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RESEARCH ARTICLE

Mining Metallothionein Gene of Stressed Nile Tilapia (*Oreochromis niloticus*) with Heavy Metals Pollutions in Idku Lake, Egypt

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Abstract

In this investigation, 30 of random juvenile starlets *Oreochromis niloticus* (*Oreochromis niloticus*) fish samples ranging in mass from 18 to 38.50 g were collected from three sectors of the Idku Lake, Egypt were used for evaluating heavy metals influence. Different heavy metals concentrations were recorded. cDNA of Metallothionein gene was amplified, sequenced and alignments as *Oreochromis niloticus* (XM_003447045.5) and *Maylandia zebra* Metallothionein (XM_024803240.1) for second and third cadmium and Lead doses respectively. Furthermore, expose to Pb reflects superior genetic polymorphism comparing with Cd and control samples 0.4 mg/L of Cadmium treatment, reflected highest polymorphism (44.4%) comparing with 0.14 mg/L of Lead treatment revealed highest polymorphism (66.6%)

Keywords: Heavy Metals; Metallothionein (MT); *Oreochromis niloticus*

Introduction

Accumulation and non-degradation were cause of serious concern worldwide about heavy metal contamination of fresh water systems and aquatic organisms (M'kandawire *et al.,* 2017). Interestingly, structural or biological functions of bio-molecular were changed by heavy metals [1].

Although some metals such as Fe, Cu, Co, Mn and Zn are essential to biological life forms, but become toxic above their threshold limits [2,3]. Natural or anthropogenic sources could be entering way for Heavy metals to aquatic systems [4]. After dissolving or suspending heavy metals in water, then, released into aquatic bodies and deposited in sediments or assimilated in aquatic biota [5]. In addition oxidative stress, physiological, biochemical, morphological and hematological damages caused by heavy metals [6].

Metallothionein (MT) plays an important role for resistance heavy metal poisoning which cleared [7]. Specific species of fish e.g. rainbow trout, are particularly susceptible to poisoning by cadmium in their aquatic environment because others e.g. roach and stone loach, are much more resistant as a results of existing Metallothionein (MT) in the tissues of these fish is unable to bind cadmium and 2) the toxic metal (in contrast to zinc) cannot switch on the gene(s) for apo-thionein production de novo. Consequently, cadmium is sequestered in the liver, kidney and gills of these fish by two low molecular weight non-metallothionein proteins for which no excretion mechanism appears to exist.

Direct influence of accumulation of heavy metals in commercial edible fish may cause health risks to fish consumers as a result of fish is a major part of the human diet in many parts of the world [8]. Tremor, ataxia, paralysis, convulsions, hemoglobinuria, and gastrointestinal disorders such as diarrhoea, vomiting and stomatitis considered direct and indirect symptoms for acute heavy metal toxicity in human [9].

This investigation was achieved to get better understanding for immune response interaction between heavy metals and Metallothionein gene which consider a major key role for heavy metals biodegradation process in *Oreochromis niloticus*. To accomplish this goal, Metallothionein was studied through end product protein and gene sequence levels.

Material and Methods

Water samples and analysis

Surface water samples were collected during summer for the year 2018 from six sampling sites, two samples of each, distributed over Idku Lake, 31°19'52"N 30°18'2"E (Figure 1) (Table 1). Physical and chemical characteristics (pH, temperature, salinity, dissolved

oxygen and Ammonia were determined. Polyethylene bottle was dipped 20 cm below the water surface [10]. For heavy metal determination, water samples were kept in ice box till transfer to lab. AA500 Atomic Absorption (Pg instruments) was applied for Cu, Zn, Cd and Pb determination as µg / L dry weight [11].

Contents	Cat. Nos. 4368813 and 4374967
10X RT Buffer, 1.0 mL	2 tubes
10X RT Random Primers. 1.0 mL	2 tubes
25X dIsITP Mix (100 mM)	1 tube, 1.0 mL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1 tube, 1.0 mL
RNase Inhibitor, 100 µL	10 tubes

Table 1: PCR component for Metallothionein (MT) gene amplification

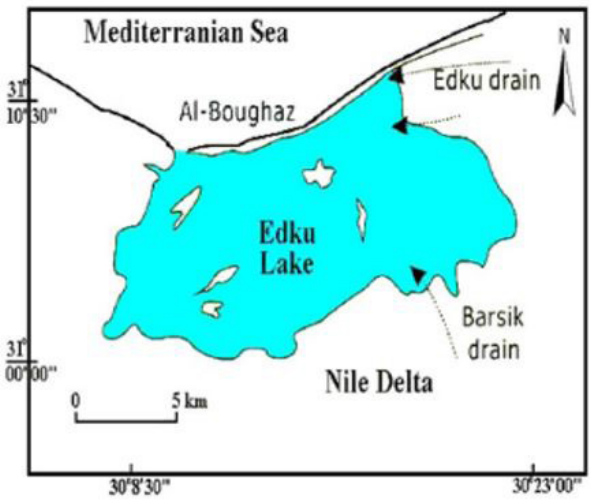


Figure 1: Idku Lake, Alexandria, Egypt

Fish samples

Total 30 of random juvenile starlets *Oreochromis niloticus* fish samples ranging in mass from 18 to 38.50 g were collected from three sectors (eastern, middle, and western) of the Idku Lake. For obtaining fish Liver, samples were dissected freshly till analysis. Liver samples were quickly removed and snap frozen at −80 °C before analysis.

Total RNA isolation and Metallothionein (MT) Semi quantification PCR

Total RNA was extracted from liver tissue samples via Trifast reagent (Peqlab, Germany) according to manufacturer protocol. RNA quality and quantity was estimated through agarose gel electrophoresis and spectrophotometric method for A260 and A280 measurements. High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) was applied to convert purified RNA to complementary DNA from purified. Complementary DNA (cDNA) products were stored at -20°C for PCR. MT primers (:5'-TTGGACACCCTGAAGT-3' and 3'-GTGGCGGAAGTAAAGT-5') were applied to amplify Metallothionein (MT) gene [12]. Semi quantified value was calculated by dividing the measured value for MT transcript by that of 18S rRNA (control with 423 bp). The PCR products with 314 bp were determined with1.5% agarose gel electrophoresis. Gel documentation system (Geldoc-it, UVP, England), was applied for data analysis using Totallab analysis software, ww.totallab.com, (Ver.1.0.1) (Table 2).

Sites		Elements					
		Mn	Zn	Fe	Ni	Cu	Pb
Locations	1	0.74	10.81	14.52	2.88	0.98	0.88
	2	1.85	9.65	10.22	3.59	1.74	N.D
	3	0.42	12.52	14.25	3.74	2.16	1.66
	4	0.95	12.54	21.55	3.21	2.33	1.24
	5	2.01	8.95	13.22	2.98	0.95	N.D
	6	1.25	11.85	20.84	2.84	1.52	0.98

ND: not detected

Table 2: Heavy metals (µg/L) Average values of in surface water of Idku Lake during 2018

Sequencing, Phylogenetic tree and multiple sequence alignment of MT

Specific DNA fragments were eluted from agarose gel through E.Z.N.A® Gel Extraction Kit (omega BioTEK, USA). Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea). Aligned sequences were analyzed on NCBI website (http://www.ncbi.nlm.nih.gov/website) using BLAST to confirm their identity. Phylogenetic tree constructed based on relationship of S-MT to MTs from other species of fishes, mammals, amphibians, and birds were computed by Pairwise Distance method using ClusteralW software analysis (www.ClusteralW.com).

Electrophoretic analysis:

SDS Polyacrylamide gel electrophoresis (SDS-PAGE) with 13% T was performed to distinguish and fragment total soluble protein and specific Metallothionein (MT) protein fragment (6 KDa) of juvenile starlets *Oreochromis niloticus* fish liver samples which purified via Tri-Fast (Peqlab, VWR company, isolation of RNA, DNA and Protein simultaneously) [12].

Metallothionein (MT) Protein expression

Western blotting technique was performed to detect MT Protein expression. Anti-Metallothionein antibody (abcam, ab192385) was applied according to manufacturer protocol. Geldoc-it, (UVP, England) gel system integrated with Total lab analysis software, ww.totallab.com, (Ver.1.0.1) was applied to evaluate MT Protein expression level.

Results

Heavy metals concentration was evaluated for Idku Lake (Table 3). Significant variation was founded among different locations.

Full-length cDNA of Metallothionein (MT) was amplified according to selected primers. Figure 2 showed amplified amplicon with two specific fragments with 314 bp for Metallothionein (MT) gene. All treated and control samples revealed specific Metallothionein (MT) amplicons with different genomic DNA content.

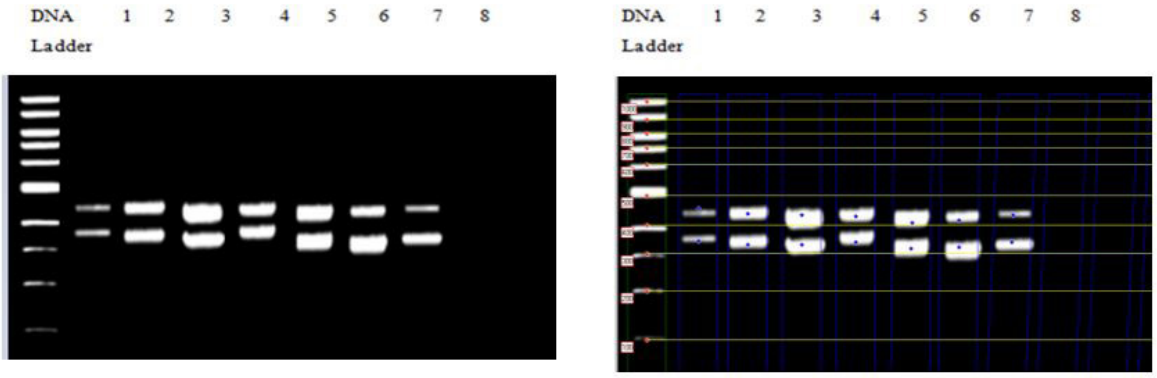
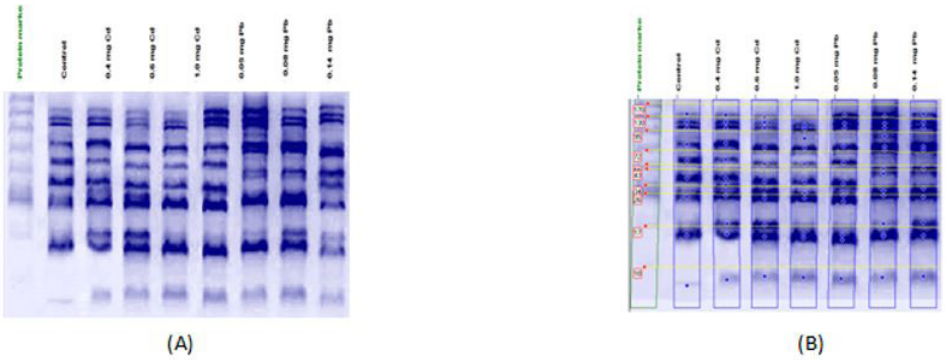


Figure 2: Metallothionein (MT) amplification products under different heavy metals doses, **(A)** amplified products and **(B)** length calculation

(A)	**(B)**
1. Cadmium 0.4 mg/L	2. Cadmium 0.6 mg/L
3. Cadmium 1.0 mg/L	4. Lead 0.05 mg/L
5. Lead 0.08 mg/L	6. Lead 0.14 mg/L
7. Control sample	8. Negative control

Generally, MT expression level was superior for liver tissue as a response to heavy metals comparing with control tissue and comparison to the 18S rRNA housekeeping gene expression. Evaluation heavy metal does reflect significant increase of expression level for second Cadmium does and third Lead dose.

To evaluate influence of heavy metals doses on *Oreochromis niloticus* samples, SDS-PAGE was applied. Protein fractions revealed distinguishable protein variation (Figure 3, 4 and Table 3). Generally, exposed to Pb reflects superior genetic polymorphism comparing with Cd and control samples. As shown by Figure 3, 0.4 mg/L of Cadmium treatment, reflected highest polymorphism (44.4%) comparing with 1.0 mg/L which showed low polymorphism (22.2%). Interestingly, treated with 0.6 mg/L didn't reflect any polymorphism. By contrary, Pb treatment caused clear polymorphism at protein level. Genetic polymorphism was increased exponentially for Pb treatment doses. 0.05, 0.08 and 0.14 Pb mg/L reflected 37.5, 45.4 and 66.6 of polymorphism percentage. Common Metallothionein (MT) protein fraction with 6 KDa was excised in all samples. Lowest protein content was evaluated for untreated sample comparing with treatment samples.

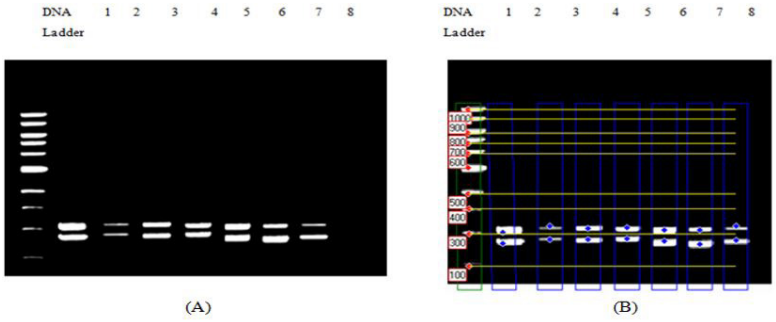


(A) (B)

	Control	Cd			Pb		
		0.4	0.6	1.0	0.05	0.08	0.14
1	-	-	-	-	-	-	144
2	137	137	-	-	137	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	120
5	114	-	-	-	114	114	-
6	-	-	-	-			-
7	-	-	102	-	102	102	-
8	-	-	-	-	-	87	-
9	-	-	-	83	-	-	-
10	-	78	-	-	-	-	-
11	76	-	-	-	-	-	76
12	-	-	74	74	-	-	-
13	-		-	-	-	72	-
14	-	65	-	-	-	-	-
15	62	-	-	-	-	-	-
16	-	-	58	58	-	-	-
17	-	-	-	-	-	-	45
18	-	-	-	-	-	37	-
19	-	36	-	-	-	-	-
20	-	-	35	35	-	-	-
21	-	-	-	-	34	-	-
22	-	-	-	-	-	-	32
23	-	-	-	-	-	30	-
24	-	-	-	27			-
25	-	24	-		24	24	-
26	23	-	23	23		-	-
27	-	-			20	-	-
28	-	-			-	-	-
29	-	16			-	16	-
30	-	15		15	-	15	-
31	14	-	14	-	-	14	-
32	-	-	-	-	13	-	-
33	-	-	-	-	-	-	-
34	-	-	-	8	-	8	-
35	-	7.8	-	-	-	-	-
36	6	6	6	6	6	6	6

(C)

Figure 3: Nile tilapia Protein electrophoretic under different doses of heavy metals, (A) protein electrophoretic patterns, (B) molecular weight detection and (C) protein fragments polymorphism



(A) (B)

1. Cadmium 0.4 mg/L
2. Cadmium 0.6 mg/L
3. Cadmium 1.0 mg/L
4. Lead 0.05 mg/L
5. Lead 0.08 mg/L
6. Lead 0.14 mg/L
7. Control sample
8. Negative control

Figure 4: Metallothionein (MT) amplification products under different heavy metals doses, (A) amplified products and (B) length calculation.

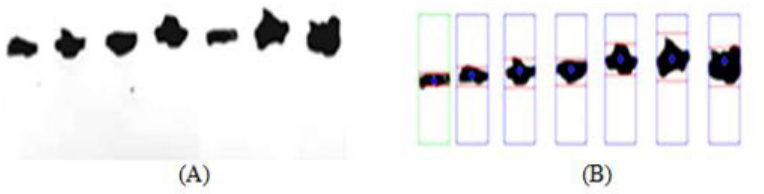
Treatments		Total protein bands	Polymorphic bands	Monomorphic bands	Polymorphism %	Average Polymorphism %
Control		7	**1**	**6**	**14.2**	**14.2**
Cd	0.4	9	4	5	44.4	22.2
	0.6	7	0	7	0	
	1.0	9	2	7	22.2	
Pb	0.05	8	3	5	37.5	49.8
	0.08	11	5	6	45.4	
	0.14	6	4	2	66.6	

Table 3: Total protein bands, Polymorphic bands, Polymorphism % and Average Polymorphism % for Nile tilapia Protein electrophoretic under different doses of heavy metals

Based on western blotting findings, Cadmium and Lead heavy metals stress reflects varied Metallothionein expression level (Figure 5 and 6). Lowest Metallothionein expression level was recorded for *Oreochromis niloticus* samples which didn't exposure to heavy metals. Generally, Metallothionein expression level after exposure to lead stress was superior on expression level after exposure to Cadmium stress (56.93 and 80.71 respectively). For Cadmium stress, exposure to second dose (0.6 mg/L) cause the highest Metallothionein expression level comparing with high and low doses (0.4 and 1.0 mg/L respectively). By contrary, highest lead dose (third dose) reflected superiority of Metallothionein expression level. Our obtaining data could be explained in the light of variation of degradation metabolic process between lead and cadmium.

Samples		Homology sequences	GenBank number	Identity %
Metallothionein (MT) sequence under second cadmium dose	1	PREDICTED: Oreochromis niloticus metallothionein (LOC100696451), mRNA	XM_003447045.5	94
	2	Oreochromis aureus Metallothionein gene, complete cds	EU005543.1	93
	3	Oreochromis aureus Metallothionein (MT) gene, complete cds	AY257201.1	93
	4	PREDICTED: Astatotilapia calliptera Metallothionein (LOC113027503), mRNA	XM_026177121.1	96
	5	PREDICTED: Maylandia zebra Metallothionein (LOC101479115), mRNA	XM_004569146.4	96
Metallothionein (MT) sequence under third Lead dose	6	PREDICTED: Haplochromis burtoni Metallothionein (LOC102305612), mRNA	XM_005935788.2	96
	7	PREDICTED: Maylandia zebra Metallothionein (LOC112435134), mRNA	XM_024803240.1	97
	8	Histiodraco velifer Metallothionein 2 (MT-2) mRNA, complete cds	FJ870690.1	91
	9	Cygnodraco mawsoni Metallothionein 1 (MT-1) mRNA, complete cds	FJ870687.1	91
	10	Pleuragramma antarcticum Metallothionein 1-like (MT-1) mRNA, partial sequence	FJ870668.1	91

Table 4: Highest homology Metallothionein (MT) sequences



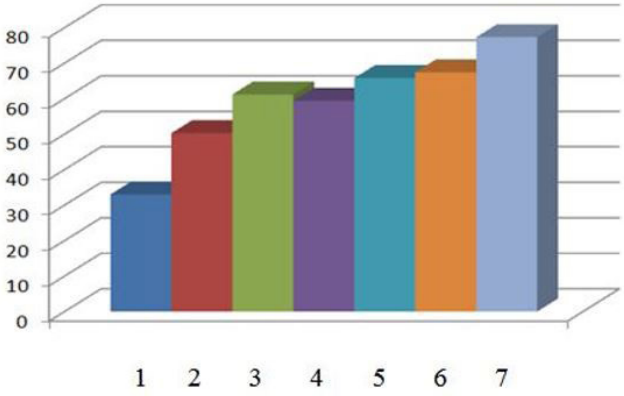
(A) (B)

	Control	Cd			Pb		
		0.4	0.6	1.0	0.05	0.08	0.14
Metallothionein protein expression level	32.98	50.25	61.17	59.38	65.77	67.43	77.32

(C)

Figure 5: Metallothionein western blotting data, (A) blotting membrane, (B) computerized expression quantification and (C) Metallothionein expression level

Biochemical and genetical studies was carried out to evaluate influence of heavy metals on Metallothionein (MT) gene. To achieve this goal based on western blotting data for most influenced heavy metals doses (second Cadmium dose and third Lead dose), Metallothionein (MT) gene was studied at end product expression level and gene sequence level. Metallothionein (MT) gene for stressed Oreochromis niloticus samples with second and third cadmium and Lead heavy doses were sequenced (Figure 7). Alignments two Metallothionein (MT) sequences reflected different Identity % (Figure 8) (Table 4) with superior identity % for Metallothionein (MT) under second cadmium dose comparing with Metallothionein (MT) under third Lead dose which clear distinguish influence of Lead comparing with Cadmium. Figure 9 showed phyllogenetic tree for Metallothionein (MT) sequences under second and third cadmium and Lead doses alignments with highest identity % templates.



1. Cadmium 0.4 mg/L
2. Cadmium 0.6 mg/L
3. Cadmium 1.0 mg/L
4. Lead 0.05 mg/L
5. Lead 0.08 mg/L
6. Lead 0.14 mg/L
7. Control sample
8. Negative control

Figure 6: Metallothionein expression level under different heavy metals stress

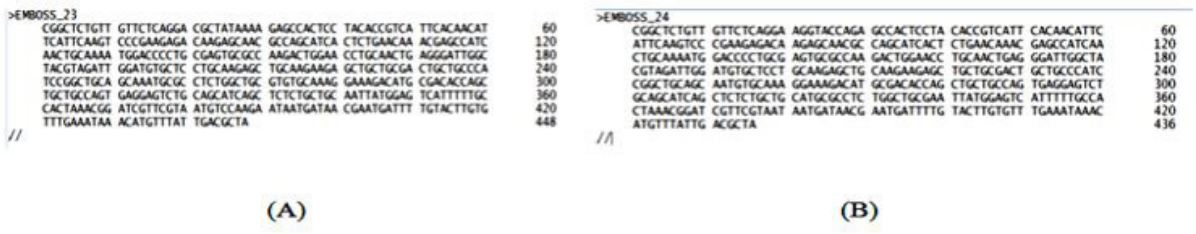


Figure 7: Metallothionein (MT) gene sequences of treated samples with (A) second cadmium dose, (B) third Lead dose

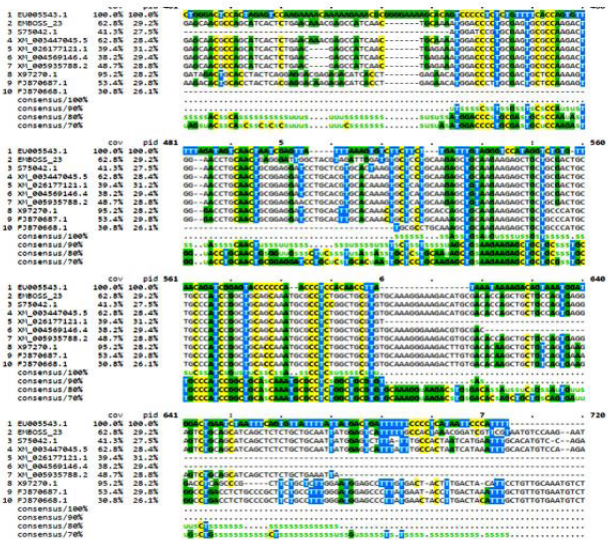


Figure 8: Alignments data for Metallothionein (MT) of treated samples with (A) second cadmium dose, (B) third Lead dose

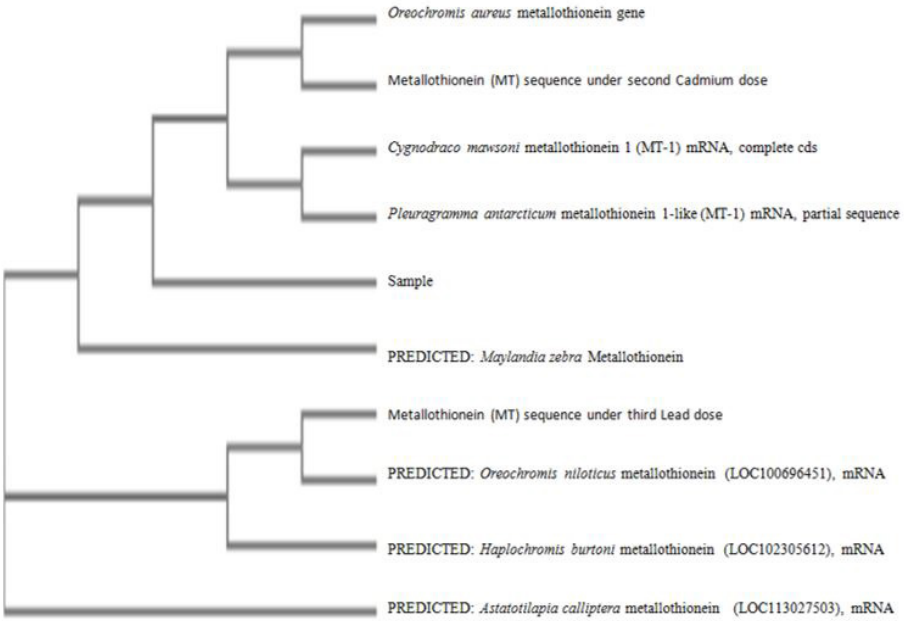


Figure 9: Phyllogenetic tree for Metallothionein (MT) sequences

Discussion

More support was added to our findings for heavy metals evaluation of Idku Lake by Hassan, *et al*. They found that highest concentrations of Lead and Cadmium were found in water sample from Idku Lake, and the highest concentrations of Cobalt and Zinc were found in water samples from Tromp at and Kafer El-Zyate, respectively. Correlation between dramatic decrease of water quality and high heavy meal concentrations was cleared in the light of many studies which cleared that concentrations of water metals were influenced by temperature, salinity and dissolved oxygen; while the concentrations of sediments metals were influenced by water metal levels [13]. Highly significant accumulated heavy metals in north Egyptian lakes specially Idku due to two main agents, the natural agents (Siltation problems a result of the combination of sand transport in the long shore as well as cross- shore directions characteristic of most of the coastal lakes and low lying deltaic Idku area) as well as the anthropogenic activities which combined with different items, land reclamation; pollution resulted from agricultural and industrial projects as well as land irrigation and domestic activities [14].

Our finding for heavy metals concentration variation for each location was supported by many studies which indicated heavy metals concentration variation for water samples which collected from six sampling sites and fish samples were collected from the three sectors (eastern, middle, and western) of the lake [15]. Our findings for Metallothionein expression level were in accordance with study which cleared highly significant expression of Metallothionein was in liver tissue which related to heavy metal pollution [16]. More support was added to our results by indicating induced heavy metals and transcriptionally competent extra MT gene copies [17]. Our findings for heavy metals influence and applied Tilapia fish as a heavy metals pollution bio marker was supported findings of highly influence of Cu on Tilapia fish total protein comparing with Fe, Pb and Zn and founded electrophoretic pattern variation for heavy metals accumulation between wild and farmed *Oreochrom isniloticus* [18]. Variation for Metallothionein expression level among different heavy metals doses was cleared by applied western blot analysis for detecting MT proteins in three of these four sampling sites. Besides, of all 15 species tested only two species had detectable MT protein bands [19]. Also, more support was added to our findings through increased MT expression levels in Cd treated CATFISH *ARIUS ARIUS* liver tissue when compared with control tissues. Additionally, increasing MT protein after exposer to Cd in the light of cytosolic MT localization [20]. Furthermore, the lysosomal population of hepatocytes also exhibited a strong MT labeling after Cd-exposure. The lysosomes constitute a major compartment for metal accumulation and sequestration allowing a reduction of the toxic availability of Cd, at least transiently [21]. In addition, lysosomes can contain degradation products of MTs and serve as a final storage site of degraded MTs and possibly, of other metal-binding proteins [22-24].

Conclusion

Varied Cadmium and Lead concentrations were detected for sixlocations of Idku Lake, Egypt. Metallothionein gene were identified for second and third cadmium and Lead doses respectively as *Oreochromis niloticus* (XM_003447045.5) and *Maylandia zebra* Metallothionein (XM_024803240.1). Significant genetic polymorphism was evaluated after exposure to cadmium and Lead doses. 0.4 mg/L of Cadmium treatment, reflected highest polymorphism (44.4%) comparing with 0.14 mg/L of Lead treatment revealed highest polymorphism (66.6%).

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