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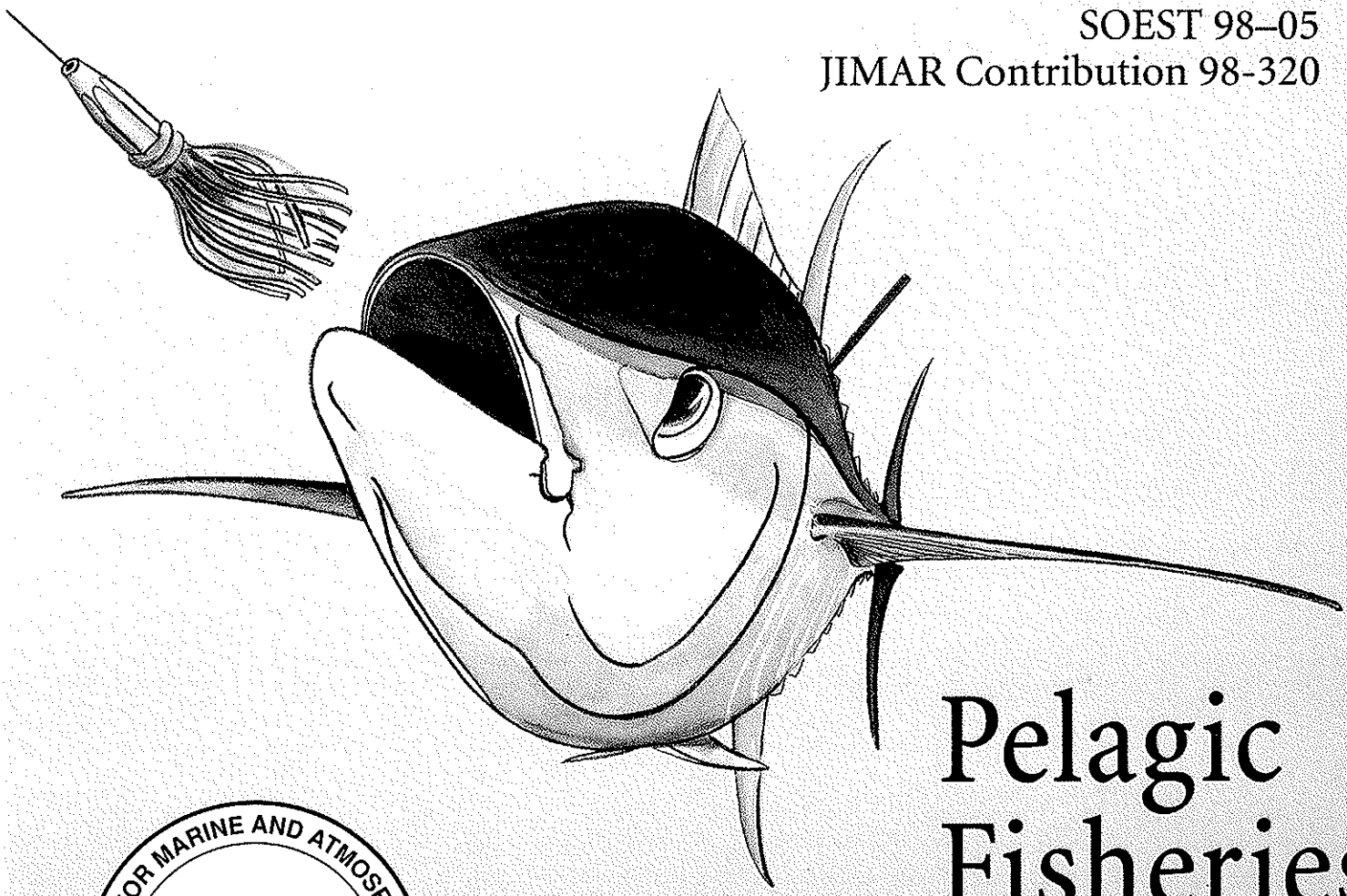

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Pelagic Fisheries Research Program

An Assessment of Bigeye (*Thunnus obesus*) Population Structure in the Pacific Ocean, Based on Mitochondrial DNA and DNA Microsatellite Analysis

Peter M. Grewe
CSIRO Marine Research
GPO Box 1538
Hobart, Tasmania, Australia
email: peter.grewe@marine.csiro.au

John Hampton
Secretariat of the Pacific Community
B.P. D5, Noumea Cedex
New Caledonia

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Contents

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Abstract

Nine collections of bigeye tuna from different regions of the Pacific Ocean, with individual sample sizes ranging from 69 to 105 specimens, were examined for genetic variation in mitochondrial DNA (mtDNA) and microsatellite loci. Eight microsatellite loci were examined in approximately 70 fish from the two geographically most separated collections—Ecuador and the Philippines; none of these loci showed evidence of significant allele frequency differentiation after Bonferroni correction. Four microsatellite loci were examined in 664-806 fish from the nine collections; again, none showed evidence of significant allele frequency differentiation after Bonferroni correction. The two geographically most separated collections—Ecuador and the Philippines—showed some evidence of differentiation for one microsatellite locus following Bonferroni correction. The mtDNA data showed some indications of significant differentiation among the collections, but this became nonsignificant if the mtDNA analyses are considered as one of the several genetic tests of differentiation. It is concluded that there is some evidence for restricted gene-flow between Ecuador and the Philippines, but that otherwise the data fail to allow the null hypothesis of a single panmictic Pacific-wide population of bigeye tuna to be rejected. Tagging data generally supports this conclusion. In order to further resolve the issue of bigeye population structure in the Pacific Ocean, larger sample sizes will be needed than those examined here.

1 Introduction

Knowledge regarding population subdivision is central to sustainable fishery management. Uncertainty regarding bigeye tuna stock structure seriously restricts the confidence that scientists and fisheries managers can place in the regional assessments that have been carried out to date. At a national or subregional level, fisheries managers need to have a better idea of the broader surrounding population of bigeye from which the fish in their fisheries are drawn.

Examination of mitochondrial DNA (mtDNA) markers is now an established technique for elucidating population genetic structure. While there is little differentiation among yellowfin tuna populations for mtDNA variants (Scoles and Graves, 1993; Ward *et al.*, 1994; Ward *et al.*, 1997), there are specific mtDNA polymorphisms that are known to differentiate bigeye tuna from the Atlantic and Indian Oceans (Alvarado-Bremer and Ely, unpublished data[1]). MtDNA variation can be used for both population structure analysis and confirmation of species identification. Usually around 1-5% (but sometimes as high as 30%) of tuna samples we receive have been misidentified by collectors, but all can be unequivocally identified by mtDNA examination (Chow and Inoue, 1993).

Until recently, variation in the much larger nuclear genome has been chiefly assessed through allozyme electrophoresis, but more powerful methods are now available. Pre-eminent among these is the detection and analysis of microsatellite variation. Microsatellites show high levels of genetic variation and high mutation rates, meaning that populations are likely to diverge not only by genetic drift but by mutation as well. Microsatellite markers also have the important advantage over allozyme markers in being able to be screened in alcohol-stored tissue or even fin-clip samples. This greatly simplifies sampling logistics.

Microsatellite analysis is a new technique, and while there have as yet been few studies on fish populations, microsatellite screening in cod has revealed substantially more about population structure than either allozyme or mtDNA analysis (Wright and Bentzen, 1994; O'Connell and Wright, 1997).

Initial results from examination of DNA microsatellites in yellowfin tuna indicate more population subdivision is present in the western Pacific than is apparent from either allozyme or mtDNA analysis (Grewe and Ward, unpublished data.). DNA microsatellite data were examined among yellowfin tuna samples from five locations in the Pacific Ocean (Philippines, Coral Sea, Solomon Islands, Fiji, and California). Analysis of data from four DNA microsatellite loci indicated significant heterogeneity, on one locus between samples collected in the western and eastern Pacific, and on a second locus between samples collected in the Philippines and Solomon Islands and those collected in Fiji and the Coral Sea. Although preliminary, these data are the first indications of genetic structure within what has been assumed previously to be a single Western Pacific yellowfin tuna stock. The data collected thus far suggest a finer-scale resolution of yellowfin tuna population structure within the Pacific Ocean than has hitherto been achieved with allozyme and mtDNA markers. Further sampling is being undertaken to confirm (or refute) these early impressions.

These same microsatellite polymorphisms from yellowfin tuna can be examined in bigeye tuna, substantially reducing the time and cost of the development phase associated with microsatellite analysis.

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[1] FISHTEC Genetics Lab, Dept. Bio. Sci., Univ. South Carolina, Columbia, SC 29208

The present study examines variation of mitochondrial DNA and DNA microsatellite markers among bigeye tuna sampled from various regions throughout the Pacific Ocean. Comparison of these marker frequencies among sampling sites provides an assessment of bigeye population structure in the Pacific Ocean.

2 Methods

2.1 Sampling Logistics

Samples of bigeye tuna were collected from nine locations within the Pacific Ocean (Figure 1). Between 69 to 105 fish were examined from each location (Table 1). All samples were collected between August and December 1995. Approximately 0.5 grams of tissue sampled from individual fish was preserved in ethanol and transported to the CSIRO Marine labs for analysis. DNA from these tissues was extracted using a modified CTAB extraction protocol described in Ward *et. al* (1994).

Figure 1. Approximate capture locations (circles) of bigeye comprising the nine samples analysed for mtDNA and DNA microsatellite allelic variation. The sample sizes (n) and approximate size range of the sampled fish are also given.

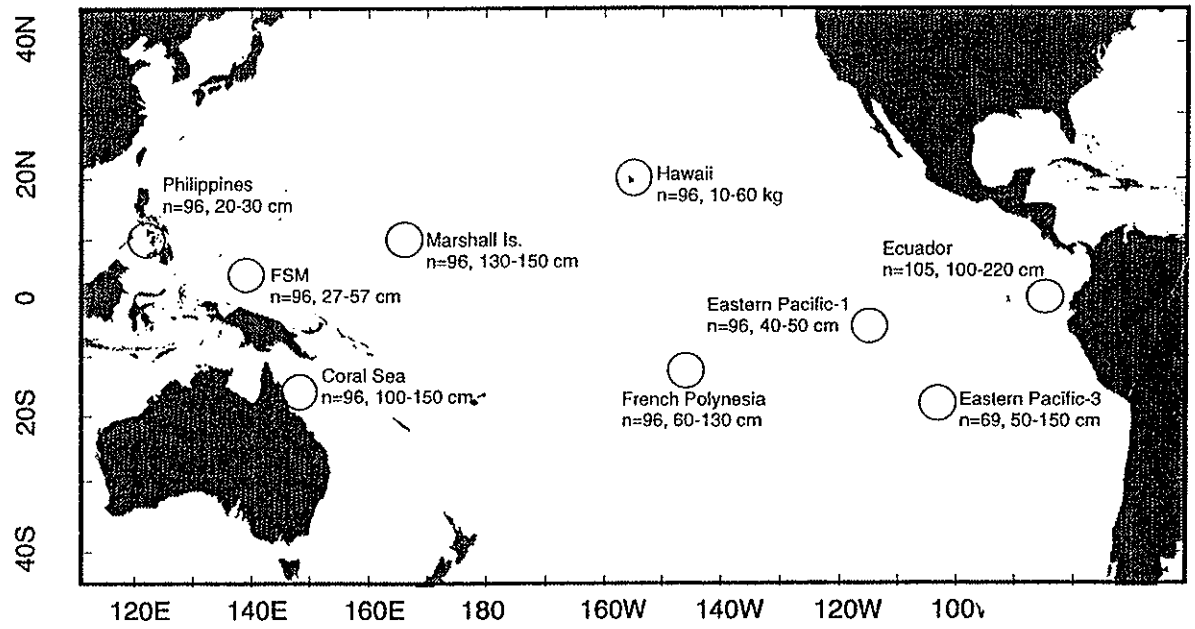


Table 1. Location and approximate number of individuals (n) of fish collected from each of the sample sites examined by the current study. Approximate longitude and latitude are given for each location. Size range of fish is given as either fork length in centimeters or as weight in kilograms. FSM = Federated States of Micronesia. All samples were collected between August and December 1995.

Location	n	Lat. ; Long.	Size
Philippines	96	10°N ; 122°E	20-30 cm
FSM	96	3-5°N ; 137-141°E	27-57 cm
Coral Sea	96	16°S ; 147°E	100-150 cm
Marshall Islands	96	10°N ; 166°E	130-150 cm
Hawaii	96	20°N ; 155°W	10-60 kg
East Pacific-1	96	5°S ; 115°W	40-50 cm
East Pacific-3	69	(see Fig.1)	50-150 cm
French Polynesia	96	6-21°S ; 142-150°W	60-130 cm
Ecuador	105	0° ; 85W	100-220 cm

2.2 DNA Analysis

Genetic analysis involved the assessment of mitochondrial DNA and nuclear DNA microsatellite variation. MtDNA variation was examined through restriction digestion of two selected fragments of the mitochondrial DNA genome, ATCO and DL19-12s. The ATCO fragment contains the flanking region between ATPase-6 and cytochrome oxidase subunit III genes and was amplified via the polymerase chain reaction (PCR) using primers described by Chow and Inoue (1993). The restriction enzyme *Mse*I was used to produce diagnostic patterns to confirm species identity. The more variable DL19-12s fragment contains the control region or d-loop region of the mitochondrial genome and is flanked by the transfer RNA proline gene and the 12sRNA gene. This fragment, digested with *Hin*fI (double digested with *Bgl*I) and *Taq*I restriction enzymes, was used to examine variation within and among bigeye samples.

DNA microsatellite loci examined in this study were previously isolated from yellowfin tuna (Grewe, unpublished data). Locus designations were derived from the clonal isolate number from which each locus was sequenced. Primers used to assay fish in the current study were synthesized for ten microsatellite loci. One primer from each locus pair was end-labeled with the fluorescent dye 6-FAM. PCR amplifications were carried out using standard conditions in a PE-Applied Biosystems 9600 thermocycler. Microsatellite fragment products for each locus were separated on a PE-Applied Biosystems ABI-377 automated DNA sequencer and sized using GENESCAN collection software. Genotyping was then completed using ABI GENOTYPER software.

2.3 Statistical Analysis

Variation in microsatellite and mtDNA allele frequencies among sub-populations was assessed using standard Monte Carlo chi-square approaches as suggested by Roff and Bentzen (1989). Such methods obviate the need to pool rare alleles. The program CHIRXC described by Zaykin and Pudovkin (1993) was used to test the probability that sampled areas are part of the same gene pool. Five thousand randomizations of the data were used to estimate P values. Significant differences in frequencies would indicate that collection localities represent areas that contain genetically distinct groups. The extent of such differentiation, if found and if the populations are assumed to be at genetic equilibrium, allows an estimate to be made of the number of genetically-effective migrants exchanged between such groups.

Tests for agreement of genotype numbers to Hardy-Weinberg proportions again used Monte Carlo procedures to avoid having to pool rare alleles. The program CHIHW described by Zaykin and Pudovkin (1993) was used. This not only tests goodness-of-fit to Hardy-Weinberg expectations but also computes an index of heterozygote deficiency or excess D, where $D = [H_O - H_e] / H_e$, where H_O = observed heterozygosity and H_e = Hardy-Weinberg expected heterozygosity. Whether or not D was significantly different from zero was also again tested using 5000 randomizations of the data and Monte Carlo procedures.

Bonferroni adjustments of significance levels, to correct for multiple tests, were carried out by dividing the original significance level, 0.05, by the number of tests in order to derive a corrected significance level. More conservative adjustments, the sequential procedure advocated by Hochberg (1988), produced the same conclusions in all cases. For these adjustments, tests are ordered according to their probability value. The highest probability value, P_m, is compared with the significance value a. Here we initially set a = 0.05. If $P_m > a$, that test is judged to be nonsignificant, and comparisons continue with subsequent probabilities, each compared with a modified significance level = $a/(1+i)$, where i is the number of tests already performed. When a test is significant, it and all subsequent tests are deemed significant.

3 Results

3.1 Mitochondrial DNA Analysis

Examination of the ATCO fragment revealed three restriction patterns. One of these patterns was diagnostic for yellowfin tuna while the others were diagnostic for bigeye. One of the two bigeye patterns was rare and present in only two of the 96 individuals from Hawaii and one of 96 Philippine fish.

One fish from the Philippines (1.1%), two of the Coral Sea (2.1%), and 10 of the East Pacific-1 (10.4%) individuals were identified as yellowfin tuna. Fish which had the mtDNA pattern diagnostic for yellowfin tuna also contained DNA microsatellite genotypes which were common (frequency > 0.50) in yellowfin but rare in bigeye (frequency < 0.002). For example, individuals that were homozygous for the 96bp allele at locus 208a were always confirmed as yellowfin using the mtDNA test. The 208a*96 allele is rare in bigeye (frequency of 0.01 to 0.06) but is very common in yellowfin (frequency of 0.62 to 0.69). The expected frequency of homozygotes for this allele (estimated from the square of the allele frequency) in bigeye is low (frequency = 0.0001 to 0.0036), but is much higher in yellowfin (expected frequency = 0.38 to 0.39). Thus, the presence of individuals homozygous for this allele at locus 208a quickly indicates a possible contamination of yellowfin in the sample and these can of course be confirmed using the completely diagnostic mtDNA test. All misidentified fish were eliminated from subsequent analyses.

Primers designed to amplify the DL19-12s fragment produced a 1.4kb fragment. Digestion of this fragment with two enzymes revealed 33 bigeye tuna haplotypes (Table 2) The mtDNA assemblages of each sample were not dominated by a single haplotype. Three of the haplotypes were present at moderate frequencies with observed values ranging from 0.12 to 0.32. Three other haplotypes had lesser frequencies which ranged from 0.02 to 0.135. The remaining 27 haplotypes were present at frequencies less than 0.02. Analysis of overall diversity using CHIRXC gave a probability of homogeneity of haplotype frequencies among all populations, with a total sample size of 791 bigeye, of $P = 0.046$. Comparing the two most separated collections—Philippines and Ecuador, gave a P value of 0.070.

3.2 DNA Microsatellite Analysis

The 10 microsatellite loci used were a mixture of perfect (CA) and imperfect or mixed repeat motifs. With the exception of locus 135a, an imperfect trinucleotide repeat, between four and 35 alleles were resolved per locus (Table 3). Locus 135a was monomorphic with an equal-sized fragment of 207 base pairs amplified from both bigeye and yellowfin tunas. Subsequent comparison of this fragment using DNA sequencing revealed identical sequences for both yellowfin and bigeye. Four to 35 alleles were observed at the remaining loci. Identification of allelic state for locus 135b was unreliable with alleles separated by single base pair units. This locus was therefore dropped from further comparison. Future analyses using this locus will require new primers to be designed to eliminate ambiguous allele calls.

The remaining eight loci (102, 113, 117, 121, 125a, 144, 161 and 208a) were used to examine a subsample of 36 fish from each of two sites (Philippines and Ecuador) that represented the extremities of the sampling area of the study. With few exceptions, and then only for rare alleles, all alleles at each locus were found in both populations (Table 4). Only one locus, 208a, showed a probability of homogeneity value of less than 5% (Table 5). The P value for this locus was 0.034, but this becomes statistically nonsignificant after correcting for multiple tests.

Table 2. Mitochondrial DNA composite haplotype (c.h.) frequencies of bigeye tuna, based on examination of the DL19-12s fragment using *HinfI* and *TaqI*.

c.h.	Philipp. (n=85)	FSM (n=89)	Marshall (n=88)	Coral Sea (n=93)	Hawaii (n=93)	F. Poly (n=96)	E.Pac-1 (n=84)	E.Pac 3 (n=62)	Ecuador (n=101)
AS	0.259	0.258	0.159	0.290	0.312	0.281	0.250	0.290	0.228
AP	0.200	0.225	0.341	0.323	0.194	0.260	0.262	0.194	0.287
BP	0.188	0.124	0.182	0.151	0.215	0.219	0.167	0.226	0.139
BR	0.082	0.079	0.023	0.043	0.054	0.042	0.071	0.032	0.099
AR	0.071	0.056	0.068	0.032	0.075	0.042	0.060	0.097	0.020
AQ	0.059	0.135	0.091	0.043	0.097	0.063	0.107	0.065	0.079
DP	0.047	0.011	-	-	-	-	-	-	-
ES	0.024	-	-	-	-	-	-	0.016	-
FQ	0.024	-	-	-	-	-	0.012	-	0.010
AT	0.012	-	-	-	-	-	-	-	-
BQ	0.012	0.011	-	0.011	-	-	0.012	-	0.010
BY	0.012	-	-	-	-	-	-	-	-
BZ	0.012	0.011	0.011	0.011	-	-	0.012	-	0.020
AA_1	-	-	-	0.011	0.011	0.010	-	-	-
AC_1	-	-	-	0.011	-	-	-	-	-
AD_1	-	-	-	0.011	-	0.021	-	-	-
AE_1	-	-	-	-	-	-	-	-	0.030
AY	-	0.011	-	-	-	-	-	-	-
AZ	-	0.011	-	0.011	-	-	-	-	-
BA_1	-	-	-	0.011	-	-	-	-	-
BD_1	-	-	-	-	-	0.010	-	-	-
BS	-	0.045	0.080	0.022	0.022	0.031	0.024	0.016	0.030
CP	-	0.011	-	-	-	-	-	0.016	0.010
CW	-	-	-	0.011	-	-	-	-	-
DR	-	-	0.011	-	-	-	-	-	-
DS	-	-	-	-	-	0.010	-	-	-
EA_1	-	-	-	-	-	-	0.012	-	-
EP	-	0.011	0.034	-	0.011	-	-	0.016	0.020
EW	-	-	-	-	-	-	-	0.016	0.010
FS	-	-	-	-	-	-	0.012	-	-
GP	-	-	-	-	-	-	-	-	0.010
HP	-	-	-	-	0.011	0.010	-	0.016	-
IS	-	-	-	0.011	-	-	-	-	-

Five of these loci (102, 113, 117, 121 and 161) had no alleles with frequencies greater than 0.07, and more than half the alleles at these loci had frequencies less than 0.07 (Table 4). Such loci, where there are many alleles but none is abundant, are of limited use in population genetic analyses. Sample sizes would have to be much larger than those planned for the present project to detect small amounts of genetic differentiation, if such existed. For these reasons, four of these loci were dropped from subsequent analyses and examinations focussed on the remaining three loci plus locus 161. Locus 161 was retained because contemporary studies of this locus in yellowfin tuna had shown evidence of stock separation (Grewe, unpublished[2]).

[2] Origin of recruits is the east coast yellowfin tuna fishery of Australia, P. Grewe, CSIRO Division of Marine Research, Hobart, Tasmania 7001.

Table 3. Variation in numbers of alleles observed at ten DNA microsatellite loci examined among 400 yellowfin (Yft) (sampled from Philippines, Coral Sea, Hawaii, and California) and 72 bigeye (Bet) (sampled from Philippines and Ecuador) tuna. The motif listed is that obtained from the original yellowfin sequence used to generate amplification primers. Dashes indicate loci that were not examined for yellowfin tuna.

Locus	Motif	Yft (n=400)	Bet (n=72)
102	$(GA)_2(CA)_{32}$	-	24
113	$(CA)_{12}$	25	35
117	$(CA)_{12}$	17	27
121	$(CA)_4(TA)(CA)_7$	-	21
125a	$(CA)_{10}$	-	4
144	$(CA)_6$	-	5
135a	$(CCA)(CCG)(CCA)_4$	1	1
135b	$(CA)_{10}(TA)(CA)_9$	22	27
161	$(CA)_{19}$	29	24
208a	$(CA)_{10}$	8	25

Thus four loci (125a, 144, 161 and 208a) were used to examine larger numbers of fish and from all additional sample locations (Table 6). Loci 125a, 144 and 208a had one or more alleles with frequencies greater than 0.20, making them more suitable for population genetic analysis than loci without common alleles.

Genotype proportions in each collection and for each locus were tested for goodness-of-fit to Hardy-Weinberg expectations (Table 7) using the estimated allele frequencies for each collection (Table 6). Only two of the 35 tests of individual collections and loci showed probability values less than 5% (locus 125a, E.Pac-3, $P = 0.026$; locus 144, Marshall Islands, $P = 0.042$), and neither of these approached significance after Bonferroni adjustment for multiple tests. Only one of 35 tests of D, which compares observed and Hardy-Weinberg expected heterozygosities, had a probability value less than 0.05 (locus 144, Marshall Islands, $P = 0.004$); again, this lost statistical significance after Bonferroni adjustment for multiple tests.

Collections were then pooled for each locus to see if the pooled or total sample showed any evidence of Hardy-Weinberg deviations (Table 7). Such deviations would be expected, in the form of heterozygote deficiencies, if there were differences in allele frequencies among collections. Two of the four tests produced a probability value just less than 0.05 (locus 125a, $P = 0.040$; locus 144, $P = 0.042$), but again these were not considered statistically significant considering that four tests were performed. Furthermore, the possible deviation for locus 125a reflects the very unlikely presence of a homozygote for a rare allele in the E.Pac-3 sample. This fish also had a unusual genotype for locus 208a. Unfortunately the mtDNA diagnostic test for this fish failed, but it is more likely that it is a yellowfin tuna rather than a bigeye tuna. Excluding it renders the pooled Hardy-Weinberg probability value for this locus nonsignificant. None of the four tests of D approached significance.

These tests of Hardy-Weinberg proportions show that observed genotype numbers within collections and within the pooled sample agree very closely with numbers expected of a randomly mating population. Further, there is no evidence of heterozygote deficiencies within collections which might reflect the presence of nonamplifying or 'null' alleles, which have been postulated in some fish populations (O'Connell and Wright, 1997; Rico et al., 1997).

There was no evidence for spatial heterogeneity of allele frequencies for three of these loci: 125a, 144 and 161 (Table 8a). Locus 208a showed weak evidence of spatial heterogeneity ($P = 0.038$), but this again becomes nonsignificant after correcting for multiple tests.

Table 4. Allele frequencies observed at eight DNA microsatellite loci in approximately 36 fish from each of the Philippines and Ecuador collections.

Locus 102		
Allele	Ecuador (n=35)	Philippines (n=34)
134	0.01	-
138	0.13	0.07
142	0.01	0.01
144	0.11	0.15
146	0.04	0.03
148	0.16	0.07
150	0.06	0.06
152	0.01	0.04
154	0.04	0.07
156	0.09	0.09
158	0.11	0.04
160	0.06	0.06
162	0.01	0.01
164	-	0.04
166	-	0.04
168	0.04	0.06
170	0.01	0.04
172	0.01	0.01
174	0.01	-
176	0.03	0.01
182	0.01	0.03
186	-	0.01
192	-	0.01
202	0.01	-

Locus 113		
Allele	Ecuador (n=34)	Philippines (n=36)
105	-	0.01
113	-	0.01
115	-	0.01
117	0.03	0.04
119	0.04	0.07
121	0.01	0.03
123	0.07	-
125	0.04	0.06
127	0.03	0.03
129	0.04	0.08
131	0.07	0.04
133	0.06	0.03
135	0.07	0.04
137	0.01	0.03
141	-	0.06
143	0.07	0.06
145	0.06	0.10
147	0.06	0.03
149	0.03	0.01
151	0.01	0.03
153	0.04	0.03
154	-	0.01
155	0.07	0.03
157	-	0.06
159	0.04	0.03
161	0.01	0.01
163	-	0.01
165	0.01	-
167	-	0.01
169	0.03	-
171	-	0.01
173	0.03	-
177	-	0.01
179	0.01	-
183	-	0.01

Table 4. (continued)

Locus 117		
Allele	Ecuador (n=34)	Philippines (n=36)
164	0.01	0.03
174	0.03	-
176	0.01	0.01
178	0.07	0.01
180	0.07	0.10
182	0.12	0.08
184	0.13	0.11
186	0.03	0.04
188	0.01	-
190	-	0.01
192	0.03	0.01
194	-	0.06
196	0.06	0.06
198	0.06	0.13
200	0.06	0.06
202	0.04	0.04
204	0.06	0.04
206	0.01	0.03
208	0.04	0.03
210	0.01	0.03
213	0.03	0.03
215	0.03	0.03
217	0.04	-
219	0.01	-
221	-	0.01
225	-	0.03
229	-	0.03

Locus 121		
Allele	Ecuador (n=35)	Philippines (n=36)
248	0.01	-
264	0.01	0.01
266	0.04	0.04
268	0.13	0.07
270	0.01	-
272	0.06	0.06
274	0.16	0.11
276	0.03	0.11
278	0.09	0.08
280	0.09	0.14
282	0.13	0.07
284	0.04	0.11
286	-	0.03
288	0.10	0.08
290	0.01	0.01
292	0.04	0.01
294	0.01	-
296	0.01	0.03
298	-	0.01
300	0.01	-
304	-	0.01

Table 4. (continued)

Locus 125a

Allele	Ecuador (n=35)	Philippines (n=34)
156	-	0.015
158	0.871	0.868
160	0.129	0.103
164	-	0.015

Locus 144

Allele	Ecuador (n=35)	Philippines (n=34)
164	0.028	0.014
168	0.139	0.194
170	0.014	0.000
172	0.583	0.653
174	0.236	0.139

Locus 208a

Allele	Ecuador (n=34)	Philippines (n=34)
94	0.000	0.014
96	0.014	0.014
98	0.000	0.014
100	0.014	0.000
102	0.389	0.111
104	0.222	0.194
106	0.167	0.319
108	0.042	0.083
110	0.014	0.014
113	0.000	0.014
115	0.014	0.014
119	0.028	0.083
123	0.014	0.000
125	0.028	0.028
127	0.028	0.014
129	0.014	0.014
131	0.000	0.014
133	0.014	0.042
148	0.000	0.014

Locus 161

Allele	Ecuador (n=34)	Philippines (n=35)
172	0.000	0.014
180	0.029	0.014
182	0.029	0.000
184	0.044	0.057
186	0.000	0.029
188	0.044	0.043
190	0.074	0.057
192	0.000	0.043
194	0.044	0.057
196	0.088	0.057
198	0.015	0.071
200	0.103	0.114
202	0.015	0.043
204	0.015	0.014
206	0.044	0.071
208	0.103	0.043
210	0.044	0.071
212	0.074	0.043
214	0.015	0.029
216	0.059	0.043
218	0.029	0.043
220	0.118	0.014
222	0.015	0.014
226	0.000	0.014

Despite this lack of support for any overall spatial structuring of the bigeye Pacific populations, it was thought possible that if there were small genetic differences between the samples from the geographically most separated regions, the Philippines and Ecuador, then these might be masked in the overall tests of homogeneity by intermediate gene frequencies in the intermediate localities. Hence a final test for spatial structuring involved comparing these two collections for the four loci 125a, 144, 161 and 208a (Table 8b). This did show evidence of differentiation at two loci, 144 and 161, although only locus 161 remained significant after

Bonferroni correction for four tests (P=0.010, compared to the critical value a=0.0125). While these results need to be confirmed with larger sample sizes, it is interesting that neither of these loci approached significance in the earlier tests based on about 70 fish (Table 5). This supports the thesis that increasing the sample size still further in any subsequent investigation may well reveal differences that are not evident in the current sample sizes.

Table 5. Chi-square analysis of differentiation among the eight DNA microsatellite loci in approximately 36 fish from each of the Philippines and Ecuador collections. Note that with eight tests, the P value for significance decreases from 0.05 to 0.05/8 = 0.0063

Locus	No. of Fish	Total alleles Observed	c^2	P
102	69	24	20.37	0.681
113	70	35	36.04	0.362
117	70	27	24.05	0.622
121	71	21	19.02	0.557
125a	69	4	2.25	0.682
144	69	5	4.10	0.420
161	69	24	23.06	0.486
208a	72	19	26.03	0.034

Table 6. Allele frequencies observed at four DNA microsatellite loci in fish from each of the nine Pacific Ocean collections.

Locus 125a

Allele	Philipp. (n=92)	FSM (n=93)	Marshall (n=73)	Coral Sea (n=95)	Hawaii (n=96)	F. Poly (n=96)	E.Pac-1 (n=84)	E.Pac 3 (n=64)	Ecuador (n=103)
149	-	-	0.007	-	-	-	-	-	-
151	-	-	0.007	0.005	0.005	-	-	0.016	-
153	-	0.005	-	-	-	-	-	-	-
156	-	-	0.007	-	0.005	-	-	-	-
158	0.902	0.876	0.870	0.868	0.875	0.904	0.946	0.898	0.893
160	0.082	0.108	0.110	0.111	0.104	0.091	0.048	0.070	0.087
162	0.011	-	-	0.016	-	0.005	-	0.008	0.010
164	0.005	0.011	-	-	0.010	-	0.006	0.008	0.010

Locus 144

Allele	Philipp. (n=56)	FSM (n=92)	Marshall (n=80)	Coral Sea (n=93)	Hawaii (n=96)	F. Poly (n=96)	E.Pac-1 (n=84)	E.Pac 3 (n=64)	Ecuador (n=103)
162	-	-	-	-	0.005	0.005	0.006	0.008	-
164	-	0.005	-	-	-	0.010	-	-	-
166	0.005	-	-	-	-	-	-	-	0.010
168	0.165	0.130	0.188	0.108	0.125	0.128	0.167	0.125	0.138
170	0.005	0.005	0.019	0.022	0.010	0.020	0.018	0.023	0.029
172	0.628	0.636	0.563	0.624	0.583	0.566	0.536	0.523	0.514
174	0.197	0.223	0.225	0.242	0.276	0.265	0.274	0.320	0.310
176	-	-	0.006	0.005	-	0.005	-	-	-

Table 6. (continued)

Locus 161

Allele	Philipp. (n=56)	FSM (n=92)	Marshall (n=80)	Coral Sea (n=93)	Hawaii (n=96)	F. Poly (n=96)	E.Pac-1 (n=84)	E.Pac 3 (n=64)	Ecuador (n=103)
172	-	0.005	-	-	0.005	0.005	-	-	-
176	-	-	0.006	-	-	-	-	-	-
180	0.054	0.027	0.044	0.054	0.021	0.030	0.054	0.023	0.009
182	0.027	-	0.019	0.005	0.016	-	0.018	0.016	-
184	0.018	0.022	0.019	0.032	0.016	0.020	0.024	0.016	0.009
186	0.027	0.016	-	0.005	0.016	0.040	0.012	0.008	-
188	0.054	0.065	0.075	0.048	0.089	0.056	0.065	0.063	0.065
190	0.063	0.076	0.075	0.059	0.057	0.025	0.048	0.055	0.037
192	0.027	0.027	0.031	0.027	0.016	0.020	0.006	0.031	0.046
194	0.080	0.049	0.050	0.065	0.089	0.051	0.089	0.078	0.037
196	0.054	0.049	0.088	0.048	0.036	0.071	0.071	0.063	0.028
198	0.027	0.043	0.056	0.054	0.083	0.051	0.060	0.055	0.065
200	0.071	0.130	0.056	0.070	0.078	0.096	0.071	0.078	0.083
202	0.036	0.060	0.050	0.054	0.052	0.030	0.048	0.055	0.037
204	0.054	0.043	0.063	0.038	0.036	0.061	0.065	0.016	0.046
206	0.080	0.071	0.044	0.059	0.057	0.076	0.083	0.086	0.046
208	0.036	0.054	0.038	0.097	0.078	0.056	0.060	0.070	0.102
210	0.134	0.130	0.081	0.118	0.125	0.152	0.125	0.094	0.148
212	0.009	0.011	0.044	0.038	0.047	0.030	0.018	0.039	0.019
214	0.045	0.038	0.031	0.048	0.031	0.025	0.012	0.047	0.093
216	0.027	0.027	0.038	0.032	0.010	0.040	0.042	0.031	0.019
218	0.045	0.016	0.056	0.032	0.016	0.030	0.012	0.023	0.037
220	0.027	0.038	0.013	0.005	0.026	0.010	0.012	0.031	0.046
222	0.009	-	0.019	0.011	-	0.015	0.006	0.008	-
224	-	-	-	-	-	0.010	-	0.016	0.019
226	-	-	0.006	-	-	-	-	-	0.009

Table 7. Summary of analyses testing for agreement to Hardy-Weinberg expectations. Hobs = observed heterozygosity, Hexp = expected heterozygosity, *P* (HW) = probability of fit to Hardy-Weinberg expectations, D = deviation of observed heterozygosity from expected heterozygosity (see text), *P* (D) = probability that D is significantly different from zero (one-tail test).

	Philipp.	FSM	Marshall	Coral Sea	Hawaii	F. Poly	E.Pac-1	E.Pac-3	Ecuador	TOTAL
125a										
n	92	93	73	95	96	99	84	64	103	799
Hobs	0.163	0.226	0.205	0.210	0.229	0.192	0.107	0.172	0.184	0.189
Hexp	0.179	0.220	0.231	0.233	0.223	0.174	0.102	0.188	0.194	0.195
P (HW)	0.106	1	0.460	0.365	1	0.640	1	0.026	0.344	0.040
D	-0.091	0.025	-0.111	-0.098	0.026	0.100	0.051	-0.083	-0.051	-0.033
P (D)	0.176	0.605	0.196	0.173	0.584	0.386	0.800	0.302	0.298	0.170
144										
n	94	92	80	93	96	98	84	64	103	804
Hobs	0.511	0.576	0.463	0.527	0.625	0.582	0.631	0.625	0.612	0.572
Hexp	0.554	0.529	0.597	0.540	0.568	0.592	0.610	0.607	0.617	0.583
P (HW)	0.192	0.230	0.042	0.186	0.090	0.283	0.156	0.934	0.805	0.042
D	-0.077	0.089	-0.226	-0.025	0.101	-0.018	0.035	0.029	-0.009	-0.018
P (D)	0.137	0.176	0.004	0.407	0.116	0.421	0.406	0.473	0.461	0.231
161										
n	56	92	80	93	96	99	84	64	54	718
Hobs	0.929	0.946	0.950	0.925	0.958	0.949	0.952	0.953	0.944	0.946
Hexp	0.937	0.930	0.944	0.938	0.932	0.933	0.933	0.941	0.928	0.940
P (HW)	0.995	0.994	0.995	0.994	0.996	0.993	0.994	0.994	0.996	0.275
D	-0.009	0.017	0.006	-0.014	0.028	0.018	0.021	0.013	0.018	0.006
P (D)	0.364	0.430	0.629	0.272	0.256	0.403	0.544	0.578	0.543	0.310
208a										
n	57	92	83	96	95	98	84	n.d.	59	664
Hobs	0.789	0.870	0.843	0.906	0.758	0.816	0.786		0.864	0.830
Hexp	0.788	0.807	0.819	0.844	0.811	0.811	0.801		0.809	0.819
P (HW)	0.998	0.371	0.899	0.990	0.997	0.663	1		0.998	0.675
D	0.002	0.077	0.029	0.073	-0.065	0.006	-0.019		0.069	0.013
P (D)	0.636	0.071	0.373	0.060	0.062	0.563	0.360		0.187	0.247

n.d. = not determined

Table 8a. Chi-square analysis of differentiation at four microsatellite loci among the nine Pacific Ocean collections. Note that with four tests, the *P* value for significance decreases from 0.05 to 0.05/4 = 0.0125.

Locus	No. of Fish	No. of alleles	c^2	*P*
125a	796	8	57.6	0.388
144	806	8	59.4	0.325
161	764	26	202.8	0.425
208a	664	25	199.8	0.038

Table 8b. Chi-square analysis of differentiation at four microsatellite loci among the two most separated Pacific Ocean collections—Philippines and Ecuador. Note that with four tests, the *P* value for significance decreases from 0.05 to 0.05/4 = 0.0125

Locus	No. of Fish	No. of alleles	c^2	*P*
125a	195	4	0.292	0.980
144	199	5	10.917	0.022
161	159	24	38.521	0.010
208a	116	22	21.579	0.271

4 Discussion

4.1 Mitochondrial DNA

The analysis of the ATCO fragment provided insufficient variation to test for heterogeneity of mtDNA haplotype frequency differences among sample locations but it did permit identification of non-bigeye individuals. The presence of yellowfin among the bigeye collected for this project, at frequencies from 0% to 10.4%, indicates the necessity of genetic testing for identification of each individual. Sizes of misidentified fish were between 40 and 60 cm in length. Interestingly, the converse has also occurred: some tunas collected in this size range for a concurrent yellowfin study have been misidentified in the field as yellowfin when the mtDNA tests indicate that they are bigeye (at frequencies from 0% to 30%). This suggests discrimination of these two tunas becomes problematic at these lengths and that further clarification and description of key morphological characters for visual identification may be required to minimize collection of non-target species.

Assessment of population heterogeneity by examining variation in the mitochondrial genome was possible using the DL19-12s fragment. DL19-12s contains the d-loop and had much higher levels of variation than ATCO as was evidenced by resolution of 33 haplotypes (Table 2). The convenient size (1,400 base pairs) of this fragment permitted simple assays using standard agarose gels stained with ethidium bromide. Analysis of mtDNA for population structure revealed a weakly significant P value of 0.046. However, this value becomes nonsignificant in an integrated Bonferroni test in which the mtDNA locus is included as an independent test along with the four DNA microsatellite loci. The P value for significance then falls from 0.05 to $0.05/5 = 0.010$. Thus, the null hypothesis of a single population of bigeye in the Pacific cannot be clearly rejected.

4.2 DNA Microsatellites

Analysis of microsatellite allele variation did not provide characters which were completely species-specific and could be used unambiguously for species identification. This was due to overlap of allelic states between bigeye and yellowfin. However, microsatellites did offer some alleles which were rare in bigeye but common in yellowfin. Homozygotes for such alleles (e.g. 208a*96) were always confirmed to be yellowfin using the mtDNA test.

Six of the eight loci examined had more than 20 alleles segregating in each population (Tables 4 and 5). The number of alleles present at each locus did not appear to be related to the type of repeat motif (i.e., perfect or imperfect) but seemed linked to the number of repeat units in the original clone isolated from yellowfin tuna. This perhaps reflects the likelihood that the most common allele will be cloned during construction of the microsatellite library. Thus, when a small repeat was cloned it usually corresponded to a locus for which there was limited size variation and only a few alleles were present in the population. Correspondingly, if an allele with many repeats was cloned and sequenced, this usually meant that a large number of alleles would be found segregating at that locus.

Large numbers of alleles ($n>25$) observed at some loci will require sample sizes greater than 36 fish to achieve desired precision on estimates of allele frequency. This degree of precision will in turn affect the statistical power of assessments of the degree of differentiation among populations, if differentiation is indeed present. There is thus limited use for loci such as 113 and 117 with 35 and 27 alleles respectively. Such loci have still less power when allele frequencies are partitioned evenly across size categories and all are at frequencies less than 0.10.

The large numbers of alleles observed for the majority of loci examined indicates that large sample sizes ($n\geq 80$) are required to assess variation among the Pacific-wide bigeye sample locations. With the exception of loci 125a and 144, there were more than 20 alleles segregating

at each locus. Thus, the lack of significant differentiation for any of the eight loci examined between the Philippine and Ecuador collections, given the initial sample sizes of only 36 fish per population, was hardly surprising.

Analysis of the four loci for which sample sizes examined were greater than 56 fish per location still did not produce good evidence of population subdivision. There was no evidence of heterozygote deficiencies for any of the four loci in the pooled sample, which might have indicated population differentiation, and there was no evidence for significant differentiation in allele frequencies among the Pacific-wide collections.

However, there was evidence of small genetic differences among the two most separated collections—Philippines and Ecuador. Thus there may be other real but subtle differences among the Pacific populations, which could only be revealed by larger sample sizes than those deployed here. This is particularly true of the two loci 208a and 161 which have more than 20 alleles segregating, and especially for the latter locus where allele frequencies were all quite low and fairly evenly distributed across all allelic classes. For 208a, three of the alleles (102, 104, and 106) had frequencies which were reasonably common (ranging from 0.15 to 0.27) and which together accounted for more than 60% of the total alleles observed in any one population. However, analysis of results from loci 125a and 144, which both had only eight alleles segregating, also failed to indicate population subdivision on a broad scale. This further suggests that if there is differentiation among populations then it must be very small and undetectable with the current loci and sample sizes.

4.3 Comparison of Genetic Results with Tagging Data

The results of the genetic analyses are broadly consistent with the results of the SPC's tagging experiments on bigeye tuna. Many bigeye tagged in locations throughout the western tropical Pacific have displayed movements of up to 4 000 nautical miles over periods of one to several years (Figures 2 and 3). Specific instances of movement from the genetics sampling location in the Coral Sea to the vicinity of French Polynesia, from Micronesia to Hawaii, and from the Coral Sea (Papua New Guinea) to the Philippines have been recorded (Figure 2a). While the observation of such movements does not necessarily imply that individual tagged bigeye were spawning in both release and recapture locations, they do demonstrate that gene flow can potentially occur across these distances at least. The widespread distribution of bigeye spawning throughout the tropical Pacific and the greater longevity of bigeye relative to other tropical tunas, such as yellowfin (Hampton et al., in prep.[3]), are also consistent with a high potential for gene flow.

[3] Hampton, J, M. Labelle and K. Bigelow. In prep. A summary of current information on the biology, fisheries and stock assessment of bigeye tuna in the Pacific Ocean, with recommendations for future research and data collection.

Figure 2. a. Displacements >100 nautical miles of bigeye tuna tagged by the SPC's Regional Tuna Tagging Project (RTTP). b. The cumulative distribution of all RTTP tagged bigeye displacements having accurate location data.

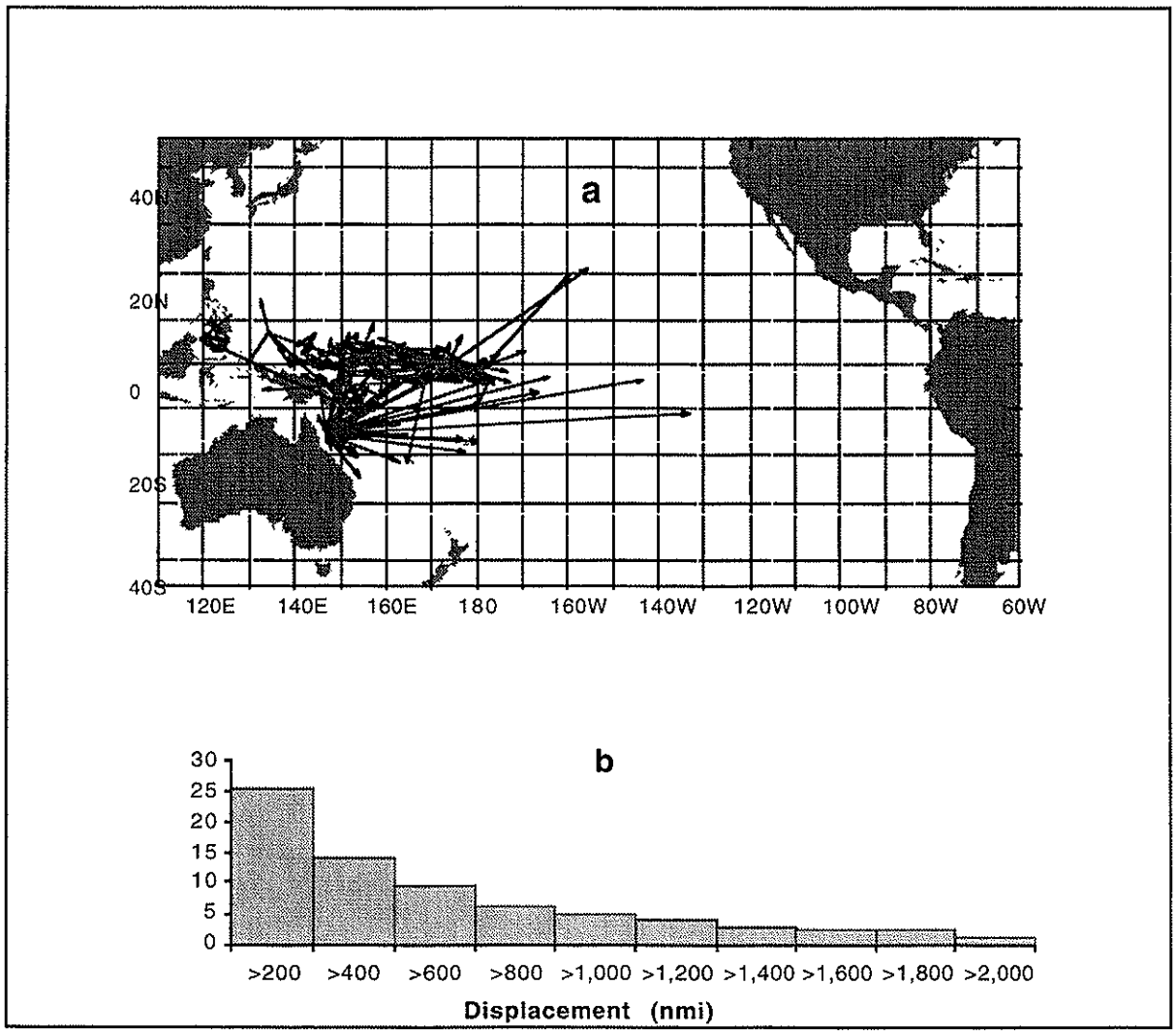
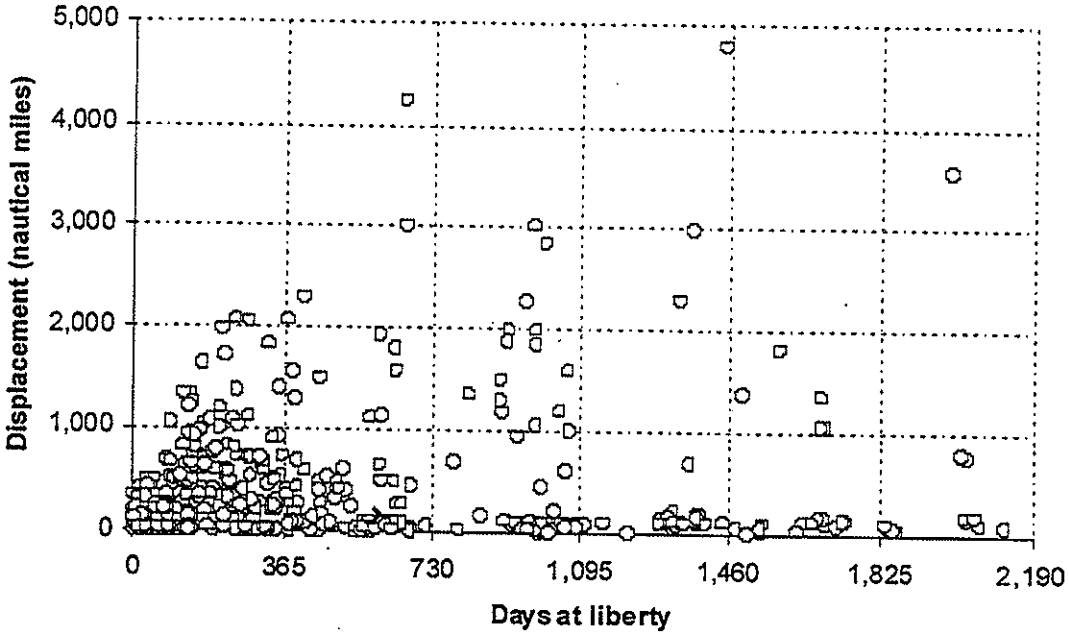


Figure 3. Plot of displacement versus time at liberty for bigeye tagged by the SPC's Regional Tuna Tagging Project.



5 Summary and Recommendations for Further Work

Analysis of mtDNA and DNA microsatellites in nearly 800 bigeye tuna failed to reveal significant evidence of widespread population subdivision within the Pacific Ocean. However, there was some evidence of restricted gene flow between the two most widely separated collections, Philippines and Ecuador. The large number of alleles present for each of the marker loci requires that a substantially larger number of individuals be surveyed in order to detect the small amount of differentiation that may in fact be present. Alternatively, the general uniformity of genetic markers within the Pacific Ocean basin might reflect sufficient exchange of individuals or genes that the collections sampled are but subsamples of an essentially Pacific-wide population.

Future research should address the sample size issue by focusing on increased sample sizes (n>100—we would recommend 200), and by increasing the numbers of loci examined. Assessing temporal variation may also give a stronger indication of subtle differences if they exist. Resolution of stock structure and demonstrating differentiation among populations is also a function of the number of loci examined, especially where differentiation is expected to be small. Thus, additional loci should also be developed to maximize the probability of finding loci which might demonstrate heterogeneity.

Comparison of genetic variation between bigeye from the Atlantic and Indian Oceans would complement and help to further determine the relative gains in resolution of Pacific Ocean population structure that can be achieved through examination of larger samples sizes and additional loci. Significant population subdivision has been recently demonstrated between Indian and Atlantic Oceans through examination of mtDNA (Alvarado-Bremer and Ely, unpublished data[1]). Comparison of these populations with those sampled from the Pacific will help indicate the relative degree of population divergence of geographically well-separated populations and may assist in determining what sample sizes and numbers of loci will be needed to address further the issue of the extent of population subdivision of bigeye tuna in the Pacific Ocean. The present study provides some support for the hypothesis that gene flow between the Philippines and Ecuador is restricted but does not otherwise indicate spatial structuring within the Pacific Ocean.

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