Mitochondrial D-loop Phylogeography of the Northwest Atlantic Porbeagles (Lamna nasus) in Comparison to the Southwest Pacific porbeagles

by

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ABSTRACT

Porbeagles (Lamna nasus) are a highly commercially important shark species that is threatened with extinction due to overfishing. Mitochondrial DNA (mtDNA) displacement loop (D-loop) sequence data from 18 Northwest (NW) Atlantic and 30 Southwest (SW) Pacific porbeagles reveal that these regional populations have been genetically separated between 1.39 and 1.25 million years ago (MYA), a time frame which correlates with the end of the earthâ€™s last cooling period. There is far greater genetic differentiation (FST = 0.835) between the NW and SW populations than among sharks within each population supporting a very high level of divergence. A lack of gene flow probably stemming from their limited distribution to cold water temperatures (-1oC to 15oC) has led to their genetic divergence. The NW Atlantic population exhibited fewer haplotypes than the SW Pacific population (2 vs 4). The mean nucleotide diversity value of the NW Atlantic population was also 50% lower (0.00143 vs. 0.00228). Male and female NW Atlantic individuals reflected virtually identical mean population diversity values (0.00393 vs 0.00399); however, females were prevalent near shorelines while the males were more often found in open waters. Of the three age groups within the NW Atlantic population, the immature individuals exhibited the greatest mean nucleotide diversity (0.00452), followed by the sub-adult group (0.00293) and the mature group (0.00288), suggesting that dispersion starts earlier in their life cycle and reduces as they get older. The porbeagle population biology, as revealed by D-loop sequence information, may have significant implications for the conservation efforts of this species. As differences in age-based and sex-based dispersion exist, it is important to

understand the relative contributions of gene flow by adults of both sexes in order to implement more effective conservation strategies.

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INTRODUCTION

Porbeagle sharks play a critical role in the ecosystem by maintaining the species below them in the food chain and serving as an indicator for ocean health (Edmunds & Meredith 2011). According to a 2013 Appendix to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), the porbeagle shark is heavily fished for its fins and meat, which has resulted in the IUCN Red List of Threatened Species™ classifying the porbeagle as vulnerable globally and critically endangered in the NW Atlantic (Curtis et al. 2016). Unsustainable fishing and stock collapses of porbeagle are particularly well documented for the North Atlantic (Anderson 1990; Walker 1998; Stevens et al. 2000; Campana et al. 2002). The lack of sufficient harvest information makes it difficult to determine the current population numbers and structure, causing confusion and discrepancies with conservation and population management (Fields et al. 2015, González et al. 2020). Conducting population genetics studies is vital for accurate and effective species population management, which involves genetic conservation and wildlife management (Shivji et al. 2002). Through elucidating evolutionary history and the main patterns of intraspecies structure formation, researchers and government agencies can implement informed and effective conservation strategies. Mitochondrial DNA (mtDNA) analysis is a favored genetic strategy for uncovering population structure because it accurately reflects genealogical relationships and genetic composition, and figures prominently in conservation genetic management strategies, with much attention, focused on the use of the displacement loop (D-Loop) region (Dalton & 3 Kotze 2011) due to the region's propensity to accumulate mutations relative to the rest of the mtDNA (Clayton 2000). Based on the D-loop sequence data of the

porbeagle sharks from the NW Atlantic Ocean, this study examined their genetic structure and composition and compared them to their SW Pacific Ocean counterparts. Therefore, this study is extremely valuable for the genetic conservation and management of this shark species. The objectives of this study include the validation of a method of DNA extraction, amplification, and sequencing of the mtDNA D-loop for the analysis of population structure among NW Atlantic porbeagle sharks. The incorporation of previously published data from porbeagles caught in the SW Pacific provides a more comprehensive view of the current genetic conservation status of this species.

MATERIALS AND METHODS

Forty NW Atlantic porbeagle fin clips were obtained from the Canadian Atlantic Shark Research Laboratory Fisheries & Oceans (Canada Bedford Institute of Oceanography, PO Box 1006, Dartmouth, NS, B2Y 4A2). The sampling locations are presented in Table 1 and Figure 1. Approximately 100mg of tissue was subsampled from the fin clips for DNA extraction following Fields et al. (2015). At the Kanthaswamy DNA Laboratory at ASU, the Qiagen (Hilden, Germany) QIAmp DNA and Blood Mini Kit was used for the extraction process following the manufacturer's recommendation. Samples were incubated in proteinase K overnight for the greatest DNA yield, as suggested in the manufacturer's manual. The DNA extracts were quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's manual. NCBI Primer Blast online software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to generate custom primer pairs built on the porbeagle mitochondrial reference genome 4 available in NCBI GenBank (NC_033911.1) and the D-loop coordinate range (15,975-16,643) for sandbar sharks (Carcharhinus plumbeus) (Blower et al. 2013). Several sets of custom primers were generated and after close evaluation of the primer pairs for specificity, non-specific PCR products, primer-dimers, length of the targeted amplicon, and amplification conditions, the primer set that was chosen included the forward primer (5'-TAA GAA CAT CGC ATC CCG CT-3') and reverse primer (5-ATA TGT CCG GCC CTC GTT TT-3') which generated the longest amplicon, approximately 668bp. PCR amplification of the DNA samples was conducted using the following amounts of reagents in each reaction: 0.25uL dNTP, 1.25uL reaction buffer, 0.35uL MgCl 2 , 0.25uL forward primer,

0.25uL reverse primer, 0.06uL Taq polymerase, 4.09uL DI water, and 8uL 5ng/uL DNA. The total reaction volume was 14.5uL. Thermocycling conditions were 94oC for 5 minutes, 94oC for 30 seconds, 60.10oC for 30 seconds, 72oC for 30 seconds, 72oC for 7 minutes, and 4oC until removed for storage for a total of 30 cycles. PCR amplification was confirmed as successful with 1% agarose gel electrophoresis using the GeneRuler 50 ladder (Thermo Fisher Scientific) for sizing and approximate quantification of double-stranded DNA in the range of 50 to 1000bp, which is optimal for the detection of the 668bp products anticipated in this study. Sanger sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Nimagen, BV, the Netherlands) through DNASU at the Biodesign Institute at ASU. DNASU used the Applied Biosystems Genetic Analyzer 3730xl (Thermo Fisher Scientific) to generate all sequence data for this study. For quality control of the sequence data, sequences with a Phred quality score of 20 or more were used for subsequent analysis, meaning the probability of an incorrect nucleotide identification is 1 in 100, and the nucleotide identification is 99% accurate (https://www.thermofisher.com/blog/behindthebench/sanger-sequencing-by-ce-4-bioinformatics/ 5). The quality validated sequences were then aligned in the forward 5' to 3' direction using the CLUSTALW alignment (Edgar 2004) software program included in the MEGA-X package (Kumar et al. 2018). Phylogenetic and molecular evolutionary analyses were conducted using MEGA-X. A phylogenetic tree was generated using the maximum likelihood (ML) method using a bootstrap analysis with 100 replicates (Wilgenbusch & Swofford 2003). The ML method relies on a full parametric approach to estimate the tree by choosing the tree with the highest probability of occurring given the data. The method ignores all evolutionary models and searches for

the tree with the least number of mutations along its branches needed to explain the data. A D-loop sequence from a great white shark (Carcharodon carcharias; GenBank accession no: MK088126.1) was used as an outgroup to help root the phylogeny and determine which porbeagle lineages on the tree are the oldest and which character states are ancestral. A shortfin mako shark (Isurus oxyrinchus; GenBank accession no: MF537044.1) and a salmon shark (Lamna ditropis; GenBank accession no: KF962053.1) were also used as outgroups after using NCBI Blast to compare the complete mitochondrial genomes with novel sample POR001 to determine the D-loop ranges for each species. To illustrate the genealogical relationships at the intraspecific level and to make inferences about biogeography and history of porbeagle populations, a median-joining (MJ) haplotype network (Forster et al. 1996) was constructed using Network 4.612 (Bandelt et al. 1999). To help define the population structure of the NW Atlantic porbeagles more clearly, published D-loop sequence data from 30 Southwest (SW) Pacific individuals were also used in a separate phylogenetic analysis (González et al. 2020). MrBayes 3.2.7 was used for analyzing NW Atlantic, SW Pacific, and outgroup population divergence times (Huelsenbeck & Ronquist 6 2001). A relaxed clock model was used with an established rooting of the great white, shortfin mako, and salmon shark outgroups. A Markov chain Monte Carlo simulation with 100,000 generations was used to produce a tree with estimated divergence times in millions of years for each sequence, with each divergence time for the NW Atlantic, SW Pacific, and outgroup populations documented through node dating. The partition of genetic variation within and among the NW Atlantic and SW Pacific populations was assessed via nested analysis of molecular variance (AMOVA) using Arlequin 3.5.2.2 (Excoffier & Lischer 2010). The same

program was used to compute D-loop sequence differentiation (pairwise FST) among the NW Atlantic, SW Pacific, and outgroup samples.

RESULTS

Eighteen samples generated forward and reverse D-loop sequences that met Phred scores of 20. These samples consisted of six female and 12 male sharks, including two sub-adult, ten immature, and six mature individuals. Multiple alignments of the 530 bp of the porbeagle D-loop revealed that among the three outgroup species, Lamna ditropis was the closest related outgroup species to the porbeagle shark while the Carcharodon carcharias was the least related (Figure 2). Based on the 530 bp sequence analysis, five NW Atlantic females and ten males exhibited haplotype A1, while one female and two other males exhibited haplotype A2. However, the sequence diversity among the NW Atlantic males and females was identical ($\pi = 0.00393$ vs. 0.00399). Nine immature, one subadult, and five mature NW Atlantic individuals belonged to haplotype A1, and an immature, a subadult, and a mature individual belonged to haplotype A2. The immature age group exhibited the highest diversity ($\pi = 0.00452$), followed by subadults ($\pi = 0.00293$) and mature adults ($\pi = 0.00288$). The mean diversity values within subpopulations were comparable between the sex and the age categories ($\pi = 0.00396$ vs 0.00344). Figure 1 7 shows the number of samples per NW Atlantic haplotype with frequency pie charts representing each sampling location. These findings suggest that there could be an increase in genetic diversity as the sampling locations moved farther from the shoreline. There was an approximately 230 bp overlap between the 18 lab-generated NW Atlantic Porbeagle sequences and the 30 SW Pacific porbeagle sequences. To ensure that the π estimations were not affected by the sample size difference between NW Atlantic and SW Pacific data sets, π values from 18 random samples from the SW Pacific were estimated and compared against the entire sets of NW Atlantic as well as

SW Pacific sequences. The 30 as well as 18 SW Pacific sample sets exhibited much greater mean diversity (π = 0.00228 and 0.00363, respectively) than the NW Atlantic group (π = 0.00143). Because the subset of 18 SW Pacific sequences showed the highest π value, it was concluded that there was no effect of sample size on all π estimations. Based on this conclusion, the NW Atlantic population highlighted a slightly lower level of differentiation than the SW Pacific population, with a mean π of 0.00143 compared to 0.00228 (Table 2). The partition of genetic variation within and among the NW Atlantic and SW Pacific porbeagle populations was investigated using hierarchical AMOVA. According to this analysis, the level of variation within the regional populations of porbeagle, i.e., differences among the NW Atlantic and SW Pacific D-loop lineages, respectively, were only 16.51% (Table 3). However, much higher genetic variation (83.49%) was attributable to genetic differences between the NW Atlantic and SW Pacific lineages (Table 3). The pairwise FST estimate of 0.835 (Table 4) also concurs with the AMOVA estimate that there is a high level of differentiation between the two oceanic populations. The results corroborate their divergence time between 1.39 and 1.25 MYA (Figure 3). Concordant with the phylogenetic trees (Figure 2 and 3), the D-loop 8 network's topology (Figure 4) also supported that the outgroups were highly distinct from both porbeagle populations. The time of divergence estimated in the present study suggests that the NW Atlantic and SW Pacific populations likely separated from the outgroup population at approximately 1.54 MYA (Figure 3). The network in Figure 3 showed that the individuals from the NW Atlantic sources fell into two well-supported clusters, i.e., A1, and A2, to the exclusion of all those from the SW Pacific. Among the two NW Atlantic clusters, A1 was the largest cluster with 28 haplotypes, followed by A2

with six haplotypes. Only one mutational step separated the A1 and A2 haplotypic clusters. Those from the SW Pacific sources were subdivided into four separate haplotypic clusters. The largest P2 comprised 24 haplotypes, followed by P1 with three haplotypes, P3 with two haplotypes, and P4 with only one haplotype. The 24 P2 haplotypes created a pitchfork branching pattern in Figure 3, suggesting that these haplotypes are all equally closely related. The smaller clusters in the network depicted in Figure 4 appeared as genetic isolates as they did not cluster with any of the major clades. Upon closer examination, P1 and P2, as well as P2 and P3 were separated by one mutation step, respectively, while P4 was the most divergent cluster from the remaining ones with two mutation steps separating it from P1 and four from P2.

DISCUSSION

The study herein represents the first population genetic analysis of the NW Atlantic porbeagle shark population. Based on the mtDNA D-loop sequence variation observed in this study, the NW Atlantic and SW Pacific porbeagles exhibited a haplotype composition that was reciprocally monophyletic. The concomitantly high pairwise differentiation of 0.835 suggests that the NW Atlantic and SW Pacific porbeagle populations do not interbreed and are effectively 9 genetically differentiated from each other. This genetic split between NW Atlantic and SW Pacific control region mtDNA is further supported by the presence of up to five hypothetical mutational steps, whereby only as few as three mutations are required for demonstrating reproductive isolation (Blanckaert et al. 2020). Other studies on Northern and Southern porbeagle mtDNA that involved areas around the coast of Chile, the Mediterranean, the Bay of Biscay and the Celtic Sea, North and South Atlantic, and North and South Pacific have also revealed this northern and southern hemispheric genetic divide (Kitamura & Matsunaga 2010; Testerman 2014). Warmer waters are not part of the natural habitat of porbeagles; typically, these sharks prefer waters that are -1°C to 15°C (Kitamura & Matsunaga 2010). Although the porbeagle is an endotherm, its habitat selection could be due to the limited distribution of their poikilothermic prey (Joyce 2002). According to González et al. (2020), porbeagles migrate latitudinally between 30° and 60° in the northern and southern hemispheres and avoid tropical regions. The genealogy of the porbeagle D-loop haplotypes reveals that the NW Atlantic and SW Pacific populations probably became reproductively isolated at least 1.25 MYA. This estimated time of divergence coincides with the gradual cooling of the earth's climate until around that time (Snyder 2016). The

porbeagles were likely more widely distributed geographically when the oceans were cooler. As a dispersal barrier of warm equatorial waters gradually emerged, it effectively reduced their territory to cooler climes and separated the populations into genetically distinct hemispheric groups. Although most samples from both populations in this study belonged to a specific haplotype in the NW Atlantic (A1) and the SW Pacific (P2), the SW Pacific population was characterized by a slightly higher haplotypic diversity than the NW Atlantic population. 10 Although the SW Pacific population was also slightly more structured, there was a clear genetic subdivision observed among the NW Atlantic porbeagles between individuals near the shorelines and those in the open waters. For instance, the number of porbeagles exhibiting different haplotypes (A1 or A2) increased further away from the shoreline. While five females and 10 male individuals exhibited haplotype A1, only one female and 2 male individuals exhibited haplotype A2. The difference in the π between females (0.00399) and males (0.00393) was negligible even though adult females tended to stay closer to the shorelines as adult male dispersed into the open waters. This differential sex-based distribution has probably resulted in sex-biased gene flow in the different habitats due to sex-specific territorial feeding and reproductive behaviors. These findings, however, are not unique to porbeagles as similar population dynamics have been reported in great white sharks (Pardini et al. 2002). A similar sex-based haplotype comparison could not be made on the SW Pacific samples due to the unavailability of sex information in González et al.'s (2020) study. All immature individuals in NW Atlantic belonged to haplotype A1, except for one that carried haplotype A2. Among all the NW porbeagles that were examined, the D-loop sequences from immature sharks showed the greatest mean π (0.00452), followed by

those from subadults (0.00293) and mature sharks (0.00288), respectively. This implies that immature porbeagles are more prone to dispersal until they reach adulthood, where the adult females become less migratory than their male counterparts (Corrigan et al. 2018). The presence of an immature individual at each sampling location also provides additional support that immature porbeagles are widely dispersed. Both male and female immature individuals were similarly dispersed throughout the sampling region in this study. Based on the D-loop sequence data, genetic differentiation increases as the distance from 11 the NW Atlantic coastline increases. All female individuals in this study exhibited haplotype A, which was found more commonly close to shorelines as a whole. Female geographic ranges were 43.001 to 47.905 latitude and -50.136 to -61.288 longitude. The prevalence of female porbeagles along the shorelines suggests these areas may serve as potential natal and breeding grounds, which has been documented in this species in other locations. For example, Biais et al. 2017 found PSAT tagged female porbeagles migrating away from the Bay of Biscay at the end of summer and returning the following spring to give birth potentially. While pregnancy was not confirmed at the time of tagging, the presence of young of the year sharks shortly after that further suggests the possible importance of nearshore areas at natal grounds and the need to protect these regions from anthropogenic activities (Keeney et al. 2005). Immature white sharks also exhibited a proclivity to stay close to their nursery area, with some of the individuals returning to the same area the following spring, which further supports that those areas prevalent with young of the year individuals are likely natal and nursery grounds (Curtis et al. 2018). However, a much larger sample size representing a wider distribution of the porbeagle belonging to different age cohorts would be needed for such

an analysis based on the data presented herein. The analysis of mtDNA from NW Atlantic female and male porbeagles, including immature, sub-adult, and mature individuals in the present study, has shed some light on the matrilineal genetic composition and structure in that region. It will be of great interest to determine if gene flow in the NW Atlantic is male- or female-biased, along with longitudinal haplotype frequency data across the Atlantic. Females being more prone to stay in natal grounds gives support to gene flow being male-biased. Male-biased gene flow has different conservation implications than female-biased gene flow; for instance, as porbeagle males are the more dispersive sex, they are more likely products of bycatch or other human activities. Suppose male 12 dispersion is responsible for promoting gene flow among natal and breeding sites. In that case, the removal of males may lead to genetic subdivisions among these sites and loss of genetic diversity. While mtDNA is very useful for population, biogeographic and phylogenetic studies, it is not without shortcomings. For example, due to its transmission along matrilines, it cannot be used to quantify male-biased gene flow (Oliveira et al. 2017). Consequently, mtDNA is strictly a marker for assessing female porbeagles' contribution toward the species' historical processes. If male and female porbeagles exhibit different life histories, then this matrilineally transmitted marker would not reflect the complete evolutionary history of the species. Determining rates of male- or female-biased gene flow, particularly when they are quite different, is important for understanding the relative importance of gene flow and other sex-specific evolutionary factors (Hedrick 2007). For example, in bonnethead sharks (Sphyrna tiburo), female philopatry fosters the sorting of locally adaptive variation, while male-biased dispersal promotes the movement of potentially adaptive variation among different

locations and environments (Portnoy et al. 2015). As such, further analyses using male-specific Y-chromosomes, a natural counterpart to mtDNA, are indispensable for a more comprehensive analysis of porbeagle phylogeographic and population genetic patterns. Currently, Y-chromosome analysis remains underexplored in porbeagle population genetic studies, as well as population genetic studies over other shark species.

Figure 1

Map of the sampling locations based on GPS coordinates. Also shown are the haplotypes of the individual sharks and the number of haplotypes per location. Haplotype A1 is red, and haplotype A2 is blue.

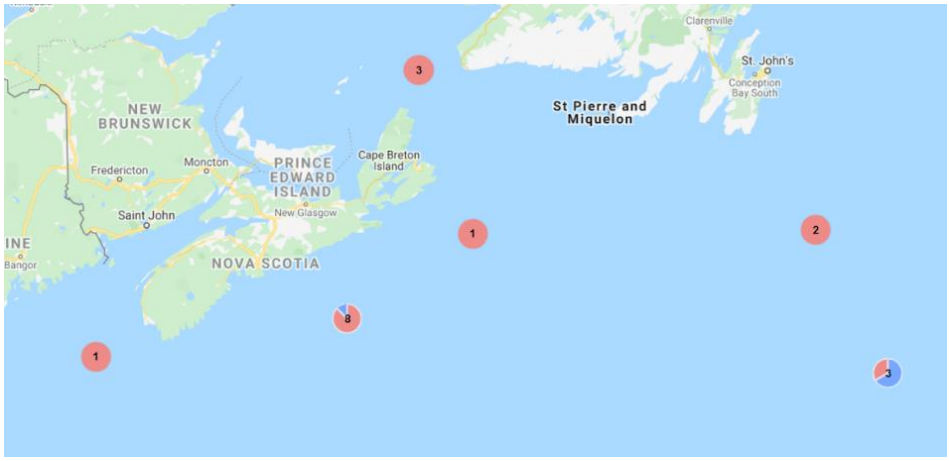

Figure 2

Phylogenetic tree of Atlantic and outgroup populations based on the 530bp overlapping D-loop sequences, generated using the maximum likelihood method with 100 bootstrap replicates.

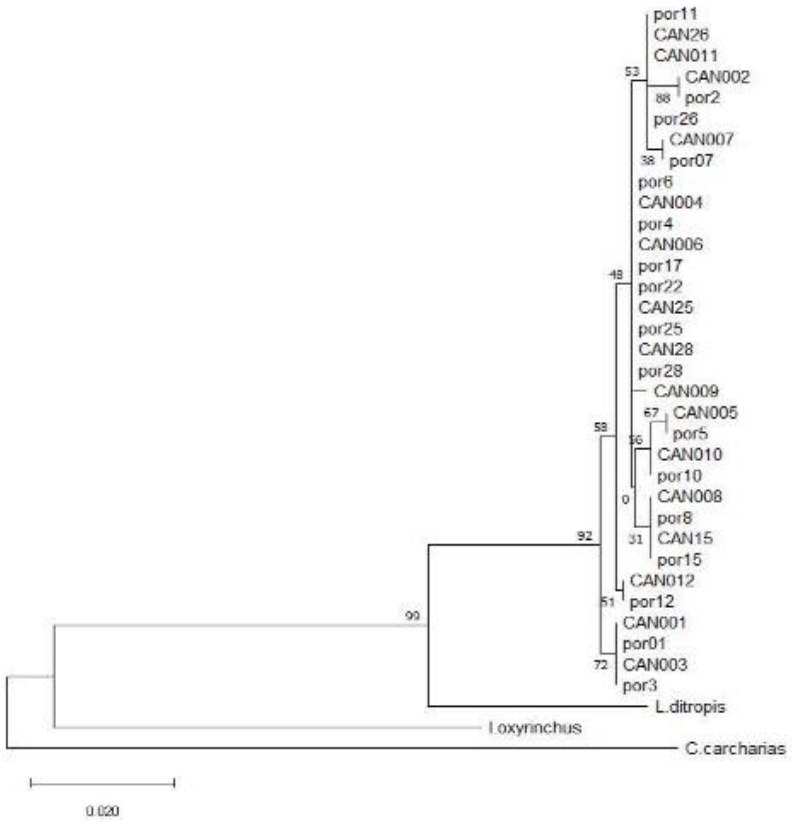

Figure 3

Phylogenetic tree including the NW Atlantic, SW Pacific, and outgroup populations based on the 223bp overlapping D-loop sequences, generated using the maximum likelihood method with 100 bootstrap replicates.

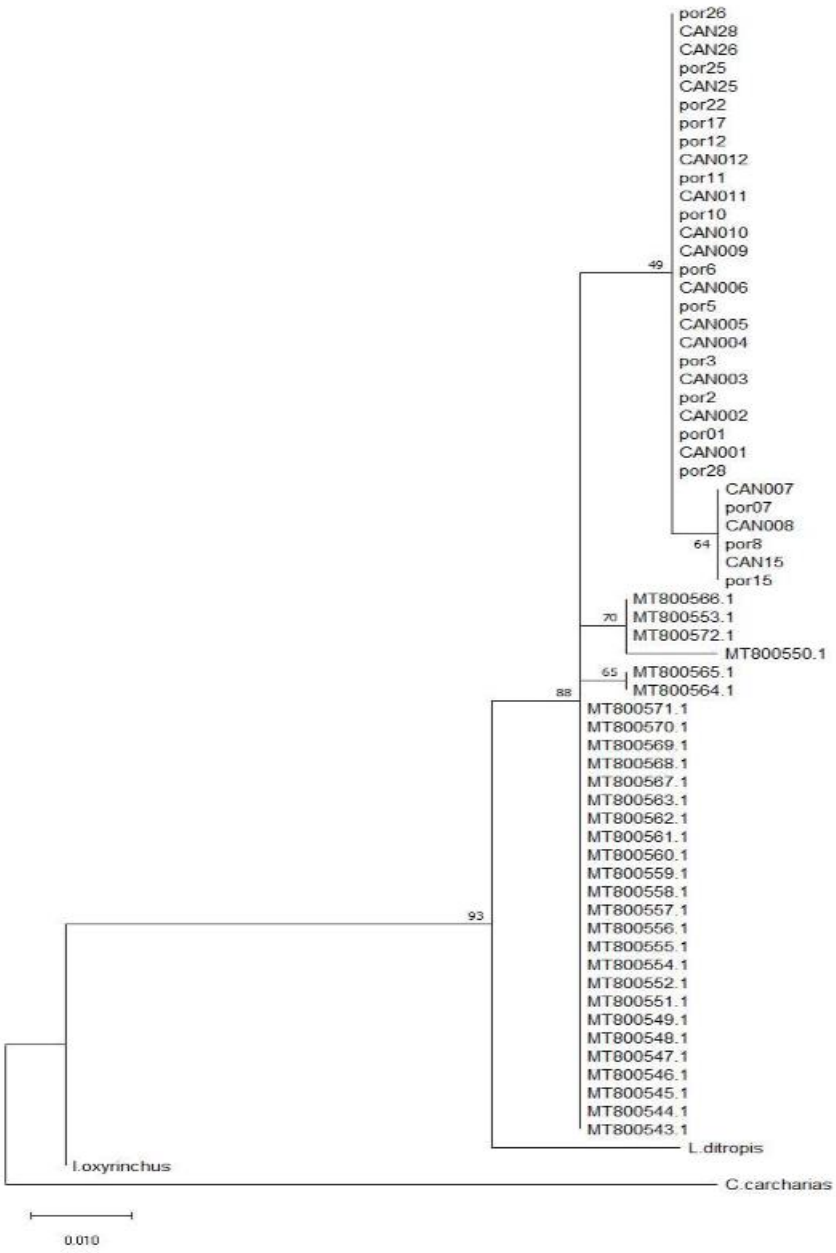

Figure 4

Network analysis of the NW Atlantic, SW Pacific, and outgroup populations based on the 530 bp overlapping sequences. A1 and A2 represent the NW Atlantic population, and P1 represents the SW Pacific population, respectively; outgroups represent the salmon shark (LD), the mako shortfin shark (IO), and the great white shark (CC), respectively. The size of the circle indicates the frequency of the haplotype, and the dashed lines indicate the genetic distance between haplotypes. The small red dots on the nodes show median vectors representing hypothetical connecting sequences or haplotypes, calculated with a maximum parsimony method. The number of haplotypes within each clade is as follows: LD with 1; IO with 1; CC with 1; P1 with 3; P2 with 24; P3 with 2; P4 with 1; A1 with 28, and A2 with 6 haplotypes, respectively.

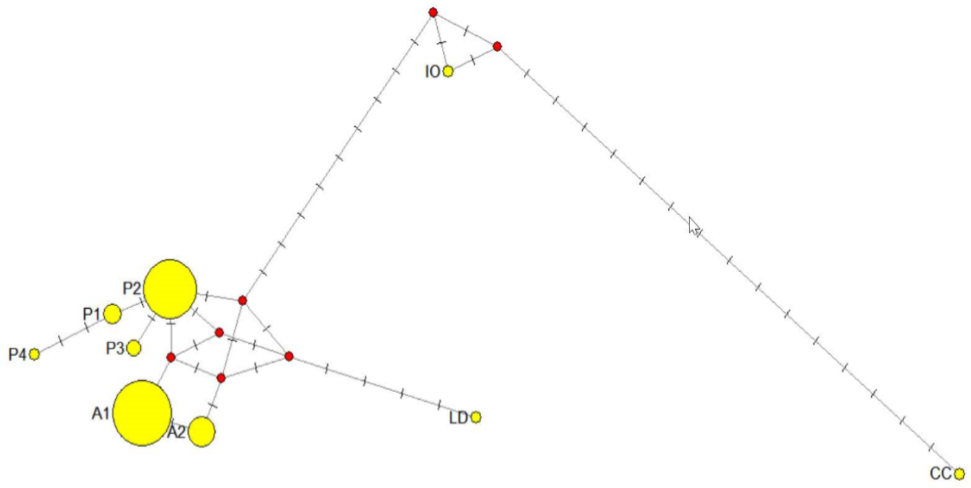

Table 1

Latitude and longitude coordinates of individual shark sampling locations.

Individual ID	Latitude	Longitude
1	44.101	-61.448
2	45.217	-51.695
3	43.155	-61.288
4	47.418	-60.410
5	44.391	-62.442
6	47.418	-60.410
7	44.391	-62.442
8	44.391	-62.442
9	43.001	-50.136
10	47.905	-59.866
11	44.824	-58.993
12	44.101	-61.448
15	43.001	-50.136
17	45.217	-51.695
22	43.155	-61.288
25	43.155	-61.288
26	43.001	-50.136
28	42.953	-67.157

Table 2

Comparisons of population diversity of the 223bp overlapping D-loop sequences.

	Mean Diversity in Entire Population	Mean Diversity Within Subpopulation	Mean Interpopulation Diversity
Atlantic	0.00405		
Female	0.00399		
Male	0.00393		
Both sexes		0.00396	0.0000924
SA	0.00293		
Immature	0.00452		
Mature	0.00288		
All ages		0.00344	0.000612
SW Pacific/NW Atlantic	0.00657	0.00185	0.00472
SW Pacific	0.00228		
NW Atlantic 223bp	0.00143		
SW Pacific 18	0.00363		
SW Pacific 18/NW Atlantic	0.00634	0.00217	0.00417

Table 3

Outline of the AMOVA framework for the Atlantic and Pacific populations with the degrees of freedom.

Source of variation	Degrees of freedom	Variance components	Percentage of variation
Among Porbeagle populations	1	1.023	83.49
Within NW Atlantic and SW Pacific populations (Among regional D-loop lineages)	60	0.202	16.51
F_{ST} = 0.835	61	1.226	

Table 4

Population pairwise F_{ST} estimates among the NW Atlantic, SW Pacific, and outgroup populations.

	NW Atlantic	SW Pacific
SW Pacific	0.835	-
Outgroup	0.880	0.846

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