GENETIC POLYMORPHISM ANALYSIS OF AHR2 GENE IN CYPRINUS CARPIO AFTER EXPOSURE TO BENZO (GHI) PERYLENE AND BENZO(A)PYRENE

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Abstract

There is growing concern about the presence of mutagenic and carcinogenic pollutants in the aquatic environment as they pose a great risk to the health of aquatic organisms, and pollution of aquatic ecosystems is one of the main risk factors for human health. Polycyclic aromatic hydrocarbons (PAHs) are one of the most influential and prevalent organic pollutants. One of the main receptors that represents the main pathway to stimulating the metabolism of these pollutants is the aryl hydrocarbon receptor (AhR2). This study is the first to detect the mutational ability of benzo(a)pyrene B(a)P and benzo(ghi)pyrene B(ghi)P compounds in the AhR2 gene in common carp fish. 30 common carp weighing an average of 35 g were divided into three groups: The first group, the control, was not given any of the test solutions, while the second group was given three doses of B(a)P with a concentration of 10µg/kg, and the third group was given three doses of B(ghi)P with a concentration of 10µg/kg. The results revealed B(a)P's high ability to mutate, causing 13 mutations in the amplified part of the AhR2 gene and 12 changes in amino acids, leading to the emergence of four new haplotypes. B(ghi)P's mutational ability was also detected, but it was less than that of B(a)P as it caused only four mutations and three changes in amino acids and the emergence of four new haplotypes. The installation of three-dimensional proteins has also changed in both groups.

Keywords: benzo(a)pyrene, benzo(ghi)pyrene, AhR2 gene, polymorphism, common carp.

1.Introduction

Aquatic ecosystems face the risk of contamination with organic and inorganic substances, as these pollutants can affect the survival of species in the short and long term which have risks on human health across the food chain (Langan et al., 2018; Reeves, 2015). This is what justifies the global interest in industrial and oil pollution, which leads to the deposition of large levels of polycyclic aromatic hydrocarbons (PAHs) (Habibullah-Al-Mamun et al., 2019), Consisting of several thousand persistent organic pollutants (POPs) that contain stable mutagenic and carcinogenic formulations (Abdel-Shafy and Mansour, 2016). Insert B(a)P and B(ghi)P from among the 16 compounds

registered as priority pollutants by the U.S. Environmental Protection Agency. B(a)P consists of five rings and is classified as carcinogenic of the first degree by the International Agency for Research on Cancer (Bukowska et al., 2022; Kim et al., 2022). The B(ghi)P consists of six rings and has not been widely studied due to its lack of classification as a carcinogen for humans (Mandal and Das, 2018; Zaragoza-Ojeda et al., 2022). But some studies have shown that B(ghi)P not only forms adducts with DNA, but also acts synergistically with B(a)P to enhance carcinogenic effects (Hughes and Phillips, 1993;Cherng et al., 2001). Evidence shows that although B(ghi)P does not have the "typical" region of the bay, it does have genotoxicity. Research reports show that B(ghi)P can damage the cell membrane, stimulate aryl hydrocarbon receptor (AhR) transposition,and induce gene transcription (Zaragoza-Ojeda et al., 2016). The AhR pathway is the main factor stimulating the metabolism of PAH (Vogeley et al., 2022). The cytosolic AhR is a protein that belongs to the basic helix-loop-helix per-ARNT-SIM (bHLH-PAS) family of proteins. Sim (single-minded) nuclear translocator for the period-AhR Canonical AhR signaling involves the receptor becoming activated in response to a ligand, moving into the nucleus, and joining the aryl hydrocarbon receptor nuclear translocator (Arnt) to create a dimer. This heterodimer interacts with xenobiotic response elements (XRE) upstream of Ahr target genes, which are particular DNA binding sites. Regulates the transcription of various enzymes involved in the biotransformation of xenobiotic, including cytochrome P450 1A (CYP1A) (Aranguren-Abadía et al., 2020; Dai et al., 2022; Rejano-Gordillo et al., 2022). AhR found in all vertebrates, mammals have one AhR gene, while fish have multiple AhR genes as they have two AhR1 and two AhR2 that originated by genomic duplication that occurred 350 million years ago, Piscine AhR1 is orthologue to mammalian AhR, while clade AhR2 is a new form in teleost fishes. Many PAHs are clearly highly affinity-bonded to AhR2 in fish (Prasch et al., 2003; Song et al., 2020). The aim of this study is to identify the mutagenic ability of the B(a)P and B(ghi)P compounds in the Ahr2 gene of common carp.

2. Material and Methods
2.1. Study sites
The study was conducted at Animal House and Genetic Engineering Laboratory in the biology department of the College of Science-Misan University

2.2. Test chemicals
B(a)P (Cas No.: 50-32-8, purity >96%) and B(ghi)P (Cas No.: 191-24-2, purity >97%) was obtained from Shanghai Macklin Biochemical (China). Dimethyl sulfoxide (DMSO, purity >99%; Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to dissolve the reagents in order to create test solutions.

2.3. Test species

Thirty common carp fish at 10 individuals (average weight 35 gm/fish) per aquarium (60 cm × 30 cm × 30 cm) were randomly distributed in 3 glass aquaria filled with 46 liter of dechlorinated tap water after treatment with a 1 ppm potassium permanganate solution for 90 minutes to get rid of external parasites (Protection, 1997). It was provided with 24-hour ventilation pumps, as well as temperature gauges, and filled with dechlorinated liquefied water, and its water was replaced by 50% per day with water kept in a large tank (capacity of 1 m³) equipped with an organized electric heater after leaving the water for 24 hours to ensure the removal of chlorine and maintain a temperature suitable for the life and growth of fish. The temperature was maintained at approximately 24 ± 1 degree using central air heating, and the amount of dissolved oxygen was maintained. At approximately 5 mg per liter and at a rate of lighting 12 hours a day, each of the was glass aquaria covered with a separate mesh cover to ensure that the fish remained in their designated and did not move to neighboring glass aquaria.

2.4. Xenobiotic exposures

Dosing solutions consist of B(a)P and B(ghi)P dissolved directly in DMSO separately. The first group (control) did not receive any of the test solutions. The second group was given a B(a)P solution with a concentration of 10 µg/kg. The third group was given a B(ghi)P solution with a concentration of 10 µg/kg. The amount of dose was 5 µL of test solution per gram of fish, according to (Gerger and Weber, 2015). It was given orally using automatic micropipettes in three doses for each fish, separating between one dose and the other for 48 hours. Then blood was drawn on the tenth day of dosing. All test fish had 0.5 ml of blood drawn from the caudal vein. These samples were gathered in EDTA tubes and stored at -18 °C in the freezer until the DNA was extracted.

2.5. Genomic DNA isolation and column purification(PCR)

DNA was extracted from whole blood using the Geneaid Company's (Taiwan) gSYNC™ DNA Extraction Kit in accordance with the kit's protocol. The samples' DNA was extracted, and the DNA genome's presence was verified by electrophoresis on 1% agarose before the nanodrop instrument was used to calculate the samples' DNA concentration. The range of 1.70 to 1.96 was found for the absorbance ratio of A260/280. Primer was designed for molecular detection and identification of mutations in the AhR2 gene, the primer was provided by the Korean company Macrogen, and its sequences are shown in Table 1. PCR reactions were of 25 µL; Table 2 shows the reaction mixture and its amounts; the primer works; and the reaction takes place according to specific conditions shown in Table 3.

Table1: The sequence of AhR2 primer and length

Gene		Sequences (5`→ 3`)	Size (bp)	References
AhR2	**Exon10**	**F:** GATATACCCACCCGCACCTG	665	**Current Study**
		R: CTTTTAGCTGCCCCACTGGA		

Table 2: Materials used in PCR technology and their quantities

Chemical	PCR master mix	Genomic DNA template	Primer		Sterile distilled water	Final volume
			Reverse	**Forward**		
Volume (µl)	13	4	1	1	6	25

Table 3: Program for PCR-DNA Primers for AhR2 Gene

PCR steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	5Min	1
Denaturation	95	30 Sec	
Annealing	55	45Sec	35
Extension	72	45 Sec	
Final extension	72	10 Min	1
Final hold	4	5Min	-

2.6. Statistical analysis

Bioinformatics software (BioEdit, DnaSP 5.10, and Phyre2 V.2.0) was used to detect mutation sites, determine total haplotypes (H), and draw the 3D protein.

3. Results and Discussion

The results of the electrophoresis on the gel at a agarose concentration of 1% showed the success of the DNA amplification process, and the results of the electrophoresis on the gel at a agarose concentration of 1.5% showed the success of the PCR amplification process for the AhR2 gene, as the primer AhR2 shown in Table 2 gave a package of 665 base pairs as in Figure 1.

Figure1: Electroplating of PCR product for AhR2 gene (665base pairs) using 1.5% agarose gel with 70V and 85 mA.

Some nucleotide sequences studied in exon 2 of the AhR2 gene were recorded for common carp fish at the National Center for Biotechnology Information (NCBI), the DNA Data Bank of Japan (DDBJ), and the European Nucleotide Archive (ENA) under the following accession numbers: LC768696, LC768698, LC768699, and LC768700.

3.1. Genetic Diversity

The results of the genetic diversity of the group B(a)P10µg/kg showed that the number of sites was 560 bp and the number of total haplotype (H) were 4 haplotypes resulting in 13 genetic polymorphism (NH), and the values of haplotype diversity (HD) and nucleotide diversity values (π) were 0.819 and 0.00974, respectively. The results of the genetic diversity of the group B(ghi)P10µg/kg showed that the number of sites was 560 bp and the number of sites were 560 bp and the number of total haplotype (H) were 4 haplotypes resulting in 4 genetic polymorphism (NH), and the values of haplotype diversity (HD) and nucleotide diversity (π) were 0.818 and 0.00317, respectively, as shown in Table 4.

Table 4: Genetic Diversity of AhR2 Gene among Fish Populations Treated with B(ghi)P at (1, 10 µg/kg) Concentrations

Compound	B(a)P10µg/kg	B(ghi)P 10µg/kg
Number of Site (N)	560	560
Haplotype (H)	4	4
Number of polymorphic (NH)	13	4
Haplotype diversity (HD)	0.819	0.818
Nucleotide diversity (π)	0.00974	0.00317

3.2. Single nucleotide polymorphisms

The results of the Sequencing analysis of the control group showed The absence of any change in the nitrogen bases

3.2.1. B(a)P10µg/kg group

The results of the analysis of the nucleotide sequence (sequencing) of the kg/B(a)P10µg group showed changes in the nitrogenous bases in the tenth axon, specifically at sites 1238, 1310, 1387, 1401, 1457, 1466, 1493, 1497, 1507, 1510, 1530, 1537, and 1694 of the AhR2 gene, as the base changed from C to G (C1238G), from G to T (G1310T), from T to A (T1387A), from C to C (C1401A) and from C to T C1457T (C1457T), A to C (A1466C), T to A (T1493A), A to T (A1497T), C to A (C1507A), A to T (A1510T), T to C (T1530C), C to T (C1537T), T to G (T1694G), as shown in (Table 5) Table 14 also showed that the formations C1238G, G1310T, T1387A, C1401C, A1466C, T1493A, A1497T, C1507A, A1510T, C1537T and T1694G led to a change in genetic codes, which led to a change in amino acids at the protein level resulting from Alanine (A) to Glycine (G), from Glycine (G) to Valine (V), from Phenylalanine (F) to Isoleucine (I), from Histidine (H) to Glutamine (Q), from Alanine (A) to Valine (V), from Lysine (K) to Threonine (T), Methionine (M) to Lysine (K), Glutamic Acid (E) to Aspartic acid (D), from (Q) to Glutamine Lysine (K), from Isoleucine (I) to Phenylalanine (F), from Leucine (L) to Phenylalanine (F), from Leucine (L) to Tryptophan (W) on the mutations were influential, either T1530C, although the genetic code changed from ATT to ATC, it gave the same amino acid isoleucine (I), meaning that the mutation is ineffective (silent) and returns This is because this amino acid has more than one genetic

Table 5: Nucleotide changes, mutation types, amino acid changes resulting, and their effect on the process of translating the AhR2 gene to the group B(a)P10µg/kg.

Gene	Site [a] of SNP	Nucleotides (SNPs)	Triple code	Amino acids	Types of mutation	Effect of mutation on translation	Missense %	Silent %
AhR2	1238	C>G	GCT>GGT	A>G	Transversion	Missense	92	8
	1310	G>T	GGC>GTC	G>V	Transversion	Missense		
	1387	T>A	TTC>ATC	F>I	Transversion	Missense		
	1401	C>A	CAC>CAA	H>Q	Transversion	Missense		
	1457	C>T	GCT>GTT	A>V	Transition	Missense		
	1466	A>C	AAG>ACG	K>T	Transversion	Missense		
	1493	T>A	ATG>AAG	M>K	Transversion	Missense		
	1497	A>T	GAA>GAT	E>D	Transversion	Missense		
	1507	C>A	CAG>AAG	Q>K	Transversion	Missense		
	1510	A>T	ATT>TTT	I>F	Transversion	Missense		
	1530	T>C	ATT>ATC	I>I	Transition	Silent		
	1537	C>T	CTT>TTT	L>F	Transition	Missense		
	1694	T>G	TTG>TGG	L>W	Transversion	Missense		

a: nucleotide locations of the AhR2 gene based on accession number XM_042749540.1 obtained from NCBI
SNP: single nucleotide polymorphism; C: Cytosine; T:Thymine; A: Adenine ;G : Guanine; G: Glycine; A: Alanine; V: Valine; F: Phenylalanine; I:Isoleucine; H: Histidine; Q: Glutamine; K: Lysine; M: Methionine; T: Threonine; E: Glutamic Acid; D: Aspartic acid; L:leucine; W :Tryptophan;

B(a)P is considered biologically inert and requires metabolic activation from the practice of genotoxicity. B(a)P undergoes phase I (activation) and phase II (conjugation/detoxification) metabolic reactions in xenobiotic, driven by its interaction with aryl hydrocarbon receptors (Ahr) (Moffat et al., 2015). B(a)P metabolites, such as B(a)P -7,8dihydrodiol-9,10-epoxide (BPDE), can form covalent bonds with DNA and proteins. In addition, the production of ROS as a by-product of B(a)P metabolism (of the B(a)P catechol cycle, the redox cycle of quinone) may result in DNA oxidation (Lan et al., 2004). This may include error-free repair of DNA damage and resumption of normal cell function, or, in severely damaged cells, cell death by apoptosis and necrotic processes of cell death. Additives that escape repair can cause mutations during reproduction or DNA breaks leading to chromosomal aberrations. There is ample evidence that B(a)P causes mutations in animal tissues and that B(a)P products cause mutations in cultured human cells. BaP metabolites and DNA approaches have been discovered in cells isolated from humans exposed to B(a)P (Rojas et al., 2000).

The results showed a high ability of the B(a)P compound to cause mutations, as it was found that 13 mutations occurred, and the reason for this is due to the ability of the metabolites of the B(a)P compound to interact with DNA and form complexes that represent pre-mutation changes, which in most cases are identified and processed by repair systems, and in the event of obstacles that have not been removed, a point mutation begins in the form of replacement or deletion, where DNA complexes can induce mutations (Alexandrov and Thompson, 1977; Melendez-Colon et al., 1999) This corresponds to his study (Yadetie et al., 2018), which confirmed an increase in the expression of the AhR2 gene mainly in the cardiovascular system of Atlantic cod (Gadus morhua) embryos and larvae after treatment with B(a)P. Mutations have been reported in multiple rodent studies after exposure to B(a)P (Malik et al., 2012; Sakai et al., 2014). in humans at p53 mutational "hotspots" (G in codons 157, 248, and 273), BPDE-DNA adducts were found. Nonsmokers exposed to BaP-containing PAH mixtures had lung cancers with mutations in the p53 gene and the proto-oncogene K-Ras. In nonsmokers exposed to BaP, p53 tumor suppressor gene mutations were found (DeMarini et al., 2001; Denissenko et al., 1996; Reeves, 2015; Waters et al., 2010). B(a)P caused cell transformation in vitro and cancer induction in experimental animals, as laboratory studies in which mice were treated with B(a)P orally or by diet showed an increase in the incidence of tumors in various organs such as the tongue, liver, lung, stomach, esophagus, lymphoid tissue, and hematopoietic tissue (Agrawal et al., 2018; Deng et al., 2018; Estensen et al., 2004; Sparnins et al., 1986).

3.2.2. B(a)P10μg/kg group

The results of the analysis of the sequence of nucleotides (sequencing) of the group B(ghi)P10μg/kg showed changes in the nitrogenous bases in the tenth axon, specifically at sites 1218, 1493, 1547, and 1694 of the AhR2 gene, where the base changed from C to T (C1218T) and from T to A at sites (T1493A) and (T1547A) and from T to G (T1694G) as shown in Table 6. Table 6 also showed that formations 1493, 1547, and 1694 led to a

change in genetic codes, which led to a change in amino acids at the level of protein resulting from Methionine (M) to Lysine (K), from Phenylalanine (F) to Tyrosine (Y), from Leucine (L) to Tryptophan (W), and C1218T, although the genetic code was changed from ACC to ACT, gave the same amino acid Threonine (T), meaning that the mutation is ineffective (silent) due to the fact that the amino acid Threonine (T) has more than one genetic code.

Table 6: Nucleotide changes, mutations type, amino acid changes resulting and their effect on the process of translating the AhR2 gene to the group B(ghi)P10µg/ kg.

Gene	Site[a] of SNP	Nucleotides (SNPs)	Triple code	Amino acids	Types of mutation	Effect of mutation on translation	Missense %	Silent %
AhR2	1218	C>T	ACC>ACT	T>T	Transition	Silent	75	25
	1493	T>A	ATG>AAG	M>K	Transversion	Missense		
	1547	T>A	TTT>TAT	F>Y	Transversion	Missense		
	1694	T>G	TTG>TGG	L>W	Transversion	Missense		
a: nucleotide locations of the AhR2 gene based on accession number XM_042749540.1 from NCBI								
SNP: single nucleotide polymorphism; C: Cytosine; T: Thymine; A: Adenine; G: Guanine;								
T: Threonine; M: Methionine; K: Lysine; F: Phenylalanine; Y: Tyrosine; L:leucine; W :Tryptophan								

The results showed the ability of B(ghi)P to mutate, where 4 mutations were found , and this is consistent with several studies that provided evidence of the ability of B(ghi)P to form DNA complexes in vitro and in vivo (Hughes and Phillips, 1993;Cherng et al., 2001; Dai et al., 2022; Labib et al., 2016; Long et al., 2016; Pan et al., 2013), This is consistent with(Zaragoza-Ojeda et al., 2022) which confirmed DNA damage in human bronchial cells three hours after exposure to B(ghi)P and through induction of the Aryl hydrocarbon receptor AhR pathway. As confirmed (Cherng et al., 2001) with a laboratory study on human liver cells where B(ghi)P induced gene expression of CYP1A1 by activation of the Ahr2 pathway.

The final results of this study showed that the B(a)P compound is more mutated than B(ghi)P, where the number of mutations for B(a)P was 13 mutations, while the total number of mutations for B(ghi)P was 4 mutations, and this may be due to the difference in the structural structure of the two compounds, where B(a)P has a bay region, while B(ghi)P lacks a bay region and instead has a K region, and therefore cannot be converted Metabolically, such as B(a)P into dihydrodiol epoxides, which are mutation-causing final metabolites, instead of which K regions of B(ghi)P are converted into arene oxides, resulting in 3,4-oxide and 3,4,11,12-bisoxides,the 3,4-oxide playing an important role as the final mutagenic metabolite of B(ghi)P(Pan et al., 2013; Platt and Grupe, 2005).

3.3. Three-Dimensional Protein Structure (3D)

The alignment of the amino acid sequence in the AhR2 gene of the B(a)P10μg/kg and B(ghi)P10μg/kg groups with the joining number XM_042749540.1 (Figure 2) There are 12 changes in the B(a)P10μg/kg group and 3 changes in the B(ghi)P10μg/kg group. Bioinformatics software was used to predict the composition of the three-dimensional protein and determine where the amino acids in AhR2 change in the aforementioned groups, as these formations led to changes in the structure of the protein containing different regions of functional importance, as shown in (Figure 3).

Figure 2: Comparison of amino acid sequence in AhR2 gene for aggregates B(a)P10μg/kg, B(ghi)P10μg/kg with accession number XM_042749540.
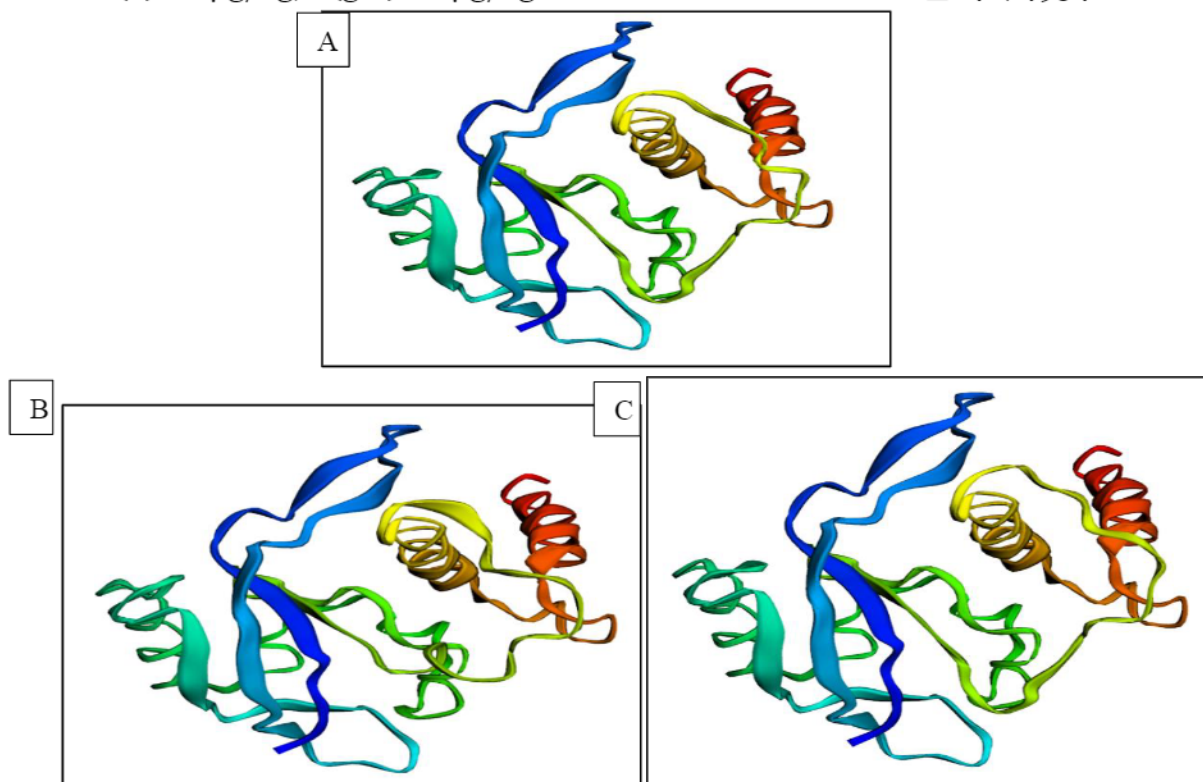

Figure 3: Three-dimensional forms of the AhR2 protein in (A) accession number XM_042749540.1, (B) B(a)P10μg/kg, (C) B(ghi)P10μg/kg

3.4. Haplotype Network

The results showed that the number of total individual patterns (haplotype) of the AhR2 gene in the B(a)P10µg/kg group is equal to 4 individual types, as in the (figure 4), and in the B(ghi)P10µg/kg group is equal to 4 individual patterns, as in the (figure 5).
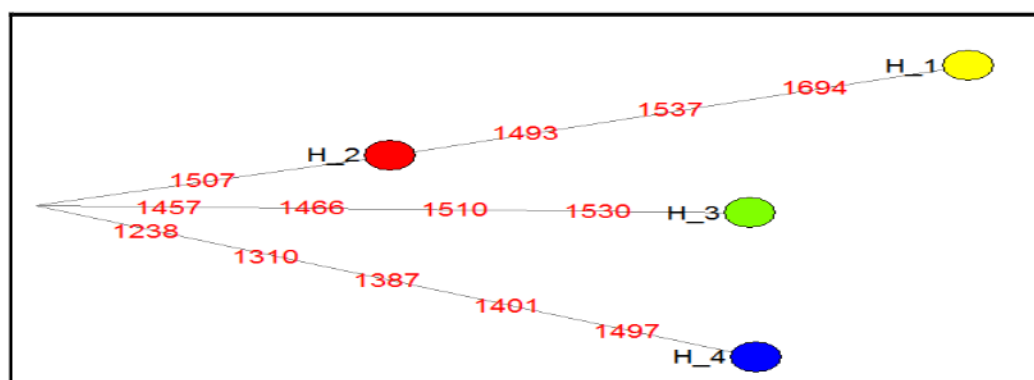

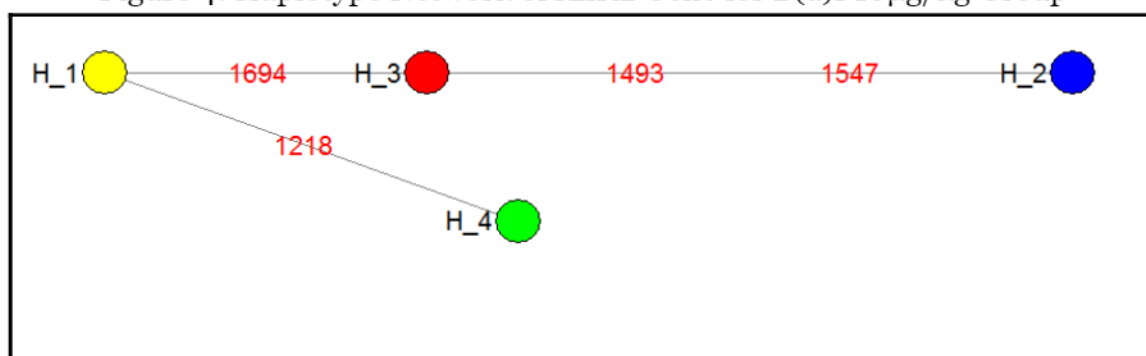Figure 4: Haplotype Network of AhR2 Gene for B(a)P10µg/kg Group

Figure 5: Haplotype Network of the AhR2 gene in the B(ghi)P10µg/kg group

4. Conclusion

This is the first study in which the effect of the B(a)P and B(ghi)P compounds is determined at the genetic level in the AhR2 gene of common carp fish, as it was found that both compounds are mutagenic, which led to changing some amino acids and changing the shape of the three-dimensional protein. The results showed that B(a)P is more mutagenic than B(ghi)P ratio of more than 3 to 1.

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Authors' contributions

Each author agreed to be accountable for every part of this work and contributed to data analysis, writing, and revision of the manuscript.

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