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Usage of new decamer primer MBl-9 to discriminate six *Barbus* fish species (Cyprinidae: Teleostei) in Iraqi freshwaters

Mustafa Sami Faddagh
Dept. Aquaculture and Marine Fisheries, Marine science Center, University of Basrah.
faddagh2003@yahoo.com
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Abstract

The most similar commercial six *Barbus* fish species in Iraqi waters are *Barbus xanthopterus, B. kersin, B. barbulus, B. grypus, B. sharpey and B. luteus*. To discriminate molecularly among them using random amplification polymorphic DNA with in polymerase chain reaction (RAPD-PCR) technique. Single decamer primer MBl-9 is designed locally to scan the genome and amplify DNA fragments. The PCR products are tested on 2% agarose gel electrophoresis with 100 bp ladder (standard molecular marker). The result of the study revealed that the six Barbus spp. Responded to the primer and gave different patern while the profile of DNA fragments showed 26 scorable bands for all six *Barbus* spp. and *Cyprinus carpio* distributed from one to six bands per species while their sizes ranged from 64 bp which we can consider it as dignostic band to this group till 807 bp in *B. barbulus*. The approximate values of DNA fragments calculated by using the linear equation of trendline of the standard curve plot between natural logarithm of 100 bp ladder bands size versus the distance migrated by the bands. In addition histogram is drawn as profile analog to the DNA fragment. The study concluded that this single decamere primer MBl-9 is suitable to discriminate among the most similer six native *Barbus* spp. and to confirm RAPD-PCR technique is efficient to differentiate among Iraqi freshwater fish species.

Keywords: RAPD, PCR, Barbus, Fish, Iraq.

1. Introduction

The random amplification polymorphic DNA (RAPD) protocol is a method based on the polymerase chain reaction (PCR) which uses single 10-meres primers for blind screening of genomes {1}.

The product of RAPD is a genetic fingerprint which usually contains multiple markers when separated on agarose gels stained with ethidium bromide dye (2, 3). The advantages of RAPD fingerprinting lies, in fact, that is a sequence independent approach and each primer annealing will produce a different spectrum of fragments from the PCR generating a species-specific fingerprint (4).

Welsh and McClelland (5) also used the arbitrary primer with 20-meres bases and resulted in DNA bands informative pattern. While McClelland and Welsh (6) described the decamere primer creating DNA

fingerprint in organisms. The RAPD protocol is capable of revealing nuclear variation in an extremely conserved species (7).

The RAPD method is very easy to set up, a useful tool for quick studies on lesser-known or even completely unknown genomes and proved to be more discriminatory, accurate and efficient than other methods (4).

There are several applications of RAPD fingerprinting in molecular ecology including the determination of taxonomic identities, the detection of interspecific gene flow, the assessment of kinship relationships, the analysis of mixed genome samples and the production of specific probes (8). These are advantages of RAPD: it needs no prior information about the template DNA and relatively low cost than other techniques, it targets many sequences in the DNA of the sample, producing DNA patterns that allow the comparison of many loci simultaneously, simplicity, relatively low cost and need only nanograms of template DNA (7)

The first applications of this technique in cyprinid research were the identification of markers sharing high levels of polymorphism among zebra fish strains (3) and the creation of a genetic linkage map based entirely on RAPD markers for the zebra fish *Danio rario* (9), whereas Barman *et al.* (10) used RAPD technique for studying genetic relationships and diversities in four species of Indian major carps belong to family Cyprinidae and to evaluate the use of the RAPD assay as a source of genetic markers to generate species-specific RAPD profiles.

Callejas and Ochando (11) reported that RAPD technique is a very advantageous and useful tool for species and population levels of *Barbus* fish. However, Perez *et al.*(12) study the evaluation of RAPD fragment reproducibility.

In Iraq this technique has been used to investigate native *Barbus* species and some other cyprinid species and DNA fingerprints were carried out by Fadddagh *et al* (13).

RAPD is also used to detect nDNA variation in fish populations (14, 7, 15, 16, 17; 18).

The genus *Barbus* belong to family Cyprinidae containing some important native fish species encompassing *Barbus xanthopterus, B. kersin, B. barbulus, b. grypus, B. sharpeyi and B. luteus* whereas common carp *Cyprinus carpio* was added for comparison.

The study aimed to design and achieve RAPD primer to discriminate among six *Barbus* species and *Cyprinus carpio* using genomic DNA and PCR method.

1. Materials and Methods:

Samples of cyprinids contain *Barbus xanthopterus, B. kersin, B. barbulus, B. grypus, B. sharpeyi, B. luteus* and *Cyprinus carpio* (as outgroup species) were collected from Shatt Al-Arab River in governorate of Basrah.

Specimens were preserved in cool box filled with ice and transferred to the laboratory. They identified according to Almaca (19) key and (20). Pieces of dorsal muscle and caudal fin were cut and preserved in vials filled with 95% ethanol alcohol.

Genomic DNA was extracted from 2-5 gm of dorsal muscle or fin tissue according to Sambrook and Russel (21) with little modifications. The tissue was analysed with SDS-Proteinase K enzyme (Promega) andDNA isolated using Chloroform-Isoamyl alcohol (24:1) and precipitated with isopropanol then dried and resuspended in 10 mM Tris(pH:8), 1 mM EDTA. The Genomic DNA integration was analyzed using electrophoresis with agarose gel 0.8% stained with ethidium bromide after mixing the DNA sample with bromophenole blue dye. Then it was incubated in chiller (-20°C) until the PCR experiment

1.1. RAPD-PCR

RAPD decamere primer was designed locally Mitochondrial *Cyt b* of *Barbus luteus* partial sequence was recovered from National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov. The accession No. AF145944. Reports *Barbus luteus* mtDNA *cyt b* [gi:19073744]. We designed decamere primers using Gene Fisher software (22) http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/gf_submit_old?mode=STARTUP&qid=na&sample=dna**,**

Ten oligodecamer primers was chosen from the long list shown in the result and purchased from Alpha (Canada) including , MBl -9 and tested. RAPD-PCR was carried out in 25 µl reaction volume contains 12.5 µl master mix, 2 µl of single decamere primer, 4 µl of template DNA and 6.5 µl distilled water 25 ng of DNA template, 5 pmoles of primer, 0.1 mM of each dNTPs, 4 mM $MgCl_2$, 0.5 U of Taq polymerase and master mix buffer solution. The reaction conditions were one cycle for initial denaturation at 94^o C for 5 min., 45 cycle at 94^o C for 1 min., 36^o C for 1 min., 72^o C for 1 min. and final extension at 72^o C for 6 min.

The PCR products were analyzed using electrophoresis with agaros gel 2% in Tris (pH:8.2)- Acetic acid-EDTA stained with ethidium bromide. The samples migrated with the 100 bp ladder (promega) under 60V for 50 min. The Agarose gel profile was checked bu UV light transilluminator and documented using the gel documentation composed of dark hood and Canon camera with UV light filter.

1.2. Data analysis:

The Microsoft office excel was used to plot the distance migrated by each band on X axis versus the natural logarithms (ln) of the size of DNA ladder bands (Promega) revealed on the horizontal agarose gel on Y axis to get a linear standard curve and equation of the trendline of the curve in order to apply all the band distance values (X) to the equation which resulted in *ln* size of the targeted band and to get the value of size of each band by inverse natural logarithm**.** Histogram was drawn by Microsoft Office Excel for the amplified bands.

2. Results and Discussion

Designing primers relating to specific function or particular gene is often complicated due to the volume and mystery of genome in vertebrate. The experiments would prove this designated primer useful or not. At the same time, the suggested primer may not be compatible to all species included in the test. The tested primers except MBl-9 were not respondents to six *Barbus* species (Table 1).

The profile of MBl -9 shows clearly the difference in band separation patterns among the six *Barbus* and *C. carpio* , the band size ranged from 64 bp to 807 bp while the primer created variant number of markers per species.

So, this new primer was efficient to screen the *Barbus* and *C. carpio* genome as the profile revealed. As well as the test with this new primer showed a highly genetic variation among studied species. This primer created a diagnostic marker of this *Barbus* spp.

Table 1 List of designed RAPD primer sequences and products.

No.	symbol	sequence	product
1	MBl-1	CCGAGACGTA	compatible partially
2	MBl-2	ATCCGCCGTA	Same single fragment
3	MBl-3	CGAAACAGGA	compatible partially
4	MBl-4	CCTCCACGAA	compatible partially
5	MBl-5	CACTAGTCGA	compatible partially
6	MBl-6	CACTAGTCGA	compatible partially
7	MBl-7	CCTGGTCCAA	compatible partially
8	MBl-8	TAACGGAGCA	No product
9	MBl-9	GTAGGCTTGC	Compatible for all spp. & multiple bands
10	MBl-10	CGTAGGCTTG	compatible partially

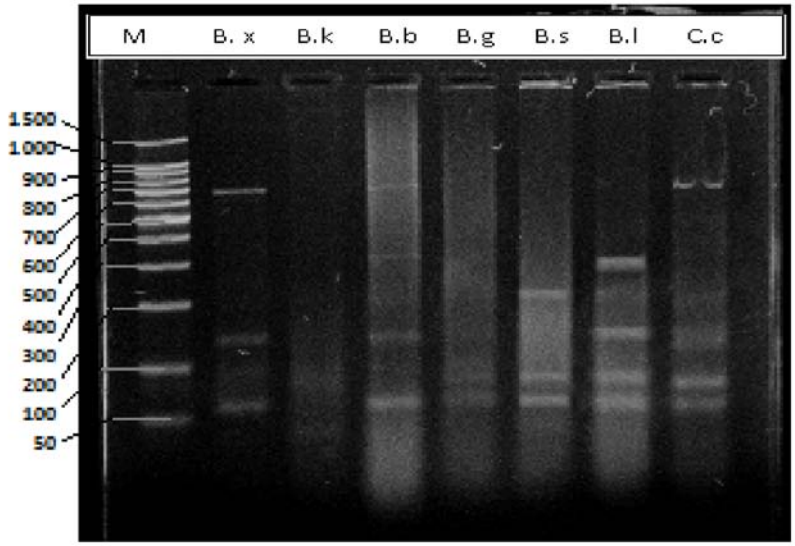

Figure (1) DNA profile of six *Barbus* species migrated on 0.2% agarose gel electrophoresis. M: Ladder, B.x: *Barbus xanthopterus*, B.k: *Barbus kersin*, B.b: *Barbus barbulus*, B.g: *Barbus grypus*, B.s: *Barbus sharpeyi*, B.l: *Barbus luteus*, C.c: *Cyprinus carpio*.

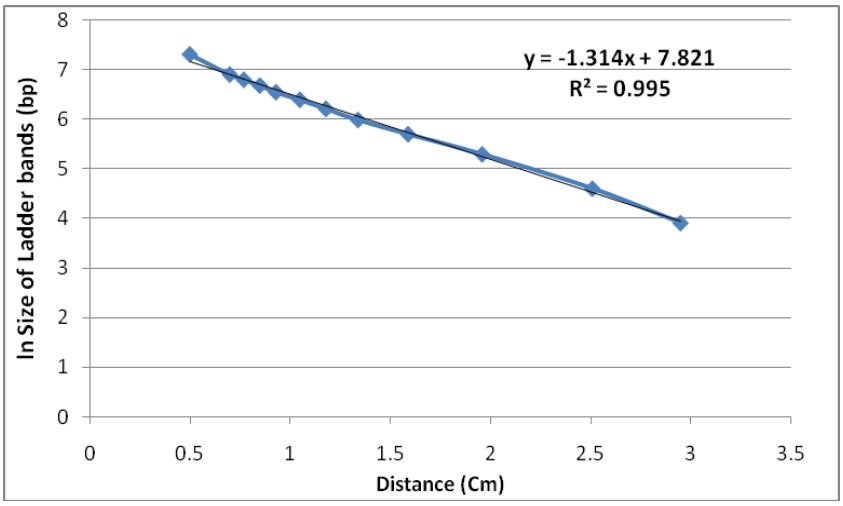

Figure (2) Standard curve of linear relationship and equation between natural logarithm (ln) of DNA band size (bp) and distance migrated on agarose gel electrophoresis.

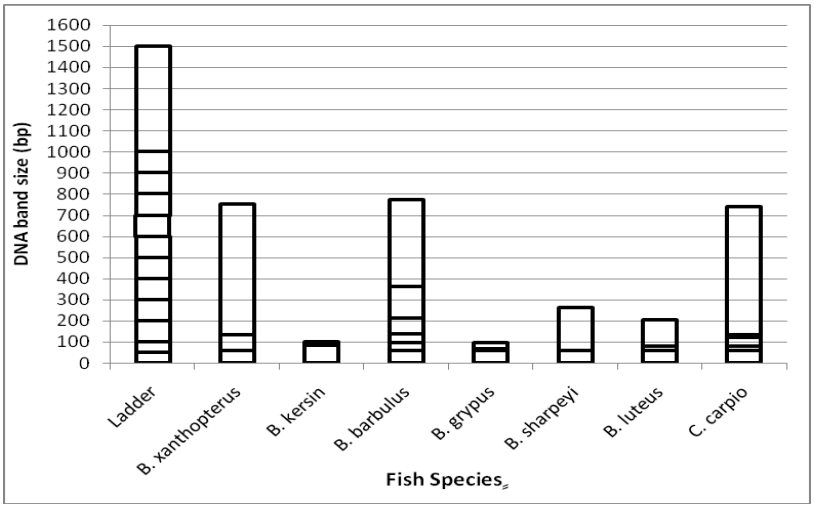

Figure (3) Histogram showing the band size (bp) of six *Barbus* species and *Cyprinus carpio.*

The technique is less labour intensive and faster than other DNA procedures, many samples may be analyzed simultaneously and large areas of the nuclear genome scored; the entire procedure from DNA isolation to visualization of amplified DNA on a gel can be performed without the use of hazardous radioactive chemicals and within 24 h; only minute amounts of DNA are required for analysis; and a priori information about the DNA sequence is not required. The technique therefore potentially offers a relatively easy and inexpensive method of examining population genetics.

However, to date, it has not been widely used in fish population studies, in part because of a number of drawbacks recognized for this technique. For example, homozygotes cannot be differentiated from heterozygote, precluding the possibility of allelic frequency analyses (23). The RAPD amplification procedure is particularly sensitive to changes in reaction conditions which may affect the reproducibility of amplification products (1); there may be subjectivity in band scoring (8); and the analysis of RAPD markers assumes that they are independent and that distinct amplified fragments of similar size do not comigrate. DNA amplification products from primer were consistently reproducible, band scores by three independent readers were highly consistent and amplifications with pairs of primers demonstrated at least some independence of information.

Furthermore, the RAPD profiles in this study displayed a high degree of polymorphism revealing species-specific (26) and diagnostic markers which indicated a population structure entirely consistent with that obtained from the analysis of RFLP markers in the same fish (27).

To date, few studies have compared the results of RAPD markers with mtDNA markers (7).

The consistency in population structuring based on several analyses of both RAPD and RFLP markers, seen in this study, supports the reliability of the interpretations and confirms the suitability of RAPD markers for discrimination of flyingfish stocks (24). Also, the identification of specific DNA markers identified here provides useful information on the choice of molecular tools for the examination of genetic variation in this species. In the other side RAPD-PCR method has some limitations, such as sensitivity to reaction conditions, occasionally non reproducible amplification product and possible comigration of amplified fragments (11).

Its disadvantages are that most RAPD markers are dominant/recessive; they usually do not work in every strain/

population (25). Nevertheless, these problems can be solved by following a strict protocol with standardized conditions, repeating the amplification reactions in order to see and record clearing reproducible bands, and increasing the resolution of band separation (8).

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أستخدام باديء عشري جديد مصمم محليا للتمييز بين اسماك جنس *Barbus* في المياه العذبة العراقية (Cyprinidae: Teleostei)

مصطفى سامي فداغ

قسم الأستزراع المائي والمصائد البحرية- مركز علوم البحار- جامعة البصرة.

الخلاصة

ان أكثر أسماك الشبوطيات تشابها مظهريا في المياه العذبة العراقية من جنس *Barbus* هي الأنواع الستة *Barbus xanthopterus, B. kersin, B. barbulus, B. grypus, B. sharpey and B. luteus*. وللتمييز فيما بينها بطريقة جزيئية استخدمت طريقة التضخيم العشوائي للحامض النووي الرايبوزي منقوص الأوكسجين DNA باتباع تقنية التفاعل البوليميريزي المتسلسل PCR-RAPD واضيفت سمكة الكارب الشائع المحلي *Cyprinus carpio* كنوع خارجي لغرض المقارنة. أستخدم الباديء MBI-9 المصمم محليا لمسح وتضخيم قطع من الذخيرة الجينية للأنواع قيد الدراسة. فحصت نواتج التفاعل باستخدام تقنية الترحيل الكهربائي على هلام الأكروز 2% مع مسطرة جزيئية تشير لكل 100 زوج قاعدي. حسبت القيمة التقريبية باستخدام معادلة الخط المستقيم الناتجة من علاقة اللوغارتم الطبيعي لحجم الحزم والمسافة التي قطعتها ورسم مخطط اعمدة مماثل لصورة الترحيل الكهربائي للحزم. اوضحت النتائج استجابة جميع انواع الأسماك المفحوصة للباديء MBI-9 واعطت نتائج جزيئية متغايرة من الحزم وقد احتوت على 26 حزمة لكل انواع *Barbus* الستة وسمكة الكارب الشائع وقد توزعت على الأنواع من حزمة واحدة الى ست حزم تبعا للأنواع بينما تراوحت بالحجم من 64 زوج قاعدي (والتي اعتبرت حزمة تشخيصية لأسماك هذه المجموعة) الى 807 زوج قاعدي في سمكة النباش. استنتجت الدراسة ان الباديء المستخدم كفوء للتمييز بين اسماك الجنس *Barbus* المحلية. واكدت الدراسة ان تقنية التضخيم العشوائي مناسبة للتفريق بين أنواع أسماك المياه العذبة العراقية.

كلمات مفتاحية: التفاعل البوليميريزي المتسلسل, طريقة التضخيم العشوائي للحامض النووي, الشبوطيات, أسماك, العراق.