CA, USA, ABI 377 or Prism 3130) with ABI Genescan 3.1 Software, and scored allele sizes with ABI's 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.

**Statistical analyses**

We assessed number of alleles (*A*), observed heterozygosity (*H<sub>o</sub>*), and expected heterozygosity (*H<sub>e</sub>*) with GENEPOP (Raymond & Rousset, 1995) and number of alleles corrected for sample size (allele richness, *A<sub>R</sub>*) with FSTAT 2.9.3.2 (Goudet, 1995). We used GENEPOP for tests of linkage disequilibrium and exact tests of Hardy-Weinberg equilibrium (HWE). We used MICRO-CHECKER (van Oosterhout *et al.*, 2004) to check for sources of scoring error and

**Table 1** Refuge abbreviations and 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	Borrego Springs, CA
AZBP	Anza Borrego Desert State Park: Palm Spring	San Diego Co., CA
AZBV	Anza Borrego Desert State Park: Visitor Center	Borrego Springs, 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, 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, CA
LD2	The Living Desert Zoo and Gardens; Oasis Pond	Indio, 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, AZ
SS	Salton Sea State Recreation Area	Riverside Co., CA
TCP	Tohono Chul Park	Tucson, AZ

to estimate null allele frequencies via Brookfield's (1996) Method I. For multiple tests applied to the same hypothesis we used the sequential Bonferroni correction (Rice, 1989) to reduce Type I errors (overall  $\alpha = 0.05$ ). We used Arlequin (Schneider, Roessli & Excoffier, 2000) to compute pairwise  $F_{ST}$  values among populations and for analyses of molecular variance (AMOVA). For refuges, we used a hierarchical AMOVA to partition among-population variation into among- versus within-lineage components.

We used the partial Mantel test in the ZT software package (Bonnet & Van de Peer, 2002) to test for association between change in diversity ( $H_e$  and  $A_R$ ) and number of founders, refuge size and time since founding for the refuge populations of *C. macularius*. The small number of populations precluded tests of association for *C. eremus* and the two species were not tested in a single analysis because they differed in loci examined. To correct for differences among parent populations, we expressed differences in expected heterozygosity and allele richness, respectively,  $H_{e(DIFF)}$  and  $A_{R(DIFF)}$ , as the absolute difference divided by the parental value for the variable (= standardized  $H_e$  and  $A_R$  herein).

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 diversity in *C. macularius*. Following Dunham & Minckley (1998),  $H_{e(SOURCE)}$  is the ratio of the absolute difference in  $H_e$  divided by the wild-source  $H_e$ . We used groups of populations as wild sources because the exact source-locality generally was not known. We used sites 1–3 and 6–9 to represent the wild source for refuge populations derived from, respectively, the Salton Sea area and the lower Colorado River delta. We treated each composite of wild-source populations as a single population with heterozygosity equal to the average for the individual populations. In computing wild-source  $A_R$ , we included these composite samples in a single F-STAT analysis that also included refuge populations.

## Results

Error rates estimated from blind re-genotyping were zero except for GATA 26 (3%). Numbers of alleles per locus ranged from 23 to 35 for the four loci assayed in *C. macularius* and from 19 to 29 for the four assayed in *C. eremus*. The composite of all refuge populations (= global population) of *C. macularius* had 89% of the alleles detected in the global wild population. The global refuge population of *C. eremus* had more alleles (103%) than the global wild

**Table 2** Attributes of refuge populations of *Cyprinodon macularius*

Source/refuge population	Number of founders	Surface area (log m <sup>2</sup> )	Years since founding	Founding steps from the wild
<i>C. macularius</i>				
Lower delta				
DNFH98	280	2.30	22	1
DNFH05	280	2.30	22	1
DBG	250	2.95	18	2
AHS	100	1.02	1	2
DVH	300	2.90	8	2
IWM	150	1.60	2	3
LE	820	3.04	8	3
FWJ	90	1.32	19	3
BT	1450	3.78	20	3 <sup>a</sup>
PZ	400	0.69	19	2
ASU1	50	2.48	17	3
CNWR	37	1.70	6	3
PVH	25	1.48	6	3
INWR	23	1.40	5	4
BWR	200	1.70	0.6	3
Salton Sea				
LD1	10 <sup>b</sup>	2.67	20	1
LD2	40	1.67	33	2
AZBC	375	2.02	24	1
OS1	77	1.26	28	1
OS2	20	1.15	26	2
AZBP	45	1.18	24	2
AZBV	20	1.88	26	2
SS	203	1.45	23	2
DP1	395	3.00	15	4
DP2	198	2.70	15	4
<i>Cyprinodon eremus</i>				
Quitobaquito Springs				
SDM	Unk	1.16	28	2
TCP	Unk	0.70	18	3
ASU2	80	1.18	16	1
SCC	50	1.78	5	2
OPC	235	1.10	0.2	1
Río Sonoyta				
FT	150	2.30	3	2

Population abbreviations are described in Table 1.

<sup>a</sup>Average for the two lineages contributing to the founding of this population (see text).

<sup>b</sup>An additional 250 wild fish were added, 2 years after establishment of the population.

population, and the percentage remained high (95%) after excluding OPC, which was founded from Quitobaquito Springs only 2 months before we made our collection.

There was no evidence of linkage disequilibrium among loci, and, with the Bonferroni correction, no instances of HWE deviation. Without the correction, there were four heterozygote excesses and nine deficiencies among the 136 tests ( $P = 0.003\text{--}0.040$ ). There were four instances of significant null-allele frequencies: GATA2, 13–14% at AHS and FT; GATA5, 11% at DP2 and GATA39, 9% at DNFH05.

**Table 3** Population genetic statistics averaged over four microsatellite loci in wild and refuge populations of *Cyprinodon macularius*

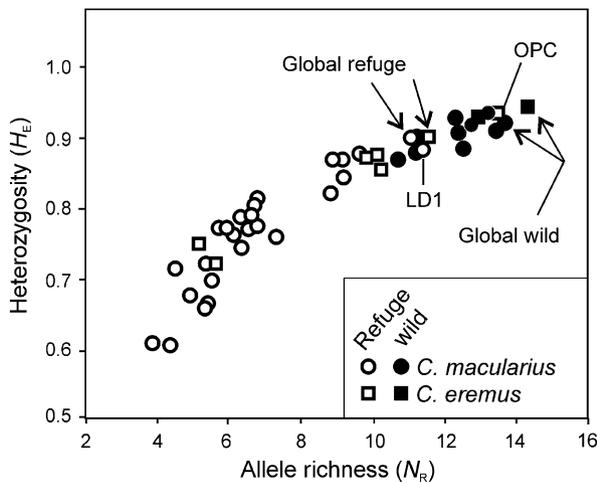
Populations	<i>n</i>	<i>H<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>A</i>	<i>A<sub>R</sub></i>
Wild populations					
Global population	183–186	0.92	0.88	28.3	13.7
CLD	19–20	0.87	0.86	12.8	10.8
SFC	21–22	0.93	0.88	15.0	12.4
SPSS	25	0.91	0.90	16.3	12.4
CP	18–19	0.90	0.85	12.0	11.3
LS	20	0.88	0.93	13.3	11.3
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 (average)	20.5	0.90	0.88	14.7	12.3
Refuge populations					
Global population	577–581	0.90	0.75	25.3	11.1
Source: Lower Colorado River Delta (LCRD)					
DNFH lineage	140–142	0.81	0.77	12.0	7.0
DNFH98	32–33	0.76	0.81	8.3	6.2
DNFH05	24–25	0.78	0.80	7.3	6.4
DBG	25	0.82	0.90	8.3	6.9
AHS	21–22	0.80	0.67	7.5	6.7
DVH	24–25	0.77	0.71	6.8	5.8
IWM	19–20	0.77	0.72	7.3	6.6
LE	25	0.77	0.79	6.8	6.0
WLM lineage	163–165	0.78	0.69	11.8	6.9
FWJ	24–25	0.74	0.67	7.5	6.4
PZ	25	0.72	0.75	6.0	5.4
ASU1	24–25	0.60	0.59	5.0	4.4
CNWR	24	0.67	0.72	6.5	5.5
PVH	25	0.66	0.59	6.0	5.4
INWR	20	0.72	0.73	4.8	4.5
BWR	20–21	0.68	0.78	5.3	5.0
BT mixed lineage	24–25	0.84	0.82	11.3	9.3
LCRD (average)	24	0.74	0.74	7.0	6.0
Source: Salton Sea area					
PC lineage	100	0.87	0.77	17.8	9.9
LD2	25	0.78	0.80	8.0	6.9
AZBP	25	0.70	0.64	6.5	5.6
AZBV	25	0.87	0.87	11.0	8.9
SS	25	0.79	0.75	7.8	6.7
CVP lineage	47	0.82	0.75	13	8.8
DP1	25	0.82	0.78	10.8	8.9
DP2	22–25	0.76	0.71	9.5	7.4
OS lineage	25	0.8	0.72	12.3	8.3
OS1	25	0.88	0.84	11.5	9.8
OS2	25	0.61	0.59	4.5	3.9
Single population lineages					
AZBC	25	0.87	0.86	11.0	9.2
LD1	25	0.88	0.81	14.8	11.5
Salton Sea (average)	24.9	0.80	0.77	9.5	7.9
All refuges (average)	24.4	0.76	0.75	8.0	6.8

With few exceptions, allele richness ( $A_R$ ) and heterozygosity ( $H_e$ ) were lower in local refuges than in wild populations (Tables 3 and 4; Fig. 3). The primary exceptions were

**Table 4** Population genetic statistics averaged over four microsatellite loci in wild and refuge populations of *Cyprinodon eremus*

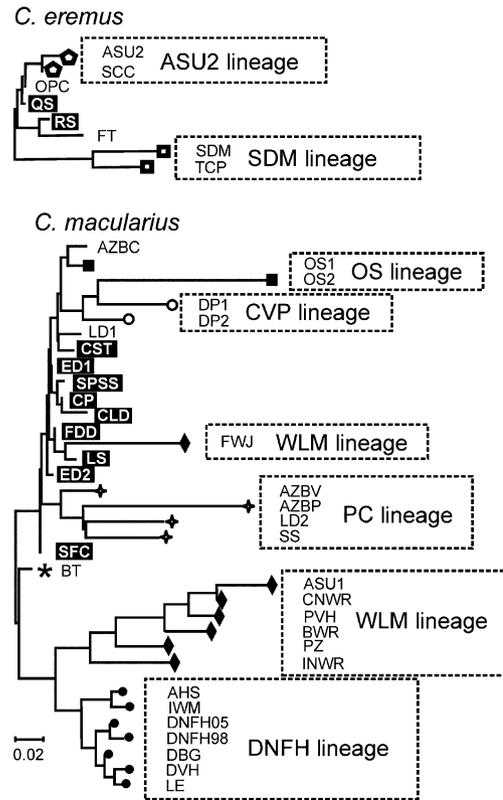
Populations	<i>n</i>	<i>H<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>A</i>	<i>A<sub>R</sub></i>
<b>Wild</b>					
Global population	42–43	0.94	0.84	21.8	14.4
QS	22–23	0.93	0.90	15.3	13.5
RS	20	0.94	0.93	16.8	13.1
All wild (average)	21.25	0.94	0.92	16.1	13.4
<b>Refuge</b>					
Global Population	123–131	0.90	0.81	22.5	11.5
SDM lineage	44–45	0.77	0.70	8.5	6.5
SDM	24–25	0.72	0.67	6.5	5.6
TCP	20	0.75	0.75	5.5	5.2
ASU2 lineage	42–50	0.88	0.86	16.5	10.6
ASU2	24–25	0.86	0.86	13.5	10.3
SCC	17–25	0.87	0.87	12.3	10.1
<b>Single population lineages</b>					
OPC	16	0.94	0.92	14.8	13.5
FT	19–20	0.87	0.79	11.3	10.0
All refuges (average)	20.92	0.84	0.81	10.7	9.1

Locality abbreviations are as in Table 1 for refuge populations and as in Fig. 1 for wild populations. The recently established OPC refuge was not included in the global estimate or the average. Remainder of legend as in Table 3.



**Figure 3** Expected heterozygosity versus allele richness in refuge and wild populations of the desert pupfish complex. The recently founded OPC refuge was not included in the computation of global heterozygosity and allele richness of refuge populations of *Cyprinodon eremus*.

the recently founded OPC and the one refuge population (LD1) that received wild fish subsequent to founding. Twenty-one of the 25 refuge collections for *C. macularius* and two of the six for *C. eremus* had  $A_R$  values that were  $\leq 60\%$  (minimum = 32%) of the average for the wild populations of the respective species. These included all populations in the WLM, DNFH and SDM lineages, two of three in the PC lineage, and one of two in the OS lineage. The percentage of wild  $A_R$  for the remaining eight refuges



**Figure 4** Neighbor-joining dendrograms summarizing pairwise  $F_{ST}$  values among populations of *Cyprinodon eremus* and *Cyprinodon 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.

ranged from 72% (AZBV and DP1) to 84% (ASU2), except for the two from *C. eremus* that were within the range of variation for wild populations (LD1 = 94%; OPC = 110%). The values for wild populations of *C. macularius* ranged from 88 to 111% of their average, and those for the two wild populations of *C. eremus* ranged from 99 to 102% of their average.

The matrices of pairwise divergence ( $F_{ST}$ ) among populations are summarized in Fig. 4. With the Bonferroni correction, all except two of the 312 comparisons of wild versus 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 parental wild stock at Quitobaquito Springs.

Among refuge populations, nearly all pairwise comparisons 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 (Fig. 4) populations within

the DNFH lineage. The one comparison involving the same refuge sampled in two separate years, DNFH in 1998 and 2005, was not significant ( $F_{ST} = 0.009$ ;  $P = 0.054$ ).

Pairwise genetic divergence was notably less common among wild populations. The test of the two wild populations (QS and RS) of *C. eremus* was marginally significant ( $F_{ST} = 0.02$ ,  $P = 0.0001 =$  critical  $P$  with the Bonferroni correction). For *C. macularius*, 17 of 36 (47%) comparisons of wild populations were significant with  $F_{ST}$  values ranging from 0.04 to 0.05.

Differences among populations accounted for 11.5 and 16.5% of total diversity in refuge populations of, respectively, *C. eremus* and *C. macularius* ( $P < 0.00001$  for both), with differences among lineages accounting for, respectively, 7.5 and 7.6% ( $P < 0.00001$  for both). In contrast, for wild populations, differences among populations accounted for only 2.1 and 1.8% of the diversity in, respectively, *C. eremus* and *C. macularius* ( $P < 0.00001$  for both).

Mantel tests were marginally significant for association between lineage age (time since founding from the wild) and divergence from the wild populations in both heterozygosity ( $H_{e(SOURCE)}$ ;  $r = 0.11$ – $0.12$ ;  $P = 0.07$ – $0.09$ ) and allele richness ( $A_{R(SOURCE)}$ ;  $r = 0.16$ – $0.17$ ;  $P = 0.05$ – $0.06$ ) and between number of founding steps and  $H_{e(SOURCE)}$  ( $r = 0.13$ ;  $P = 0.06$ ) but not  $A_{R(SOURCE)}$  ( $r = 0.10$ ;  $P = 0.12$ ). Using number of founders as a covariate with lineage age, and vice versa, had little effect, nor did other covariates (number of founders of the immediate population, refuge size and number of supplements to the refuge).

Differences between daughter and parent refuge in standardized allele richness ( $A_{R(DIFF)}$ ) were significantly associated with refuge size ( $r = 0.57$ ;  $P = 0.01$ ) and marginally associated with number of founders ( $r = 0.41$ ;  $P = 0.06$ ); using these variables as covariates had little effect on the results. There were no significant associations between standardized heterozygosity ( $H_{e(DIFF)}$ ) and the independent variables ( $r = -0.00$ – $0.07$ ;  $P = 0.21$ – $0.65$ ). A highly significant association was found between refuge size and number of founders for the population ( $r = 0.88$ ;  $P = 0.0002$ ).

## Discussion

The global estimates of genetic diversity for the refuge programs were within the range of diversity among individual wild populations and only moderately lower than the estimates for global wild populations of the two species. This was achieved without inoculation with individuals from the wild, and essentially with no interchange among refuge lineages, both of which are recommended for avoiding unwanted change in managed stocks (Allendorf, 1986). In contrast with the global refuge programs, the estimates of diversity for individual lineages and local refuge populations were, with few exceptions, well below those of wild populations. This signals reduced diversity, particularly of rare alleles, throughout the genome, including loci affecting 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 adapt-

ability of local populations (Lesica & Allendorf, 1995; Frankham, Ballou & Briscoe, 2002).

Factors causing losses in genetic diversity generally conformed to theoretical expectations, with declines associated with increasing lineage age and number of founding events, and with decreasing refuge size, number of founders and number of supplementations with pupfish from outside the refuge. Allele richness was more sensitive to these factors than was heterozygosity, also as predicted from theory (Nei, Maruyama & Chakraborty, 1975; Allendorf, 1986). It is worth emphasizing that these losses in diversity occurred despite the aforementioned aspects of pupfish biology that promote rapid rebound from population bottlenecks, a factor that should moderate such losses (Nei *et al.*, 1975).

The tendency for refuge populations to cluster by lineage suggests signatures of strong genetic drift early in lineage histories. Reduced diversity in the DNFH lineage seems to have occurred in the wild-source population before founding of the lineage. Dunham & Minckley (1998) attributed low allozyme diversity in the parent DNFH refuge and one of its descendant refuges (DBG) to founder effect during establishment of the wild-source population in a recently dug pool. Since translocation to DNFH, the stock has consistently remained above 500 adults (M. Ulibarri, pers. comm.). Although the size of the founding stock was large (280 fish), it apparently had low diversity that was passed on to its descendants. 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 the WLM lineage largely reflects genetic drift in the parent (= source) refuge sometime between 1991 and the founding of the lineage in 1976. The one descendant population of a WLM transplant in 1977 (FWJ) was markedly less divergent from wild populations than it was from a cluster of six refuge populations, five from a WLM transplant in 1991 and one from a WLM transplant in 2002. The WLM lineage originated with 64 fish from several springs and was kept in a small backyard pool (<4 m<sup>2</sup> 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.). The population was estimated to have 'persisted in the low hundreds' (Dunham & Minckley, 1998, p. 10), but 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.). 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 BT appears to show the effect of lineage mixing, together with population size. This large refuge (~6000 m<sup>2</sup>), 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 Fig. 4 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 with 10 wild fish in 1985 and received an additional 250 in 1987 (S. Keeney, pers. comm.). It is the only refuge that received supplementation from the wild, and it is one of the largest refuges ( $470 \text{ m}^2$ ) in our study. The success in retaining diversity after two decades probably is a result of the size of the refuge, together with inoculation from the wild, 2 years after it was founded with an inadequate number of fish.

In conclusion, the global refuge program has been reasonably successful at maintaining the original diversity in wild populations. However, the majority of local refuge populations have markedly low levels of microsatellite diversity. Fortunately, wild populations still exist and managers have a number of options. At one extreme, some of the existing refuge stocks could be destroyed and replaced with stocks from the wild. Alternatively, all existing stocks could be retained and managed in a way to increase levels of diversity. Translocation of a few individuals from genetically diverse populations into refuge populations can have an immediately large effect on diversity (Yamamoto *et al.*, 2006), particularly if coupled with prior removal of a portion of the refuge population. This is logistically simple, especially for smaller refuges where a single seine haul can remove a large proportion of the adults.

The prognosis for wild populations of the desert pupfish complex is not good (Dunham & 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 habitat loss and interactions with non-native fishes (Hendrickson & Varela-Romero, 1989; Varela-Romero *et al.*, 2002; Martin & Saiki, 2005). It appears, therefore, that refuge management will continue to be critical to conservation of the desert pupfish complex.

The success, to this point in time, of the global refuge program in preserving genetic diversity in *C. macularius* and *C. eremus* clearly has been facilitated by establishment of multiple lineages. Losses of alleles have occurred in most lineages, but with some complementation in the suites of alleles retained by others, an effect long recognized as a retardant to overall loss in diversity (Lacy, 1987). With continued losses, however, overall diversity could decline to unacceptable levels. This, and other problems, such as the potential for unwanted adaptation to local conditions can be minimized with management for increased effective population size and a program of genetic exchange (Mills & Allendorf, 1996). We emphasize that such a program should

be developed with careful attention to the potential negative effects of such exchange (Kinnison, Hendry & Stockwell, 2007). Regardless, with relatively minor alterations in management the refuge program appears adequate to preserve a large proportion of the wild genetic diversity.

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