Molecular and Genetic Variation among *Aphanius dispar* and *Aphanius fasciatus* (*cyprinodontidae*) using RAPD-PCR, Protein and Isozymes Electrophoresis

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Abstract: Family Cyprinodontidae is represented by two species, *Aphanius dispar* (Arabian killifish) and *Aphanius fasciatus* (Mediterranean killifish). *A. dispar* is distributed in brackish water as well as marine water. In view of this adaptive versatility, their chromosomal complement was examined to study the type of chromosomes. The use of the electrophoresis heretic analysis of the muscle proteins patterns of the two species and their hybrid indicated that each of both valid species have distinct protein patterns. High genotypic diversity was found within populations. Among the two *Aphanius* species 21 banding positions, were resolved, the electrophoresis showed a species-specific pattern and in some cases these patterns differ of the same species. There are few characters have been detected by morphological analysis. The general genetic polymorphisms and structure of variability among the two species cyprinodont fishes and to carry out genetic variation studies based on modern techniques. To study some biological and genetic variations among *Aphanius* species we used a protein-banding patterns muscle proteins, esterase isozyme polymorphisms and RAPD-PCR DNA markers. Restriction endonucleases of the genomic DNA have been used to detect the genetic variability among and within fish populations. It was found that these are the most successful and accurate methods. The relative from, molecular weight (MW) and band frequency fingerprints that generated by the 3 primers revealed unique for each *Aphanius* species in terms of number and position of RAPD bands.

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1. Introduction

Cyprinodonts, are small-size fishes usually known as tooth-carps or Killifish. They are adaptable little fishes, able to tolerate extreme environmental conditions of heat, salinity and drought that would be fatal to most other groups of fishes (Chaouai and Hassine, 1998).The Killifish *Aphanius dispar* is a cyprinodont fish inhabiting brackish waters and is a good target species for ecological and evolutionary studies. It is indeed quite common in coastal brackish habitats of the Mediterranean and Red sea area (Cognclti and Maltagliati, 2000) and able to tolerate a wide range of variations of relevant chemical-physical parameters such as temperature and salinity.

Cyprinodontidae contains about 50 genera (e.g. *Adinia, Aphanius, Aphyosemion, Crenichthys. Cynolehias, Cyprinodon, Fundulus, Oryziaz, Pantanodon and Rivulus*) with at least 300 species (Maltagliati, 2002). The largest sizes found were 45mm versus 38mm (Varas and De-Sostoa, 1997). The distribution of the genus *Aphanius* give rise to different hypotheses on origin and evolution of its species. The genus *Aphanius* also has to tolerate a wide range of day and night differences in temperature, about 21 °C difference. Because of these specialized environmental conditions, the distribution of different *Aphanius* species has a natural situation (Maltagliati, 1999).

The conditions in the Red sea were favorable for both species where the area had swamp marshes which cause a disturbance in the salinity (increase in salinity) which in return unfavorable for the Mediterranean less tolerant *A. fasciatus*. The Unusual environmental conditions of the Red Sea appear to have affected the distribution of *A. dispar* , and *A. fasciatus*. These conditions (particularly fluctuations in salinity from brackish to the hypersaline) seem unfavorable to species migrating from adjacent water of both Red Sea and Mediterranean. Al- Akel*et al*.(1987) reported about selective feeding of Arabian freshwater fish, *A. dispar*.

The hybrids between both species were naturally found in the area. But the hybridization between these two species along the course of the Red sea itself. The use of protein variation in the study of species or intraspecies relationships in *Aphanius* has been applied by several workers. Some authors have mentioned the occurrence of natural hybridization between *A. dispar* and *A. fasciatus* at the northern part of the Suez canal in Egypt (Dufresne and Bernatchcz, 2002). Many papers reported the effectiveness of RAPD markers in discriminating between species, subspecies and populations in a wide range of organisms, including

fish (Mamuris*et al*., 1998;Partis and Wells, 1996;Reichenbacher*et al*.,2009;Gonzalez *et al*., 2014).

RAPD-PCR DNA markers are the most widely used molecular markers in fish species systematic and has become a convenient tool in studying genetic structure and phylogenctic relationships (Williams *etal*., 1998).The differences among fish populations by polyacrylamide gel electrophoresis (PAGE) of total soluble proteins were estimated. There are several methods were used to separate the protein mixture to determine its molecular weight and polymorphisms such as sodium dodecylsulphatpolyacrilamid gel electrophoresis (SDS-PAGE).

Random Amplified polymorphic (RAPD) DNA has been applied for the identification of fish species (Partis and Wells, 1996; Williams *et al.*, 1990). RAPD - PCR analysis had a value to assay polymorphisms within and between populations of two species of *Aphanius*. RAPD technique was also used to estimate the phylogenetic relationships and to calculate the similarity values among and within fish population (Callejas and Ochando, 1998). Although the RAPD polymorphism is presumed to be located at the annealing site of the 10-mcr primer, it has been shown that the primer-binding sites are often identical between two samples showing polymorphic bands (Bowditch *et al*., 1994).This method has been applied to the discovery of genetic markers for mapping studies (Postlethwait *et al*., 1994).

2. Materials and Methods

Collection of Samples

Samples were selected depending on their morphological features. Ten samples were collected from each morphological specific group. There were three groups, the first group characterized with 2-3 dark lines on the anal fin which was *Aphanius dispar*. The second group was characterized by dark demarcation surrounding the dorsal fin which was *A. dispar*. The third group was characterized by the presence of three dark strips on the tail fin and a dark strips on the circumference of the dorsal fin.

A. fasciatus and *A.dispar* hybrid were collected from one location at the Red sea, Saudi Arabia.

Preparation of samples(Isozyme polymorphism)

SDS-PAGE electrophpresis: 4 *A. fasciatus*, and 4 *A. dispar* were used to study the electrophoresis protein analysis as well as isozyme according to Lacmmli (1970) with some modifications. For the 0.85% NaCl and 70% ethanol soluble proteins 20 and 40 uL were added to it of SDS sample buffer, (pH 8.8). Control wells were loaded with standard protein markers (133, 116, 108, 97, 68, 66 and 48 KDa).Extraction of isozyme, electrophoresis conditions, gel preparation, staining and distaining were conducted according to the standard methods(Tanksley andOrton, 1983).

Ten individuals of *A. dispar* were analyzed by allozyme electrophoresis for the study concerning genetic structure and gene flow among populations. Gel electrophoresis for fish-stock identification (Smith, 1990). Muscle samples of *A. dispar*, *A. fasciatus* and hybrids were obtained from alive fish and the muscle samples were homogenized in the buffer and centrifuged after sample preparation gel. The SDS-PAGE gel was used in the separation. Following the separation, the gel was removed, using solution of Coomassie Brilliant Blue and destained according to the procedures used by Williams *et al*.(1990). Standard molecular weight marker proteins (wide range) obtained from Sigma Chemical Company, was applied as sample to the gel. method of Laemmli (1970). Standard molecular. All gels resulted from protein and DNA electrophoresis, were scanned and the analysed Protien and isozynics gels were photographed.

Dendrogram Construction

For constructing a combined dendrogram dealing with genetic relationships among the species studied, the data generated from, protein banding pattern and molecular markers were introduced to SPSS package program according to binary values (1.0). The output results involved both different hierarchical pair distance (UPGAMA) and constructed dendrogram.

Chromosomal analysis

The chromosome complement of *A. dispar, A. fasciatus* and their hybrid were examined to study the type of their chromosomes. Metaphase chromosomes were investigated and counted under microscope oil immersion lens (XI000). Photographs were taken from the best metaphases and karyotypesand were prepared for each of the two studied species and their hybrid according to Al-Hawary and Al-Saleh, (1989). Chromosomal analysis including the fundamental number (FN).Samples of cyprinodontid fish *A. dispar* from three locations were examined. Nei's genetic distance values (Nei and Tajima, 1981) were the characteristic of populations within species.

Genomic DNA Isolation

Genomic DNA was extracted from pectoral fin clips of fish following the methods of Maniatis*et al.* (1982) with some modifications. Pectoral fin was lysed in 50 mL of 50mml/1 Tris buffer (pH 8.0).The genomic DNA pellet was (resuspended) dissolved in 500 uL TE buffer, and (10uL) was aliquoted for PCR concentration determined.

PCR Amplification

Samples were used directly for PCR (Shears *et al*., 1991) with different primers. The PCR products were run in 0.8 % agarose gels by electrophoresis. Amplification and electrophoresis conditions were carried according to (Williams *et al* ., 1990). Primer codes and sequences used for amplification are presented in Table (1).

Approximately 15 uLpf amplification products were separated on 1.5% agarose until the marker bands give a good resolution. The gel was immediately photographed using Polaroid camera. 15 uL of DNA implied product was loaded in each well and 1 Kb (Sigma) DNA marker (23, 9.4, 6.5, 4.3, 2.3, 0.56 Kbp mix was used as standard DNA. The banding patterns of individual were compared among fish species. Bands were scored as positive (+) if it is present or negative (-) if it is absent.

Table 1. Primer codes and sequences used for DNA amplification

Primer code	Primer Sequence '5----------3'
Al	CAGGCCCTTC
A2	TGCCGAGCTG
A5	AGGGGTCGTT

3. Results and Discussion

The karyotypes are distinguishable on the basis of chromosome morphology. The species showed 48 (2n) chromosomes with an NF-96 (Figure1). Results demonstrate that both of *A. dispar* and *A. fasciatus* as well as their hybrids have a diploid number of 48. Chromosomes were classified into four categories namely metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (t). Features that characterize the karyotype of *A. dispar* are the presence of one pair of metacentric. In hybrids, the arrangement of chromosomes in pairs shows an over abundance of acrocentric chromosomes (with one minute arm). It is possible that acrocentric chromosomes are more suited to handle extremes of variations in environmental salinity (Siddiqui and Ahmed, 1999).

The results of studying protein polymorphisms of 10 individuals from *Aphanius* species were sampled from the three groups. Electrophoretic protein patterns are shown in Figure (2) and summarized in Table (2) cleared the Intra-specific variations between different species. The SDS-PAGE gel showed the separation of the muscle proteins both of *A. dispar* and *A. fasciatus* as well as their hybrids. There are some proteins patterns appeared only in the case of hybrids (116, 97, 48 KDa).

The results obtained were in close agreement with Comparini *et al*. (1983), who examined population samples of *A. dispar* at five localities from Mediterranean, Red Sea and Dead Sea by standard methods of starch gel electrophoresis. They concluded that *A. dispar* was extant in the Mediterranean before the construction of the Suez Canal based on the genetic similarity between all possible pairs of these populations. They used the protein electrophoresis to confirm the identity of the two species and their hybrids where there are few characters have been detected by morphological analysis.

The results of electrophoretic analysis of muscle protein cleared that each of the examined species had its own protein pattern which could distinguish not only between the two species and also between the same species and the hybrid has more of its protein pattern. The results showed a high degree of polymorphisms, especially in the range of molecular weight from 133 -48 KDa among the two species and their hybrids. The two species were approximately similar. The protein variations among *Aphanius* species, cleared characteristic protein bands for each species were only detected in the specific protein bands at MW (KDa) were *A. dispar* 133, AM 16, 97, 48 Table (2).

Table 2. Characteristic protein bands for the two species and their hybrids

M.W.KDa	1 hybrid	2 hybrid	1 *A. fasciaius*	2 *A. fasciaius*	3 *A. fasciatus*	4 *A. fasciaius*	1 *A. dispar*	2 *A. dispar*	3 *A. dispar*	4 *hybrid*
133	-	-	-	-	-	-	-	-	-	+
116	+	-	-	-	-	-	-	-	-	-
108	-	-	+	+	+	+	+	+	+	+
97	+	-	-	-	-	-	-	-	-	-
86	-	+	-	+	+	+	+	+	+	+
66	+	-	-	-	-	-	-	-	+	+
48	+	-	-	-	-	-	-	-	-	-

Results in Table (2) and Figure (2) showed a total of 21 bands which were labeled according to their relative fronts along the gel. The total number of bands in each lane ranged from 9 -21 bands. The band frequencies were calculated and ranged from 1 to 0.87. The average of similarity values within hybrid population was 0.96.Comparative electrophoresis of *Aphanius* species of proteins aimed at the identification of species or their hybrid markers has been described by Dufresne *et al*. (2002).

Among the two *Aphafiius* species *A. dispar, A. fasciatus* and their hybrid 15 banding positions were resolved. Isozyme are functionally similar but separable forms of enzymes, encoded by one or more gene loci. Fish geneticists had been used by protein (isozyme) electrophoresis as their primary tool to characterize population genetic - level variation fish species (Waples, 1990; Gonzalez *et al.*, 2014).

Allozyme electrophoresis as been used to analyze and to compare the genetic structure of ten populations

of the killifish *A. fasciatus* (Roberta *et al*., 2003). Allozyme markers in general showed co-dominant inheritance patterns (Appleyard and Mather, 2000). Esterase are enzymes that characterized by their common activity on many naphthylester substrates. Some isozymesystems have good values in fish characterization over other systems such as esterase isozyme system. Consequently, each band of esterase activity reflects a structure of one polypeptide chain. Hence, each band on the gel represents the end product of one locus (allele). Many authors have repeatedly mentioned the occurrence of natural hybridization between *A. fasciatus* and *A. dispar*. assumed that this hybridization occurred along the course of the Red sea itself (Dufresne *et al*., 2002).

Samples of *A. dispar* and *A. fasciatus* as well as their hybrids were investigated for gene loci. Four of these loci were found to be monomorphic in all the studied samples. The remaining 7 loci were polymorphic and their allele frequencies are shown in Figure (3).

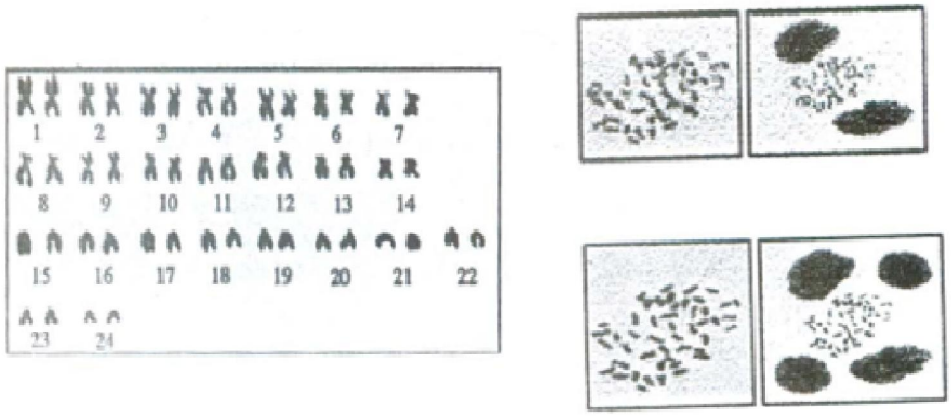

Figure 1. Karyotype *Aphanius* species 2n = 24 pair chromosomes

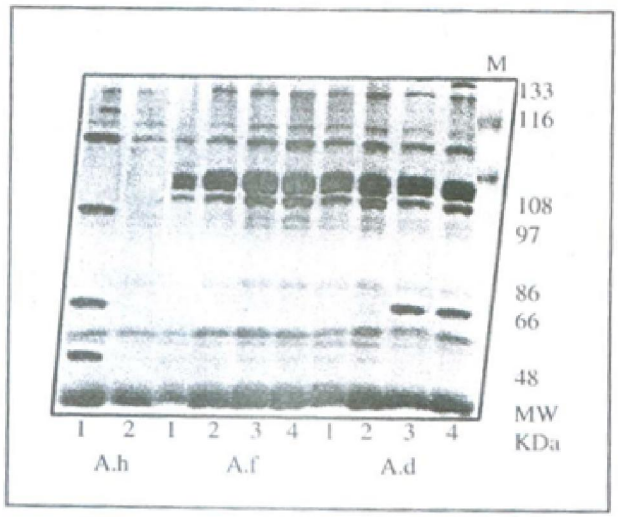

Figure 2. SDS-PAGE of two *Aphanius* species (*A.dispar*,*A.fasciatus* and their hybrid. Electrophoretic protein patterns)

Workers concluded that the electrophoresis showed a species-specific pattern and in some cases these patterns differ of the same species (Dufresne *et al*., 2002). Highest value of similarity within population was obtained for *A. fasciatus* and the lowest was for *A. dispar*, the RAPD fingerprints under identical amplification and electrophoretic conditions are highly reproducible for any given primer-template combination (Dinesh, 1995). Fishes and other marine organisms have shown, in many cases, a close

correlation between genetic polymorphism and ecological response to habitat challenge (Cognetti and Maltagliati, 2000). *A. dispar* is more tolerant than *A. fasciatus* and hybrid, thus it is dominant in the saline (Nevo, 2001;Reichenbacher*et al.*, 2009).

The results of the overall genetic diversity of the species is almost completely determined by the among population rather than within population genetic variability and was agreed with Maltagliati (2002). A highly significant coancestry coefficient value was found in that study (p<0.001) which agreement with result of Maltagliati (1999).

In the present study, and to recognize the genetic variation techniques of RAPD-PCR were used. From the 7 random primers used only three primers (Al, A2, A5) were succeeded in matching and amplifying DNA of all (three) populations and RAPD-PCR proceeding. Band patterns were clear and could be scored with confidence Table (3). These primers produced 14 variable bands that ranged in size from 4,7 to 0.6 Kbp. The relative from molecular weight (MW) and band frequency fingerprints generated by the 3 primers revealed unique for each *Aphanius* species in terms of number and position of RAPD bands.

From table (3) the number of fragments generated by each primer was ranged from (1-8) in the case of hybrid from (1-3) in *A. fasciatus* and from (1-3) in *A. dispar* respectively as displayed in Table (4). The phylogenetic relationship based on DNA markers was presented in Figure (4). This indicates that the highest degree of variability was that between *A. fasciatus* and *A. dispar*. Many specific Ah DNA markers were generated by the 3 primers From. Table (3), hybrid had 8 specific DNA markers. These markers at molecular weight of 4.7, 1.8, 0.7 Kbp (primer Al), 2.8, 1.9, 1.2, 0.7 Kbp (primer A2), and 1.3 Kbp (primer A5) respectively.

With regard to *A. fasciatus* it had 3specific DNA markers. These markers were distributed among 3 primers, these primers were Al (one marker at 0.82 Kbp), A2 (markers at 3.9 Kbp) A5 (marker at 0.8 Kbp) respectively.The third applied *Aplutnius* species (*A. dispar*) had 3 Specific DNA markers, (one fragments generated by the primer Al (3.8 Kbp) and two fragments generated by the primers A2, A5 (0.51, and 0.6 Kbp) respectively.

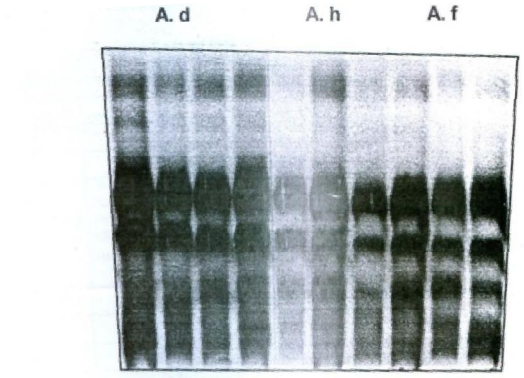

Figure 3. Electrophoretic patterns of esterase isozyme polymorphisms of (*A. Fasciatus* and *A. Dispar*and their hybrid (*A.h*). generated by α - aphthylacetate

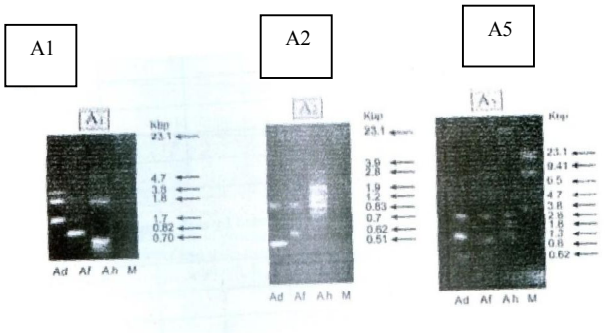

Figure 4. DNA polymorphisms using RAPD markers generated by three random primers (A1, A2, A5)

Table (3): Specific DNA markers that were generated by the three primers

BF	A1Bn	R.f	Mw(Kbp)	hybrid	*A. fasciatus*	*A. dispar*
0.33	1	0.7	4.7	+	-	-
0.33	2	0.29	3.8	-	-	+
0.33	3	0.41	1.8	+	-	-
0.33	4	0.46	1.7	-	+	+
0.33	5	0.56	0.82	-	+	-
0.33	6	0.66	0.70	+	-	-
0.33	1	0.33	3.9	-	+	-
0.33	2	0.41	2.8	+	-	-
0.33	3	0.45	1.9	+	-	-
0.33	4	0.56	1.2	+	-	-
1	5	0.62	0.83	+	+	+
0.33	6	0.66	0.7	+	-	-
1	7	0.73	0.62	+	+	+
0.66	8	0.81	0.51	-	-	+
1	1	0.42	2.8	+	+	+
0.66	2	0.40	1.8	-	-	+
0.33	3	0.52	1.3	+	-	-
0.33	4	0.58	0.8	-	+	-
0.33	5	0.66	0.6	-	-	+

Table 4. Number of DNA fragments generated by each primer for the the two species and their hybrid.

Primer code	*A. dispar*	*A. fasciatus*	hybrid
A1	1	1	3
A2	1	1	4
A5	1	1	1
Sum	3	3	8

From Table (4) The number of fragments generated per primers varied between 5 to 8. All primers gave specific patterns. The subspecies specific patterns were for some but not all primers.Among the three populations, hybrid has a closer affinity to *A. fasciatus*than to *A. dispar*. It showed that *Aphanius*, sp was distant related to *both A. dispar*and *A. fasciatus*. and their hybrid Figure (5).

Figure (5) showed the results of dendrogram construction entrance coefficients between popular. It showed the close affinity of *A. fasciatus* and *A. dispar* and their hybrid. A slight separation of the *A. dispar* and the closer affinity hybrid, shows with *A. dispar* than with *A. fasciatus*. Intra-and inter spcci fie variations in banding patterns were therefore expressed as presence / absence data and a pair wise matrix based on bands shared was estimated by RAPDPLOT program (Kambhampati*etal.*, 1992).

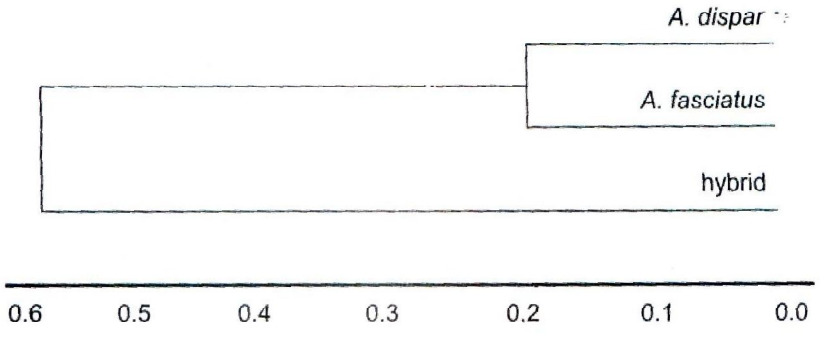

Figure (5): Dendrogram.of genetic relationship among the two species of *A. tliypttr*, *A. fasciatus*and their hybrid based on protein polymorphism.

Conclusions:

Molecular weight (MW) and band frequency fingerprints generated by the three primers revealed unique for each *Aphanius* species in terms of number and position of RAPD bands.

References

1. Al- Akel, A.S.; Shamsi, M.J.K. and AL-Kahem,H.F.(1987):Selective Feeding Behaviour of the Arabian Freshwater Fish, *Aphaniusdispar*. Pakistan. J. Zool. 19:211-215.
2. Al-Hawary, B.A. and Al-Saleh, A.A. (1989): Cytogenetic effects of jacarbazine on mouse marrow cells *in vivo*. Mut. Res. 223: 259-266.
3. Appleyard, A.S. and Mather, B.P. (2000): Investigation into the mode of inheritance of allozyme and random amplified polymorphic DNA markers in Tilapia *Oreochromismossambicus* (Peters). Aquacult-Res. (31)5: 435-445.
4. Barton, N.H. and Turelli, M. (1990): Evolutionary quantitative genetics : how little do we Know?. Ann. Rev. Genet. 23: pp.337-370.
5. Bowditch, B.M.; Albright, D.G.; J. Williams and M.J. Braun (1994): Use of random amplified polymorphic DNA markers in comparative genome studies. Meth.Enzymol. (224): 294 -309.
6. Callejas, C. and Ochartdo, M. (1998): Identification of Spanish barbell specie's using the RAPD technique of fish biology. 53:208-215.
7. Chaouai, B. and Hassine, O.K.B. (1998): The status of fish biodiversity in Ichkeul lagoon, Tunisia . J. Zool.Vol.659, pp 303 -304.
8. Cognetti, G. and Maltagliati, F. (2000): Biodiversity and adaptive mechanisms in brackish water fauna. Mar. Poll. Bull. 40, pp.7-14.
9. Comparing A.; Scattolin, N. and Rodino, E. (1983): Genetic differentiation among some populations of the cyprino-dontAphaniusfasciatus. Nardo. Novo Thalassia, Vol. 6, Supp, 1983-1984: pp.261-268.
10. Dinesh, K.; W. Chan; T. Urn; V. Pkang (1995): RAPD markers in fishes: An evaluation of resolution and reproducibility. ASIAPAC. J. MOL. fciol. Biotech. (3)2: 112-118.
11. Dufresne, F.f Bourget, E. and Bernatchez, L. (2002): Differential patterns of spatial divergence in micro satellite and alloryme alleles: Further evidence for locus-specific selection in the acorn barnacle, Semibalonusolonoides?.Mbl. Ecol. 11, pp. 113-123.
12. Gonzalez E.G, Pedraza-Lara C, Doadrio I (2014).Genetic diversity and population history of the endangered killifish *Aphaniusbaeticus*. J Hered. 105(5):597-610.
13. Hartl, D.L.; Dykhuize, D.E. and Dean, A.M. (1985): Limits of adaptation:the evollution of selective neutrality. Geneticsl 11, pp. 655-674.
14. Kambhatnpati, S.; Black, W.C. and RaiK, S. (1992): Random amplified polymorphic DNA of Mosquito species and populations. J. Medical. Entomology 29: 939-945.
15. Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-258.
16. Maltagliati, F. (1999): Genetic divergence in natural populations of the Mediterranean brackish-water kilHfish*AphaniusFasciatus*. Mar. Ecol. Prog. Scr. 179: nn 155-162.
17. Maltagliati, K (2002): Genetic monitoring of brackish-vvatcr populations: the Mediterranean toothcarp*AphaniusFasciatus* (Cyprinodontidae) as a model. Mar. Ecol. Prog. Ser. 235, pp. 257-262.
18. Mamuris, Z.; Apostolidis, A.P.; Theodorou, A.J. and Triantaphyllidis, C. (1998): Application of random amplified polymorphic DNA (RAPD) markers to evaluate intraspecific genetic variation in red mullet (*Mullusbarbatus*). Mar. Biol. 132, pp.m-178.
19. Maniatis, T.; Fritsch, E.F. and Sambrook, J. (1982): Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).
20. Nei, M. and Tajima, F. (1981): DNA polymorphism detectable by restriction endonuclcases. Genetics 105: 207-217.
21. Neva, E. (2001): Evolution of genome-phenome diversity under environmental stress.
22. Partis, L. and Wells, R.J. (1996): Identification of fish species using random amplified polymorphic DNA (RAPD). Molecular and cellular probes 10 (6): 435-440.
23. Postlethwait, S.L. Johnson: C.N. Midson; W.S. Talbot; M. Gates; E. W. Ballinger; D. Africa; R* Andrews; T. Carl; J. Eisen; S. Home; C. Kimmel; M. Hutchinson; M. Johnson and A. Rodriguez (1994): Agentic linkage map for the zcbrafish. Science. (264): 699-703.
24. Reichenbacher B, Feulner GR, Schulz-Mirbach T.(2009):Geographic variation in otolith morphology among freshwater populations of Aphaniusdispar (Teleostei, Cyprinodontiformes) from the southeastern Arabian Peninsula.J. Morphol.,270(4):469-84.
25. Roberta, C.; Fabrizio, S,; Fabio, L. and Giuseppe, N. (2003): Genetic diversity and environmental stress in Italian populations of the Cyprinodont fish *AphaniusFasciatus*. Occanologica Ada. Volume 26, pp.101-110.
26. Shears, M.A.; Fletcher, G.L.; Hew, C.L.; Gauthier, S. and Oavies, P.L. (1991): Transfer,

expression and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmosalar*). Mol Marine Biol Biotechnology 1: 58-63.
27. Siddiqui, G. and Ahmed, M. (1999): Karyotype of the hardy Killifish *Aphaniusdispar* occurring in the tidal backwaters of Karach i (Pakistan). J. zool (Pak, J, Zool) Vol. 31, No.3: pp. 293-295.
28. Smith, P.J, (1990): Proteine electrophoresis for identification of Australian fish stocks. Aust.J.MAR. Freshwat. RES. 41.6: pp 823-833.
29. Tanksley, S. and Orton, T.(1983): Isozymes in plant genetic and breeding. Part (B). Elsevier Science Bub b.V. Amesterdam
30. Varas, M.J.; De-Sostoa, A. (1997): Life-history pattern of the Iberian toothcarp*Aphanius Iberian* (Pisces Cyprinodont idae) from a Mediterranean estuary, the Ebro Delta (Spain). J.Zool.vol.47, no2:, pp143-160.
31. Waples, R.S. (1990): Temporal changes of allelic frequency in Pacific salmon: implications for mixed stock fishery analysis. Can. J. Fish.Aquat. Sci. 47: 968-976.
32. Williams, D.J.; S. Kazianis and R.B. Walter (1998): Use of Random Amplified Polymorphic DNA (RAPD) for identification of large mouth bass subspecies and their intergrades.Transactions of the American Fisheries Society 127(5): 825.
33. Williams, J.G.; A.R. Kubelik; K.J. Livak; J.A. Rafalski and S. V. Tingy (1990): DNA polymorphism amplified by arbitrary primers are used as genetic markers. Nucleic Asid Res., 18:6531-6535.

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