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SHORT PAPER

Development and inheritance of molecular markers in the kelp bass *Paralabrax clathratus*

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While molecular tools have transformed fisheries biology, their development and implementation can necessitate the use of delicate equipment, be expensive, and require extensive optimization [1]. Counting only consumables, we have calculated that in-house sequencing of 500–700 bases on a Beckman CEQ 8000 (Beckman Coulter, Fullerton, CA, USA) costs ~$4–5 per sample under ideal conditions. It is a process not amenable to isolated or rudimentary lab conditions and is not easily performed as a quick-return assay. Loci scored as dye-labeled fragments on an automated sequencer are also expensive [2]; they require development of a microsatellite library and cost commercially ~US$12,500 [3]. Confirmation of Mendelian inheritance in the marker set is yet another cost burden and represents an important but infrequently performed prerequisite for statistical testing. A recent survey found 1 in 15 microsatellite loci, as expressed in their data scores, had non-Mendelian patterns [4].

The reporting of methodologies and inheritance studies that are cost effective and have minimal equipment requirements can therefore be invaluable to a new research program or a monitoring effort that requires fast turn-around times. A set of markers will be available for the species in question and frequently its congeners; the strategies for developing the markers, such as our inheritance methodology below, can have even wider application. Based on this rationale, this short paper presents genetic methods developed for the kelp bass, *Paralabrax clathratus,* that confirm the robustness of markers and allow for economical and expeditious assays. Kelp bass are a temperate serranid found abundantly from Point Conception, CA, to just south of Punta Eugenia, Mexico [5], and are an important component of the $300 million California sport fishing industry [6, 7]. *P. clathratus* occurs in current and proposed southern California marine reserves and has been the subject of a diversity of research [8–11].

We document a restriction fragment length polymorphism (RFLP) assay of the mitochondrial control region that can assess a substantial amount of the sequence diversity with only a thermal cycler and an agarose gel box. We also present a series of controlled crosses to confirm Mendelian inheritance for previously published microsatellite markers [12], a task generally difficult to perform with many marine fish [13].

Development of RFLPs

Sequences for the mitochondrial control region of 105 kelp bass from ten locations (Genbank accession numbers DQ192295–DQ192399) were used. Sequences were aligned in Sequencher 4.5 (Gene Codes, Ann Arbor, MI, USA) and surveyed with the program's restriction enzyme library. Pairwise Φ_{ST} values for the ten locations using sequence (trimmed to 499 base pairs to include all individuals) and RFLP data were calculated with Arlequin 3.01

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[14]. Correlation between the two data types was calculated with a Mantel test.

Sequence data represented 51 polymorphisms, 25 of which were found in only one individual. Enzymatic digestion could identify 34 of the polymorphisms; our RFLP assay, which cost $2–3 per sample (as compared to the $4–5 sequencing price tag noted previously), recognized 14 of the substitutions, including the three most polymorphic (Fig. 1). Digestions of a band (~ 1,100 bases; primers "K" and "12RH" [15]) with a mixture of *Aci*I and *Dde*I were scored on a Spreadex acrylamide gel (EL1200, Elchrom Scientific, http://www.elchrom.com "Accessed 4 Feb 2009".) run in a 600-ml horizontal agarose gel box. A separate digestion of PCR product with α*Taq*I was run on a denser gel, the EL400. Because the most abundant polymorphism (26/105 samples; cut at position 237) could be

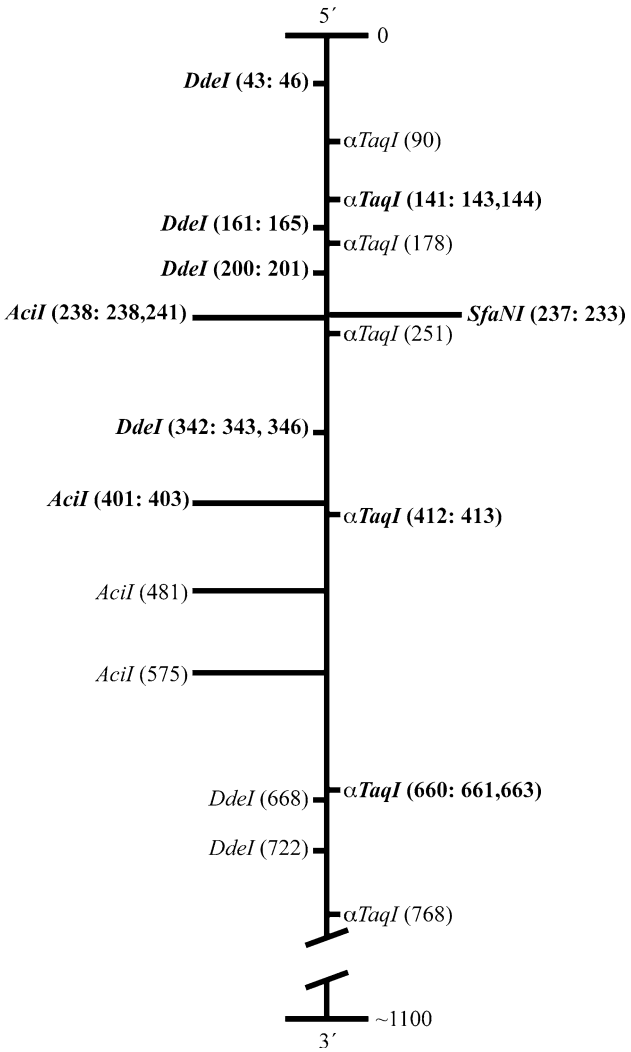

Fig. 1 Enzymes used in RFLP assay of the mitochondrial control region of *Paralabrax clathratus*. Enzymes in *boldface* indicate polymorphic recognition sites; the *number before the colon* is the cut site, *numbers after the colon* are locations of identified polymorphisms

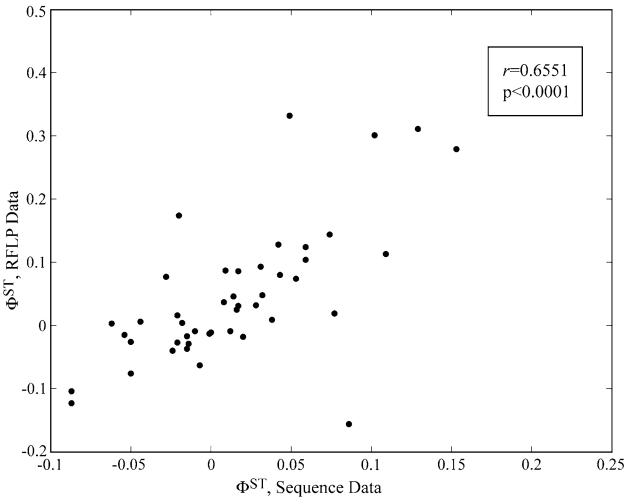

Fig. 2 Plot of Φ_{ST} scores for ten populations of *Paralabrax clathratus* with sequence and RFLP data

recognized only by the expensive enzyme *SfaN*I, a new primer (237C, 5′-CGATTAGGCGTGKCGACATG-3′) was designed to pair with "K" and lay down on the 3′ edge of the variable site. The 18th base of the primer does not match the strand sequence and creates a change (polymorphism is underlined) from 5′-GCAT***C***TAC-3′ to 5′-GCAT***G***TAC-3′. A cheaper enzyme, *Afl*III, was then used to assay adenine substitutions at 233 (product was double-loaded into *Aci*I and *Dde*I gels).

Pairwise differences (Φ_{ST}) of the two data types were significantly correlated ($r = 0.6551$, $P < 0.0001$) (Fig. 2). Because reduced haplotype diversity in RFLP data (0.758 from 0.9081) lowered within-location distances (analogous to H_S), the global Φ_{ST} value was higher with RFLP data (0.0374) than sequence data (0.0144); 4/45 pairwise comparisons with sequence data and 6/45 with RFLP data were significant. Although this method provides a tool comparable to sequence data for assaying genetic differences between sample locations, it cannot be considered to be phylogenetically informative in that roughly a third of the polymorphisms cannot be identified.

Inheritance patterns

Previously reported microsatellites [12] were tested for Mendelian inheritance. An improved reverse primer (5′-TG TATTTAACTTTTAGCCCC-3′) was created for *AV*115, which appeared prone to stutter patterns.

Four males and four females (1.5–7 kg) were held in 1,000-gallon running seawater tanks at the Wrigley Institute for Environmental Studies on Santa Catalina Island, CA. Two injections (the second after 24 h) of 20 μg/kg body weight of luteinizing hormone releasing hormone

Table 1 Results from scoring of eight microsatellites for parents and offspring of three crosses

Microsatellite	$\bar{x}$ (±SE)	Expected no. of genotypes	Measured no. of genotypes	No. of significant χ^2 tests
AV6	13.3 (2.91)	12	11	0/3
AV15	13.3 (2.73)	12	12	0/3
AV17	22.7 (1.33)	4	4	1/3
AV88	15.0 (2.65)	12	12	0/3
AV115	19.0 (3.06)	12	12	0/3
Gag010	19.0 (0.58)	5	5	0/3
Mbo066	19.0 (2.08)	3	3	0/3

Table shows marker name, mean sample size and standard error, expected and observed number of genotypes over the three crosses, and the number of significant χ^2 tests of expected versus observed genotype counts

analog (LHRH-a) were used to induce spawning behavior. Of the eight fish, two males and two females responded and were stripped, with eggs and milt mixed in 2 gal of seawater for 5 min. Fertilized eggs were added to bubbling, 15-gal tanks and harvested after 12 h (development to the blastula stage and beyond was checked by microscope) for immediate DNA extraction with phenol chloroform. Publication of methodologies that perform a genetic assay on such an early stage of fish larvae could not be found by the authors, but it was felt that this method avoids the potentially selective complications of raising larvae past metamorphosis. Mendelian segregation was checked with contingency tests of genotype counts (using a χ^2 distribution to ascertain significance).

Seven microsatellite loci were amplified from offspring and parents of three crosses (Table 1). One cross had a reduced number of successful DNA amplifications because of low DNA quality. In only a single instance was an anticipated genotype not found (microsatellite *AV6*), which occurred in the poor cross. Crosses produced no unexpected genotypes, as might be expected were null alleles segregating or amplifications nonspecific. After sequential Bonferroni corrections, there were no significant differences between expected and observed genotype counts (1/24 tests was significant before correction) and no significant evidence of linkage (4/36 tests were significant before correction, all in the poor cross).

The crosses provide confidence in the efficacy of results using these loci. Analyses that assume Mendelian transmission such as estimation of variance effective population size (N_e) [16] or geographic population structure (F_{ST}) [17], for example, could be validly run. Combined with the mitochondrial marker protocol, the loci offer a suite of tools for assessing the population dynamics of *P. clathratus*, even in situations where sequencing is not an option. The microsatellite primers can also be used in congeners such as *P. nebulifer* (G. Benavides, personal communication). Further, the strategies for identifying inexpensive restriction enzymes and performing crosses with materials found easily in a running seawater laboratory can also be applied to many other marine organisms not surveyed typically with large sample sizes or Mendelian-confirmed markers.

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