# Species identification of *Anguilla* eels by polymerase chain reaction /restriction fragment length polymorphism analysis of the gonadotropin II-beta subunit gene

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## Abstract

Gonadotropin II beta-subunit gene (GTH II-β DNA) fragments of nine eel species of the genus *Anguilla* were amplified with specific oligonucleotides by polymerase chain reaction (PCR), and a single product was obtained from each species. In subsequent restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified GTH II-β DNA, the restriction endonuclease *Hpa II*, *Sph I* and *Nsi I* gave restriction patterns that differed among 3 species, e.g., *Anguilla japonica*, *A. bicolor bicolor* and *A. anguilla*, with other six species of anguillid eels, e.g., *A. rostrata*, *A. australis australis*, *A. reinhardti*, *A. celebesensis*, *A. marmorata*, and *A. bicolor pacifica*. Our results suggest that the PCR/RFLP analysis provides a rapid and convenient method to discriminate eel species.

**Key words:** genus *Anguilla*, gonadotropin II beta-subunit gene (GTH II-β), PCR, RFLP, restriction enzyme, *Hpa II*, *Sph I*, *Nsi I*.

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## Introduction

The life history of the catadromous eel, genus *Anguilla*, is very complicated and distinctive. The eel spawns in the deep tropical/subtropical ocean. Their leaf-like larvae, leptocephali, are transported and dispersed by ocean currents, and metamorphose into glass eels after several months of drifting. Glass eels become pigmented elvers in the estuary and grow in freshwater until silvering. Eels undergo a complex series of morphological and physiological changes as they metamorphose from freshwater yellow form to seagoing silver form in preparation for spawning migration (for reviews, see Tesch, 1977).

According to Ege (1939), Castle and Williamson (1974), and Watanabe (2001), anguillid eels were classified into 18 species and subspecies based on the skin color, morphometric characters, dentition, and vertebral counts: i.e., *A. japonica* Temminck and Schlegel 1846, *A.anguilla* (Linnaeus, 1758), *A. rostrata* (Le Sueur, 1817), *A. dieffenbachii* Gray, 1842, *A. reinhardti* Steindachner, 1867, *A. marmorata* Quoy and Gaimard, 1824, *A. celebesensis* Kaup, 1856, *A. borneensis* Popta, 1924, *A. mossambica* (Peters, 1852), *A. interioris* Whitley, 1938, *A. obscura* Günter, 1871, *A. bicolor bicolor* McClelland, 1844, *A. bicolor pacifica* Schmidt, 1928, *A. australis australis* Richardson, 1841, *A. australis schmidti* Phillips, 1925, *A. nebulosa labiata* Peters, 1852, and *A. nebulosa nebulosa* McClelland, 1844. Two are found in the northern Atlantic region and the rest in the Indo-Pacific zone.

Four species of Anguillid eels have ever been reported in Taiwan by morphometric index, e.g., *A. japonica* Temminck and Schlegel, *A. marmorata* Quoy and Gaimard, *A. bicolor pacifica* Schmidt, and *A. celebesensis* Kaup (Tzeng, 1982, 1983a, 1983b; Tzeng and Tabeta, 1983; Shen, 1997). *A. japonica* is the most abundant, which contributes to 93-99% of the total catch of elvers, followed by *A. marmorata* (1-7%) (Tzeng *et al*., 1995a). The other two species are very rare and are recognized only in elvers (Tzeng, 1982, Tzeng and Tabeta, 1983).

Japanese eel is a commercially important species for aquaculture in Taiwan. The elvers for aquaculture need are supplied totally by catch in the estuaries, as the

artificial cultivation of the eel larvae is still unsuccessful till now. Since the catch of Japanese eel elvers being insufficient to meet the demand of eel aquaculture, the elvers of exotic eel species, such as European eel (*A. anguilla*) and American eel (*A. rostrata*), were thus imported since 1969 and 1977, respectively (Li, 1997). We had found that the human-introduced exotic American eels have inhabited in the natural waters of Taiwan by DNA sequence identification of the mitochondrial cytochrome b gene (submitted).

However, since the external morphology of Japanese eel, European eel, and American eel is quite similar to each other, it is necessary to come up with a more sensitive and rapid method for eel species identification. In this paper, we set up the PCR/RFLP profiles of gonadotropin II beta-subunit gene of nine species of the genus *Anguilla*, which could be used to distinguish *A. japonica*, *A. anguilla*, *A. bicolor bicolor* from other six eel species.

## Materials and methods

Nine species of anguillid eels in the present study were collected from the Pacific, Indian and Atlantic Oceans (Table 1), either in fresh or ethanol-preserved form. The genomic DNAs of 31 individuals were extracted according to the method of Smith *et al.* (1987). Briefly, the ethanol-preserved muscle tissues were washed with L15 medium (GIBCO, BRL) for three times, and then incubated for 3 hours at 4℃. These procedures were omitted in fresh tissues. The tissues were lysed at 65℃ for 3 hours in a buffer containing 0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, 0.1 M NaCl, 1% SDS, and 0.32 mg/ml proteinase K. The DNAs were extracted by phenol/ chloroform standard protocol and precipitated with isopropanol. Genomic DNA pellets were then dissolved in 100 µl sterile water.

GTH II-β subunit genes of the 9 species of anguillid eels were amplified by using sense and antisense primers designed based on the GTH II-β subunit sequence of *A. anguilla* (Querat *et al*., 1990). Sense primer (5'-G<u>GAATTC</u>ATGTCAGTCTAT(C)C CAGAG(A)TGT(C)AC-3') is a 5' forward degenerated oligonucleotide comprising the initiated translation codon of GTH II-beta precursor, and anchors an *EcoR I* restriction site in the 5' end. Antisense primer (5'-GC<u>TCTAGA</u>CTCGAGTATG TGCACAATGTGATTGC-3') is a 3' reverse oligonucleotide located within 3' untranslated region of the gene, and anchors an *Xba I* restriction site in the 5' end (Fig. 1). The reaction mixtures for PCR contain 4 µl of dNTP mixtures (2.5 mM each), 5 µl of 1 µM sense primer, 5 µl of 1 µM antisense primer, 5 µl of 10x Ex Taq buffer, 2.5 U Ex Taq (TaKaRa, Japan, a high fidelity Taq DNA polymerase with 3'→5' exonuclease proofreading activity), sterile water added to 50 µl, and then overlay with mineral oil (Sigma). PCR reactions were carried out in a Perkin-Elmer DNA Thermal Cycler 480 using the cycle profile of 1 cycle of 95℃ for 1'45'', 30 cycles of 94℃ for 30 sec, 58 ℃ for 1min, and 72℃ for 1 min. Elongation was extended to 10 min at 72℃ after the last cycle. 2-5 µl PCR products were taken directly for restriction endonuclease digestion with the enzymes *Hpa II*, *Sph I*, and *Nsi I*. The restriction enzyme digested PCR products were then analyzed by 1.0 % agarose gel electrophoresis.

## Results

### GTH II-β gene of the eel

For the nine Anguillid eels, PCR amplification of the GTH II-β subunit gene fragment gave a single product from each species about 963-980 base pairs (Fig. 1, Table 2). The exon/intron organization of the GTH II-β subunit genes was defined by comparing with the cDNAs of the *A. anguilla* reported previously (Querat et al. 1990). The exon 1 encoded a signal peptide followed by the pre-half part of the mature GTH II-β subunit. One intron, started with the dinucleotide GT and ended with the dinucleotide AG, interrupted the exon 1 and 2. The exon 2 encoded the post-half part of the mature GTH II-β subunit followed by the 3' UTR. The sequences of mature GTH II-β subunit were 116 amino acids long, and were totally identical for the nine eel species.

### Patterns of RFLP of GTH II-beta gene

Digestion of the PCR products with the restriction endonuclease *Hpa II* generated three different RFLP patterns for he nine eel species studies (Fig. 2, Table 2). *Hpa II* cut the GTH II-β subunit DNA of *A. japonica* at the two sites, thereby generating DNA fragments of 643, 293, and 44 base pairs fragments (lane 3 in Fig. 3, Table 2). In contrast, the PCR product of the *A. bicolor bicolor* could not be cleaved by *Hpa II*, and remained intact (978 bp) after *Hpa II* digestion (lane 16 in Fig. 3, Table 2). The other seven species of anguillid eels were cleaved by *Hpa II* at only one site, generating DNA fragments of 687 and 176-293 bp (lane 4,7,8,11,12,15, and 19 in Fig. 3, Table 2).

Moreover, digestion of the PCR products with the restriction endonuclease *Sph I* or *Nsi I* generated two different RFLP patterns for the nine eel species (Fig. 2, Table 2). The GTH II-β subunit gene of *A. anguilla* possess a 15 base pairs deletion in 3' noncoding region, which contained the Sph I and Nsi I restriction site. Therefore, *Sph I* and *Nsi I* restriction enzymes could not cut the PCR products of the *A. anguilla* and remained intact DNA fragment (963 bp) under enzyme digestion (lane 3 and 15 in Fig. 4, Table 2). The 980 bp fragments of GTH II-β subunit DNA could be cleaved into 875-879, 101 bp (*Sph I* restriction), and 879-881, 99 bp (*Nsi I* restriction) in all other

eels. *A. anguilla* (lane 1,2,4–8,10,11, and 13-16 in Fig. 4, Table 2).

Combined analysis of restriction maps of PCR-amplified GTH II-β subunit gene fragments indicated that the three endonucleases *Hpa II*, *Sph I* and *Nsi I* are valuable for distinguishing *Anguilla japonica*, *A. bicolor bicolor*, and *A. anguilla* from other six species of anguillid eels (Fig.2, Table 2).

## Discussion

Traditionally, the eel species were usually identified by morphological characters, e.g. vertebral counts, skin mottle, long-finned/short-finned, and the patterns of vomerine teeth (Ege, 1939). However, the morphological markers are not quietly sufficient for systematic analysis, for these characters are usually overlapped with each other, and may be not significant in elver stage. Thus, eel identification needs to be supported by molecular characterization. DNA sequencing provides a tool for detailed and accurate identification of eel species, but it is expensive and time-consuming. PCR/RFLP analysis, on the other hand, constitutes a simpler and more rapid alternative. Aoyama *et al.* (2000) used PCR/RFLP analysis of the mitochondrial 16S rRNA domain to discriminate genus *Anguilla*. Zhang *et al.* (1999) also used this method with mitochondrial cytochrome b gene fragment for the discrimination of *A. japonica*, *A. marmorata*, and *A. anguilla*. However, high intraspecies polymorphism of mitochondrial DNA may result in the polymorphism of PCR/RFLP patterns. In this study, we amplified the highly conserved GTH II-β subunit gene fragments to reduce the frequency of intraspecies polymorphism, and thus increased the accurate rate of PCR/RFLP patterns. Among the individuals of the same species, only one nucleotide base difference was found in *A. marmorata*, but not effect the result of restriction digestion.

In this study, we found that PCR/RFLP analysis of GTH II-β subunit gene could be able to distinguish *A. japonica*, *A. bicolor bicolor*, and *A. anguilla* from the other six eel species, *A. rostrata*, *A. australis australis*, *A. reinhardti*, *A. celebesensis*, *A. marmorata* and *A. bicolor pacifica*. It can be used to screen the potential exotic *A. anguilla* and *A. rostrata* population in the natural waters of Taiwan. We have only exam the PCR/RFLP patterns of nine species of total 18 eel species and subspecies, and thus could not ruled out the possibility that some other eel species may have their specific patterns or have the same ones as indicated. However, since the other natural eel species of Taiwan are easily distinguished with Japanese eel, and those long-finned eels without marble are neither naturally existing nor commercially cultured in Taiwan, it is thus reliable to detect the exotic American and European eels using this method.

In conclusion, the PCR/RFLP analysis of GTH II-β subunit gene fragment with the restriction endonuclease *Hpa II*, *Sph I* and *Nsi I* are potentially of value for identification of *A. japonica*, *A. bicolor bicolor*, and *A. anguilla*. However, further studies of other eel species are needed for the validation of PCR/RFLP patterns of each eel species

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## Figure legend

**Fig. 1 Schematic structure of the GTH II-β subunit genomic DNA and deduced partial mRNA transcribed from the PCR products.** Arrowheads represent the primers. Broken lines represent the DNA fragments that are not amplified. Exon and intron are noted within the boxes.

**Fig. 2 A summary of PCR/RFLP analysis of GTH II-β subunit gene fragments in nine eel species.** Three different *Hpa II* and two different *Sph I* and *Nsi I* restriction patterns were detected among the nine PCR products of the anguillid eels. *A. japonica* possesses two *Hpa II* restriction sites and one *Sph I* and *Nsi I* restriction site. *A.anguilla* possesses one *Hpa II* restriction site but no *Sph I* / *Nsi I* restriction site. *A. bicolor bicolor* possesses one *Sph I* / *Nsi I* restriction site but no *Hpa II* restriction site. The other six eel species, *A. rostrata*, *A. australis australis*, *A. reinhardti*, *A. celebesensis*, *A. marmorata*, and *A. bicolor pacifica*, possess one *Hpa II* restriction site and one *Sph I* / *Nsi I* restriction site, respectively.

**Fig. 3 *Hpa II* RFLP map of the GTH II-β subunit gene of the nine eel species.** Three *Hpa II* restriction patterns were detected among the nine eel species; Lane 1: molecular weight marker; lane 2 and 3: *A. japonica*; lane 4 and 5: *A. rostrata*; lane 6 and 7: *A. anguilla*; lane 8 and 9: *A. australis australis*; lane 10 and 11: *A. reinhardti*; lane 12 and 13: *A. celebesensis*; lane 14 and 15: *A. marmorata*; lane 16 and 17: *A. bicolor bicolor*; lane 18 and 19: *A. bicolor pacifica*. The lane 3,4,7,8,11,12,15,16, and 19 were treated with *Hpa II* restriction enzyme, and the other lanes were untreated. Asterisks indicated different restriction patterns from that of the other seven eel species.

**Fig. 4 *Sph I* and *Nsi I* RFLP map of the GTH II-β subunit gene of the nine eel species.** Two restriction patterns were detected by *Sph I* and *Nsi I* among the nine eel species. The *Sph I* restriction patterns were shown from lane 1 to lane 8, with the order of *A. japonica, A. rostrata, A. anguilla, A. australis australis, A. reinhardti, A. celebesensis, A. marmorata,* and *A. bicolor bicolor*, respectively. The *Nsi I* restriction patterns were shown from lane 10 to lane 16, with the order of *A. japonica, A. rostrata, A. anguilla, A. australis australis, A. reinhardti, A. celebesensis,* and *A. bicolor bicolor*, respectively. *A. anguilla Sph I* and *Nsi I* restriction maps were marked with asterisks, which were different from that of the other eel species. Lane 9 is molecular weight marker.

# 以促性腺激素 II-β 基因片段之聚合酶連鎖反應/限制酶片段長度多形性分析法鑑別鰻魚種類

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## 摘要

將 9 種鰻魚之促性腺激素 II-β 基因片段以聚合酶連鎖反應放大後，每一種鰻魚皆可得到單一之 DNA 產物。利用限制酶 *Hpa II*, *Sph I* 以及 *Nsi I* 進行限制酶片段長度多形性分析，可得到日本鰻 (*A. japonica*)、雙色鰻 (*A. bicolor bicolor*)、以及歐洲鰻 (*A. anguilla*) 三種不同之限制酶圖譜，可與其他 6 種鰻魚—美洲鰻 *(A. rostrata)*、澳洲鰻 (*A. australis australis*)、東澳鰻 (*A. reinhardti*)、西里伯斯鰻 (*A. celebesensis*)、鱸鰻 (*A. marmorata*)、以及太平洋雙色鰻 (*A. bicolor pacifica*) 所得之相同限制酶圖譜區隔開。本結果提供一快速方便之聚合酶連鎖反應/ 限制酶片段長度多形性分析方法，用以鑑別鰻魚種類。

**關鍵詞**：鰻魚屬，促性腺激素 II-β 基因，聚合酶連鎖反應，限制酶片段長度多形性，限制酶，*Hpa II*，*Sph I*，*Nsi I*。

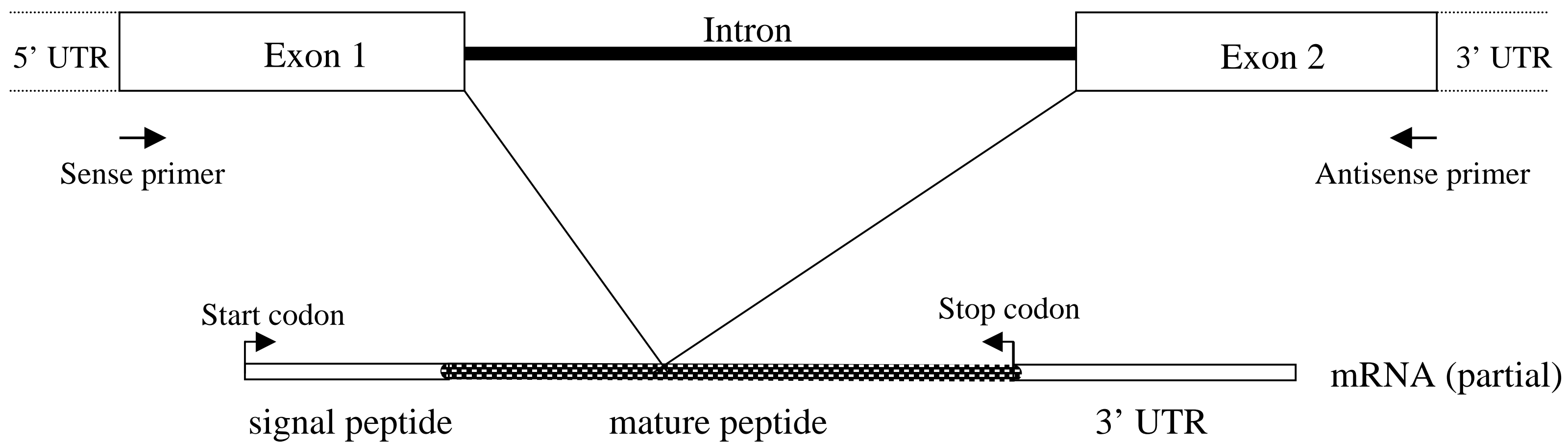



Fig. 1

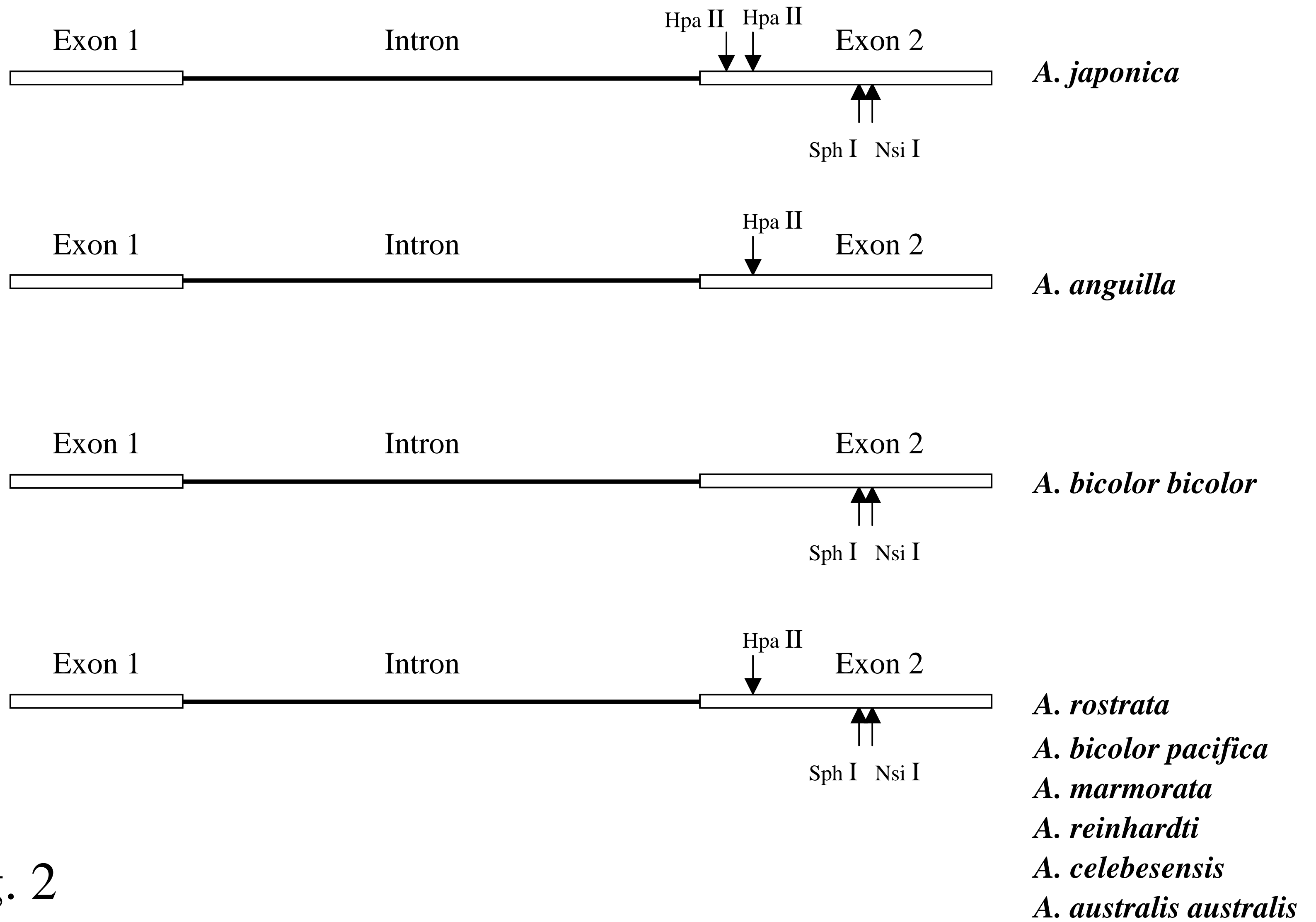
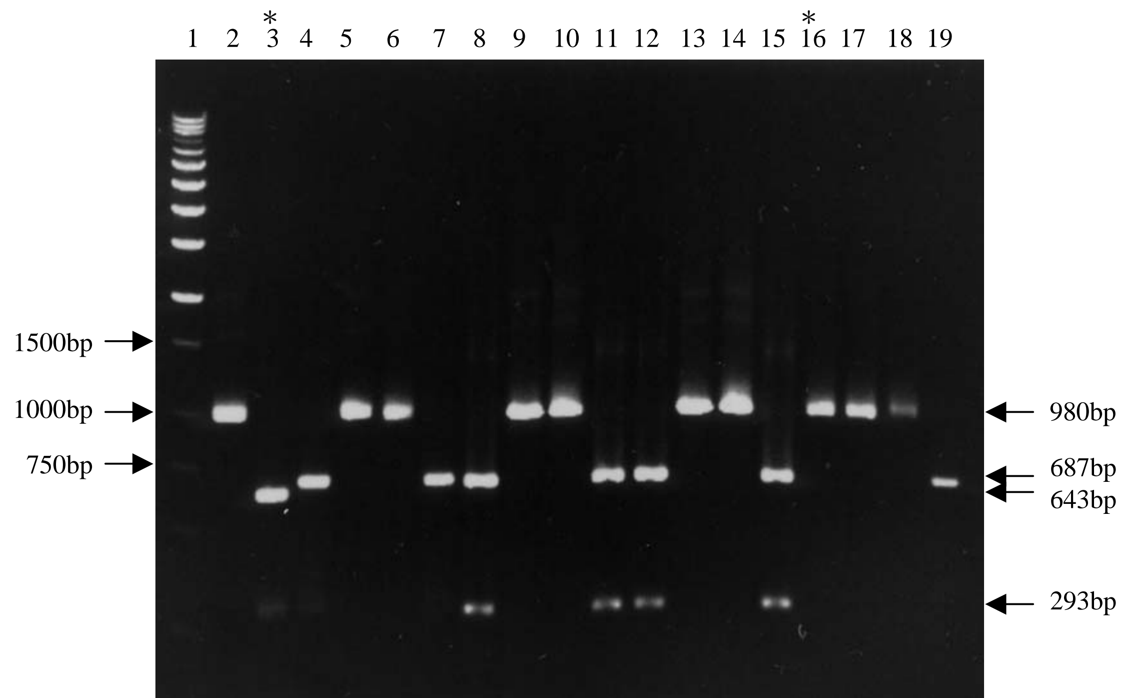



Fig. 2




# Fig. 3

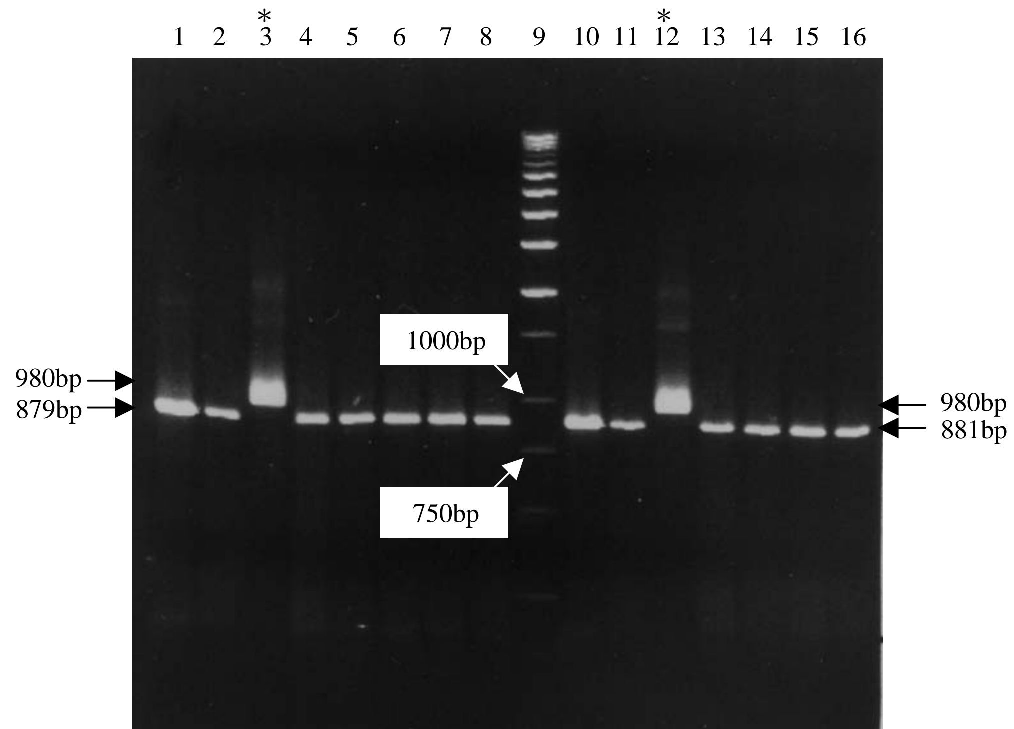



**Fig. 4**