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Molecular identification and Phylogeny of the tow freshwater fish *Bagrus bajad* and *Bagrus docmac* in Egypt

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ABSTRACT

In Egypt, two species of *Bagrus* fish have been described based on morphometric, meristic and cytotaxonomical characteristics. These species are *Bagrus bajad* and *Bagrus docmac*. The accurate identification of these fishes is complicated by the high variation in their characters, similarity among species and in some cases by the size of the fish. In this paper, we used polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) analysis of the mitochondrial cytochrome *b* gene (*Cyt b*) for molecular identification of *Bagrus* spp. in Egypt. The present study aimed at evaluating such advanced molecular biological approaches for identification of *Bagrus* spp. Genomic DNA was extracted from the two species of *Bagrus*. About 1120 bp *Cyt b* gene was amplified by PCR using specific primers. The technique of restriction fragment length polymorphisms was used to identify the specific *Cyt b* gene for each species. *Acc*I, *Apo*I and *Eae*I restriction endonucleases would differentiate *Cyt b* sequences of *Bagrus bajad* and *Bagrus docmac* into one group. *Bse*RI, *Bsm*BI, *Dra*I, *Bsa*OI, *Xma*III, *Aat*I, *Bam*HI and *Eco*RI restriction endonucleases differentiated the two species as a polyphylogenetic relationship.

Key words: Identification, *Bagrus*, *Cyt b*, RFLPs, PCR, Polymorphism.

INTRODUCTION

Fish is the cheapest source of animal protein and is estimated to account for over 50 percent of the animal protein supply. Fish has traditionally been the most affordable source of animal protein.

Bagrus bajad and *Bagrus docmac* (Family: Bagridae) are well distributed in the River Nile, Lake Naser and freshwater parts of other lakes. Their economic importance is due to their well marketable size, good taste flesh and acceptability by the Egyptian people (Boulenger, 1907; Bishai and Khalil, 1997). Moreover, these fish could be used in fish farming.

The relationships among members of Bagridae have been the focus of cladistic analyses of morphological (Mo, 1991; De Pinna, 1993; Ng, 2004) and biochemical data (Okazaki *et al.*, 1999; Peng *et al.*, 2002; Watanabe and Nishida, 2003). However, the taxonomic sampling of these studies has been

influenced by the historical treatment of this non-monophyletic group of catfishes and this makes their results difficult to interpret.

Catfishes (Order: Siluriformes) are a wonderful diverse clade that has more than 3000 valid living species plus an estimated 1750 undescribed species (Sabaj *et al.*, 2004). In the first six years of the 21st century 332 new catfish species were described, and among these are the first representatives of nine new genera and one new family (Eschmeyer *et al.*, 2004). As indicated by several complex anatomical synapomorphies (Fink and Fink, 1981, 1996; Arratia *et al.*, 2003) there is no doubt about the monophyly of Siluriformes. The broadest studies of interrelationships among catfish families are those of Mo (1991); de Pinna (1993, 1998); Britto (2003); Diogo (2004) and Rodiles-Hernández *et al.* (2005) using morphological data, and Hardman (2002, 2005) using molecular data.

Mitochondrial gene cytochrome *b* (cyt *b*) has been widely used to infer phylogenetic relationships within and among taxonomic categories of Bagridae, ranging from populations to classes (Murphy and Collier, 1996; Lydeard and Roe, 1997; Briolay *et al.*, 1998; Akihito *et al.*, 2000; Lavoué *et al.*, 2000; Lovejoy and de Araújo, 2000; Waters *et al.*, 2000; Farias *et al.*, 2001; Derome *et al.*, 2002; Orrell *et al.*, 2002; Reed *et al.*, 2002), and is one for which comparative and biochemical information is available (Esposti *et al.*, 1993; Lydeard and Roe, 1997).

MATERIAL AND METHODS

Collection of specimens:

Bagrus bajad and *Bagrus docmac* fish were collected from El-Riah El-Tawfequi stream (a branch of the River Nile) at El-Kanater city and transported to the laboratory in Faculty of Science, Benha University, Benha, Egypt. By using the morphometric analysis, *Bagrus* spp. were laboriously differentiated into *Bagrus bajad* and *Bagrus docmac*. Liver pieces of the fish were stored for one week in a freezer until DNA extraction.

Extraction of genomic DNA:

Total DNA was extracted from the two species of *Bagrus* spp. using the UNSET lysis solution. Liver pieces of each species were homogenized and resuspended in 500 µl of UNSET (Lysis solution; 8M urea, 2% sodium dodecyl sulphate, 0.15M NaCl, 0.001M EDTA, 0.1M Tris pH 7.5) (Hugo *et al.*, 1992). Phenol-chloroform extraction was used two to three times to separate the organic and aqueous phases. To precipitate the nucleic acid, iced absolute ethanol was added (2:1 v/v), and incubated at -20 °C for 24 to 72 hours. The nucleic acids (nuclear and mitochondrial DNA) were recovered by centrifugation at ~5,000 rpm for 15 minutes. The pellet was dried and resuspended in 40 µl of sterile H_2O. One µl of the resuspended pellet was cheked by 0.8% gel electrophoresis for the presence of DNA, as in Fig. 1.

Determination and amplification of Cytochrome b (*Cyt b*) gene by polymerase chain reaction (PCR):

To amplify the complete mitochondrial *Cyt b* gene, one µl of whole-cell DNA template was used plus oligonucleotide primers complementary, either to 5′ and 3′ ends of the gene (*Cyt b*1 and *Cyt b*2). The standard PCR reaction mixture was used (Kessing *et al.*, 1989). The entire *mtCyt b* gene was amplified using the primers *Cyt b*1 (5′-TAA TGG CGT GAA AAA CCA CCG TTG T-3′) and *Cyt b*2 (5′-TCT TTT CTG GCG CTA GGG AGG-3′) (Amresco) anchored respectively in the conserved extremities of the *Cyt b* gene (Hardman, 2005).

The standard PCR program for amplification of *Cyt b* gene was: 30 – 35 cycles; one minute at 94°C; two to three minutes at 45°C; and three minutes at 72°C.

Deoxynucleotides triphosphates (dNTP; dATP, dGTP, dTTP and dCTP) were obtained from Promiga. The *Taq* DNA polymerase used for the *Cyt b* gene was obtained from Boehringer Mannheim Biochemica.

PCR products were isolated after separation by agarose gel electrophoresis (0.8 g agarose; BRL ultrapure electrophoresis grade/100 ml 1xTAE [Tris base, glacial acetic acid and EDTA]). Ethidium bromide was used to stain PCR products in the gel (50 µg/100 ml 1xTAE) for ten minutes. The PCR products (bands) were visualized under UV lamp and then cut from the gel. Glassmilk DNA purification was used to purify the gene from the agarose gel. Three microlitres of the amplification products were visualized on 0.8% ethidium bromide stained agarose gels to check the quality of amplification. The remaining 7 µl were mixed with 53 µl of water, and divided into 10 µl aliquots for enzyme digestion.

The Mitochondrial *Cyt b* gene RFLPs Profiles:

In the initial experiments, the enzymes *Acc*I, *Apo*I and *Eae*I restriction endonucleases (Amersham, Life Science) were evaluated for there ability to differentiate the two *Bagrus* spp. Additional enzymes were tested including *Bse*RI, *Bsm*BI and *Dra*I (Boehringer Mannheim) *Bsa*OI, *Xma*III, *Aat*I, *Bam*HI and *Eco*RI (Sigma Co.). One microlitre (10 – 12 units) was used for each digestion reaction, together with 1.2 µl of the respective enzyme buffer for ~3.5 hours at ~37°C, and the digestion products were evaluated on 2% TBE – agarose (FMC Bioproducts) gels and stained with ethidium bromide. Bands were detected under ultraviolet transillumination and photographed (35 mm Kodak Film, England).

RESULTS

DNA genome was extracted from *Bagrus bajad* and *Bagrus docmac* (Fig. 1). The full-length of cytochrome b (*Cyt b*) gene PCR products resulted in a product of approximately 1120 bp (Fig. 2).

*Acc*I, *Apo*I and *Eae*I restriction endonucleases did not differentiate between the *Cyt b* genes of the two species. *Acc*I restriction enzyme digested the

Cyt b genes of the two samples into five restriction fragments (~40, ~230, ~250, ~270 and ~330 bp; Fig. 3 and Table 1). *Apo*I restriction endonuclease cut the gene of each of the two samples into five restriction bands (~80, ~200, ~230, ~250 and ~360 bp; Fig. 4 and Table 2). Also, *Eae*I restriction enzyme fragmented the gene of each of the two individuals into three fragments (~190, ~290 and ~640 bp; Fig. 5 and Table 3).

Table (1): The length of *Cyt b* gene fragments, resulted from digestion with *Acc*I enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 3).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~40	~230	~250	~270	~330
docmac	~40	~230	~250	~270	~330

Table (2): The length of *Cyt b* gene fragments, resulted from digestion with *Apo*I enzyme in *Bagrus bajad* and *Bagrus docmac*. (see Fig. 4).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~80	~200	~230	~250	~360
docmac	~80	~200	~230	~250	~360

Table (3): The length of *Cyt b* gene fragments, resulted from digestion with *Eae*I enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 5).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~190	~290	~640	-------	-------
docmac	~190	~290	~640	-------	-------

*Bse*RI, *Bsm*BI and *Dra*I restriction endonucleases clustered the two individuals into two clusters with some differences. *Bse*RI restriction enzyme digested *Cyt b* gene of *Bagrus bajad* into five restriction fragments (~100, ~150, ~250, ~300 and ~320 bp) and *Bagrus docmac* into three fragments (~100, ~320 and ~700 bp) (Fig. 6 and Table 4). *Bsm*BI restriction endonuclease fragmented the gene of *Bagrus bajad* into three restriction bands (~200, ~350 and ~570 bp) and *Bagrus docmac* gene into two fragments (~200 and ~920 bp; Fig. 7 and Table 5). *Dra*I enzyme differentiated the two individuals into two clusters when digested the gene of *Bagrus bajad* into three restriction fragments (~50, ~500 and ~570 bp) and *Bagrus docmac* into two bands (~50 and ~1070 bp); Fig. 8 and Table 6.

Table (4): The length of *Cyt b* gene fragments, resulted from digestion with *Bse*RI enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 6).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~100	~150	~250	~300	~320
docmac	~100	~320	~700	--------	--------

Table (5): The length of *Cyt b* gene fragments, resulted from digestion with *Bsm*BI enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 7).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~200	~350	~570	--------	--------
docmac	~200	~920	--------	--------	--------

Table (6): The length of *Cyt b* gene fragments, resulted from digestion with *Dra*I enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 8).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~50	~500	~570	--------	--------
docmac	~50	~1070	--------	--------	--------

*Bsa*OI and *Xma*III restriction endonucleases cut uniquely the gene of *Bagrus bajad* but did not digest the gene of *Bagrus docmac*. *Bsa*OI restriction enzyme digested the *Cyt b* gene of *Bagrus bajad* into three fragments (~270, ~290 and ~560 bp; Fig. 9 and Table 7). Also, *Xma*III restriction endonuclease cut the gene of *Bagrus bajad* into three cuts (~280, ~300 and ~540 bp; Fig. 10 and Table 8).

Table (7): The length of *Cyt b* gene fragments, resulted from digestion with *Bsa*OI enzyme in *Bagrus bajad* and *Bagrus docmac*. (see Fig. 9)

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~270	~290	~560	--------	--------
docmac	--------	--------	--------	--------	--------

Table (8): The length of *Cyt b* gene fragments, resulted from digestion with *Xma*III enzyme in *Bagrus bajad* and *Bagrus docmac*. (see Fig. 10)

Band # *Bagrus* sp.	1	2	3	4	5
bajad	~280	~300	~540	--------	--------
docmac	--------	--------	--------	--------	--------

Moreover, *Aat*I, *Bam*HI and *Eco*RI restriction endonucleases digested, uniquely, the gene of *Bagrus docmac* but did not digest the gene of *Bagrus bajad*. *Aat*I restriction enzyme digested the gene of *Bagrus docmac* into four

restriction bands (~130, ~160, ~330 and ~500 bp; Fig. 11 and Table 9). *Cyt b* gene of *Bagrus docmac* was digested into two fragments when treated with *Bam*HI enzyme (~500 and ~620 bp; Fig. 12 and Table 10). Also, *Eco*RI restriction enzyme fragmented the gene of *Bagrus docmac* into two fragments (~150 and ~970 bp; Fig. 13 and Table 11).

Table (9): The length of *Cyt b* gene fragments, resulted from digestion with *Aat*I enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 11).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	--------	--------	--------	--------	--------
docmac	~130	~160	~330	~500	--------

Table (10): The length of *Cyt b* gene fragments, resulted from digestion with *Bam*HI enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 12).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	--------	--------	--------	--------	--------
docmac	~500	~620	--------	--------	--------

Table (11): The length of *Cyt b* gene fragments, resulted from digestion with *Eco*RI enzyme in *Bagrus bajad* and *Bagrus docmac* (see Fig. 13).

Band # *Bagrus* sp.	1	2	3	4	5
bajad	--------	--------	--------	--------	--------
docmac	~150	~970	--------	--------	--------

DISCUSSION

Species identification based on morphological criteria and protein analysis was the most reliable and widely used method. Species-specific banding patterns are typically generated by iso-electric focusing. This technique has proven to be reliable (Rehbein *et al.*, 1995). Protein-based identification techniques become less reliable with fish. However, in some cases, it is still possible to generate a banding pattern which enables identification (Hsieh *et al.*, 1997). As an alternative to protein analysis, DNA-based identification techniques have been proposed and applied. The molecular techniques based on PCR-RFLP analysis of the srDNA have been extensively used for many analyses of fish (Chow *et al.*, 1993; Chow and Inoue, 1993; Ram *et al.*, 1996 ; Céspedes *et al.*, 1998; Quinteiro *et al.*, 1998; Fernandez, 2001). Englander and Moav (1989), Wright (1989), Franck *et al.* (1992), Seyoum and Kornfield (1992), Agnese *et al.* (1997), Rognon *et al.* (1997) and Farias *et al.* (1999) used restriction fragment length polymorphisms of nuclear and mitochondrial DNA PCR products (RFLPs\PCR) as a basis for examining relationships among fishes.

*Acc*I, *Apo*I and *Eae*I restriction endonucleases would not differentiate *Cyt b* sequences of *Bagrus bajad* and *Bagrus docmac*. This indicated that the two species of *Bagrus* may follow the same species, or monophylogenetic species. It has been found that every one of the two could be differentiated from the other by RFLPs applied by using specific endonucleases to digest *Cyt b* PCR products. Three restriction enzymes (*Bse*RI, *Bsm*BI and *Dra*I) indicated that *Bagrus bajad* and *Bagrus docmac* may be polyphylogenetic. There were two restriction endonucleases (*Bsa*OI and *Xma*III) that could cut uniquely the gene of *Bagrus bajad* but did not digest the gene of *Bagrus docmac*. Also, *Aat*I, *Bam*HI and *Eco*RI restriction endonucleases digested, uniquely, the gene of *Bagrus docmac* but did not digest the gene of *Bagrus bajad*.

Sequencing PCR fragments has become a standard technique in laboratories applying recombinant DNA technologies. Because of its high reproducibility, it might well become the advisable method for constructing such databases. They could be used to establish the authenticity of a sample unambiguously (e.g., at the species or subspecies level). Yet, several groups of researchers who compared the sequencing option with the RFLP option for analyzing fragments, claimed that the RFLP option would be considerably simpler and faster (Ram *et al.*, 1996; Céspedes *et al.*, 1998; Quinteiro *et al.*, 1998). In addition, the RFLP technique is less costly. Ram *et al.* (1996) calculated that the RFLP option for analyzing fragments was about seven times lower in cost with respect to the sequencing option.

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LEGEND OF FIGURES

Figure 1: Total DNA genome from *Bagrus bajad* and *Bagrus docmac*.

Figure 2: Full-length of *Cyt b* gene of *Bagrus bajad* and *Bagrus docmac*.

Figure 3: The length of *Cyt b* genes fragments, resulted from digestion with *Acc*I enzyme in the investigated species.

Figure 4: The length of *Cyt b* genes fragments, resulted from digestion with *Apo*I enzyme in the investigated species.

Figure 5: The length of *Cyt b* genes fragments, resulted from digestion with *Eae*I enzyme in the investigated species.

Figure 6: The length of *Cyt b* genes fragments, resulted from digestion with *Bse*RI enzyme in the investigated species.

Figure 7: The length of *Cyt b* genes fragments, resulted from digestion with *Bsm*BI enzyme in the investigated species.

Figure 8: The length of *Cyt b* genes fragments, resulted from digestion with *Dra*I enzyme in the investigated species.

Figure 9: The length of *Cyt b* genes fragments, resulted from digestion with *Bsa*OI enzyme in the investigated species.

Figure 10: The length of *Cyt b* genes fragments, resulted from digestion with *Xma*III enzyme in the investigated species.

Figure 11: The length of *Cyt b* genes fragments, resulted from digestion with *Aat*I enzyme in the investigated species.

Figure 12: The length of *Cyt b* genes fragments, resulted from digestion with *Bam*HI enzyme in the investigated species.

Figure 13: The length of *Cyt b* genes fragments, resulted from digestion with *Eco*RI enzyme in the investigated species.

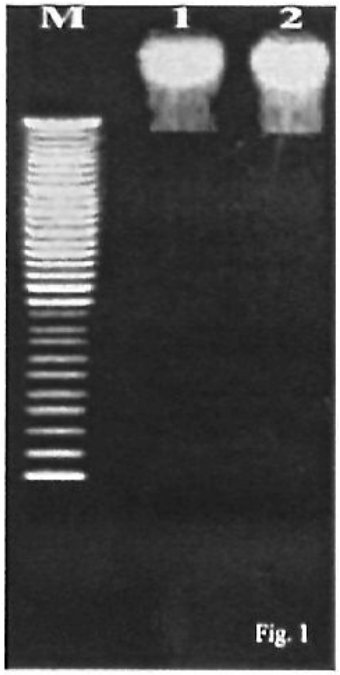


Fig. 1

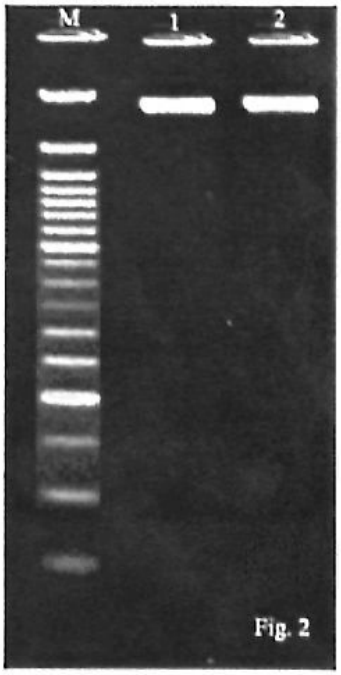
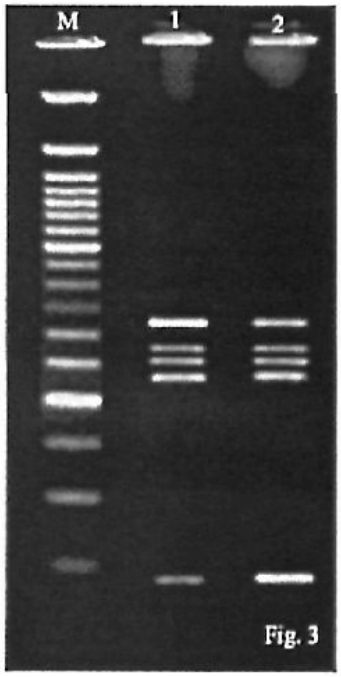


Fig. 2



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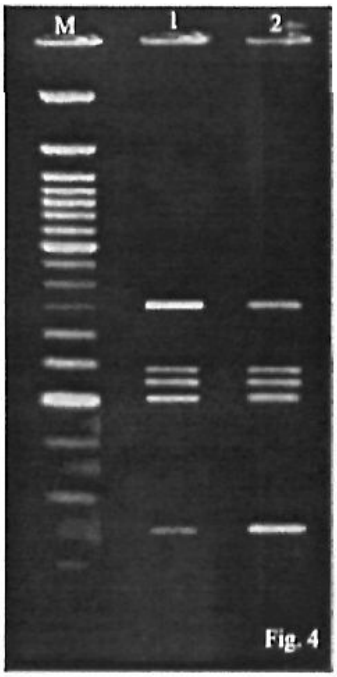

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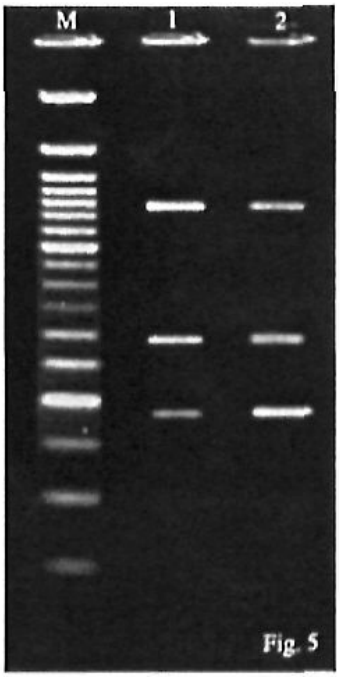
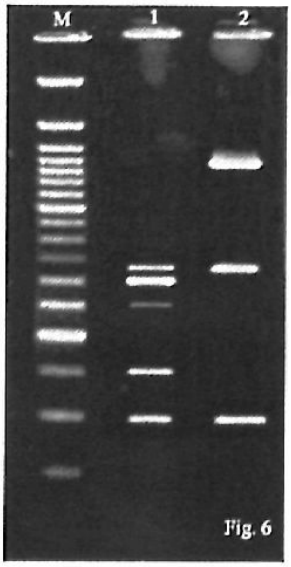


Fig. 5



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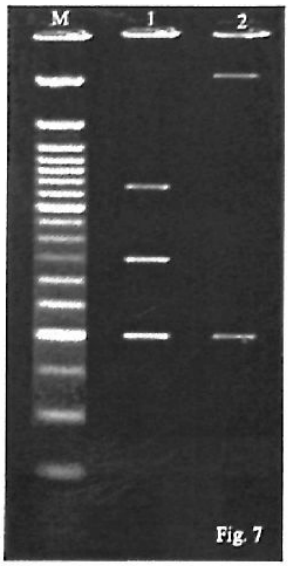

Fig. 7

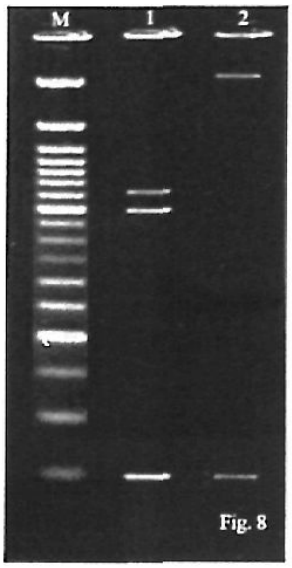

Fig. 8

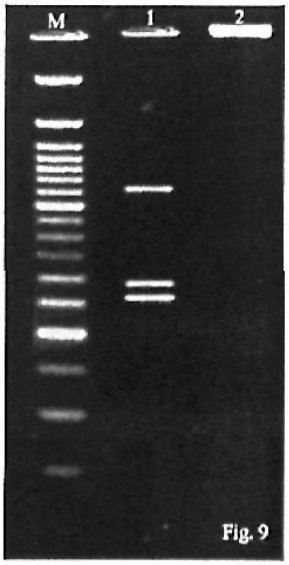

Fig. 9

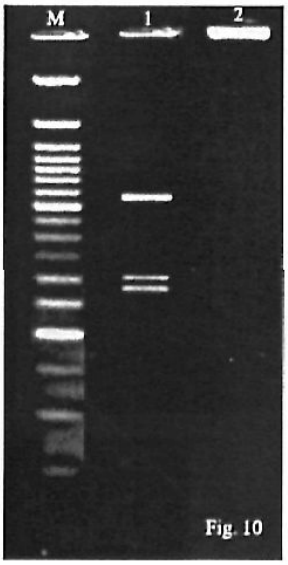

Fig. 10

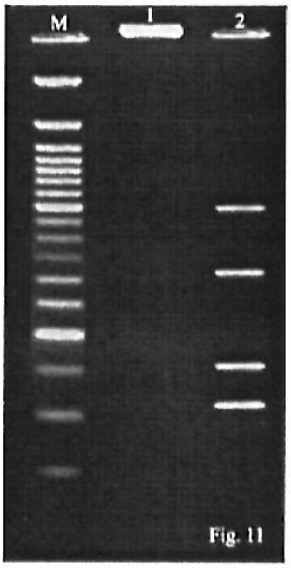

Fig. 11

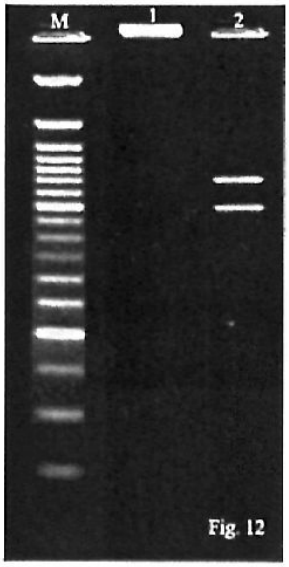

Fig. 12

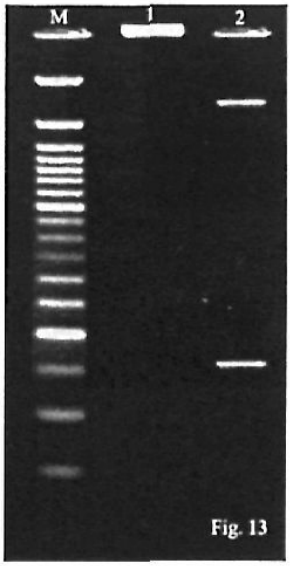

Fig. 13