Identification of Two Evolutionary Significant Units based on mitochondrial DNA and microsatellite evidence in the Pelagic Thresher Shark (*Alopias pelagicus*) in the Pacific Ocean

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Abstract

There has been an increasing concern about shark overexploitation in the last decade. For some open ocean shark species, there is a paucity of data about their life histories and population dynamics. Here, we present the population structure of *Alopias pelagicus* analyzing 329 samples from six different locations across the Pacific Ocean. Using data from mitochondrial DNA COI sequences and seven microsatellite loci we found evidence for two Evolutionary Significant Units with almost no geneflow between them. In addition, we report the first evidence of a long-term migration for this species in the Pacific Ocean. Moreover, a recent bottleneck was found for the Western Pacific clade, which highlights the increased need for management and conservation measures. Furthermore, our results may indicate the existence of a new cryptic species in the Pacific Ocean. Finally, The Evolutionary Significant Units found in this study should be considered in management plans and initiatives such as the different National Actions Plans for the Conservation of Elasmobranch Species in all nations with jurisdiction in the Pacific Ocean.

Key Words: Evolutionary Significant Units, microsatellite loci, mitochondrial DNA, population structure, pelagic thresher shark, *Alopias pelagicus*

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Introduction

Sharks of the open ocean are highly migratory species that routinely cross national borders, therefore knowledge of population genetics is crucial in order to conserve and manage shark fisheries on an international level. The fact that these species have a wide range of geographical distribution, may lead to the misconception that these marine resources are unlimited and resistant to localized depletion. When population subdivision exists, these misconceptions lead to an unsustainable use of the resource, ending in the depletion of the population under pressure. If severe enough, these population depletions may cause a significant drop in genetic diversity, which may be reflected in the reduce ability of a population to recover to a changing environment(Kuussaari *et al.* 1998; Westemeier 1998).

The use of population genetic analyses allows the identification of evolutionary significant units (ESUs, (Ryder 1986)), information that is essential for the design and adoption of effective conservation and management measures of important and heavily exploited resources. The definition of ESUs has been described as the presence of reciprocal monophyly for organelle haplotypes (e.g. mitochondrial DNA) and significant divergence of allele frequencies at nuclear loci (e.g. microsatellites, (Moritz 1994)). In addition, ESUs must have a significant level of reproductive isolation from other conspecific population units (Moritz 1994).

Highly migratory open ocean shark species that routinely move cross multiple marine biogeographic provinces are expected to show little to no population heterogeneity. As expected, previous population genetic studies on such epipelagic sharks, such as the shortfin mako shark *Isurus oxyrinchus* (Schrey & Heist 2003), the basking shark *Cetorhinus maximus* (Hoelzel *et al.* 2006), the whale shark *Rhincodon typus* (Castro *et al.* 2007; Schmidt *et al.* 2009), and the blue shark *Prionace glauca* (Ovenden *et al.* 2009) show low to no genetic structuring among ocean basins.

The pelagic thresher shark (*Alopias pelagicus)* is a large (up to 330 cm TL) aplacental viviparous epipelagic shark with a distribution restricted to the Indian and Pacific Oceans. Fecundity is very low with litters of only 1-2 pups (Liu *et al.* 1999), with an unknown gestation period, but assumed to be around a year or less (Camhi *et al.* 2009). The pelagic thresher shark is one of the most abundant open ocean sharks in the Eastern Tropical

Pacific (ETP) and Western Pacific Ocean (WP), and one of the most exploited shark species in commercial, artisanal and illegal fisheries of these regions (Caldas & Castro-González 2010; Tsai *et al.* 2010; Caballero *et al.* 2011). Despite a high level of exploitation little is known regarding their life history, population dynamics, and overall abundance.

Between 2009 and 2011, tissue samples collected in Colombian fishing ports and during seizures of illegal shark finning vessels were sent to the Universidad de Los Andes for molecular identification. We found that more than 95% of those samples were *A. pelagicus,* suggesting that perhaps exploitation rates in this region are higher than previously thought and that there is a need to gather more information on this species for its effective management (Caballero *et al.* 2011). Furthermore, Tsai et al. (2010) suggested, using a stochastic stage-based model, that the northwest Pacific stock of *A. pelagicus* is overexploited. The combined levels of exploitation, low fecundity and lack of population information led this species to be classified as Vulnerable on the IUCN Red List since 2004.

Here, we examine the population structure and molecular ecology of the pelagic thresher shark across the Pacific Ocean by analyses of mitochondrial and nuclear molecular markers.

Methodology

Tissue Collection and DNA Extraction

A total of 349 samples were collected between 1997 and 2010 (Fig.1). Samples were identified by experts in the field (NOAA and INCODER), and for the samples that identification was uncertain, we implemented the molecular identification protocol described by Caballero *et al*. (2011). DNA was extracted using 200 µl of 10% Chelex (BioRad) and heating at 60° C for 20 minutes, followed by heating at 103°C for 25 minutes followed by a brief centrifugation and storage at 4°C (Hyde *et al.* 2005).

Cytochrome Oxidase I amplification and analyses

A portion of the mitochondrial COI gene (655 bp) was amplified using the universal primers FishCoxI F (5' TCWACCAACCACAAAGAYATYGGCAC) and FishCoxI R (TARACTTCWGGGTGRCCRAAGAATCA) modified from Ward *et al*. 2005. The PCR

profile was as follows: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 40 s, with a final extension of 72 °C for 10 min. PCR products were sent to Macrogen Inc, Korea, for sequencing.

All forward and reverse sequences were checked and edited manually using Geneious Pro version 3.6.1 created by Biomatters (http://www.geneious.com/), and aligned using Seaview 4.4.1 software (Gouy *et al.* 2010). Haplotypes were defined using MacClade (Maddison & Maddison 2000). A haplotype network was constructed in the software TCS v. 1.21 (Clement *et al.* 2000) using the statistical parsimony methodology. This method estimates an unrooted tree, providing a 95% plausible set for all sequence type linkages within the tree and considering gaps as a fifth character state. We tested the model of substitution using JModelTest v.2.3.1 (Posada 2008) and the best fit model (GTR+I) was used in the setting for the phylogenetic reconstructions performed in Beast v.1.7.5 (Drummond & Rambaut 2007). We also included two haplotypes from India, one from Malaysia, and one from Mexico downloaded from Genbank to reconstruct the phylogenetic relationships among haplotypes across the entire distribution range of *A. pelagicus.* A coalescent tree was reconstructed with a molecular clock using sequences from other Lamniformes species, *I. oxyrinchus*, *Carcharias taurus*, *A. vulpinus*, and *A. superciliosus* downloaded from Genbank with first appearance of a taxon in the fossil record used as the minimum time for evolutionary change along that lineage (Martin *et al.* 1992). In addition, we estimated the mutation rate using 18 *A. pelagicus* COI haplotype sequences and one *C. taurus*. The rate of nucleotide substitution per lineage per year (λ) was estimated using the equation $\lambda=d/2t$, where d is the adjusted divergence and t is the time since divergence (Li 1997). We used the Tamura-Nei model (Nei & Kumar 2000) in Mega 5.2.2 to estimate d, with a pairwise gamma-corrected Tamura-Nei distance. The date of divergence between *Alopias* spp. and *C. taurus* lineages was estimated based on the fossil record (Martin 1995), to be between 74 and 83 MYA (million years ago). After calculating λ and d between EP and WP clades mean distances, we calculated the divergence time between both clades.

Haplotype and nucleotide diversity calculations were performed in Arlequin v.3.5.1 software (Excoffier *et al.* 2005) and restricted to 655 bp of the cytochrome oxidase I gene. We also performed an analysis of molecular variance (AMOVA), based on conventional F statistics, using 10,000 random permutations, and population units were established *a piori*

based on haplotype frequencies found in previous analyses (Eastern Tropical Pacific, Baja, Hawaii and Taiwan). The Eastern Tropical Pacific population unit comprised all samples from Colombia (112), Ecuador (31) and Central America (5).

Microsatellite loci amplification and analyses

Nine microsatellite loci [Iox-01, Iox-30, Iox-12 (Schrey & Heist 2002), AV-H8, AV-H110, AV-H138, AV-I11, Iox-M36 and Iox-M115 (D. Kacev unpublished data)] were amplified separately in 29 μl PCR reactions. Two thermocycling profiles were implemented. The first profile consisted in an initial denaturation at 94 ^{o}C for 2 min, followed by 35 cycles of 94 ^{o}C for 30 s, 60 s at specified annealing temperature (Table 1), and 72 ^{o}C for 90 s, with a final extension of 72 ^{o}C for 30 min. The second profile consisted of the same temperatures with a final extension of 60 min (Table 1).

PCR products were run on either an ABI3500 automated sequencer at Universidad de Los Andes (Colombia) or an ABI 3130 Genetic Analyzer at the NOAA SWFSC (La Jolla, CA), and visualized using ABI PRISM GeneMapper Software 4.0 (Applied Biosystems). All authors scored all samples for all loci individually in order to minimize scoring errors across readers.

We estimated patterns of genetic structure in the Pacific Ocean using Structure 2.3.4 (Pritchard *et al.* 2009), which assigns individuals to groups using a Bayesian model-based method that minimizes linkage disequilibrium and deviations from Hardy-Weinberg expectations. We used the admixture model with correlated allele frequencies, and employed sampled locations as prior information (Hubisz *et al.* 2009). We inferred the best *K* value from the log probability LnP(D), by using an *ad hoc* statistic (ΔK, (Evanno *et al.* 2005)) that calculates the second order rate of change of LnP(D) between the values of *K*. We used a 5000 step burn-in followed by 50,000 simulations to test K= 1-10 with 20 repetitions each.

We assessed genetic differentiation and diversity among population units and between EP and WP clusters with population pairwise F_{ST} and R_{ST} values. We also tested for linkage disequilibrium and Hardy-Weinberg equilibrium (H-W) deviations using Arlequin v.3.5.1 (Excoffier *et al.* 2005). We calculated the number of effective migrant per generation (*Nm*) using the equation $Nm= 1/4(1/F_{ST}-1)$ assuming Wright's island model.

We conducted an analysis using the program Bottleneck (Piry *et al.* 1999) in order to test for evidence of a recent population size reduction in the EP and WP clusters. The program was run under the two-phase model (TPM) with the default values for variance and probability. We selected this model since microsatellite loci are generally believed to mutate according to this model, where mutations result in the addition or loss of a single repeat motif (Peery *et al.* 2012). The Wilcoxon signed-rank test, which is the most powerful and commonly used approach (Cornuet & Luikart 1996), was conducted to test the significance of the analysis.

Results

Mitochondrial DNA COI analyses

A total of 323 sequences were successfully obtained from individuals sampled in the Pacific Ocean. A 655 bp fragment of the COI gene was analyzed and from each of the 323 individuals which resulted in the identification of 24 variable sites that defined a total of 18 unique haplotypes, nine were observed in only one sample (Table 2).

The haplotype network derived from the TCS analysis showed two well differentiated phylogeographic clusters, roughly corresponding to the EP and WP populations (Fig. 2). We excluded haplotype 10, due to missing data. Ten haplotypes were observed in a single geographical region (H2, H11, H12, H16, H17, and H18 in the ETP region, H13 and H15 in Baja, and H7 and H14 in Taiwan) with the rest being observed across multiple regions. Haplotype H1 showed the highest frequency in the EP region, and the analysis suggested it to be the ancestral haplotype in this region. Haplotype H3 was the most common in the WP region and only seven samples from the EP shared that haplotype. Haplotype H9 was shared between Hawaii and Baja, although it belongs to the WP cluster.

The coalescent tree resulting from the Beast v.1.7.5 (Drummond & Rambaut 2007) analysis showed reciprocal monophyly between the EP and WP clades (Fig. 3) and the molecular clock in our genealogy suggested that the EP and WP clades shared a common ancestor 7,8 MYA. In addition, we found a rate of 0,7% sequence divergence per million years and a divergence time between the EP and WP clades between 4,66 and 4,12 MYA.

Indo-Pacific haplotypes were grouped within the WP clade and the previously published

haplotype from Mexico was grouped within the EP clade. In addition, *A. pelagicus* appeared to be the sister group of the clade formed by *A. vulpinus* and *A. superciliosus*.

Due to the reciprocal monophyly found between EP and WP clades (Fig. 3), we performed a non-hierarchical AMOVA analysis for each clade separately. For the EP population, we found no significant differences in pairwise comparisons for both F_{ST} and Φ_{ST} levels (Table 3) among population units. Haplotype diversity was similar in each sampled area and the lowest nucleotide diversity was found in the ETP region. For the WP population, we found no significant differentiation among population units at the pairwise F_{ST} and Φ_{ST} levels, except for Hawaii (Table 4). Hawaii was significantly different from all other population units at both F_{ST} and Φ_{ST} and had the highest nucleotide diversity. Taiwan showed the lowest haplotype diversity compared to all other population units.

Microsatellite loci analyses

Microsatellite fragments were analyzed from a total of 331 individuals across the Pacific Ocean. Following scoring of all loci, Iox-01 and Iox-30 loci were found to be monomorphic and were eliminated from all analyses.

After testing for the best *K* value for our data (*K*=2), Structure (Pritchard *et al.* 2009) analysis showed a clear separation of EP and WP clusters (Fig 4). Interestingly, approximately half of the samples from Hawaii were assigned to the WP cluster and the other half to the EP cluster. We also found three individuals sampled in Baja with assignment probabilities higher than 0,80 to the WP cluster.

We performed a non-hierarchical AMOVA analysis among population units. We found no significant differences in pairwise comparisons at the F_{ST} and R_{ST} between Baja and ETP population units (Table 5). Hawaii and Taiwan were significantly different from all other populations units at both F_{ST} and R_{ST} across the Pacific Ocean. The EP and WP clusters were significantly different between them (F_{ST}= 01245, *P*< 0,0001; R_{ST}= 0,153, *P*< 0,0001). Genetic diversity values, such as expected heterozygosity (H_E) and observed heterozygosity (H_O), were obtained for seven loci in all populations units and for the EP and WP clusters, along with deviations from H-W equilibrium. H_E and H_O varied among population units at different loci. Furthermore, Hawaii and Baja showed the largest differences between H_O and H_E, and Baja showed the lowest values of H_o in six of seven

loci (Table 6). The level of geneflow found between EP and WP clusters, based on the number of effective migrants per generation (*Nm*) was 0,2189.

Wilcoxon signed-rank test analysis performed in Bottleneck (Piry *et al.* 1999) showed that the WP cluster presented a significant deficiency in heterozygosity (*P*= 0,008), which is a signal for a recent population bottleneck. The EP cluster showed no significant deficiency or excess of heterozygosity (P= 0,938).

Discussion

This study presents the first extensive analyses of the molecular ecology of *A. pelagicus* in the Pacific Ocean using mitochondrial and nuclear molecular markers. Both markers showed similar results with respect to the clear population structure found between the EP and WP. These results support our hypothesis of the existence of two Evolutionary Significant Units (ESUs), based on the definition presented by Morizt (1994). *A. pelagicus* showed reciprocal monophyly for mitochondrial DNA, significant divergence of allele frequencies at microsatellite loci, and a significant level of reproductive isolation from other conspecific population units in the Pacific Ocean. The reason for this marked population division remains unclear. There was previous evidence of this divergence between EP and WP clades using mitochondrial DNA control region (Trejo 2005).

We found two different divergence times between the WP and WP clades of 7,8 and between 4,66 and 4,12 MYA. The first estimate is solely based on the fossil record, while the second estimate is more likely when comparing our estimates to those found in previous studies (Duncan *et al.* 2006). Therefore, the divergence between these two ESUs could be a consequence of the closure of the Panama Isthmus. Numerous studies have demonstrated that the closure of the Panama Isthmus created different Ocean circulation patterns (e.g. Central American Seaway; (Nisancioglu *et al.* 2003; Schmidt 2007)). In addition, several studies have shown that epipelagic sharks normally use Ocean currents to migrate across Ocean basins (Stevens 1976; Casey 1985; Stevens 1990). Hence, it is feasible that *A. pelagicus* used these currents to migrate across the Pacific Ocean in the late Miocene increasing geneflow between distant populations. To date, there is no evidence of a similar case in an epipelagic shark species in the Pacific Ocean. Previous population genetic studies have shown low levels of genetic structure in several epipelagic shark species at

both global and regional levels (Schrey & Heist 2003; Castro *et al.* 2007; Ovenden *et al.* 2009; Schmidt *et al.* 2009).

Another interesting element is the results found in the central Pacific around Hawaii. Hawaii shared haplotypes from both ESUs at high frequencies and these individuals assigned with high probability to respective WP and EP groups in the microsatellite loci analyses. These results leave some new questions open; are both ESUs found year-round in this region or do they seasonally migrate here? Is there spatial or temporal separation between these groups that allow for reproductive isolation? Future satellite migration studies should be conducted in the Pacific Ocean in order to answer these questions. Even though we did not use any electronic devices, our results show the first evidence of migration across the Pacific Ocean basin for this species. Structure software (Pritchard *et al.* 2009) analysis showed the presence of five individuals from the WP ESU in Baja, California, USA (Fig. 4). There has been evidence of migrations detected using molecular markers in other taxa. For example, the Hector's dolphins were found to migrate more than 400 km into the distribution range of its sister species the Maui's dolphin in New Zealand (Hamner *et al.* 2013).

Levels of H_O and H_E were similar for all population units, although were higher than genetic diversity values for other oceanic species (Pardini *et al.* 2000; Schmidt *et al.* 2009; Daly-Engel *et al.* 2012), and similar to those found in *I. oxyrinchus* (Schrey & Heist 2003). In general, genetic diversity was lowest in the EP compared to the WP (Table 6), and there is evidence of a recent population bottleneck in the WP. The low haplotype diversity in Taiwan could be a consequence of the sampling methodology in that region. Almost all of the samples from Taiwan came from a single sampling event. Thus, it is possible that there is some population structure present in the WP region and that by sampling only one group of animals we are missing additional diversity.

Taxonomic and conservation implications

Our results provide key information to scientists, resource managers and governmental agencies regarding management and conservation of this vulnerable species. We presented evidence of two ESUs of *A. pelagicus* in the Pacific Ocean and the genetic differentiation presented here is the highest found in the literature for an epipelagic shark. Moreover,

considering the slow mutation rate of sharks compared to other vertebrates (Martin 1995), the strong genetic differentiation found in *A. pelagicus* in the Pacific Ocean, and the almost nonexistent geneflow, may be an indicator of the existence of a cryptic species. Due to conflicts with the species concept found in the literature, we decided to leave our findings at the ESU level, although future research should include morphological data and more genetic markers. The ESUs found in this study should be considered in management plans and initiatives such as the different National Actions Plans for the Conservation of Elasmobranch Species. In addition, due to the bottleneck found in the WP, this ESU should be considered for stronger conservation initiatives and management regulations.

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FIGURES

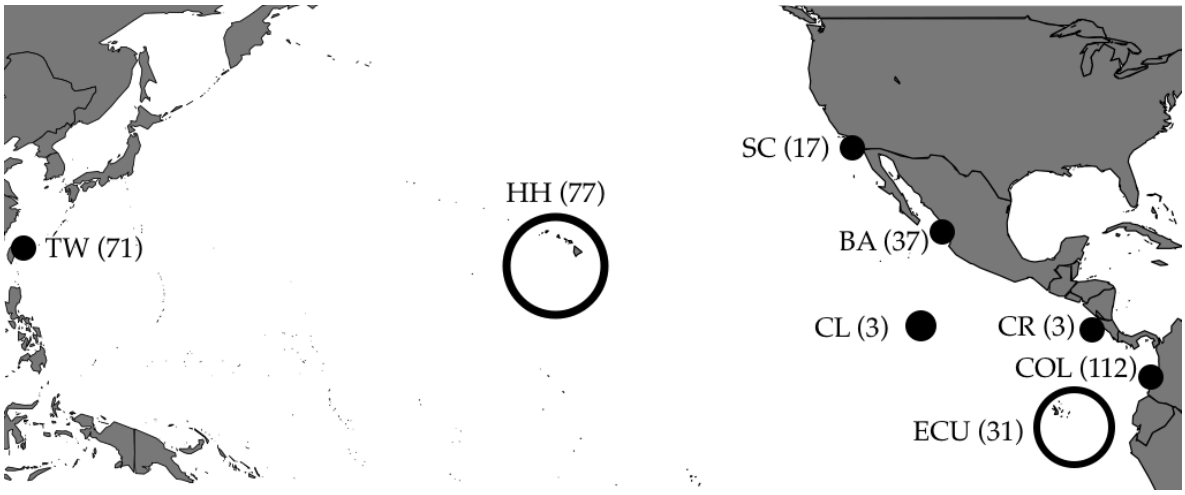

Figure 1. Map indicating sampling locations in the Pacific Ocean. TW= Taiwan, HH= Hawaii, BA= Baja, SC= South California, CL= Clipperton Island, CR= Costa Rica, COL= Colombia, ECU= Ecuador. Sample numbers for each location are shown in parenthesis.

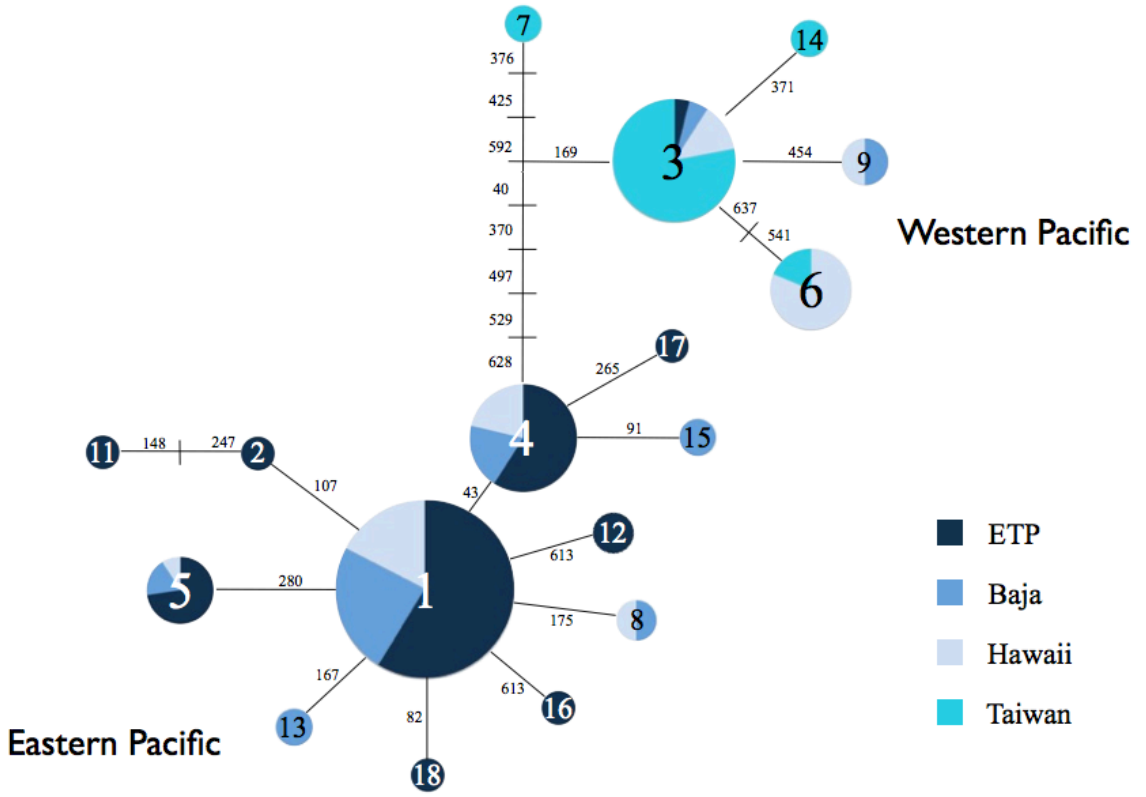

Figure 2. Haplotype network obtained from the TCS analysis. The size of the circle represents the frequency of each haplotype. Numbers represent substitutions between haplotypes and small lines represent hypothetical haplotypes not observed in this study.

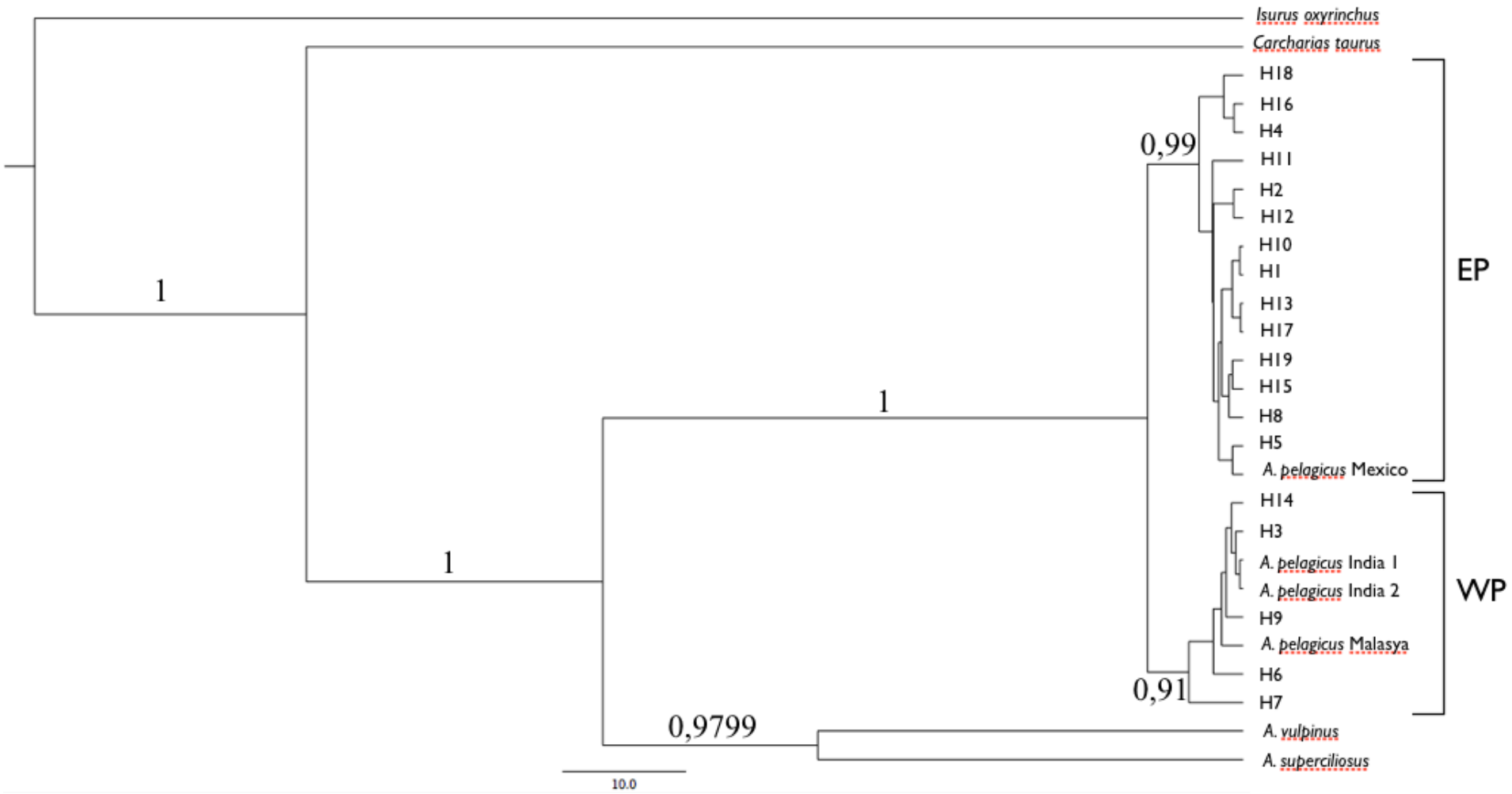

Figure 3. Coalescent tree showing the reciprocal monophyly between haplotypes from the EP and WP clades. Posterior values are shown as branch labels

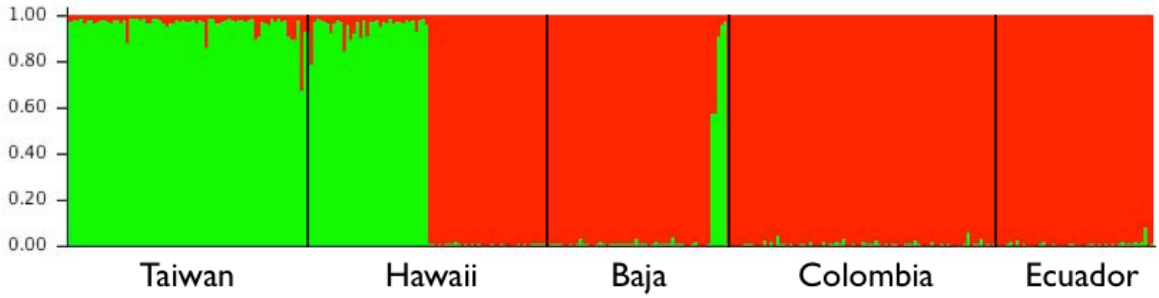

Figure 4. Bar plot showing the assignment probabilities of each genotyped individual of *A. pelagicus* from the different sampling locations in the Pacific Ocean.

TABLES

Table 1. Loci name, PCR profile number and annealing temperature for each locus

Loci	**PCR Profile**	**Annealing Temp. (°C)**
Iox-01	2	56
Iox-12	2	64
Iox-30	2	56
AV-H8	2	58
AV-H110	1	65
AV-H138	1	58
AV-I11	1	58
Iox-B3	2	62
Iox-M36	2	66
Iox-M115	2	68

Table 2. Twenty-four variable sites over 655 bp of the mitochondrial COI gene determining 17 Pacific *Alopias pelagicus* haplotypes

	Variable Sites																								
Haplotypes	40	43	82	91	107	145	167	169	175	247	265	280	370	371	376	425	454	497	529	541	592	613	628	637
H1	G	G	A	T	A	A	G	A	T	T	T	T	G	G	C	T	T	C	C	A	C	T	G	T
H2	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	A	A	-	-	-	-	-	G	-	-	-	-	A	-	-	-	-	T	T	-	-	-	A	-
H4	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
H6	A	A	-	-	-	-	-	G	-	-	-	-	A	-	-	-	-	T	T	G	-	-	A	C
H7	A	A	-	-	-	-	-	-	-	-	-	-	A	-	T	C	-	T	T	-	T	-	A	-
H8	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	A	A	-	-	-	-	-	G	-	-	-	-	A	-	-	-	C	T	T	-	-	-	A	-
H11	-	-	-	-	G	G	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	?	?
H13	A	A	-	-	-	-	-	G	-	-	-	-	A	A	-	-	-	T	T	-	-	-	A	-
H14	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H15	-	A	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
H17	-	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-

(?) denotes missing data

Table 3. Pairwise Fst (below diagonal) and Φst (above diagonal) values for COI gene from the Eastern Pacific Ocean clade of *Alopias pelagicus*.

Φ_{ST} / F_{ST}	ETP (n=128)	Baja (n=49)	Hawaii (n=39)
ETP	π=0,106% h=0,573±0,035	0,00308 (*0,2774±0,0049*)	0,01763 *(0,08791±0,0029)*
Baja	-0,00806 (*0.68795±0,0050*)	π=0,183% h=0,572±0,066	-0,01682 (*0,85298±0,0036*)
Hawaii	-0,00981 (*0,68340±0,0045*)	-0.01337 (*0,69864±0,0050*)	π=0,1966% h=0,582±0,0568

Probability values based on 10.000 permutations are shown in italic. Significant different values (p<0,05) in bold. Haplotype (h) and nucleotide (π) % ± standard deviation (SD) diversity values are shown in the diagonal of each population unit. Numbers of samples per location are shown in parenthesis.

Table 4. Pairwise Fst (below diagonal) and Φst (above diagonal) values for COI gene from the Western Pacific Ocean clade of *Alopias pelagicus*.

Φ_{ST} / F_{ST}	ETP (n=3)	Baja (n=6)	Hawaii (n=38)	Taiwan (n=71)
ETP	π=0,000% h=0,000±0,000	0,04000 (*0,4919±0,0044*)	0,45053 ***(0,04138±0,0020)***	-0,15951 (*0,9999±0,000*)
Baja	0,04000 (*0,50589±0,005*)	π=0,0814% h=0,533±*0,1721*	0.49708 ***(0,00158±0,0004)***	0,08442 (*0,10167±0,0027*)
Hawaii	0,48760 ***(0,02396±0,002)***	0,39156 ***(0,00297±0,001)***	π=0,1512% h=0,4723±0,0718	0,45833 ***(0,0000±0,0000)***
Taiwan	-0.12378 (*0,9999±0,0000*)	0,14431 (*0,06069±0,003*)	0,49943 ***(0,0000±0,0000)***	π=0,1181% h=0,2793±0,0669

Probability values based on 10.000 permutations are shown in italic. Significant different values (p<0,05) in bold. Haplotype (h) and nucleotide (π) % ± standard deviation (SD) diversity values are shown in the diagonal of each population unit. Numbers of samples per location are shown in parenthesis.

Table 5. Population differentiation between pairwise populations with seven microsatellite loci

Rst / Fst	Taiwan	Hawaii	Baja	ETP
Taiwan	0,00000	**0,10088****	**0,25233****	**0,10672****
Hawaii	**0,03758****	0,00000	**0,11172***	**0,03044****
Baja	**0,12532****	**0,03193***	0,00000	-0,00706
ETP	**0,11309****	**0,03099****	-0,00610	0,00000

Significant scores are in bold and probability values are based on 10.000 permutations. Below diagonal Fst values and above diagonal Rst values. Degrees of significance: *0,001 and **0,0001.

Table 6. Genetic diversity for seven microsatellite loci in all sampling locations analyzed

Locus	Taiwan	HH WP	HH EP	Baja WP	Baja EP	SC EP	CAM	Colombia	Ecuador
	N= 71	N= 39	N= 37	N= 5	N= 14	N= 35	N= 5	N= 94	N= 31
Iox-12	n= 19	n= 20	n= 24	n= 4	n= 16	n= 14	n= 8	**n= 37**	n= 26
	H_o= 0,971	H_o= 1,000	H_o= 1,000	H_o= 1,000	H_o= 1,000	H_o= 1,000	H_o= 0,800	**H_o= 0,956**	H_o= 1,000
	H_e= 0,922	H_e= 0,924	H_e= 0,952	H_e= 0,867	H_e= 0,956	H_e= 0,949	H_e= 0,955	**H_e= 0,944**	H_e= 0,958
	P= 0,051	P= 0,993	P= 0,799	P= 1,000	P= 1,000	P= 0,220	P= 0,235	**P= 0,002**	P= 0,544
Iox-M36	n= 3	n= 5	n= 4	n= 3	n= 4	n= 3	n= 3	n= 3	n= 4
	H_o= 0,471	H = 0,571	H_o= 0,588	H_o= 0,500	H_o= 0,700	H_o= 0,185	H_o= 0,400	H_o= 0,372	H_o= 0,429
	H_e= 0,405	H_e= 0,575	H_e= 0,580	H_e= 0,714	H_e= 0,642	H_e= 0,174	H_e= 0,711	H_e= 0,397	H_e= 0,419
	P= 0,246	P= 0,192	P= 0,685	P= 0,315	P= 0,203	P= 1,000	P= 0,111	P= 0,780	P= 0,230
AV-I11	n= 4	n= 3	n= 3	n= 2	n= 2	n= 3	n= 2	**n= 2**	**n= 4**
	H_o= 0,338	H_o= 0,289	H_o= 0,486	H_o= 0,200	H_o= 0,429	H_o= 0,733	H_o= 0,400	**H_o= 0,617**	**H_o= 0,645**
	H_e= 0,339	H_e= 0,298	H_e= 0,509	H_e= 0,200	H_e= 0,423	H_e= 0,662	H_e= 0,533	**H_e= 0,494**	**H_e= 0,569**
	P= 0,577	P= 0,708	P= 1,000	P= 1,000	P= 1,000	P= 0,197	P= 1,000	**P= 0,020**	**P= 0,008**
AV-H8	n= 32	n= 25	n= 30	n= 5	n= 12	**n= 20**	n= 6	**n= 40**	**n= 22**
	H_o= 0,969	H_o= 0,933	H_o= 0,970	H_o= 1,000	H_o= 0,778	**H_o= 0,882**	H_o= 0,800	**H_o= 0,947**	**H_o= 0,967**
	H_e= 0,955	H_e= 0,959	H_e= 0,973	H_e= 0,933	H_e= 0,922	**H_e= 0,959**	H_e= 0,844	**H_e= 0,967**	**H_e= 0,956**
	P= 0,384	P= 0,476	P= 0,830	P= 1,000	P= 0,063	**P= 0,017**	P= 0,798	**P= 0,002**	**P= 0,014**
AV-H138	n= 16	n= 11	n= 19	n= 7	n= 13	n= 16	n= 9	n= 34	n= 24
	H_o= 0,958	H_o= 0,868	H_o= 0,865	H_o= 0,800	H_o= 0,857	H_o= 0,965	H_o= 0,800	H_o= 0,883	H_o= 0,935
	H_e= 0,872	H_e= 0,850	H_e= 0,887	H_e= 0,867	H_e= 0,860	H_e= 0,913	H_e= 0,978	H_e= 0,905	H_e= 0,937
	P= 0,365	P= 0,251	P= 0,298	P= 0,612	P= 0,948	P= 0,739	P= 0,118	P= 0,165	P= 0,073
AV-H110	n= 16	n= 16	n= 18	n= 5	n= 13	n= 18	n= 5	n= 21	n= 18
	H_o= 0,900	H_o= 0,889	H_o= 0,919	H_o= 0,750	H_o= 0,917	H_o= 0,926	H_o= 0,800	H_o= 0,903	H_o= 0,900
	H_e= 0,913	H_e= 0,887	H_e= 0,910	H_e= 0,857	H_e= 0,946	H_e= 0,933	H_e= 0,822	H_e= 0,905	H_e= 0,905
	P= 0,547	P= 0,136	P= 0,930	P= 0,650	P= 0,683	P= 0,697	P= 0,897	P= 0,094	P= 0,203
Iox-M115	n= 11	n= 9	**n= 12**	n= 5	n= 8	**n= 5**	n= 4	n= 9	n= 8
	H_o= 0,844	H_o= 0,850	**H_o= 0,645**	H_o= 1,000	H_o= 0,857	**H_o= 0,222**	H_o= 1,000	H_o= 0,778	H_o= 0,679
	H_e= 0,802	H_e= 0,845	**H_e= 0,706**	H_e= 0,933	H_e= 0,901	**H_e= 0,752**	H_e= 0,733	H_e= 0,747	H_e= 0,660
	P= 0,426	P= 0,581	**P= 0,034**	P= 1,000	P= 0,235	**P= 0,003**	P= 0,392	P= 0,380	P= 0,852

N= simple size for each locus; n= total numer of alleles, Ho= observed heterozygosity, He= expected heterozygosity. Loci out of equilibrium are shown in bold.