Advancing White Shark Conservation in the Monterey Bay, CA through eDNA-based Population Monitoring and Community Education

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Abstract

White sharks (*Carcharodon carcharias*) are important to the ecological stability of the northeastern Pacific, serving as top predators and shaping the size and distribution of other marine animal populations ("White Shark"). However, they are difficult to detect and quantify because of their low density, large ecological niche, and high mobility (Merson et al.). Environmental DNA (eDNA), the DNA derived from the cells organisms shed into their surroundings, have the potential to supplement current surveying methods (visual surveys and acoustic tagging) and provide more robust information on where white sharks tend to occur and aggregate. Though the current literature has identified eDNA as a non-invasive means of detecting the presence of white sharks, its use in quantifying the number of white sharks in an area remains largely unexplored (Merson et al.). We aim to support the development of this technology through the synthesis of white shark-specific primers, which target and amplify regions of the genome (200-400 base pairs) that contain identifying information for individuals. We also aim to engage communities in white shark conservation awareness, creating educational content for the Monterey Bay White Sharks project. By approaching conservation through this two-pronged approach, the purpose of this work is to support the protection of these critical species along the California coast and the northeastern Pacific.

Background

Surveying White Sharks in the Northeastern Pacific Ocean

White sharks (*Carcharodon carcharias*) are among the most iconic and well-studied species in marine biology. Known for their massive size (up to 21 feet in length and 4,500 lbs in mass), torpedo-shaped bodies, and white underbellies, these sharks are found in subtropical and temperate regions across the globe ("White Shark"). The Northeastern Pacific ocean contains a genetically distinct sub-population of these white sharks, who tend to migrate between regions along the western coasts of California and Mexico and the pelagic waters farther west (Jorgensen et al.). Previous survey-based studies have also indicated that these sharks display high site fidelity, aggregating at select regions for feeding, mating, and other life activities (Anderson et al.).

However, white shark populations are difficult to quantify and monitor due to their low density, large ecological niche, and high mobility (Merson et al.). Current monitoring methods rely primarily on visual surveys and acoustic tagging in white shark aggregate areas (Jorgensen et al.). While these approaches do provide scientists insight into the size, distribution, and movement patterns of white sharks in the Northeastern Pacific, they are costly to implement, invasive for the animals (as the tags are physically implanted into the sharks' skin), and biased to the sharks that are close to the water at the time of tagging and surveying. These limitations have sparked interest in genetics-based monitoring as a cost-effective, non-invasive supplement to current surveying means (Merson et al.).

Environmental DNA

Environmental DNA (eDNA) is the DNA derived from the cells organisms shed into their environments. This includes, but is not limited to: hair, skin, nails, blood, mucus, scales, and dermal

denticles. eDNA was first introduced in 1987 as a means to study microbial diversity in soil samples (Ogram et al.). In recent years, however, there has been interest in extending this technology to population size and distribution studies for marine macrofauna.

In a marine context, eDNA analysis begins with collecting water samples from a site of interest. After isolating the nucleic acids from other solid material (proteins, fats, debris, etc.), species-specific DNA primers are added to the sample and a polymerase chain reaction (PCR) is run to amplify and detect target DNA. Currently, eDNA analysis has been used to detect the presence/absence of white sharks in water samples (Merson et al.). However, its use in quantifying the number of white sharks in an area remains largely unexplored.

Identifying Individuals: Assembling Microhaplotype Panels

Simply detecting the presence of white sharks through eDNA is a feat in itself. The filtering and extraction process isolates all nucleic acid material contained in the water sample, meaning that the genetic material of small microorganisms, plants, and even other macrofauna are also present. The Monterey Bay National Marine Sanctuary (located in the Northeastern Pacific) presents an additional challenge, as several related shark species—namely, shortfin mako sharks (*Isurus oxyrinchus*), salmon sharks (*Lamna ditropis*), and blue sharks (*Prionace glauca*)—are also found in and around sampling areas (Burton and Lea). Amplifying only white shark DNA in the polymerase chain reaction requires DNA primers that bind regions of the genome that are unique to white sharks.

Distinguishing between individual sharks of the same species, however, takes this a step further. White sharks share around 99.9% of their DNA between individuals, necessitating primers with even higher specificity (Wagner et al.). To differentiate white sharks from one another, we must target short regions of high intraspecific genetic variation. This variation is found through SNPs, or single nucleotide polymorphisms. These are sites in the genome with a single nucleotide base difference (Wagner et al.). White sharks have tens of millions of SNPs in their nuclear DNA, and combinations of these variable sites are unique to each individual.

To improve efficiency of capturing these SNPs, recent studies on other marine macrofauna have targeted microhaplotypes, which are short regions of the genome (around 200-400bps) that contain three or more SNPs (Meenakshisundaram et al.). Since these points of variation are located so closely together, they are linked and inherited together on the chromosome. Identifying multiple sites of high genetic variation allows us to assemble a microhaplotype panel, which provides enough information to form a genetic "barcode" that is unique to each individual shark (Kidd and Speed).

Targeting the Nuclear Genome

Though prior intraspecies diversity studies have primarily targeted the mitochondrial DNA (mtDNA) of species, for the purposes of this analysis, we are assembling microhaplotypes within the nuclear genomes of white sharks (Jorgensen et al.). Mitochondrial DNA was originally used for eDNA studies due to their high copy number per cell, meaning there is a higher chance that the primers introduced during PCR will bind to and amplify the target region (Andres et al.). The limitations of mtDNA in

assembling microhaplotype panels, however, are that they are haploid and nonrecombining (inherited through the mother). Therefore, they would not be able to distinguish between siblings, as they would likely have the same mtDNA. Though nuclear DNA is more difficult to recover in an eDNA sample due to its lower copy number, it contains more genetic diversity and thus has more potential to tell individuals in a population apart (Andres et al.).

Methods

Generating Primers via Primer-BLAST

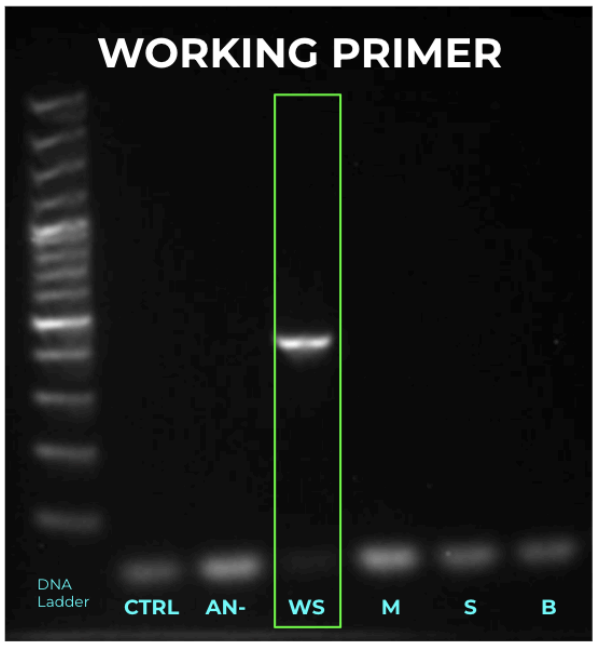
SNP-dense regions were first identified in the white shark genome, then run through the NIH Primer-BLAST software. A combination of the Primer 3 and genome BLAST programs, Primer-BLAST streamlines the process of identifying appropriate DNA primers for target regions and ensuring their specificity to the species of interest (National Library of Medicine). The software allows for users to input their sequence of interest, select parameters for the amplicon and associated primers, and view results in a CSV format. From here, we cross-checked each amplicon with our list of white shark SNPs to determine the number of variable sites captured within each primer.

Testing Primer Specificity via Touchdown PCR

After identifying potential primers through Primer-BLAST, each was tested in-lab using a touchdown polymerase chain reaction (Touchdown PCR or TD-PCR) to ensure specificity to white sharks. TD-PCR is a modified polymerase chain reaction aimed at providing greater specificity and sensitivity

for the target amplicon (Korbie and Mattick). It achieves this by employing a higher initial annealing temperature (around 5-10°C greater than the primer melting temperature, T_m), then lowering the temperature in each successive cycle. This provides an early competitive advantage to the target amplicon site, as the primers will only bind and duplicate the DNA at these high temperatures if it is a 100% match (Korbie and Mattick). Thus, by the time the temperature "touches down" at a value equal to or slightly less than T_m, the target amplicon will be at a higher concentration than other non-target sequences in the sample.

To check our primers, we ran a TD-PCR reaction with a control sample of nuclease-free water, an environmental negative sampled from the aggregate site Año Nuevo during a season when white sharks are not present, white shark (*Carcharodon carcharias*) tissue, salmon shark (*Lamna ditropis*) tissue, shortfin mako shark tissue (*Isurus oxyrinchus*), and blue shark (*Prionace glauca)* tissue.



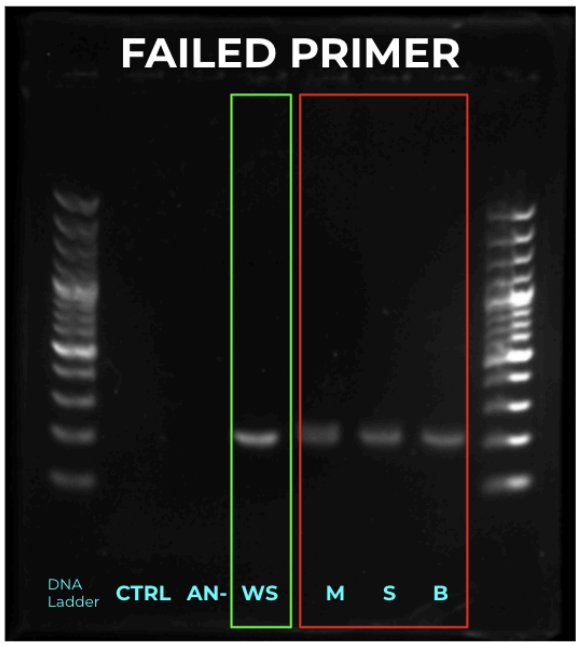


Fig. 1. Gel electrophoresis results for a working primer versus a failed primer. From left to right, the samples loaded in each well for the PCR reaction are (a) DNA ladder, (b) control (nuclease-free water), (c) white shark negative environmental control, (d) white shark tissue, (e) shortfin mako shark tissue, (f) salmon shark tissue, and (g) blue shark tissue.

Results

Effective Number of Alleles

Of the 11 primer pairs (including the forward and reverse primers) tested in the lab through TD-PCR, we identified two new viable primer sets that are (a) SNP dense and (b) specific to a one region of the white shark genome. These primers, located on scaffolds 3 and 153 of the white shark genome, were added to the existing microhaplotype panel assembled by Stanford PhD student Raksha Doddabele.

To assess the quality of each microhaplotype in providing identifying information, we calculated the effective number of alleles (Ae). This value provides a more accurate measure of the genetic variation contained within a locus by accounting for the frequency of each possible allele in the population (Kidd and Speed). In short, commonly occurring alleles are given more weight than rarer alleles.

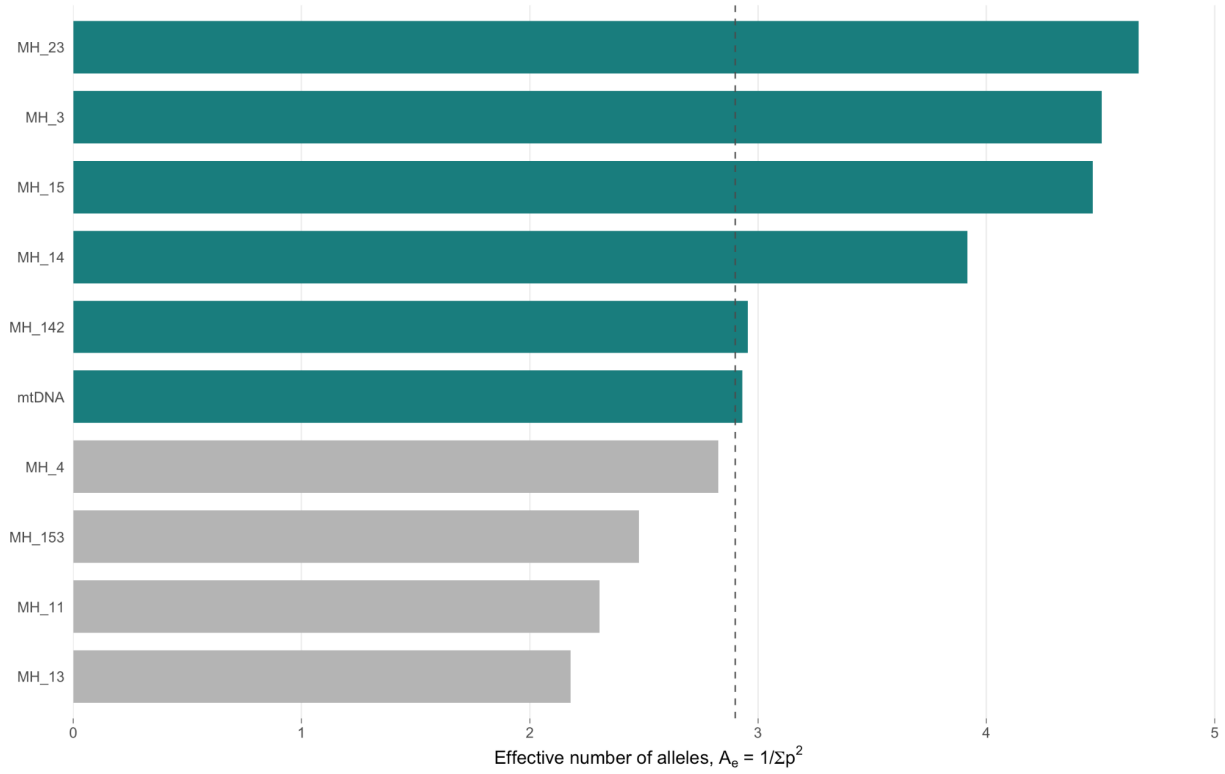


Fig. 2. Effective number of alleles across microhaplotypes. The threshold for ideal microhaplotypes was set to 2.9 in the graph to capture all microhaplotypes with effectively 3 alleles (ie. values > 2.9).

Previous studies indicate that the ideal microhaplotype for mixed sample detection and deconvolution should contain 3 or more SNPs (Kidd et. al.). In calculating the effective number of alleles for the nine microhaplotypes currently included in the panel, we can see that six microhaplotypes effectively meet or exceed this threshold (Fig. 2). However, the four other microhaplotypes with Ae values between 2 and 2.9 are still useful to include in the panel because they provide additional identifying information, increasing the cumulative probability of distinguishing between individual sharks.

Probability of Identity

To assess the quality of the microhaplotype panel in identifying individual white sharks, we calculated the probability of identity (PID) for the set of target loci. PID is defined as the probability that two randomly chosen individuals from a population will have the same genotype across a set of loci ((Kidd and Speed)). Mathematically, it is the inverse of Ae, meaning as the number of effective alleles in a set increases, the probability of identity decreases (it is less likely that two individuals will have the exact same genotype). The mitochondrial DNA (mtDNA) microhaplotype was omitted from this calculation as it is haploid and nonrecombinant.

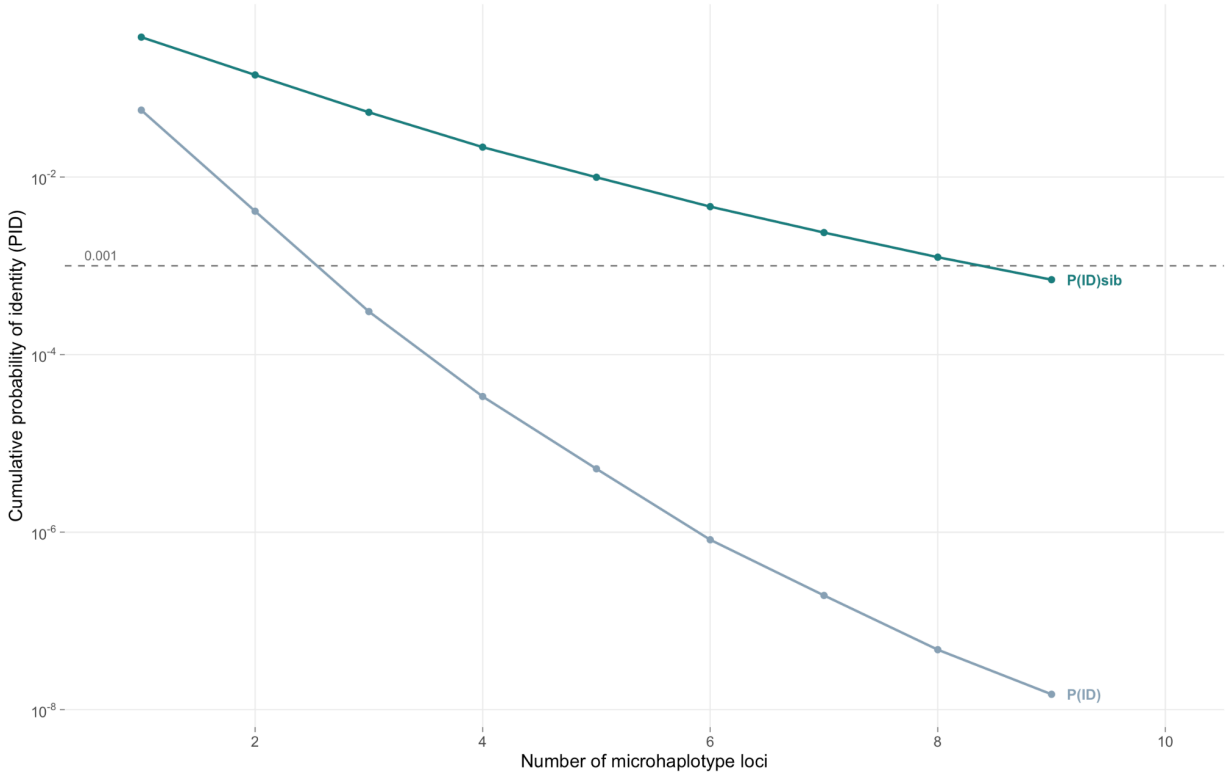


Fig. 3. Probability of Identity (PID) for current microhaplotype panel.

Since the population of white sharks in this region of the Northeastern Pacific is estimated to be 200-300 individuals, the threshold for significance was set to 0.001 or 1/1000 (Kanive et al.). This

ensures with high confidence that the microhaplotype panel is able to tell individual sharks apart. However, this population also contains several siblings, which presents an additional challenge because they share around 50% of their variable regions. To account for this, we calculated the probability of identity for siblings (PIDsib) alongside the probability of identity for unrelated individuals (PID). In observing the results, we can see that the PID drops below the 0.001 threshold after adding the third microhaplotype, while the PIDsib crosses the threshold after the addition of the ninth microhaplotype (Fig. 3). This indicates the need for additional microhaplotypes to account for sibling relationships and complete the microhaplotype panel.

Discussion

Limitations

The process of selecting white shark-specific primers did present challenges, decreasing our success rate (2/11 working primer sets) in the lab phase. The main roadblock stemmed from limitations in available genomic data for related species of sharks, which reduced the effectiveness of Primer-BLAST. The "core_nt" reference database used for the specificity-checking part of the program only contains the shortfin mako shark (*Isurus oxyrinchus*) nuclear genome: the salmon shark (*Lamna ditropis*) and blue shark (*Prionace glauca*) nuclear genomes are currently unassembled. Thus, Primer-BLAST is unable to check if the primers created map to parts of the salmon shark or blue shark genomes.

Next Steps

After assembling the microhaplotype panel, the next step in the process is to test the compatibility of these primers in a multiplex PCR reaction. A multiplex PCR is another modified polymerase chain reaction in which multiple target DNA sequences are duplicated in the same reaction ("What is multiplex PCR?"). This is important for our eDNA analysis because it allows us to selectively amplify all of the target microhaplotypes in one PCR reaction, then sequence them to identify the SNPs. Thus, factors like similar melting temperatures (T_m) between primers are important to note because it can interfere with the specificity and efficacy of the Touchdown PCR procedure. Though we accounted for compatibility of primers in our online primer design, it is important to test its efficacy in the lab as there may be interactions that are not accounted for in the algorithm. If found to be effective, however, this microhaplotype panel could be an important step towards conducting genetics-based population studies on white sharks in the Northeastern Pacific.

Supplementing Conservation Science with Community Education

In conjunction with the genetics portion of this project, we also created educational content for the Monterey Bay White Sharks Project (MBWS). It is a collaboration between the Block Lab at Stanford Hopkins Marine Station, the Costa Lab at the University of California Santa Cruz, the Garza Lab at the University of Washington, and the Center for Blue Economy at the Middlebury Institute at Monterey. The project was created to better understand and communicate the interactions between oceanic conditions, marine mammal distribution, and white shark occurrence in the Northeastern Pacific ocean. As part of their community education initiative, we curated content for their social media accounts, posting three times a week. The content posted ranged from team member spotlights

and reviews on new papers/projects to white shark features and fun facts. Our goal was to (a) counteract the fear and aversion around white sharks due to popular culture by providing more educational, desensationalized content, and (b) make conservation science (and scientists) more accessible to people of all backgrounds. This community-engaged component was an important supplement to the genetics work completed as part of the experience, as it helps with information sharing and creating constructive dialogue outside the scientific sphere.

Conclusion

We identified two new white shark-specific DNA primers that amplify microhaplotypes in the white shark genome. These were added to the microhaplotype panel assembled by Raksha Doddabele, bringing the PID and PID(sib) values of the panel below the target threshold of 0.001. If found to be successful in a combined multiplex PCR reaction, the assembly of this panel could be an important step towards quantifying white shark populations in the Monterey Bay National Marine Sanctuary through environmental DNA (eDNA) analysis.

To improve the process of identifying and selecting primers on Primer-BLAST, a publicly available assembly of the salmon shark (*Lamna ditropis*) and blue shark (*Prionace glauca*) nuclear genomes would be helpful. This would allow the program to more accurately detect if the DNA primers synthesized have any matches to these related shark genomes.

As a complement to the genetics research conducted through this project, we also worked on creating educational content through the Monterey Bay White Sharks (MBWS) project. By taking this two-pronged approach to conservation science, the intent is to equip scientists with tools to effectively monitor populations while making conservation more accessible and engaging to the broader public.

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