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Genetic evidence for two evolutionarily significant units of White Sands pupfish

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Summary

White Sands pupfish (*Cyprinodon tularosa*) are endemic to southern New Mexico and occur in only four localities: Malpais Spring, Salt Creek, Mound Spring and Lost River. Recently reported historical accounts indicate that the latter two populations were derived from translocations. Their limited distribution and complicated history suggest that knowledge of population genetic structure would be useful for the development of a sound conservation strategy. Mitochondrial DNA (mtDNA) sequences for a segment of the control region showed little variation. Variation was observed for microsatellite and allozyme loci with 37% attributable to divergence among populations. The mean genetic distance between Malpais Spring and the other three populations was high (allozymes: D_{arc} = 0.541; microsatellites: R_{st} = 0.684) compared with the mean distance among the other three populations (D_{arc} = 0.161, R_{st} = -0.016). There were fixed or nearly fixed differences in allele frequency between the Malpais Spring population and the other three populations at one allozyme locus (*hexokinase*) and two microsatellite loci (*WSP-02* and *WSP-11*). We suggest the recognition of two evolutionarily significant units (ESUs) for the White Sands pupfish: Malpais Spring and Salt Creek. Our data indicate that the Lost River and Mound Spring populations descended from translocations from the Salt Creek population. Therefore, conservation efforts should focus on the Malpais Spring population.

INTRODUCTION

Ryder (1986) introduced the concept of the *evolutionarily significant unit* (ESU) to help guide conservation efforts for captive breeding programs. This concept was subsequently extended to management of wild populations (Waples, 1991) where decisions must be made regarding the allocation of scarce resources to the conservation of rare species. Waples (1991) defined ESUs as populations that are reproductively isolated and constitute an important component of the evolutionary legacy of the species.

Operational definitions for ESUs typically rely on molecular genetic data but may also include ecological, behavioral, or other data (Dizon *et al.*, 1992; Vogler *et al.*, 1993; Moritz, 1994; Vogler & DeSalle, 1994; Barlow, 1995; Stauffer *et al.*, 1995; Legge *et al.*, 1996). Evaluation of population genetic structure provides an efficient means for the identification of ESUs. Consideration of geographic genetic structure of targeted species in the development of conservation plans has been widely discussed (Allendorf & Phelps, 1981; Vrijenhoek, Douglas & Meffe, 1985; Avise & Nelson, 1989; Echelle, 1991; Avise, 1994; Quattro *et al.*, 1996). The advent of numerous molecular genetic techniques (Avise, 1994; Carvalho & Pitcher, 1995) has facilitated the recognition of ESUs, an especially pressing concern for allopatric populations of threatened species that have not been extensively studied.

In contrast to many taxa of the cyprinodontid fishes (Miller, 1948, 1981; Minckley, Meffe & Soltz, 1991), the White Sands pupfish (*Cyprinodon tularosa*) was only recently scientifically described (Miller & Echelle, 1975) and has received relatively little attention (Jester & Suminski, 1982; A. A. Echelle, Echelle & Edds, 1987). The White Sands pupfish occurs in only four localities in southern New Mexico: Salt Creek, Malpais Spring and Mound Spring located on White Sands Missile Range and Lost River located on Holloman Air Force Base (Fig. 1), and is classified as endangered by the State of New Mexico.

Genetic data have been used to guide the conservation efforts for this rare species. A. A. Echelle *et al*. (1987) examined allozyme variation in the White Sands pupfish and found only two out of 28 loci to be polymorphic.

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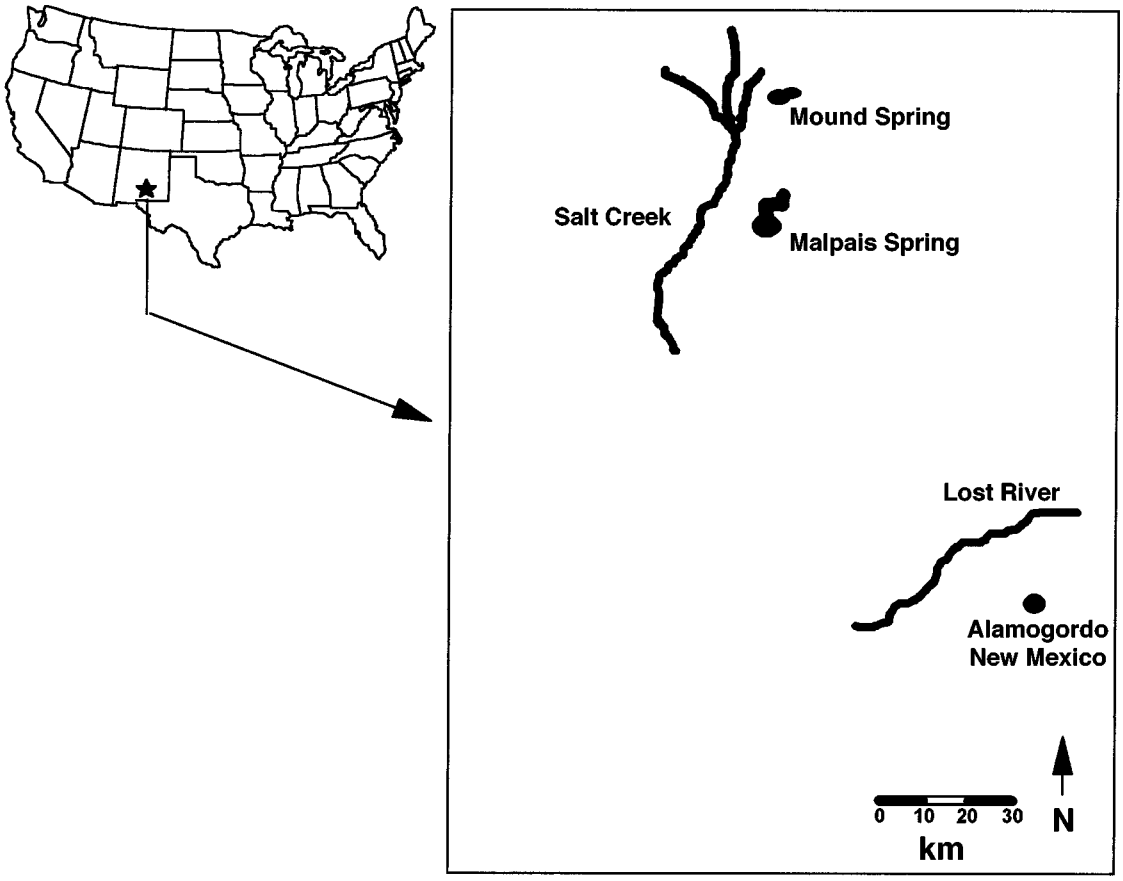

Fig. 1. The distribution of the White Sands pupfish is shown. Fish were collected at: Lost River (confluence of Malone Draw and Ritas Draw), Salt Creek (lower end, i.e. south), Malpais Spring (approximately 50 m below the head spring), and the lower pool at Mound Spring.

Approximately 19% of the observed variation occurred among populations (A. A. Echelle *et al.*, 1987). The Mound Spring population was the most divergent; whereas Lost River and Salt Creek populations were the most similar (A. A. Echelle *et al.*, 1987).

The apparent management implications of these data have been widely discussed (Meffe & Vrijenhoek, 1988; Sublette, Hatch & Sublette, 1990; Meffe & Carroll, 1994). Meffe & Vrijenhoek (1988) suggested that White Sands pupfish populations should be managed as independent units and based their recommendations largely on the available genetic data. Because of its restricted range, Johnson & Jenson (1991) suggested that additional refuge populations be established. The Lost River population has been presumed to be an introduced population based on anecdotal reports of fish translocation, and allozyme data presented by A. A. Echelle *et al.* (1987) have been used to argue that the Salt Creek population provided the founding stock (Sublette *et al.*, 1990). Because of its genetic uniqueness (see A. A. Echelle *et al.*, 1987) and relatively small habitat, the Mound Spring population was given the highest priority in the species management plan, and for translocation to create a 'replicate' population (Pittenger & Springer, in press).

These management recommendations should be re-evaluated, because of recently reported information regarding the history (Pittenger & Springer, in press) and genetics of White Sands pupfish. Recently obtained historic information indicates that the populations at both Mound Spring and Lost River are not native (Pittenger & Springer, in press). Further, Echelle & Echelle (1992) recently revised their genetic data to indicate that only one locus was polymorphic, calling into doubt interpretations based on the original data.

The present study was undertaken to provide additional data regarding genetic divergence and relationships in the White Sands pupfish to facilitate identification of ESUs and to update management strategies. We apply allozymes, microsatellites and sequence analysis of the control region of mtDNA to assess the genetic structure of the four populations of the White Sands pupfish.

METHODS

During August 1995, fish were collected with minnow traps at Malpais Spring, Salt Creek and Lost River (Fig. 1). In April 1996, 50 fish were collected at Mound Spring (Fig. 1). These sites are near localities sampled by A. A. Echelle *et al.* (1987). Fish were stored on dry ice and shipped to the Savannah River Ecology Laboratory.

Allozymes

Fifty fish per population were prepared for allozyme electrophoresis following the methods described by A. A. Echelle *et al.* (1987). Three tissues were dissected from the fish: (1) eyes and brain, (2) liver and (3) muscle and were stored at -70°C. We examined the gene products of 28 loci reported by A. A. Echelle *et al.* (1987) in 30 fish; 10 fish from each of the Lost River, Malpais Spring and Salt Creek populations. The results from this survey were in agreement with Echelle & Echelle (1992); all but one locus, phosphogluconate dehydrogenase (*Pgdh-A*; EC 1.1.1.44), were monomorphic. We surveyed pupfish for additional polymorphic loci and resolved nine additional loci. Buffers, tissue source and corresponding protein systems were: (1) tris citrate pH 8.0; (Selander *et al.*, 1971) for hexokinase (*Hk-A*; EC 2.7.1.1; liver), L-iditol dehydrogenase (*Iddh-A*; EC 1.1.1.14; liver), xanthine dehydrogenase (*Xdh-A*; EC 1.1.1.204; liver), aconitate hydratase (*Acoh*; EC 4.2.1.3; muscle), and malate dehydrogenase ($NADP^+$) (*mMdhp-A*, *sMdhp-A*; EC 1.1.1.40; muscle), and (2) tris borate EDTA pH 8.6 (Selander *et al.*, 1971) for esterase (*Est-1, 2, 3*; EC 3.1.1; muscle). We examined 30 fish from each of the four populations for these nine loci plus *Pdgh-A* (liver tissue on Tris citrate pH 8.0). Of these 10 loci, three were polymorphic: *Pgdh-A*, *Hk-A* and *Xdh-A*. Finally, to improve the estimates of allele frequencies, we collected data on these three polymorphic loci for a total of 50 fish per population. The most common allele in the Salt Creek population was designated '100' and other alleles were designated based on their mobility relative to the standard and the origin '0'.

Gels stained for hexokinase activity showed a polymorphic locus with two alleles, which displayed the double-banded heterozygote pattern indicative of a monomeric protein. Products of this locus were also observed when gels were stained for phosphogluconate dehydrogenase. Turner (1974) reported a similar co-staining phenomenon for other pupfish species. Xanthine dehydrogenase displayed a pattern typical of gene products with a dimeric structure.

DNA assessments

Whole genomic DNA was extracted using phenol-chloroform from the muscle tissue of 30 fish from each population. These fish were an arbitrary subset of the fish examined for allozymes. Six microsatellite primer pairs were designed, four of which yielded consistent amplification products (Table 1). Alleles were named according to the size of the amplified product. Two loci, *WSP-03* and *WSP-07*, were monomorphic in our population samples (Table 1), and will not be considered further. The polymerase chain reaction (PCR) was performed in 10 µl reaction volumes containing 1× Promega *Taq* buffer, 1.5 mM $MgCl_2$, 0.15 µM of each primer, 0.1 mM of each dNTP and 0.5 units of Promega *Taq* polymerase. Thermal cycling consisted of 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, preceded by 2 min at 94°C and followed by 4 min at 72°C. One primer was end-labeled with 1 µCi [γ–^{32}P] ATP per 5 pmol of primer. Products were resolved by electrophoresis on 6% polyacrylamide sequencing gels followed by overnight autoradiography.

Ten individuals were examined for control region sequence variation; three each from Lost River and Salt Creek and two each from Malpais Spring and Mound Spring. The 482 bp segment of mtDNA control region was amplified by PCR using primer L15926 (5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′; Kocher *et al.*, 1989) and primer H16498 (5′-CCTGAAC-TAGGAACCAGATG-3′; Shields & Kocher, 1991). The former primer is located in the threonine tRNA gene adjacent to the control region, and the latter primer is located in a conserved central region of the control region (Fajen & Breden, 1992). Approximately 100 ng of whole genomic DNA was used in a 50 µl reaction solution containing 250 µM of each dNTP, each primer at 0.5 µM, 1 unit of *Taq* polymerase (Perkin Elmer Cetus, Inc.), and 10 µl 5× optimizer kit buffer F (Invitrogen™). Reactions were amplified for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Double-stranded DNA was purified using Microcon 100™ filters and used as template for automated sequencing following Applied Biosystems protocol.

Table 1. Microsatellite loci from *C. tularosa*

Locus [sequence motif]	Primer sequences (5′ → 3′)	n[a]	Number of alleles
WSP-02 $[(CAA)_3(CA)_{14}CCAA(CA)_4]$	GCGCGTCAGCCAAAACAACAAT GCGTGCAACCCTGGAGGAAG	120	6
WSP-03 $[(GT)_9]$	TAAGTGCGTTTGAACTCTGAAT AGGGTGGGCTTTCTCAATA	120	1
WSP-07 $[(CA)_7(TA)_3CG(TA)_5]$	AAGGAGCTGCAAGCACAGTT TTAGGCGGAGAAAAAGCTAGA	120	1
WSP-11[b] $[(TA)_6TC(TA)_2(TC)_{18}(TA)_{15}]$	AACAAATCCAATAATGTATTAGAA CCCCTGCTGCCTCAAAG	120	10

[a] Thirty adults were assayed from each of the four extant *C. tularosa* populations for a total of 120 individuals.
[b] The primer sequences shown for *WSP-11* are the redesigned primers which do not suffer from non-amplifying alleles. For the original primer sequences see Jones *et al.* (in press).
The microsatellite sequence motifs in the original cloned fragment, primer sequences, number of individuals assayed (n), and numbers of alleles are shown.

Analyses

BIOSYS-I (Swofford & Selander, 1981) was used with allozyme and microsatellite data to calculate allele frequencies, mean observed heterozygosity (H_{obs}), mean expected heterozygosity (H_{exp}), mean number of alleles per locus (A) and F statistics. Because standard distance metrics may not be appropriate for analysis of microsatellite data (Goldstein *et al.*, 1995; Slatkin, 1995), we conducted separate genetic distance analyses with the allozyme data and microsatellite data. Allozyme data were analyzed for genetic distance using the D_{arc} distance metric (Cavalli-Sforza & Edwards, 1967) and microsatellite data were analyzed using the R_{st} pairwise distance metric (Goodman, 1997). Departures from Hardy–Weinberg expectations were tested via χ^2 analyses for all cases where expected numbers were at least three. Tests for linkage disequilibrium were performed using GENEPOP (version 1.2; Raymond & Rouset, 1995). Control region sequences were assembled and aligned by Sequencher™ and visually examined for alignment. Sequences were analyzed using PAUP 3.1 (Swofford, 1993).

RESULTS

Genotype scores are presented in the Appendix. No significant departures of genotype frequencies from

Table 2. Allele frequencies for four populations of White Sands pupfish

	Malpais Spring	Salt Creek	Lost River	Mound Spring
Locus				
Pgdh-A				
110	0.551	0.200	0.090	0.125
100	0.449	0.630	0.900	0.500
91	–	0.170	0.010	0.375
n	49	50	50	48
Hk-A				
100	0.053	1.000	1.000	1.000
91	0.947[a]	–	–	–
n	47	50	50	49
Xdh-A				
107	0.011	–	0.010	0.052
100	0.989	1.000	0.990	0.948
n	46	50	50	48
WSP-02				
220	0.933	0.183	–	0.250
222	0.033[a]	–	–	–
224	0.033	0.700	1.000	0.583
228	–	0.050	–	0.033
230	–	0.067	–	0.133
n	30	30	30	30
WSP-11				
173	0.083[a]	–	–	–
179	0.167[a]	–	–	–
181	0.317[a]	–	–	–
187	0.233[a]	–	–	–
189	0.017[a]	–	–	–
194	–	0.317	0.383	0.383
196	–	0.050[a]	–	–
198	–	–	0.017[a]	–
200	–	0.633	0.600	0.617
202	0.183[a]	–	–	–
n	30	30	30	30
Mean heterozygosity[b]				
Overall				
Direct count (H_{dc})	0.282 ± 0.127	0.311 ± 0.127	0.151 ± 0.103	0.343 ± 0.124
Expected (H_{exp})	0.308 ± 0.146	0.304 ± 0.125	0.141 ± 0.096	0.354 ± 0.127
Allozymes				
Direct Count (H_{dc})	0.192 ± 0.131	0.173 ± 0.173	0.073 ± 0.064	0.215 ± 0.166
Expected (H_{exp})	0.208 ± 0.148	0.180 ± 0.180	0.068 ± 0.058	0.233 ± 0.186
Microsatellites				
Direct count (H_{dc})	0.417 ± 0.283	0.517 ± 0.017	0.267 ± 0.267	0.533 ± 0.100
Expected (H_{exp})	0.459 ± 0.331	0.491 ± 0.014	0.251 ± 0.251	0.534 ± 0.054
Mean alleles per locus				
Overall	3.0	2.4	2.0	2.4
Allozymes	2.0	1.7	2.0	2.0
Microsatellites	4.5	3.5	2.0	3.0

[a] Private alleles.
[b] Mean ± standard deviation.

Table 3. *F* statistics are shown for: (a) all four populations, and (b) without the Malpais Spring population

Locus	F_{is}	F_{it}	F_{st}	χ^2	*P*
(a) Four populations					
Pgdh-A	0.052	0.206	0.162	139.7	< 0.001
Hk-A	–0.056	0.926	0.930	365.0	< 0.001
Xdh-A	–0.042	–0.019	0.022	8.7	0.033
WSP-02	–0.107	0.403	0.461	164.6	< 0.001
WSP-11	0.032	0.225	0.199	251.4	< 0.001
Mean	0.005	0.377	0.374	929.5	< 0.001
(b) Excluding Malpais Spring[a]					
Pgdh-A	0.037	0.140	0.107	52.5	< 0.001
Xdh-A	–0.047	–0.021	0.025	7.5	0.024
WSP-02	–0.113	0.015	0.115	32.0	< 0.001
WSP-11	–0.003	0.000	0.004	8.5	0.201
Mean	–0.021	0.052	0.071	100.6	< 0.001

[a] *Hk-A* is monomorphic for these three populations.
Heterogeneity among populations is tested by χ^2 analysis (Swofford & Selander, 1981).

Hardy–Weinberg expectations were observed. Linkage disequilibrium was detected for only one out of 21 tests; *WSP-02* and *WSP-11* in the Mound Spring population. Such a result is expected by chance.

Both direct count and expected multi-locus heterozygosity were relatively low in the Lost River population compared to the other three populations (Table 2). The lower level of heterozygosity in the Lost River population was in part due to reduced allelic diversity (alleles per locus) in this population. Lost River was monomorphic at *WSP-02* and relatively invariant at *Pgdh-A*; two loci that were relatively polymorphic in the other three populations (Table 2).

Allozyme allelic diversity was similar among the four populations (Table 2). In contrast, the highest level of microsatellite allelic diversity occurred at Malpais Spring, followed by Salt Creek, Mound Spring and, finally, Lost River. Much of this variability was due to a suite of private alleles at the *WSP-11* locus in the Malpais Spring population. Malpais Spring had eight private alleles; whereas Salt Creek, Lost River and Mound Spring populations each had one, one and zero private alleles, respectively.

Genetic divergence among populations was substantial (Table 3(a)); approximately 37% of the variation occurred among populations. This was due to the fixed or nearly fixed difference in allele frequency at *Hk-A*, *WSP-02* and *WSP-11* among populations (Table 2). The *Hk-A*^{100} allele was at 100% frequency in Salt Creek, Mound Spring and Lost River samples, whereas this allele was uncommon at Malpais Spring (5.3%; Table 2). The *WSP-02*^{220} allele was most common in the Malpais Spring population but was absent or at low frequency in the other three populations (Table 2). The *WSP-02*^{224} allele was at low frequency at Malpais Spring but at high frequency or fixed in the other populations (Table 2).

Allele frequencies at *WSP-11* were very similar among the Salt Creek, Mound Spring and Lost River populations, but there were no shared alleles between these populations and the Malpais Spring population (Table 2). Our initial survey detected a high frequency null allele in the Malpais Spring population, but this problem was circumvented by redesigning one of the PCR primers and genotyping all individuals with the new primer pair (Jones *et al.*, in press). Additional molecular dissections revealed that the original failure to amplify some sequences from this microsatellite locus was due to a 4-bp deletion in one of the original priming sites (Jones *et al.*, in press). Amplifications with redesigned primers revealed five distinct alleles (173, 179, 181, 187 and 189) unique to the Malpais Spring population originally identified as the original *WSP-11* null allele (Jones *et al.*, in press). All genotypes reported for *WSP-11* are derived from the redesigned primer pair which did not suffer from non-amplifying alleles. Thus, direct comparisons of allele sizes are still possible. For example, allele 173 differs from allele 202 by the 4-bp null-allele-causing deletion as well as an additional 25 missing base-pairs of unknown composition, probably including microsatellite repeats and, perhaps, flanking sequences.

Malpais Spring was the most divergent population as indicated by two measures. First, the variance among populations (F_{st} = 0.374; Table 3(a)) was considerably lower when the Malpais Spring population was excluded from analyses (F_{st} = 0.071; Table 3(b)). Second, the mean genetic distances (D_{arc}: Cavalli-Sforza & Edwards, 1967; R_{st}: Goodman, 1997) between Malpais Spring and the other three populations were high (D_{arc} = 0.541, R_{st} = 0.684) compared with the mean distances among the other three populations (D_{arc} = 0.161, R_{st} = -0.016, Table 4). Negative R_{st} values can arise for populations which show no differentiation. Under these circumstances, the between-population component of variation can be much smaller than the within-population component leading to a negative value for R_{st}. This is effectively due to statistical noise (S. Goodman, pers. comm.).

For a 482 bp segment of the d-loop two base-pair substitutions were observed (0.001% sequence divergence) giving rise to three haplotypes among the 10 fish examined (GenBank number AF067556–AF067565). The

Table 4. Genetic distances among the four populations of White Sands pupfish are shown

	Malpais Spring	Salt Creek	Lost River	Mound Spring
Malpais Spring	—	0.530	0.530	0.564
Salt Creek	0.652	—	0.145	0.122
Lost River	0.795	–0.014	—	0.217
Mound Spring	0.605	–0.016	–0.017	—

The arc distance (D_{arc}) of Cavalli-Sforza & Edwards (1967) for allozyme data, and R_{st} for microsatellite data (Goodman, 1997) are above and below the diagonal, respectively.

distribution of haplotypes did not fit any particular geographic pattern. One Lost River fish had a haplotype which was due to a transition, and two fish (Salt Creek and Lost River) shared the same haplotype due to a transversion. The remaining seven fish shared the most common haplotype. The greatest uncorrected distances among all White Sands pupfish was 0.004. Control region sequences available in GenBank for *C. variegatus* and *C. diabolis* (Parker & Kornfield, 1995) were compared for a 341 bp segment homologous with the segment in White Sands pupfish. Distances between the White Sands pupfish and *C. diabolis* and *C. variegatus* were 0.077 and 0.041, respectively. The greatest genetic distance (0.080) was observed between *C. variegatus* and *C. diabolis*.

DISCUSSION

The Malpais Spring population was the most genetically divergent of the White Sands pupfish populations. Our data are consistent with recently obtained historical information indicating that the Mound Spring and Lost River populations are not native (Pittenger & Springer, in press). In the early 1900s, fish were present at Malpais Spring (Herrick, 1900) and at Salt Creek (Pittenger & Springer, in press), but fish were not reported from Mound Spring or Lost river until the early 1980s (Jester & Suminski, 1982; A. A. Echelle *et al.*, 1987). Apparently, pupfish were absent at Mound Spring during the period from 1967 to 1973, when this spring was excavated. A documented introduction of approximately 30 fish occurred at the lower end of Lost River in the early 1970s (Pittenger & Springer, in press). Our molecular genetic data suggest that the Lost River and Mound Spring populations were derived from the Salt Creek population.

The low levels of variation in the control region of White Sands pupfish are consistent with low levels of genetic diversity observed in allozymes for this species (A. A. Echelle *et al.*, 1987; this study). The mitochondrial control region has provided population-level phylogenetic resolution for some fish species (Fajen & Breden, 1992; Brown, Beckenbach & Smith, 1993; Meyer, 1993), but has been relatively uninformative in other recently diverged taxa (Meyer *et al.*, 1990). For White Sands pupfish, low variation and small sample sizes made the control region uninformative for estimates of population structure.

Allozyme and microsatellite data suggest these populations have been isolated long enough to have diverged at three loci. The lack of divergence in the control region can be used to estimate a crude upper limit for time of divergence for these populations. If 8.5% sequence divergence is expected per million years (Vigilant *et al.*, 1989; Fajen & Breden, 1992), then the observed lack of divergence would indicate that these populations diverged much less than 100 000 years ago. Indeed, Miller & Echelle (1975) suggested that these populations were most likely isolated at the end of the Pleistocene.

Based on their strong genetic and geographic structure, we suggest that the White Sands pupfish comprises two evolutionarily significant units: the Salt Creek and Malpais Spring conservation units. Loss of either of these conservation units would result in a substantial reduction in allelic diversity; 32–36% as suggested by our data. The ESU status of the Salt Creek and Malpais Spring populations is further warranted by the divergent ecological attributes of their respective habitats. Salinity was relatively high at Salt Creek ($\bar{x}$ = 15.9 parts per thousand (ppt)) compared with Malpais Spring ($\bar{x}$ = 5.9 ppt) (J. Pittenger, New Mexico Department of Game and Fish, unpublished data). Mound Spring has a low salinity ($\bar{x}$ = 3.0 ppt), whereas salinity at Lost River is exceptionally high ($\bar{x}$ = 27.1 ppt) and has been recorded as being as high as 100 ppt (Turner, 1987).

Salinity may have direct and indirect effects on fish (see Stockwell & Mulvey, in press). Invertebrate diversity is noticeably lower at the two saline sites (Salt Creek and Lost River) compared with Malpais Spring and Mound Spring. Snails (*Physa* sp.) are present at both Malpais Spring and Mound Spring, but absent at Salt Creek and Lost River. These physids can only tolerate salinity below 9 ppt (C. A. Stockwell, unpublished data). *Physa* acts as an intermediate host to parasitic diplostome trematodes. Therefore, fish at Malpais Spring and Mound Spring are exposed to those parasites, whereas fish from Salt Creek and Lost River escape this parasitism (C. A. Stockwell, unpublished data). A white grub (presumably *Posthodiplostomum minimum*; J. Landye, pers. comm.) had high prevalence in fish from Malpais Spring and Mound Spring (up to 100%), but was absent from fish examined from Salt Creek and Lost River.

Thus, the Salt Creek and Malpais Spring populations confront different ecological challenges; another factor that warrants their recognition as separate ESUs (see Vogler *et al.*, 1993; Legge *et al.*, 1996). Isolation of other cyprinodontids in divergent environmental conditions is associated with rapid diversification in this group (Miller, 1948, 1981).

Others have pointed out limitations of the ESU concept (Mayden & Wood, 1995; Pennock & Dimmick, 1997). However, given the vagaries of taxonomy below the species level and the general problems associated with species concepts (Mayden & Wood, 1995), the ESU provides an ideal concept by which to manage the White Sands pupfish. Our findings would elevate the status of Salt Creek and Malpais Spring populations of White Sands pupfish to that accorded to the numerous taxa of pupfishes, many of which have official protection (Williams *et al.*, 1989; Minckley *et al.*, 1991). For

instance, there are five extant subspecies of *Cyprinodon nevadensis*, and three of these subspecies are protected as endangered or threatened (Williams *et al.*, 1989). However, an inspection of allozyme data (Echelle & Echelle, 1993) shows that differences among these taxa are no greater than those observed between the Salt Creek and Malpais Spring populations of White Sands pupfish. Thus, the ESU concept avoids the taxonomic morass and provides recognition of divergent populations. Loss of either of the two ESUs of White Sands pupfish would result in a substantial loss of the evolutionary legacy of this species.

According to Moritz (1994), ESUs must be reciprocally monophyletic with respect to mtDNA and must exhibit significant differences in allele frequencies at nuclear loci. This operational definition provides a simple set of criteria that can be easily applied to molecular data, but, as noted by Moritz (1994), strict adherence to this definition may seem overly restrictive in some instances. In the case of White Sands pupfish, for example, the Malpais Spring population is identified by a number of unique alleles including one private allozyme allele which is nearly fixed. Further, this population occupies a habitat with different ecological attributes from the other native population at Salt Creek. Under a strict interpretation of Moritz's ESU definition, these populations would be Management Units (Moritz, 1994), because they exhibit significant allele frequency differences at nuclei loci, yet have not been shown to be reciprocally monophyletic for mtDNA. However, given the substantial genetic and ecological differences between the Malpais Spring and Salt Creek populations, we argue they should be recognized as separate ESUs.

The translocation and replication of the Salt Creek population in Lost River and Mound Spring provides an opportunity to evaluate the effectiveness of population translocation as a management strategy and its potential effects on genetic diversity. The number of founders for the Mound Spring population was not documented, but the Lost River population was founded with approximately 30 fish (Pittenger & Springer, in press). Compared with the putative source population at Salt Creek, heterozygosity and allelic diversity were reduced in the Lost River population, but not in the Mound Spring population (Table 2). Thus, these results are consistent with theoretical expectations, as well as empirical work which has shown that genetic diversity is often, but not predictably, compromised in translocated populations (Allendorf & Ryman, 1987; Stockwell, Mulvey & Vinyard, 1996; Dunham & Minckley, 1998). Loss of the Malpais Spring, Salt Creek, Lost River or Mound Spring populations would result in a reduction of White Sands pupfish allelic diversity of 36, 4.5, 4.5 or 0%, respectively. This illustrates how replication of conservation units increases the security of overall allelic diversity. As with other species that exhibit significant genetic structure, maximal genetic diversity is best preserved by conserving numerous populations (Allendorf & Leary, 1988; A. F. Echelle, Echelle & Edds, 1989; Leary, Allendorf & Forbes, 1993).

Current conservation plans for the White Sands pupfish call for the creation of additional 'refuge' populations. Clearly, replicating populations of the Salt Creek strain would be a costly and redundant effort. Results of this study have led to a revision of conservation plans so that attention is now focusing on replicating the Malpais Spring population (J. Pittenger, New Mexico Department of Game and Fish, pers. comm.). A number of sites, most of which are characterized by low salinity, are currently being considered as refuge sites for the Malpais Spring ESU. A number of considerations, such as geography and local ecology, will also be considered in the selection of a refuge site. Pupfish are exceptionally numerous at Malpais Spring, therefore the replicate populations can be founded with a large number of individuals (hundreds), a luxury not often afforded for endangered species. Further, we advocate the establishment of one-way gene flow between the parental population at Malpais Spring and the refuge population.

Gene flow could also be established between the Salt Creek population and the two introduced populations at Mound Spring and Lost River. Alternatively, the Mound Spring and Lost River populations could be managed independently as management units (Moritz, 1994; Britten *et al.*, 1997). The Mound Spring and Lost River populations are genetic 'replicates' of the Salt Creek strain. However, only the Lost River population can be considered a true ecological 'replicate' because of the much lower level of salinity at Mound Spring. We suggest the establishment of one-way gene flow from Salt Creek to Lost River, and that the Mound Spring population be considered a separate Management Unit. Artificial gene flow could serve to replenish the allelic diversity in the Lost River population so that it more closely represents the Salt Creek ESU. Further, independent management of the Mound Spring population is warranted because the ecological attributes of the habitat at Mound Spring are different from Salt Creek. Gene flow may retard the opportunity for this population to adapt to local conditions (Stearns & Sage, 1980).

In the long term, creation of refuge populations may also serve to preserve taxa from extinction (Miller & Pister, 1971; Hendrickson & Brooks, 1991; Minckley *et al.*, 1991; Echelle & Echelle, 1997). Conservation and replication of both ESUs of the White Sands pupfish should ensure the overall security of this rare species.

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APPENDIX

Raw genotype scores for allozymes (*Pgdh-A, Hk-A, Xdh-A*) and microsatellites (*WSP-02, WSP-11*) are given

Population	Sample	*Pgdh-A*	*Hk-A*	*Xdh-A*	*WSP-02*	*WSP-11*
Malpais Spring	ML01	100/100	91/91	100/100	220/220	187/187
Malpais Spring	ML02	110/110	91/91	100/100	220/220	179/202
Malpais Spring	ML03	110/110	91/91	100/100	220/220	181/187
Malpais Spring	ML04	100/110	91/91	100/100	220/220	181/187
Malpais Spring	ML05	100/100	91/91	100/100	220/220	187/187
Malpais Spring	ML06	100/110	91/91	100/100	220/220	179/202
Malpais Spring	ML07	100/110	91/91	100/100	220/220	181/181
Malpais Spring	ML08	100/110	91/91	100/100	220/220	181/181
Malpais Spring	ML09	110/110	91/91	100/100	220/220	173/187
Malpais Spring	ML10	100/110	91/91	100/100	220/220	202/202
Malpais Spring	ML11	100/110	91/91	100/100	220/220	181/189
Malpais Spring	ML12	100/100	91/91	100/100	220/220	187/202
Malpais Spring	ML13	100/110	91/91	100/100	220/220	173/181
Malpais Spring	ML14	100/100	91/91	100/100	220/220	187/202
Malpais Spring	ML15	110/110	91/91	100/100	220/220	181/187
Malpais Spring	ML16	100/110	91/91	100/100	220/220	181/187
Malpais Spring	ML17	100/110	91/91	100/100	220/222	179/202
Malpais Spring	ML18	110/110	91/91	100/100	220/220	181/181
Malpais Spring	ML19	100/100	91/91	100/100	220/220	181/187
Malpais Spring	ML20	100/110	91/100	100/100	220/220	173/179
Malpais Spring	ML21	100/100	91/91	100/100	220/220	187/202
Malpais Spring	ML22	100/110	91/91	100/100	220/220	173/181
Malpais Spring	ML23	100/110	91/91	100/100	220/220	173/181
Malpais Spring	ML24	110/110	91/91	100/100	220/222	202/202
Malpais Spring	ML25	100/110	91/91	100/107	220/220	179/181
Malpais Spring	ML26	110/110	91/91	100/100	220/224	179/179
Malpais Spring	ML27	100/100	91/100	100/100	220/224	181/181
Malpais Spring	ML28	110/110	91/91	100/100	220/220	179/202
Malpais Spring	ML29	100/110	91/91	100/100	220/220	179/181
Malpais Spring	ML30	100/100	91/91	100/100	220/220	179/187
Malpais Spring	ML31	100/110	91/91	100/100		
Malpais Spring	ML32	100/110	91/91	100/100		
Malpais Spring	ML33	100/110	91/91	100/100		
Malpais Spring	ML34	100/110	91/91			
Malpais Spring	ML35	100/110	91/91	100/100		
Malpais Spring	ML36	100/110	91/100	100/100		
Malpais Spring	ML37	100/110	91/91	100/100		
Malpais Spring	ML38	110/110	91/91	100/100		
Malpais Spring	ML39	110/110	91/91	100/100		
Malpais Spring	ML40	110/110	91/91	100/100		
Malpais Spring	ML41	100/100	91/91	100/100		
Malpais Spring	ML42	100/110	91/100	100/100		
Malpais Spring	ML43	110/110	91/91	100/100		
Malpais Spring	ML44	110/110	91/100	100/100		
Malpais Spring	ML45	100/100		100/100		
Malpais Spring	ML46	110/110	91/91	100/100		
Malpais Spring	ML47	110/110		100/100		
Malpais Spring	ML48	100/100	91/91	100/100		
Malpais Spring	ML49	110/110	91/91	100/100		
Malpais Spring	ML50		91/91			

Population	Sample	*Pgdh-A*	*Hk-A*	*Xdh-A*	*WSP-02*	*WSP-11*
Salt Creek	SC01	100/100	100/100	100/100	224/224	194/194
Salt Creek	SC02	100/100	100/100	100/100	220/224	200/200
Salt Creek	SC03	91/100	100/100	100/100	224/224	200/200
Salt Creek	SC04	110/110	100/100	100/100	220/224	200/200
Salt Creek	SC05	100/100	100/100	100/100	224/224	194/200
Salt Creek	SC06	91/100	100/100	100/100	220/224	194/200
Salt Creek	SC07	100/110	100/100	100/100	224/224	194/200
Salt Creek	SC08	100/110	100/100	100/100	224/230	194/194
Salt Creek	SC09	91/110	100/100	100/100	224/224	194/200
Salt Creek	SC10	100/110	100/100	100/100	224/224	194/200
Salt Creek	SC11	100/100	100/100	100/100	224/224	194/200
Salt Creek	SC12	100/100	100/100	100/100	220/224	196/200
Salt Creek	SC13	100/100	100/100	100/100	224/224	194/200
Salt Creek	SC14	91/91	100/100	100/100	224/230	194/194
Salt Creek	SC15	100/100	100/100	100/100	224/224	200/200
Salt Creek	SC16	100/100	100/100	100/100	224/230	194/196
Salt Creek	SC17	100/100	100/100	100/100	224/228	200/200
Salt Creek	SC18	100/110	100/100	100/100	224/224	194/200
Salt Creek	SC19	91/110	100/100	100/100	224/224	194/200
Salt Creek	SC20	91/100	100/100	100/100	224/224	200/200
Salt Creek	SC21	100/110	100/100	100/100	224/224	200/200
Salt Creek	SC22	100/110	100/100	100/100	220/220	200/200
Salt Creek	SC23	91/110	100/100	100/100	220/224	194/200
Salt Creek	SC24	100/100	100/100	100/100	220/224	194/200
Salt Creek	SC25	91/100	100/100	100/100	220/224	200/200
Salt Creek	SC26	100/110	100/100	100/100	224/230	200/200
Salt Creek	SC27	100/110	100/100	100/100	220/224	196/200
Salt Creek	SC28	100/110	100/100	100/100	224/228	200/200
Salt Creek	SC29	100/110	100/100	100/100	224/228	194/200
Salt Creek	SC30	100/110	100/100	100/100	220/224	200/200
Salt Creek	SC31	100/100	100/100	100/100		
Salt Creek	SC32	100/100	100/100	100/100		
Salt Creek	SC33	100/100	100/100	100/100		
Salt Creek	SC34	100/100	100/100	100/100		
Salt Creek	SC35	100/100	100/100	100/100		
Salt Creek	SC36	91/110	100/100	100/100		
Salt Creek	SC37	91/100	100/100	100/100		
Salt Creek	SC38	91/100	100/100	100/100		
Salt Creek	SC39	91/100	100/100	100/100		
Salt Creek	SC40	100/100	100/100	100/100		
Salt Creek	SC41	91/110	100/100	100/100		
Salt Creek	SC42	110/110	100/100	100/100		
Salt Creek	SC43	100/100	100/100	100/100		
Salt Creek	SC44	91/100	100/100	100/100		
Salt Creek	SC45	91/100	100/100	100/100		
Salt Creek	SC46	100/100	100/100	100/100		
Salt Creek	SC47	100/100	100/100	100/100		
Salt Creek	SC48	91/100	100/100	100/100		
Salt Creek	SC49	100/100	100/100	100/100		
Salt Creek	SC50	100/100	100/100	100/100		

Population	Sample	*Pgdh-A*	*Hk-A*	*Xdh-A*	*WSP-02*	*WSP-11*
Lost River	LR01	100/100	100/100	100/100	224/224	194/200
Lost River	LR02	91/100	100/100	100/100	224/224	194/200
Lost River	LR03	100/100	100/100	100/100	224/224	200/200
Lost River	LR04	100/100	100/100	100/100	224/224	194/200
Lost River	LR05	100/100	100/100	100/100	224/224	194/200
Lost River	LR06	100/100	100/100	100/100	224/224	194/200
Lost River	LR07	100/110	100/100	100/100	224/224	194/194
Lost River	LR08	100/110	100/100	100/100	224/224	200/200
Lost River	LR09	100/100	100/100	100/100	224/224	194/200
Lost River	LR10	100/100	100/100	100/100	224/224	200/200
Lost River	LR11	100/100	100/100	100/100	224/224	200/200
Lost River	LR12	100/100	100/100	100/100	224/224	198/200
Lost River	LR13	100/100	100/100	100/100	224/224	194/200
Lost River	LR14	100/100	100/100	100/100	224/224	194/200
Lost River	LR15	100/100	100/100	100/100	224/224	194/194
Lost River	LR16	100/100	100/100	100/100	224/224	194/200
Lost River	LR17	100/100	100/100	100/100	224/224	194/194
Lost River	LR18	100/100	100/100	100/100	224/224	200/200
Lost River	LR19	100/100	100/100	100/100	224/224	194/200
Lost River	LR20	100/100	100/100	100/100	224/224	200/200
Lost River	LR21	100/100	100/100	100/100	224/224	200/200
Lost River	LR22	100/100	100/100	100/100	224/224	194/200
Lost River	LR23	100/100	100/100	100/100	224/224	200/200
Lost River	LR24	100/100	100/100	100/100	224/224	194/200
Lost River	LR25	100/100	100/100	100/100	224/224	200/200
Lost River	LR26	100/100	100/100	100/100	224/224	200/200
Lost River	LR27	100/100	100/100	100/100	224/224	194/200
Lost River	LR28	100/100	100/100	100/100	224/224	194/194
Lost River	LR29	100/100	100/100	100/100	224/224	194/200
Lost River	LR30	100/100	100/100	100/100	224/224	194/200
Lost River	LR31	100/100	100/100	100/100		
Lost River	LR32	100/100	100/100	100/100		
Lost River	LR33	100/110	100/100	100/100		
Lost River	LR34	100/100	100/100	100/100		
Lost River	LR35	100/110	100/100	100/100		
Lost River	LR36	100/100	100/100	100/100		
Lost River	LR37	100/100	100/100	100/100		
Lost River	LR38	100/110	100/100	100/100		
Lost River	LR39	100/100	100/100	100/100		
Lost River	LR40	100/100	100/100	100/100		
Lost River	LR41	100/110	100/100	100/107		
Lost River	LR42	100/110	100/100	100/100		
Lost River	LR43	100/100	100/100	100/100		
Lost River	LR44	100/100	100/100	100/100		
Lost River	LR45	100/110	100/100	100/100		
Lost River	LR46	100/100	100/100	100/100		
Lost River	LR47	100/100	100/100	100/100		
Lost River	LR48	100/100	100/100	100/100		
Lost River	LR49	100/110	100/100	100/100		
Lost River	LR50	100/100	100/100	100/100		

Population	Sample	*Pgdh-A*	*Hk-A*	*Xdh-A*	*WSP-02*	*WSP-11*
Mound Spring	MO01	91/100	100/100	100/100	224/224	194/200
Mound Spring	MO02	91/110	100/100	100/100	224/230	194/200
Mound Spring	MO03	100/100	100/100	100/107	220/224	200/200
Mound Spring	MO04	91/110	100/100	100/100	224/230	194/194
Mound Spring	MO05	100/110	100/100	100/100	220/224	194/200
Mound Spring	MO06	91/100	100/100	100/100	220/224	194/200
Mound Spring	MO07	91/91	100/100	100/100	220/224	200/200
Mound Spring	MO08	100/100	100/100	100/100	224/224	194/200
Mound Spring	MO09	91/91	100/100	100/100	224/224	194/200
Mound Spring	MO10	91/100	100/100	100/100	220/230	200/200
Mound Spring	MO11	91/100	100/100	100/100	228/230	194/200
Mound Spring	MO12	91/110	100/100	100/100	224/224	194/200
Mound Spring	MO13	91/91	100/100	100/107	224/228	200/200
Mound Spring	MO14	100/100	100/100	100/100	224/224	194/194
Mound Spring	MO15	91/100	100/100	100/107	224/224	194/194
Mound Spring	MO16	100/110	100/100	100/100	224/224	194/200
Mound Spring	MO17	91/100	100/100	100/100	220/220	194/200
Mound Spring	MO18	100/100	100/100	100/100	224/230	200/200
Mound Spring	MO19	100/100	100/100	100/100	220/224	200/200
Mound Spring	MO20	100/110	100/100	100/100	220/224	200/200
Mound Spring	MO21	100/100	100/100	100/100	224/224	194/194
Mound Spring	MO22	100/110	100/100	100/100	224/230	194/194
Mound Spring	MO23	100/100	100/100	100/100	224/224	194/200
Mound Spring	MO24	100/100	100/100	100/100	220/224	194/200
Mound Spring	MO25	100/100	100/100	100/100	224/224	194/200
Mound Spring	MO26	100/100	100/100	100/100	220/230	200/200
Mound Spring	MO27		100/100	100/100	220/224	200/200
Mound Spring	MO28	91/100	100/100	100/100	220/224	200/200
Mound Spring	MO29	91/110	100/100	100/100	220/230	200/200
Mound Spring	MO30	91/100	100/100	100/100	220/224	200/200
Mound Spring	MO31	91/91	100/100	100/100		
Mound Spring	MO32	91/100	100/100	100/100		
Mound Spring	MO33	91/110	100/100	100/100		
Mound Spring	MO34	91/91	100/100	100/100		
Mound Spring	MO35	100/110	100/100	100/100		
Mound Spring	MO36	91/100	100/100	100/100		
Mound Spring	MO37	91/100	100/100	100/100		
Mound Spring	MO38	100/100	100/100	100/100		
Mound Spring	MO39		100/100	100/100		
Mound Spring	MO40	100/110	100/100	100/100		
Mound Spring	MO41	100/100	100/100	100/107		
Mound Spring	MO42	91/100	100/100			
Mound Spring	MO43	91/100	100/100	100/107		
Mound Spring	MO44	91/91	100/100	100/100		
Mound Spring	MO45	100/100	100/100	100/100		
Mound Spring	MO46	91/91	100/100	100/100		
Mound Spring	MO47	100/100	100/100	100/100		
Mound Spring	MO48	91/91	100/100	100/100		
Mound Spring	MO49	91/100	100/100	100/100		
Mound Spring	MO50	91/110	100/100	100/100		