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RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs) OF THE SMALL-SUBUNIT RIBOSOMAL DNA AS A TOOL FOR IDENTIFICATION OF *TILAPIA* SPP.

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Key words: Identification, *Tilapia*, nsrDNA, RFLPs, PCR, Polymorphism.

ABSTRACT

In Egypt, four species of *Tilapia* have been described based on morphometric, meristic and cytotaxonomical characteristics. These species are *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus*. The accurate identification of these fishes is complicated by the high variation in these characters, similarity among species and in some cases by the size of the fish. In this paper, we examined the use of polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) analysis of the nuclear small subunit ribosomal RNA gene (srDNA) for molecular identification of *Tilapia* spp. in Egypt. The present study aims to evaluate such advanced molecular biological approach for identification of *Tilapia* spp. Genomic DNA was extracted from the four species of *Tilapia*. About 2000 bp 18S ribosomal DNA was amplified by PCR using specific primers. The technique of restriction fragment length polymorphisms was used to identify the specific 18S rDNA for each species. *O. niloticus* rDNA RFLPs species-specific pattern is proved by digestion with restriction endonucleases *AlwNI* and *AvaI*. On the other hand, digestion of amplified 18S rDNA with endonuclease enzymes *SmaI*, *XmaI* and *SstII* produced species-specific patterns for *T. zillii, O. aureus* and *S. galilaeus* respectively. This indicates the efficiencies of these restriction endonuclease enzymes in species-specific identification of *Tilapia* spp. Restriction fragment length polymorphisms of the nuclear srDNA (RFLPs\PCR) proved to be a good tool for checking the relationships among species and their subspecies, even more than the morphological analysis.

INTRODUCTION

Tilapia is the common name for over than 70 species of perch-like fishes (family Cichlidae) native to the fresh waters of tropical Africa (Trewavas, 1983 and Stiassny, 1992). The term is derived from the African native Bechuana word "thiape," meaning fish . They were first bred thousands of years ago in ancient ponds and aquaria for the tables of Egyptian pharaohs (Naylor *et al.*, 2001). They include the mouth-brooding genera *Sarotherodon* [*Sarotherodon galilaeus* (Günthe,r 1862; Lortet, 1883 and Boulenger, 1899)] and *Oreochromis*, [*Oreochromis niloticus* (Linnaeus, 1757; Günther, 1864; Steindachner, 1864; Boulenger, 1898 and Trewavas, 1983) and *Oreochromis aureus* (Steindachner, 1864; Boulenger, 1899; Daget, 1954; Blache & Miton, 1960 and Trewavas, 1965)] and substrate spawning *Tilapia* [*Tilapia zilliii* (Smith, 1840; Gervais, 1848; Günther, 1859; 1862; 1864; Steindachner, 1870 and Boulenger, 1899)]. These fishes have been widely distributed in tropical and subtropical countries. Tilapia are one of the most economical cultured fished in Egypt and several other countries.

The morphological identification of *Tilapia* spp. is greatly complicated by the extensive intraspecific variation of the morphological characteristics used for classical identification (Albertson *et al.*, 1999). To overcome this obstacle, the use of molecular techniques as additional tools for the identification of these *Tilapia* spp. has been proposed.

Analysis of the PCR fragments. which are all approximately the same size, can either be based on restriction analysis (RFLP: restriction fragment length polymorphism) or on more sophisticated analytical procedures like DNA sequencing or on SSCP (single-stranded conformational polymorphism) or on all of the above. Analysis of PCR fragments by RFLP has been feasible for identifying Atlantic snappers (Chow *et al.*, 1993), tuna species (Chow and Inoue, 1993), tuna and bonito species (Ram *et al.*, 1996 and Quinteiro *et al.*, 1998) and flatfish species (Céspedes *et al.*, 1998). The advantages of this method are due to its simplicity (Quinteiro *et al.*, 1998).

The possibility of using sequence polymorphism in the small subunit ribosomal RNA gene (srDNA; 18S rRNA gene) of the DNA of the *Tilapia* spp. (four species) by means of PCR amplification and digestion with different restriction enzymes was investigated. This method was successfully employed before to construct the molecular

key for *Aedes* species (West *et al.*, 1997). It was also used to distinguish closely related parasitic worms and other different organisms (Wu *et al.*, 1999).

The aim of this study was to investigate the feasibility of DNA-based approaches in addressing problems of identification of the four species of *Tilapia sp.*, isolated from the River Nile by using RFLPs of the small subunit region of rDNA.

MATERIAL AND METHODS

Collection of Specimens: Live *Tilapia* fish samples were collected from El-Tawfiqi Stream (a branch of the Nile river) and transported to the laboratory in Faculty of Science- Zagazig University- Benha- Egypt. By using the morphometric analysis and the merestic analysis, the *Tilapia* spp. were laboriously differentiated into *Tilapia zillii (Tilapia), Oreochromis niloticus* and *Oreochromis aureus (Oreochromis)* and *Sarotherodon galilaeus (Sarotherodon).* The lives of the fish were terminated and liver pieces were stored in the freezer or in 95% ethanol until the DNA extraction started within one week.

Extraction of genomic DNA: Total DNA was extracted from the four species of the *Tilapia sp. (Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus)* 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 left to incubate at -20^0C for 24 to 72 hours. The nucleic acids 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 cheeked by 0.8% gel electrophoresis for the presence of DNA, as in Figure 1.

Determination and amplification of rDNA by polymerase chain reaction (PCR)—To amplify the complete nuclear srRNA gene, one µl of whole-cell DNA template was used plus oligonucleotide primers complementary either to the 5' and 3' ends of the gene (ssu1 and ssu2). The standard PCR reaction mixture was

used (Kessing *et al*., 1989). The entire nuclear srDNA was amplified using the primers SSU1 (5'-CGACTGGTTGATCCTGCCAGTAG-3') and SSU2 (3'-TCCTGATCCTTCTCAGGTTCAC-5') (Amresco) anchored respectively in the conserved extremities of the 18S ribosomal gene (Stohard & Rollinson, 1997).

The standard polymerase chain reaction program for amplification of nuclear srRNA was: 30-35 cycles; one minute, at 94^0C; two to three minutes, at 45^0C; and three minutes, at 72^0C.

Deoxynucleotide triphosphates (dNTP, dATP, dGTP, dTTP, and dCTP) were from Promega. The taq DNA polymerase used for the nuclear rDNA was 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 a 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 Nuclear rDNA-18S RFLPs Profiles: In the initial experiments the enzyme *Bgl1* and *EcoRI* (Amersham, Life Science) was evaluated for its ability to differentiate all *Tilapia* species. Additional enzymes were tested including *SacII, ApuI* and *AvaI* (Boehringer Mannheim) *SmaI, AlwNI, XmaI* and *SstII* (Sigma Co, USA). One microlitre (10-12 units) was used for each digestion reaction, together with 1.2 µl of the respective enzyme buffer for a final volume of 12.2 µl. The digestion was performed for ~3.5 h at ~37°C, and the digestion products were evaluated on 2% TBE-agarose (FMC Bioproducts) gels and stained with ethidium bromide. Bands were detected upon ultraviolet transillumination and photographed (35mm Kodak Film, England).

RESULTS

Both morphometric and meristic analysis differentiated the collected samples into four *Tilapia* species. Two species belonged to genus *Oreochromis: Oreochromis niloticus* and *Oreochromis aureus.*

The third specis was *Tilapia zillii* and the fourth was *Sarotherodon galilaeus*. Advanced molecular biological tools were applied in order to confirm the differentiation of these species taking into consideration the morphometric and meristic analysis. Total cellular DNA was extracted from liver of *Tilapia sp. (Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus)* and represented in Figure 1; lanes 1-4 represented *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* DNA genome, respectively. The sizes of the PCR products of the nuclear srDNA were ~2000 bp (Figure 2). The PCR product (the nuclear srDNA) of the *Tilapia* spp. was digested with *ApaI* restriction endonuclease producing the same fragment lengths (three bands; ~250, ~800, and ~950 bp) with the four species *T. zillii, O. niloticus, O. aureus* and *S. galilaeus*, respectively (Figure 3). Also, the small subunit ribosomal RNA gene of the four species when digested with *BglI* restriction endonuclease, gave two restriction patterns (~750 and ~1250 bp) (Figure 4). *EcoRI* endonuclease, when digested the nuclear srDNA, produced the same restriction fragment sizes with the four species of the *Tilapia sp.* (two bands; ~350 and ~1650 bp) (Figure 5). At the same time, the nuclear srDNA when digested with *ScaII* endonuclease produced three restriction fragments (~350, ~650 and ~1000 bp) with the four species of *Tilapia* spp. (Figure 6).

AlwNI and *AvaI* restriction endonucleases differentiated *Oreochromis niloticus* only when digested the nuclear srRNA gene of the four species. *AlwNI* restriction enzyme cut the nuclear srDNA of the species *O. niloticus* uniquely into two fragments (~300 and ~1700 bp) without digestion of the nuclear rDNA of the other three species (Figure 7). On the other hand, *AvaI* restriction endonuclease digested the nsrDNA of *O. niloticus* to six restriction fragments (~100, ~150, ~250, ~350, ~500 and ~650 bp) and the other strains (*T. zillii, O. aureus* and *S. galilaeus*) into five restriction patterns (~200, ~250, ~300, ~550 and ~700 bp) (Figure 8).

Tilapia zillii was digested uniquely by *SmaI* restriction endonuclease into two bands (~950 and ~1050 bp), whereas the other three species (*O. niloticus, O. aureus* and *S. galilaeus*) were not digested (Figure 9). *XmaI* restriction enzyme digested distinctively the nsrDNA of the *Oreochromis aureus* into two restriction fragments (~900 and ~1100 bp), but the nsrDNA of *T. zillii, O. niloticus* and *S.*

galilaeus were not digested at all with that restriction enzyme (Figure 10). Also, *Sarotherodon galilaeus* nsrDNA was digested uniquely by *SstII* restriction endonuclease into two restriction bands (~400 and ~1600 bp), while *T. zillii, O. niloticus* and *O. aureus* were digested into three restriction fragments (~350, ~600 and ~1050 bp) (Figure 11).

DISCUSSION

Species identification based on morphological criteria and protein analysis is 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 investigated. 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 and 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 *Tilapia* spp. and finding out if the four species monophylogenetic or polyphylogenetic species and discovering specific enzymes to identify individual subspecies.

ApaI, Bg1I, EcoRI and *ScaII* would differentiate nsrDNA sequences of *T. zillii, O. niloticus, O. aureus* and *S. galilaeus* into one group. This indicated that the four species of *Tilapia* may follow the same species, or monophylogenetic species. It has been found that every one of the four could be differentiated from the others by RFLPs applied by using specific endonucleases to digest nsrDNA PCR products. Two restriction enzymes (*A1wNI* and *AvaI*) indicated that *O. niloticus* may be polyphylogenetic when compared to the other three subspecies. There were three restriction endonucleases (*SmaI, XmaI* and *SstII*) that gave unique RFLPs for *T. zillii, O. aureus* and *S. galilaeus*, respectively, through digestion of nsrDNA. This showed that *Tilapia zillii, Oreochromis niloticus, Oreochromis*

aureus and *Sarotherodon galilaeus* may be different subspecies and have polyphylogenetic relationships.

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, claim that the RFLP option would be considerably simpler and faster (Ram *et al.*, 1996; Céspedes *et al.*, 1998 and 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 in consumables with respect to the sequencing option.

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Fig. 1: DNA genome from *Tilapia sp.*. Lane M is the 1 kb DNA ladder (Gibco/Gaithersburg). Lanes 1 – 4 represent the DNA genome of *Tilapia zillii, Oreochromis niloticus, Oreochromis auraeus* and *Sarotherodon galilaeus* respectively.

Fig. 2: Symbolized full-segment srDNA (~2000 bp) of *Tilapia sp.*. Lane M is the 1 kb DNA ladder (Gibco/Gaithersburg). Lanes 1 – 4 represent srDNA of *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* respectively.

Fig. 3: shows the representative RFLPs patterns from *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* with *ApaI* restriction endonuclease, which produced roughly the same fragments (three bands, ~250, ~800 and ~950 bp, for all).

Fig. 4: shows the representative RFLPs patterns from *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* with *BglI* restriction endonuclease, which produced roughly the same fragments (two bands, ~750 and ~1250 bp, for all).

Fig. 5: shows the representative RFLPs patterns from *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* with *EcoRI* restriction endonuclease, which produced roughly the same fragments (two bands, ~350 and ~1650 bp, for all).

Fig. 6: shows the representative RFLPs patterns from *Tilapia zillii, Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* with *ScaII* restriction endonuclease, which produced roughly the same fragments (three bands, ~350, ~650 and ~1000 bp, for all).

Fig. 7: shows *AlwNI* restriction enzyme digested the nsrDNA of *Oreochromis niloticus* uniquely into two different band sizes (~300 and ~1700 bp, lane 2) whereas *Tilapia zillii, Oreochromis aureus* and *Sarotherodon galilaeus* nuclear genes were not digested at all by this restriction enzyme (lanes 1, 3 and 4).

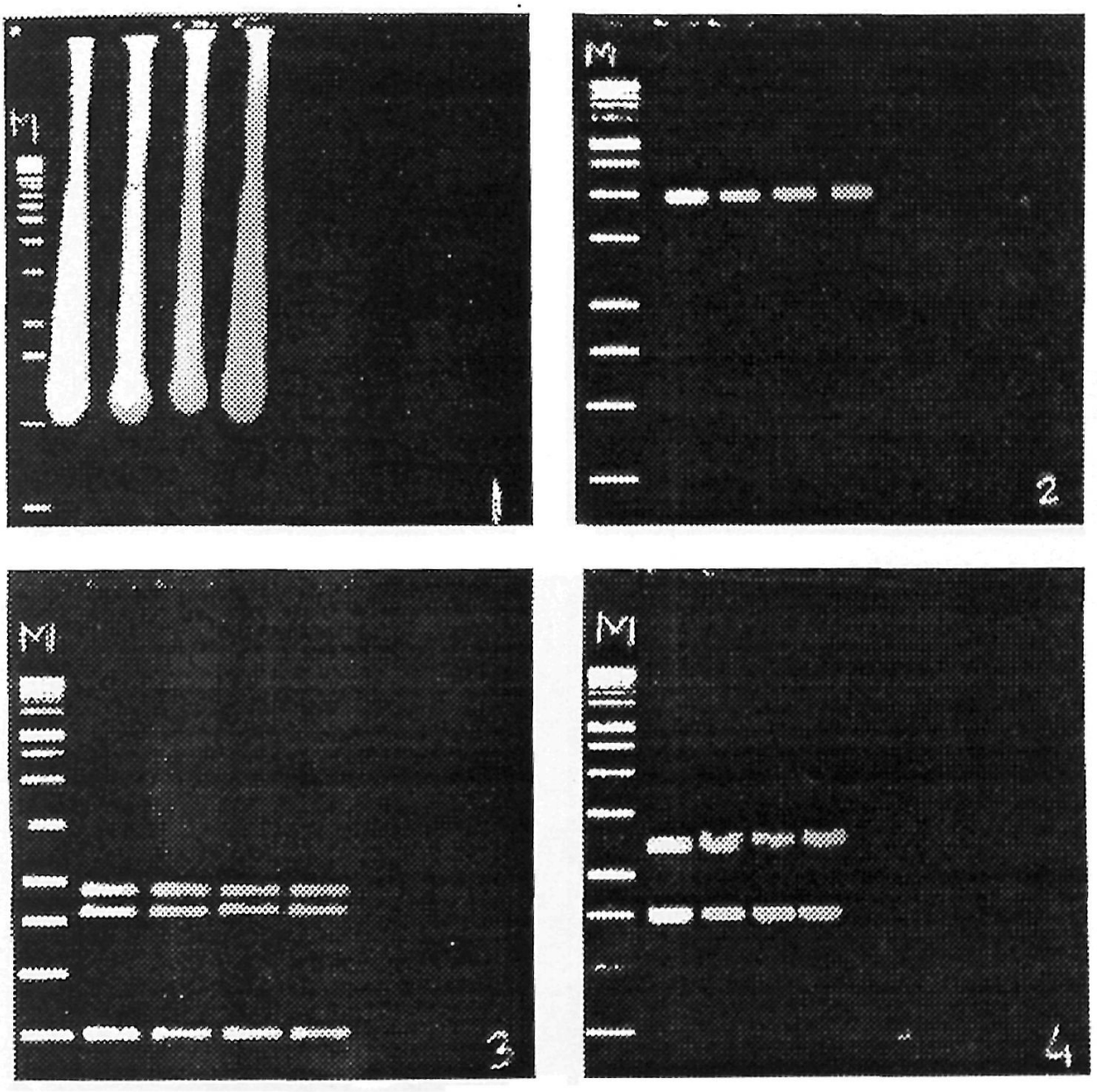
Fig. 8: shows *AvaI* restriction enzyme digested the nsrDNA of *Oreochromis niloticus* to six different band sizes (~100, ~150, ~250, ~350, ~500 and ~650 bp, lane 2) whereas *Tilapia zillii, Oreochromis aureus* and *Sarotherodon galilaeus* nuclear genes cut into five

restriction patterns (~200, ~250, ~300, ~550 and ~700 bp; lanes 1, 3 and 4).

Fig. 9: shows *SmaI* restriction enzyme digested the nsrDNA of *Tilapia zillii* uniquely into two different band sizes (~950 and ~1050 bp, lane 1) while *Oreochromis niloticus, Oreochromis aureus* and *Sarotherodon galilaeus* nuclear genes were not digested (lanes 2, 3 and 4).

Fig. 10: shows that *XmaI* restriction enzyme digested distinctively the nsrDNA of the *Oreochromis aureus* into two restriction fragments (~900 and ~1100 bp; lane 3), but the nsrDNAs of *T. zillii, O. niloticus* and *S. galilaeus* were not digested at all with that restriction enzyme (lanes 1, 2 and 4).

Fig. 11: shows that *Sarotherodon galilaeus* nsrDNA was digested uniquely by *SstII* restriction endonuclease into two restriction bands (~400 and ~1600 bp; lane 4), whereas *T. zillii, O. niloticus* and *O. aureus* nsrDNAs were digested into three restriction fragments (~350, ~600 and ~1050 bp; lanes 1, 2 and 3).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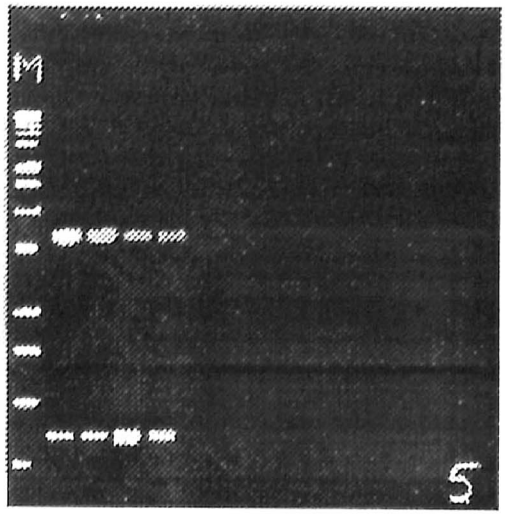
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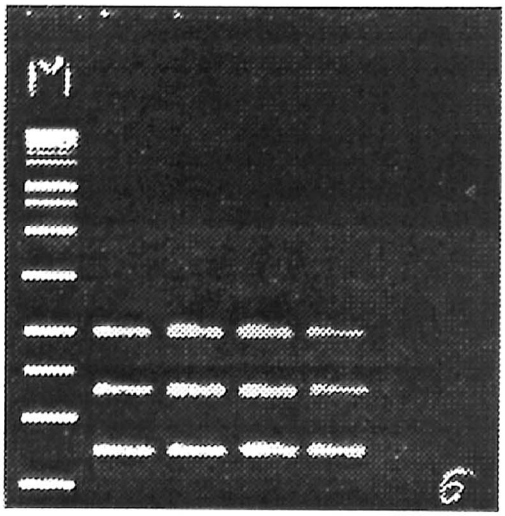
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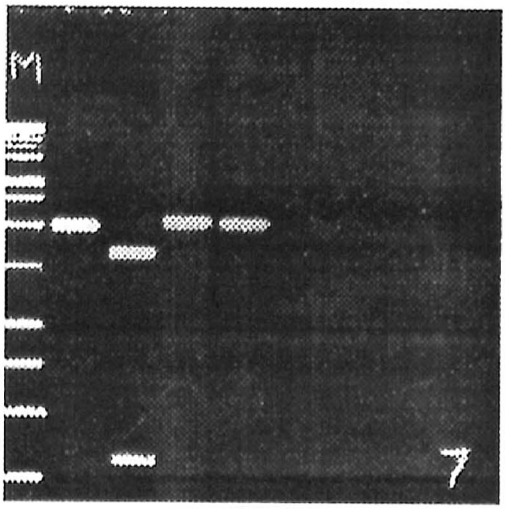
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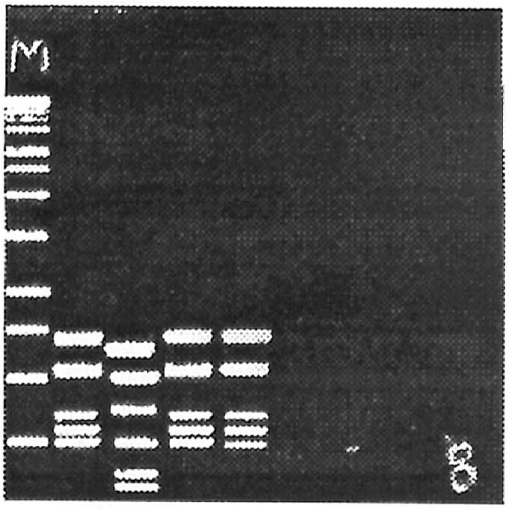
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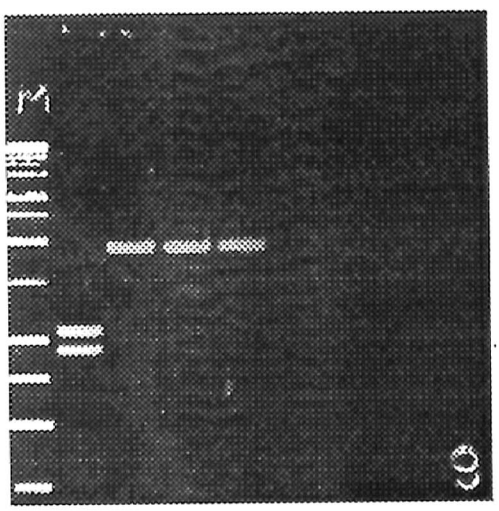
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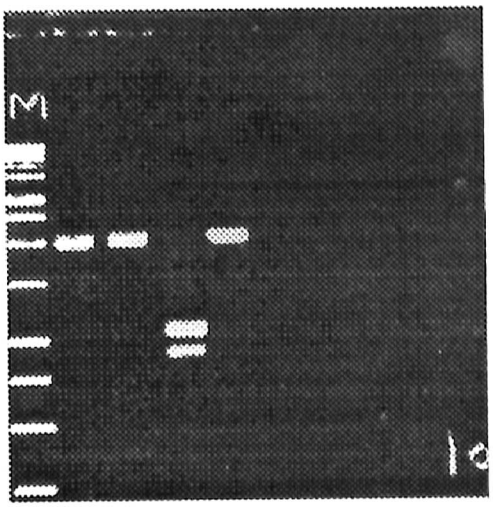
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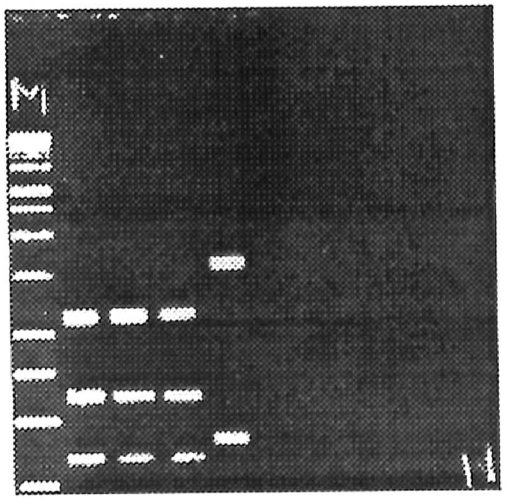
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11