UDC 575
DOI: 10.2298/GENSR1503131A
Original scientific paper

USING CYTOGENETIC ANALYSIS RAPD IN DETERMINATION OF GENETIC VARIATIONS AMONG FOUR SPECIES OF ORNAMENTAL FISHES OF FAMILY: Poecilidae (Order: Cyprinodontiform)

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Abu-Almaaty,A. H.,.M. Welson Zekry and Y. A. Essa (2015): *Using cytogenetic analysis and RAPDin determination of genetic variations among four species of ornamental fishes of family: Poecilidae (order: Cyprinodontiform).*- Genetika, Vol 47, No. 3, 1131-1148.

The karyological and molecular analysis of four fresh water fish species of Family: Poecilidae and their genetic relationships have been studied. *Xiphophorus maculates* and *Xiphophorus hellerii* have the same diploid chromosome number 2n=48, but they were different in their karyotypes. *Poecilia sphenops* and *Poecilia reticulata* have the same diploid chromosome number 2n=46 and the same fundamental number FN=46, also the same karyotype one group of acrocentric chromosomes. Nine RAPD primers, showed monomorphic bands, were used for the construction of the dendrogram and a similarity matrix. A total of 65 bands were obtained; 39 of them were monomorphic bands. Similarity values among the studied samples ranged from 21% to 38%. High similarity value was obtained between *Xiphophorus maculates* and *Xiphophorus hellerii.* (38%) and the low similarity values were obtained between *Xiphophorus hellerii* and *Poecilia reticulata* (21%). The cluster analysis clearly differentiated *Xiphophorus maculates* and *Xiphophorus hellerii* from *Poecilia sphenops* and *Poecilia reticulata*. RAPD analysis confirmed that the four species under study are genetically different from each other. These cytogenetic data obtained can be applied for further studies in cytotaxonomy and evolutionary relationships of fishes.

Key words: Cytogenetics, karyotype, RAPD,PCR,DNA,Fishes,Poecilidae *Xiphophorus, Poecilia*.

INTRODUCTION

Guppy (*Poecilia reticulata*), black molly (*P. sphenops*), red fin swordtail (*Xiphophorus helleri*) and red fin platy (*X. maculatus*) are freshwater fishes found in East America to South

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America, Africa and Madagascar. They are classified in the family Poeciliidae which contains about 30 genera and 293 species (NELSON, 1994). Based on morphology alone, many fishes have been misidentified. Current knowledge about cytotaxonomy, immunology and molecular biology plays important roles in fish identification (BUTH *et al*., 1991; ARAI, 1982).

The studies on the chromosomes of fishes have not been successful or widespread as in other vertebrate groups. Fish karyotypes are generally characterized by a large number of small chromosomes, discouraging researchers from pursuing fish-karyotype analysis. Therefore karyological data on fish are available only for a small percentage (about 10%) of some 25,000 species taxonomically known so far.

Cytogenetics refers to the study of heredity through the study of chromosomes (the bearers of the genes) and the cytological mechanisms of inheritance. Procedures involving preparations of mitotic chromosomes from actively dividing somatic tissues of live specimens or from embryos have been the most widely used among fish cytologists and have the dual advantages of being rapid and inexpensive. The soft organs (kidney, spleen, and liver) have proved to be fine sources of chromosomes. The discipline of "cytogenetics," along with its practical application, has yet to be used extensively in fish breeding or fish culture (GOLD, 1979). Cytogenetic studies provide important basic knowledge which may have applications for many other studies, such as for the detection of ploidy in fishes (PRADEEP *et al*., 2011, 2012). In many vertebrate groups, the study of karyotypes and genome size has contributed along with analyses of mitochondrial and nuclear gene sequences to the resolution of challenges in biology systematics and evolution. However, in fishes, the most diverse of all vertebrate groups, higher taxa traditionally have been classified largely by morphology and paleontology, with a much smaller input of cytogenetic information. In part, karyotypes can be obtained only from living specimens, tissues, or cells, which make it challenging to study the karyotypes of fishes that are difficult to collect alive. Of course, even fresh material provides no guarantee that reliable chromosome figures can be obtained easily (ARAI, 2011).

Karyotypes describe the number of chromosomes, and what they look like under a light microscope. Attention is paid to their length, the position of the centromeres, banding pattern, any differences between the sex chromosomes, and any other physical characteristics, the preparation and study of karyotypes is part of cytogenetic (KING *et al*., 2006). Karyotyping is the process of pairing and ordering all the chromosomes of an organism, thus providing a genome-wide snapshot of an individual's chromosomes. Karyotypes are prepared using standardized staining procedures that reveal characteristic structural features for each chromosome (O'CONNOR, 2008). Most fishes studied have a diploid complement of 48 acro-centric chromosomes (O'CONNOR, 2008; KLINKHARDT *et al*., 1995; SOLA *et al*., 1998). However, in some taxa, close species have been reported showing changes in chromosome numbers and formula (BRUM, 1996; MEDRADO *et al*., 2012).

OZOUF-COSTAZ *et al*., (1997) reported that the diploid chromosome number in most fishes varies from 2n=22 to 2n = 260. A karyotype composed of 48 acrocentric chromosomes is considered to be ancestral for all teleosts (OHNO, 1974). The technique of random amplified polymorphic DNA (RAPD) marker (WELSH and MC CELLAND, 1990; WILLIAMS *et al*., 1990) has been successfully exploited for stock identification and population analysis in fish (DONG and ZHOU, 1998; BARTFAI *et al*., 2003; EL-ZAEEM *et al*., 2006); where Genetic information assists in solving problems of identity and defining conservation units for species (FRANKHAM *et al*., 2004).

Chromosomal studies in recent years gained a considerable importance, concerning species characterization, evolution and systematic (BARAT *et al*., 2002).

Due to the lack of karyotypic data of *Poecilia reticulata*, *P. sphenops*, *Xiphophorus helleri* and *X. maculatus*, we therefore aimed to study the karyotypes, molecular genetic variations and phylogenetic relationships among these fishes. The findings of this study can be further applied in research areas of fish cytotaxonomy and evolutionary relationships.

MATERIALS AND METHODS

A total of four species of freshwater fishes were collected from sahl el-tina in Port-said, *Poecilia reticulata*, *Poecilia sphenops*, *Xiphophorus helleri* and *Xiphophorus maculates* of family Poecilidae, then they were transported to the lab and kept alive until processed.

Mitotic chromosomes were prepared from head kidney, spleen and gills as described by (NIRCHIO, 1998). Each specimen was injected with 0.05% colchicines (1ml / 100g fish weight) the fish were maintained in a well aerated aquarium and after 2hr they were sacrificed. The kidneys, spleen and gills were removed and placed in a hypotonic solution of 0.56% kcl after nearly 30 min, the tissues immersed three times in a ethanol-acetic acid glacial mixture 3:1 every time was taken 20min, then the tissues squashed in 60% acetic acid , three droplets of the cellular suspension was dropped on a clean microscope slide, previously chilled in a freezer, from a height of 50 cm. the slides were briefly passed over a flame and then allowed to air-dry.for conventional karyotype the preparations were stained during 40 min with 5% Giemsa in phosphate buffer ph 6.8. The slides were examined under a research light microscope using ×10 or ×15 eyepieces, together with × 15 objectives for chromosomal analysis. Karyotypes were made from good spreads of chromosome. Classification of chromosomes in karyotype studies relating to centromeric index was done according to LEVAN, (1964).

DNA was extracted by using "Quick Genomic DNA Extraction Kit" Cat. No.1112. Nine primers (Alpha DNA, Montreal, Quebec, Canada) were used in RAPD – PCR analysis to study the difference between four specimens of family *Poecilidae*, the code and sequences of these primers are shown in Table 1

Table 1. Primers and primer sequences used for amplification and sequencing in this study

No.	Primer code No.	Nucleotide sequence (5` to 3`)	Annealing Tm °C /Sec	GC%
1	**OPA-9**	**GGGTAACGCC**	**42.70**	**70**
2	**OPA-11**	**CAATCGCCGT**	**38.60**	**60**
3	**OPG-2**	**GGCACTGAGG**	**42.70**	**70**
4	**OPM-2**	**ACAACGCCTC**	**38.60**	**60**
5	**OPM-5**	**GGGAACGTGT**	**38.60**	**60**
6	**OPM-17**	**TCAGTCCGGG**	**42.70**	**70**
7	**OPO-2**	**ACGTAGCGTC**	**38.60**	**60**
8	**OPO-4**	**AGGTCCGCTC**	**42.70**	**70**
9	**OPO-6**	**CCACGGGAAG**	**42.70**	**70**

Each sample was analyzed in agarose gel prepared in 10 mM tris-HCL (PH 7.6), 10 mM EDTA, 0.005% bromophenol blue, 0.005% xylene cyanide and 10% glycerol. The gel was stained with ethidium bromide though adding 5 µl of this stain/100 ml buffer of agarose gel and photographed under ultraviolet light for visualizing the resulted bands. The banding patterns of DNA fragments were analyzed by Gene profiler computer software program showing the molecular weight and the intensity of each band. The marker is composed of 10 chromatography purified individual DNA fragments of molecular weight of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and100 bp, respectively.

The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient (SNEATH *et al*., 1973). Dice formula: GSij = 2a/ (2a+b+c),Where GSij is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The similarity matrix was used in the cluster analysis. At the first step, when each accession represents its own cluster, the distances between these accessions are defined by the chosen distance measure (Dice coefficient). However, once several accessions have been linked together, the distance between two clusters is calculated as the average distance between all pairs of accessions in the two different clusters. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA).

RESULTS

Four species of ornamental fishes, belonging to family Poecilidae, were cyto- and molecule-genetically studied, using air drying technique and RAPD-PCR analysis. The chromosomal numbers of all species under the study were between 2n=46 and 2n=48, but differ in their karyotypes in some species. Nine single 10-mer primers (OPA-9, OPA-11, OPG-2, OPM-2, OPM-5, OPM-17, OPO-2, OPO-4 and OPO-6) - with G+C contents of 60% and 70% - were used in the present investigation to determine the genetic differences among four species of family Poecilidae, *Poecilia reticulata*, *P. sphenops*, *Xiphophorus helleri* and *X. maculates*. The DNA fragments generated by the nine primers from the genomic DNA of the four species were separated using Agarose gel electrophoresis. The banding patterns of these DNA fragments were analyzed by Gene profiler computer software program. Following are the kayotypes and amplification results of the four species obtained from this study.

Poecilia reticulata

The chromosomal analysis of this fish indicated that the diploid chromosome number 2n=46 and the fundamental number is FN=46 (Fig.1), the chromosomes are arranged in one group: group A composed of twenty three acrocentric pairs of chromosomes with relative lengths ranged from 2.86 % to 6.99 %, arm ratio ∞ and centromeric index is zero. All these measurements are shown in Table (2).

Random amplified polymorphic DNA (RAPD) technique was used to examine the genetic variability on *P. reticulata* with all primers except OPO-2 and OPO-6 produced different RAPD band patterns of number of 22 bands ranged approximately from 160 bp by the primer OPG-2 to 1100 bp by the primer OPA-11. The generated bands ranged in number from 1 by the primer OPA-11 to 6 by the primer OPM-2.

Table 2. Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Poecilia reticulata

Chromosome Number	Chromosome Length			Relative Length %			Arm ratio	Centro meric Index	Classification
	Long arm	Short arm	Total	Long arm	Short arm	Total			
	Mean ±S. D	Mean ±S. D	Mean±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	
1	0.71± 0.03	Zero	0.71± 0.03	6.99±0.27	Zero	6.99±0.27	∞	Zero	Acro.
2	0.57 ± 0.07	Zero	0.57 ±0.07	5.61±0.31	Zero	5.61±0.31	∞	Zero	Acro.
3	0.56 ± 0.09	Zero	0.56 ±0.09	5.52 ±0.30	Zero	5.52±0.30	∞	Zero	Acro.
4	0.55 ± 0.04	Zero	0.55 ±0.04	5.42 ±0.27	Zero	5.42±0.27	∞	Zero	Acro.
5	0.53 ± 0.03	Zero	0.53 ± 0.03	5.22 ±0.33	Zero	5.22±0.33	∞	Zero	Acro.
6	0.51 ± 0.06	Zero	0.51 ± 0.06	5.02 ±0.19	Zero	5.02±0.19	∞	Zero	Acro.
7	0.50 ± 0.07	Zero	0.50 ± 0.07	4.93 ±0.21	Zero	4.93±0.21	∞	Zero	Acro.
8	0.47 ± 0.02	Zero	0.47 ± 0.02	4.63 ±0.16	Zero	4.63 ±0.16	∞	Zero	Acro.
9	0.47 ± 0.04	Zero	0.47 ± 0.04	4.63 ±0.20	Zero	4.63 ±0.20	∞	Zero	Acro.
10	0.46 ± 0.05	Zero	0.46 ± 0.05	4.53 ±0.18	Zero	4.53 ±0.18	∞	Zero	Acro.
11	0.44 ± 0.04	Zero	0.44 ± 0.04	4.33 ±0.16	Zero	4.33 ±0.16	∞	Zero	Acro.
12	0.43 ± 0.06	Zero	0.43 ± 0.06	4.24 ± 0.17	Zero	4.24 ±0.17	∞	Zero	Acro.
13	0.42 ± 0.07	Zero	0.42 ± 0.07	4.14 ± 0.14	Zero	4.14 ±0.14	∞	Zero	Acro.
14	0.41 ± 0.06	Zero	0.41 ± 0.06	4.04 ±0.12	Zero	4.04 ±0.12	∞	Zero	Acro.
15	0.40 ± 0.08	Zero	0.40 ± 0.08	3.94 ±0.19	Zero	3.94 ±0.19	∞	Zero	Acro.
16	0.38 ± 0.03	Zero	0.38 ± 0.03	3.74 ±0.14	Zero	3.74 ±0.14	∞	Zero	Acro.
17	0.37 ± 0.05	Zero	0.37 ± 0.05	3.64 ±0.13	Zero	3.64 ±0.13	∞	Zero	Acro.
18	0.36 ± 0.07	Zero	0.36 ± 0.07	3.55 ±0.16	Zero	3.55 ±0.16	∞	Zero	Acro.
19	0.35 ± 0.03	Zero	0.35 ± 0.03	3.45 ±0.18	Zero	3.45 ±0.18	∞	Zero	Acro.
20	0.34 ± 0.02	Zero	0.34 ± 0.02	3.35 ± 0.15	Zero	3.35 ±0.15	∞	Zero	Acro.
21	0.33 ± 0.05	Zero	0.33 ± 0.05	3.25 ± 0.15	Zero	3.25 ±0.15	∞	Zero	Acro.
22	0.30 ± 0.03	Zero	0.30 ± 0.03	2.95 ± 0.16	Zero	2.95 ±0.16	∞	Zero	Acro.
23	0.29 ± 0.07	Zero	0.29 ± 0.07	2.86 ±0.13	Zero	2.86 ±0.13	∞	Zero	Acro.
Sum			10.15± 0.27						

Poecilia sphenops

The metaphase spreads of *Poecilia sphenops* giving a diploid chromosome number 2n=46 and fundamental number of FN=46 (Fig.2). The karyotype was composed of one group all of them are acrocentric pairs of chromosomes, with relative lengths varies from 2.40 % to 6.83 %, arm ratio is ∞ and centromeric index is zero. All these measurements are shown in Table (3).

The RAPD analysis of *P*. sphenops illustrated that all primers except OPM-17 andOPO-6 gave fragments of 23, one band by the primer OPO-2 and six bands by the primer OPG-2. The size of fragments ranged from 220 bp by the primer OPG-2 to 1100 bp by the primer OPM-2.

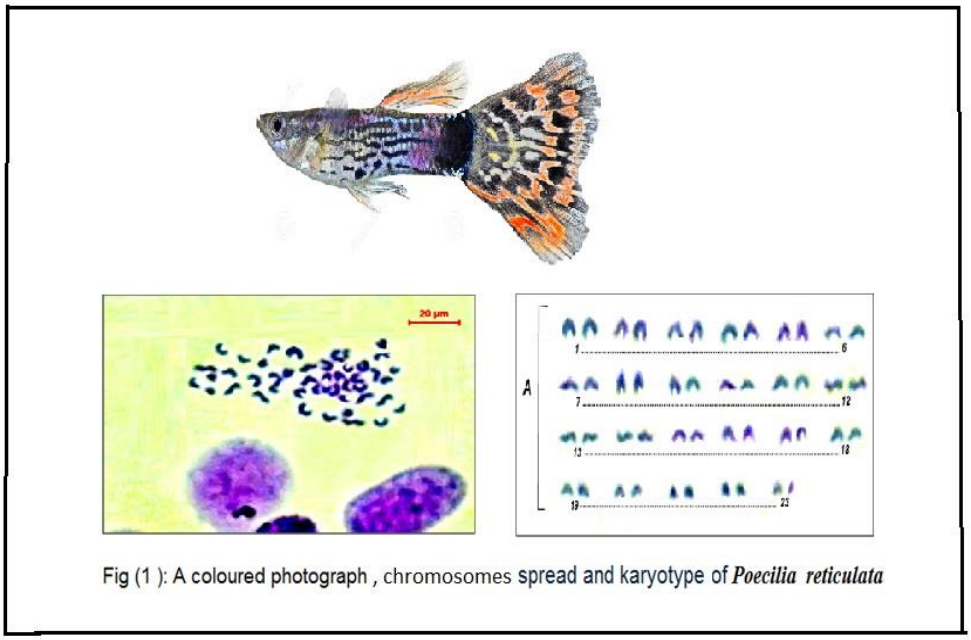


Fig (1): A coloured photograph , chromosomes spread and karyotype of *Poecilia reticulata*

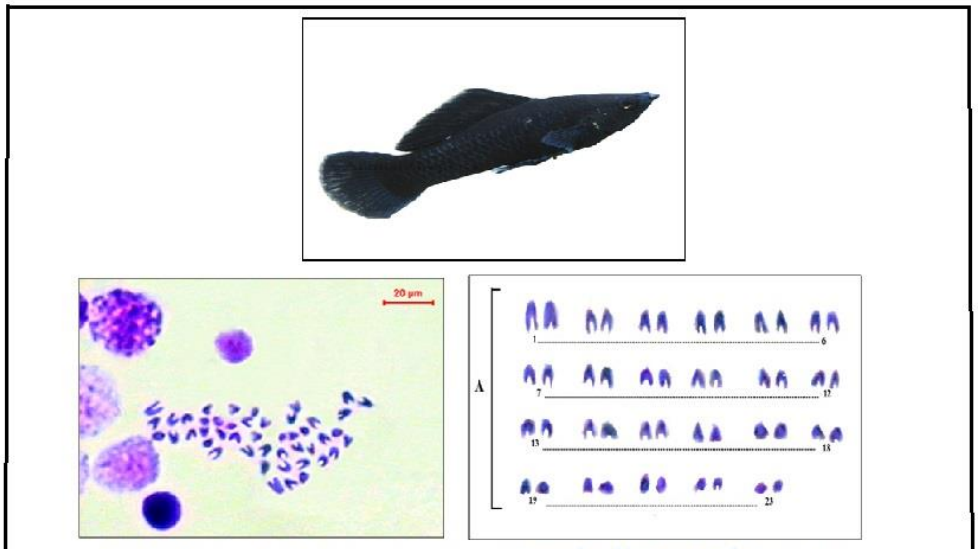


Fig (**2**): A coloured photograph , chromosomes spread and karyotype of *Poecilia sphenops*

Table 3. Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Poecilia sphenops

Chromosome Number	Chromosome Length			Relative Length %			Arm ratio	Centromeric Index	Classification
	Long arm	Short arm	Total	Long arm	Short arm	Total			
	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	
1	0.54 ± 0.08	Zero	0.54 ±0.08	6.83 ± 0.38	Zero	6.83 ±0.38	∞	Zero	Acro.
2	0.50 ± 0.05	Zero	0.50 ±0.05	6.33 ±0.28	Zero	6.33 ±0.28	∞	Zero	Acro.
3	0.45 ± 0.02	Zero	0.45 ±0.02	5.70 ±0.24	Zero	5.70 ±0.24	∞	Zero	Acro.
4	0.44 ± 0.04	Zero	0.44 ±0.04	5.57 ± 0.31	Zero	5.57 ±0.31	∞	Zero	Acro.
5	0.44 ± 0.01	Zero	0.44 ±0.01	5.57 ±0.31	Zero	5.57 ±0.31	∞	Zero	Acro.
6	0.43 ± 0.06	Zero	0.43 ±0.06	5.44 ± 0.33	Zero	5.44 ±0.33	∞	Zero	Acro.
7	0.42 ± 0.07	Zero	0.42 ±0.07	5.32 ± 0.27	Zero	5.32 ±0.27	∞	Zero	Acro.
8	0.40 ± 0.03	Zero	0.40 ±0.03	5.06 ±0.21	Zero	5.06 ±0.21	∞	Zero	Acro.
9	0.36 ± 0.04	Zero	0.36 ±0.04	4.56 ± 0.18	Zero	4.56 ±0.18	∞	Zero	Acro.
10	0.36 ± 0.05	Zero	0.36 ±0.05	4.56 ± 0.26	Zero	4.56 ±0.26	∞	Zero	Acro.
11	0.35 ± 0.04	Zero	0.35 ±0.04	4.43 ±0.14	Zero	4.43 ±0.14	∞	Zero	Acro.
12	0.35 ± 0.02	Zero	0.35 ±0.02	4.43 ±0.14	Zero	4.43 ±0.14	∞	Zero	Acro.
13	0.33 ± 0.05	Zero	0.33 ±0.05	4.18 ±0.16	Zero	4.18 ±0.16	∞	Zero	Acro.
14	0.32 ± 0.01	Zero	0.32 ±0.01	4.05 ± 0.19	Zero	4.05 ±0.19	∞	Zero	Acro.
15	0.31 ± 0.09	Zero	0.31 ±0.09	3.92 ±0.10	Zero	3.92 ±0.10	∞	Zero	Acro.
16	0.30 ± 0.08	Zero	0.30 ±0.08	3.80 ± 0.13	Zero	3.80 ±0.13	∞	Zero	Acro.
17	0.27 ± 0.02	Zero	0.27 ±0.02	3.42 ±0.11	Zero	3.42 ±0.11	∞	Zero	Acro.
18	0.26 ± 0.03	Zero	0.26 ±0.03	3.29 ±0.15	Zero	3.29 ±0.15	∞	Zero	Acro.
19	0.25 ± 0.04	Zero	0.25 ±0.04	3.16 ±0.20	Zero	3.16 ±0.20	∞	Zero	Acro.
20	0.22 ± 0.01	Zero	0.22 ±0.01	2.78 ±0.18	Zero	2.78 ±0.18	∞	Zero	Acro.
21	0.21 ± 0.05	Zero	0.21 ±0.05	2.66 ± 0.13	Zero	2.66 ±0.13	∞	Zero	Acro.
22	0.20 ± 0.03	Zero	0.20 ±0.03	2.53 ± 0.10	Zero	2.53 ±0.10	∞	Zero	Acro.
23	0.19 ± 0.07	Zero	0.19 ±0.07	2.40 ± 0.17	Zero	2.40 ±0.17	∞	Zero	Acro.
Sum			7.90 ±0.22						

Xiphophorus maculates

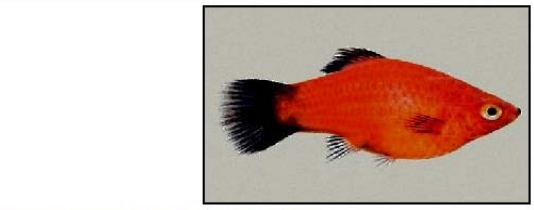
The mitotic metaphase spread of *Xiphophorus maculates* were found to have a diploid chromosome number of 2n=48 and fundamental number 50, spread and karyotype are shown in (Fig.3). These chromosomes were arranged in two groups; group A contains one metacentric pair of chromosomes with relative length 5.50 %, arm ratio 1.26 and centromeric index 44.26; group B which composed of twenty three acrocentric pairs of chromosomes with relative lengths ranged from 3.25 % to 5.86 %, arm ratio is ∞ and centromeric index is zero. All these measurements are shown in Table 4.

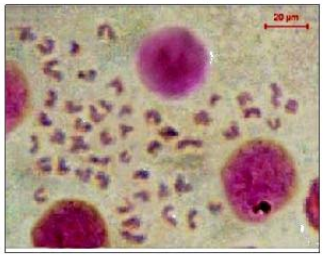
All primers amplified yielded distinct RAPD pattern with *Xiphophorus maculates* except with primer OPA-9, the nine primers generated 35 fragments, ranging from 1 by the primer OPO-6 to

8 by the primer OPG-2 and the size of these fragments arranged from 160 bp by the primer OPG-2 to1600 bp by the primer OPO-6.

Table 4. Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Xiphophorus maculates

Chromosome Number	Chromosome Length			Relative Length %			Arm ratio	Centromeric Index	Classification
	Long arm	Short arm	Total	Long arm	Short arm	Total			
	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	Mean ±S. D	
1	0.34 ± 0.05	0.27 ± 0.03	0.61 ± 0.07	3.07 ± 0.30	2.43 ± 0.18	5.50 ± 0.21	1.26 ±0.12	44.26 ± 1.11	M
2	0.65 ± 0.07	Zero	0.65 ± 0.07	5.86 ± 0.27	Zero	5.86 ±0.27	∞	Zero	Acro.
3	0.65 ± 0.05	Zero	0.65 ± 0.05	5.86 ± 0.18	Zero	5.86 ±0.18	∞	Zero	Acro.
4	0.64 ± 0.03	Zero	0.64 ± 0.03	5.77 ± 0.30	Zero	5.77 ± 0.30	∞	Zero	Acro.
5	0.61 ± 0.02	Zero	0.61 ± 0.02	5.50 ± 0.22	Zero	5.50 ±0.22	∞	Zero	Acro.
6	0.60 ± 0.08	Zero	0.60 ± 0.08	5.41 ± 0.19	Zero	5.41 ± 0.19	∞	Zero	Acro.
7	0.56 ± 0.06	Zero	0.56 ± 0.06	5.05 ±0.17	Zero	5.05 ± 0.17	∞	Zero	Acro.
8	0.51 ± 0.04	Zero	0.51 ± 0.04	4.60 ± 0.26	Zero	4.60 ± 0.26	∞	Zero	Acro.
9	0.50 ± 0.02	Zero	0.50 ± 0.02	4.51 ± 0.15	Zero	4.51 ±0.15	∞	Zero	Acro.
10	0.47 ± 0.05	Zero	0.47 ± 0.05	4.24 ±0.16	Zero	4.24 ±0.16	∞	Zero	Acro.
11	0.46 ± 0.03	Zero	0.46 ± 0.03	4.15 ± 0.10	Zero	4.15 ± 0.10	∞	Zero	Acro.
12	0.46 ± 0.07	Zero	0.46 ± 0.07	4.15 ±0.11	Zero	4.15 ± 0.11	∞	Zero	Acro.
13	0.45 ± 0.04	Zero	0.45 ± 0.04	4.06 ± 0.24	Zero	4.06 ±0.24	∞	Zero	Acro.
14	0.45 ± 0.02	Zero	0.45 ± 0.02	4.06 ±0.21	Zero	4.06 ±0.21	∞	Zero	Acro.
15	0.44 ± 0.07	Zero	0.44 ± 0.07	3.97 ±0.18	Zero	3.97 ±0.18	∞	Zero	Acro.
16	0.44 ± 0.05	Zero	0.44 ± 0.05	3.97 ± 0.14	Zero	3.97 ± 0.14	∞	Zero	Acro.
17	0.43 ± 0.02	Zero	0.43 ± 0.02	3.88 ± 0.12	Zero	3.88 ±0.12	∞	Zero	Acro.
18	0.43 ± 0.01	Zero	0.43 ± 0.01	3.88 ± 0.17	Zero	3.88 ±0.17	∞	Zero	Acro.
19	0.42 ± 0.04	Zero	0.42 ± 0.04	3.79 ± 0.20	Zero	3.79 ± 0.20	∞	Zero	Acro.
20	0.41 ± 0.06	Zero	0.41 ± 0.06	3.70 ± 0.25	Zero	3.70 ± 0.25	∞	Zero	Acro.
21	0.41 ± 0.05	Zero	0.41 ± 0.05	3.70 ± 0.11	Zero	3.70 ±0.11	∞	Zero	Acro.
22	0.40 ± 0.03	Zero	0.40 ± 0.03	3.61 ± 0.19	Zero	3.61 ± 0.19	∞	Zero	Acro.
23	0.38 ± 0.02	Zero	0.38 ± 0.02	3.43 ±0.10	Zero	3.43 ± 0.10	∞	Zero	Acro.
24	0.36 ± 0.08	Zero	0.36 ± 0.08	3.25 ±0.10	Zero	3.25 ±0.10	∞	Zero	Acro.
Sum			11.09 ±0.17						





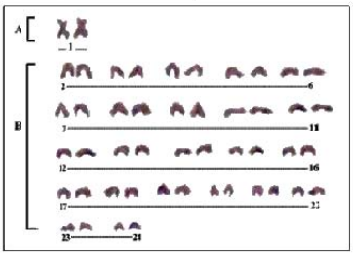
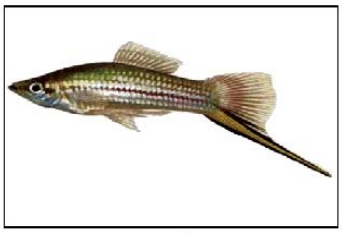
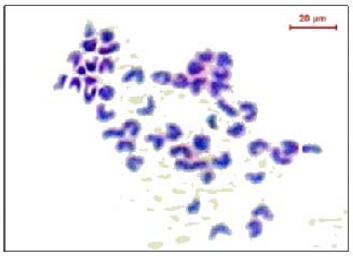


Fig (3): A coloured photograph , chromosomes spread and karyotype of ***Xiphophorus maculatus***





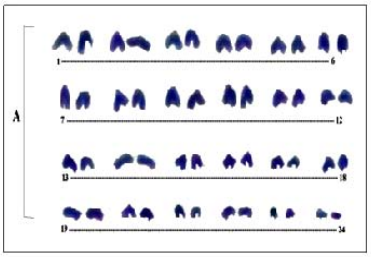


Fig (4): A coloured photograph , chromosomes spread and karyotype of ***Xiphophorus hellerii***

Table 5. Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Xiphophorus hellerii

Chromosome Number	Chromosome Length			Relative Length %			Arm ratio	Centromeric Index	Classification
	Long arm Mean ±S. D	Short arm Mean ±S. D	Total Mean ±S. D	Long arm Mean ±S. D	Short arm Mean ±S. D	Total Mean ±S. D	Mean ±S. D	Mean ±S. D	
1	0.73 ± 0.03	Zero	0.73 ± 0.03	6.02 ± 0.28	Zero	6.02 ± 0.28	∞	Zero	Acro.
2	0.71 ± 0.02	Zero	0.71 ± 0.02	5.86 ±0.22	Zero	5.86 ± 0.22	∞	Zero	Acro.
3	0.67 ± 0.02	Zero	0.67 ± 0.02	5.53 ±0.18	Zero	5.53 ±0.18	∞	Zero	Acro.
4	0.64 ± 0.04	Zero	0.64 ± 0.04	5.28 ± 0.19	Zero	5.28 ± 0.19	∞	Zero	Acro.
5	0.61 ± 0.01	Zero	0.61 ± 0.01	5.03 ± 0.30	Zero	5.03 ± 0.30	∞	Zero	Acro.
6	0.61 ± 0.06	Zero	0.61 ± 0.06	5.03 ±0.14	Zero	5.03 ±0.14	∞	Zero	Acro.
7	0.60 ± 0.07	Zero	0.60 ± 0.07	4.95±0.12	Zero	4.95±0.12	∞	Zero	Acro.
8	0.56 ± 0.08	Zero	0.56 ± 0.08	4.62 ±0.16	Zero	4.62 ±0.16	∞	Zero	Acro.
9	0.55 ± 0.04	Zero	0.55 ± 0.04	4.54 ± 0.11	Zero	4.54 ± 0.11	∞	Zero	Acro.
10	0.55 ± 0.05	Zero	0.55 ± 0.05	4.54 ± 0.17	Zero	4.54 ±0.17	∞	Zero	Acro.
11	0.51 ± 0.04	Zero	0.51 ± 0.04	4.21 ± 0.10	Zero	4.21 ±0.10	∞	Zero	Acro.
12	0.50 ± 0.02	Zero	0.50 ± 0.02	4.13 ± 0.12	Zero	4.13 ±0.12	∞	Zero	Acro.
13	0.50 ± 0.02	Zero	0.50 ± 0.02	4.13 ± 0.12	Zero	4.13 ±0.12	∞	Zero	Acro.
14	0.50 ± 0.01	Zero	0.50 ± 0.01	4.13 ±0.10	Zero	4.13 ±0.10	∞	Zero	Acro.
15	0.50 ± 0.09	Zero	0.50 ± 0.09	4.13 ± 0.19	Zero	4.13 ± 0.19	∞	Zero	Acro.
16	0.43 ± 0.08	Zero	0.43 ± 0.08	3.55 ± 0.15	Zero	3.55 ±0.15	∞	Zero	Acro.
17	0.42 ± 0.02	Zero	0.42 ± 0.02	3.47 ± 0.14	Zero	3.47 ±0.14	∞	Zero	Acro.
18	0.41 ± 0.03	Zero	0.41 ± 0.03	3.38 ± 0.18	Zero	3.38 ± 0.18	∞	Zero	Acro.
19	0.40 ± 0.01	Zero	0.40 ± 0.01	3.30 ±0.20	Zero	3.30 ±0.20	∞	Zero	Acro.
20	0.40 ± 0.01	Zero	0.40 ± 0.01	3.30 ±0.24	Zero	3.30 ± 0.24	∞	Zero	Acro.
21	0.36 ± 0.05	Zero	0.36 ± 0.05	2.97 ± 0.16	Zero	2.97 ± 0.16	∞	Zero	Acro.
22	0.33 ± 0.03	Zero	0.33 ± 0.03	2.72 ± 0.10	Zero	2.72 ± 0.10	∞	Zero	Acro.
23	0.32 ± 0.07	Zero	0.32 ± 0.07	2.64 ±0.13	Zero	2.64 ±0.13	∞	Zero	Acro.
24	0.31 ± 0.09	Zero	0.31 ± 0.09	2.56 ± 0.19	Zero	2.56 ± 0.19	∞	Zero	Acro.
Sum			12.12 ± 0.32						

Xiphophorus hellerii

A diploid chromosome number of 2n=48, and fundamental number also 48 were found in the mitotic metaphases. The spread and karyotype are shown in (Fig.4). The chromosomes were arranged in one group of acrocentric with relative lengths ranged from 2.56 % to 6.02 %, arm ratio is ∞ and centromeric index is zero. All these measurements are shown in Table 5.

The RAPD DNA analysis indicated that OPA-9, OPA-11, OPG-2, OPM-2 and OPM-5 primers produced 17 fragments with this fish, varied from 1 by the primer OPM-5 and to 6 by the primer OPG-2, the size of these fragments varies from 310 bp by the primer OPG-2 to 1240 bp by the same primer OPG-2.

The size of the amplified fragments also varied with different primers, the size of fragments ranged from 160 bp (OPG-2) 1600 bp (OPO-6), Table 6. The number of fragments amplified per primer varied between 1 (OPO-6) and 12 (OPG-2 and OPM-2). A total of 65 DNA bands were generated by the 9 primers for all studied fishes, out of these DNA bands. 39 of them were monomorphic bands. The tested primers generated 23 bands in *Poecilia sphenops*; 35 bands in *Xiphophorus maculates*; 17 bands in *Xiphophorus hellerii*; 22 bands *Poecilia reticulata*. Table (7).

Table 6. Survey of RAPD Markers using nine primers. (1-Xiphophorus maculates, 2-Xiphophorus hellerii, 3-Poecilia sphenops, 4- Poecilia reticulata), where (1) means present and (0) means absence.

		OPA-9						OPA-11			
Band No.	RAPD Marker bp.	1	2	3	4	Band No.	RAPD Marker bp.	1	2	3	4
1	1200	1	1	0	0	1	1200	0	1	0	0
2	790	1	1	0	0	2	1100	0	0	0	1
3	700	1	1	0	0	3	840	0	1	1	0
4	560	1	0	0	0	4	600	0	0	1	0
5	540	1	1	0	0					OPM-2	
6	470	1	0	0	0	Band No.	RAPD Marker bp.	1	2	3	4
7	420	0	0	1	0	1	1100	0	0	1	0
8	370	0	0	1	0	2	820	1	1	1	1
9	260	1	0	1	0	3	740	0	0	1	0
10	250	0	0	0	1	4	680	1	0	0	0
11	230	0	0	0	1	5	610	0	1	0	1
		OPG-2				6	560	1	0	0	0
Band No.	RAPD Marker bp.	1	2	3	4	7	520	0	1	0	1
1	1240	0	1	0	0	8	480	0	0	0	1
2	980	1	1	0	0	9	430	0	1	0	0
3	760	1	1	0	0	10	400	0	0	0	1
4	650	1	1	0	0	11	380	0	0	1	0
5	610	0	0	1	0	12	330	1	0	0	1
6	540	1	1	1	0					OPM-17	
7	430	0	0	1	0	Band No.	RAPD Marker bp.	1	2	3	4
8	400	1	0	0	1	1	450	1	0	0	1
9	350	1	0	1	1	2	290	1	0	0	0
10	310	1	1	1	1					OPO-2	
11	220	0	0	1	0	Band No.	RAPD Marker bp.	1	2	3	4
12	160	1	0	0	1	1	880	0	0	1	0
		OPM-5				2	780	1	0	0	0
Band No.	RAPD Marker bp.	1	2	3	4	3	610	1	0	0	0
1	1000	0	0	0	1	4	380	1	0	0	0
2	860	1	0	0	1					OPO-4	
3	620	0	0	0	1	Band No.	RAPD Marker bp.	1	2	3	4
4	580	0	1	1	0	1	1139	1	0	0	0
5	440	0	0	1	0	2	983	0	0	1	1
6	410	1	0	0	0	3	748	0	0	1	1
7	400	0	0	0	1	4	677	1	0	0	0
8	310	1	0	1	0	5	592	1	0	1	0
9	200	1	0	0	0	6	565	0	0	0	1
		OPO-6				7	541	1	0	0	0
Band No.	RAPD Marker bp.	1	2	3	4	8	410	1	0	0	1
1	1600	1	0	0	0	9	322	1	0	0	0
						10	304	0	0	1	0

Table 7. Number of amplified and monomorphic DNA-fragments in the four species.

No.	Primer Code	1	2	3	4	Total Amplified Bands	No. of monomorphic Bands	Polymorphism %
1	**OPA-9**	**7**	**4**	**3**	**2**	**11**	**6**	**55**
2	**OPA-11**	**0**	**2**	**2**	**1**	**4**	**3**	**75**
3	**OPG-2**	**8**	**6**	**6**	**4**	**12**	**4**	**33**
4	**OPM-2**	**4**	**4**	**4**	**6**	**12**	**8**	**67**
5	**OPM-5**	**4**	**1**	**3**	**4**	**9**	**6**	**67**
6	**OPM-17**	**2**	**0**	**0**	**1**	**2**	**1**	**50**
7	**OPO-2**	**3**	**0**	**1**	**0**	**4**	**4**	**100**
8	**OPO-4**	**6**	**0**	**4**	**4**	**10**	**6**	**60**
9	**OPO-6**	**1**	**0**	**0**	**0**	**1**	**1**	**100**
Total		**35**	**17**	**23**	**22**	**65**	**39**	**60**

(*1-Xiphophorus maculates, 2-Xiphophorus hellerii, 3- Poecilia sphenops, 4- Poecilia reticulata*).

Table 8. Genetic similarity values calculated from the DNA fragments amplified from Poecilia reticulata, Poecilia sphenops, Xiphophorus helleri and Xiphophorus. maculates, using nine OPERON primers.

	Xiphophorus maculates	*Xiphophorus hellerii*	*Poecilia sphenops*	*Poecilia reticulata*
Xiphophorus maculates	100			
Xiphophorus hellerii	38	100		
Poecilia sphenops	24	25	100	
Poecilia reticulata	32	21	22	100

Data of the presence / absence of DNA fragments of *Poecilia reticulata*, *P. sphenops*, *Xiphophorus helleri* and *X. maculates*, were used to calculate the genetic similarity, based on the calculated genetic similarity presented in Table 8. and dendrogram as in Figure 14, Similarity values among the studied samples ranged from 21% to 38%. The description of this similarity coefficient is not simple, especially when more than one character is involved in the same cluster. Thus *Xiphophorus maculates* and *Xiphophorus hellerii* are found to have a similarity coefficient of 38%, but between *Xiphophorus hellerii* and *Poecilia sphenops* is 25%, while between *Poecilia sphenops* and *Poecilia reticulata* is 22%. High similarity values were obtained between *Xiphophorus maculates* and *Xiphophorus hellerii.* (38%) and the low similarity values were obtained between *Xiphophorus hellerii* and *Poecilia reticulata* (21%).

In spite of species of family *Poecilidae, Xiphophorus maculates* and *X. hellerii* have the same diploid chromosome number 2n=48, and *Poecilia sphenops* and *P. reticulata* have the same diploid chromosome number 2n=46 and the same fundamental number FN=46, also the same karyotype one group of acrocentric chromosomes, this study concluded that these four species have molecular variations between each other by using the RAPD-PCR technique as showing in the relationship between *Xiphophorus maculates, X. hellerii, Poecilia sphenops* and *P. reticulata* is 38%, 24% and 32% respectively, also between *Xiphophorus hellerii*, Poecilia sphenops and *P. reticulata* is 25% and 21% respectively, finally between *Poecilia sphenops and P. reticulata* is 22%.

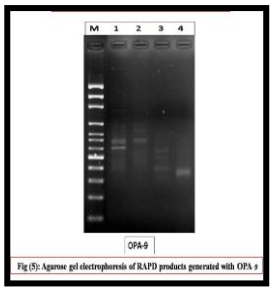


Fig (5): Agarose gel electrophoresis of RAPD products generated with OPA 9

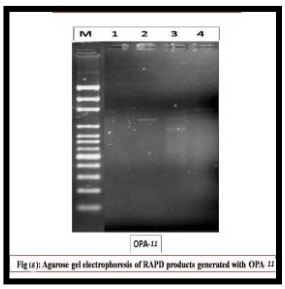


Fig (6): Agarose gel electrophoresis of RAPD products generated with OPA 11

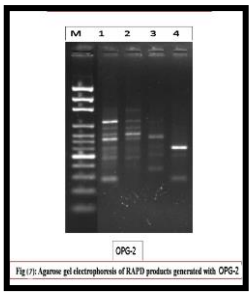


Fig (7): Agarose gel electrophoresis of RAPD products generated with OPG-2

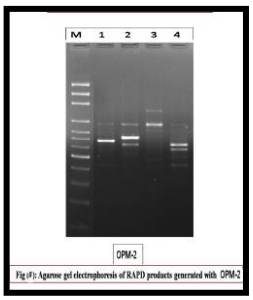
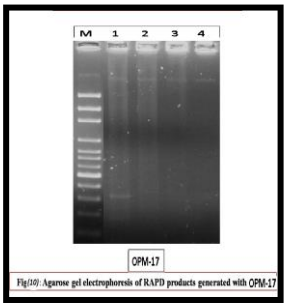
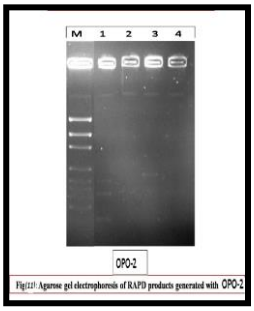


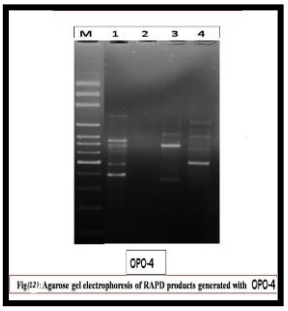
Fig (8): Agarose gel electrophoresis of RAPD products generated with OPM-2



Fig(10): Agarose gel electrophoresis of RAPD products generated with OPM-17



Fig(11): Agarose gel electrophoresis of RAPD products generated with OPO-2



Fig(12): Agarose gel electrophoresis of RAPD products generated with OPO-4

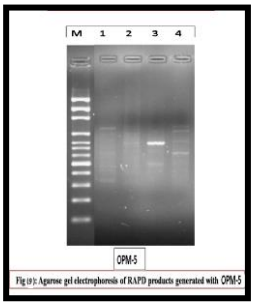
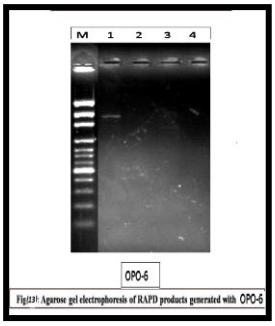


Fig (9): Agarose gel electrophoresis of RAPD products generated with OPM-5



Fig(13): Agarose gel electrophoresis of RAPD products generated with OPO-6

1-Xiphophorus maculates, 2-Xiphophorus hellerii, 3- Poecilia sphenops and 4- Poecilia reticulata

Fig.14. Dendrogram demonstrating the relationship among *Poecilia reticulata*, *P. sphenops*, *Xiphophorus helleri* and *X. maculates*, based on data recorded from polymorphism of RAPD markers. 1- *Xiphophorus maculates* 2- *Xiphophorus hellerii* 3-*Poecilia sphenops* 4- *Poecilia reticulate.*

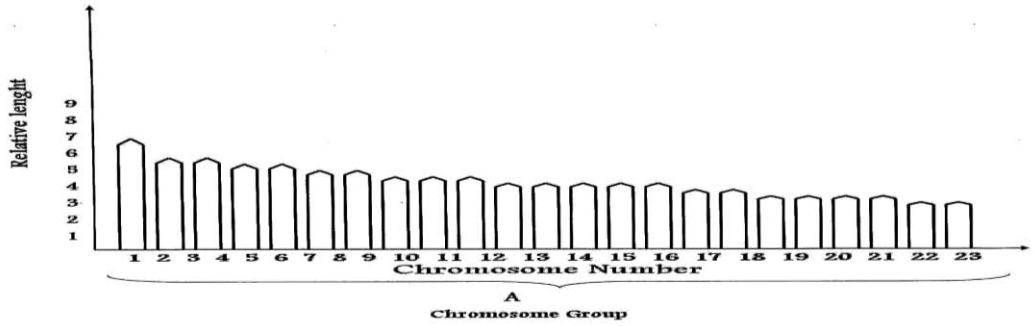


Fig. 15. Ideogram of chromosomes of *Poecilia reticulata* which constructed in respect to the relative length

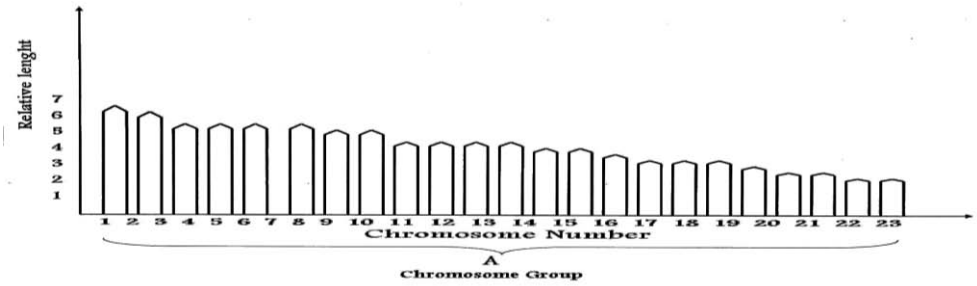


Fig. 16. Ideogram of chromosomes of *Poecilia sphenops* which constructed in respect to the relative length

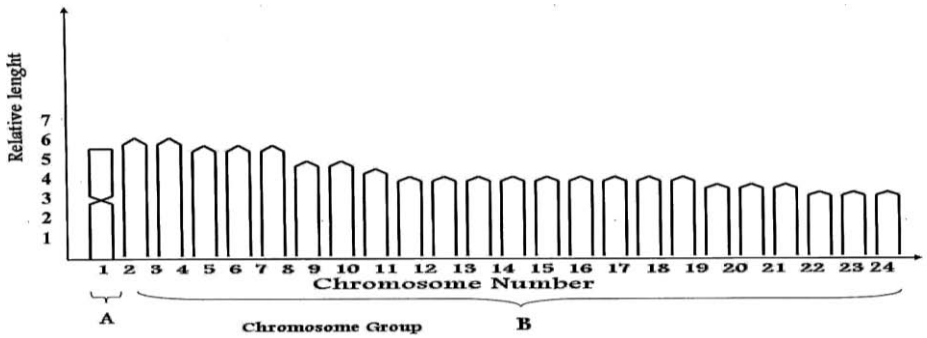


Fig. 17. Ideogram of chromosomes of *Xiphophorus maculates* which constructed in respect to the relative length

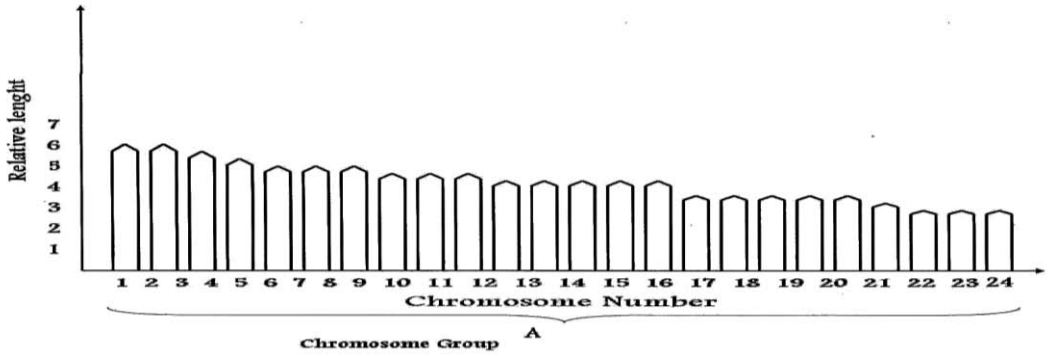


Fig. 18. Ideogram of chromosomes of *Xiphophorus hellerii* which constructed in respect to the relative length

DISCUSSION

Cyprinodontiform fishes comprise approximately 850 species, of which only 67 Species have cytogenetic information (COSTA, 1998; OLIVEIRA *et al*., 2009). This dearth of information from cytogenetic data is mainly due to the low Commercial importance and the small size of specimens that make up the cyprinodontiforms, limiting our understanding of their chromosomal organization and karyotype evolution.

Most of the Poeciliidae species are with diploid chromosomes number 48 (Rab, 1984; OLIVEIRA *et al*., 2007). This diploid number has been found in around 51% of the species currently described and it is considered modal number for the order Cyprinodontiformes (SCHEEL, 1972; OLIVEIRA *et al*., 1988; GARCIA *et al*., 2001).

Numbers of chromosomes have been reported in fishes such as *Poecilia sphenops* (2n = 46) and *Xiphophorus helleri* (2n = 48) (PREHN and RUSH, 1969; POST 1965). The present work

showed that the chromosome number of *Poecilia reticulate* (2n=46), *Xiphophorus helleri* (2n=48) and *X. maculates* (2n=48).

This study reports on the use of RAPD markers for studying genetic similarity among the four species (Family: Poecilidae). The RAPD assay has been used to construct phylogenetic trees for resolving taxonomic problems in many organisms (BARDAKCI and SKIBINSKI, 1994; GREEF and TRIEST, 1999; ALI, 2003). RAPD bands in this study were always variant (i.e. strong, faint, fuzzy and sharp bands) generated with each primer because one or more copies of DNA may exist per genome or may be attributed to the varying of the annealing process between the primer and the DNA, this problem of mixed bands shows the well known sensitivity PCRs (BIELAWSKI *et al*., 1995).

The RAPD method was successfully used to detect the variation between the different species of fishes. The results obtained in this study showed that RAPD could be used to generate useful fingerprints characteristic of fish species and for genotyping of individuals within the species. Thus, it provides an efficient and sensitive method which can be used to estimate genetic variability, relatedness, inbreeding levels, pedigree analyses, detection of economic traits and in other maker based studies in fishes (SHAIR *et al*., 2011).

In conclusion, the results of this study indicated that *Xiphophorus maculates* and *X. hellerii* were identical in 2n=48, but they were different in their karyotypes, while *Poecilia sphenops* and *P. reticulata* were identical in 2n= 46 and in their karyotypes. In addition, to the results indicated that each species has different molecular genetic characteristics. The cluster analysis clearly differentiated *Xiphophorus maculates, X. hellerii* from *Poecilia sphenops* and *P. reticulata*. The molecular genetic taxonomic relationship among four species of Poecilidae fishes (*Xiphophorus maculates, X. hellerii, Poecilia sphenops* and *P. reticulata*) were investigated using cytogenetic analysis and RAPD markers for first time in Egypt. The findings of this study can be further applied in research areas of fish cytotaxonomy and evolutionary relationships.

Received May 20th, 2015
Accepted September 05th, 2015

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PRIMENA CITOGENETIČKIH ANALIZA I RAPD MARKERA U ODREĐIVANJU GENETSKE VARIJACIJE ČETIRI VRSTE UKRASNIH RIBA PORODICE *Poecilidae* (Red: Cyprinodontiform)

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Izvod

Kariološka i molekularna analiza, kao I međusobni genetski odnosi četiri slatkovodne vrste riba, familije *Poecilidae*, bile su predmet proučavanja u ovom radu. Vrste *Xiphophorus maculates* i *Xiphophorus hellerii* imaju isti diploidan broj hromozoma 2n=48, ali se razlikuju njihovi kariotipi. *Poecilia sphenops* i *Poecilia reticulata* imaju isti diploidan broj hromozoma 2n=46 i takođe isti kariotip. Devet RAPD prajmera sa monomorfnim trakama, korišćeno je za konstrukciju dendrugrama i matrice sličnosti. Ukupno je dobijeno 65 traka, od toga 39 monomorfnih. Sličnost ispitivanih uzoraka je varirala od 21% do 38%. Visok nivo sličnosti je dobijen između *Xiphophorus maculates* i *Xiphophorus hellerii.* (38%), dok je nizak nivo sličnosti (21%) dobijen između vrsta *Xiphophorus hellerii* and *Poecilia reticulate*.RAPD analiza je potvrdila da se ispitivane vrste genetički razlikuju. Dobijeni citogenetski rezultati mogu biti primenjeni u daljim istraživanjima u oblasti citotaksonomije I evolutivnih odnosa riba.

Primljeno 20 V. 2015.
Odobreno 05. IX. 2015.