

N22

April 30, 1996

Dr. Anthony A. Echelle
Oklahoma State University
College of Arts and Sciences
Department of Zoology
430 Life Sciences West
Stillwater, Oklahoma 74078-0459

Dear Dr. Echelle:

Thank you for submitting your proposal to do research on genetic diversity in the desert pupfish (*Cyprinodon macularius*). We appreciate your interest in conducting research at Organ Pipe Cactus National Monument. After review of the materials you provided, we wish to grant approval for your project in Organ Pipe Cactus, contingent on the conditions of a research/collection permit and stipulations. Also, we would like to receive a final signed copy of the grant agreement between you and the Fish and Wildlife Service, to add to your research file here. A monument research/collection permit for 1996 is enclosed, as well as permit stipulations and conditions. Please review these documents, then sign page one of the permit and return it to the monument. Retain page two, and if you have any assistants identified on the permit, please make them each a copy of this permit and the enclosed stipulations. When at Organ Pipe Cactus National Monument doing field work, be sure you have the permit in your possession. Also as a reminder, all research findings must be reported to the monument.

Also enclosed is general "Information for Researchers." Please review this information for additional issues to keep in mind when doing work in the monument. In particular, note the importance of contacting the monument prior to any research visits, preferably seven days before a visit. This allows the Resource Management Division to notify other monument staff of your activities, if appropriate. Also, this will help you become aware of any logistical constraints that may affect your planned visit. For example, construction projects, rains, and other factors have caused some monument roads to be closed intermittently. Finally, by contacting the monument early we may be able to provide staff to accompany you for technical assistance and for their own exposure to your research. Also enclosed is a yellow "Researcher" sign to be placed on your dashboard if you leave your vehicle parked on monument roads. This will help our law enforcement officers, Border Patrol, and others recognize your vehicle.

Finally, you may wish to keep in mind that the monument's Resource Management Division conducts its annual pupfish census in late August or early September. Some incidental mortalities are inevitable; these could be made available as a source for your 30 specimens.

Thank you again for your interest in doing research in Organ Pipe Cactus National Monument. If you have any questions, please contact Chief of Resources Management Jim Barnett or Research Coordinator Tim Tibbitts on (520) 387-7662, extensions 7100 or 7114, respectively.

Sincerely,

/s/ Harold J. Smith

Harold J. Smith
Superintendent

Enclosures

cc: Research Coordinator, ORPI
Field Supervisor, Fish and Wildlife Service, Arizona Ecological Services, 2321 West Royal Palm Road, Phoenix, 85021

bcc: Files
Reading
Superintendent
Chief, Resources Management

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Oklahoma State University

COLLEGE OF ARTS AND SCIENCES

Department of Zoology
430 Life Sciences West
Stillwater, Oklahoma 74078-0459
405-744-5555

7 March 1996



Tim Tiddits
Organ Pipe Cactus National Monument
Route 1, Box 100
Ajo, Arizona 85321

Dear Tim:

As per our recent telephone conversation, I am requesting permission to collect 30 specimens of Quitobaquito pupfish *Cyprinodon macularius eremus* from Quitobaquito Spring.

Included with this application is a grant agreement between Oklahoma State University and the U.S. Fish and Wildlife Service. This agreement explains the purposes of the research.

I hope to make the collections sometime in the summer or fall of 1996, depending on the timing of collections elsewhere in the range of the species (we are still waiting on a permit from Mexico, and it is not clear when this will arrive). I hope that it will be possible to keep the exact time of collection flexible with regards to the park.

I have applied for the appropriate federal permit through Region 2 of the U.S. Fish and Wildlife Service and am in the process of applying for a permit from the State of Arizona.

Please let me know if you need further information.

Thanks for your help in this matter.

Sincerely,

A handwritten signature in dark ink, which appears to read "Anthony A. Echelle".

Anthony A. Echelle
Professor of Zoology



FWS Agreement No: 1448-00002-95-_____

DCN: _____

Charge Code: _____

Amount Obligated: \$25,000

GRANT AGREEMENT

between

U.S. Fish and Wildlife Service, Region 2
P.O. Box 1306
500 Gold Avenue SW, Suite 5108
Albuquerque, New Mexico 87103-1306

and

Oklahoma State University
Department of Zoology
430 Life Science West
Stillwater, Oklahoma 74078-0459

I. GRANT AGREEMENT RECIPIENT

Oklahoma State University
Department of Zoology
430 Life Science West
Stillwater, Oklahoma 74078-0459

Recipient Class: Institute of Higher Learning CFDA Number: _____

II. AUTHORITY

This agreement between the U.S. Department of the Interior, Fish and Wildlife Service (hereinafter referred to as the "Service") and the Oklahoma State University (hereinafter referred to as the University) is hereby entered into under the authority of the Fish and Wildlife Coordination Act, 16 USC 661 to 666C.

III. PURPOSE

FWS Agreement No: 1448-00002-95-

The parties involved have a mutual interest in determining the genetic diversity of the endangered desert pupfish in the Southwest desert ecosystem of southern Arizona. The parties involved have the resources available to prepare a genetics management plan, following a thorough genetic survey of the species. Such studies are considered vital for the ultimate benefit of the recovery of this endangered species. Specifically, this agreement will:

1. Use protein electrophoresis ~~and analyses of mitochondrial DNA~~ to describe genetic diversity in the desert pupfish (Cyprinodon macularius).
2. Write a genetics management plan for the species, that will be used as a tool in the recovery and restoration of this endangered fish.
3. Coordinate and provide data to be used at management discretion for consideration in future implementational strategies by the Fish and Wildlife Service's Ecosystem Management Teams.

IV. BACKGROUND

See attached proposal which provides a detailed plan for this project, Attachment A, incorporated herein and made a part hereof.

V. SCOPE

A. The Service Shall:

1. Provide supervision, staff, facilities, and funding for the purposes established in this agreement and Attachment A.
2. To the extent and manner approved by the Unit Leader of the Arizona Fishery Resources Office - Parker Unit, allow the University personnel to use the Service's facilities, office equipment, and reference libraries as resources allow, to accomplish the project described in Attachment A.
3. Attachment A contains the equipment, materials, and staff support and specific responsibilities of the Services for completion of this project, excluding vehicles.

B. The University Shall:

1. Assist the Service in the project described in Attachment A, utilizing Service funds of \$25,000.
2. Assay a minimum of 20 specimens of the desert pupfish from the 20 different natural ~~populations~~ for variations in genetic composition. Approximately 30-

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35 gene loci will be examined for genetic variations. Additional samplings of Cyprinodon nevadensis and C. radiosus would also be analyzed for outgroup perspective on relationships among populations of the desert pupfish (C. macularius).

3. Using computer programs BIOSYS and FREQPARS, the protein electrophoretic data would be subjected to a variety of analyses, including tests of between-sample heterogeneity in allele frequencies, a hierarchical analysis of genetic diversity, and relationships among populations.

4. Perform Restriction Fragment Length Polymorphism (RFLP) analyses on mitochondrial DNA of a minimum of 20 specimens from each of the 20 known natural populations of the desert pupfish to determine genetic variations in the populations.

5. Using computer programs REAP and HAPLOID, those mitochondrial DNA data would be examined for haplotype diversity per sample, significance tests of between sample differences in haplotype frequencies, geographic heterogeneity in haplotype frequencies, and a gene diversity analysis of within versus between-sample diversity.

6. Collection of specimens will occur in September and October, 1995. Final report will be submitted to the Service by September 30, 1996.

7. Genetic management plan for the desert pupfish will address the following items:
- A) Number and identity of evolutionarily significant units among desert pupfish populations.
 - B) A protocol for establishing captive stocks.
 - C) A plan for future genetic monitoring of populations of desert pupfish.

C. Both parties mutually agree:

- 1. To cooperate in efforts to further the protection and conservation of fish and aquatic resources.
- 2. All assistance and services performed under this agreement will be carried out in full compliance with Service and Coop Unit objectives, policies, and responsibilities.

VI. PERIOD OF PERFORMANCE

The overall period of performance of this agreement is a period of 16 months beginning on the date of last signature below.

VII. AWARD AMOUNT

Financial contribution by the Service to carry out this project is \$25,000 for the period of

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performance identified above.

VIII. PAYMENT PROVISIONS

- A. Upon acceptance of the terms and conditions of this agreement, the recipient may submit requests for payment, either an invoice or a Standard Form 270, Request for Advance or Reimbursement, no more frequently than monthly. Electronic Fund Transfer (EFT) may be used in lieu of SF-270 if available.
- B. The original and two copies of each invoice/payment request shall be submitted to the Service Project Officer identified in Section X.A of this agreement for review and recommendation for payment to be forwarded to the Service Administrative Officer for approval and submission for disbursement. In accordance with U.S. Treasury regulations, payments will be made within 30 calendar days after receipt and approval of a proper invoice. Payment of the final invoice will be made available after the Service Project Officer accepts the final report/deliverable.
- C. Should the recipient be unable to complete the provisions of this agreement, all monies provided by the Service which prove to be cancelable obligations or unallowable in accordance with OMB Circular A-122 ("Cost Principles for Nonprofit Organizations") or the approved budget, shall be refunded to the Service.
- D. This agreement is intended to support a particular project for a specific period of time. Any portion of funds not expended at the completion of the period of performance of this agreement shall be returned to the Service, along with any interest earned on that amount.

IX. ADMINISTRATIVE OFFICER

- | | |
|---|--|
| A. U.S. Fish and Wildlife Service
Reymundo Aragon, Contracting Officer
P.O. Box 1306
Albuquerque, New Mexico 87103-1306
(505) 766-3085 FAX (505) 766-1261 | B. Oklahoma State University
Oklahoma Cooperative Fish
And Wildlife Research Unit
Dr. David Leslie, Unit Leader |
|---|--|

X. PROJECT OFFICERS

- | | |
|---|---|
| A. U.S. Fish and Wildlife Service
[Name(s) and Address(es)]
[Telephone Number(s)] | B. [Recipient Name]
[Name(s) and Address(es)]
[Telephone Number(s)] |
|---|---|

FWS Agreement No: 1448-00002-85-

C. Principle Investigator
Anthony A. Echelle
Professor of Zoology
Oklahoma State University
430 Life Science West
Stillwater, OK 74078-0459
(405) 744-5555

Principle Investigator
Chuck Minckley, Unit Leader
Arizona Fishery Resources Office -
Parker Unit
60911 Highway 95
Parker, AZ 85344
(620) 667-4785

XI. REPORTING and/or DELIVERY REQUIREMENTS

- A. The University (Principle Investigator) shall submit a quarterly progress report to the Service Project Officer by the 10th day of the month following the period reported upon.
- B. Within 90 days after the completion of this agreement the University (Principle Investigator) shall forward to the Service Project Officer a final report summarizing all project accomplishments under this award. One copy of the final report shall also be forwarded to the Service Administrative Officer.
- C. Within 90 days after completion of this award the University shall submit to the Service Administrative Officer a final Financial Status Report (Standard Form 269).

XII. TERMS AND CONDITIONS

A. Minority Business Enterprise/Woman Business Enterprise (MBE/WBE) Reporting:

- 1. In accordance with OMB Circulars, Recipients are required to take specific affirmative actions to ensure that minority business enterprises receive a fair share of subcontracts which result from Federal funds. The Recipient of this Agreement must report all subcontracting awards in excess of \$10,000.00 which involve the procurement of supplies, equipment, construction, or services executed under this Agreement.
- 2. The Recipient is required to submit a written report to the Service Administrative Officer (Identified in Section IX above) within one month following the end of each Federal fiscal year quarter during which any procurement in excess of \$10,000 is actually executed under this assistance Agreement. The report shall include the following: 1) FWS Agreement Number; 2) Number of subcontracting awards; 3) Dollar amount awarded to Minority Business Enterprises and/or Woman Business Enterprises; and 4) Signature of authorized Recipient representative.
- 3. ME-WBE utilization is based on Executive Orders (EOs) 11626, 12138, and 1243, and it is the policy of the Service to comply with the intent of the EOs, by enforcing the requirement for recipients to submit this information to the Service, when applicable.

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Procurement is defined as the acquisition through order, purchase, lease, or barter of supplies, equipment, construction or services needed to accomplish Federal assistance programs. A minority business enterprise is a business concern that is (1) at least 51 percent owned by one or more minority individuals, or in the case of a publicly owned business, at 51 percent of the stock is owned by one or more individuals; and (b) whose daily business operations are managed and directed by one or more of the minority owners. There is no standard definition of minority individuals used by all Federal financial assistance agencies. However, recipients shall presume that minority individuals include Black Americans, Hispanic Americans, Native Americans, Asian Pacific Americans, or other groups whose members are found to be disadvantaged by the Small Business Act or by the Secretary of Commerce under section 6 of Executive Order 11625. A woman business enterprise is a business concern that is, (c) at least 51 percent owned by one or more women, or, in the case of a publicly owned business, at least 51 percent of the stock is owned by one or more women; and, (d) whose daily business operations are managed and directed by one or more of the women owners.

B. Applicable Administrative and Audit Requirements:

1. General Provisions dated September 1993 applicable to recipients which are institutions of higher education, hospitals, or other nonprofit organizations as defined in OMB Circular No. A-110 are hereby included as Attachment B.
2. Pursuant to Section 310 of the Department of the Interior and Related Agencies Appropriations Act, 1995, Public Law (96 Approp.), 107 Stat. 1312, please be advised that the following is applicable to Agreements with State, Local, or Indian Tribal Governments: In the case of any equipment or product that may be authorized to be purchased with financial assistance provided using funds made available in this Act, it is the sense of the Congress that entities receiving the assistance should, in expending the assistance, purchase only American-made equipment and products. Applicable to purchases with an aggregate value of \$500,000.00 or above.
3. * Include if applicable. Patents. Any assistance agreement awarded for the purpose of research and/or development, is subject to the Government-wide patent policies in Department of Commerce regulations at 37 CFR Part 40. Under these regulations, all assistance agreements for experimental, developmental, or research projects should include the standard patent rights clause in Section 401.14. An alternate clause may be used only if the recipient is not located in the U.S. or is subject to the control of a foreign government, or if the Service has determined that limitations on the recipient's right to retain title will better promote the purposes of 35 U.S.C. 202-206.

In accordance with Public Law 98-620, all assistance agreements for experimental, developmental, or research projects include the standard patent rights in 37 CFR,

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Section 401.14, with the following exceptions:

- a. The recipient is not located in the U.S. or is subject to the control of a foreign government, or
 - b. The Service has determined that limitations on the recipient's right to retain title will better promote the purposes of 35 U.S.C. 202-206.
- C. Certifications. The certifications applicable to Federal Grants and Cooperative Agreements must be signed by an authorized representative of the recipient prior to award of this agreement. Certifications applicable to this agreement are included as Attachment ____.
- D. Publications Produced. The U.S. Department of Interior (DOI) Manual, Part 505 requires that two copies of each publication produced under a grant or cooperative agreement be sent to the DOI, Natural Resources Library with a transmittal that identifies the sender and the publication. If applicable, the [Recipient Name] Project Officer will provide the Service Project Officer three copies of the publication. The Service Project Officer will retain one copy and forward two copies to the Natural Resources Library.

XIII. MODIFICATION

Amendments or renewals may be proposed at any time during the period of performance by either party and shall become effective upon approval by both parties. This Grant Agreement, unless otherwise amended or renewed, is scheduled for completion on September 30, 1996. No change to this agreement shall be binding upon the Service or Recipient unless and until reduced to writing and signed by both parties.

- A. The results of any studies or investigations accomplished under this agreement may be published jointly by the parties or by either party separately. Appropriate credits to the United States Department of Interior, Fish and Wildlife Service, shall be included in any formally published article providing the Service does not otherwise deem it appropriate to issue a disclaimer. Authorship shall not incur any privileges of copyright or restriction on distribution.
- B. Any research data collected under this agreement shall be jointly owned by the parties to this agreement. Both parties shall have complete and unlimited access to all such data.
- C. News releases and other publicity issued by either party concerning this agreement will give due credit to cooperators to this agreement and is subject to approval prior to release by the Service's Regional Public Affairs Office.
- D. No member of, or delegate to, Congress or resident commissioner, shall be admitted to any share or part of this agreement; or to any benefit that may rise therefrom. This provision



Donk

Organ Pipe Cactus National Monument
INVESTIGATOR'S ANNUAL REPORT - 1996

Region	Park	NRSP # or RMP Project #	Year
(Leave Blank)	ORPI	(Leave Blank)	1996

Project Title *Genetic diversity in the desert pupfish
(Cyprinodon maculatus)*

Field of Study
Conservation genetics

Principal Investigator
Anthony A. Echelle

Co-investigators
Alice F. Echelle and C. O. Minckley

Estimated Funding

\$	National Park Service
\$ 20,000	Other Federal Agency - USFWS
\$	State/Local Agency
\$	Academic Institution
\$	Non-profit Institution SPMA
\$	Personal/Volunteer
\$	Total

Date Started

1 Aug. 1996

Anticipated End Date

31 Aug. 1998

Percent Done

2%
Because of problems
with Mexican permits
the project just got
started.

Project Objectives

To describe genetic diversity in Cyprinodon maculatus
To write a genetics management plan for the species.

Progress to Date

collections have been taken from Sonora Mexico.
The biochemical work just got started.

Significant Findings

None as yet

Specimen Status

None yet taken from Organ Pipe Cactus Nat'l Mon.

Reports Produced

None

Plans for Next Year

To make the collections from Quitobaquito Spring
and southern California, and to complete most
of the genetic survey

Species Studied

Cyprinodon macularius

Family/Order Studied

Cyprinodontidae / Cyprinodontiformes



Department of Zoology
430 Life Sciences West
Stillwater, Oklahoma 74078-3052
405-744-5555

10 April 1999

Tim Tiddits
Organ Pipe Cactus National Monument
Route 1, Box 100
Ajo, Arizona 85321

Dear Tim:

Enclosed is a final report for a study of mitochondrial DNA variation in the desert pupfish complex. A modified version has been submitted for publication in *Copeia*. As noted in the abstract of the enclosed report, we believe the Quitobaquito/Rio Sonoyta populations should be referred to as a separate species (*Cyprinodon eremus*) from the more wideranging form (*C. macularius*).

We detected no significant variation in frequency of different mtDNAs between the Quitobaquito pupfish and populations from the Rio Sonoyta. However, if the current understanding of how long the two have been isolated is correct, then this probably will change when additional genes are examined. Also, more than one population from Rio Sonoyta probably should be looked at. I am presently looking for funds to study variation in frequency of rapidly evolving nuclear markers (microsatellites) in the complex. Do you think the Park Service might be sufficiently interested to provide some support? We could do the work for about \$5,000.

Starting on page 9, I have made some general recommendations for genetics management of the members of the complex. If you have any questions, I would be glad to try answering them.

Thanks for your help with collection permits.

Sincerely,

A handwritten signature in cursive script, reading "Anthony A. Echelle".

Anthony A. Echelle
Professor



FINAL REPORT

TO: C. O. Minckley, Project Coordinator, Arizona Fishery Resources Office, Parker Unit, U.S. Fish and Wildlife Service

FOR: NAFTA Borderlands Funds, contract #1448-00002-95-0890

TITLE: Genetic Variation in Desert Pupfish

FROM: Anthony A. Echelle, Zoology Department, Oklahoma State University, Stillwater, Oklahoma 74078

DATE: 22 March 1999

ABSTRACT:

Variation in mtDNA was assessed in a captive stock and 11 wild populations (n = 259) from throughout the native range of desert pupfish *Cyprinodon macularius* as traditionally understood. Using PCR-SSCP, 18 composite haplotypes were identified from a 333-bp segment of the mitochondrial D-loop and two segments of the ND2 gene (333 and 325 bp). Representatives of each haplotype were sequenced for the entire ND2 gene and the 337-bp segment of the D-loop. Phylogenetic analyses revealed reciprocal monophyly between populations in two general regions, Río Sonoyta/Quitobaquito Springs and Salton Sea/Colorado River Delta. This, with previous observations on morphology, color pattern, and geological history, supports recognition of the Río Sonoyta/Quitobaquito populations as a separate species, the Quitobaquito pupfish *C. eremus* Miller and Fuiman, from the more widespread desert pupfish *C. macularius* Baird and Girard. More than 70% of mtDNA diversity across all populations was attributable to differences between the two species. Within species, the average local population contains 94% and 97% of the diversity in, respectively, *C. eremus* and *C. macularius*. Differences between the Salton Sea and Colorado River Delta populations of *C. macularius* explain a small (3.7%), but statistically significant, portion of mtDNA diversity in this species. This and the history of connections between Salton Sea and the delta suggest that the two regions should be managed separately with no intermixing of pupfish other than what occurs when the present, human-regulated hydrology is overcome by natural flooding. Haplotype frequencies in *C. eremus* from Quitobaquito Springs and Río Sonoyta were not significantly different. However, the potentially long history of isolation between these two populations and evidence of some degree of morphological divergence indicate a need for conservative management with no intermixing. The captive stock exhibited reduced mtDNA variation relative to its wild parent population from a locality on the delta.

INTRODUCTION:

The desert pupfish *Cyprinodon macularius* is listed as an endangered species by the International Union for Conservation of Nature and Natural Resources (Miller, 1979) and by the United States government (U.S. Dept. of the Interior, 1986). In this paper, we use mitochondrial DNA (mtDNA) variation to describe the genetic structure of populations traditionally classified as *C. macularius* and to provide a basis for recommendations regarding conservation genetics of the species. The results, together with geological history and previous observations on color patterns, indicate that populations grouped as *C. macularius* represent two evolutionarily divergent entities that should be recognized as separate species. We hereafter refer to the two species as the "desert pupfish complex."

The historic range of the desert pupfish complex once extended from Gila River tributaries in southeastern Arizona and northern Sonora, westward to the Salton Sea area of southern California and southward into the Colorado River Delta region in Sonora and Baja California (Miller, 1943). Across this region, Miller and Fuiman (1987) recognized one wide-ranging subspecies *C. m. macularius*, and a local endemic, *C. m. eremus*, restricted to Quitobaquito Springs, Organ Pipe Cactus National Monument, southern Arizona. The wide-ranging form has disappeared from the Gila and lower Colorado river areas of Arizona/California (Minckley, 1973; Moyle, 1976), and the remaining natural populations are much smaller and more fragmented than in earlier times (Hendrickson and Varela, 1989; Dunham and Minckley, 1998). This decline is a result of interactions with introduced species and habitat losses resulting from reservoir construction, surface-water diversion projects, groundwater depletion, and other factors (Miller and Fuiman, 1987; Shoenherr, 1988; Hendrickson and Varela, 1989).

Mexican populations of the desert pupfish complex have been somewhat neglected in previous genetic surveys, although protein electrophoretic studies of geographic variation in the species have included one or two samples of transplanted Mexican populations founded by individuals taken from the Colorado River Delta in Sonora (Turner, 1983; Dunham and Minckley, 1998). Other genetic studies that included desert pupfish were aimed primarily at resolving evolutionary relationships among western pupfishes and included samples from only one or two localities (Turner, 1974; Echelle and Dowling, 1992; Echelle and Echelle, 1993).

MATERIALS AND METHODS:

In 1997-1998, collections of the desert pupfish complex were made at 11 sites ($n = 19-25$) representing all major populations of the complex (Fig. 1; Appendix 1). We also included 33 specimens from a captive population at Dexter National Fish Hatchery and Technology Center in New Mexico. Specimens were initially frozen in liquid nitrogen or dry ice and stored in the lab at -75°C until processed for analysis.

DNA was extracted from muscle tissue following the method of Longmire et al. (1997) and the polymerase chain reaction (PCR) and single-stranded conformational polymorphism (SSCP) analysis (Orita et al., 1989; Fan et al., 1993) were used to screen all

specimens for variation in portions of the mitochondrial D-loop and ND2 gene. For ND2, we used primers ND2B-L and ND2E-H (T. E. Dowling, pers. comm.) to amplify the entire gene, used an ABI automated sequencer to directly sequence the resulting amplicon, and developed internal primers for a 333-bp segment at the 5' end (positions 8 to 340) and a 325-bp segment at the 3' end (positions 661 to 986); the primer sets were, respectively, (1) 5'-CCTTCCTTTGCTAATGAACC-3' and 5'-CCAATTTTAAGTGCCAGGG-3' and (2) 5'-ATACCTCGCCACCTCTTG-3' and 5'-AGCCAGATTGTTGCGGAG-3'. For SSCP analysis of the D-loop, we used primers L15926 (Kocher et al., 1989) and H16498 (Meyer et al., 1990) to amplify a portion of mtDNA extending from the threonine tRNA gene through the first 337 bp of the D-loop; these are primers E and K in Lee et al. (1995). To screen for SSCP variation, the three mtDNA segments from each specimen were PCR-amplified from genomic DNA in separate reactions and in the presence of α -³²P-dCTP, 2.0 μ l of each diluted PCR product was electrophoresed in a 5% polyacrylamide gel (40:1 acrylamide:bis acrylamide; 10% glycerol) at 300 V (3 W) for 22 h, and bands were visualized by autoradiography.

For phylogenetic analysis, the entire ND2 gene (1047 bases; primers ND2B-L and ND2E-H) and the 337-bp fragment of the mtDNA D-loop (primers L15926 and H16498) in 35 specimens representing all composite haplotypes detected by SSCP analysis of the three mtDNA segments were sequenced directly using either an ABI 373 or 377 automated sequencer. All haplotypes occurring at more than one locality were sequenced in representatives from 2 to 6 localities, depending on distribution of the haplotype; for example, one haplotype occurred in 9 of the 11 samples and was sequenced in nine specimens from six different localities. To test the monophyly of haplotypes in desert pupfish, we included GenBank sequences provided by Duvernell and Turner (1998) for three other pupfishes, two species grouped with the desert pupfish complex in the "western pupfish clade" (Echelle and Dowling, 1992), Amargosa pupfish *C. nevadensis* (GenBank accession numbers: ND2, AF028308; D-loop, AF028290), and Owens pupfish *C. radiosus* (ND2, AF028311; D-loop AF028293), and a rather distantly related (Echelle and Echelle, 1998) outgroup species, sheepshead minnow *C. variegatus* (ND2, AF028313; D-loop, AF028299). The mtDNA of *C. radiosus* seems to be the sister-group to the mtDNA lineage in the desert pupfish complex (Echelle and Dowling, 1992). Duvernell and Turner's (1998) sequences included the entire ND2 gene and they used the same primers for D-loop amplification that we employed.

Sequences for each composite haplotype identified by SSCP from the desert pupfish complex and the sequences from the other three species were aligned with CLUSTAL W (Thompson et al., 1994) and subjected to phylogenetic analysis with PAUP (version 3.05; Swofford, 1991). Sequences were coded as discrete, unordered characters and the most parsimonious tree(s) were searched for using heuristic search algorithms with 10 iterations of a random input order of taxa. To evaluate the robustness of the most parsimonious tree(s) we performed a heuristic bootstrap analysis (Felsenstein, 1985) of 100 iterations with 10 replications of random input order and tree-bisection-reconnection for each iteration. Gaps representing four indels in the control region were treated as missing data.

Haplotype frequencies and sequences for the three mtDNA segments assayed for SSCP variation were used in analyses of population genetic structure. We used Arlequin version 1.1 (Schneider et al., 1997) to compute within-sample estimates of haplotype diversity

(h) and nucleotide diversity (π , from Tajima-Nei distances, no gamma correction), pairwise F_{ST} and Φ_{ST} statistics, exact tests (Raymond and Rousset, 1995) of difference in haplotype frequencies, and an analysis of molecular variance (AMOVA; Excoffier et al., 1992). The AMOVA partitioned molecular variance into proportions resulting from within-sample variation, differences among samples within regions, and differences among regions. We designated three regions for this analysis: the Salton Sea area (sites 1-3, Fig. 1), the Colorado River Delta (sites 4-9), and the Río Sonoyta/Quitobaquito area (sites 10-11). The AMOVA was performed on haplotype frequencies alone (producing F-statistics) and on frequencies plus sequence divergences (producing Φ -statistics; Excoffier et al., 1992). Also using Arlequin, we obtained significance tests for individual, pairwise F_{ST} and Φ_{ST} values and for the variance components in the AMOVAs (non-parametric permutation method described by Excoffier et al. [1992]). For multiple pairwise tests, we used the stepwise Bonferroni correction (Rice, 1989) to obtain a Type I error less than 0.05.

RESULTS:

SSCP analysis of haplotype variation among 258 specimens of desert pupfish identified 10 variants for the 5' end of the ND2 gene and 5 for the 3' end of the gene, producing 13 composite ND2 haplotypes; 9 variants were detected for the D-loop, giving 18 composite haplotypes across the three segments (Table 1). All SSCP-identified haplotypes for each of the three segments had at least one unique base substitution. In three instances, haplotypes identical on the basis of SSCP differed by one base substitution in the mid-portion of the ND2 gene that was not surveyed for SSCP variation; these variants were ignored in the analysis because additional sequencing would have revealed other base substitutions for other portions of the mtDNA.

Genetic structure.--The 18 haplotypes detected by SSCP analysis varied at a total of 18 sites (15 transitions, 3 transversions) across the two ND2 segments surveyed: 11 in the 333-bp segment at the 5' end and 7 in the 325-bp segment at the 3' end. Five variable sites (all with transitions) were detected in the ND2 segments not included in the SSCP analysis (389 bp). For the 337-bp D-loop fragment, there were 12 variable sites and 13 substitutions (8 transitions, 5 transversions). Individual haplotypes differed at 1 to 10 sites. The measures of haplotype and nucleotide diversities showed no geographic pattern of variation and no consistent pattern of variation between ND2 and the D-loop (Table 1).

All populations had two to five SSCP variants except the DNFH sample, which was monomorphic for haplotype A. The Río Sonoyta and Quitobaquito populations were similar in haplotype frequencies and showed no significant differences (Tables 1 and 2). These populations shared no haplotypes with samples from the Salton Sea and the Colorado River Delta areas. Thus, all pairwise tests of F_{ST} and Φ_{ST} and the corresponding exact tests of differences in haplotype frequency were highly significant ($P < 0.0001$) in comparisons of Río Sonoyta/Quitobaquito samples with Salton Sea and Colorado River Delta samples. All Salton Sea and Colorado River Delta samples had high frequencies (60-89%) of haplotype A, and,

with the Bonferroni correction, there were no significant differences among paired samples within or between the two regions. There was, however, evidence of significant divergence between the captive stock we examined and its parent population (site 6, Fig. 1; $F_{ST} = 0.31$, $P < 0.00001$; $\Phi_{ST} = 0.21$, $P < 0.00001$).

The AMOVA based on F-statistics indicated that 34.3% of the variation in haplotype frequencies is attributable to differences among the three regions (Río Sonoyta/Quitobaquito, Salton Sea, and Colorado River Delta), 64.8% to variation within samples, and only 0.9% to differences between samples within regions. The corresponding values for Φ -statistics ("total diversity;" i.e., haplotype frequencies plus sequence divergences) were, respectively, 75.8%, 23.6%, and 0.6%. The difference in proportions between the two AMOVAs shows that a large amount of the among-region genetic variance results from sequence differences and not just haplotype frequencies. For both analyses, there were highly significant differences among regions ($P < 0.00001$), but not among samples within regions ($P = 0.11$ - 0.15). More than 70% of the total diversity reflects differences between samples from the Río Sonoyta/Quitobaquito region and those from the Salton Sea and Colorado River Delta regions. When the Río Sonoyta/Quitobaquito populations were excluded from the analysis, only 3.7% of total diversity was attributable to differences between the Salton Sea and Colorado River Delta regions (Table 2). The corresponding value for differences between the samples from Río Sonoyta and Quitobaquito Springs was 2.9%.

Considering only haplotype frequencies, roughly equivalent proportions of gene diversity were attributable to differences among regions for the ND2 gene and the D-loop sequences (Table 2). However, when sequence divergence was included in the AMOVA, the among-region component of diversity was much greater for ND2 than for the D-loop (87% versus 11%; Table 2). Within regions, the two sequences are roughly equivalent in proportion of genetic diversity attributable to differences among samples.

The AMOVAs on the combined ND2/D-loop data for the Salton Sea and Colorado River Delta regions indicated a small (3.7%), but statistically significant among-region component of variation when sequence data were included in the analysis, but not when they were excluded (Table 2). The two regions share the same common haplotype (A), and the difference in sequence information is attributable to the remaining, relatively uncommon ("secondary") haplotypes in the two regions (Table 1). The Cerro Prieto population (Site 4, Fig. 1), which is geographically intermediate between Salton Sea and the other sample sites on the delta, shared one secondary haplotype (E) with Salton Sea populations and none with the other populations on the delta.

Phylogenetic analysis.--The 1384 sites available for phylogenetic analysis of the ND2/D-loop sequences included 191 variable sites, 54 of which were parsimony-informative (40 ND2 sites, 14 D-loop sites); i.e., two or more (but not all) haplotypes in the ingroup differed from the outgroup (*C. variegatus*) haplotype by a shared base substitution. Only 13 sites (12 ND2, 1 D-loop) were informative regarding relationships among haplotypes of the desert pupfish complex. The heuristic search for the shortest tree with the combined ND2/D-loop data produced two equally parsimonious topologies (230 steps; $CI = 0.89$) after collapsing all nodes with no synapomorphies. The consensus tree was identical to the 50% majority-rule

consensus tree from the bootstrap analysis (Fig. 2). These trees, and similar trees produced by analysis of ND2 separately, supported monophyly of the desert pupfish complex and the previously reported (Echelle and Dowling, 1992; Duvernall and Turner, 1998) close relationship between haplotypes of the complex and those of *C. radiosus* (Fig. 2). Separate analysis of the D-loop data supported monophyly for the desert pupfish complex (bootstrap support = 99%).

Haplotypes of the desert pupfish complex formed two separate, monophyletic clades, one comprising four haplotypes restricted to the Río Sonoyta/Quitobaquito area, and the other comprising 14 haplotypes restricted to the Salton Sea/Colorado River Delta area (Fig. 2). The Salton Sea/Colorado River Delta clade included one subclade containing haplotypes B (widespread in the Colorado River Delta) and F (widespread in Salton Sea area) and one containing all other Salton Sea/Colorado River Delta haplotypes. Haplotype A of the latter subclade was both the most plesiomorphic and the most widespread member of this group of 12 haplotypes (Fig. 2; Table 1); the other 11 haplotypes in this group differed from haplotype A by only 1-3 bases. These 12 haplotypes formed a large polytomy with no phylogenetic resolution except that haplotypes C and D from the Salton Sea area formed a monophyletic pair, as did haplotypes I and N from the Colorado River Delta.

The bootstrap analysis of the ND2 data alone produced a topology (180 steps; CI = 0.90) identical to that for the combined ND2/D-loop data except that it did not resolve the clade containing haplotypes B and F; instead these haplotypes clustered as separate parts of the large polytomy in Figure 2 that includes the remaining Salton Sea/Colorado River Delta haplotypes. Bootstrap support for most internal nodes of the tree were greater with the ND2 data alone than with the combined ND2/D-loop data (Fig. 2).

Notes on nuptial coloration in males. - Males from Río Sonoyta/Quitobaquito and those from the remainder of the historic range of the desert pupfish complex apparently are diagnostically different in male breeding coloration. Miller (1943:4, 11) reported that breeding males of *C. macularius* from the Salton Sea and from a location near our sites 8 and 9 in Sonora had “bright yellow to brilliant yellow orange” on the caudal peduncle and caudal fin. Miller’s (pers. comm.) fieldnotes from April 1950 mention similar colors in males from a locality on the Río San Pedro, a tributary of the Gila River in northern Sonora and southeastern Arizona. Thus, the now-extirpated eastern populations apparently had the bright yellows seen in the extant populations in the Salton Sea and the Colorado River Delta. In contrast, males from Quitobaquito Springs and the associated pond do not exhibit bright yellow in the caudal region (Liu, 1969; Loiselle, 1982; M. R. Douglas, pers. comm.; pers. obs.), although Miller and Fuiman (1987:599) reported “yellow to olive-yellow” in the caudal fin and “just encroaching onto the posterior third of caudal peduncle.” Recent observations by one of us (COM) and by P. Unmack indicate that males from the Río Sonoyta population do not exhibit bright yellow colors, and this is supported by R. R. Miller’s fieldnotes for 14 April 1950: “color of males [from a Río Sonoyta locality] essentially same as at Quitobaquito [Springs] . . . No yellow on body, but some males with yellowish caudal [fin] and caudal [fin] base.”

DISCUSSION:

Reciprocal monophyly between the mtDNA haplotypes of the Río Sonoyta/Quitobaquito populations and those of the Salton Sea/Colorado River Delta populations suggests long, mutually exclusive evolutionary histories (Neigel and Avise, 1986) for the two groups of populations in the desert pupfish complex. Reciprocal monophyly and the consistent difference in male breeding colors, considered together or separately, are sufficient for recognition of separate species under a variety of species concepts (Frost and Hillis, 1990; Mayden and Wood, 1995). Thus, we recommend classifying the Río Sonoyta/Quitobaquito populations as a separate species, the Quitobaquito pupfish *C. eremus* Miller and Fuiman, from the more wideranging desert pupfish *C. macularius* Baird and Girard. The two species are divergent in morphometric and meristic variables, but with considerable overlap, primarily between *C. macularius* and the Río Sonoyta population of *C. eremus* (Miller and Fuiman, 1987).

Geological history supports the suggestion from the phylogenetic analysis that the taxonomy proposed here more accurately reflects pattern of descent than does the previous classification (Miller and Fuiman, 1987) in which the Río Sonoyta population was considered a morphologically divergent representative of the wide-ranging subspecies, *C. m. macularius*. Isolation of the ancestral Río Sonoyta/Quitobaquito drainage from the Colorado River Delta probably occurred sometime in the last 100,000 years when lava beds resulting from eruptions of the Sierra Pinacate Volcanic Field diverted the flow of the Río Sonoyta southward to the Gulf of California and away from its previously westward course toward the Colorado River Delta (Ives, 1964; Donnelly, 1974; Turner, 1983). More recent separation of the Río Sonoyta and Quitobaquito Springs populations would explain the lack of a significant difference in haplotype frequencies between our samples from these areas. Despite this high degree of similarity, there is no evidence of surface-water contact in historic times and the two populations may have been isolated for thousands of years (Miller and Fuiman, 1987). This predicts that additional DNA markers would reveal significant divergence between these two populations.

Recent speciation explains the observation (Turner, 1983) that *C. macularius* and the Quitobaquito population of *C. eremus* had no fixed allele differences for eight polymorphic proteins, and it explains the low estimate from RFLP data for overall mtDNA sequence-divergence (0.34%; Echelle and Dowling, 1992) between the two groups. This level of mtDNA divergence is similar to that (0.16-0.64%; $\bar{x} = 0.42$) within a monophyletic group of pupfish haplotypes in which those of *C. nevadensis* are paraphyletic with respect to the Devils Hole pupfish *C. diabolis*, a morphologically divergent species, that, as Miller (1981) suggested, may have evolved within the last 20,000 years.

Rapid evolution of the markedly high morphological divergence of *C. diabolis* compared with that between *C. eremus* and *C. macularius* probably is a result of the unique, cavernous habitat in Devils Hole and the consistently small size of the resident pupfish population (Miller, 1950). The species flock of five pupfishes in Lake Chichancanab on Mexico's Yucatan Peninsula is another example of rapid speciation with little allozyme divergence (Humphries 1984) and a lack of reciprocal monophyly among mtDNAs of the

various species (Strecker et al., 1996). In this example, speciation and striking morphological divergence (Humphries and Miller, 1981) might have occurred in as little as 8,000 years (Strecker et al., 1996), possibly as a result of among-pupfish, competitively driven, intralacustrine speciation (Humphries, 1984).

Recent gene flow probably explains the lack of significant genetic divergence among pupfish populations within the Salton Sea area and among those in the Colorado River Delta. Dunham and Minckley (1998) reported homogeneous allele frequencies for four polymorphic allozyme loci in their samples of *C. macularius* from four sites in the Salton Sea area. Samples from two captive stocks, including the DNFH stock we examined, gave indirect evidence of divergence among populations on the delta (Dunham and Minckley, 1998), but this might reflect genetic drift in captivity. The DNFH stock began with 280 founders captured in 1983 from our Santa Clara Slough site (Dunham and Minckley, 1998; their "Canal Sanchez Toboada"). In our survey, this stock had only the common haplotype of *C. macularius*, whereas all samples from the wild had two to five haplotypes. This suggests founder effect or subsequent genetic drift during the 15 years of captivity. Correspondingly, the population also showed reduced allozyme variation relative to other samples of the species (Dunham and Minckley, 1998; 1 polymorphic locus vs 3 to 4). In contrast with our results for the Salton Sea area and the allozyme homogeneity reported by Dunham and Minckley (1998), Turner (1983) found significant heterogeneity for four of eight polymorphic allozyme loci in samples from the area. Dunham and Minckley (1998:12) suggested that the present homogeneity may reflect "a recent dramatic increase in pupfish abundance and gene flow."

A significant, although rather small, among-region component of mtDNA genetic diversity in *C. macularius* occurs between the Salton Sea area and the Colorado River Delta. Similarly, Turner (1983) and Dunham and Minckley (1998) reported allozyme divergence between wild populations in the Salton Sea area and captive stocks derived from the delta, although this might reflect genetic drift in the captive stocks. The low level of molecular divergence detected for mtDNA probably reflects the dynamic nature of the regional hydrology. Although extant populations are separated by large expanses of desert, there has been ample historic opportunities for gene flow. For example, two of the more isolated areas, the Salton Sea and Laguna Salada, are in topographic sinks that, through time (including several periods in recorded history), have experienced repeated cycles of flooding and dessication as the river meandered across its deltaic plain (Carpelan, 1961). The last flow of the river into the Salton Sink occurred from 1905 to 1907 when the Colorado River breached an irrigation-canal system and created the present Salton Sea (Carpelan, 1961). Laguna Salada last received flow from the River during high flows in 1983 and 1984 (Hendrickson and Varela, 1989). These flows apparently were associated with dispersal and increased abundance of pupfish populations on the delta (Hendrickson and Varela, 1989).

The dynamics of populations in the Salton Sea/Colorado River Delta area include periods of "boom and bust" (Dunham and Minckley, 1998:12), with population expansion and dispersal alternating with population declines, isolation, and local extinctions. This effect is particularly pronounced for fishes in the arid regions of western North America (Minckley et al., 1986). For *C. macularius*, the decline, isolation, and local-extinction phase of the cycle has been greatly exaggerated by human activities. In such instances, populations should be

managed to simulate historic patterns of dispersal (Meffe and Vrijenhoek, 1988), with periodic intermixing and, when conditions are favorable, transfers into areas of extirpation. Dunham and Minckley (1998) also recommended manipulation of local habitats to provide the harsh, unstable conditions favoring pupfish over the usually less tolerant non-native species.

The significant among-region component of variation in *C. macularius* suggests that the Salton Sea and the Colorado River Delta regions should be managed as separate units. The distribution of secondary mtDNA haplotypes indicates a lack of wholesale intermixing of populations between these two regions despite periodic surface-water connections. Under natural conditions in the past, brief periods of surface-water connection between Salton Sea and the delta were separated by decades or longer with no connection (Sykes, 1937). A conservative management approach would be to avoid intermixing of pupfish between the two regions beyond what might occur when the present human-regulated hydrology fails under the forces of nature. Within these regions, neither the available genetic data nor the morphological analysis by Miller and Fuiman (1987) provide a basis for partitioning populations into separate units of genetic conservation. A similar conclusion might be reached for *C. eremus* on the basis of mtDNA variation alone, but the potentially long history of isolation between these two populations and evidence of some degree of morphological divergence (Miller and Fuiman, 1987) indicate a need for conservative management with no artificial intermixing of the populations in Quitobaquito Springs and Río Sonoyta.

CONSERVATION GENETICS RECOMMENDATIONS:

1. Perform a nuclear DNA survey of genetic diversity in natural populations of *C. eremus* and *C. macularius*. The present knowledge is based on mtDNA and partial information from allozymes. A survey of hypervariable nuclear DNA markers, such as microsatellites, would provide greater confidence in the distribution of genetic variation. The following recommendations are based on the present state of knowledge.
2. Maintain at least one, preferably more, captive stocks of *C. eremus* and *C. macularius* at state or federal facilities with personnel trained in appropriate practices for conserving genetic diversity. These are the only captive populations that should be used in re-introductions to the wild. Any such re-introductions should include large numbers of specimens (200-300 minimally, and approximately a 1:1 sex ratio), supplemented, if possible, by about 10% with samples from wild populations at the time of the re-introduction.
3. The following are minimal recommendations for numbers of state or federally maintained captive stocks: *C. eremus*, one stock from Río Sonoyta and one from Quitobaquito Springs/Pond; *C. macularius*, one stock from Salton Sea, one from the lower Colorado River delta (LCRD). If only one stock is maintained, then decisions must be made regarding concerns such as which natural populations are most vulnerable to genetic losses.
4. Evaluate existing captive or semi-natural stocks for genetic structure and proceed

appropriately. Replace the existing stock at DNFH with a new stock from a wild population. The DNFH stock appears to have lost at least some of its genetic variability during 15 years of captivity without supplementation from wild populations. The most advisable solution is to eradicate the present stock and re-establish it with new founders from the wild. Other captive and semi-natural stocks need such evaluation.

5. Establish a system of control in which managers of captive or semi-natural populations would receive guidance in terms of conservation genetics. The three-tier approach suggested in the recovery action plan for the species (Marsh and Sada, 1991) would be a good framework for the establishment of a controlled system of captive and semi-natural stocks.

6. Founding stocks for all captive populations should be taken from large, natural populations known to have relatively high levels of genetic diversity and that have not experienced any recent bottlenecks. Ideally, the founding stocks should comprise several hundred individuals.

7. Captive and semi-natural stocks should receive infusions from wild populations representing the source of the original founding stock. The timing and size of such infusions is a complex problem affected by the size of the captive stock and circumstances, such as bottlenecks in size of the captive stock. Allendorf and Ryman (1987) recommend a 10% contribution by wild fish every second or third generation. This will guard against unwanted change; however, much smaller contributions (as low as one "effective" individual) per year class is adequate to counter the effects of genetic drift on allele diversity, and would help retard change due to genetic drift or selection. Minimally, each captive stock of these small, short-lived fishes should receive an infusion of wild fish once a year if possible. However, managers must be alert to the possibility that wild populations have been genetically contaminated by introduced pupfishes.

8. Obtain a genetic description of the founding stock or the earliest possible generation of their descendants. This is worthy of considerable effort, particularly for stocks being maintained at state or federal facilities as gene banks or for future re-introductions. This assay provides the baseline for comparison of all descendant generations in captivity. Ideally, each founding member would be genotyped. With present DNA-analysis technology and the resulting non-lethal sampling methods, this is a more realistic recommendation for small fishes than it would have been in the past. The analysis should include both mtDNA and nuclear markers. The inclusion of mtDNA is recommended because it is particularly sensitive to genetic drift; nuclear markers such as microsatellites will provide a greater diversity of allele variation, allowing a more accurate assessment of allele losses.

9. Monitor population structure of captive and semi-natural stocks, particularly those maintained at state or federal facilities. At a minimum, records should be maintained on at least crude assessments of population size, age/size structure, and breeding structure (number of territorial males, in particular); all efforts should be made to keep the effective size of the population as large as possible.

10. Monitor genetic structure of captive and semi-natural stocks maintained in state or federal facilities. Unwanted genetic changes in stocks might result from genetic drift, selection, or introductions of hybridizing species (other pupfish). This monitoring would use the same approach(es) used to characterize the founding stock. The schedule and number of fish to be assayed depends in part on how critical the captive population is for survival of the gene resources it represents. If wild fish remain available to supplement captive stock, then the primary concern is one of avoiding any drastic genetic change in the captive broodstock. For this kind of monitoring, a sample of 30 fish would be adequate. Such monitoring should be done every two or three generations, which for pupfish would mean sampling at least every other year or so. With a number of successive samples, one can estimate effective population size under the captive regime (Waples, 1989); such information would help guide future management.

11. Consider implementing a program of genetic exchange among wild populations. Whether this is necessary or not depends on the population(s) being considered and local features of the landscape and hydrology; e.g., projections regarding future opportunities for natural gene flow.

A suggested program: It seems impractical and inadvisable to mobilize a large proportion of available resources in developing and executing an ultra-refined model that simulates “natural” (pre-modern) patterns of gene flow. The primary concern should be to maintain overall allele (or haplotype) diversity within the major units of concern, and not a detailed attempt to simulate past patterns or amounts of gene flow. Based on patterns of geographic variation, there seem to be four such units, two for *C. eremus* (Quitobaquito Springs/Pond and Río Sonoyta) and two for *C. macularius* (the Salton Sea area and the lower Colorado River delta); programs of gene transfer should occur only within these units.

Although this needs further consultation, the basic guidelines for a program of gene transfer might be the following: (1) transfers into local populations should be rather small (e.g., 10% or less of the receiving population); (2) such transfers should be biased toward exchange with neighboring populations; (3) the schedule of genetic infusion for any one population might not have to be any more frequent than once every few years; (4) in a system such as the Colorado River Delta, most gene flow might have occurred in pulses over a large area, so the schedule might include local one-time “hopping” from one large source population to several recipient populations, and with a lesser amount of “back-hopping.”

For *C. eremus*, there seems to be no immediate need for any program of genetic exchange. The Quitobaquito form seems large and relatively stable and it is the only representative of its type (assuming on the basis of morphology that the Río Sonoyta population is genetically divergent). The Río Sonoyta occasionally floods, and, presumably, there would be natural intermixing of local populations, at least during major storm events.

Because of genetic divergence between the populations of *C. macularius* in the Salton Sea area and the LCRD, it seems generally advisable to avoid intermixing between these regions. It appears that some mtDNA haplotypes have mutually exclusive distributions between these two areas. However, the Cerro Prieto population on the LCRD might be an exception because of its complement of secondary mtDNA haplotypes (the sample shared one

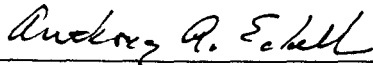
secondary haplotype with two samples from the Salton Sea area and none with samples from the lower Colorado River delta). Because of its location, this population might represent an area of transition between populations in the two regions. A further analysis, with larger sample sizes and a variety of nuclear markers might help resolve this question.

The decision on whether to implement genetic exchanges among populations within the two major regions occupied by *C. macularius* and the protocol for such exchanges requires information on absolute and relative sizes of populations, degree of ongoing connectedness, and prospects for future connections due to flooding by streams and tidal events or population expansions. Presently, there is insufficient data for such judgments.

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TABLE 1. NUMBER OF OCCURRENCES FOR 18 MTDNA HAPLOTYPES IN THE DESERT PUPFISH COMPLEX, WITH HAPLOTYPE DIVERSITY (h) AND NUCLEOTIDE DIVERSITY (π) FOR THE ND2 GENE, THE D-LOOP, AND BOTH COMBINED. Locality numbers correspond with those in Figure 1 and Appendix 1.

Area/Locality	Haplotypes																		<i>h</i>			π			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	ND 2	D-loop	Both	ND2	D-loop	Both	
Salton Sea area																									
1. Shoreline Pool	18	1	2	3	1														0.48	0.42	0.48	0.00087	0.00179	0.00118	
2. San Felipe Creek	16	1	2		2														0.42	0.27	0.42	0.00084	0.00057	0.00075	
3. County Line Drain	17	3																	0.27	0.27	0.27	0.0082	0.00161	0.00108	
Colorado River Delta																									
4. Cerro Prieto	16				2					1									0.29	0.20	0.29	0.00062	0.00000	0.00041	
5. Laguna Salada	14						3	3											0.49	0.27	0.49	0.00123	0.00241	0.00163	
6. Santa Clara Slough	12						4		3		1								0.42	0.61	0.61	0.00118	0.00407	0.00215	
7. Flor del Desierto	17						1		1										0.11	0.20	0.20	0.00032	0.00126	0.00064	
8. El Doctor 1	17						1					1	1						0.19	0.19	0.28	0.00046	0.00120	0.00071	
9. El Doctor 2	13						3	1	1					1	1				0.51	0.35	0.57	0.00143	0.00271	0.00186	
Río Sonoyta/Quitobaquito Spring																									
10. Río Sonoyta																10	5	4	1	0.34	0.68	0.68	0.00051	0.00248	0.00118
11. Quitobaquito																11	2	8		0.50	0.60	0.60	0.00075	0.00202	0.00118
Captive DNFH population ^a	33																		0.00	0.00	0.00	0.00000	0.00000	0.00000	

^a DNFH = Dexter National Fish Hatchery and Technology Center; population was founded by 280 specimens from locality 6 (Fig. 1; Appendix 1) in 1983 (Dunham and Minckley, 1998)

TABLE 2. PERCENTAGE OF GENETIC VARIATION EXPLAINED BY DIFFERENT LEVELS OF GEOGRAPHIC STRUCTURE IN DESERT PUPFISH. RESULTS OF HIERARCHICAL ANALYSES OF GENETIC DIVERSITY FOR DIFFERENT SEGMENTS OF MTDNA. Asterisks signify probability levels: * < 0.05, ** < 0.01, *** < 0.0001.

Source of variation	ND2 and D-loop					
	Haplotype frequencies only			Haplotype frequencies and sequences		
	Salton Sea and Colorado River Delta regions	Rio Sonoyta/ Quitobaquito Region	All three regions	Salton Sea and Colorado River Delta regions	Rio Sonoyta/ Quitobaquito Region	All three regions
Among regions	1.5	--	34.3**	3.7*	--	75.8*
Among populations within regions	1.9	0.0	0.9	2.1	2.9	0.6
Variation within populations	96.6 ^a	100.0	64.8	94.2	97.1	23.6
ND2 only						
Among regions	1.9*	–	44.6**	3.5**	–	86.8*
Among populations within regions	1.3	2.9	0.8	2.0	2.9	0.3
Variation within populations	96.7	97.1	54.6	94.5	97.1	12.9
D-loop only						
Among regions	2.0	–	40.9*	3.8	–	10.6***
Among populations within regions	2.1	0.0	0.8	2.3	2.9	2.1
Variation within populations	95.9 ^a	100.0	58.3	93.9 ^a	97.1	87.4

^a A significant ($P < 0.05$) portion of the genetic variation was attributable to differences among samples, but the differences were not consistently among or within regions.

APPENDIX 1. SAMPLE LOCATIONS FOR 11 NATURAL POPULATIONS OF THE DESERT PUPFISH COMPLEX. Locality numbers correspond with those in Figure 1 and Table 1.

Salton Sea area: (1) irrigation drain, 100 m from shore of Salton Sea near boundary between Riverside and Imperial Counties, 3 km N Desert Shores, Imperial Co., California; (2) San Felipe Creek at Highway 86 bridge, 18 km SE Salton City, Imperial Co, California; (3) shoreline pool of Salton Sea near Trifolium 20A drain, about 15 km NW of Westmorland, Imperial Co., California.

Colorado River Delta: (4) a slough near Cerro Prieto and 0.8 km N of a geothermal power plant, Baja California; (5) a seep area at E edge of Laguna Salada, 20.6 km S 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 NW of locality 8.

Río Sonoyta/Quitobaquito area: (10) Quitobaquito Springs/Pond, Organ Pipe Cactus National Monument, Pima Co., Arizona; (11) Río Sonoyta 11.5 km W, 1.5 km S Sonoyta, Sonora.

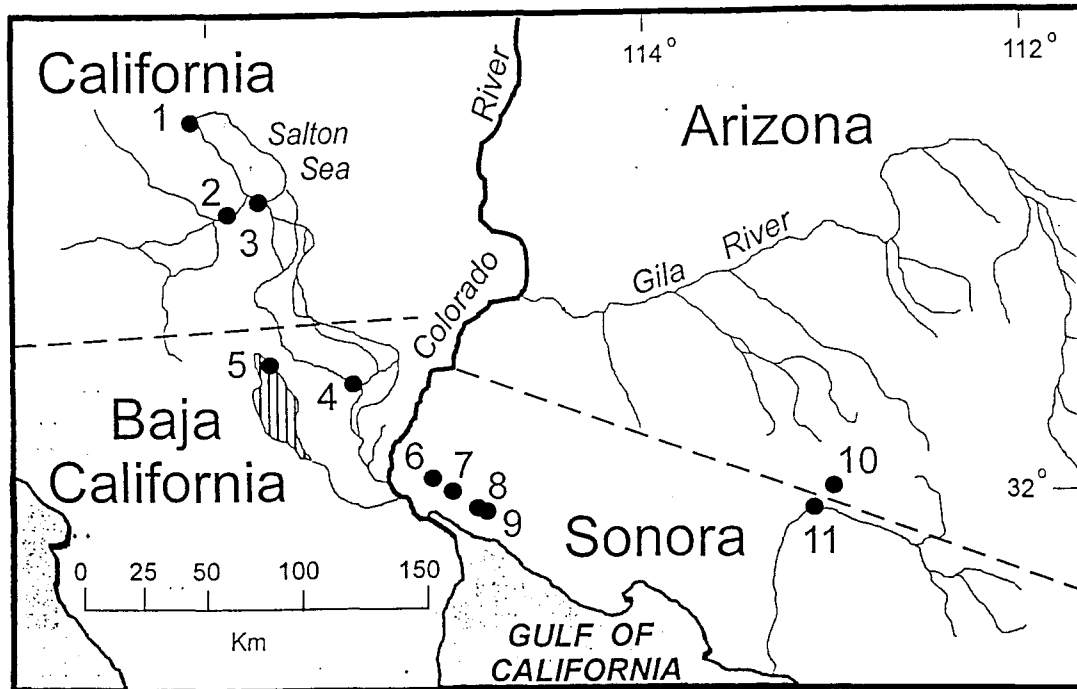


Fig. 1. Localities sampled for mtDNA variation in the desert pupfish complex. The sample sites represent the three major areas occupied by extant populations: the Salton Sea area (sites 1-3), the Colorado River Delta (sites 4-9), and the Río Sonoyta/Quitobaquito Springs area (sites 10 and 11). Now-extirpated populations are known to have occurred in waters associated with the lower Colorado River in Arizona and from the Gila River Basin at scattered localities from near the confluence with the Colorado River to the San Pedro River (= Río San Pedro) in southeastern Arizona and northern Sonora, somewhat beyond the eastern limit of this map (Minckley, 1973; Miller and Fuiman, 1987).

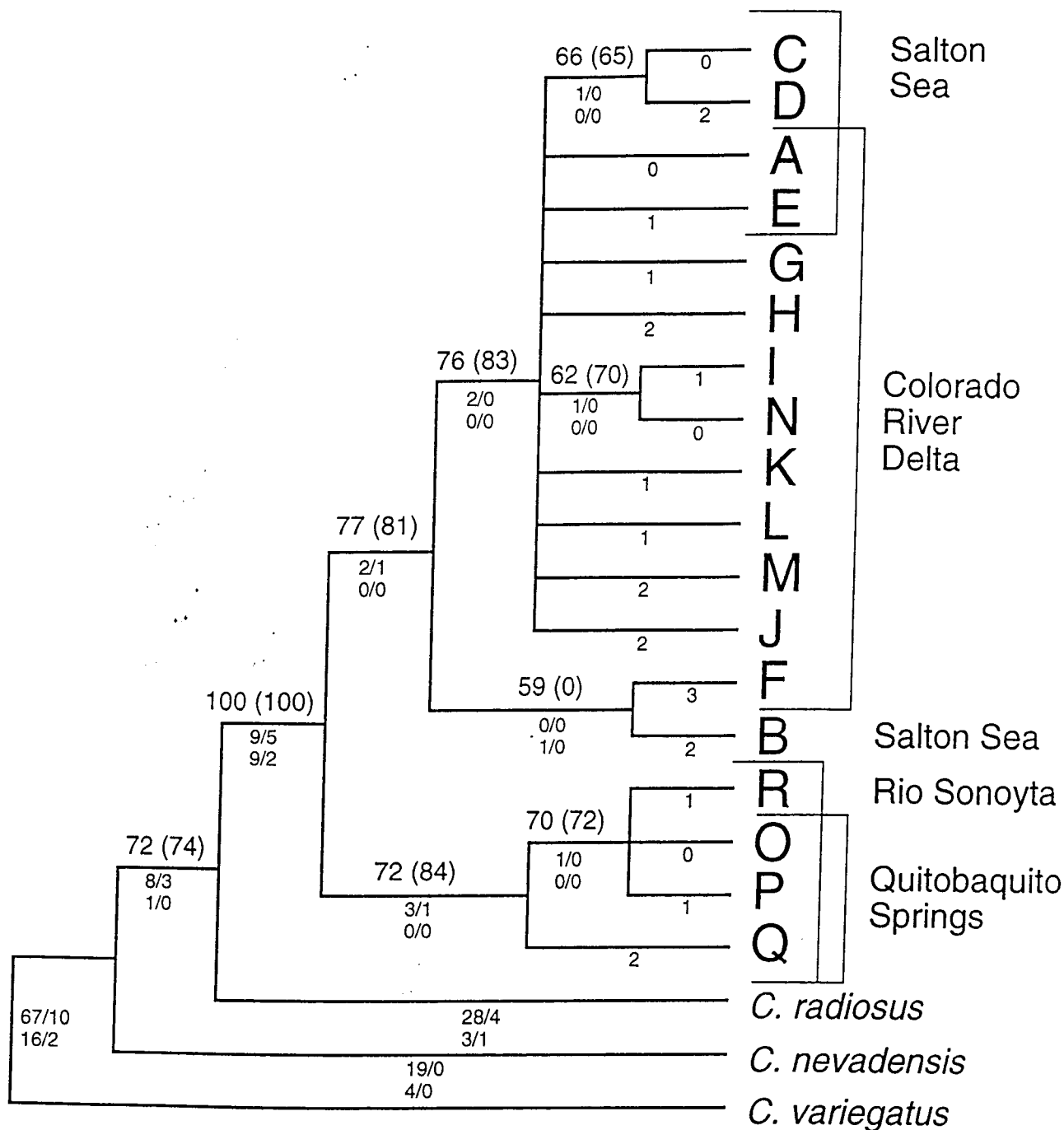


Fig. 2. Phylogenetic relationships among 18 mtDNA haplotypes (A to R) from desert pupfish and haplotypes from two other western pupfishes, *C. nevadensis* and *C. radiosus*. *Cyprinodon variegatus* was the designated outgroup. Numbers above each node indicate percent of trees supporting all nodes receiving greater than 50% support in a maximum parsimony bootstrap analysis (outside the parentheses are results for combined ND2/D-loop data; inside parentheses are results for ND2 alone). Numbers below each interior node indicate number of synapomorphies for the ND2 gene (top) and the D-loop (bottom); for each, number of transitions is to the left of the slash mark and number of transversions is on the right. Only the total number is given for autapomorphic changes.

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