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RESEARCH ARTICLE

THE USE OF RAPD-PCR AND PCR-RFLP MOLECULAR MARKERS TO GENETICALLY DISTINGUISH THE MORPHOLOGICALLY CLOSE TWO SPECIES OF METAPENAEUS GENUS FROM TWO DIFFERENT ENVIRONMENTAL LOCATIONS(KAUR ABDULLAH & SHATT AL ARAB) IN WATERS SOUTHERN OF IRAQ.

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Abstract

RAPD-PCR and RFLP–PCR molecular markers were used to study the Genetic Characterization for two members in Metapenaeus genera of Penaeidae shrimps from two different environment southern of Iraq. Metapenaeus affinis which is in kaur Abdullah (salty water) but the second is suspected member (shrimp) is in Shatt al-Arab(freshwater) While the marine closely related organism in freshwater bodies. Unlike the marine pawn, it is small but has similar morphological characteristics like the marine counterpart. The results indicate that although Metapenaes genus share considerable external features except that the genetic heterogeneity at the DNA level was high in the two organisms. RAPD marker used 12 universal primers to detect monomorphic and polymorphic patterns to genetically distinguish differences between the two organisms whereas in RFLP marker the voucher DAAPV F7 in 16S rRNA gene was amplified using PCR technique the desired gene was digested by using the enzymes (TagI , SmI and HindIII) which produced different size and numbers of bands. Thus, the voucher DAAPV F7 in 16S rRNA gene proved to be a useful molecular marker to differentiate the studied Metapenaeus species , which makes the task easier of telling apart species that are morphologically very similar.

Introduction:-

Shatt al-Arab (River of the Arabs") is a river in Southwest Asia of some 200 km (120 mi) in length, formed by the confluence of the Euphrates and the Tigris in the town of al-Qurnah in the Basra Governorate of southern Iraq. The southern end of the river constitutes the border between Iraq and Iran down to the mouth of the river as it discharges into the Arabian Gulf. It varies in width from about 232 metres (761 ft) at Basra to 800 metres (2,600 ft) at its mouth. It is thought that the waterway formed relatively recently in geologic time, with the Tigris and Euphrates originally emptying into the Arabian Gulf via a channel further to the west. Indeed ,the marshlands in Iraq were drained in the early 1990s in order to increase government control over the Arab Shiites (Marsh Arabs) who lived there. Restoration of the marshlands began in 2003, following the invasion of Iraq by Anglo-American forces, but only half the area has been restored. The river supplies fresh water to Iraq and Kuwait but the construction of dams and the demand for water upstream has led to a greatly increased salt content. The Shatt al Arab is navigable for oceangoing vessels as far as Basra, Iraq's chief port .In Iraqi south waters there are two main areas are considered

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important regions for study and research ongoing because of environmental and life changes that occur from time to time for several reasons, which are (Khour Abdullah and Shatt al-Arab)which is always the focus of attention of scientists and researchers, particularly in the south of Iraq there are aquatic organisms variety. Aquatic organisms living in salty water environment and aquatic organisms live in fresh water environment and the other can live in salty and fresh water environment which are called (euryhaline) . Often most scientists suspect of phenotypic classification of some aquatic organisms and which are too closed , scientist classified it according to potentially classification. Through present study resorted to solve this problem genetically .In many countries , Metapenaeus genus classified into several species , some countries relied on phenotypic classification and other countries adopted genetically classification but other countries relied on both. Iraq has been registered only one species of Metapeaeus genus (Metapenaeus affinis) which lives in salty water, (Khour Abd Allah or Al-faw) but the puzzle in species lives in fresh water (Shatt al-Arab, or marsh) which is closed metapenaeus affinis species in number of phenotypic traits**.** The diversity of organisms is influenced by multiple evolutionary factors, a situation that can affect the morphology, ecosystems and other biological behaviors of a plant or animal. Biological diversity is evident in a clear majority of species and leads to individual variations in numerous characteristics (Andi 781). Differences in species because of diversity is reflected in the genetic differences and environmental factors, or a combination of both. *Metapeneus* is a genus of pawns and has been classified into different species including the *Metapenaus affinis,* a marine water pawn (Nisha 557). While the marine pawn has been defined and named, a second closely related organism has been discovered in freshwater bodies. Unlike the marine pawn, it is small but has similar morphological characteristics like the marine counterpart (Thanh 144). To compare the genetic diversity, different molecular biology techniques, apart from the current conventional genetic analyzer capillary sequencer can be used (Caijing 49). Understanding the genetic diversity of the two organisms is critical in categorizing them and creating a new taxonomic characterization of the freshwater species.

Molecular Biology Techniques in Genetic Diversity of *Metapenaeus* Species:-

Molecular characterization of different species is gene dependent, an approach that focuses on the differences in DNA code at different loci. *Metapenaus* species may have arisen from the same organism but experienced environmental pressures, leading to mutations which may have enabled the unknown species to be smaller and to survive in freshwater bodies. Gene mutation or chromosomal changes are common contributors of genetic diversity and are associated with biological events such as meiosis and fertilization. To understand the molecular biodiversity of the two organisms, the differences in DNA base sequence or the amino acid of different protein can be assessed. Different techniques have so far been developed that can be used for the molecular characterization of diversity between the two species. While DNA sequencing is the most commonly used technique today, another cost-effective and non-laborious techniques also exist. Rapid Amplified Polymorphic DNA (RAPD) technique is one method that can be adopted in the diversity characterization of the organisms (Arif 274). RAPD data used and supported by RFLP-PCR marker which used 16s rRNA gene. In this method, genomic DNAwas amplified using specific primers to identify possible polymorphic markers in the absence of prior information on the mitochondrial genetic loci, according to Alex and Kochzius (6). To define the genetic difference between the two organisms of shrimp using RAPD, many steps were followed. First, DNA was extracted from the two organisms according to standard procedure (Williams*et al.*, 1990). The process was avoid potential breakdown of the DNA which can affect the amplification and identification of polymorphic sites (Patrick 241). Once DNA extracted, the next step involves the introduction of single arbitrary primers. Arbitrary primer amplification is a technology that has traditionally been used in the classification of *Bacillus* strains when DNA extraction is done from bacterial colonies with single templates (Hong 27). Primers are developed arbitrarily without the specific target of loci. Knowledge of the genetic composition of *Metapenaus* marine species is used in the development of primers (Mahoney 59). Furthermore, the primers specified to target specific conserved sequences to define homology and demonstrate an area of evolutionary diversion.Once primers introduced, the next step was the amplification through polymerase chain reaction (PCR). The amplification process duplicates the genetic loci that bind the primers, further increasing the number of copies. Once the fragments were amplified, separation for the different samples was undertaken using gel electrophoresis, a process that exploits the charge and size of the fragments (Ceren and Bilgen 571). A kilo base lambda DNA was used in the gel to help in the determination of the fragment sizes (Kacee 124). Once the fragments separated on 1% agarose gel in the presence of ethidium bromide, visualization was done under UV light. The band that moves highest has the least size and comparison was done for the two organisms (Nguyen 147). Size fragments were compared in the gel to determine the presence of microsatellites and polymorphic sites which act the molecular markers. The use of RAPD identified potential polymorphic markers that can differentiate the two *Metapenaeus* organisms and help demonstrate the difference in ecosystems. The method was convenient for this characterization due to its quick, simple and efficient nature. The process was only dependent on the thermocycling

machine and the agarose gel electrophoresis equipment (Cyrus 761). However, prior knowledge on the size of conserved sequences may be needed to compare and identify points of polymorphic diversion (Jimenez and Amaya 246).

Materials and Methods:-

Species collection and identification:-

A total of thirty five specimens of Metapenaeus affinis species as well as the same number of suspected organism were collected from two different locations, Khour Abd Allah and Shatt al-Arab which situated in waters southern of Iraq(north of Arabian Gulf). Metapenaeus affinis situated in Khour Abd Allah water (salty water) whereas the suspected shrimp is situated in Shatt al-Arab (freshwater) as shown in a figure (1.1).

Fig (1.1):- (A) Species of Metapenaeus affinis exist in Khour Abd Allah (salty water)
(B) Unknown species (suspected shrimp)located in Shatt al-Arab(Freshwater).

Genomic DNA isolation:-

Total genomic DNA was isolated from a piece of pleopod of each shrimp using a phenol-chloroform-proteinase K method (Klinbunga et al., 1996) / DNeasy Tissue Kit (Germany). DNA concentrations were spectrophotometrically determined at using a NanoDrop; 1000 Spectrophotometer at the absorbance of 260 (A260) and 280 nm (A280). The purity of extracted DNA was determined by using A260/A280 ratio. 1% Agarose Gel Electrophoresis was performed to detect the genomic DNA using Gel documentation .The DNA was diluted using TE buffer to a final concentration of µg/ml for RAPD analysis. It was then supported by using the PCR-RFLP technique .Twelve random oligonucleotide primers were used for RAPD-PCR analysis and specific primers were used to amplified the voucher DAAPV F7 in 16s rRNA gene for PCR-RFLP marker. The sequences of primers for RAPD -PCR technique were given in table (1.1).

For (RAPD-PCR) : PCR reactions were performed in 25 µl reaction volumes containing 1x PCR buffer (100 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 50 mM , KCl, 0.2 µM primer,100 µm each of dATP, dCTP, dGTP and dTTP 0.75 U of *Taq* DNA polymerase 20 ng of template DNA . Thermo cycler conditions were as follows ; for initial denaturation at 94ºC for 3 min, followed by 35ºC cycles at 94ºC for 1 min, 36ºC for 1 min and 72ºC for 2 min, one cycle at 72ºC for 10 min, and then 4ºC soak. The amplified samples were stored at – 20ºC for further analysis. RAPD reaction products were visualized on 1.5% agarose Gel , amplified fragments were identified by its size in base pairs and its associated primer. For each primer , PCR amplified products were scored on the gel images for monomorphic or polymorphic bands for each sample of the two organisms. Then RAPD-PCR analysis was supported by PCR-RFLP marker which used voucher DAAPV F7 in16S rRNA gene. The 16s rRNA gene fragment here seems suitable for examining phylogenetic relationships at the species or genus levels in Crustaceans. The 16s rRNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. Strongly conserved regions often flank these hypervariable regions. The Polymerization chain reaction (PCR)was used to amplify the voucher DAAPV F7 in 16s rRNA gene, in order to search for

molecular markers that could discriminate both species. The primers were designed based on rRNA gene sequences found in GenBank where nucleotide sequences of the 16S rRNA gene were aligned using Clustal W (Thompson et al., 1994). A pair of primers primed at the conserved regions of the voucher DAAPV F7 in 16S rRNA was designed and tested against 70 shrimp individuals. Namely as following :

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	67	20	60.12	50.00	3.00	3.00	CCGTGCGAAGGTAGCATAAT
RIGHT PRIMER	302	20	59.92	50.00	4.00	1.00	TATATTCTCGTCGCCCCAAC

The target DNA fragments (voucher DAAPV F7) for both organisms were amplified a 517bp segment of the 16s rRNA gene .PCR reaction was carried out in Eppendorf Thermo Cycler .(Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R.1994) .The reactions in volumes of 50 µL containing 4µl of DNA template, 4 µl of each primers, 25 µl of master mix completed the size with 18 µl of dd.w. Thermo cycler conditions were as follows: 5 min at 95°C for pre-running, then 35 cycles at 95°C for denaturation,40s at 50-52 °C for annealing, and 40 s at 72 °C for extension followed by 10 min at 72 °C for a final extension. Amplicons were visualized on 1.2% agarose Gel as shown in figure (1.14). Then voucher DAAPV F7 in 16s rRNA gene amplification products were digested with TaqI (TCGA), Sm II (TCYRAG) and Hind III (AAGCTT) . The restricted products were electrophoresed through 2.0% agarose gel and visualized under a UV transilluminator after ethidium bromide staining (maniatis et.,al 1982).

Results and Discussion:-

PCR was carried out using standers conditions for twenty random oligonucleotide primers on gDNA samples of two organisms (Metapenaeus affinis & suspected shrimp).Out of twenty primers ,twelve primers produced high reproducibility and consistent with RAPD profile and yielded monomorphic as well as polymorphic fragments ,while 8 primers yielded either weak amplifications with ambiguity or no amplification at both. The present RAPD study had shown a higher level of polymorphism in the two members of the Metapenaeus genus. Twelve random primers yielded a total of 82 fragments, of which, 50 bands were polymorphic whereas 32 fragments were monomorphic as were given in table (1.2). The number and size of the amplified products varied depending on genetic characterization of the species. A unique band of 1892 bp obtained for *Metapenaeus affinius* which are highly species specific for primer OPF-09 and not both organisms shared in this primer . Maximum of 11 bands were obtained from OPA-13 primer and the least of three bands from OPC-02 primer .The highly polymorphic bands were obtained with the primer OPA-13. When the size of the fragment is concerned, the highest range of 164-1892 obtained from the OPF-09 whereas the lowest range from OPE-02 (269-1207). RAPD profiles obtained by twelve primers as were shown in the figures 1.2,3,4,5,6,7,8,9,10,11,12 and 13 . Species-diagnostic markers from DNA segments should exhibiting low genetic polymorphism within a particular species but showing high genetic divergence between different species (Thaewnon-ngiw et al., 2004). Profile of RAPD obtained by OPA-08 primer as shown in the figure (1.2). The size of the amplified products ranged from 163-1346bp. The smallest fragment belongs to suspected shrimp and the biggest one belongs to both members. A total of 6 bands were scored. Of these bands, two were monomorphic with 803bp and 1346bp and shared both members (species). Out of 7 bands, 4 bands were polymorphic and shared by both species. RAPD profile obtained by OPA-13 primer as shown in the figure (1.3). The size of the amplified products ranged from 273bp-1354bp . The smallest fragment belongs to *Metapenaeus* affinis and the biggest one belongs to unknown member. A considerable amount of polymorphism was detected with this primer. A total of 11 bands were scored. Of these bands, four were monomorphic with (524,782,891, 1045)bp and shared by both species. Out of 11 bands, 7 bands were polymorphic and shared by both species.

Profile of RAPD obtained by OPC-02 primer was depicted in the fig. (1.4). The size of the amplified products ranged from 351-1800 bp. The smallest fragment belongs to unknown member whereas both members had the largest fragment. Figure (1.5) shows RAPD profile obtained by OPD-08 primer. The size of the amplified products ranged from 257bp-1335 bp. The smallest fragment was observed for unknown member and the largest to *Metapenaeus affinis*. Four of polymorphism was detected with this primer. And 3 of monomorphic of 7 total fragments were scored. RAPD profile of OPE-02 primer was depicted in the fig. (1.6).The size of the amplified products ranged from 269bp-1207bp. unknown member with the largest band and both members with smallest monomorphic.A total of 8 fragments were scored. Of these fragments, three were monomorphic band with (269,407 and 932) bp shared by both species.The size of the amplified products of RAPD profile obtained by OPE-03 primer ranged from 346-1303 bp. Both members had small fragment whereas the biggest one observed in unknown member. A considerable amount of polymorphism was detected with this primer. Of the eight fragments scored, two were monomorphic (346 &535bp) shared by both species and six fragments were polymorphic and shared by

both species as shown in the figure (1.7). while figure (1.8) was shown RAPD profile obtained by OPE-06 primer. The size of the amplified products ranged from 162-1257bp. Metapenaeus affinis had the smallest fragment and it also had the biggest fragment. A total of 8 fragments were scored, of these fragments, three were monomorphic with (209,739 &1005)bp shared by both species. five fragments were polymorphic and shared by both species. RAPD profile obtained by OPF-05 primer was presented in the fig. (1.9), the size of the amplified products ranged from 442-1254bp ,unknown member had smallest fragment as well as it had the biggest one . A total of 5 fragments were scored of these fragments, two were monomorphic band with (775 and 966) bp shared by both species. Out of 5 fragments, three were polymorphic , shared by both species while figure(1.10) was shown RAPD profile obtained by OPF-06 primer. The size of the amplified products ranged from(284-1476)bp ,unknown member had the smallest fragment and metapenaeus affinis species had the biggest fragment. A total of 7 fragments were scored, of these fragments, one was monomorphic with 1245bp shared by both species whereas six fragments were polymorphic and shared by both species. RAPD profile obtained by OPF-09 primer was depicted in the fig. (1.11). The size of the amplified products ranged from (164-1892)bp. This profile had some differences with others such as number of monomorphic bands that it was three with (164,694 and977)bp, the polymorphic was also three bands. The smallest fragment belongs to both members and Metapenaeus affinis species had the biggest one . RAPD profile obtained by OPG-16 primer was depicted in the fig. (1.12). The size of the amplified products ranged from (135-1798) bp. Unknown member had the smallest whereas the biggest one observed in Metapenaeus affinis. Of the seven fragments scored, three were monomorphic (523,845 ,1662)bp shared by both species and four fragments were polymorphic and shared by both species. The number and size of the amplified products obtained by OPG-17 primer was represented in the fig.(1.13). The size of the amplified products ranged from (558-1634) bp , the smallest fragment of belongs to both members Metapenaeus affinis and unknown member, the biggest one observed in Metapenaeus affinis . Four fragments were scored, of the three were monomorphic (558,721, 1325)bp and shared by both species. Only one fragment was polymorphic in Metapenaeus affinis species. Genetic diversity and the genetic heterogeneity at the DNA level was high in the two organisms from different populations by using RAPD-PCR. After voucher DAAPV F7 in 16s rRNA gene amplification ,the segment of 16s rRNA (517bp) for both Metapenaeus affinis and suspected shrimp were digested with three suitable restriction enzymes(**TaqI , SmII** & **HindIII**). **TaqI** restriction enzyme was cut the gene of Metapenaeus affinis at three sites , at 501bp , 447bp & 426bp while **SmII** restriction enzyme was cut it at two sites at 316bp and at 258bp whereas HindIII restriction enzyme cut the gene at only one site at 215bp . Size pieces which were cut result in differing size and differing numbers of patterns Whereas another species which suspected to be Metapenaeus affinis morphologically gave different results , (TaqI)restriction enzyme cut the gene at two sites **342bp & 401bp** while SmI restriction enzyme uncut any site of the gene whereas HindIII cut voucher DAAPV F7 in 16s rRNA segment at three sites at 525bp, 475bp & 301bp. Restriction patterns of TaqI , SmI & Hind III digested voucher DAAPV F7 in 16S rRNA gene clearly differentiate both Metapenaeus affinis and suspected one, as shown in the figure (1.15).. Overlapping patterns between different species should not be observed . Both molecular markers (RAPD and RFLP/PCR) were facilitated the task of distinguishing species that are morphologically close species and very useful in determining the distribution of the exotic species .

Table 1.1:-Names and sequences of the Primers used for Random Amplified Polymorphic DNA assay**.**

No	Primer	Primer Seq 5′ -3′	Mer	MW	Tm	%GC
1	OPE-02	GGTGCGGGAA	10	3133	44.9	70
2	OP E-03	CCAGATGCAC	10	3180	36.7	60
3	OPE-06	AAGACCCCTC	10	2967	31.9	60
4	OPG-16	AGCGTCCTCC	10	2964	37.1	60
5	OPG-17	ACGACCGACA	10	3006	34.8	60
6	OPF-05	CCGAATTCCC	10	2948	38.3	60
7	OPF-06	GGGAATTCGG	10	3108	38.3	60
8	OPF-09	CCAAGCTTCC	10	2948	33.9	60
9	OPC-02	GTGAGGCGTC	10	3084	33.4	60
10	OPD-08	GTGTGCCCCA	10	3004	40.6	70
11	OPA-8	GTGACGTAGG	10	3108	22.9	70
12	OPA-13	CAGCACCCAC	10	2942	34.8	70

Table – 1.2:- List of Monomorphic and Polymorphic fragments in RAPD analysis for each primer along with fragments and Molecular size

No	Primer	Total bands	Size range (in bp)	Monomorphic Bands	Polymorphic bands
1	OPE-02	8	269-1207	3	5
2	OPE-03	8	346-1303	2	6
3	OPE-6	8	162-1257	3	5
4	OPG-16	7	135-1798	4	3
5	OPG-17	4	558-1634	1	3
6	OPF-05	5	442-1254	2	3
7	OPF-06	8	285-1476	3	5
8	OPF-09	6	164-1892	3	3
9	OPC-02	3	351-1800	1	2
10	OPD-08	7	257-1335	3	4
11	OPA-8	7	163-1346	3	4
12	OPA-13	11	273-1354	4	7

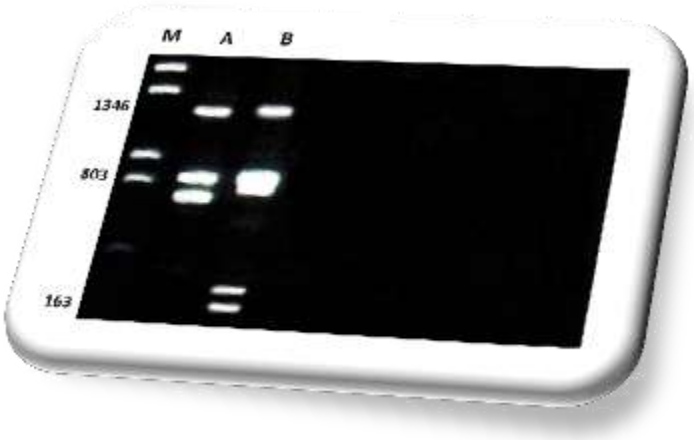

Figure 1.2:- RAPD profile obtained by OPA-08 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

Figure 1.3:- RAPD profile obtained by OPA-13 primer
M - DNA Ladder (2kb)

A – Metapenaeus affinis species
B - Suspected shrimp

Figure 1.4:-RAPD profile obtained by OPC-02 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

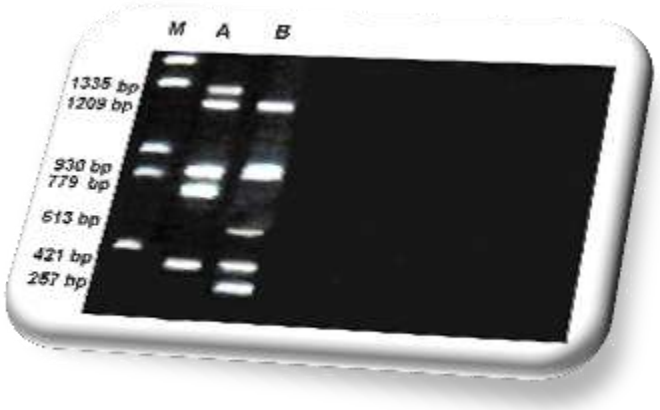

Figure 1.5:-RAPD profile obtained by OPD-08 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

Figure 1.6:-RAPD profile obtained by OPE-02 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

Figure 1.7:-RAPD profile obtained by OPE-03 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

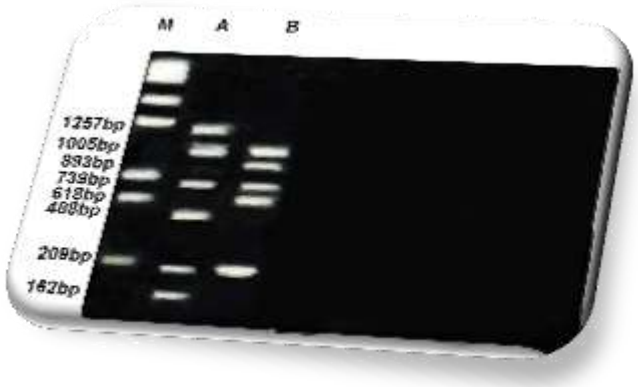

Figure 1.8:-RAPD profile obtained by OPE-06 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

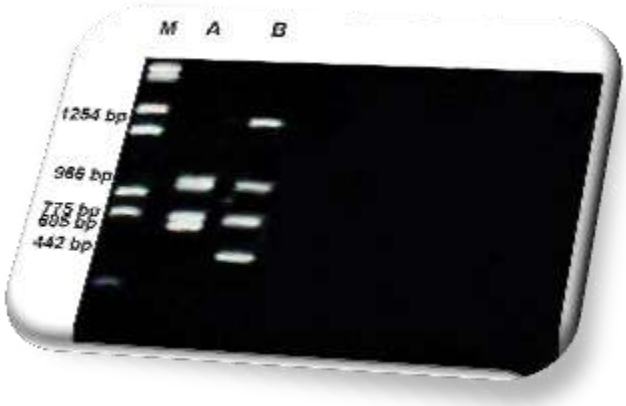

Figure 1.9:-RAPD profile obtained by OPF-05 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

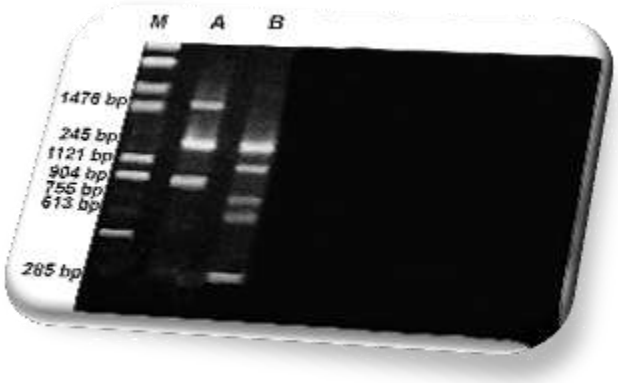

Figure 1.10:-RAPD profile obtained by OPF-06 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

Figure 1.11:-RAPD profile obtained by OPF-09 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

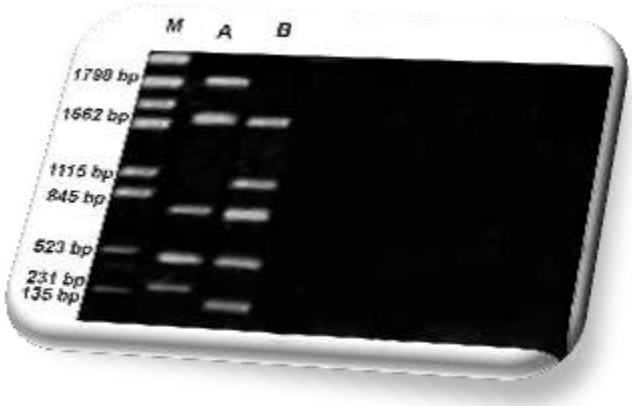

Figure 1.12:-RAPD profile obtained by OPG-16 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

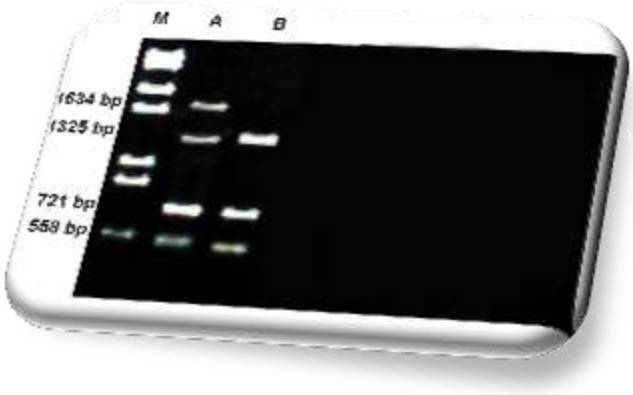

Figure 1.13:-RAPD profile obtained by OPG-17 primer.
M - DNA Ladder (Molecular weight)
A – Metapenaeus affinis species
B - Suspected shrimp

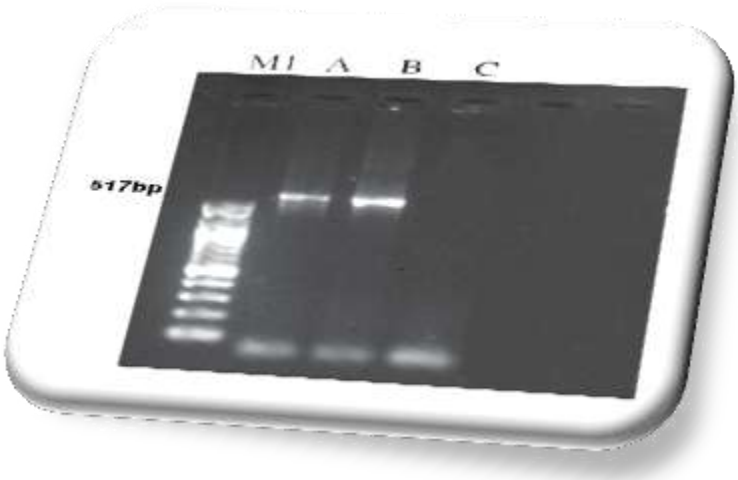

Figure (1.13):-PCR products for the 16s rRNA gene. M) Molecular Marker 100 bp BioLabs; A) *Metapenaeus affinis* ; B) Suspected shrimp; C)Negative Control; onto a 1.2 % agarose gel, ethidium bromide stained.

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Figure (1.15):-RFLP analysis of the voucher DAAPV F7 in 16s rRNA gene ; A) Metapenaeus affinis ; B) Suspected shrimp. onto a 1.2% agarose gel, ethidium bromide stained.

Conclusion:-

Metapenaus species were commonly found in the marine water ecosystem. In the current study, a morphologically similar but smaller freshwater version of Metapenaeus species discovered. To understand the genetic diversity and potential source of evolutionary diversion, RAPD analysis was used then it supported by RFLP-PCR marker by using 16s rRNA mitochondrial gene. Rapid Amplified Polymorphic DNA used in the genetic diversity characterization of the organism due to its low cost and ease of use. RAPD analysis gives more accurate estimates between closely related populations and less accurate estimates for distantly related populations. RAPD data used

for phylogenetic studies and generally supported existing taxonomies based on morphology, isozymes and RFLPs. Conclude from RAPD & RFLP -PCR analysis evidence of genetic mismatch between the two organisms of Metapenaeus genus from different populations and therefore these results indicate that the two organisms is not from the same species.

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