Population Genetic Structure of the Armorhead, *Pseudopentaceros wheeleri*, in the North Pacific Ocean: Application of the Polymerase Chain Reaction to Fisheries Problems

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Martin, A. P., R. Humphreys, and S. R. Palumbi. 1992. Population genetic structure of the armorhead, *Pseudopentaceros wheeleri*, in the North Pacific Ocean: application of the polymerase chain reaction to fisheries problems. Can. J. Fish. Aquat. Sci. 49: 2386–2391.

Armorhead (*Pseudopentaceros wheeleri*) occur in the subarctic, epipelagic habitats of the northern Pacific Ocean and are known to reproduce on seamounts in the central Pacific. Over the last few decades, overexploitation of seamount populations led to dramatic declines in abundances of reproductive populations. We undertook a study of the population genetics of armorhead to test whether distinct stocks exist in association with specific seamounts. We used the polymerase chain reaction (PCR) and a combination of DNA sequencing and restriction fragment length polymorphism (RFLP) analysis to analyze mtDNA variants for individuals collected from three localities: two seamounts and from the open ocean. We discovered that mtDNA haplotypes are not partitioned geographically, refuting the hypothesis that different seamounts harbor genetically distinct populations. Furthermore, genetic similarity of seamount and open-ocean fish supports the hypothesis that armorhead migrate between the central and northern Pacific Ocean for reproduction and feeding, respectively.

L'espèce *Pseudopentaceros wheeleri* vit dans les habitats subarctiques épipélagiques de la région septentrionale de l'océan Pacifique et on sait qu'elle se reproduit sur les monts sous-marins de la région centrale de cet océan. Au cours des dernières décennies, la surexploitation des populations des monts sous-marins a conduit à des déclins spectaculaires de l'abondance des populations reproductrices. Nous avons entrepris une étude de la génétique des populations de *P. wheeleri* afin d'examiner s'il existe des stocks distincts en association avec des monts sous-marins particuliers. Nous avons utilisé la réaction en chaîne de la polymérase (RCP) et une combinaison de séquençage d'ADN et d'analyse du polymorphisme de la longueur des fragments de restriction (PLFR) pour analyser les variants d'ADNmt de sujets prélevés en trois endroits : deux monts sous-marins et la haute mer. Nous avons découvert que les haplotypes d'ADNmt ne suivent pas une compartimentation géographique, ce qui réfute l'hypothèse selon laquelle des monts sous-marins différents seraient peuplés par des populations génétiquement distinctes. En outre, la similarité génétique des poissons des monts sous-marins et des poissons de haut mer corrobore l'hypothèse selon laquelle *P. wheeleri* migre entre la région centrale et la région septentrionale de l'océan Pacifique respectivement à des fins d'alimentation et de reproduction.

Received September 3, 1991
Accepted May 26, 1992
(JB209)

Reçu le 3 septembre 1991
Accepté le 26 mai 1992

The hypothesized life history of armorhead (*Pseudopentaceros wheeleri*) is extraordinary. Eggs and larvae are planktonic. Larvae rapidly develop and assume a nektonic habit. At this stage, juveniles move away from the seamounts and subadult fish are most often observed in the cold, nutrient-rich waters of the North Pacific (Boehlert and Sasaki 1988). After feeding for at least 1–3 yr, individuals are believed to migrate to the seamounts in the central Pacific Ocean, take up a demersal habit, spawn, and die (Humphreys et al. 1989).

Armorhead have been recorded from over a dozen seamounts in the central North Pacific Ocean (Borets 1979; Humphreys and Tagami 1986). There are often differences in morphology among individual fish captured in the vicinity of a particular seamount as well as among fish captured at different seamounts. Such variation led Hardy (1983) to recognize two distinct species: *P. pectoralis* and *P. wheeleri*. Subsequent work by Humphreys and Tagami (1986) and Humphreys et al. (1989) showed that the morphological variation exhibited by armorhead is associated with the transition from a pelagic to demersal existence coupled with the onset of reproduction. Just after transition from the pelagic stage, individuals have high fat reserves, and ratios of body depth to fork length are usually greater than 0.25. Over time, individuals become leaner as fat reserves are used up, exhibiting body depth to fork length ratios

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of 0.20 and less. Regional differences in morphology among fish are thought to be due to differences in age at recruitment to the demersal stage.

The existence of a single species of armorhead in the North Pacific is supported by allozyme data that show no allele frequency differences between morphologically distinguishable fish from the Hancock Seamount in the Northern Hawaiian Ridge (NHR) (Humphreys et al. 1989). However, Humphreys et al. analyzed fish from only a single seamount, leaving open the possibility that fish at other seamounts, differing in latitude, oceanographic characteristics, size, proximity to other seamounts, and productivity (Uchida et al. 1986), are genetically different.

Elucidation of genetic relationships among the different seamount populations is warranted because armorheads were once (and may again be) commercially important. Furthermore, only the southernmost seamounts (Hancock Seamounts) lie within U.S. waters whereas the remainder are in international waters. If independent gene pools are associated with different physiogeographically distinct seamounts, then efforts to manage the exploitation of these fish can be implemented on a seamount-by-seamount basis. On the other hand, lack of differentiation among fish from different seamounts would suggest that there is a single panmictic population that is widely distributed throughout the North Pacific. In this case, exploitation of these fish anywhere in the North Pacific may impact the entire stock, and management tactics should be implemented accordingly.

We investigated the population genetics of *P. wheeleri* collected from the two seamounts separated by approximately 500 km and from the open ocean (Fig. 1) to determine if there was evidence for genetic differentiation among fish captured near different seamounts. We also determined haplotypes of fish captured in the open ocean and compared them with haplotypes of fish captured near seamounts to provide indirect evidence for migration between sites of reproduction in the central Pacific Ocean and the hypothesized feeding grounds in the North Pacific Ocean. Finally, we compared the haplotypes for morphologically different fishes to test whether there is a relationship between morphological and genetic differentiation and if there is genetic evidence for two species in the North Pacific Ocean. Our approach employed a combination of DNA sequence and restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified gene regions of mitochondrial DNA (mtDNA). These techniques for population genetic analysis of *P. wheeleri* can be applied to other species of interest.

Methods and Materials

Collection of Fish

Demersal adult fish were caught by pelagic longline from two seamounts, Koko and southeast Hancock in the central North Pacific (Fig. 1). Pelagic-phase juveniles were obtained from a region bounded by latitudes 41–44°N and longitudes 157–165°W (Fig. 1) by foreign drift-net vessels. For the fish caught at the seamounts, portions of the liver and oocytes (when present) were initially frozen in liquid nitrogen and then stored at −70°C until analyzed. For the pelagic fish, 1-g samples of

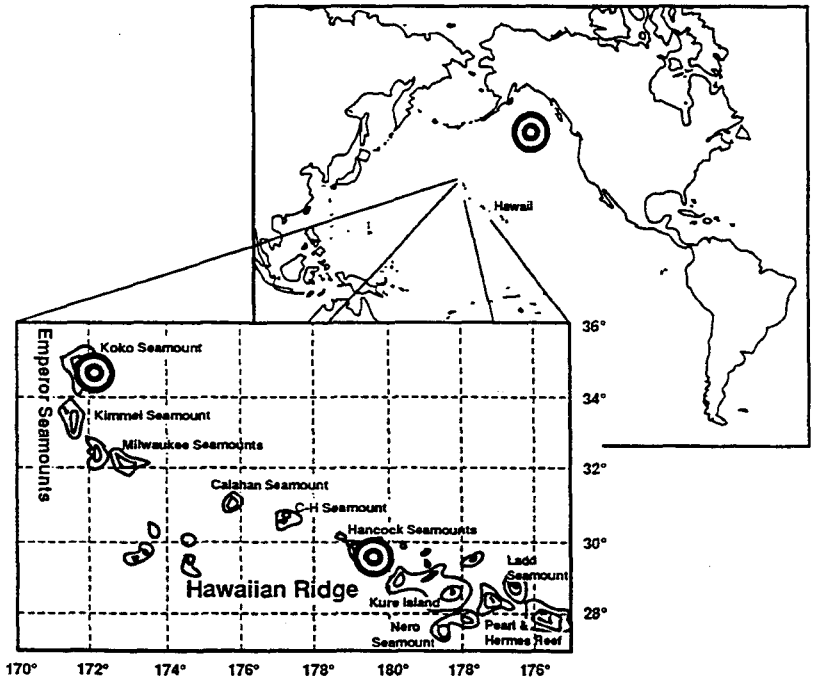

FIG. 1. Map of the Northwestern Hawaiian Islands – Southern Emporer Seamounts showing the location of collection sites for armorheads. Collections were obtained from Koko Seamount, Hancock Seamount, and for pelagic-phase fish from a region bounded by 41–44°N latitude and 157–165°W longitude.

TABLE 1. Primers used for PCR amplification. H and L refer to heavy or light strand, respectively. Numbers correspond to the location of the primer sequence in the human genome sequence (Anderson et al. 1981). Primer sequences are given 5′ to 3′. Y = C and T. See Palumbi et al. (1991b) for more information.

Primer	Sequence
Cytochrome oxidase I	
COI-L6569	CCTGCAGGAGGAGGAGAYCC
COI-H7110	CCAGAGATTAGAGGGAATCAGTG
Hypervariable D-loop	
CB3-L15560	CATATTAAACCCGAATGATATTT
12SA-H1067	ATAATAGGGTATCTAATCCTAGTTT
Ribosomal genes	
12SA-L1067	AAACTGGGATTAGATACCCCACTAT
16SA-H2492	ATGTTTTTGATAAACAGGCG

muscle were placed in homogenization buffer (see below) and stored at 4°C.

Extraction of DNA

Approximately 1 g of tissue was homogenized in 2–3 mL of buffer (250 mM sucrose, 50 mM EDTA, 50 mM Tris, pH 7.5) using a Teflon-glass homogenizer and 1 mL of the homogenate was placed into a 1.5-mL Eppendorf tube. The homogenate was centrifuged for 2 min at 1000*g* at room temperature. The supernatant was transferred to a new tube and centrifuged at 8000*g* for 3 min. The resulting pellet was resupended in 600 μL of fresh buffer and lysed by the addition of sodium dodecyl sulfate (an ionic detergent) to a concentration of 1%. The lysate was extracted once with phenol, once with phenol–chloroform (1:1), and once with chloroform – isoamyl alcohol (24:1). The nucleic acids were precipitated with ethanol and 2.5 M ammonium acetate (pH 7.5), vacuum dried, resuspended in 50 μL of 0.1 × TE, and stored at −20°C. When oocytes were present in individuals, a simpler and quicker method of DNA extraction was substituted. Like most species of teleosts, the oocytes of armorhead are small (<1 mm in diameter). Two to 10 oocytes were suspended in 50 μL of buffer (50 mM KCl, 10 mM Tris, pH 8.3, 1 mM EDTA) and the cells lysed by the addition of NP40 (a nonionic detergent) to a concentration of 1%. The sample was boiled at 95°C for 5 min and sterile distilled water added to a final volume of 100 μL.

Amplification of Mitochondrial Gene Regions

Specific gene regions of the mitochondrial genome were amplified using the PCR (Erlich 1989; Palumbi et al. 1991b). Double-stranded DNA was amplified in 50 μL reactions containing 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.01% Triton-X, 0.01% NP40, 1 μM each of oligonucleotide primer, 200 μM each of dNTP, 0.5 unit of Taq polymerase, and 1 μL of the DNA sample. Thermal cycling parameters were as follows: denaturation at 94°C for 40 s, annealing at 55°C for 25 s, and elongation at 72°C for 1–2 min. For the purpose of sequencing, single-stranded templates were generated by adding 1 μL of the double-stranded reaction to a 100-μL reaction containing only a single primer and all of the amplification reagents. The same conditions for single-stranded amplification were used as in double-stranded amplifications. Primers were synthesized on a Biosystems PCR Mate Oligonucleotide Synthesizer.

We routinely amplified three distinct gene regions of the mitochondrial genome of *P. wheeleri*: a 450-bp region of the cytochrome oxidase I gene, a 1425-bp region comprising most of the 12S rRNA gene and the 5′ half of the 16S rRNA gene, and an approximately 2000-bp fragment containing the entire D-loop including flanking gene regions on both sides. Primer sequences are given in Table 1.

DNA Sequencing

Single-stranded templates were purified by centrifugal ultrafiltration through Centricon 30 tubes. DNA sequencing of the cytochrome oxidase fragment was performed using the Sequenase system (USBiochemicals). Fragments were separated through 6% acrylamide, 7 M urea buffer – gradient gels (Palumbi et al. 1991b) and the radioactive bands from the sequencing reaction were visualized by exposure to X-ray film for 24–48 h (Fig. 2).

Endonuclease Restriction Analysis

The ribosomal genes and D-loop region were amplified as described and 12 μL of the amplified sample subjected to endonuclease digestion using the four-base recognition enzymes *Hae* III, *Mbo* I, and *Msp* I. Digestions were performed directly in the PCR buffer at 37°C for 4–6 h. The DNA fragments were separated in 1–1.5% agarose gels, stained with ethidium bromide, and the fragments made fluorescent by exposure to ultraviolet light and photographed (Fig. 3). The presence or absence of restriction sites was inferred from comparisons of fragment patterns (Palumbi et al. 1991a), and composite genotypes were assigned to each individual.

Genetic Relationship among Genotypes

The topology of relationships among genotypes was determined using the principle of parsimony (PAUP, Swofford 1989), treating the restriction site changes as Dollo character types. Restriction sites in the ribosomal and D-loop fragment were assigned weights of 5 and 1, respectively, reflecting the fact that ribosomal genes evolve considerably slower than the D-loop (Cann et al. 1987). Equal weights were also considered. We tested for differentiation among the seamount and open-ocean fish by standard contingency analysis and the G_{ST} test of Takahata and Palumbi (1985). Significance levels were determined using randomization tests (Roff and Bentzen 1989; Palumbi and Wilson 1990). In addition, average nucleotide diversity was estimated following Takahata and Palumbi (1985). The estimation of migration rate, *Nm*, was done using the method of Slatkin and Maddison (1989) based on the inferred topology of the relationships among distinct alleles.

Results

DNA Sequence Analysis

DNA sequences were determined for a 353-bp region of the cytochrome oxidase I gene for 10 individuals from each of the two seamounts (Fig. 2). Only a single C–T transition nucleotide substitution at a silent site (third "wobble" position of a codon) was found that distinguishes two mtDNA genotypes. This sequence variant was observed in four individuals: three from Koko and one from southeast Hancock Seamounts, respectively. None of the pelagic fish were sequenced. Based on all pairwise sequence comparisons for this gene region, the average (±1 SD) nucleotide diversity of 0.10 ± 0.14%. However, because most of the nucleotide positions (approximately two thirds) of the cytochrome oxidase I gene are invariable due to selective constraints on the protein, a more

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CTT GGC TAC ATA GGC ATA GTG TGA GCT ATA ATA GCA ATC GGC CTC CTA
T.. ..A ... ... ..T ..G ..C ... ... ..G ... T.. ..T ... T.. ...

GGG TTT ATT GTC TGG GCC CAC CAC ATG TTC ACA GTG GGA ATA GAC GTA
... ... ..C ..G ..A ..A ... ..T ..A ..T ... ..A ... ... ... ...

GAC ACC CGT GCC TAC TTC ACG TCC GCC ACT ATA ATT ATC GCA ATT CCC
... ..A ..A ..A ..T ... ..C ... ..T ..C ... ..C ... ..T ..C ...

ACC GGT GTT AAA GTT TTC AGC TGA CTC GCC ACC CTC CAT GGG GGA AAC
... ..C ..C ... ..A ..T ... ... ... ... ..A ... ..C ..A A.C ..T

ATC AAA TGA GAC ACA CCA CTT CTA TGA GCC CTA GGC TTT ATC TTC CTC
..G ... ... TCT G.T G.. G.G ..C ... ... ... ..A ..C ... ..T ..T

                                                    *
TTT ACA GTA GGG GGC TTA ACA GGG ATT ATC CTT GCC AAY TCT TCT TTA
..C ..C ... ..T ... C.G ..T ..C ... G.A T.A ..A ..C ..A ..A C..

GAC ATT GTG CTC CAT GAC ACA TAC TAT GTA GTA GCC CAC TTC CAC TAC
... ..C ..A ..A ..C ... ..G ... ..C ..T ... ... ... ... ... ..T

GTA CTA TCA ATA GGA GC
..C ... ... ... ... ..
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FIG. 2. A 353-bp DNA sequence for a region of the cytochrome oxidase I gene from armorhead (above) aligned with the homologous region from human (below). The only variable nucleotide position detected from analysis of 20 individuals is a C–T transition mutation represented as a Y in the armorhead sequence and marked by an asterisk. The human cytochrome oxidase I sequence begins at position 6705 in the published sequence (Anderson et al. 1981).

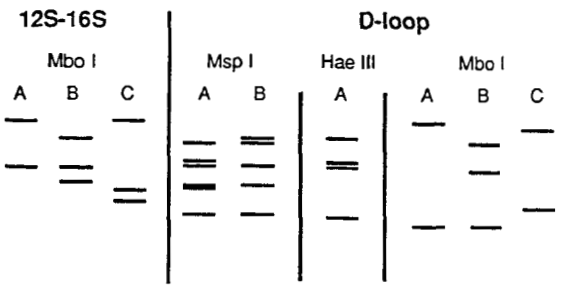

FIG. 3. Fragment pattern profiles for endonuclease digestions of the 12S–16S ribosomal gene regions and the D-loop.

informative estimate is the average percent sequence divergence at silent, third codon ("wobble") positions. Based on these data, this value is 0.30 ± 0.42%.

Endonuclease Restriction Site Analysis

A 1425-bp fragment containing large portions of the 12S and 16S rRNA genes as well as the lysine transfer RNA was amplified for all individuals. Although this region is conserved in all metazoans examined to date, there are short variable regions that are useful for describing intraspecific relatedness. Digestion of this fragment with *Mbo* I detected three different fragment patterns inferred to be the result of polymorphisms at two restriction sites (Fig. 3). Digestion profiles for *Hae* III and *Msp* I revealed four and one restriction sites, respectively, that were monomorphic for 17 of the individuals analyzed by sequencing and thus were not used for the remainder of the samples. (Three individuals that were sequenced were omitted from restriction site analysis because PCR amplification and subsequent endonuclease digestions yielded uninterpretable results.) For the 17 individuals surveyed with three 4-bp recognition enzymes, nucleotide diversity in the ribosomal fragment was estimated as 0.64%.

We also developed primers that routinely amplify an approximately 2000-bp fragment containing the entire D-loop and adjacent flanking regions. We focused on this region because most intraspecific mtDNA nucleotide variation occurs in this region (Cann et al. 1987). The hypervariability of the D-loop is thought to be due in part to frequent length mutations and lack of constraint on sequence change (Saccone et al. 1987).

TABLE 2. Comparison of estimates of nucleotide diversity based on direct DNA sequence analysis and indirect restriction site analysis. *D* is the average percent nucleotide diversity. Length represents the approximate amount of the mtDNA genome (in base pairs) represented by the gene region. *N* is the average number of nucleotide differences between two individuals estimated as ($D \times$ length)/100. S = sequencing and R = restriction site analysis.

Gene region	*D*	Length	*N*	Method
Protein coding	0.10	12 000	12	S
Ribosomal	0.64	2 700	17.3	R
D-loop	1.87	1 000	18.7	R
Total		≈16 000	48	

Initial screening of this region with *Hae* III, *Mbo* I, and *Msp* I for the same 17 individuals analyzed for ribosomal gene variation and cytochrome oxidase I sequences showed an average nucleotide diversity for this region of 1.87%, greater than 10 times the amount of variation detected by sequencing a small portion of the cytochrome oxidase I gene (Table 2).

Population Differences

For both ribosomal and D-loop regions, an additional 67 individuals were surveyed using restriction digestion analysis. A total of 10 distinct mtDNA types were detected. PAUP analysis yielded two most parsimonious trees depicting the relationships among the mitochondrial haplotypes. We used the strict consensus option in PAUP to construct a topology which summarizes the information contained in the two most parsimonious trees (Fig. 4).

The most common type (designated A) was identified in over 64% of the fish surveyed. The frequency of occurence of the next most common type was only 9.2%. The remaining types were rare, with frequencies of occurrence of 5% or less. Based on the restriction site data for all individuals combined, the overall average percent sequence diversity in the ribosomal and D-loop regions was 1.2%.

No significant differences in allele frequency were detected among the three populations surveyed (chi-square = 14.4, df = 16, $p > 0.5$). G_{ST} analysis revealed that 6.7% of the genetic variation is distributed among groups. Bootstrap analysis indicates that this level of partitioning of genetic variation is not significantly different from random ($p > 0.25$). Using the approach of Slatkin and Maddison (1989), we estimated that, on average, greater than 10 mitochondrial haplotypes have been exchanged among the three populations (Koko, southeast Hancock, and pelagic phase) per generation ($Nm > 10$). (This result also holds for the topology generated from unweighted data.) Theory suggests that for Nm values greater than unity, there is sufficient gene flow among populations to inhibit genetic differentiation by drift. Thus, the genetic data do not provide evidence for significant population structure of armorhead in the North Pacific.

The individual fish sampled from the two seamounts were identified as being either lean, intermediate, or fat types according to the ratio of fork length to body depth categories defined by Humphreys et al. (1989). A contingency test showed no significant genetic differentiation among the three morphological classes (chi-square = 18.3, df = 20, $p = 0.57$).

Discussion

Variability of mtDNA in Armorhead

The cytochrome oxidase I sequence data indicated a low level of intraspecific nucleotide variability in this species. Only a single variable position was observed in 7020-bp surveyed (20 individuals × 351-bp). To our knowledge, this is the lowest level of mtDNA nucleotide variability documented for a vertebrate species using these techniques. By contrast, there is appreciable levels of nuclear gene diversity (average heterozygosity = 0.077 (Humphreys et al. 1989)). If the low level of mtDNA diversity is a real phenomenon, then this discrepancy in estimates of genetic variation may indicate that populations of these fish underwent a transient bottleneck event, perhaps relatively recently (Wilson et al. 1985).

It is important to point out, however, that at this time we are unable to directly compare the estimate of nucleotide diversity in armorhead with other taxa because we sampled only a small section of a single gene whereas most other studies of mtDNA variability have employed RFLP analysis of whole mtDNAs (e.g. Graves et al. 1984; Avise et al. 1987). Although we surveyed 117 silent sites (the third "wobble" positions of codons), this amount of sequence is insufficient for reliable estimation of genetic divergence between mtDNAs (Martin

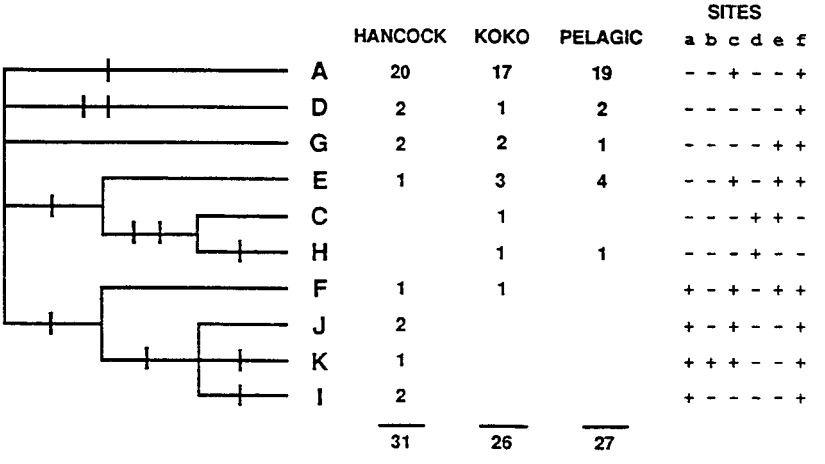

FIG. 4. Inferred topology of the relationships among the 10 mtDNA types identified by endonuclease restriction analysis. Individual tick marks represent site gains or losses. Sites are presented as gains (+) or losses (−) of restriction sites based on the fragment patterns in Fig. 3. Numbers of individuals possessing a particular mtDNA types are given. Site designations are as follows (see Fig. 3 for reference): a, 12S–16S *Mbo* I site gain from A to B; b, 12S–16S *Mbo* I site gain from A to C; c, D-loop *Msp* I site loss from A to B; d, D-loop *Mbo* I site gain from A to C; e, D-loop *Mbo* I site loss from B to A; f, D-loop *Mbo* I site loss from A to C. Consistency index, excluding uninformative sites, is 0.70.

et al. 1990). Thus, the low level of mtDNA sequence variability detected may be a real phenomenon or reflect the limited number of base pairs sampled; therefore, the transient bottleneck hypothesis is highly speculative.

Population Structure

The mtDNA data indicated lack of differentiation between fish collected at different seamounts and from the open ocean. These data provide support for the hypothesized life history and suggest that individuals born at the southernmost seamounts, for example, are equally as likely to reproduce at northern seamounts as at southern seamounts in the next generation. However, we detected few genetic markers that afforded limited resolution of within-species geneology making it possible that further analysis of armorhead may reveal significant genetic differentiation among fish collected near different seamounts.

One or Two Species?

Based on analysis of the cytochrome oxidase I sequence data and restriction digestion profiles, there is no evidence for genetic differentiation between the three morphotypes of armorhead, identified as lean, intermediate, and fat using the ratio of body depth to mean fork length (Humphreys et al. 1989). In addition to analyses of morphology and allozyme variation, the lack of mtDNA differentiation between morphotypes provides convincing evidence for the existence of only a single species of North Pacific armorhead.

D-loop Amplification for Rapid Genetic Analysis of Fish Populations

DNA sequence analysis provides high-resolution data for population genetic studies because individual nucleotide substitutions can be identified. Unfortunately, DNA sequencing is more expensive and time consuming than indirect but lower resolution methods like restriction site analysis. We find that restriction site analysis of amplified D-loop fragments allowed detection of more nucleotide variation than direct sequence analysis of a 353-bp region of the cytochrome oxidase I gene (Table 2). Although DNA sequence data of D-loops provide superior resolution of genetic relationships among individuals (Meyer et al. 1990; di Rienzo and Wilson 1991), it is possible to distinguish mtDNA types using our technique of PCR amplification of the hypervariable D-loop and endonuclease restriction analysis. This method is rapid, does not require freezing tissue samples to $-70°C$, and does not involve radioactivity for visualization of DNA, enabling analysis of large numbers of individuals for only a fraction of what sequencing would cost. In addition, variant genotypes identified by restriction digestion can be sequenced directly from the amplification reaction (Palumbi et al. 1991b) in order to ascertain exact sequence divergence.

Acknowledgements

This work was supported by National Science Foundation grants to S.R.P. and by the National Marine Fisheries Service, NOAA. We thank W. O. MacMillan and J. Brown for assistance in the laboratory.

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