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Comparative study of *cyp1a* expression in *Oreochromis aureus* from different regions of Lake Manzala, Egypt

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ABSTRACT

The *cytochrome P450* superfamily (*cyp*) is a unique, diverse group of enzymes that catalyze the oxidation of organic pollutants such as polycyclic hydrocarbons (PAHs). Both levels of *cyp1a* expression in *Oreochromis aureus* organs and the accumulation of PAHs in water were determined in five different locations of Lake Manzala; namely, El-Inaniya, El-Serw, El-Temsah, El-Gamil, and El-Bashtir during December 2011. The area of Al-Qanater was included as a reference site from the River Nile. The *cyp1a* gene was highly expressed in the liver of all fish populations followed by gill, testis, ovary and muscle. The liver and gonads of the fish population in the El-Gamil location exhibited the greatest *cyp1a* gene expression, while the gills and muscles in the El-Serw fish population were the highest in the expression pattern among Lake Manzala regions. A higher accumulation profile of PAHs in water was found in the El-Gamil location followed by El-Serw, El-Bashtir, El-Inaniya and El-Temsah. The correlation between PAHs and *cyp1a* expression of fish organs showed a significant positive correlation. The highest positive correlation was observed between the gill and liver based on the expression of *cyp1a*. The obtained cDNA of the *cyp1a* gene from *O. aureus* was 569 base pairs, and it exhibited 86.8% of the identity with *cyp1a* of *O. niloticus*. The phylogenetic tree of *cyp1* family genes showed a closer relationship between *cyp1a* and *cyp1d* subfamily compared to *cyp1b* or *cyp1c* subfamily members. The results showed the sensitivity of *cyp1a* expression in fish as a biomarker for PAHs concentrations in the regions of Lake Manzala.

INTRODUCTION

Most factors disrupting the ecosystem are attributed to the release of toxic chemicals capable of interacting with the environment **(Abumourad *et al*., 2013)**. The toxic chemicals produce stress on aquatic organisms including fish **(Slaninova *et al*., 2009)**. Aquatic organisms cannot avoid exposure to chemicals which are dissolved or suspended in the surrounding water, being less than land animals to move to suitable

regions to avoid harmful conditions **(Elghobashy *et al*., 2001)**. The water of Lake Manzala has been subjected to several changes including an increase in wastewater discharges in the lake. All changes have strongly impacted both the chemical and physical characteristics of the lake **(Ahmed and Donia, 2007)**.

Several researchers studied the problems of pollution in Lake Manzala that have highly increased in the past years. Those problems involve the presence of high concentrations of pesticides and heavy metals in both water and various organs of fish that affected fish fertility, reproduction and production. Most of those toxic substances exceed the accepted international threshold levels in foodstuffs, which definitely have anadverse effect on the health of human fish consumption **(Dowidar & Hamza, 1983; Khalil & Bayoumi, 1988; El-Ghobashy, 1990; Khalil, 1990; Said, 1992; El-Bokhty, 1996; Frihy *et al*., 1998; Abdel-Satar, 2001; Fathi *et al.*, 2001; Flower, 2001; El-Enani, 2004; Gad, 2005)**; however, genetic studies on the effects of the pollution in Lake Manzala were somewhat rare.

The *cyp1a* subfamily of cytochromes P450 has been identified in a broad range of fish. The enzymes of cytochrome P450 gene family 1 (*cyp1*) catalyze the metabolic activation of many polycyclic aromatic hydrocarbons and other carcinogens **(Powell *et al*., 2004)**. *CYP1* family members are inducible by aromatic hydrocarbons acting through the aryl hydrocarbon receptor (AHR) **(Androutsopoulos *et al*., 2009)**. Numerous studies of the Cyp1a isoform in fish observed its role in aquatic pollution **(Williams *et al*., 1998; Fent, 2003; Moore *et al*., 2003; Sakamoto *et al*., 2003; Brammell *et al*., 2010)**.

Oreochromis aureus is a highly tolerant species to several environmental variables, including a wide range of salinity, in addition to their potential to live and reproduce in brackish waters **(Froese & Pauly, 2015)** such as the water of Lake Manzala. This ability allows us to find the type of fish from all the studied regions of the Lake.

The present study aimed to compare the expression of *cyp1a* gene in organs of *O. aureus* in five different locations of Lake Manzala (in addition to Al-Qanater location as a reference site). Additionally, this work was focused to assess the polycyclic hydrocarbons levels in water, addressing its impact on the expression profile of *cyp1a* gene, investigating a novel partial *cyp1a* cDNA of *O. aureus,* and determining the phylogenetic relationship of newly identified *cyp1a* gene with the other *cyp1* family members.

MATERIALS AND METHODS

Sample collection

Thirty individuals of *Oreochromis aureus* were collected from the five afore- mentioned locations of Lake Manzala during December 2011. Fig. (1) shows the location of sampling as follows: 1- El-Inaniya, 2- El-Serw, 3- El-Temsah, 4- El-Gamil and 5- El-Bashtir. Simultaneously, samples of the studied fish with comparable weight and conditions were collected from Al-Qanater as a reference site from the River Nile, which

is considered an unpolluted area according to the studies of **Mohamed (2002)** and **Abd-El-Kader (2006)** and it is within the permissible limits set by the FDA and the Egyptian standards for fish and shellfish **(El-Kady *et al*., 2007)**.

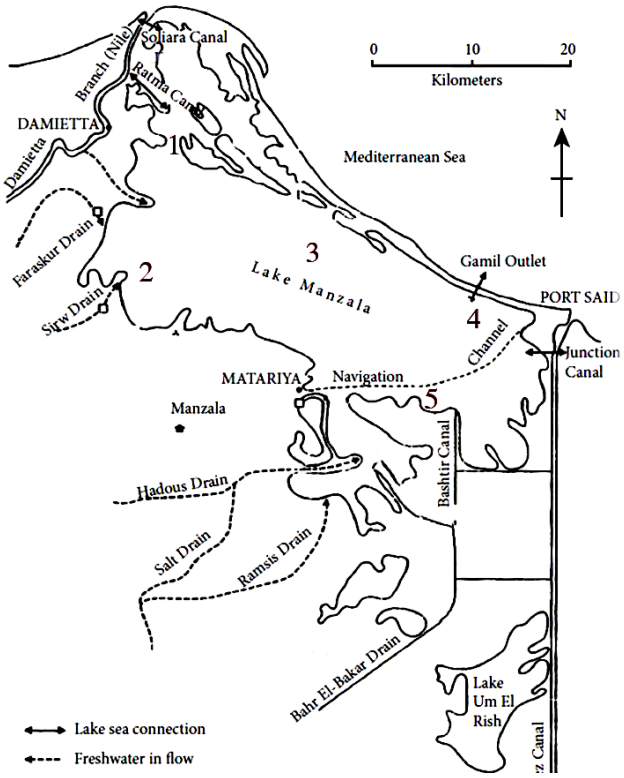

Fig. 1. Sampling regions from Lake Manzala; 1: El-Inaniya, 2: El-Serw, 3: El-Temsah, 4: El-Gamil, and 5: El-Bashtir

CYP1A gene expression

Total RNA was extracted from the liver, gill, muscle, testes, and ovary of *O. aureus* using the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method of **Chomczynski and Sacchi (1987)**. For the quantitative PCR analysis, the extracted RNA samples should be highly pure with no genomic DNA using DNAseI. The quality of RNA was verified using spectrophotometer measurements (A260/A280 ratio) and visual on an agarose gel. For designing CYP1A specific primer to *O. aureus*, cytochrome P450 1A (*cyp1a*) mRNA of *O. niloticus* (acc. No. **FJ389918.2)** was used. The beta-actin gene was used as an internal control. All data were expressed relative to beta-actin to

normalize expression levels. All gene expressions were presented in triplicates. Primers are provided in Table (1).

Table 1. Sequences, start, stop, and GC% contents using CYP1A and β-actin primers for competitive RT-PCR

		Nucleotide Sequence (5'->3')	Start	Stop	GC%
CYP1A	**F**	GCAAATGGCTGCTGCTTGTCA	1912	1932	52.38
	R	GTGTATCAAGGGTTCATGCCCT	2479	2458	50.00
β-actin	**F**	GAGTTACGAGCTGCCTGACGGA	5	26	59.05
	R	GTGGACAATGCTGGGGCCGGA	404	384	66.67

The total RNA concentrations of samples were diluted to 500 ng/ µl. To perform Q-RT-PCR, SCRIPT One-Step RT-PCR Kit (Jena Bioscience - Jena, Germany) was used. PCR condition detail was performed, as noted in Table (2).

Table 2. PCR reaction details

Reverse transcription	50°C	30-60 min	
Initial denaturation	95°C	5 min	
Denaturation	95°C	10 sec	35x
Annealing	67[a]-62[b]°C	20 sec	
Elongation	72°C	1 min	
Final extension	72°C	5 min	

a: The annealing temperature for *β-actin* primer

b: The annealing temperature for *cyp1a* primer

PCR products were visualized on 4% non-denaturing polyacrylamide gels at 20 V cm^{-1} following the method of **Rees *et al*.** (**2005**). Product size concentrations were determined based on the concentration of O'GeneRuler 100 bp plus DNA Ladder (**Fermentas**) using the Gelanalyzer 2010a program. To observe the relative gene expression, quantification analysis of gene expression was determined stepping the method of **Raeymaekers (1995)**.

Phylogenetic analysis of CYP1A sequences

The resulting PCR products of the *cyp1a* gene were sequenced using primers complementary to the forward and reverse tags on the primary PCR primers using dye fluorescence terminator chemistry. The obtained sequences were deposited in the GenBank (accession no. KX024472).

For the determination of homology among the obtained *cyp1a* sequence and *CYP1* family cDNAs sequences, sequences with the following GenBank accession numbers of various species were used from the database [Acc. No. FJ389918.2 (Nile tilapia *cyp1a*),

AJ251913.1(European seabass *cyp1a*), GU373806.1(Japanese sea perch *cyp1a*), JQ622220.1(Atlantic croaker *cyp1a*), AF022433.1(golden grey mullet *cyp1a1*), GQ281041.1(large yellow croaker *cyp1a*), AB020414.1(Japanese eel *cyp1a9*), AF420257.1 (European eel *cyp1a*), AF011223.1 (gilthead seabream *cyp1a*), AB048939.1 (common carp *cyp1a*), U19855.1 (butterfly fish *cyp1a*), NM012540.2 (Norway rat *Cyp1a1*), K03191.1 (human *CYP1A1*), NM009993.3 (Mouse *Cyp1a2*), AB048944.1 (Nile tilapia *cyp1b*), HQ829968.1 (Nile tilapia *cyp1b1*), AJ249074.1 (European plaice *cyp1b1*), HQ829969.1 (Nile tilapia *cyp1c1*), AY437776.1(common carp *cyp1c1*), AY444748.1(Japanese eel *cyp1c1*), AY437777.1(common carp *cyp1c2*), NM001007310.1(Zebrafish *cyp1d1*), FJ786961.1(Killifish *cyp1d1*)]. Sequence alignments were performed by the CLUSTALW method **(Thompson *et al.*, 1994)** using the Lasergene Megalign program (**Ver. 5, 2001, DNASTAR Inc**). The phylogenetic tree was constructed by the neighbor-joining method **(Felsenstein, 2002)** of the previously reported *CYP1* family sequences.

Measurements of Polycyclic aromatic hydrocarbons (PAHs) in water samples

All water samples were analyzed by a Hewlett Packard 5890 series II GC gas chromatograph equipped with a flame ionization detector (FID) according to **El Deeb *et al*. (2007)**.

Sulfur removal

In order to remove sulfur that interferes in GC-FID analysis, the copper powder was used following the steps of **Sundberg *et al*. (2005)**.

Blank preparation

Eighty ml of methylene chloride was poured into a rotary evaporator flask and evaporated to dryness at 30 ºC according to **Parson *et al.* (1985)**. The residue was dissolved in 1 ml of n-hexane before fractionation.

Statistical analysis

By using the SPSS program (Ver.15), the results of gene expression levels were analyzed using one-way ANOVA, and the correlation coefficient between gene expression and PAHs levels was determined (two-tailed). By using Tukey's multiple comparison test, significant differences were noted by different letters ($p < 0.05$) among the different regions of Lake Manzala and the reference region.

RESULTS

1. **The expression of cyp1a gene**

All profiles of PCR products were used to compare among fish populations in different regions of Lake Manzala based on the *cyp1a* expression in the organs of *O. aureus*. In each origin of Lake Manzala, the highest value was detected in the liver

followed by the gill, testis, ovary, and muscle in the El-Gamil fish population. In the El-Serw fish population, the liver and gill were nearly equal in *cyp1a* gene expression. Moreover, the expression in the ovary and muscle was almost equal. In the El-Bashtir fish population, the highest value was observed in the liver followed by the gill, while the testis and ovary were almost equal, and the lowest value was noted in muscles. For the fish population in El-Temsah, the highest value was observed in the liver followed by the testis and gill, while the ovary and muscle were almost equal in the increasing levels of *cyp1a* expression. In the El-Inaniya fish population, the liver recorded the highest value, followed by the gill, testis, ovary and muscle. In general, the highest value of *cyp1a* gene expression was determined in the liver in all populations followed by the gill, testis, ovary, and the muscle of *O. aureus*.

Among Lake Manzala regions, the liver of the El-Gamil fish population showed the highest *cyp1a* expression level, followed by the El-Serw, El-Bashtir, El-Temsah, and El-Inaniya fish populations as shown in Fig. (2). On the other hand, the gill of the El-Serw and El-Gamil fish population exhibited the greatest level of *cyp1a* gene expression, followed by the El-Bashtir, El-Temsah, and El-Inaniya fish populations as shown in Fig. (2). The highest level of *cyp1a* gene expression was observed in the testis of El-Gamil, followed by El-Serw, El-Temsah, El-Bashtir, and El-Inaniya fish populations. In addition, *cyp1a* gene expression of the ovary in the El-Gamil fish population was higher than El-Serw, El-Bashtir, El-Inaniya, and El-Temsah fish populations.

Generaly, *cyp1a* expression levels were higher in the testis than in the ovaries. The highest level of *cyp1a* expression in the muscle was detected in the El-Serw fish population, followed by the El-Gamil, El-Inaniya, El-Bashtir, and El-Temsah fish populations. The muscle was lower compared to the other organs in the increasing level of *cyp1a* expression. The El-Gamil and El-Serw fish populations exhibited the highest levels of *cyp1a* expression compared to other sites of Lake Manzala as shown in Fig. (2).

2. **Polycyclic aromatic hydrocarbons in water of Lake Manzala regions**

The analysis of water by gas chromatograph (GC) showed that the El-Gamil region had the highest values of indeno (1,2,3- cd) pyrene, fluoranthene, benzo(a)pyrene and pyrene. The El-Serw region had the highest values of acenaphthylene. In addition, El-Bashtir region had the highest values of benzo(a)anthracene, chrysene and dibenz(a, h)anthracene. Moreover, the El-Inaniya region possessed the highest values of benzo(b)fluoranthene and benzo(ghi)perylene. The El-Gamil region had the highest values of polycyclic aromatic hydrocarbons (PAHs) and was followed by El-Serw, El-Bashtir, El-Inaniya, and El-Temsah regions as noted in Table (3).

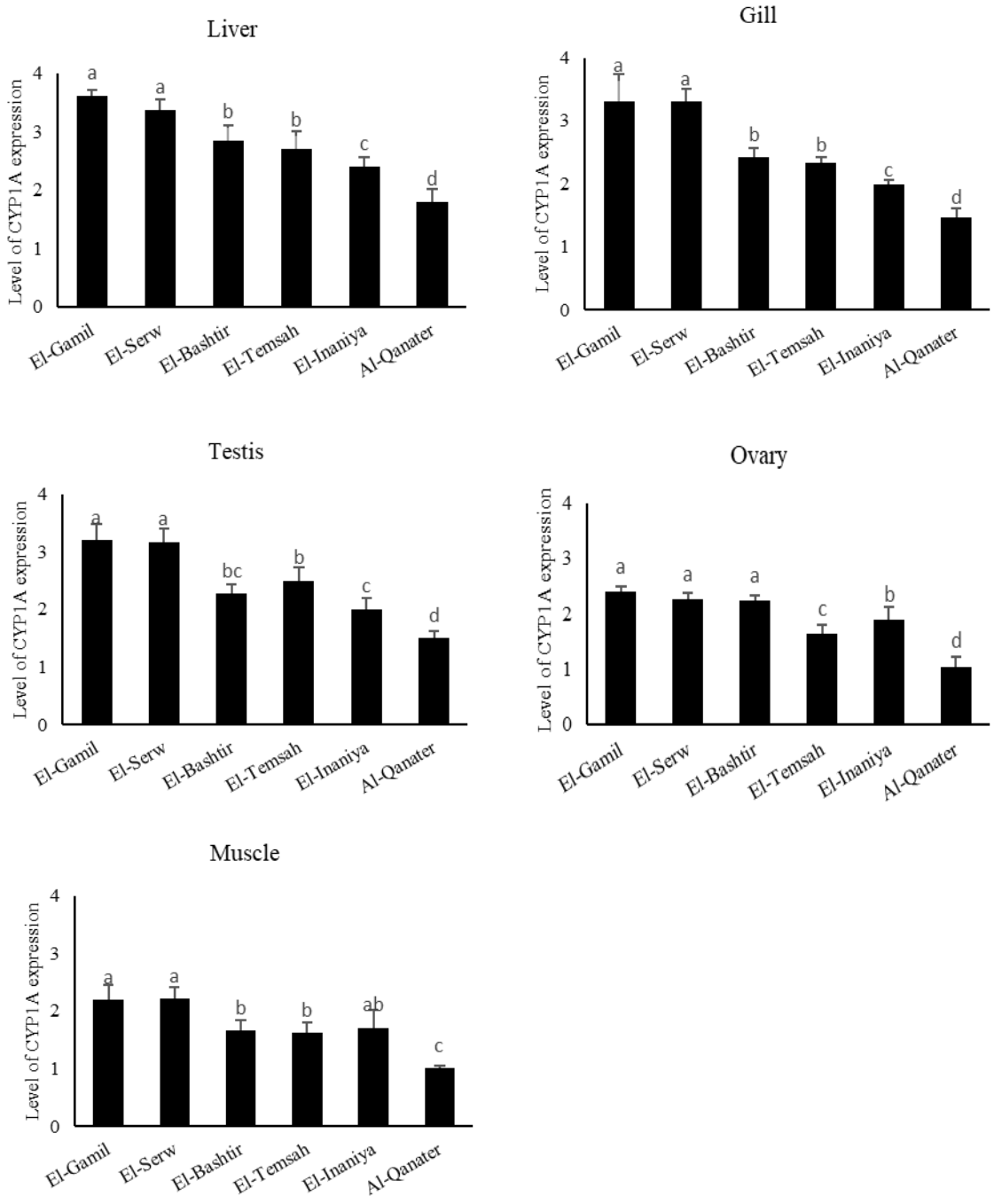

Fig. 2. Levels of CYP1A expression in the liver, gill, testis, ovary and muscle of *O. aureus* in different regions of Lake Manzala. CYP1A expression levels were normalized against β-actin.

Table 3. The analysis of water samples by a Hewlett Packard 5890 series II Gas Chromatograph (concentration unit is µg/l)

PAHs / Region	Ace	Ant	Flu	Pyr	BaA	Chr	BbF	BaP	InP	DBA	Bghi
El-Gamil	24.5998	0.278	2.744	1.4906	9.806	0.408	7.571012	0.578	17.64283	0.3992	0.0632
El-Serw	33.6898	0.3732	2.081	0.9238	15.3384	0.825	1.319	0.1224	0.2022	0.3048	0.0792
El-Bashtir	9.8958	0.4176	0.1444	0.6528	18.8722	1.0406	3.5184	0.1506	0.7964	0.664	0.0354
El-Temsah	8.608	0.1738	0.5492	0.4748	2.6072	0.3704	0.447	0.0384	5.003298	0.0434	0.0258
El-Inaniya	0.1762	0.8306	0.8336	0.899	9.12	0.2518	15.81261	0.0922	0.0454	0.041	1.0784
Al-Qanater	1.8076	1.5262	0.413	1.1268	8.9206	0.552	0.3812	0.0312	0.0476	0.3266	0.0474

Note: Polycyclic aromatic hydrocarbons (PAHs), Acenaphthylene (Ace), Anthracene (Ant), Fluoranthene (Flu), Pyrene (Pyr), Benzo(a)anthracene (BaA), Chrysene (Chr), Benzo(b)fluoranthene (BbF), Benzo(a)pyrene (BaP), Indeno(1,2,3-cd)Pyrene (InP), Dibenz(a,h)anthracene (DBA), Benzo(ghi)perylene (BghiP).

A positive correlation was observed among the expression of the *cyp1a* gene in fish organs. The highest correlation was denoted between the gill and liver in *cyp1a* expression, while the lowest correlation was between the testis and ovary. Moreover, the highest correlation was detected between PAHs and *cyp1a* expression levels in the ovary followed by testis, gill, liver, and muscle as presented in Table (4). The correlation between the *cyp1a* expression and PAHs levels in the regions of Lake Manzala was an indicator of the pollution effect on El-Gamil and El-Serw regions, while the lowest effect was depicted in the El-Temsah region compared to the reference site (Al-Qanater)

3. **Sequencing of cyp1a gene and phylogenetic tree**

The partial sequencing of *cyp1a* mRNA of *O. aureus* was 569 bp, and the identity percentage of partial sequencing of *cyp1a* mRNA of *O. aureus* with *O. niloticus* was 86.8% (Fig. 3). The identity of *cyp1a* between *O. aureus* and other fish was closer than mammals. The phylogenetic tree showed a closer relationship of the *cyp1a* of *O. aureus* to the *cyp1a* subfamily, followed by *cyp1d*, *cyp1c* and *cyp1b*. Whereas, *cyp1a* and *cyp1d* subfamily shared in the same branch to be more correlated to each other than *cyp1b* and *cyp1c* subfamily members (Fig. 4).

Table 4. Pearson correlation between PAHs concentration and *cyp1a* gene expression in the organs

	PAHs	Liver	Gill	Muscle	Testis
Liver	0.871*	1			
Gills	0.880*	0.992**	1		
Muscles	0.844*	0.958**	0.978**	1	
Testes	0.889**	0.976**	0.988**	0.953**	1
Ovaries	0.892**	0.894**	0.888**	0.891**	0.829*

(*) indicate a significant at the 0.05 level and (**) at the 0.01 level.

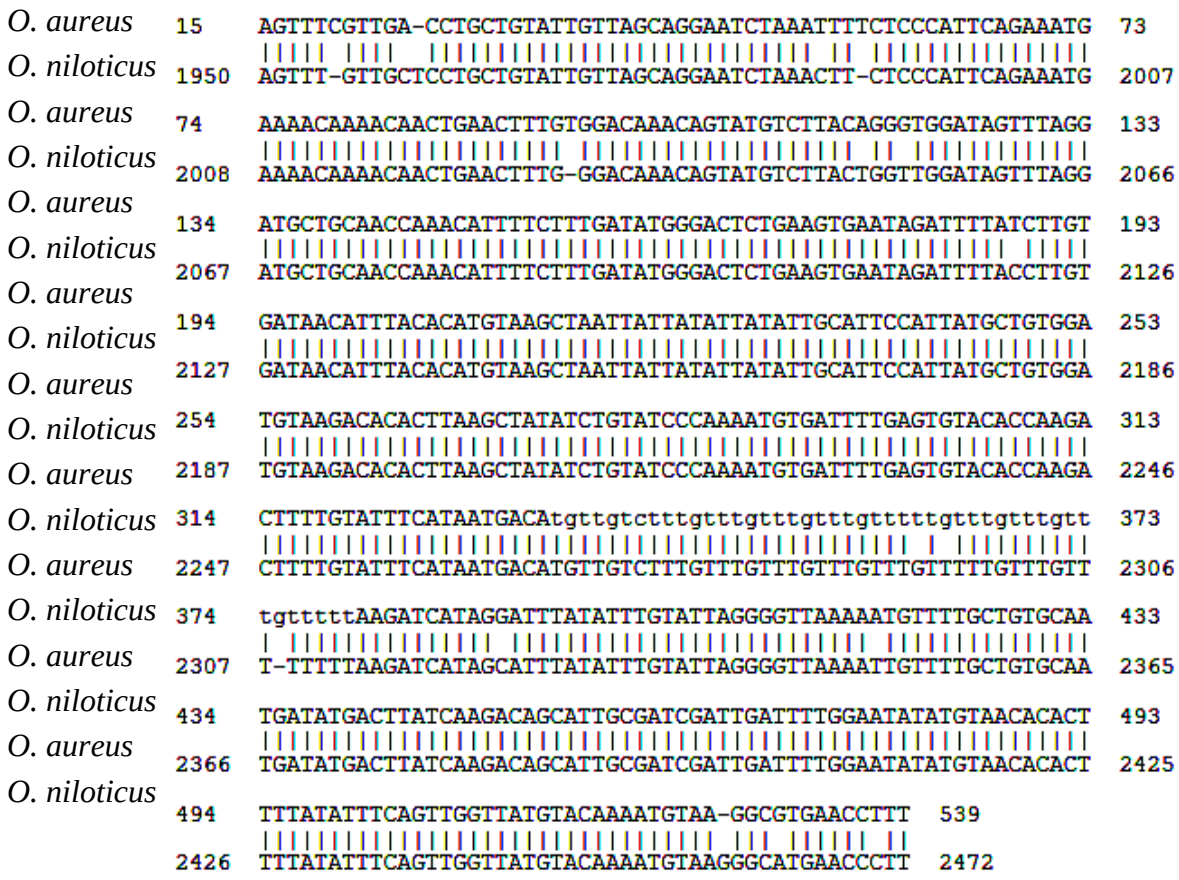

Fig. 3. The alignment of partial sequence of *cyp1a* mRNA of *O. aureus* with *O. niloticus cyp1a* mRNA

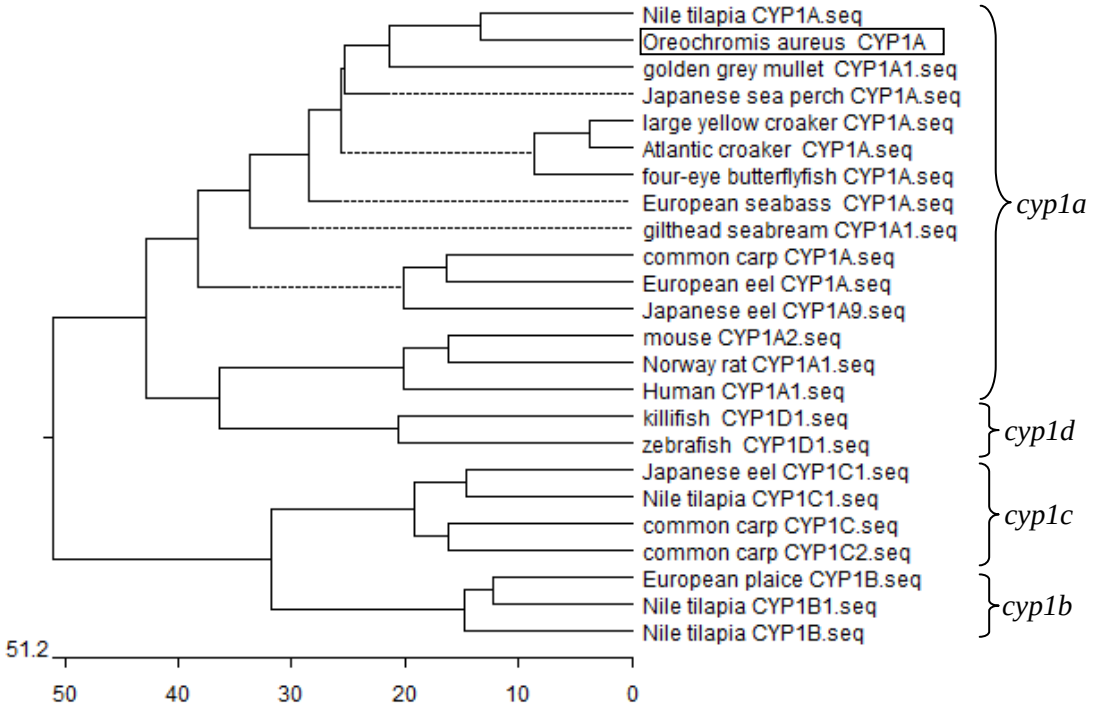

Fig. 4. Phylogenetic tree of *cyp1* family genes constructed by the neighbor-joining method **(Felsenstein, 2002)** using cDNAs sequences of mammals and teleosts

DISCUSSION

Cytochrome P450 enzymes are central to the metabolic activation of polychlorinated biphenyls (PCBs) and arylamines (**Gelboin, 1980**). They are most active in hydroxylating of assorted environmental pollutants, including: arylamines, planar polychlorinated biphenyls, and polyaromatic hydrocarbons (PAH). Thus, they play a vital function in the toxicology of pollutants **(Leaver & George, 2000)**. In addition, *cyp1a* showed wide specificity and recorded the highest rates of metabolic for almost all substrates **(Scornaienchi *et al*., 2010)**.

The present study used the level of *cyp1a* expression in *O. aureus* as a biomarker of pollutants to set a comparison among Lake Manzala regions. This matched with the induction of *cyp1a* expression by exposure to PAHs and other chemical pollutants of fresh and marine water habitats **(Kim *et al*., 2008)**. Additionally, a vital part of risk analysis and modern risk characterization makes use of *cyp1a* monooxygenase activity as a bio-indicator to evaluate xenobiotic exposure in the aquatic environment **(Goksoyr & Forlin, 1992; Holdway *et al*., 1995; Buhler & Wang-Buhler, 1998)**. In the present study, *cyp1a* was successfully used to detect drugs and compare among different regions of Lake Manzala based on fish organs. Several studies regarded the induction of *cyp1a* as a biomarker of pollution to monitor fish exposure to organic contaminants **(Payne, 1976)**,

acting as a bio-indicator for possible exposure to dioxins and PCBs in teleost fish **(Rees *et al*., 2005)**. This forms a useful model for environmental toxicology **(Yamazaki *et al*., 2002)** and an effective biomarker of organic pollutant exposure in Lepomis species **(Brammell *et al*., 2010)**. In goldfish, *cyp1a* offered basic information as a biomarker for the exposure to benzo(a)pyrene (BaP) in organs **(Oh *et al*., 2009)**. Furthermore, it is supporting the extracts' ability to induce *cyp1a* correlated with the content of PCBs measured in the same fish that showed *cyp1a* staining in the kidney, heart, gill, and/or liver of many species **(Stegeman *et al*., 2001)**.

In all the studied fish populations, *cyp1a* gene expression was extrahepatic and detected in fish organs. This observation agrees with those of several studies, among which the study of **Wang *et al*. (2006)** is considered. The previous authors detected *cyp1a* expression in the brain, spleen, eye, gonad, heart, gill, and liver of killifish. Moreover, the expression of *cyp3a30*/*56* mRNA was observed in the liver, intestine, gill, spleen, kidney, brain, and the ovary of killifish **(Hegelund & Celander, 2003)**. In goldfish, BaP induced the *cyp1a* expression in extrahepatic organs, including the intestine and gill, which are sensitive to aqueous and dietary exposure to AHR agonists **(Oh *et al*., 2009)**. In the current study, the liver and gill had the highest expression of *cyp1a*. This finding coincides with that of **Kim *et al*. (2008)** who detected an induction of *cyp1a* expression in the gill and liver in response to βNF exposure. **Leaver and George (2000)** spotted an increase of *cyp1a* expression in fish tissues with the greatest response in gill followed by liver. The gill and liver of *Gasterosteus aculeatus* had a strong *cyp1* expression, giving rise to a high and persistent induction by PCB 126 **(Gao *et al*., 2011)**.

The highest value of *cyp1a* gene expression was observed in the liver in all populations followed by the gill, testis, ovary, and muscle of *O. aureus*. Similarly, the greatest basal expression of the *cyp1a* gene was in the liver of *T. obscurus* in response to beta-naphthoflavone. The liver is the major place of xenobiotics metabolism in fish, and therefore, the expression of *cyp1a* is high in this organ **(Arukwe, 2002; Zapata-Perez *et al*., 2002; Lee *et al*., 2005; Miranda *et al*., 2006)**. In addition, gills are the primary organ of toxicants' absorption; hence, inductions of cytochrome P450-dependant enzymes are observed in the gills **(Evans, 1987; Wilson & Laurent, 2002; Jonsson *et al*., 2003)**. This explains the highest expression of *cyp1a* in the liver and gill noted in the present study at different locations. In this study, the expression of *cyp1a* in fish organs matches with the concentrations of heavy metals in different organs of *Clarias gariepinus* from Lake Manzala that are greatly detected in the liver followed by the kidney, gill, gonad, brain and muscle **(Abdel Baky, 2001)**. Results showed that muscles had less expression of *cyp1a*; a result that concurs with the observation of **Kim *et al.* (2008**). It is worthy to mention that, this tissue is the least important for the metabolism of xenobiotics in fish **(Monostory *et al*., 1996)**.

Each *cyp1a* and PAHs levels detected in the Lake Manzala regions were highly increased in the El-Gamil region followed by El-Serw, El-Bashtir, El-Inaniya, and El-

Temsah regions. Likewise, the persistent organic pollutants were highly detected in El-Gamil, followed by El-Degwa, El-Bashtir, El-Barghotya (closer to El-Inaniya), El-Temsah **(Barakat *et al*., 2012)**.

A positive correlation between *cyp1a* expression and PAHs in the present study matches with the induction of *cyp1c1* expression by Alpha-naphthoflavone (ANF), antagonized 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and BaP **(Bugiak & Weber, 2009)**. Pharmaceutical mixtures are detected in the aquatic environment and adverse effects related to interactions among drugs, including the *CYP* effect by these mixtures may be of concern **(Smith *et al*., 2012)**. This supports that the correlation between gene expression and each chemical component of PAHs separately was inexpressive because some chemical components overlapped in the environment on their impact on *cyp1a* gene expression. Moreover, the *CYP1* family had a different response for PAHs component, such as *cyp1b1* and *cyp1c2* had the highest rates of BaP metabolism in zebrafish. In addition, *cyp1a* had the highest metabolic rates for nearly all substrates **(Scornaienchi *et al*., 2010)**.

In the present study, *cyp1a* of *O. aureus* and *cyp1d* subfamily resulted in the clustering of both genes in the same branch to be more correlated to each other than any *cyp1b* or *cyp1c* subfamily members. Similarly, the *cyp1a*s and *cyp1d*s together constitute one clade, while the *cyp1b*s and *cyp1c*s constitute another clade, **(Goldstone *et al*., 2009)**.

CONCLUSION

Remarkably, this is the first detection of the partial *cyp1a* gene of *O. aureus*. The *cyp1a* expression was highly induced in the liver of all fish populations followed by the gill, testis, ovary and muscle. Higher levels of *cyp1a* and PAHs were observed in the El-Gamil region, followed by the El-Serw, El-Bashtir, El-Inaniya and El-Temsah. The correlation between PAHs and *cyp1a* expression of fish organs was a significant positive correlation among the Lake Manzala regions. The level of *cyp1a* gene expression in fish provided an indicator of aquatic pollution.

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