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Genetic characterization of the tidewater goby using microsatellites

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GENETIC CHARACTERIZATION OF THE TIDEWATER GOBY USING
MICROSATELLITES

A Thesis

Presented to

The Faculty of the Department of Biology

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

by

Holly Lynn Mendonca

March 2001

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ABSTRACT

GENETIC CHARACTERIZATION OF THE TIDEWATER GOBY USING MICROSATELLITES

by Holly Lynn Mendonca

The first microsatellite loci for the endangered tidewater goby, *Eucyclogobius newberryi*, are described. A partial tidewater goby genomic library was constructed in pUC19 plasmid. Microsatellite containing clones were isolated, sequenced and amplified. Out of 12 positive clones sequenced, three were polymorphic and proved useful in a preliminary genetic screen of three well-separated populations (50 individuals each) along the central California coast. The number of alleles per locus ranged from two to three. This initial study suggests a population bottleneck had occurred in one of the populations.

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Thanks to Jerry Smith, the brave soul who “took me on” as a graduate student in Spring 1995, when he already had his quota of graduate students. I signed up for his Ichthyology course at San Jose State University in Fall 1994 to jump-start my brain after taking several years off from school. My bachelor’s degree was in Marine Biology, but I had a full-time job in a Cancer Biology lab. I had never taken an Ichthyology course and to put an end to this embarrassment, I signed up for the course, just to learn some tidbits about fish. I had no intention of trying to get a Masters degree! He taught me in his class that the skills I had acquired in the workforce (in a molecular biology lab) were currently being used to answer ecological questions. When I started to entertain the idea of doing a thesis, he was the one who knew the ecological questions to ask, and the purpose of this thesis was to answer, at least in part, Jerry’s question of “could microsatellites be used to characterize local tidewater goby genetics?” He was the brains behind knowing which goby populations to target, having done many sampling “freebies” for U. S. Fish and Wildlife Service. He was also the first to put on his waders, with seine in hand, and was indispensable in the sampling done for this study. Thanks also for his many hours spent editing the manuscript that was prepared from this work.

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Thanks to Dr. Keith Laderoute at SRI International in Menlo Park, California, for the use of equipment and laboratory space in the SRI Cancer Biology Laboratory for conducting this work and for allowing the research to be done during the off-hours at my job at SRI. Keith was also my first mentor in the field of molecular biology. His meticulous lab etiquette and approaches, and his efficient way of processing data into the written word taught me what it takes to be a good scientist. I hope I have incorporated some of what he taught me in my day to day activities in the laboratory.

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INTRODUCTION

The tidewater goby (*Eucyclogobius newberryi*) is a small (< 5 cm) benthic fish inhabiting coastal lagoons and estuaries along the coast of California from Del Norte County to San Diego County. The only member of its genus, *Eucyclogobius* is almost unique among Pacific coast fishes in its preference for low-flow, shallow, brackish water (Swift et al., 1989). The one year life span of *Eucyclogobius* is marked by seasonal and year to year population explosions and crashes making this fish particularly vulnerable to extirpation due to habitat alteration by weather-related events such as drought and flooding, as well as anthropogenic influences such as coastal development activities (U.S. Fish and Wildlife Service, 1994; Lafferty et al., 1999). Disappearance of *Eucyclogobius* from numerous sites, particularly in southern California where healthy populations had been reported within the last 15 years, raised concern among conservation biologists (Swift et al., 1989). In 1994, *Eucyclogobius* gained federal protection as an endangered species (U.S. Fish and Wildlife Service, 1994).

Swift et al. (1989) recognized the need to monitor and characterize existing *Eucyclogobius* populations. Because *Eucyclogobius* lacks a pelagic marine life history phase, limiting its dispersal capacity in all but wet years, populations can become isolated and differentiate genetically. Lafferty et al. (1999) investigated and described possible metapopulation structures of *Eucyclogobius* in numerous southern California sites, and emphasized the importance of understanding metapopulation dynamics in developing management strategies such as restocking of extirpated populations.

The importance of identifying differences among stocks and conserving local adaptations in management practices has been a central issue in conservation biology (Fontaine et al., 1997; Nielsen, 1998). Molecular genetic analysis, especially when combined with other disciplines such as ecology, provides additional insight for management plans of endangered species (O'Brien, 1994). The development of molecular tools to identify the genetic structure of populations of *Eucyclogobius* along its range could aid in implementing beneficial management practices.

Using allozyme analysis, Crabtree (1985) found limited gene flow between southern populations of tidewater gobies, suggesting a long period of isolation in the study areas. The northern part of the tidewater goby range has not been characterized genetically. While allozymes have been used extensively by biologists, the large tissue requirements precluded use of this method on a small endangered species for this study. In addition, a tool with finer resolution was preferable for detection of genetic differences within closely related populations.

Recent studies comparing DNA methods for genetic characterization within and between populations have shown remarkable agreement between different classes of molecular markers, including different classes of neutral nuclear loci (allozymes, microsatellites and random amplified polymorphic DNAs) and mitochondrial DNA (Scribner et al., 1994; FitzSimmons et al., 1997; Cagigas et al., 1999; Goldstein et al., 1999; Ross et al., 1999). It has been suggested, however, that microsatellites offer the finest resolution available for studies within populations (FitzSimmons et al., 1997; Patton et al., 1997; Cagigas et al., 1999; Ross et al., 1999) because they have the highest

mutation rate found in nuclear DNA (Angers and Bernatchez, 1997) and are not biased by female mediated gene flow as in mitochondrial DNA analysis (FitzSimmons et al., 1997; Ross et al., 1999).

Microsatellites, tandemly repeated DNA sequences with repeat units of 1-6 base pairs (bp) in length and 10-100 units per locus (Charlesworth et al., 1994), are very abundant throughout the eukaryotic genome, and they behave in Mendelian fashion (Gjetvaj et al., 1997; Banks et al., 1999; Stoner et al., 1997). Genetic patterns detected in populations of island foxes (*Urocyon littoralis*) on the Channel Islands using microsatellites showed concordance with biogeographic data for these populations (Goldstein et al., 1999). Since microsatellite analysis is a polymerase chain reaction (PCR) based method, it requires only minute, non-lethal amounts of tissue for analysis, making it particularly advantageous for studies of endangered species where tissue requirements are of special concern. Genetic analysis using microsatellites has been well documented for many aquatic species including chinook salmon (*Oncorhynchus tshawytscha*) (Banks et al., 1999), Atlantic salmon (*Salmo salar*) (Fontaine et al., 1997), sea bass (*Dicentrarchus labrax*) (Garcia de Leon et al., 1995), water snakes (*Nerodia sipedon sipedon*) (Prosser et al., 1999), lobsters (*Homarus americanus*) (Tam and Kornfield, 1996), and rockfish (*Sebastes spp.*) (Wimberger et al., 1999).

In the current study, microsatellites were identified in *Eucyclogobius* and primers were designed which flanked these microsatellite loci. Loci that amplified reproducibly by PCR were used in a preliminary study to determine whether they were polymorphic in

three distinct populations along the central California coast and therefore useful as markers in the genetic characterization of *Eucyclogobius*.

MATERIALS AND METHODS

Construction of Partial Genomic Library and Primer Design

DNA extraction. A single tidewater goby was collected at Baldwin Creek in San Mateo County in October, 1992 and stored at -70°C until DNA extraction. A standard DNA extraction protocol for obtaining genomic DNA was used (Ausubel et al., 1987). The posterior third of the fish (0.64 g) was ground to a bulky powder with liquid nitrogen in an autoclaved and UV-sterilized chilled porcelain mortar, then scooped into a 15 ml conical centrifuge tube containing pre-warmed lysis buffer [100 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml Proteinase-K, 10 mM Tris-Cl (pH 8),] at 1.2 ml/100 mg tissue and incubated overnight at 50°C with gentle rotation in a hybridization oven. The DNA was extracted with an equal volume of phenol/chloroform (1:1), inverted several times, then spun at $1700 \times g$ in a swinging bucket centrifuge for 5 minutes. The aqueous phase was extracted again with an equal volume of chloroform, inverted several times, and spun for 5 minutes at $1700 \times g$, then the aqueous phase was precipitated with 2.5 M ammonium acetate (final) and 2 volumes (of original volume) 100% ethanol. The mixture was allowed to precipitate at -20°C for 1 hour, then spun at $1700 \times g$ for 2 minutes. The pellet was rinsed with 1 ml 70% ethanol, transferred to a 1.5 ml microfuge tube, and spun at $1700 \times g$ in a Tomy

refrigerated microfuge. The pellet was dried, then resuspended in 200 μ l TE buffer [1 mM EDTA, 10 mM Tris-Cl (pH 8)]. The DNA pellet was allowed to hydrate at 4°C for >1 hour, treated with 1 μ g/ml DNase-free RNase (Gibco/BRL, Grand Island, New York) at 37°C for 30 minutes, then extracted and precipitated as previously described. After drying the DNA pellet and resuspending in TE buffer, 2 μ l were diluted and evaluated in a spectrophotometer and quantitated using the conversion [1 A_{260} unit = 50 μ g/ml double stranded DNA]. The DNA was also evaluated on a 1% agarose gel, stained with 0.5 μ g/ml ethidium bromide and photographed with a FisherBiotech™ Photodocumentation System (312 nm UV light source/ type 667 Polaroid film, 3000 ISO).

DNA digestion. Enzymes used previously for similar studies (Tam and Kornfield, 1996) were initially tested on goldfish DNA to determine which enzyme or combination of enzymes would best be suited for this study. It was determined that a single 4 base cutter, *Alu* I, which cuts at AG*CT, generated the appropriate size range of fragments for the partial genomic library. Several aliquots of DNA (5 μ g in 25 μ l final volume) were digested with a four-fold excess of *Alu* I (Gibco/BRL) in 1X Promega Buffer B [50 mM NaCl, 6 mM MgCl₂, 1 mM dithiothreitol (DTT), 6 mM Tris-HCl (pH 7.5)] for 1 hour at 37°C. DNA loading buffer (30% glycerol, 0.25% bromophenol blue, 0.24% xylene cyanol) was added and the samples were loaded onto a 1.5% agarose gel. DNA was loaded (5 μ g x 6 lanes) with 100 bp standards (Gibco) and the gel was run at 100 V until a smear was visualized down the length of the gel in the digested samples. The gel was then stained with ethidium bromide. Size fractions of DNA (400 $\pm$ 50 and 600 $\pm$ 50 bp) were excised from the gel with a sterile scalpel blade. DNA was eluted from the agarose

blocks with a syringe elution technique as described by Lu et al. (1994). In brief, a 3 ml sterile syringe barrel (with plunger removed) was connected to a sterile single use, low protein binding Acrodisc® syringe filter (0.2 micron, Gelman, Ann Arbor, Michigan) and pre-wetted with 50 µl TE buffer. The agarose blocks were scooped into the barrel (size ranges kept separate) and the plunger was inserted which forced the agarose onto the syringe filter. "Squeezate" (liquid which squeezed out of the agarose plugs) was collected in a sterile 1.5 ml microfuge tube (50-100 µl). The filter was then washed two times by adding 100 µl TE buffer, inserting the plunger and collecting the eluate. This eluate was then ethanol precipitated by adding 0.1 volume 3 M sodium acetate (pH 5.2) and 2 volumes 100% ethanol. Precipitation occurred at -20°C for 2-16 hours. Samples were spun at 15,000 x g at 4°C for 15 minutes, then the supernatant was discarded and the pelleted DNA was dried down and resuspended in TE.

Ligation of pUC19 with recovered DNA fragments. Five µg pUC19 vector (Bayou Biolabs, Harahan, Louisiana) were digested with an 8-fold excess of *Sma* I (Gibco) in 1X *Sma* I buffer (U. S. Biochemical) in a final volume of 50 µl at 25°C for 4 hours, producing a blunt cut at the restriction site, CCC*GGG, which linearized the plasmid. Uncut and *Sma* I digested plasmid were run side by side on an agarose gel to confirm that the plasmid had been linearized. The 5' termini of the vector were then dephosphorylated with alkaline phosphatase (to minimize vector recircularization during ligation). Calculations for this procedure are shown in Appendix A. To the remaining 45 µl (4.5 µg) of *Sma* I digested pUC19 DNA, 10 µl 10X calf intestinal alkaline phosphatase (CIAP) buffer, 2 µl diluted CIAP enzyme (0.1 unit) and 88 µl water were added. The

reaction proceeded at 37°C for 1 hour. The reaction was stopped with 2 µl 0.5 M EDTA and was stored overnight at -20°C. The digest was phenol extracted twice, then phenol/chloroform extracted and ethanol precipitated as previously described. The plasmid was resuspended in TE at 0.1 µg/µl.

Optimal vector:insert molar ratios were determined to be 3:1 (vector:insert) in a separate ligation and transformation experiment. Calculations for conversion of molar to mass ratios are shown in Appendix B. Ligations were performed with 900 ng vector (pUC19) and 30 ng tidewater goby genomic DNA size fractions in 1X Gibco ligase reaction buffer [10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% polyethylene glycol-8000, 50 mM Tris-HCl (pH 7.6)] with 1 µl (1 U/µl) Gibco DNA ligase in a total reaction volume of 20 µl at 14°C for 16 hours, then were kept at 4°C until used in a transformation.

Transformation of E. coli cells with ligation products. DH5α[™] competent cells (subcloning efficiency, Gibco/BRL) were used for the transformations. Molten Luria broth (LB) agar containing 40 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Gibco/BRL) and 100 µg/ml ampicillin was poured into 150 mm sterile Petri dishes and allowed to set. The plates were inverted and dried several hours in a 37°C incubator prior to plating. Transformation was done according to manufacturer's directions (Gibco). In brief, 2 µl (~50 ng) of ligation reaction for each size fraction were used in separate transformations with 50 µl and 100 µl competent cells. Negative controls (pUC19 alone and no DNA) were included. Competent cells and DNA were mixed in 1.5 ml microfuge

tubes and incubated on ice for 30 minutes. Then the cells/DNA were heat shocked by placing the tubes in a 37°C water bath for either 20 seconds (for 50 µl transformation) or 45 seconds (for 100 µl transformation). The cells/DNA were then put on ice for 2 minutes. Room temperature Luria broth (0.9 or 0.95 ml) was added and the tubes were shaken (225 rpm) at 37°C for 1 hour. One quarter to one half of the volume was plated onto the prepared LB agar plates, then the plates were inverted and incubated overnight in a 37°C incubator.

Screening of the genomic DNA digest with dinucleotide probes. The oligonucleotides (GT)₁₅ and (GA)₁₅ were previously shown to work for identifying microsatellite loci in other species (Tam and Kornfield, 1996). These probes were synthesized by Midland Certified Reagent Company (Midland, Texas). Concentrated stocks (150 ng/µl) were made in water. The presence of these repeats in tidewater goby DNA was first tested using a Southern blot (Ausubel et al., 1987) of the original *Alu* I digest of the genomic DNA. Undigested tidewater goby DNA, *Alu* I digested and *Hae* III digested tidewater goby DNA, aliquots of the 400 and 600 bp size fractions used in the ligation reactions, and *Alu* I digested goldfish DNA were loaded and run on a 1% agarose gel, then stained with ethidium bromide and photographed as described previously, then incubated with 0.25 N HCl for 10 minutes (or until the dye front changed color from blue to yellow). The gel was rinsed with water, then incubated with denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 30 minutes with shaking (dye front returned to blue color). The gel was rinsed again with water and then incubated with neutralization buffer [1.5 M NaCl, 1 mM EDTA, 0.5 M Tris (pH 7.2)] for 15 minutes with shaking. The gel was

rinsed with water and incubated again with neutralization buffer for 15 minutes with shaking. The blot was assembled (Ausubel et al., 1987) and the DNA was allowed to transfer to Hybond™-N nylon membranes (Amersham Life Science Inc., Cleveland, Ohio) overnight in 10X standard saline citrate (SSC). The DNA was fixed to the membrane with a Stratalinker® UV crosslinker (Stratagene, La Jolla, Calif.) and prehybridized with 5X SSC, 0.1% SDS at 65°C for at least 1 hour. The oligonucleotides were end-labeled together as described in the *Colony lift and hybridization* section, and allowed to hybridize to the blot in 5X SSC, 0.1% SDS at 65°C for 16 hours. Two 15 minute washes and one 30 minute wash with 5X SSC, 0.1% SDS were done at 65°C, and the membrane was exposed to Kodak XOMAT™-AR autoradiography film for 2 days at -80°C.

Colony lift and hybridization. Approximately 5000 colonies were lifted onto Hybond™-N nylon membranes (Amersham Life Science Inc., Cleveland, Ohio) and prepared for Southern blotting according to manufacturer's directions. In brief, agar plates with colonies stored at 4°C were allowed to come to room temperature over several hours. Nylon membranes were laid onto the plates for approximately 1 minute. India ink was injected with needle and syringe in an identifiable pattern (e.g., 4 o'clock) through the membrane and into the agar, marking both the membrane and agar. Membranes were lifted off the plates with forceps and placed face up on 3 MM Whatman paper. The bacterial agar plates were returned to the 37°C incubator to re-grow for at least 8 hours and then stored at 4°C for later use. Membranes, colony side up, were transferred with forceps to 3 MM Whatman paper soaked in 10% SDS for 3 minutes. Then the membrane

was transferred, colony side up, to separate 3 MM Whatman papers soaked in denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 7 minutes; neutralization solution [1.5 M NaCl, 1 mM EDTA, 0.5 M Tris (pH 7.2)] for 3 minutes; fresh neutralization solution for 3 minutes; and 2X SSC for 5 minutes. (Note: 3 MM Whatman papers should not be allowed to get too saturated with solution or the colonies may float off the membrane and alignment with agar plate and autoradiogram will be compromised). Membranes were air dried for 1 hour, UV crosslinked and continued to air dry overnight.

Prehybridization buffer [5X SSC, 0.01% SDS, 5X Denhardt's solution (5 Prime-3 Prime, Boulder, Colorado)] was prepared (10 ml/blot) and the blots prehybridized at least 1 hour at 60°C. (GT)₁₅ and (GA)₁₅ oligonucleotides, (300 ng each) were end-labeled in one reaction using T4 polynucleotide kinase (Gibco/BRL) according to manufacturer's directions with 40 µCi [γ -³²P]ATP (10 mCi/ml, >5000Ci/mmol, Amersham). Labeled probes (5x10⁶ cpm/ml) were heated to 70°C for 5 minutes, then added to 10 ml hybridization solution (5X SSC, 0.01% SDS, 5X Denhardt's solution) and added to the blots. Hybridization proceeded >16 hours at 65°C in a hybridization oven. The blots were washed (5X SSC, 0.01% SDS) twice for 15 minutes and once for 30 minutes at 65°C. The blots were enclosed in hybridization bags and exposed to Kodak XOMAT™-AR autoradiography film for 2 hours at room temperature.

Resulting autoradiograms were lined up with the original bacterial agar plates, matching the 4 o'clock pattern of the membrane with the India ink marks embedded in the agar. At least 25 positive colonies were pin-pointed that were well isolated from their neighbors. A total of 26 positive colonies were picked and grown up in Luria broth (3 ml)

containing 100 µg/ml ampicillin. These cultures were grown overnight in 14 ml Falcon® snap-cap polystyrene culture tubes at 37°C with shaking (225 rpm). Aliquots were frozen in 10% glycerol and stored at –80°C. Some of the liquid culture from each sample was re-streaked onto fresh LB agar plates containing X-gal and ampicillin as described in the *Transformation of E. coli cells with ligation products* section for better isolation and to confirm positive status. After overnight incubation at 37°C single colonies were picked, grown up as described in the *Colony lift and hybridization* section, then dot-blotted according to manufacturer's directions (Amersham) and analyzed by hybridization with the labeled oligonucleotides as described. Liquid cultures of 15 positive colonies were grown in LB containing ampicillin for 16 hours and then purified for plasmid DNA. Aliquots of the cultures (1 ml) were frozen in 10% glycerol and stored at –80°C.

Plasmid purification. A plasmid purification protocol was taken from Ausubel et al. (1987). Overnight cultures (2 ml) were spun at 12,000 x g for 30 seconds in a refrigerated Tomy microcentrifuge. The supernatants were poured off and the bacterial cell pellets were resuspended by vortexing in 100 µl "Solution 1" [50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8)]. Two-hundred µl "Solution 2" (0.2 N NaOH, 1% SDS) were added and the tubes were inverted several times. Then 150 µl ice cold "Solution 3" (3 M potassium acetate, 11.5% glacial acetic acid) were added and the tubes were vortexed and left in an inverted position for 10 seconds. The tubes were placed on ice for 3-5 minutes and then centrifuged at 12,000 x g for 5 minutes. The supernatants were transferred to new tubes, phenol/chloroform extracted and ethanol precipitated. The dried DNA pellets were dissolved in 30 µl TE containing 20 µg/ml DNase-free RNase.

Each sample was quantitated using a spectrophotometer as described in the *DNA extraction* section.

DNA (10 µg) from each sample was digested with *EcoR* I and *Xba* I in 1X Multicore buffer (Promega) with a 10-fold excess of enzyme in a total volume of 20 µl for 2 hours at 37°C. The digests were run side by side with undigested plasmid DNA from the clones on 1% agarose gels and stained with ethidium bromide, in order to determine if an insert was present. The gel was Southern blotted and probed as described in the *Screening of the genomic DNA digest with dinucleotide probes* section.

Double stranded DNA sequencing. Double stranded DNA from positive clones was denatured according to an alkaline denaturation protocol from Amersham. DNA (20 µg) was denatured with 0.1 volume [2 N NaOH, 2 mM EDTA] for 30 minutes at 37°C. The solution was neutralized with 0.1 volume 3 M sodium acetate (pH 5.2) and then precipitated with 2 volumes ethanol for 10 minutes on dry ice. The DNA mixture was spun (15,000 x g, 15 minutes) in a microcentrifuge, then the pellet was washed with 70% ethanol, spun again, then dried and redissolved in water to 1 µg/ml.

Clones were sequenced using a T7-Sequenase™ kit (Amersham Life Sciences, Inc.) according to manufacturer's directions. In brief, 3-5 µg denatured double stranded DNA were incubated at 65°C for 2 minutes with 1X Sequenase reaction buffer [20 mM MgCl₂, 50 mM NaCl, 40 mM Tris-HCl (pH 7.5)] with forward (5'-GGG TAA CGC CAG GGT TTT-3') and reverse (5'-AGC GGA TAA CAA TTT CAC ACA GGA-3') pUC19/M13 sequencing primers (obtained from Dr. Chris Green, SRI, International, Menlo Park, Calif.) in a total reaction volume of 10 µl. The mix was cooled slowly to

room temperature in order to anneal, then spun to remove condensation, then put on ice. Four termination mixes (80 μ M dATP, dCTP, dGTP, dTTP and 50 mM NaCl) containing either 8 μ M ddATP, 8 μ M ddCTP, 8 μ M ddGTP or 8 μ M ddTTP were aliquoted (2.5 μ l each) to separate 500 μ l thin walled tubes. The mixes were prewarmed to 37°C.

The following components were added to the ice cold annealed DNA mix: 1 μ l 0.1 M DTT, 2 μ l 0.75 μ M nucleotide mix (80 μ M each dATP, dCTP, dGTP, dTTP and 50 mM NaCl), 0.5 μ l 32 P-dCTP (10 mCi/ml. Amersham), 2 μ l T7 Sequenase™ version 2.0 DNA polymerase diluted 1:8 in Sequenase dilution buffer [5 mM DTT, 10 mM Tris-HCl (pH 7.5)]. The final reaction volume was 15.5 μ l. This mixture was incubated 2-5 minutes at room temperature. The labeled mix (3.5 μ l) was then added to each termination tube and incubated 5 minutes at 37°C. Four μ l stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were then added to each reaction, and the mixtures were stored at -20°C for up to 3 days. The sequencing reactions (forward and reverse) were run on 4.5% polyacrylamide denaturing gels in 0.5X Tris borate-EDTA (TBE) buffer and run at 25 mA for 1.5-3 hr, then dried and exposed to Kodak XOMAT™-AR autoradiography film overnight at room temperature.

Ligation of inserts with M13 phage for single stranded sequencing. Twelve positive clones were digested with *Xba*I/*Eco*RI to remove the insert as described in the *Plasmid purification* section and the insert was cloned into *Xba*I/*Eco*RI digested M13 (1 μ g, mp19) vector using standard procedures (Maniatis et al., 1982). The digestion of M13

with these enzymes removed a small fragment from the multicloning site. All digested samples were run on 1% SeaPlaque® gels (FMC BioProducts, Rockland, Maine) and stained with ethidium bromide. The inserts and M13 were excised from the gel, then heated to 70°C to melt the agarose. Ligations were performed with equal volumes of M13 and insert, in 1X Gibco ligase reaction buffer with 1 µl (1 U/µl) DNA ligase (Gibco) in a total reaction volume of 20 µl. These reactions proceeded overnight at 4°C.

Transformation of DH5αF'IQ™ competent cells (MAX efficiency, Gibco/BRL) with the M13 ligation mixes was performed according to manufacturer's directions. In brief, DH5αF'IQ™ cells were thawed on ice. Ligation reactions were melted at 70°C, then diluted 10-fold with 50 mM CaCl₂ and cooled on ice. The DH5αF'IQ™ cells (50 µl) were added to the ligation mixes and incubated on ice 30 minutes. The mixtures were then heat shocked for 45 seconds in a 42°C water bath, then placed on ice 2 minutes. Three ml 2YT top agar (0.8% bactotryptone, 0.5% bacto yeast extract, 0.5% NaCl, 1.5% agar), containing 50 µl Bluo-gal (halogenated indolyl-β-D-galactoside, Gibco), 10 µl 100 mM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) and 200 µl *E. coli* lawn cells [DH5α noncompetent cells (Gibco) grown in ampicillin-free Luria broth] were added to a sterile 15 ml culture tube and kept at 45°C in a heat block. Ten percent (20 µl) or 90% (220 µl) of DH5αF'IQ™ transformation mix were added to the Bluo-gal top agar mix in the culture tube. The mix was poured onto prepared LB agar plates without ampicillin, tilting the plate to spread the top agar evenly over the surface. After the top agar

solidified, the plates were incubated overnight at 37°C, then inverted and kept at 4°C for up to two weeks. Plaques were evident on all plates with varying efficiencies.

Single stranded M13 clonal DNA was grown up and purified according to Maniatis et al. (1982). An *E. coli* liquid lawn culture (2 ml) in LB without ampicillin was inoculated by touching a flamed wire loop to the plaque, then immersing the loop in the LB. The cultures were grown overnight at 37°C with shaking. Aliquots of these cultures were frozen at -80°C (no glycerol needed). One ml culture per 1.5 ml microfuge tube was spun at 14,000 x g for 1 minute. The supernatants were transferred to new tubes and 1/5 volume 25% polyethylene glycol (PEG)/2.5 M NaCl was added, mixed by inversion, then spun at 14,000 x g for 10 minutes. A white pellet was visible which contained the pelleted phage. Supernatants were removed completely, then the tubes were drained upside down for 5 minutes. The pellet was resuspended in 140 µl TEN [150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 8)] with vortexing. The mixture was phenol extracted twice, then chloroform extracted and ethanol precipitated with ethanol, then spun at 14,000 x g for 5 minutes. The supernatant was removed completely and the pellet was dissolved in 30 µl sterile water. DNA was quantitated in a spectrophotometer using the conversion, [1 A₂₆₀ unit = 33 µg/ml single stranded DNA].

Single stranded DNA sequencing. Sequencing was performed as described for double stranded sequencing using the T7-Sequenase™ kit, but 7 µl M13 DNA was used (< 1 µg) with forward M13 primer only. Sequencing products were run on 6% polyacrylamide gels in 0.5X Tris borate-EDTA (TBE) buffer and run on a genomyxLR® Programmable DNA Sequencer (Foster City, Calif.) at 60 W for 1.5 hours. Gels were

dried and exposed to Kodak XOMAT™-AR autoradiography film. Sequences were read manually.

Primers were designed based on sequence information flanking the microsatellite motifs using the program Primer3 (MIT, Cambridge, Mass.) using default parameters. Primers were synthesized by Operon Technologies, Inc. (Alameda, Calif.) and 100 µM stocks were prepared in TE. The loci were designated *Ene* 1-4, identified by genus and species (*Eucyclogobius newberryi*) of the type host.

PCR optimization. PCR was done in a Perkin Elmer GeneAmp™ PCR System 2400 (PE Biosystems, Foster City, Calif.) in 0.2 ml MicroAmp™ reaction tubes (PE Biosystems). PCR conditions were optimized on purified tidewater goby DNA used in the partial genomic library (50 ng) by varying magnesium concentration (1.5 or 3 mM) and annealing temperature (45, 50, 55, and 60°C). In general, optimal PCR conditions for *Ene* 1, 2, 3 and 4 were 1.5 mM MgCl₂, 0.15 mM each dNTP (2'-deoxynucleoside 5'-triphosphate, Gibco), 0.25 µM each primer, 1 X Taq buffer [Gibco: 50 mM KCl, 20 mM Tris-Cl (pH 8.4)] and 0.5 units Taq DNA polymerase (Gibco/BRL) in a 10 µl reaction volume. An optimized general cycling protocol for all four loci consisted of 30 cycles of 40 seconds at 94°C, 1 minute at 60°C, and 1 minute at 72°C with an initial 5 minute denaturation at 94°C and a final elongation step of 7 minutes at 72°C. Aliquots of PCR reactions were run on 1% agarose gels and stained with ethidium bromide.

Chelexed fin clip samples from the different populations (see *Population analysis* section) were analyzed using the optimized PCR conditions with all primer sets to test for polymorphisms of the alleles. PCR products from these reactions were run first on 2%

agarose and then on 5% non-denaturing TBE-based acrylamide gels. Both types of gels were stained with ethidium bromide.

Internal labeling of PCR reaction. An internal labeling protocol from Innis et al. (1990) was used, which recommended reducing the dNTP used in the PCR reaction from 0.2 mM to 0.04 mM. Purified tidewater goby DNA (50 ng) or 1 µl chelexed DNA was diluted in a 1X PCR reaction mix [3 mM MgCl₂, 0.04 mM dNTP, 0.5 µM forward and reverse primer, 0.5 U Taq polymerase, and 5 µCi ³²P-dCTP (10 mCi/ml, Amersham)] in a 20 µl reaction volume. The general optimized cycling program was used as described under PCR optimization. PCR products were run as described in the *End labeling of primer in PCR reaction* section.

End labeling of primer in PCR reaction. One µl of 100 µM primer stock (either forward or reverse) for each locus was diluted with 1X Gibco forward reaction buffer [10 mM MgCl₂, 50 mM KCl, 1 mM 2-mercaptoethanol, 70 mM Tris-HCl (pH 7.6)] then 20 µCi [γ -³²P]ATP (10 mCi/ml, >5000Ci/mmol, Amersham) and 10 U T4 polynucleotide kinase enzyme (Gibco) were added. The labeling reaction proceeded at 37°C for 30 minutes, followed by an enzyme inactivation incubation at 65°C for 15 minutes (Nielsen et al., 1994). The labeled probe was stable for up to one month at -20°C. PCR was performed using optimal PCR conditions and the general cycling protocol as described in the *PCR optimization* section, except using 0.25 µM labeled forward primer and 0.25 µM unlabeled reverse primer.

After PCR using either method (internal or end-labeling) 12 μ l formamide loading buffer [95% formamide, 20 mM EDTA (pH 8), 0.05% bromophenol blue, 0.05% xylene cyanol] were added to the PCR products. The samples were heated to 95°C for 2-5 minutes, then were loaded and run on a 6% polyacrylamide denaturing gel on the Genomix system as described in the *Single stranded sequencing* section, then dried and exposed to Kodak XOMAT™-AR autoradiography film overnight at room temperature.

Ene 1. 25 random fish representing each of the three populations were analyzed. PCR was performed on chelexed DNA (1 μ l), purified tidewater goby genomic DNA (50 ng) and negative controls (no DNA and chelexed sand sample from each site) using optimal PCR conditions as described in the *End labeling of primer in PCR reaction* section. The general cycling protocol was used as described in the *PCR optimization* section. A sequencing ladder was prepared as follows: an annealing reaction with 1 μ g M13, 0.5 pmol forward primer, T-7 Sequenase reaction buffer and water to 10 μ l, then a sequencing reaction was performed as described in the *Single stranded sequencing* section. PCR products and the sequencing ladder were heated at 95°C for 2-5 minutes, then were loaded onto 6% acrylamide denaturing gels as described in the *Single stranded sequencing* section and run at 60 W for 1.5 hours. Gels were dried and exposed to autoradiography film for overnight, room temperature exposures as described.

Ene 2 and 4. Forward primer was end-labeled (30 μ l) to process 50 samples at a time. Chelexed DNA (1 μ l) from all samples from each of the three populations were processed. PCR was performed using optimal PCR conditions as described in the *End labeling of primer in PCR reaction* section. Negative controls (no DNA and chelexed

sand sample from each site) were also prepared. The general cycling protocol was used and PCR products and a sequencing ladder were run on 6% acrylamide denaturing gels as described for *Ene 1*. In addition, internal labeling was done for selected fish as described in the *Internal labeling of PCR reaction* section and run on the same gels as the labeled primers to help confirm the alleles. The strongest signals that agreed using the two different methods were determined to be the allele(s).

Ene 3. Further PCR optimization was needed for this locus because of a stutter problem evident using the standardized procedure. Optimization with more stringent conditions was performed using annealing temperatures at 60°C, 62°C and 65°C. The number of cycles was reduced to 25 instead of 30 (Innis et al., 1990). Primers were also redesigned (Appendix C). All possible combinations of primers were used with the tidewater goby DNA used in the genomic library. Nonradioactive PCR reactions were performed first varying the parameters as described. PCR products were run on 1.5% agarose and stained with ethidium bromide.

Optimization was repeated with end-labeled primers on an acrylamide gel as described in *Ene 1* using optimal PCR conditions with 25 cycles of the general cycling protocol but with variation of annealing temperature as described. All primer combinations were used with the genomic tidewater goby DNA and one fish from each population shown to be genetically different as shown by *Ene 2*. Finally, nonradioactive reactions with 25 cycles of the general cycling protocol with optimal PCR conditions using an optimal primer pair (as shown in the preceding experiments) were run using the genomic tidewater goby DNA and one fish from each population shown to be genetically

different as shown by *Ene 2*. PCR products were run on 3-4% MetaPhor® (FMC BioProducts) agarose gels and stained with ethidium bromide. The remaining samples were analyzed using the optimal PCR conditions and the general cycling protocol as described in the *PCR optimization* section. PCR products were run on 3% MetaPhor® agarose gels using 20 and 24 well combs, then stained with ethidium bromide and photographed.

Ene 5. All optimization attempts as outlined for *Ene 3* (except for redesign of primers) were done on this locus using purified tidewater goby genomic DNA and selected chelexed DNA population samples (shown to be different genotypes by other loci).

For the purpose of obtaining images to be used in Figures for all four loci, nonradioactive PCR reactions were performed using conditions as described for each locus. Formamide loading buffer was added and the PCR products were run on 8% acrylamide denaturing gels, then stained with SYBR® Gold nucleic acid stain (0.5 µg/ml in TE, Molecular Probes, Eugene, Oregon) and scanned on a FMBIO® fluorescent imaging system (ReadImage v1.5, Analysis v8.0, Hitachi).

Population Analysis

Details of chosen sites. Samples for population analysis were collected under U.S. Fish and Wildlife Service (USFWS) permit # PRT 793640 in August 1997. Fin clips were collected from 50 fish at each of three sites from Santa Cruz and San Mateo counties (Figure 1). The chosen sites were well separated from each other and were

Figure 1. Map of sampling sites in Santa Cruz and San Mateo counties. Sampling sites are circled.

thought to have had limited historical flood or drought impacts, and have had large, relatively stable recent populations, based on sampling by Dr. Jerry Smith (personal communication). The most southern site in Santa Cruz County, Moran lagoon, is a small, urban, salty (> 20 ppt) lagoon with a small watershed, limited flood flows, and a sandbar in place every summer. Wilder Creek is 9 km further north in Santa Cruz county, has a much larger watershed and flood peaks, but has backwater refuges from floods; a sandbar forms early every summer and conditions are usually mildly brackish. San Gregorio Creek, in San Mateo County, is 40 km further north and has a large lagoon, large watershed, and has backwater refuges against floods. This site has only a partially closed sandbar for most of the summer in many years.

Method of collection. Tidewater gobies were captured at each of these three sites with a 3 mm mesh seine. With each seine haul, the seine was brought close to shore (but still submerged in lagoon water) and tidewater gobies of appropriate size (>35 mm) were removed and placed in a separate pan with lagoon water. Individual fish were placed in a sterile petri dish and a 1 mm² fin clip was excised from the caudal fin with a sterile razor blade onto 3 MM Whatman paper. Fish were returned to a separate pan with lagoon water and detained until all fish were sampled to ensure adequate recovery and to avoid repetitive sampling. Following tissue sample collection, fish were released back into the lagoon. This sampling procedure resulted in minimal distress to the fish. Collected fish swam normally in the recovery pan and retreated when released. The fin clips were air dried for several hours, then stored at room temperature with desiccant. A sample of sand (with all accompanying organic matter) was collected at each site by the fish handler so

that DNA recovered from sand from the habitat and/or the handler could be used as a negative control in the PCR reactions.

DNA preparation. DNA was extracted from fin clips with 5% Chelex® 100 resin (weight/volume) in water (BioRad, Hercules, Calif.) (100 µl/sample in sterile microfuge tubes). A portion of each fin clip (approximately half) was excised with a fresh sterile razor blade (using a dissecting microscope) and tapped into the microfuge tube containing Chelex. The tissue in Chelex was incubated at 65°C for 20 minutes, then vortexed 5-10 seconds. Then a second incubation at 95°C for 10 minutes was done, followed by vortexing for 5-10 seconds. The samples were microcentrifuged at 10,000 x g for 3 minutes then stored at -20°C. Before use in PCR reactions, the samples were incubated at 65°C until thawed, then vortexed and spun (10,000 x g for 3 minutes). The supernatant (1 µl) was used for PCR reactions.

RESULTS

Partial genomic library construction. Recovered DNA was initially contaminated with RNA which appeared as an ethidium stained smear from the 20 kb band of non-fragmented DNA and two prominent bands (as viewed on an agarose gel) representing 28S and 18S RNA molecules. RNase treatment successfully eliminated the contamination and genomic DNA >15 kb was recovered after RNase treatment as evaluated electrophoretically (Figure 2A). The absorbance curve from the spectrophotometric analysis is shown in (Figure 2B). The A₂₆₀:A₂₈₀ ratio (a determination of DNA purity,

Figure 2. Analysis of *Eucyclogobius* genomic DNA. (A) 1% agarose gel stained with ethidium bromide. Lane 1 = 1 Kb DNA ladder (Gibco). Lanes 2,3 = Proteinase-K digested tidewater goby genomic DNA >12kb after RNase treatment. (B) Absorbance curve of purified DNA.

with protein-free DNA preparations having a ratio of 1.8-1.9) was 1.64. Yield of DNA extracted from whole fish for the partial genomic library was approximately 270 µg/mg tissue.

DNA in the correct size range (400-600 bp) for the *Alu* I-digested samples was confirmed by agarose electrophoresis (Figure 3). Yield of recovered genomic DNA size fractions after digestion with *Alu* I (400 and 600 bp) was estimated based on the size of the plug of agarose excised and the length of the smear, relative to the total amount of DNA loaded on the gel (Appendix B). Loss of DNA from the elution technique was assumed to be 50%. DNA concentration recovered from the 400 and 600 bp plugs was estimated to be 32 ng/µl (Appendix B). The Southern blot confirming the presence of dinucleotide microsatellite motifs in the tidewater goby genomic DNA and the 400/600 bp plugs is shown in Figure 4.

Approximately 50% positive colonies (white versus blue) on average was observed after transformation. An example of the autoradiogram obtained from hybridization of the colony lifts with ³²P labeled dinucleotide oligonucleotides is shown in Figure 5. Transformation efficiency (CFU/µg) was calculated according to manufacturer's directions (Gibco) and ranged from 4.6 x 10⁴ to 6.4 x 10⁴. Out of approximately 5,000 colonies screened, 25 colonies were tested and found to be positive by Southern blotting followed by hybridization. Of these, 15 colonies tested positive upon re-screening. *Xba* I/*Eco* R I digests of plasmid DNA from these positive clones were Southern blotted and probed with ³²P labeled dinucleotide oligonucleotides. Inserts were visible for 12 of the 15 clones (Figure 6).

Figure 3. Ethidium bromide stained 1.5% agarose gel with *Alu* I-digested tidewater goby DNA. Lanes 1-3 = 5 µg digests. Lane 4 = 100 bp DNA ladder (Gibco). For easy reference, the 600 bp band in these markers is 2-3X brighter than the other bands in the ladder. The dark smudge at 2,072 bp is bromophenol blue and the lighter smudge at 200 bp is xylene cyanol in the loading dye.

Figure 4. Southern blot analysis of restricted tidewater goby DNA. (A) Ethidium bromide stained 1% agarose gel. Lane 1 = undigested tidewater goby DNA used in partial genomic library. Lane 2 = *Alu* I and Lane 3 = *Hae* III digested tidewater goby DNA. Lane 4 = 100 bp DNA ladder (Gibco). Lane 5, 6 = 400 and 600 bp, respectively, size fractions of tidewater goby *Alu* I digested DNA recovered as described and used in ligation reactions with pUC19 plasmid. Lane 7 = *Alu* I digested goldfish DNA. (B) Autoradiogram of the blot for this gel probed with (GT₁₅/GA₁₅) [γ -³²P]ATP labeled oligonucleotides. Arrows on the right of (B) show the location of the DNA size fractions used in the ligation (Lanes 5,6) which were not visible on the agarose gel.

Figure 5. Autoradiogram of Southern colony blot (lift of representative bacterial agar plate containing clones of transformed *E. coli* with partial tidewater goby genomic library) probed using [γ - 32 P]ATP labeled (GA) $_{15}$ and (GT) $_{15}$ oligonucleotide probes. The blot was hybridized with these probes at 65°C for 16 hours, then exposed to autoradiography film for 2 hours at room temperature. The dark dots are positive colonies containing tidewater goby microsatellite motifs.

Figure 6. Southern blot analysis of cloned microsatellite inserts. (A) Ethidium stained 1% agarose gel showing *Eco*R I and *Xba* I digests of clones from tidewater goby partial genomic library. The clones are run side by side, undigested and digested, with the digested samples showing the 400 bp DNA fragment separated from the pUC19 plasmid (2686 bp). (B) Southern blot for this gel probed with (GT₁₅/GA₁₅) [γ -³²P]ATP labeled oligonucleotides. Lanes 1,2 = uncut/cut *Ene* 3. Lanes 3,4 = clone which did not have a microsatellite. Lanes 5,6 - uncut/cut *Ene* 4. Lanes 7,8 = false positive (contained no microsatellite). Lane 9 = 1 Kb DNA ladder (Gibco). Lane 10 = 100 bp DNA ladder (Gibco). The arrows on the left (A, B) show the insert for *Ene* 3 and 4.

Sequencing and primer design. Direct sequencing from the recombinant plasmids gave short, poor quality reads (Figure 7) so single stranded sequencing with M13 vector was performed instead. This resulted in a very clear, easy to read sequence for all clones examined (Figure 8). Table 1 summarizes all microsatellite motifs identified. Two of the 12 clones were false positives, showing no microsatellite motif on sequencing. Six of the 12 clones were not useable due to vector sequence directly flanking the 3' end of the insert or hairpin artifacts. Five of the 12 clones were true positives, with imperfect or perfect microsatellite repeats. Primer sequences and microsatellite characteristics for each of these five loci are listed in Table 2. Complete sequence information is given for both clones that were used (Appendix C) and clones that were not used (Appendix D). A GenBank BLAST search confirmed that these sequences were not previously identified except for short (most likely random) sequence similarity in *Ene 3* (18 bp) with *Aquifex aeolicus* and *Arabidopsis thaliana*, and *Ene 4* (27 bp) with *Bufo bufo*. Chelexed sand from sampled sites and negative controls containing no template consistently produced no PCR product with these primer sets.

Microsatellite analysis. End labeling of primers proved to be the best method for processing the chelexed samples. Internal labeling produced a bigger signal but stutter was more evident using this method. The convenience of end-labeling primer in a single reaction which could process 50 samples at a time, combined with the smaller amount of radioactivity required by this method (resulting in less expense) were the determining factors in choosing the end labeling method to process the samples (except for *Ene 3*

Figure 7. Example of the sequence information obtained by double stranded sequencing. A microsatellite is visible in one of the clones, but flanking sequence information was limited.

Figure 8. Single stranded sequencing of microsatellite-containing clones from tidewater goby partial genomic library. *Ene* 1, 2 and 3 are shown. The other clones were either false positives (did not contain microsatellite) or were not useable due to vector sequence directly flanking the microsatellite insert.

Table 1. Summary of microsatellite clones recovered from *Eucyclogobius* partial genomic library.

<u>Clone</u>	<u>Microsatellite motif</u>	<u>Fate of clone</u>
2	(AC) ₅ AA(AC) ₁₀	Primers designed. Named <i>Ene</i> 1.
6	(AC) ₁₄	No good primers found in Primer3 Program.
7	no microsatellite	False positive.
8-1	(ATCTCT) ₅	Primers designed. Named <i>Ene</i> 2.
8-2	GT imperfect	3' end closest to the GT repeat had only 5 bp before vector sequence, thus flanking primers could not be designed.
11	(AC) ₁₂	M13 vector sequence began right at 3' end of the repeat, thus flanking primers could not be designed.
12	(GT) ₁₂	Primers designed. Named <i>Ene</i> 3.
14	(GA) ₇ G(GA) ₁₁	Vector sequence at 3' end, but there was enough goby sequence to design primers. Named <i>Ene</i> 4.
15	no microsatellite	False positive.
20	microsatellite visible	Microsatellite was visible on sequencing, but sequencing was smeary in 3 of 3 attempts, smearing mostly in the C/T lanes. This technical problem could not be overcome.
22	(GT) imperfect	A huge microsatellite. Primers were designed but the locus never performed well in PCR reactions. No alleles could be called. Named <i>Ene</i> 5.
24	(AC) ₁₂	This repeat was directly flanked on each side by (CG) ₄ , creating a potential hairpin artifact, thus primers were not designed.

Table 2. *Eucyclogobius* microsatellites.

Locus	Core Sequence	Primers	Size (bp)	Alleles	T _m (°C)
<i>Ene 1</i>	(AC) ₅ AA(AC) ₁₀	5' TGAATCTGGCATCTGCTCTG 3' 5' CACAAAATGCATGATCCCAA 3'	150	1	56-60
<i>Ene 2</i>	(ATCTCT) ₅	5' GTCGACTGGCAGTATGGGAT 3' 5' AGACTCAAAATA GTGCACACCCAC 3'	142-154	3	61-62
<i>Ene 3</i>	(GT) ₁₂	5' CTAACCTGCCCTTGACCCAAA 3' 5' GCAGAAATCAACATAAGAACTATGGAAA 3'	120-130	2	59-60
<i>Ene 4</i>	(GA) ₇ G(GA) ₁₁	5' GTGAATCTGCTCTTAGGCCG 3' 5' GATGAACTTCGGAAATGGTCC 3'	216-220	3	60-62
<i>Ene 5</i>	GT imperfect	5' CTGCTCTGCCCCTGTCTCAGT 3' 5' GGTACAGAGGGGGAGACTGC 3'	280		62

which was processed with MetaPhor® agarose gels). Internal labeling was done only on selected samples to help identify and/or confirm alleles when necessary.

Ene 1 showed no polymorphisms for 25 randomly selected samples from the three sites. The amplified band was 150 bp. Three other loci (*Ene 2*, *Ene 3*, and *Ene 4*) proved polymorphic in preliminary screening and were analyzed for all fish from the three sites. Allelic frequencies and observed heterozygosities are shown in Table 3. *Ene 2*, a hexanucleotide repeat, showed three alleles in the populations examined (142, 148 and 154 bp; Figure 9). *Ene 3*, a perfect dinucleotide repeat, showed two alleles in the populations examined (120 and 130 bp). All primer combinations produced the same result. Increasing the stringency of PCR conditions by varying the magnesium concentration in the PCR mix, varying the number of cycles in the PCR protocol, or varying the annealing temperature had minimal effect on the stutter problem. This technical difficulty was only resolved by using the MetaPhor® agarose gels. A representative MetaPhor® agarose gel used for analysis of the sample sets is shown in Figure 10A, while an example of the stutter problem evident with the standard method of analysis (acrylamide denaturing gel), is shown in Figure 10B. *Ene 4*, an imperfect repeat, showed 3 alleles (216, 218, and 220 bp; Figure 11). The third allele for *Ene 4*, 220 bp, was observed in only 3 out of 141 individuals and was present only in heterozygous individuals from Moran and Wilder lagoons. *Ene 5*, an imperfect GT repeat, never performed well enough to identify the alleles for this locus, however primer sequences are reported since further optimization on this locus might yield a functional microsatellite marker. The average heterozygosity

Table 3. Allelic frequencies and observed heterozygosities (H. obs.) of polymorphic microsatellite loci in three populations of *Eucyclogobius*.

<u>Locus</u>	<u>Allele (bp)</u>	<u>Moran</u>	<u>Wilder</u>	<u>San Gregorio</u>
<i>Ene 2</i>	142	0.34	0.47	0.00
	148	0.32	0.51	1.00
	154	0.33	0.02	0.00
n		45	50	50
H. obs.		0.60 (27/45)	0.58 (29/50)	0.00 (0/50)
<i>Ene 3</i>	120	0.16	0.59	0.08
	130	0.84	0.41	0.92
n		42	50	50
H. obs.		0.26 (11/42)	0.58 (29/50)	0.12 (6/50)
<i>Ene 4</i>	216	0.23	0.19	0.00
	218	0.74	0.80	1.00
	220	0.03	0.01	0.00
n		41	50	50
H. obs.		0.39 (16/41)	0.30 (15/50)	0.00 (0/50)
<i>Average Heterozygosity for each population:</i>		0.313	0.365	0.030

Figure 9. *Ene 2* PCR products run on 8% acrylamide denaturing gel and stained with SYBR®-Gold stain. The gel was scanned with an FMBIO® device. Arrows on the left show the location of the 3 alleles for this locus. Lanes A-D = fish from Moran Lagoon, Santa Cruz County showing 142/154 genotype (A), 148/148 (B), 154/154 (C) and 142/142 (D). Lanes E-H = fish from Wilder Creek, Santa Cruz County showing 142/148 genotypes (E), 148/154 (F), 148/148 (G) and 142/142 (H). Lanes I-N = fish from San Gregorio Creek, San Mateo County, showing the 148/148 genotype for all fish. Lane O = tidewater goby purified DNA used in partial genomic library. Lanes P, Q, R = chelexed sand PCR reactions from sampled sites. Lane S = no DNA. PCR size markers (Promega) are shown on the right.

Figure 10A. Ethidium stained 3% MetaPhor® agarose gel showing PCR products of allele types of *Ene* 3 locus. Lanes 1, 2, 4, 5, 7, 9-11 = 120/130 genotype. Lanes 3, 6, 8 = 120/120 genotype. Lane 12 = 100 bp DNA ladder (Gibco). The ladder runs differently on the MetaPhor® agarose, with compression of bands in the higher molecular weight range and good resolution of the lower molecular weight bands.

Figure 10B. *Ene 3* PCR products run on 8% acrylamide denaturing gel and stained with SYBR® Gold nucleic acid stain. The gel was scanned with an FMBIO® device. The arrows on the right show the location of the 2 alleles for this locus. Lanes A-F = fish from Moran Lagoon, Santa Cruz County showing 130/130 genotype(A, B, D-F) and 120/130 (C). Lanes G-J = fish from Wilder Creek, Santa Cruz County showing 120/130 genotype. Lanes K-P showing San Gregorio Creek, San Mateo County showing 130/130 genotype (K, L, O, P) and 120/130 (M, N). Lane Q = tidewater goby purified DNA used in partial genomic library. Size markers (Promega) on the left. The 120/120 genotype is not shown (is shown in Figure 11b). Note the high stutter/noise evident with analysis using this method, for this locus.

Figure 11. *Ene 4* PCR products run on 8% acrylamide denaturing gel and stained with SYBR®-Gold nucleic acid stain. The gel was scanned with an FMBIO® device. The arrows on the right show the location of the three alleles. Lanes A-D = fish from Moran Lagoon, Santa Cruz County showing 218/218 genotype (A), 216/216 (B and C), and 216/220 (D). Lanes E-H = fish from Wilder Creek, Santa Cruz County showing 218/218 genotype for all fish. Lanes I-N = fish from San Gregorio Creek, San Mateo County showing 218/218 genotype for all fish. Lane O = tidewater goby purified DNA used in partial genomic library. PCR size markers (Promega) are on the left.

(Hartl, 1988) for each population was calculated as: Moran Lagoon, 0.313; Wilder Creek, 0.365; and San Gregorio Creek, 0.030 (Table 3).

DISCUSSION

These are the first microsatellite markers identified and characterized for *Eucyclogobius*. Three of four loci demonstrate moderate polymorphism (2-3 alleles for *Ene 2*, *Ene 3* and *Ene 4*) in the populations examined. The fourth was not polymorphic in a partial screening (n=25), but all four loci might show more alleles with widespread geographical sampling. Preliminary studies using these microsatellite markers on archived *Eucyclogobius* from the southern portion of their range suggest this to be true (Dr. Dave Jacobs, UCLA, personal communication). While amplification of microsatellite loci for genetic characterization is a valid tool for population analysis, size of a microsatellite allele may not always indicate the true genetic structure of the individual (Angers and Bernatchez, 1997) due to point mutations. Therefore, sequencing of a subset of alleles from these fish for a more accurate analysis is warranted. In addition, the presence of a rare allele at locus *Ene 4* (allele frequency of 0.01) indicates that a larger sample size for each population could reveal more polymorphisms within the sampled populations.

These preliminary data suggest that the San Gregorio Creek population, the most northern site, is substantially less heterozygous than Moran Lagoon or Wilder Creek, indicating that this population may have experienced a bottleneck event (Miller and Kapuscinski, 1997). The San Gregorio Creek site was chosen because it is a large and

complex habitat, and severe floods or droughts should not impact it. However, it is located at the northern end of a string of more than 12 *Eucyclogobius* populations in San Mateo and Santa Cruz counties. If San Gregorio Creek were impacted by a natural or man-made disaster, natural recolonization from other populations would be unlikely because offshore currents travel downcoast (north to south).

Although three distinct populations were identified in this study, genetic analyses of temporal replicates over several generations are needed to infer genetic stability or variability of these populations (Miller and Kapuscinski, 1997; Tessier and Bernatchez, 1999). Recent efforts by the U.S. Fish and Wildlife Service to delist *Eucyclogobius* in all areas but Orange and San Diego counties (U.S. Fish and Wildlife Service, 1999) have been described as premature since many *Eucyclogobius* populations remain vulnerable to decimation by natural and man-made habitat impacts (Lafferty et al., 1999). Genetic characterization has not been done on central or northern California *Eucyclogobius* populations, and some of these populations still may be imperiled. In addition, a clearer understanding of metapopulation source and sink populations is necessary before recolonization among isolated coastal streams can be accepted as substantially improving long term *Eucyclogobius* status. Studies are in progress using these microsatellites to characterize the genetic structure of *Eucyclogobius* in other populations from San Mateo and Santa Cruz counties, which may identify metapopulation structure if it exists in the central part of the range. These studies are urgently needed for monitoring the genetic stability of *Eucyclogobius* and to aid in management decisions.

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APPENDIX A

Dephosphorylation calculations for pUC19 used in ligation.

Calf-intestinal alkaline phosphatase (CIAP) enzyme was used on linearized pUC19 vector prior to insert ligation. Dephosphorylation calculations are done according to manufacturer's directions (Gibco).

1. pUC19 plasmid (5 μg) digested with *Sma* I as described in Materials and Methods.
2. $2,686 \text{ bp (size of pUC19 plasmid)} \times (660 \text{ g/mole/bp}) = 1.77 \times 10^6 \text{ g/mole}$
3. $4.5 \text{ } \mu\text{g pUC19 (remaining in digest)} / 1.77 \times 10^6 \text{ } \mu\text{g}/\mu\text{mole} = 0.0000025 \text{ } \mu\text{mole} = 2.5 \text{ pmole}$
4. $2.5 \text{ pmole} / 100 \text{ } \mu\text{l dephosphorylation reaction volume} = 0.025 \text{ pmole}/\mu\text{l}$
5. 0.1 U CIAP used to dephosphorylate 10 pmole of 5' ends.
6. Dilute CIAP (1 U/ μl) 20-fold in 1X CIAP dilution buffer [50% glycerol, 1 mM MgCl_2 , 0.1 mM ZnCl_2 , 25 mM Tris-HCl (pH 7.6)] = (0.05 U/ μl) final enzyme concentration.
7. Add 2 μl (0.1 U/ μl) to 40 μl of pUC19 *Sma* I digest.
8. Incubate at 37°C, 30 minutes. Heat inactivate for 15 minutes at 85°C.
9. Cool to room temperature over 10 minutes, then put on ice. Store the reaction at -20°C.

APPENDIX B

Calculations for conversion of molar ratios to mass ratios for ligation of pUC19 with tidewater goby genomic DNA size fractions.

1. The length of the DNA smear of the *Alu* I digest was measured to be 28 mm.
2. Size of plugs excised and eluted from gel were 6 mm.
3. $6 \text{ mm} / 28 \text{ mm} = 21.4\%$
4. Amount of DNA loaded on gel:
 $6 \text{ lanes} \times 5 \text{ } \mu\text{g } Alu \text{ I genomic DNA digest} = (30 \text{ } \mu\text{g total DNA}) \times (0.214) =$
 $6.42 / 2 \text{ (recovered two different size fractions)} = 3.21 \text{ } \mu\text{g}$
5. 50% loss assumed from efficiency of elution technique =
 $1.605 \text{ } \mu\text{g} / 50 \text{ } \mu\text{l recovered as "squeeze-ate" from elution technique} =$
 $0.0321 \text{ } \mu\text{g}/\mu\text{l} = 32.1 \text{ ng}/\mu\text{l}$
6. Thus, 1.605 μg recovered for each size fraction, 400 and 600 bp.
7. For 600 bp:
 $600 \text{ bp} \times [(660 \text{ g/mole})/\text{bp}] = 3.96 \times 10^5 \text{ g/mole}$
 $1.605 \text{ } \mu\text{g} / (3.96 \times 10^5 \text{ } \mu\text{g}/\mu\text{mole}) = 0.000004 \text{ } \mu\text{mol} = 4 \text{ pmol}$
 $4 \text{ pmol} / 50 \text{ } \mu\text{l volume per size fraction} = 0.08 \text{ pmol}/\mu\text{l} = 80 \text{ fmol}/\mu\text{l}$
7. For 400 bp:
 $400 \text{ bp} \times [(660 \text{ g/mole})/\text{bp}] = 2.64 \times 10^5 \text{ g/mole}$
 $1.605 \text{ } \mu\text{g} / (2.64 \times 10^5 \text{ } \mu\text{g}/\mu\text{mol}) = 0.000006 \text{ } \mu\text{mol} = 6 \text{ pmol}$
 $6 \text{ pmol} / 50 \text{ } \mu\text{l volume per size fraction} = 0.12 \text{ pmol}/\mu\text{l} = 120 \text{ fmol}/\mu\text{l}$
8. pUC19, as shown in Appendix A = $0.025 \text{ pmol}/\mu\text{l} = 25 \text{ fmol}/\mu\text{l}$

9. Optimal vector: insert molar ratios as determined empirically in a separate experiment is 3:1, vector (pUC19) : insert (400 and 600 bp genomic DNA size fractions). In order to obtain approximately 1 µg per ligation reaction, approximately 300 fmol of pUC19 + 80-120 fmol insert were used together in the ligation.

10. $\text{pUC19} = (25 \text{ fmol}/\mu\text{l}) \times 6 \mu\text{l} = 300 \text{ fmol}$

11. $400 \text{ and } 600 \text{ bp insert} = 80 \text{ and } 120 \text{ fmol}/\mu\text{l}$, respectively $\times 1 \mu\text{l} = 80 \text{ and } 120 \text{ fmol}$

12. $300 \text{ fmol} : 80 \text{ or } 120 \text{ fmol} = \text{approximately } 3:1$, vector: insert molar ratios.

13. To calculate mass ratios, go back to the calculated concentration for each:

$\text{pUC19} = 0.15 \mu\text{g} / \mu\text{l} \times 6 \mu\text{l} = 0.9 \mu\text{g} \text{ or } 900 \text{ ng}$

$400 \text{ bp} = 0.0321 \mu\text{g} / \mu\text{l} \times 1 \mu\text{l} = 0.0321 \mu\text{l} \text{ or } 32.1 \text{ ng}$

$600 \text{ bp} = 0.0321 \mu\text{g} / \mu\text{l} \times 1 \mu\text{l} = 0.0321 \mu\text{l} \text{ or } 32.1 \text{ ng}$

APPENDIX C

Sequences of amplifiable microsatellite clones.

Primers used are underlined. Sequences in italics indicate M13 sequence.

Clone 2 (*Ene 1*)

5'-TTGCAATGCACATAAGCAAAAATATAGGGGAAAAAA
CGCTTTTCTGGTAGACTTTAAAACAAATTTAAATGCATA
TTTTAAATTTAAGATTGTGTCACCATTTAATTAGTAGTA
GCATTCTGCACACCAAAGTAGACATTGCGAATTGAATCT
GGCATCTGCTCTGTCTGTCTGTCTGTGTTTTATGACAAA
ATAAATGAGTCATGAGTCTCGAGTCTCTGGTGTGAGGA
AGGCAGAAAACACC(A C)₅AA(A C)₁₀AGTTGGGATCATGCA
TTTTGTGGGACATTACATGAAGGGGGATCCTCTAGAGT
CGACCTGCAGGCATGCA AGCT TGG-3'

Clone 8 (*Ene 2*)

5'-CAGTGAATTCGAGCTCGGTACCCCTGGTTTTAATAGTA
ACTTGGAGTCTCTAACAGTCATACTGCTGGTTTTAGAGT
AACGTGGTGTCTTTTGTCTTTCCAGTCGGAGTCGGAGCAG
GACGAGGAGTCGACTGGCAGTATGGGATCTGTGGCTGT
GGCTGTAAGTCGCACTACATTT CATAAACCTTTA
TTTATATCTATCTATCT(ATCTCT)₅ATATATCTTATAGTG
GTGTGCACATATTTGAGTCTTACTTTCATTAAAGGTGCA
CTATGTAAC(G T)₁₁CTCT(G T)₆CTCT(G T)₅GCTCAGGGATCC
TCTAGAGTCGACCTGCAGGCATGCAAGCTT-3'

Clone 12 (*Ene 3*). All designed primers are shown. Primers used are underlined on the sequence.

←-----

5'-AGGTGAATACTGTTGTTGTGACGCCTAACCTGCCTT

-----→

<.....>

GACCCAAATATTT (GT)₁₂CAAGATAGATTTCAGTGTAGA

ATTATTTTAACATTAAACACCTATTTCTGAATGTCTA

←-----→

<.....>

TTTCAATAGTTCTTATGTTGATTCTGC-3'

Symbol	Name	T _m (°C)
←-----→	= L1 and R1	59 and 60
<.....>	= L2 and R2	65 and 58
*****	= R3	58

Clone 14 (*Ene 4*)

5'-TTGTCTGTGAATCTGCTCTTAGGCCCGCCAGCTAACCA

CTCTGAACTCATTCTGACACAGGTGAACAGAATGTGTCT

TCTTCGCCTCTGGACAGAAAGTGAGATGGTGGCGTTTG

GTCCCCAGCGGCAGGTATCAGAG (GA)₇ G (GA)₁₁ CTGTCTA

CCCAGACCTTTGAGGCCTGGACCATTCGGAAGTTTCATCT

GTTATGGACAGGGGGATCCTCTAGAGTCGACCTC -3'

APPENDIX D

Sequences of nonamplifiable microsatellite clones and clones with primer design problems.

Clone 6

5'-CAGTGAATTCGAGCTCGGTACCCCTTTGCCAGATAAA
ACGACCTGAAAGACAAAATAAAGTCATTGTGAAAAAAC
AGAAATGAAAAAGCTTTTTTATTGTGCTTACAATTATTTT
CAAAGAAATTAAAAC(A C)₁₄CGGTAATAAATAGTCAAGA
AAGAATAAGACTGCTTT-3'

Clone 22

5' - TTTGCGGGTGCTGCAGGCGGACTGTTACCTCTGCTC
TGCCCTGTCTCAGTTACTGCTTC(G T)₁₄CT(G T)₄CT(G T)₁₃GC
(G T)₂CT(G T)₈CT(G T)₁₂CT(G T)₃CT(G T)₂CT(G T)₂CT(G T)₃CT
(G T)₂CT(G T)₂CT(G T)₂GGGT(G T)₂CT(G T)₂CT(G T)₂CT(G T)₁₅GC
ACGTGAGTCTGCTGCAGTCTCCCCCTCTGTACCATCTGCTCT-3'

Clone 24

5' - GTCTCGCGCGGAGGTCTTCTCTAAACGTGGGGAAGA
CACAACAGAATGACAGTTGAGCCGCTTTAGCCGGACGT
CAATCACTCAAACCTGAAAGCGCACGGATCCGGCGGAAT
ACTCACATCTCCGCCGCTGTCGACGCGGACACTTCCTC
CCCTAAACGCGCGCGCGT(CA)₁₂CGCGCGCGCAGCCTGT
CCGTTACAACCGGACAGCGCACGGTC(C)GGTCTCGCGT
TCTTCTTGTTGATTC-3'