# GENOTYPING HISTORIC ATLANTIC TOMCOD SAMPLES TO DETERMINE THE TIMELINE OF ONSET OF PCB RESISTANCE

A Final Report of the Tibor T. Polgar Fellowship Program

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## ABSTRACT

It has been discovered that the contemporary population of Atlantic tomcod (*Microgadus tomcod)* in the Hudson River has developed resistance to coplanar PCBs and TCDD while populations of Atlantic tomcod from cleaner locales have not. The mechanistic basis of this resistance has been traced to a single genetic polymorphism in the aryl hydrocarbon receptor 2 (AHR2) gene. This variant polymorphism is a six base pair deletion in the AHR2 locus and can be used as a DNA marker of the resistant phenotype. This study was undertaken to determine if previous generations of tomcod in the Hudson River were also resistant, in effect dating when this dramatic evolutionary change took place. Between 1947 and 1977, approximately 1.3 million pounds of PCBs were discharged into the Hudson River. It was hypothesized that tomcod collected in the Hudson River from the 1930s would have a lower frequency of the variant AHR2 allele (AHR2-1) compared to present day tomcod population. DNAs from fin clips of Hudson River tomcod collected in 1936 and preserved in formalin were isolated using a modified version of the Dayton Protocol. The isolated DNAs were PCR amplified at primers specific to the AHR2 deletion polymorphism, digested with informative restriction enzymes, and electrophoresed on agarose gels. A subset of the isolated DNAs was successfully genotyped. It was determined that in the 1936 Hudson River tomcod sample, the AHR2 deletion allele (AHR2-1) frequency was 38%. This frequency is significantly less than in the current Hudson River population (96%). It was concluded that PCBs probably contributed to the rapid evolutionary change that occurred in the Hudson River tomcod population.

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## INTRODUCTION

The Hudson River currently contains the largest federal Superfund site in the United states (200 miles long) as a result of the release of 1.3 million pounds of PCBs from two GE electrical capacitor manufacturing plants located at Fort Edward (RM195) and Hudson Falls (RM 197) between 1947 and 1977 (Wirgin et al. 2011). PCBs are highly lipophilic, very persistent in the environment, bioaccumulate and biomagnify through the food chain. Atlantic tomcod *Microgadus tomcod* is a species of estuarine fish found throughout the northeastern United States and Canada and have a high risk of PCB exposure. This is because tomcod are bottom dwelling, feed on benthic invertebrate prey, and have lipid rich livers. Additionally, tomcod are resident in their natural estuaries year-round, increasing the likelihood of their exposure to industrial and municipal pollutants that are often released into contaminated waterways. However, Atlantic tomcod in the Hudson River have been found to show dramatic resistance to coplanar PCBs and TCDD early life stage toxicities and cytochrome P4501A (CYP1A) inducibility, while tomcod in cleaner estuaries such as the Miramichi River, New Brunswick (NB), and Shinnecock Bay, New York (NY) do not exhibit resistance. The mechanism of resistance is a six base pair deletion in the aryl hydrocarbon receptor 2 (AHR2) allele. Therefore, this variant allele (AHR2-1) serves as a marker of the resistant phenotype. Since the PCBs were first introduced into the river in 1947, examining the AHR2 allele frequencies in tomcod collected in the 1930s from the Hudson River may determine if PCBs were the driving agent to this resistance.

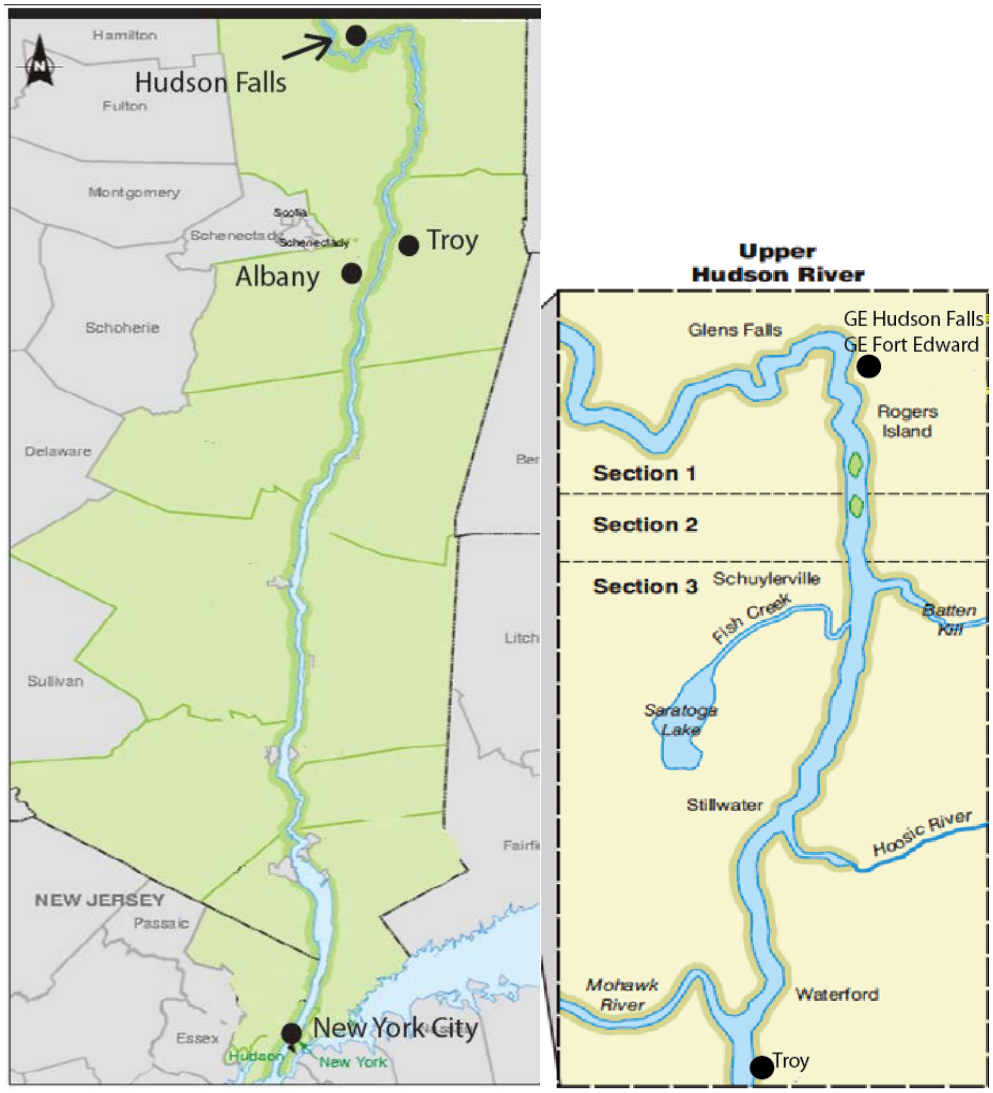



**Figure 1.** Map of the Hudson River, with an inset of the upper Hudson River indicating the two GE capacitor plants (EPA 2011)

PCBs are deposited into river sediment, and do not easily degrade naturally in the environment, which makes them very persistent in aquatic ecosystems including the Hudson River (EPA 2011). Additionally, PCBs bioaccumulate and biomagnify in the environment, which poses a severe threat for many species in the food web, particularly those at its apex, including humans (Roy et al. 2006). In 1976, it was demonstrated that many game fishes and other resource species in the upper Hudson River had been contaminated with PCBs at very high levels, which caused New York State to ban all

fishing in the northern Hudson River and limit consumption of most fishes throughout the estuary (EPA 2011). Bottom-dwelling fish species that are in direct contact with contaminated sediments and feed on benthic invertebrates are especially susceptible to PCB contamination as those are areas where PCB concentration is highest. Since PCBs are not readily degraded by the environment, remediation efforts such as dredging to remove PCBs are the most effective way to clean the river (EPA 2011). The first phase of dredging PCBs in the Hudson River took place in a six mile area of the upper Hudson River between May 15 and October 26, 2009. While this dredging successfully reached the Environmental Protection Agency's goal of removing ten percent of the contamination, there is still a significant amount of PCBs remaining in the Hudson River (EPA, 2011). The second phase of the dredging process began in May of 2011 and is scheduled for continue for at least six years.

Atlantic tomcod (*Microgadus tomcod*) are distributed in rivers throughout the northeast United States and Canada from north of the St. Lawrence River to the mouth of the Hudson River (Wirgin and Waldman, 2004). The tomcod in the Hudson River are the most southern spawning population of this species. Tomcod are estuarine, feed on small benthic invertebrates, and known to be the only winter-spawning fish species in the Hudson River (Yuan et al. 2006). Tomcod also have very lipid rich livers. This combination of diet, habitat, and liver composition of Atlantic tomcod in the Hudson River causes them to have an increased risk of exposure and bioaccumulation of PCBs (Roy et al. 2006). Their unique ecological niche and wintertime spawning make their young life stages critical prey in the Hudson River food web during the summer months (Carlson et al. 2009).

**Figure 2.** Atlantic tomcod (*Microgadus tomcod)*

While PCBs are known to cause early life-stage toxicities in many fishes and likely recruitment failure (Yuan et al. 2006), Atlantic tomcod in the Hudson River have been found to show a dramatic resistance to PCBs and dioxin-induced early life stage toxicities (Wirgin et al. 2011). In contrast, tomcod from cleaner locales are highly sensitive to the early life stage toxicities of these chemicals (Roy et al. 2006). There are usually tradeoffs to having this resistance, which can manifest as a significantly shorter lifespan, higher sensitivity to other environmental stressors, and less competency in life functions, such as reproduction (Yuan et al. 2006). It is thought that resistance is associated with severe evolutionary costs, which would be selected against under normal environmental circumstances (Wirgin et al. 2011). Therefore, as the Hudson River is remediated and the PCB concentration is decreased, there may be a rapid evolutionary shift in favor of non-PCB resistant tomcod.

Resistance to PCBs in the Hudson River tomcod population has been demonstrated to result from a single genetic polymorphism in the aryl hydrocarbon receptor (AHR2) (Wirgin, et al. 2011). This is the first identification of the mechanistic

basis of resistance to a toxicant in any vertebrate population and suggests that contaminants have evoked rapid evolutionary change in the Hudson River tomcod population by strong selection of a single variant gene at a single genetic locus. While humans and other mammals have one AHR pathway (AHR1), fish and birds have two AHRs (AHR1 and AHR2). In fish, binding efficiency assays have shown that AHR2 better binds TCDD and is likely more functionally active (Lin et al. 2001). The AHR pathway regulates genes that encode xenobiotic metabolizing enzymes such as cytochrome P4501A (CYP1A). Normally, when chemicals such as PCBs are present, this signaling pathway is activated by ligand binding to the AHR receptor in the cytoplasm. The bound complex travels to the nucleus, where it binds with aryl hydrocarbon receptor nuclear translocator (ARNT). This three part complex works to bind to specific recognition motifs, such as xenobiotic response element (XRE) and dioxin response element (DRE) in the promoter of genes in the AHR battery. This in turn activates transcription of dioxin responsive genes such a CYP1A (Roy et al. 2006). Most importantly, it has also been shown that activation of the AHR pathway co-occurs with the development of teratogenic effects in young life stages of fishes exposed to PCBs or dioxins. Furthermore, AHR knockout models are no longer sensitive to PCB induced early life stage toxicities confirming the essential role that AHR plays in mediating PCB and dioxin toxicities (Prasch et al. 2003). Consistent with this role, tomcod with the AHR polymorphism are no longer sensitive to PCB-induced early life stage toxicities or induction of the cytochrome P4501A gene (Roy et al. 2006).

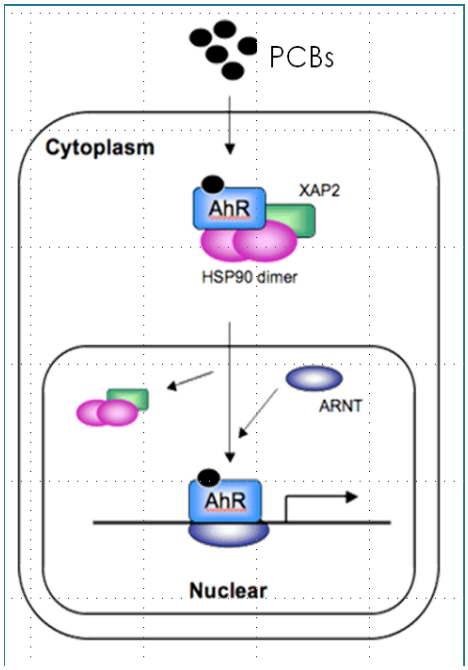



**Figure 3.** When PCBs are introduced, the AHR pathway is activated and the AHR complex translocates to the nucleus. In the nucleus, the HSP90 Dimer and XAP2 are lost, and ARNT binds to the complex. This allows the binding of dioxin response elements in the promoter of genes such as CYP1A. The complex then induces the transcription of dioxin response genes

One AHR2 polymorphism in tomcod is a six-base deletion downstream of the ligand binding domain of the AHR2 and resistance is heritable by Hudson River tomcod to at least the $F_2$ generation (Wirgin et al. 2011). It was shown that the resistant AHR2 allele is at least five times less effective than the normal AHR2 allele (AHR2-2) in binding TCDD (dioxin) and is significantly less able to drive reporter gene expression in AHR deficient mammalian cells that were treated with graded doses of PCB126 or TCDD(Wirgin et al. 2011). While there is nearly a

95% frequency of the AHR2 (AHR2-1) deletion variant allele in the Hudson River tomcod population, there is a 5% or less frequency of the AHR2-1 variant allele in Atlantic tomcod populations from other, nearby rivers. In previous studies, it was found that in the Hudson River population, only heterozygotes had the normal AHR2-2 allele and conversely, the AHR2-1 allele was only observed as heterozygotes in proximal cleaner rivers (Romeo and Wirgin 2011). This suggests that both alleles existed in all of the populations in the past in frequencies similar to what is seen today in the Miramichi River and Shinnecock Bay (Wirgin et al. 2011), and that the pollution of the Hudson River elicited a rapid evolutionary change in the tomcod population, leading to the differing observed frequencies of this allele today. Due to this, the AHR2-1 allele was considered a DNA marker of the resistant phenotype.

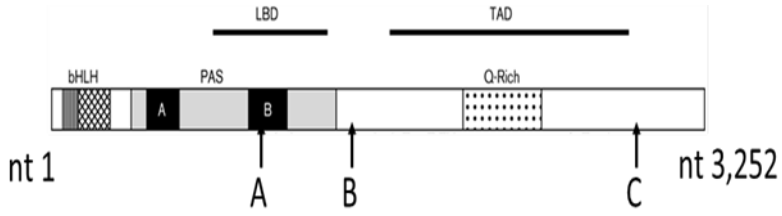



**Figure 4.** Functional domain of the AHR. A,B,and C show the three polymorphisms that all PCB-resistant fish have. “B” indicated the six base pair deletion

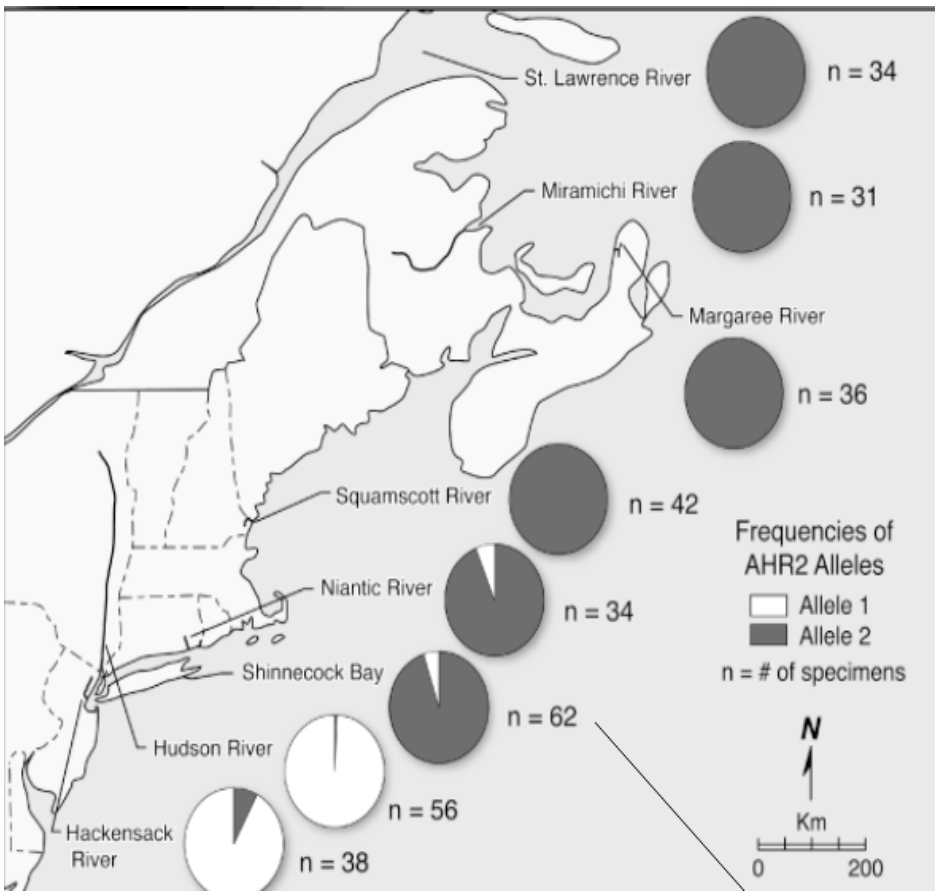



**Figure 5.** The current allelic frequencies of AHR2-1 and AHR2-2 in various tomcod populations in the northeastern United States and Canada.

tomcod population developed this resistance to PCBs is unknown. The goal of this project was to use the AHR2 DNA marker to evaluate when resistance developed in the Hudson River tomcod population. Originally, archived otoliths from tomcod collected from the Hudson River in the 1980s were to be used; however, sufficient DNA could not be obtained from these otoliths, so formalin-preserved fish collected in the 1930s from the Hudson River were used instead. It was hypothesized that fish collected from the Hudson River at earlier time points (prior to the release of PCBs) would have a reduced frequency of the AHR2-1 variant compared to tomcod collected today. This would support the notion that PCBs are the selective agents for the AHR2 variant, as well as date the onset of PCB resistance by the Hudson River Atlantic tomcod population.

Additionally, understanding the timeline by which Hudson River population tomcod became resistant to PCB toxicities can aid in the prediction and monitoring of the frequency of the resistant phenotype as remediation of the Hudson River takes place. Since PCB resistance may have evolutionary tradeoffs such as a truncated life span, increased frequency of liver tumors, and decreased life functions, it is hypothesized that resistance would be selected against as tomcod have reduced exposures to PCBs as a result of remediation. Therefore, as the Hudson River becomes cleaner, this population would quickly evolve to a genotype with a reduced frequency of the AHR2-1 variant for resistance. Measuring population samples at periodic time points during remediation would give a clear picture of the effectiveness that dredging is having on removing PCB contamination from the Hudson River.

## METHODS

*Sample Collection*

Formalin-preserved tomcod samples collected in the 1930s from the Hudson River were obtained from the collection of fishes housed at the New York State Museum in Albany, NY. Hudson River fish had been collected from Orange, Rockland, Westchester, and Dutchess counties in New York. Long island sound samples were collected in Suffolk and Nassau counties in New York. Each fish's pectoral fin was cut and a 1-2mm section was placed into 95% ethanol. There were 110 fin clips collected from Hudson River tomcod from 1936, and 60 fin clips collected from Long Island Sound tomcod from 1938. These were taken back to the NYU laboratory for analysis.

*DNA Isolation*

To extract DNA from the formalin-preserved fin clips, a modified version of the Dayton Protocol (Friedman and Desalle 2008) was used. This protocol calls for the fin clips to be crosslinked in a Stratolinker (Stratogene, Santa Clara, CA), and then soaked in a 1x GTE buffer (100 mM glycine, 10 mM Tris-HCL pH 8.0, 1mM-EDTA) for 72 hours. This extended soaking was to disassociate the formalin from the tissue. After soaking, the samples were air-dried, cut into small pieces, and homogenized. Proteinase K (15 μl) was then added, and the samples incubated in a water bath at 50-55° C overnight. The following day, a QIAGEN DNA kit was used to isolate the DNA. In this process, RNAase and a protein precipitation solution were used to purify the DNA. An alcohol precipitation process was then performed to form a DNA pellet, after which the pellet was resuspended in DNA hydration solution. Each sample was read on a Nanodrop

spectrophotometer at 260 and 280 nm to quantify their DNA concentrations and determine their purity.

*PCR and Restriction Enzyme Digestion*

PCR and gel electrophoresis were performed to genotype DNAs from each Hudson River historic sample and controls of contemporary Shinnecock Bay and Hudson River DNAs. Isolated DNAs were added to a PCR master mix containing Taq DNA polymerase enzyme. The primers that were used were 1401 (#78061722, 5'-CACGCAGATCCAGACCAG-3') and 1642 (#78061723, 5' GCTCGCCCTCCTCCTTGA-3') because the amplified segment of DNA (241 bp) contains the six base deletion in AHR2. Additionally, primers 3376(#78061724, 5'CCTGCTGAAGACAACGAT-3') and 3500 (#78061725, 5'-GGGGTTTAAGGAGACGAT-3') were used to analyze a downstream tyrosine to asparagines (T:A) substitution polymorphism in the AHR2 that is coheritable with the six base pair deletion. The PCR reactions were in 40 µl final volumes that contained 1 µl DNA, 0.5mM of each primer (IDT, Coralville, Iowa), 0.008mM dNTP (GE Healthcare Corp, Piscataway, New Jersey), 10x Taq Polymerase DNA buffer (Roche, Indianapolis, Indiana), and 1 unit of Taq Polymerase (Roche, Indianapolis, Indiana). PCR reactions were amplified in a thermocycler (MJ Research Inc, PTC-100) for 40 cycles. Each cycle consisted of fifteen seconds at 95° C for denaturing, 15 seconds at 50° C for annealing, and 30 seconds at 72° C for extension. After PCR, the samples were digested for one hour using the restriction enzyme EcoNI (New England Biolabs, #B70045) for primer set 1401-1642 or enzyme MseI(New England Biolabs) for primer set 3376-3500. The EcoNI enzyme is designed to cut the DNA at the six-base deletion site. When digested with

EcoNiI if the site was missing (AHR2-1 alleles), the DNA was not cut and remained as one large piece. If the site was present (AHR2-2 allele) the DNA was cut into two smaller fragments.

However, when digesting with MseI, the opposite results would occur due to the downstream polymorphism. Samples with the AHR2-1 variant allele would be digested, while those with the AHR2-2 common allele would remain as one large piece. After digestion, the samples were loaded into a 1.6% agarose gel and run for two hours. The gel was then stained with ethidium bromide and visualized with UV light and imaged.

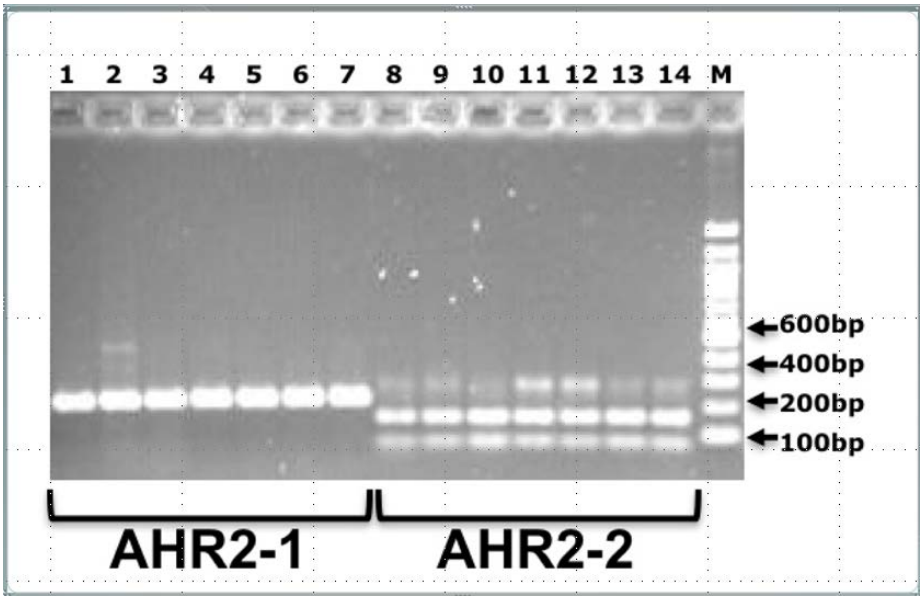



**Figure 6.** Agarose gel with tomcod DNA representative of variant AHR2 genotypes. DNAs were digested with EcoNI and electrophoresed in a 1.6% agarose gel. Lanes 1-7 indicate samples that were homozygote for the AHR2-1 variant allele, while lanes 8-14 indicate samples that were homozygote for the AHR2-2 common allele. Lane “M” indicated the marker to determine the size of the DNA bands.

For each set of samples run on the gel, negative controls were included. These were PCR blanks in which one well of the PCR plate contained the PCR master mix, but no DNA was added. Ideally, these would show a clear lane with no bands. Occasionally, a faint band would be present, and when this was the case only samples that were significantly darker than this background could be reliably scored.

*Data Analysis*

AHR2 resistance genotypes can be determined based on the digestion pattern in the gel. In tomcod that do not have the AHR2 deletion polymorphism (AHR2-2), the DNA amplicon was cut into two smaller fragments (Figure 6). However, in those samples where the AHR2 deletion was present (AHR2-1), the DNA remained as a single fragment. Samples digested with MseI for the downstream base substitution would be analyzed in the opposite way; tomcod samples with the AHR2-1 allele would be cut and digested, while those without (AHR2-2) would remain as a larger piece. Since smaller pieces run further on the gel, the samples which have the deletion, and therefore the resistance could be determined.

Each sample was scored as a “1”, “2”, or “3” genotype for each of the two AHR2 polymorphisms. For EcoNI digested samples, “1” showed no digestion, indicating it is a homozygote for the variant AHR2-1 genotype. A “2” showed complete digestion, indicating that it was a homozygote for the common AHR2-2 genotype. A “3” had both an undigested and digested band. This indicated that it was a heterozygote genotype, and had one allele for each the variant and common allele. In the case of MseI digestions,

samples were scored a “1” if they showed complete digestion, indicating that it was a homozygote for the variant AHR2-1 genotype. A “2” score showed no digestion, and corresponds to a homozygote for the common AHR2-2 genotype. A “3” score again was a heterozygote with both bands present.

To determine the genotype frequency, the occurrence of each genotype number in the archived samples was tallied, and these results were compared against the current tomcod AHR2 genotype frequencies using a chi-squared test for independence. The AHR2 allelic frequencies were then calculated from the genotype frequencies. Each homozygote variant, or “1” score, contained two variant alleles, so the value for this genotype was doubled. The amount of individuals scored under the “3” column were added to this number because each of these fish contained one variant allele. The final value is the frequency of the AHR2-1 variant allele. For the homozygote common allele frequencies, the same procedure was done except that “2” score was doubled, and the “3” score was added to it. Another chi-squared test for independence was performed to compare the AHR2-2 allelic frequencies in the archived and contemporary Hudson River samples. Differences in genotype and allelic frequencies were considered statistically significant when $p < 0.05$.

## RESULTS

In order for a sample to be considered reliable, it had to be PCR amplified and clearly analyzed three separate times. From the 110 Hudson River samples extracted and analyzed, 20 samples were successful. The results from these tomcod specimens were then used to perform the statistical analysis.

*Gel Pictures*

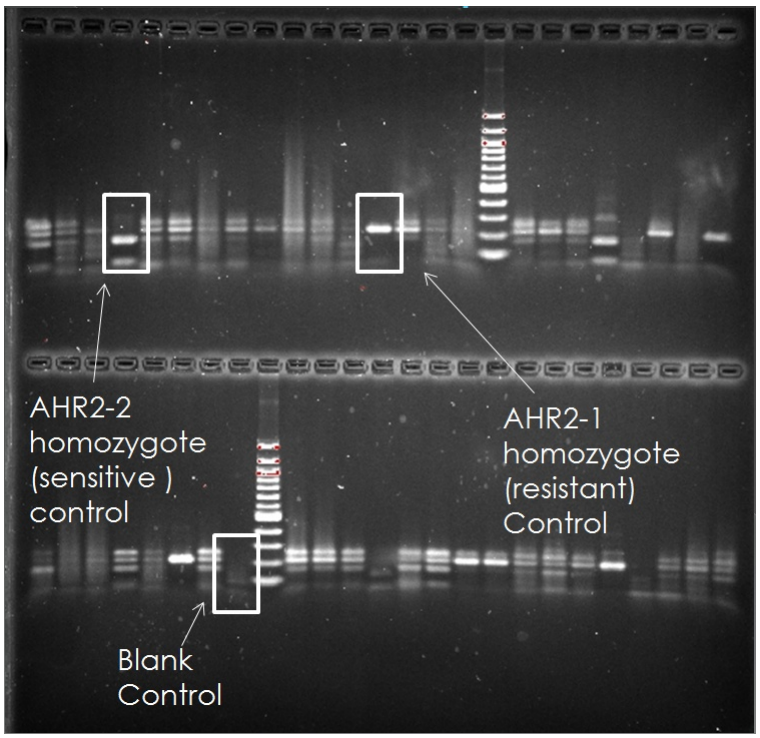



**Figure 7.** Agarose gel picture of tomcod DNA after PCR amplification for an AHR2 amplicon and digestion with EcoNI enzyme. The positive and negative controls are highlighted.

All of the tomcod analyzed were PCR amplified and digested in triplicate, both with 1419-1668 primers (EcoNI digestion) and 3376-3500 primers (MseI digestion). The gel pictures showed clear positive and negative controls, which allowed for the characterization of the archival samples. The samples (as shown in Figures 8A and 8B in

dark lettering) were then scored using the “1”,”2”, “3” designations for homozygote variant, homozygote common, or heterozygote genotype, respectively.

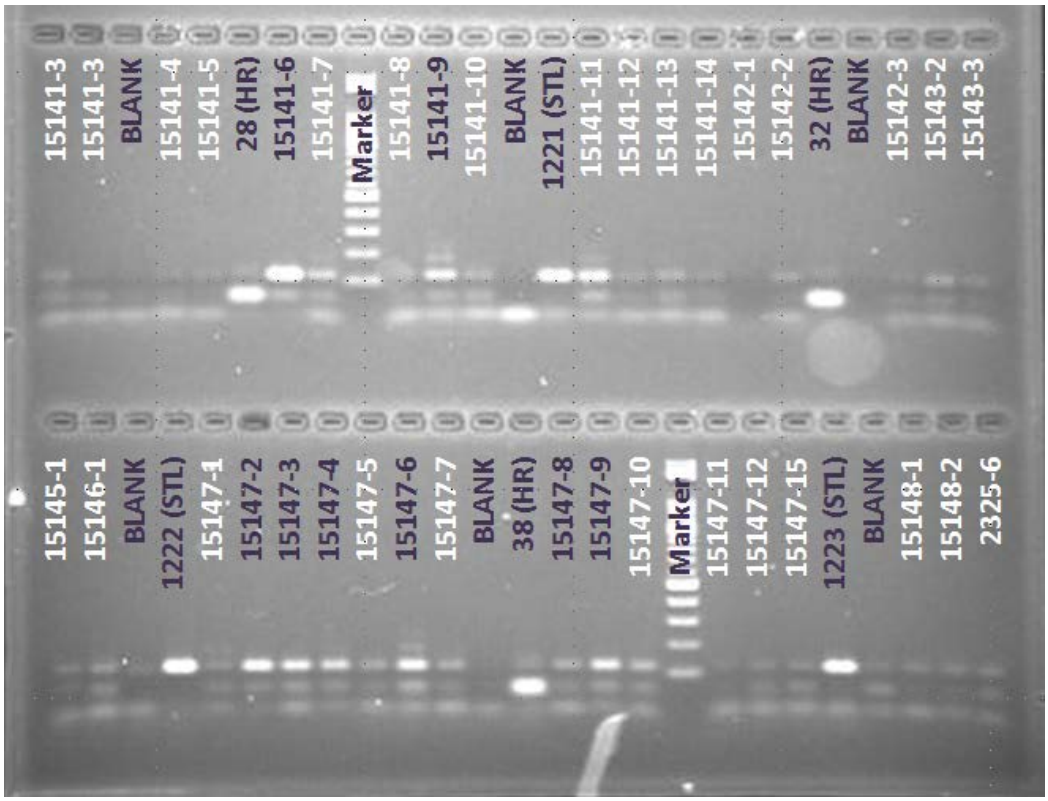
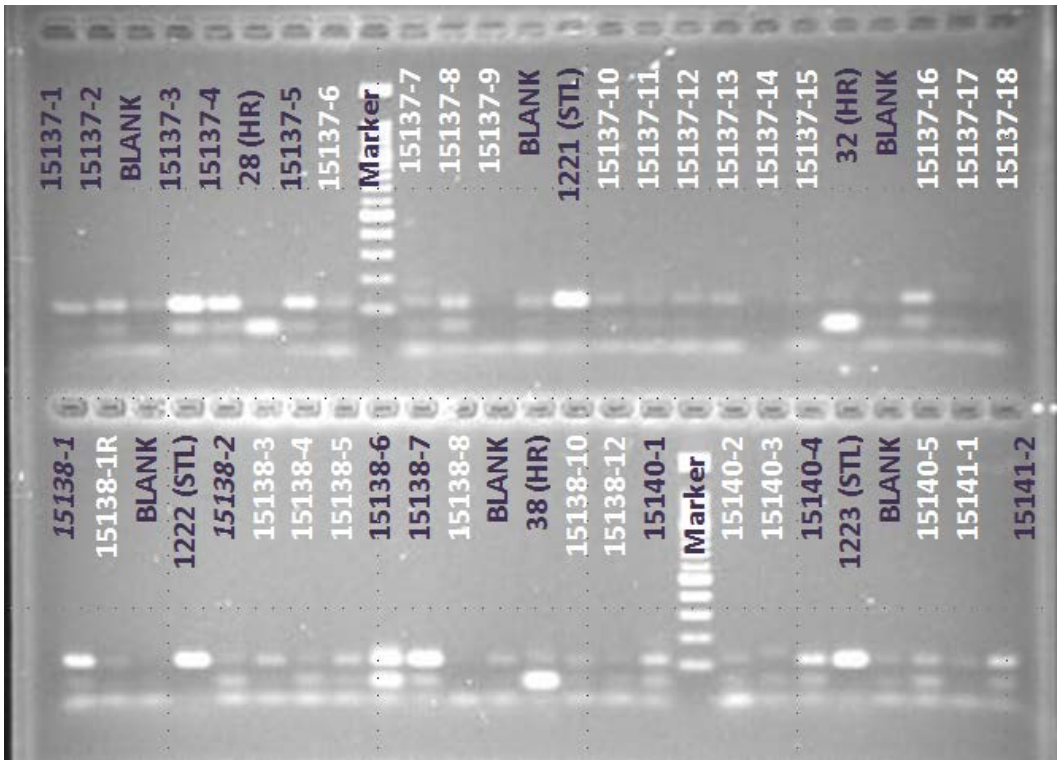



**Figure 8A and 8B.** Agarose pictures of tomcod DNAs digested with MseI restriction enzyme. Controls, markers, and successfully analyzed samples are indicated in dark lettering. White lettering indicates samples that did not successfully amplify or genotype. The “blank” lanes are PCR water blanks. These are lanes in which the PCR mixture was amplified without any DNA




*AHR2 Genotype Frequencies*

The genotype frequencies represent the number of individuals which exhibit each genotype. From this data, it can be determined that the genetic frequency of the homozygote PCB-resistant genotype, AHR2-1, in Hudson River tomcod collected in 1936 was 2, which is approximately 10% of the samples. This is in comparison to the present day population, which has a frequency of 93%, for the AHR2-1 genotype. Also of note is that the difference in the number of heterozygotes between each time point: 55% (11 of 20) of the specimens from 1936 had this genotype, while only about 7% (4 of 54) from the present day possess it. A chi-squared test to compare the 1936 Hudson River tomcod population genotypes to the present day Hudson River population showed a highly significant p-value ( $< 0.0001$).

| Location and Time | AHR2 Genotype | | |
|---|---|---|---|
| | AHR2-1 (Resistant Homozygote) | AHR2-2 (Common Homozygote) | (Heterozygote) |
| HR 1936 | 2 | 7 | 11 |
| HR Present | 50 | 0 | 4 |

**Figure 9.** AHR2 genotype frequencies in contemporary and archived Hudson River tomcod samples.

*Allelic Frequencies*

Additionally, frequencies of the variant AHR2-1 allele and the common AHR2-2 allele were calculated for the archived and contemporary Hudson River tomcod samples and statistically compared. This was done using the genotypes determined from the agarose gel pictures. In tomcod collected from the Hudson River in the 1930s the AHR2-1 allele frequency was 15 of 40, which was 37%. The allelic frequency of the variant AHR2-1 allele in present day tomcod from the Hudson River is 104 of 108, or 96%. A chi-squared test was done to compare the statistical significance of AHR2 allele frequency for the tomcod from 1936 and present day, resulting in a p-value of $<0.0001$.

| Location and Time | AHR2 Allele | | |
|---|---|---|---|
| | | AHR2-1 | AHR2-2 |
| | HR 1936 | 15 | 25 |
| | HR Present | 104 | 4 |

**Figure 10**. AHR2 allelic frequencies in contemporary and archived Atlantic tomcod collections from the Hudson River.

## DISCUSSION

From this data, it can be concluded that the frequency of the PCB-resistant genotype was significantly lower in Hudson River tomcod in the 1936 samples compared to the present day population. Additionally, it was concluded that the allelic frequency of the variant resistant AHR2-1 allele was also significantly lower in the 1936 Hudson River sample as compared to the present day population. This serves as the beginning of the process to determine a timeline of PCB resistance in the Hudson River tomcod population. The hypothesis from this project stated that the AHR2-1 allele frequency would be significantly lower in the 1936 tomcod population as compared to the present day population. The data supports this hypothesis; however, the AHR2-1 allele frequency was still higher than predicted for the 1936 Hudson River sample. Since PCBs were not released from the GE facilities into the Hudson River at the earliest until 1947, the expected allelic frequency of the variant AHR2-1 allele in 1936 would have been lower, similar to the 5% frequency that was observed from the proximal populations in the

relatively clean Niantic River, CT., and Shinnecock Bay NY. (Wirgin et al. 2011). This would lead to the conclusion that while PCBs were not the sole driving force for this resistance, their introduction in the Hudson River ecosystem probably played an important role in the rapid evolution seen in the tomcod population in the past eighty years. Alternatively, it is possible that the frequency of AHR2-1 was historically higher than seen in the other two nearby tomcod populations. With the current data, it is not possible to distinguish between the two possibilities.

However, there were limitations to this study. The archived tomcod samples were preserved in formalin, which made the DNA extracting process much longer and more difficult than for contemporary samples. The longer extraction time limited the number of samples from which DNA could be isolated and the success of their analysis. Some of the samples that were not isolated included reference ones collected from the Long Island Sound in 1938. Their genotypes would have provided an excellent positive control for this study. Due to this, not all of the samples extracted were able to provide adequate DNA to be PCR amplified.

Further research involves continuing to isolate and analyze DNA from additional archived Hudson River specimens to expand the sample size which will increase the statistical power in comparing the AHR2 genotype and allelic frequencies in archived versus contemporary Hudson River tomcod collections. Also, this work would include replicating the current samples that did not amplify multiple times to obtain a larger sample size. It will also be important to genotype archived samples collected from the Long Island Sound in the 1930s that were not available for this report. Additional research would be to genotype Hudson River tomcod collected at various time points

during the period of 1947 to the present day. Having the allelic frequencies of Hudson River Atlantic tomcod populations throughout the release of PCBs (1947-1977) as well as after the plants were closed and then when remediation began (2009) would provide a clear timeline of the resistance to PCBs in these fish.

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