MOLECULAR SYSTEMATICS OF MALARIA VECTORS :

STUDIES BASED ON RAPD PCR AND RELATED TECHNIQUES

THESIS SUBMITTED IN ACCORDANCE WITH THE

REQUIREMENTS OF THE UNIVERSITY OF LIVERPOOL

FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

BY

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OCTOBER 1997

VOLUME 2

ABSTRACT

This thesis describes a series of investigations of inter- and intra-specific DNA sequence variation in malaria vectors from Africa, the Middle East and Indian sub-continent. A variety of PCR-based techniques have been employed, particularly Random Amplified Polymorphic DNA (RAPD) PCR. With careful attention to the conditions of amplification, the technique was reliable and gave reproducible results.

With a total of four RAPD primers it was possible to differentiate and identify to species all six members of the *Anopheles gambiae* complex. Other primers revealed intra-specific variation in *An.gambiae* and *An.arabiensis* and may be useful in defining the population structure of these species. Phylogenetic trees based on RAPD data confirmed that *An.gambiae* and *An.arabiensis* are sister taxa. A cloned RAPD fragment from *An.bwambae* showed a high sequence homology with a cloned RAPD sequence from *An.culicifacies* and may include part of a ubiquitin gene. By testing parents and their progeny it was confirmed that the major RAPD bands are inherited in a dominant fashion.

Application of RAPD PCR to the two major species of the *An.culicifacies* complex showed that species A and B could readily be separated in samples from Iran, Pakistan, India and Sri Lanka. Some genetic differences were apparent between sub-populations of species B within Sri Lanka, but Indian species B appears to be genetically distinct from Sri Lankan species B. These differences reflect the vastly differing roles in malaria transmission of species B in the two countries. An Iranian population of mosquitoes identified morphologically to *An.culicifacies* s.l. proved to be comprised of two forms differing in their RAPD patterns. On sequencing the rDNA ITS2 region of these two forms, one was virtually identical in sequence to other species A populations while the other was highly divergent, (50% nucleotide divergence) suggesting that it may be a new species related to but outside the *An.culicifacies* complex.

An.fluviatilis, which is known to comprise three sibling species in India, is an important secondary vector of malaria in parts of India and Iran. Field collected specimens from Iran were examined by RAPD PCR, SSR PCR and sequencing of the rDNA ITS2 region. RAPD results suggested three populations but only two forms of ITS2 were seen, implying the presence of two distinct species within this taxon in Iran.

In India, *An.subpictus* comprises four sibling species of which two are known to occur in Sri Lanka. Samples from Sri Lanka examined by RAPD PCR fell into four population groups representing the four sample sites, one coastal and three inland. However, ITS2 sequence analysis was compatible with the existence of two groups corresponding to species A and B.

In contrast to many malaria vectors, *An.stephensi* is thought to be a single species, despite its enormous geographical range. RAPD readily distinguished seven laboratory colonies derived from collections in Iraq, Iran and India. Field samples from Iran and Tadjikistan were similar by RAPD but differed from one sample from Pakistan. Rural and urban collections from Iran differed in their RAPD patterns but it is unclear whether these two samples represent *mysorensis* and the type form. Tests with parents and their F1 progeny showed that RAPD could be used to determine paternity in this species, a feature that could be useful in evaluating experimental laboratory crosses.

Samples of the *An.maculipennis* complex from Iran and Russia were examined using RAPD. *An.maculipennis* s.s. from Iran could readily be distinguished from *An.sacharovi*. Furthermore, *An.beklemishevi* from Siberia could be distinguished from both northern and southern karyotypes of *An.messeae* from the same region. When the rDNA ITS2 region of the *An.maculipennis* complex samples from Iran was sequenced, three different types of sequence were obtained which, it is suspected, correspond to *An.atroparvus*, *An.messeae* and *An.sacharovi*.

This thesis demonstrates the power of RAPD PCR in elucidating the systematics of groups of insect vectors for which no prior DNA sequence data exist. It can serve equally to indicate genetic differences between populations or between individuals within populations. Once thus identified, it becomes possible to examine individuals or populations of particular interest using a battery of molecular techniques, including sequencing of various gene segments, particularly in rDNA or mtDNA, or carrying out RFLP or SSCP analysis. By such means the timeframe from initial recognition of genetically variant individuals or populations to resolving their taxonomic status can be radically shortened.

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CHAPTER SIX

PRELIMINARY EVIDENCE FROM DNA SEQUENCE DATA FOR TWO SIBLING SPECIES OF *ANOPHELES FLUVIATILIS IN IRAN*

6.1 ABSTRACT

An. fluviatilis James 1902 is one of the most efficient vectors of malaria particularly in the hills and foothills of India, Iran, Nepal, Pakistan. It also occurs extensively in the oriental region and parts of the West Asian subregion. On the basis of variations in density, feeding behaviour, and malaria infection rates, by several workers have postulated the existence of distinct biological forms and based on chromosomal differences it has been suggested that in India *An. fluviatilis* comprises at least three sibling species.

We have examined field collected specimens of *An. fluviatilis* from sites in Southeastern and South Iran, including the provinces of Sistan and Baluchistan, Hormozgan, the tropical areas of Kerman province and Fars. Random Amplified Polymorphic DNA (RAPD) revealed a number of population specific PCR products as well as some variation within populations.

Differences between specimens from coastal and hill areas were further investigated by sequence analysis of the ITS-2 region. There were no consistent differences between hill and coastal specimens. ITS-2 being 375bp long in all

individuals examined and most had complete identity at all nucleotide positions. However, one coastal specimen from Minab, Hormozgan province differed from others at seven bases. On the basis of the extent of sequence divergence and RAPD variation, we postulate that the forms from these two different groups represent two sibling species of *An. fluviatilis*.

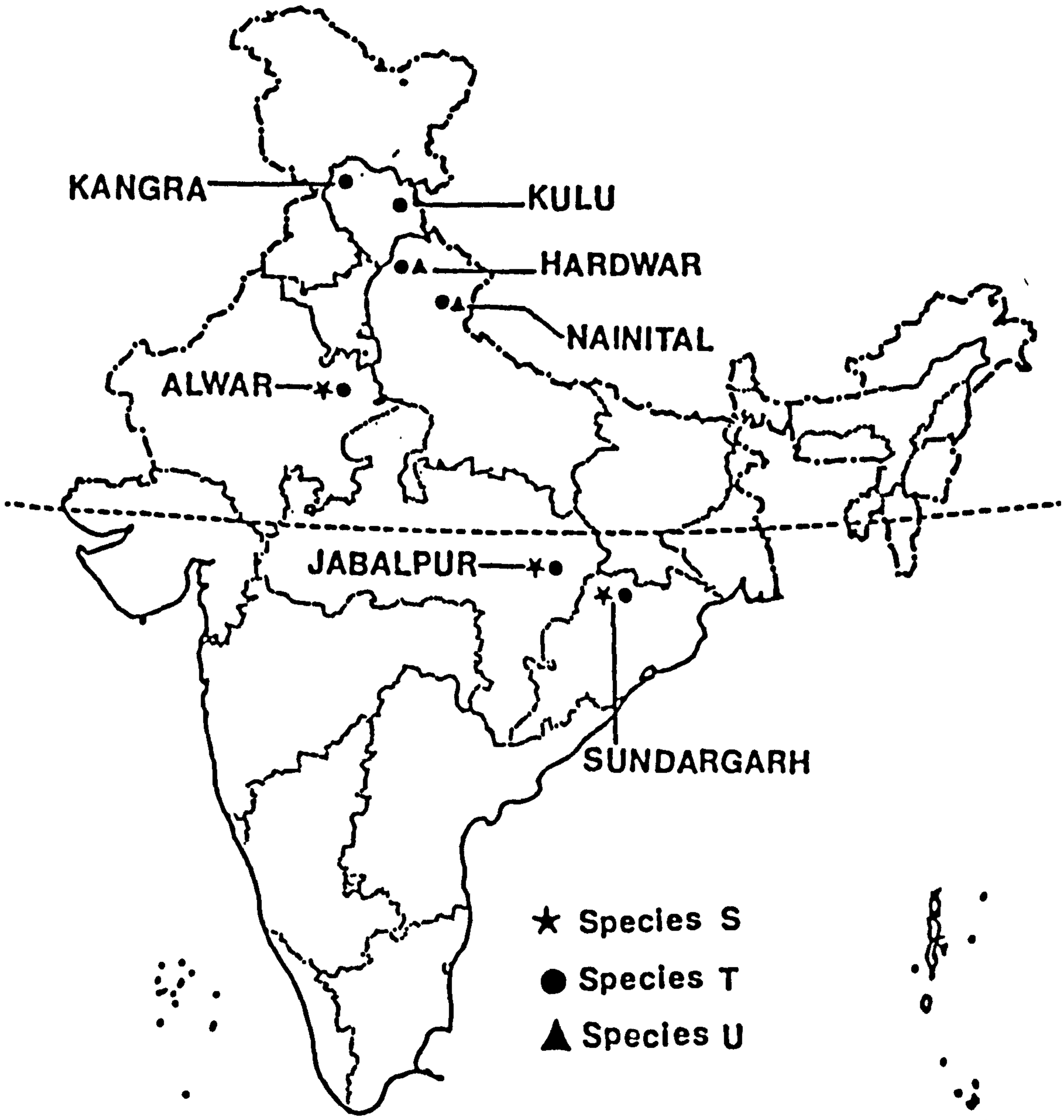
6.2 INTRODUCTION

An. fluviatilis James 1902, of the Myzomia series is one of the most efficient vectors of malaria particularly in the hills and foothills of India, Iran, Nepal, and Pakistan, although its role in malaria transmission varies from place to place (Subarao *et al.*, 1994). This species is widely distributed in the oriental regions and parts of the West Asian subregion, Afghanistan, East and South Arabia, Iran, Iraq, Kazakh, Oman, and Pakistan. In the oriental region it occurs in South China, India, Indochina, Myanmar, Nepal, Taiwan, and Thailand (Nagpal & Sharma 1995).

On the basis of variations in density, feeding behaviour, and malaria infection rates, Rao (1984) suggested the existence of two distinct biological forms. Recent studies of ovarian polytene chromosomes from nurse cells has provided evidence that *An. fluviatilis* is a complex of three reproductively isolated taxa that constitute good species (Subbarao *et al.* 1994). These have been provisionally designated as species S, T,and U, with species T and U also S and T being sympatric in the study villages in India (Sharma *et al.* 1995) (Fig. 6.1). There are also four morphological variations based on maxillary palpi and four in wings.

Adult *An. fluviatilis* usually rest in human dwellings and to a lesser extent in cattle sheds during the day. The species also rests outdoors and has been collected from an altitude of 1,060 to 3,340m in the Gum valley of Nepal (Nagpal & Sharma, 1995).

Fig. 6.1- Distribution of An. fluviatilis sibling species in India (CTD/MAL/96.1)



In studies in Nepal, *An. fluviatilis* showed a Preference for breeding places with a perceptible flow of water such as field and irrigation channels, slow-moving streams, seepage channels of dams and silted grassy margins of canals (Shukla *et al.* 1995). Breeding has also been recorded in shallow wells, water reservoirs tanks, rice fields along the foothills or undulating terrains, swamps, borrow pits during monsoon period when streams and channels are flushed by heavy rains.

Its peak biting activity occurs between 20.00 - 24.00 hrs, but there may be variation depending on season and location (Nagpal & Sharma, 1995). A wide variation in the feeding preference of *An. fluviatilis s.l.*, with an anthropophilic index ranging from 0 to 97%, has been reported (Rao 1984; Nanda *et al.* 1996).

However, studies on blood meal analysis of chromosomally identified specimens from Uttar Pradesh, India showed that despite high densities of *An. fluviatilis*, malaria incidence was very low (0.002%), and both species T and U were exclusively zoophilic (Sharma *et al.* 1995). These results are supported by bait catches which indicated no preference of *An. fluviatilis s.l.* for man, although the man:cattle ratio was high. Also in previous study, using IRMA, one out of eight specimens of *An. fluviatilis* was positive for both *P. vivax* and *P. falciparum* (Subbarao *et al.* 1992).

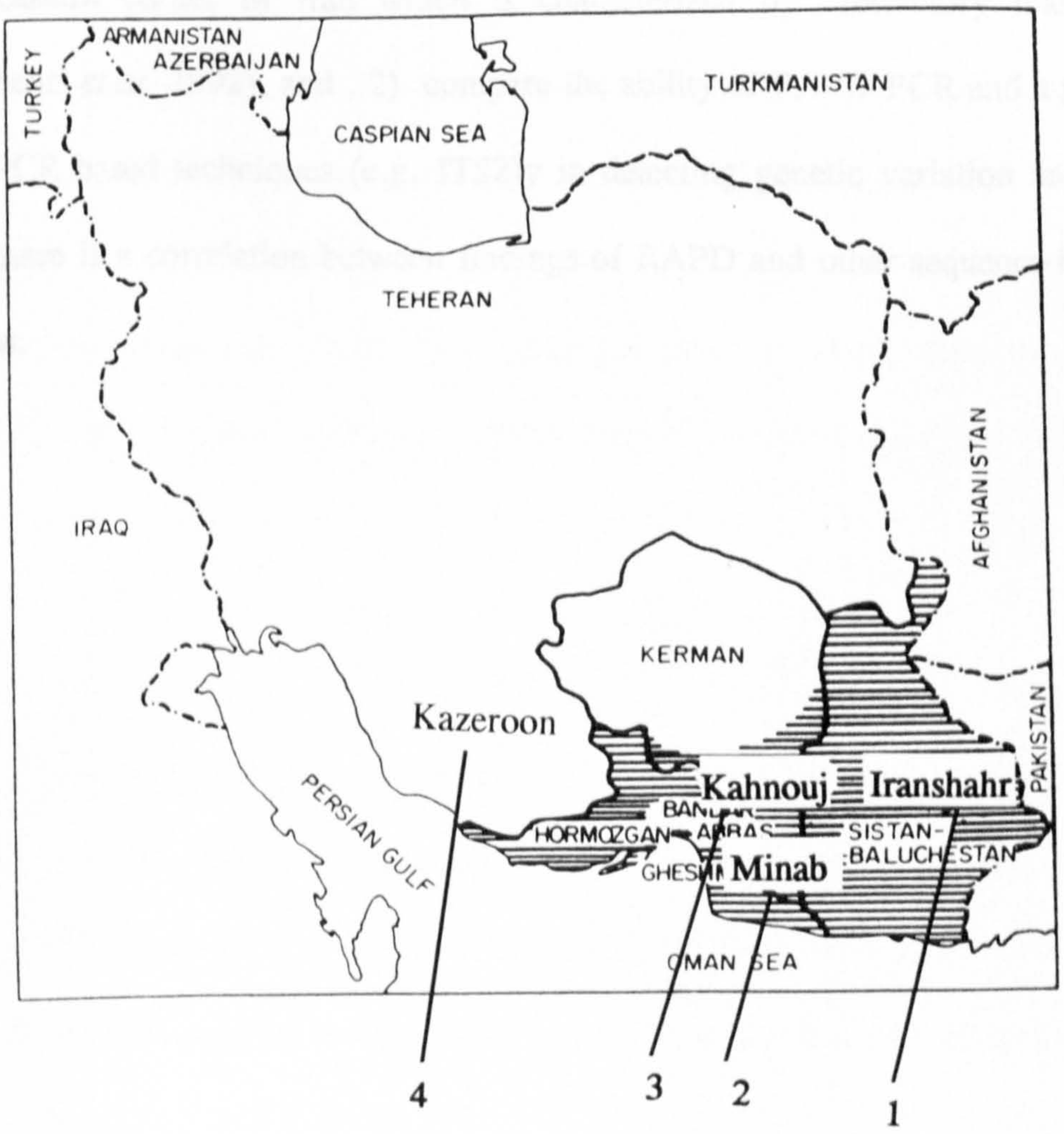
In Iran, *An. fluviatilis* has not been incriminated as a malaria vector since 1957, but the persistence of the disease even after indoor insecticide application

suggested that further field studies on this species are needed. In 1982 for the first time during dissection, *An. fluviatilis* infected with sporozoite have been found in Behbahan (Fars province) and subsequently more infected adults in Hormozgan and Kerman Provinces.

These results suggested that *An. fluviatilis* as an exophilic vector has an important role in maintaining malaria transmission specially in the foothills (Zaim and Javadian, personal communication). Recently, Kamyabi *et al.* (1996) was working on malaria vectors in Kahnouj district, reported that out of 2761 female anopheles trapped in the bednet with animal bait, *An.fluviatilis* comprised 86% of collections and out of 44 female anopheles collected in bednet trap with human bait *An.fluviatilis* comprised 95.4% of collections.

An. fluviatilis is widespread in southern Iran, and distributed from east to west in Bluchestan, Kerman, Fars, Khozestan, and Kermanshahan Provinces, in all southern area of the Zagros Range of Mountains which contain hills and foothills, and its distribution extends to coastal areas in the Persian Gulf (Fig. 6.2). The species has been collected at altitudes ranging from 50 m above sea level in Borazjan and Minab, up to 1100 m in Kazeroon. However, the highest altitude at which biting has been reported is from hilly areas. These areas have warm summers and temperate winters, and *An. fluviatilis* is known to be a vector of stable malaria.

Fig. 6.2- Four collection sites of *An. fluviatilis* in Iran:
Iranshahr (1), Minab (2), Kahnouj (3), Kazeroon (4).



Aim

The main objectives of this study were, 1) to determine inter and intra-specific variation within populations of *An. fluviatilis* as a secondary and less studied species in Southeastern corner of Iran which is characterized by "refractory malaria" (Manouchehri *et al.* 1992), and , 2) compare the ability of RAPD-PCR and a gene-specific PCR based techniques (e.g. ITS2)y in detecting genetic variation and, 3) whether there is a correlation between findings of RAPD and other sequence-based techniques.

6.3 MATERIALS AND METHODS

6.3.1 Mosquitoes

Dried field specimens of *An. fluviatilis* were collected from Kazeroon in Fars, Minab in Hormozgan, Kahnouj in Kerman, and Iranshahr in Sistan and Baluchistan province in the south, southeast, and east of Iran (Fig.1) by staff of Tehran University of Medical Sciences during 1994-95. Immediately after collection the specimens were dried and stored over silica gel prior to DNA extraction. An additional pinned redundant specimen collected from Minab (Cheloo) in 1984 by Yaghubi was also used.

6.3.2 Genomic DNA extraction

The following extraction methods were used; phenol extraction (Ballinger-Crabtree, 1992), the method of Collins *et al.* (1987) and Mini -Prep (Medina-Acosta & Cross, 1993). Air-dried pellets have been resuspended in 100 ul of double distilled H_2O or TE buffer and stored at 4°C.

6.3.3 Primers

10 RAPD primers from the UBC series (University of British Columbia, Canada) were used. These have been found useful in our laboratory in studying other anopheline species complexes. ITS2- PCR primers were based on a modification of published nucleotide sequence of *Cx. tritaeniorhynchus* 5.8S rDNA (Shimada &

Sasaki 1991) and *An. hermsi* 28S rDNA (Porter & Collins 1991). Table 6.1 lists the names, sequences and GC content of the primers for which results are presented.

6.3.4 DNA amplification and PCR product analysis

All RAPD reactions were performed in a total volume of 25 μl. Each mixture contained 2.5 ul of 10X reaction buffer, 2 mM $MgCl_2$, 10-100 ng of primer, 0.001% gelatin, 0.1 mM each of dATP, dTTP, dCTP, and dGTP, 0.5 unit of Promega Taq polymerase, and sterile double-distilled water to 25 ul. Reactions were overlaid with 50 ul of mineral oil and

amplified in a Hybaid Omnigene thermal cycler.

The RAPD programme : (1) 94°C for 5 min; (2) 15 cycles of 94°C for 30 sec, 36°C for 30 sec, 72°C for 30 sec; (3) 20 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec; (4) 72°C for 7 min (Prog 6). ITS2 PCR amplification was performed using the following program:(1) 94°C for 5 min; (2) 30 cycles of 94°C for 30 sec, 50°C for 1 min, and extension at 72°C for 30 sec (Prog 10). Following PCR, 10 ul of amplified DNA mixed with Ficol/orange G loading buffer was subjected to electrophoresis in 6% polyacrylamide gel or 1% agarose (Appligen) in TBE buffer containing ethidium bromide at 1.5 v/cm for 16 hours (large gel) or 5 v/cm for two hours (mini gel). A mixture of BglI and HinfII digest of pBR328 (Boehringer Mannheim) was used as molecular weight markers for determination of PCR product size (molecular weights 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234,

Table 6.1 - selected primers and their sequences

primer	5....sequence ....3'	GC%
M13	GTAAAACGACGGCCAGT	53
UBC-304	AGTCCTCGCC	70
UBC-306	GTCCTCGTAG	60
B13	TTCCCCCGCT	70
X19	(AGT)5GCCA	42
B3	CATCCCCCTG	70
A2	TGCCGAGCTG	70
33C1R	ATGAAACACCACGCTCTCGG	55
33C1L	TTGCGCAACAAAAGCCCACG	55
ITS2 primers:		
5.8S	ATCACTCGGCTCGTGATCG	57.8
28S	ATGCTTAAATTTAGGGGGTAGTC	39.1

234, 220, 154, 154 bp). Gels visualised were under UV light and photographed with black and white Polaroid 55 film (Hargreaves photo Ltd) or Ilford FP4 film.

6.3.5 Data analysis

The computer programs GCG (version 7.0), DNA-STAR, and Clustal V (Higgins & Sharp 1988) have been used to enter and analyze the sequences and carry out parsimony alignments.

6.4. RESULT AND DISCUSSION

6.4.1 RAPD-PCR

UBC-306

RAPD primer UBC 306 readily differentiates *An. fluviatilis* populations by three distinct patterns; two products of 1105, 720bp in Iranshahr, three of 720, 460, 430bp in Minab, and four major products of 1200, 1105, 720, 500bp in Kahnouj samples (Fig. 6.3). The 720bp fragment was consistently present in all samples of the three populations examined by this primer. Iranshahr and Kahnouj specimens shared an additional two bands; 1105 and 720bp, while individuals from Minab have another two uncommon (460 and 430bp) band with others.

UBC-304

Another RAPD primer, UBC 304 also produced a series of distinct bands specific for each population, but with a single common band (680bp) in all specimens, a shared band (475bp) between Iranshahr and Kahnouj, and a 365bp shared between Iranshahr and Minab populations (Fig. 6.4).

M13F

M13F primer produced a main 420bp common in three populations, although its intensity increase from Iranshahr to Kahnouj and Minab. A 255bp band is specific to Minab specimens while the Kahnouj and Iranshahr patterns are almost identical (Fig. 6.5).

Fig. 6.3- RAPD primer UBC-306 products in *An. fluviatilis* populations from Iran: Iranshahr (1-3), Minab (4-6), Kahnouj (8-10); marker (7); -ve (11). This primer readily differentiates three populations by specific patterns. however, the 720 bp fragment is common in all populations examined.

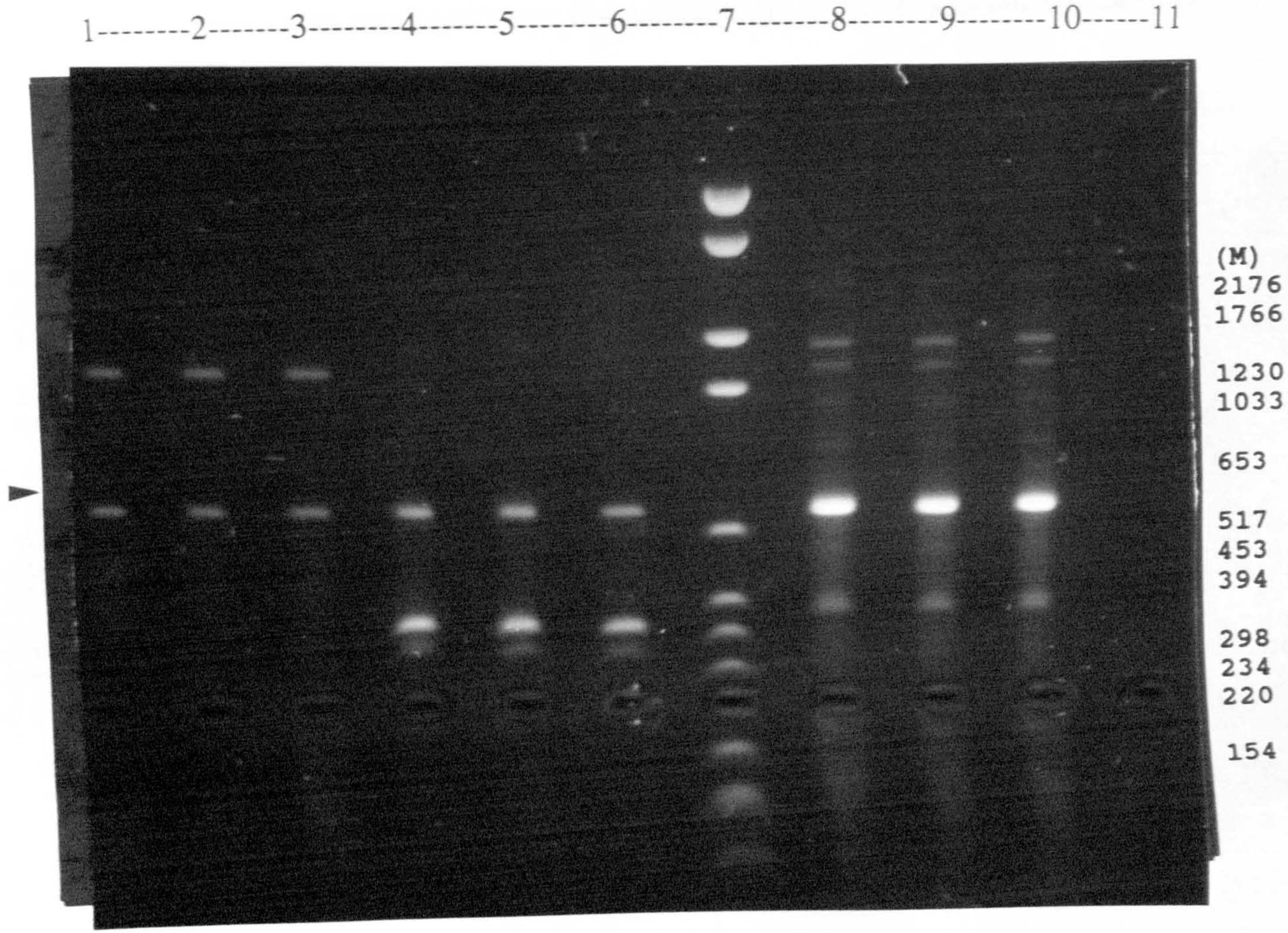


Fig. 6.4- RAPD primer UBC-304 products in *An. fluviatilis* populations from Iran: Minab (1-2), Iranshahr (4-8), Kahnouj (9-11); marker (3). This primer also produced three distinct patterns in these populations and a 680 bp common band. examined.

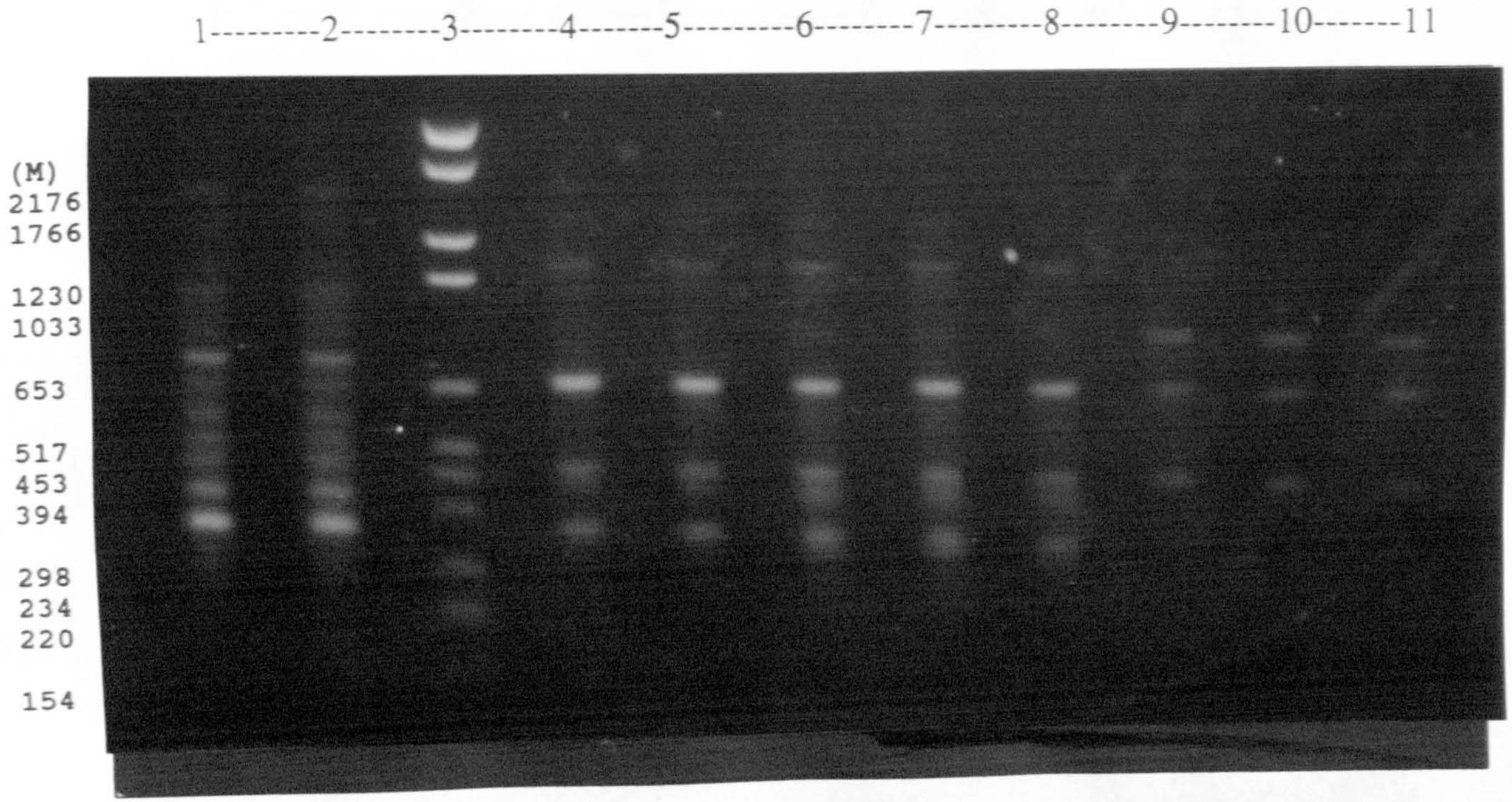
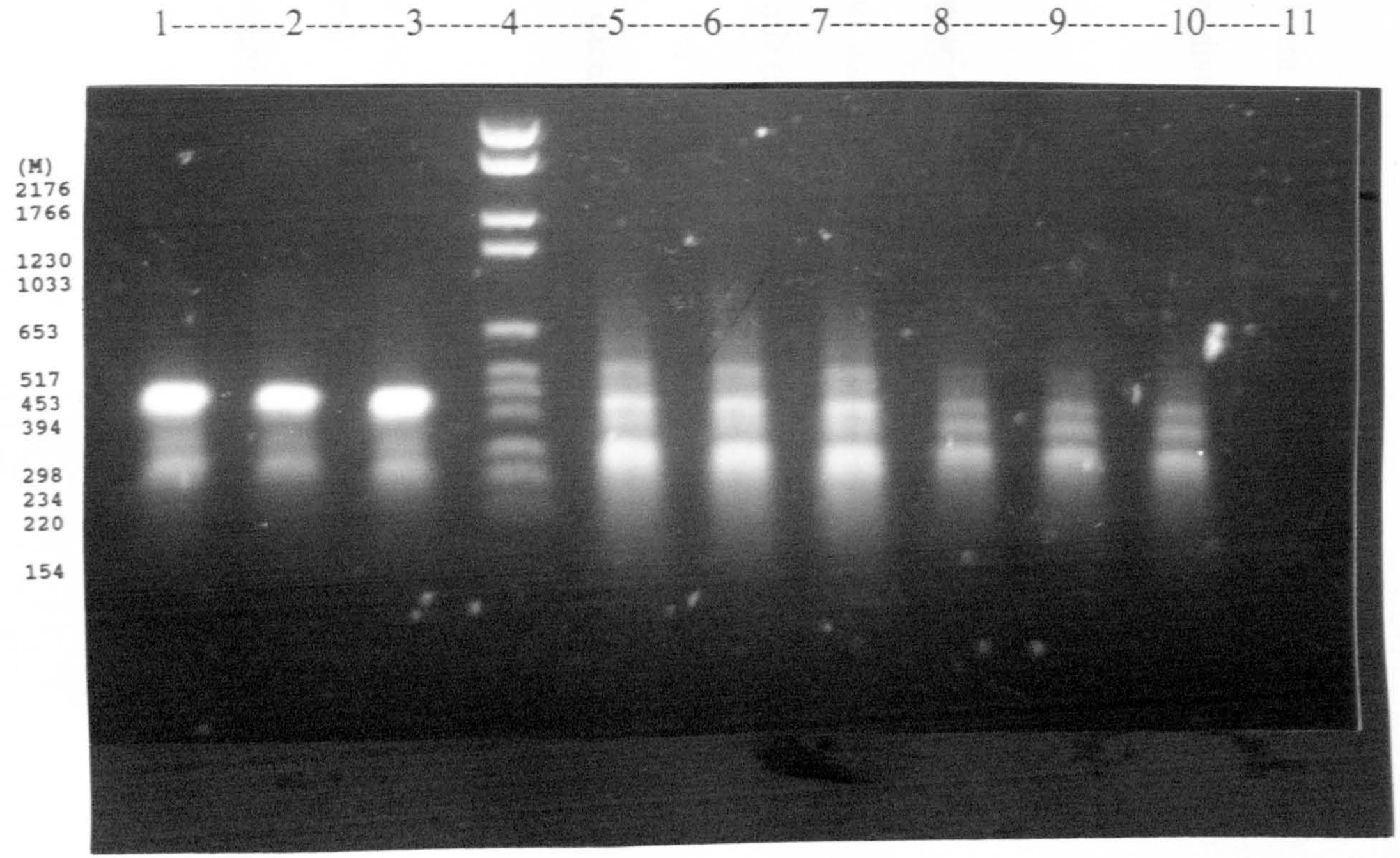


Fig. 6.5- RAPD primer M13 products in *An. fluviatilis* populations from Iran: Minab (1-3), Kahnouj (5-7), Iranshahr (8-10); marker (4); -ve (11)



B13

Primer B13 produced a 155bp species-specific band, although the fourth population from Kazeroon which showed variation within its specimens seems more similar to Minab (Fig. 6.6).

UBC-301

Primer UBC 301 has been found useful for looking for variations within populations, as it produced two distinct patterns in specimens collected from Minab (Fig. 6.7).

However, although these primers differentiated *An. fluviatilis* populations with specific markers, Iranshahr and Kahnouj specimens that were collected from hilly areas showed more common bands than Minab populations which were from a coastal area. In fact it seems that the first two populations have the same origin while they were collected some 200 km each other. Kazeroon specimens' RAPD pattern is between these two groups.

Based on RAPD patterns, it seems that the Iranshahr population is more homogeneous than those from Kahnouj, Kazeroon and Minab which showed more variation within populations with different primers.

Fig. 6.6- RAPD primer B13 products in *An. fluviatilis* populations from Iran: Kazeroon (1-4), Minab (6-7), Iranshahr (8-9), Kahnouj (10-11); marker (5)

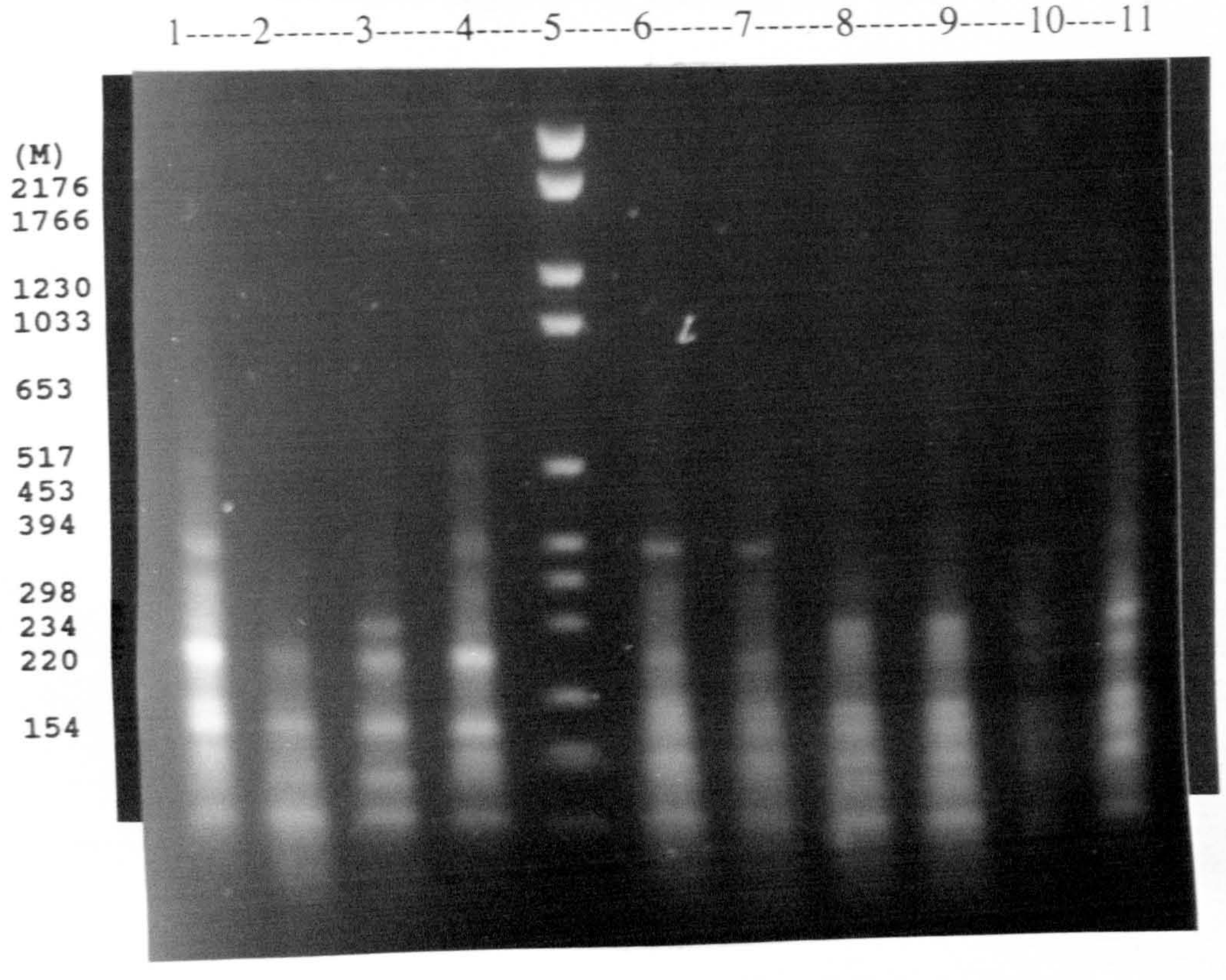
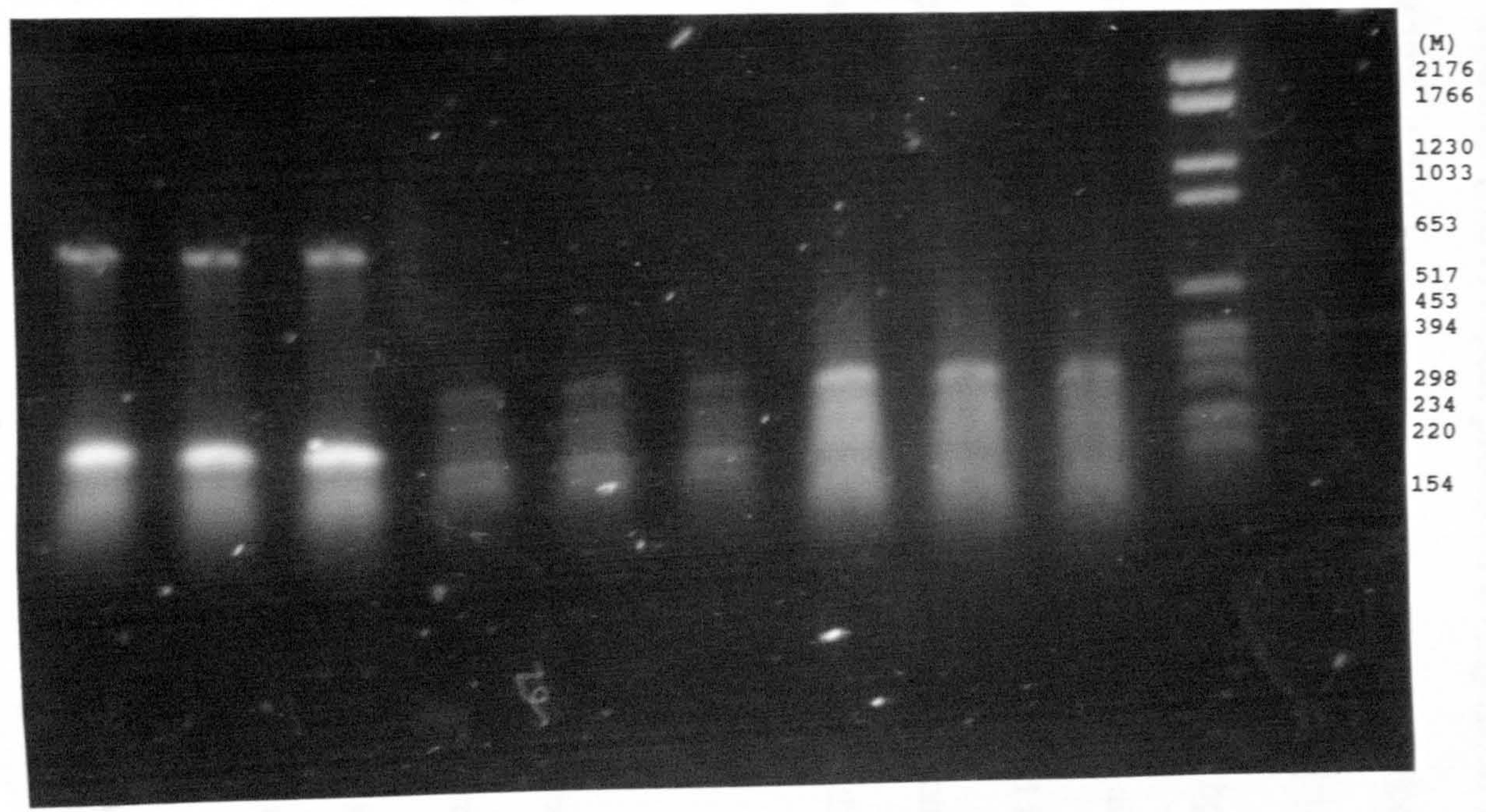


Fig. 6.7- RAPD primer UBC-301 products in *An. fluviatilis* populations from Iran: all Minab population; marker (10); -ve (11)



6.4.2 SSR-PCR

X19

As a random primer, a 3'- anchored (AGT) repeat produced a fingerprint of 8 bands, but only two of these (630 and 310 bp) were common in all populations (Fig. 6.8). Iranshahr specimens showed more similarity with the other groups, by sharing 3 (630, 530, 310bp) bands with Minab and 4 bands (1010, 630, 510, 310 bp) with Kahnouj populations. Population-specific bands revealed by this SSR primer are a 260bp for Minab; double 510 and 530bp bands in Iranshahr, and a 1170bp band in Kahnouj. However, Kahnouj populations have shown two distinct patterns; one similar to Iranshahr and another with a 800bp specific band which is absent in the others.

Variations within Kahnouj population have been tested by using this repeat primer in a mix with a microsatellite primer (33R110) which produced two main common bands of 1010 and 260bp and a strong 630bp band in some individuals (Fig. 6.9). The 260bp band seems to be amplified from a conserved region of genome because individuals of *An. culicifacies* from different localities in Iran used as positive control in same amplification, also showed this 260bp band.

Fig. 6.8- SSR primer X19 $(AGT)_5GCCA$ products in *An. fluviatilis* populations from Iran: Minab (1-3), Iranshahr (4-5), Kahnouj (6-10); marker (11)

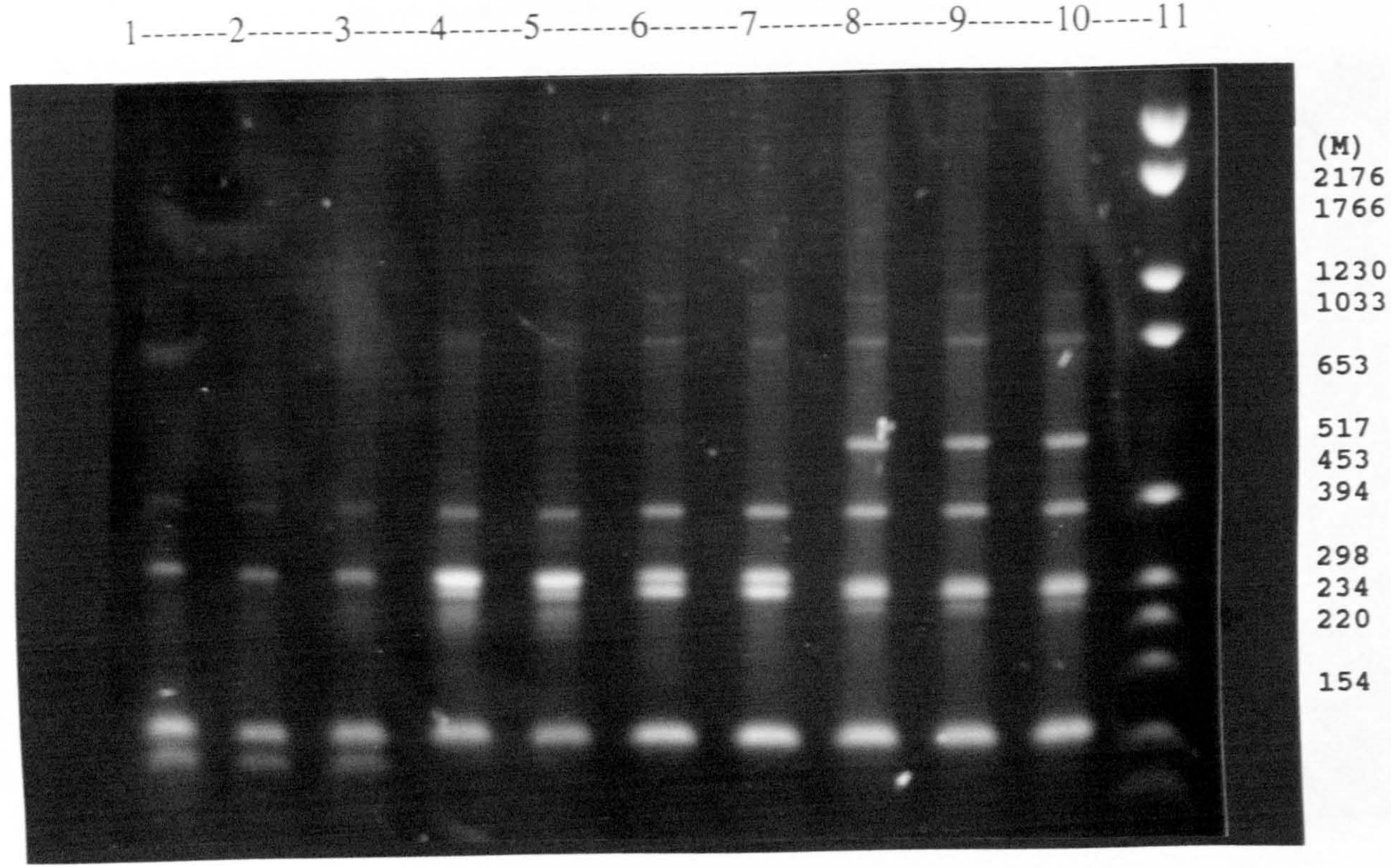
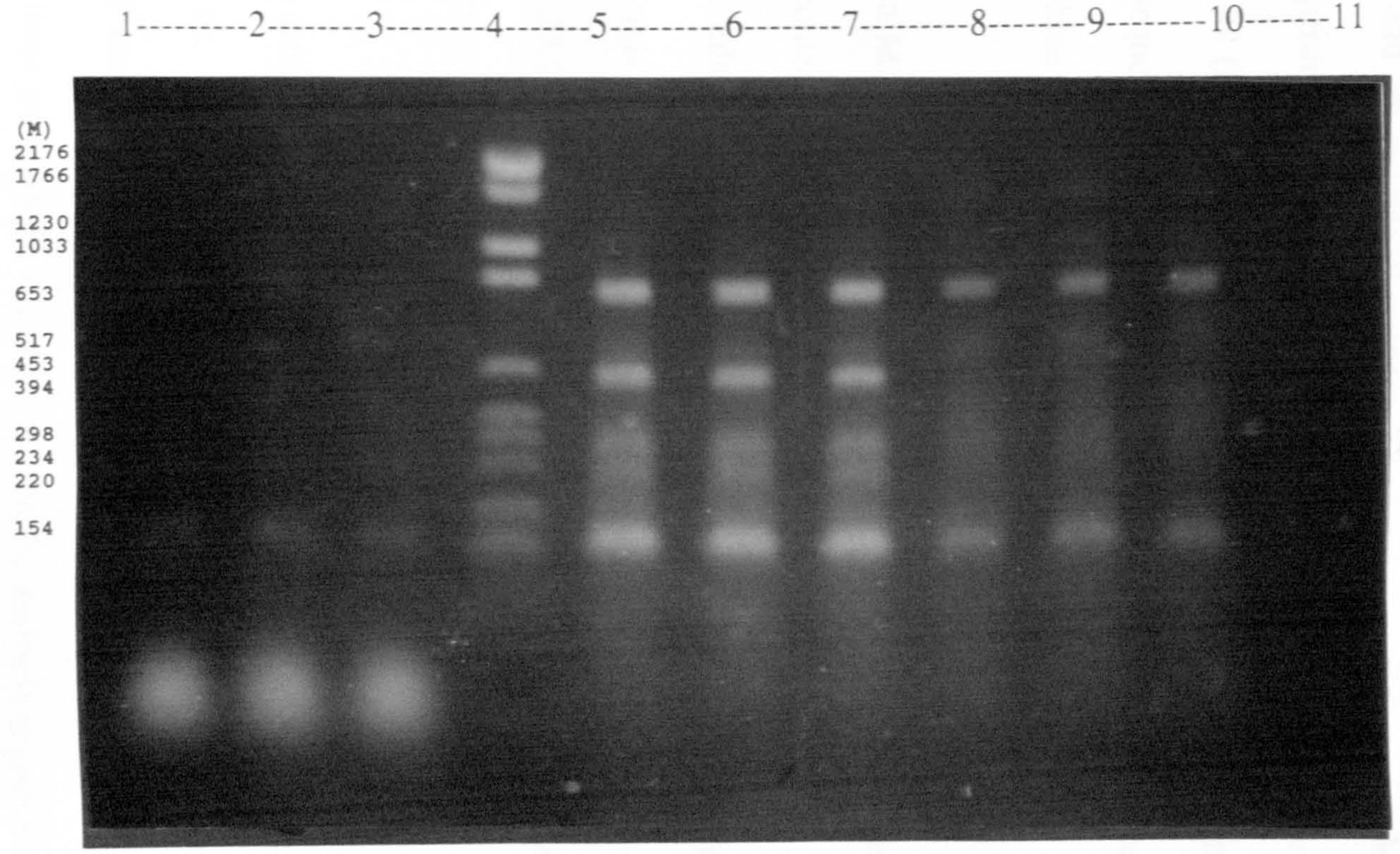


Fig. 6.9- Mixed SSR-X19 and 33R110 primers product in *An. fluviatilis* from Iran: all Kahnouj population; marker (4); -ve (11)



6.4.3 rDNA ITS-2 PCR

In order to clarify the extent of variation between four populations, and to compare RAPD results with a sequence-based technique, the rDNA ITS2 region has been amplified using 5.8s and 28s conserved primers. The total size of the amplified fragment was 562bp comprising 144bp of the 3' end of the 5.8, ITS2 (375bp) and 43bp of the 5' end of 28S (Fig. 6.10). ITS2 was 375bp long in all individuals examined with identical sequence in four populations (two individuals each from Kazeroon and Iranshahr, three from Kahnouj and 6 individuals from Minab).

One individual from Minab (Minab1) differed at seven bases from the other specimens (Table 2). GC content of ITS2 in this population is about 54.7% which is similar to the *An. subpictus* inland population (Djadid and Townson, in preparation). Single-base frequencies in this rDNA region were very similar in two groups: in Minab; A = 23.4% ; T = 23.2% ; C = 26.9% ; G = 26.4% whereas in others, A = 22.4% ; T = 22.9% ; C = 27.7% ; G = 26.9% .

The ITS2 nucleotide differences between this individual and the others is 1.8% with seven mismatches, six transversions and one transition which are not concentrated at the 5' and 3' extremities and therefore there is not a large conserved block in the ITS2 region of these two groups. Fig. 6.11 shows the multiple alignment of ITS-2 sequence in *An. fluviatilis* four populations.

Fig. 6.10- rDNA ITS2 products in *An. fluviatilis* populations (4-9) and An. culicifacies (2) from Iran; marker (1,11); -ve (3)

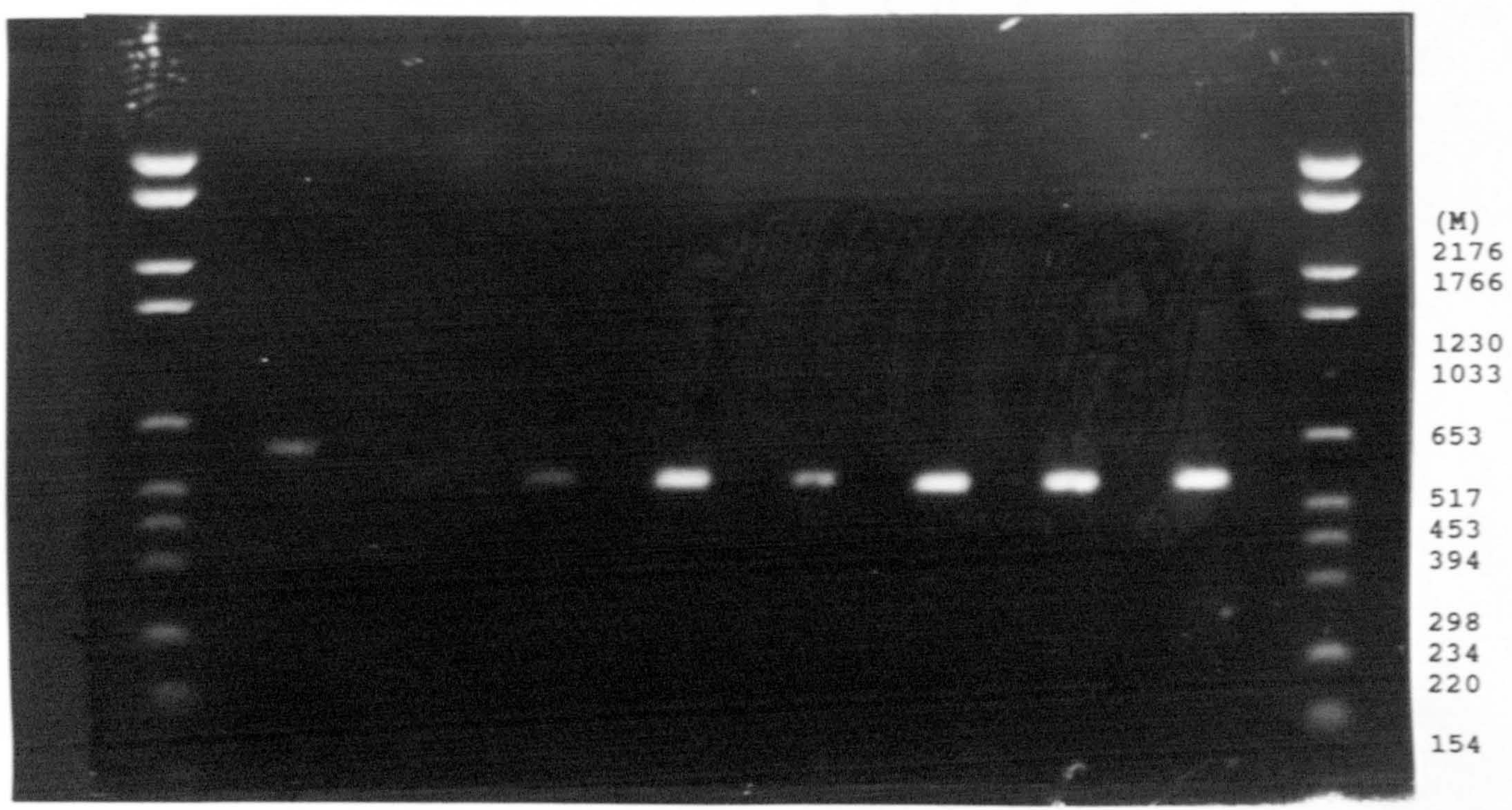


Table 6.2- nucleotide substitution in *An. fluviatilis* populations

Nt. Subst. Site	66	138	139	176	204	299	310
Irn/Kah/Kaz/Min	G	C	C	G	C	G	C
Minab1	C	A	A	C	T	A	A

Fig. 6.11- ClustalV multiple alignment of rDNA ITS2 sequence in *An. fluviatilis* populations from Iran: Kahnouj (KAH), Iranshahr (IRS), Kazeroon (KAZ), Minab (MIN).

```
KAH1        CAATTCCTTGTTACACACTATTCTAACTACATGGCGCCCGTGTACGGACG
IRS1        CAATTCCTTGTTACACACTATTCTAACTACATGGCGCCCGTGTACGGACG
KAZ1        CAATTCCTTGTTACACACTATTCTAACTACATGGCGCCCGTGTACGGACG
KAZ2        CAATTCCTTGTTACACACTATTCTAACTACATGGCGCCCGTGTACGGACG
MIN4        CAATTCCTTGTTACACACTATTCTAACTACATGGCGCCCGTGTACGGACG
MIN1        CAATTCCTTGTTACACACTATTCTAACTACATGGCGCCCGTGTACGGACG
            ************************************************

KAH1        GCATCATGGCGAGCAGCCCGCCTTCTGATGTTGCTGAATGAACACGTGAG
IRS1        GCATCATGGCGAGCAGCCCGCCTTCTGATGTTGCTGAATGAACACGTGAG
KAZ1        GCATCATGGCGAGCAGCCCGCCTTCTGATGTTGCTGAATGAACACGTGAG
KAZ2        GCATCATGGCGAGCAGCCCGCCTTCTGATGTTGCTGAATGAACACGTGAG
MIN4        GCATCATGGCGAGCAGCCCGCCTTCTGATGTTGCTGAATGAACACGTGAG
MIN1        GCATCATGGCGAGCACCCCGCCTTCTGATGTTGCTGAATGAACACGTGAG
            ********** ********************************

'KAH1       CGCACTGTGCATCATTGCGTGCAGGGCCCGTCTCCTACCGGGAACCTTGG
'IRS1       CGCACTGTGCATCATTGCGTGCAGGGCCCGTCTCCTACCGGGAACCTTGG
'KAZ1       CGCACTGTGCATCATTGCGTGCAGGGCCCGTCTCCTACCGGGAACCTTGG
'KAZ2       CGCACTGTGCATCATTGCGTGCAGGGCCCGTCTCCTACCGGGAACCTTGG
'MIN4       CGCACTGTGCATCATTGCGTGCAGGGCCCGTCTCCTACCGGGAACCTTGG
'MIN1       CGCACTGTGCATCATTGCGTGCAGGGCCCGTCTCCTAAAGGGAACCTTGG
            ************************************ **********

'KAH1       GCGCTGAAACAGGTAAGGCAGTGCAGTGTTACTGTACAATTTGGGTGGTG
'IRS1       GCGCTGAAACAGGTAAGGCAGTGCAGTGTTACTGTACAATTTGGGTGGTG
'KAZ1       GCGCTGAAACAGGTAAGGCAGTGCAGTGTTACTGTACAATTTGGGTGGTG
'KAZ2       GCGCTGAAACAGGTAAGGCAGTGCAGTGTTACTGTACAATTTGGGTGGTG
'MIN4       GCGCTGAAACAGGTAAGGCAGTGCAGTGTTACTGTACAATTTGGGTGGTG
'MIN1       GCGCTGAAACAGGTAAGGCAGTGCACTGTTACTGTACAATTTGGGTGGTG
            ************************** **********************

'KAH1       CAGCGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGATAC
'IRS1       CAGCGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGATAC
'KAZ1       CAGCGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGATAC
'KAZ2       CAGCGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGATAC
'MIN4       CAGCGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGATAC
'MIN1       CAGTGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGATAC
            *** ********************************************

'KAH1       CCGGCAGCCTACTAACACCAGGCTTGTCGATAAGGTTCCAGGGGTTACGA
'IRS1       CCGGCAGCCTACTAACACCAGGCTTGTCGATAAGGTTCCAGGGGTTACGA
'KAZ1       CCGGCAGCCTACTAACACCAGGCTTGTCGATAAGGTTCCAGGGGTTACGA
'KAZ2       CCGGCAGCCTACTAACACCAGGCTTGTCGATAAGGTTCCAGGGGTTACGA
'MIN4       CCGGCAGCCTACTAACACCAGGCTTGTCGATAAGGTTCCAGGGGTTACGA
'MIN1       CCGGCAGCCTACTAACACCAGGCTTGTCGATAAGGTTCCAGGGGTTACAA
            ************************************************ *

KAH1        ATCATCCGGCCGAGTCGTGTAACGCGTGCGACCCATACGGTGCACCCATG
'IRS1       ATCATCCGGCCGAGTCGTGTAACGCGTGCGACCCATACGGTGCACCCATG
'KAZ1       ATCATCCGGCCGAGTCGTGTAACGCGTGCGACCCATACGGTGCACCCATG
'KAZ2       ATCATCCGGCCGAGTCGTGTAACGCGTGCGACCCATACGGTGCACCCATG
'MIN4       ATCATCCGGCCGAGTCGTGTAACGCGTGCGACCCATACGGTGCACCCATG
'MIN1       ATCATCCGGACGAGTCGTGTAACGCGTGCGACCCATACGGTGCACCCATG
            ********** ********************************************

KAH1        TTTAATTGGCAACCTACCTTTACAA-
'IRS1       TTTAATTGGCAACCTACCTTTACAA-
'KAZ1       TTTAATTGGCAACCTACCTTTACAA-
'KAZ2       TTTAATTGGCAACCTACCTTTACAAT
'MIN4       TTTAATTGGCAACCTACCTTTACAA-
'MIN1       TTTAATTGGCAACCTACCTTTACAA-
            ****************************
```

This sequence difference is greater than the interspecies, intraspecies, and intra -individual variation in the *An. gambiae* species complex (0.4%-1.6%, 0.07%-0.43%, 0-0.4%, respectively) (Paskewitz *et al.* 1993) but less than those reported in the *Cx. pipiens* species complex (3%) (Severini *et al.* 1996) and in *An. culicifacies* species complex (Townson and Adeniran, in press).

The 375bp ITS2 region in *An. fluviatilis* populations is within the range of that reported in other anophelines, e.g. Cornel *et al.* 1996, Porter & Collins 1991)), *An. gambiae* species complex (426 bp, Paskewitz *et al.* 1993) and, *Culex, Aedes, Haemagogous*, and *Psorophora* thus far, which fall in the approximate range of 300-500 bp (Severini *et al.* 1996, Wesson *et al.* 1992, Paskewitz *et al.* 1993, Fritz *et al.* 1994)

The degree of sequence conservation in ITS2 (98.2% similarity) may suggest that these two groups are the result of recent speciation. Using the estimate of Sharp & Li (1989) of $16X10^{-9}$ substitutions per site per year, indicates that the two groups are separated about 1.12 million years.

So, based on RAPD data which readily differentiate these populations and have shown more similarity between Kahnouj and Iranshahr specimens than the Minab population, and ITS2 sequence differences that separated these specimens into two groups , we suggest that this study supports evidence of an *An. fluviatilis* species complex in Iran, comprising a more prevalent species which exists in hilly and coastal

areas and another species with a distribution restricted to coastal areas. The reason why only one individual with ITS2 sequence variation was detected could be due to a number of reasons, such as the lower prevalence of this group, the collection site and season or to different aspects of its ecology such as feeding habit (exophagic or endophagic), resting place and type and distance of larval breeding places from collecting sites (human and animal shelters).

Moreover, in field conditions, in response to changes in microclimate, the disappearance of adult mosquitoes and breeding sites often occurs although there are still new cases of malaria. This is in concordance with the presence of vectors at low density that are not detectable by routine collection techniques. However, despite small sample size (10-30 from each population) examined, RAPD primers' ability to detect considerable variation within populations, provide strong evidence for suggesting gene flow and creation of local forms in *An. fluviatilis* based on ecological variations in the Kahnouj, Kazeroon, and Minab population. In contrast Iranshahr specimens have shown a more identical pattern with different primers.

In summary, this study is the first DNA based investigation on molecular systematics of *An. fluviatilis* from Iran and its RAPD, SSR and ITS2 sequence data provided preliminary evidence for two sibling species of *An.fluviatilis* in Iran.

TEXT BOUND INTO

THE SPINE

CHAPTER SEVEN

INTRA-SPECIFIC VARIATION IN ANOPHELES SUBPICTUS SPECIES COMPLEX FROM SRI-LANKA REVEALED BY PCR-BASED APPROACHES

7.1 ABSTRACT

Anopheles subpictus Grassi is a major vector of malaria in Sri-Lanka. Recent studies of the polytene chromosomes from ovarian nurse cells have shown that species B is present only in coastal localities of the Island, whereas species A is found both in coastal and inland areas with a predominance in inland areas.

This species complex has been investigated in samples collected from coastal and inland areas of Sri Lanka. RAPD and SSR primers resolved these populations into four groups; one coastal and three inland. The ITS2 of the rDNA has been sequenced in samples from these four groups. ITS2 is 473bp in the coastal group and inland 1 group. In the remaining inland populations (inland 2 and 3) it is 564bp. However, there was some sequence variation within inland 2 and inland 3. A phylogenetic tree constructed on the basis of the ITS2 sequence from at least three specimens from each of the four groups clustered coastal and inland 1 in one group and inland2 and 3 in another group.

RAPD, SSR fingerprints and ITS2 sequences are in agreement with previous cytogenetic studies. Specimens collected in the coastal area of Sri Lanka have previously identified as species B, based on polytene chromosome inversions, whereas inland 2 and 3 correspond to species A. However, the result of this study confirms the presence of species B in inland area 1, whereas previous studies have reported this species only from coastal localities.

7.2 INTRODUCTION

Anopheles subpictus species complex

Anopheles subpictus Grassi 1899, comprises at least four sibling species with 11 reported morphological variations in the maxillary palpi and one in the wing. It occurs very widely in the oriental region, extending from Afghanistan in the west to the Mariana Islands in the east, and from China in the north to Sri Lanka in the south. In India, it occurs in all the mainland zones. It also has been reported from the Andaman Islands and Lakshadweep. Specimens of this species infected with *Plasmodium* spp. have been recorded from India, Indonesia and Java (Nagpal & Sharma 1995).

This group of species breed in a variety of habitats except the most highly polluted or contaminated ones. Most studies have not identified to the precise species hence it is difficult to interpret the observations reported in Nagpal and Sharma (1995). *An.subpictus s.l.* has been recorded from flowing or stagnant waters, clear or turbid waters, water with or without vegetation, unshaded or slightly shaded places, wells, borrow pits, channels, lake margins, ponds, tanks, ground pools, fallow and freshly flooded rice-fields, cement cisterns, tree holes, and both fresh and brackish waters.

An.subpictus s.l has been found very commonly in houses and cattlesheds and is often collected outdoors. It feeds predominantly on cattle and other domestic animals and bites throughout night but its peak biting is between 18.00 and 22.00 hrs. Again it is unclear how these behaviours differ between the constituent species of the complex. It has been recorded

as being resistant to DDT in India. The main identification characters of *An. subpictus* are:

1 Apical pale band nearly equal to the pre-apical dark band

2 Fore leg tarsomers with broad bands

3 Fring spot on all the veins

Works on members of this species complex started with egg morphology, salivary gland and ovarian nurse cell polytene chromosomes, and crossing experiment with *Anopheles subpictus* from coastal and inland samples, these studies strongly suggested the existence of two separate sibling species ; the type with the standard X chromosome type has been designated as species A and the inverted arrangement as species B (Suguna 1982).

Species A breeds in fresh water and occurs in inland as well as coastal localities, while species B breeds in brackish water and has previously only been recorded from the coast, mainly in backwaters (Reuben & Suguna 1983). Later, a review of cytotaxonomic studies of malaria vectors in India by Subbarao *et al.* (1988), summarized the available information on *subpictus*. Populations of this species from villages near Delhi were cytologically identified as species A, but the number of the ridges on the egg floats did not correspond with those of species A found in Tamil Nadu where the average ridge number was 33.

The fresh water Delhi population of *subpictus* identified as species A was found to support sporogeny of *P. vivax* when fed experimentally on infected blood (Nanda *et al.* 1987). Sporozoite-positive specimens of *An. subpictus s.l.* were detected in coastal villages

of Tamil Nadu (Paniker *et al.* 1981), as well as in areas where only the fresh water breeding form has been found (Kulkarni 1983).

Since both sibling species of *subpictus* are prevalent in coastal villages, while in interior villages, only species A has been found, the role of these two species in malaria transmission needs to be clarified (Subbarao *et al.* 1988b).

Paracentric fixed inversions on the X-chromosomes served to distinguish four species in the *An. subpictus* complex with no inversion heterozygotes among 4814 specimens examined: species A ($X+^a$, $+^b$), species B (Xa,b), species C (Xa, $+^b$), and species D ($X+^a$, b) (Suguna *et al.* 1994). Morphologically diagnostic characters; egg float ridge number, larval mesothoracic seta, pupal seta and the palpi of adult female have also been described. Species B breeds in saltwater and was found only in coastal villages, whereas species A, C and D immature stages breed in freshwater (Suguna *et al.* 1994).

In Sri-Lanka, *An. subpictus* was regarded as a secondary vector of malaria (Hearth *et al.* 1983). However, studies in the Mahaweli irrigation project in north eastern province have revealed the role of this species as a major vector (Amerasinghe *et al,* 1996). Recently, the distribution of *An. subpictus* sibling species has been examined in Sri Lanka, based on chromosomal characters (Abhayawardana *et al.*, 1997). Species A and B were identified based on the diagnostic inversion genotypes of species A - $X+^a$ and species B - X^a . these authors revealed that species B was present only in coastal localities of the island whereas species A was found both in coastal and inland areas with predominance in inland areas.

7.3 MATERIALS AND METHODS

7.3.1 Mosquitoes

Alcohol preserved and dried field specimens of *An. subpictus* were collected from coastal and inland areas in Sri-Lanka (Fig. 7.1, see also Fig 3.1), by Dr. W Abeyewickrema and staff of the University of Colombo, Sri-Lanka.

7.3.2 Genomic DNA extraction

The following extraction methods were used: phenol extraction (Ballinger-Crabtree *et al.* 1992), Collins *et al.* 1988), Mini -Prep (Medina-Acosta & Cross 1993). Air-dried pellets have been resuspended in 100 μl of double-distilled H_2O or TE buffer and stored at 4°C.

7.3.3 Primers :

RAPD and SSR primers

four Random primer from the UBC (University of British Columbia, Canada) and AB01 series (OLIGO Express, UK), and two 3'-anchored dinucleotide repeat primers were used. These have been found useful in studies we carried out with other anopheline species complexes (see elsewhere in this thesis).

ITS2- PCR primers

5.8S and 28S primers of the rDNA region were based on modification of the published nucleotide sequence of *Cx. tritaeniorhynchus* 5.8S rDNA (Shimada & Sasaki 1991)

Fig. 7.1- . *An. subpictus* sibling species A and B distribution and collection sites in Sri Lanka . Species A (●), Species B (■), Species A and B sympatric (▲) (after Abhayawardana *et al.*, 1996)

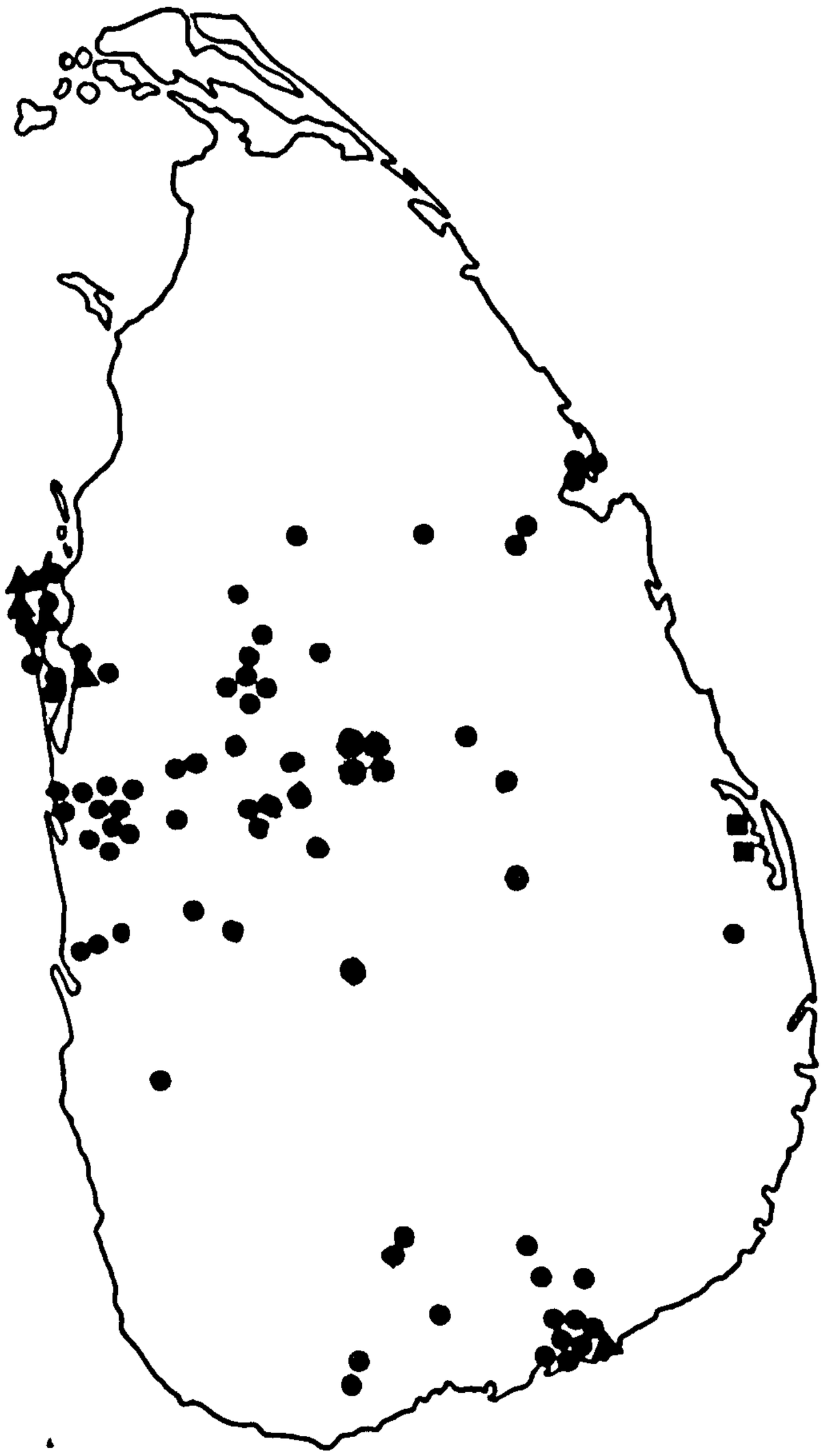


Table 7.1 - selected primers and their sequences

primer	5....sequence ....3'	GC%
AB4	GGACTGGAGT	60
AB19	ACCCCCGAAG	70
UBC-308	AGCGGCTAGG	70
UBC-353	TGGGCTCGCT	70
GT1	$(GT)_7ATCC$	50
GT2	$(GT)_7TGTA$	44
ITS2 primers:		
5.8S	ATCACTCGGCTCGTGATCG	57.8
28S	ATGCTTAAATTTAGGGGGTAGTC	39.1

and *An. hermsi* 28S rDNA (Porter & Collins 1991). Table 7.1 listed the name, sequences and GC content of the primers for which results are presented.

7.3.4 Amplification condition and PCR product gel electrophoresis

PCR amplification mix

All RAPD reactions were performed in a total volume of 25 μl. Each mixture contained 2.5 μl of 10X reaction buffer, 2 mM $MgCl_2$, 50ng of primer, 0.001% gelatin, 0.1 mM each of dATP, dTTP, dCTP, and dGTP, 0.5 unit of Promega Taq polymerase, and sterile double-distilled water to 25 μl. Reactions were overlaid with 50 μl of mineral oil and amplified in a Hybaid Omnigene thermal cycler.

RAPD programme

(1) one cycle of 94°C for 5 min; (2) 45 cycles of 94°C for 1 min, 39°C for 1 min, 72°C for 1 min; (3) one cycle of 72°C for 7 min (Prog 2). ITS2 PCR amplification was performed using Prog 10. following PCR, 10 μl of amplified DNA mixed with Ficol/orange G loading buffer and electrophoresed in 6% polyacrylamide or 1% agarose (Appligen) in TBE buffer containing ethidium bromide at 1.5 v/cm for 16 hours (large gel) or 5 v/cm for two hours (mini gel). A mixture of BglI and HinfII digest of pBR328 (Boehringer Mannheim) were used as molecular weight marker for PCR product size determination (molecular weights 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154 bp). Gels visualised under UV light and photographed with black and white polaroid 55 film (Hargreaves photo Ltd) or Ilford FP4 film.

7.3.5 Computer programs

GCG (version 7.0), DNA-STAR, and Clustal V (Higgins & Sharp 1988) have been used to enter and analyze the sequences and alignments.

7.4 RESULT:

7.4.1 RAPD-PCR

AB4 primer

This primer produced a population-specific pattern in the coastal and three inland collections of *An. subpictus* from Sri-Lanka (Fig. 7.2). All these specimens share a 700bp common band. Coastal population has another common band with inland 1 (1380bp). Inland 2 and 3 are almost identical except two 580 and 520bp bands which present in inland 3 but not in inland 2 which has a different size (530bp) fragment on that range (Table 2). This species-specific 700bp common band has been excised from the gel, and after re-amplification produces the same size product (700bp) with two other fainter 900 and 250bp fragments (Fig. 7.3).

Regarding the intensity of bands within AB4 products, *An. subpictus* population could be clustered into three groups; coastal, inland 1 and finally third group consisting of inland 2 and inland 3 (Table 7.2- underlined fragments).

UBC-308

This primer produced 8-10 fragment in each population, with more bands common between inland 1 and coastal. Population-specific fragments are: a 478bp band in inland 2,3 specimens, a 420bp in coastal and 380bp in inland1 (Fig. 7.4).

Fig.7.2- RAPD primer AB4 products in *An. subpictus* populations from Sri Lanka: coastal (1-4), inland1 (6-9), inland2 (10-13), inland3 (15-18); marker (5,14); -ve control (19). The main population-specific bands are: 950bp in coastal, 875bp in land1, 810 and 725bp in inland2 & inland3

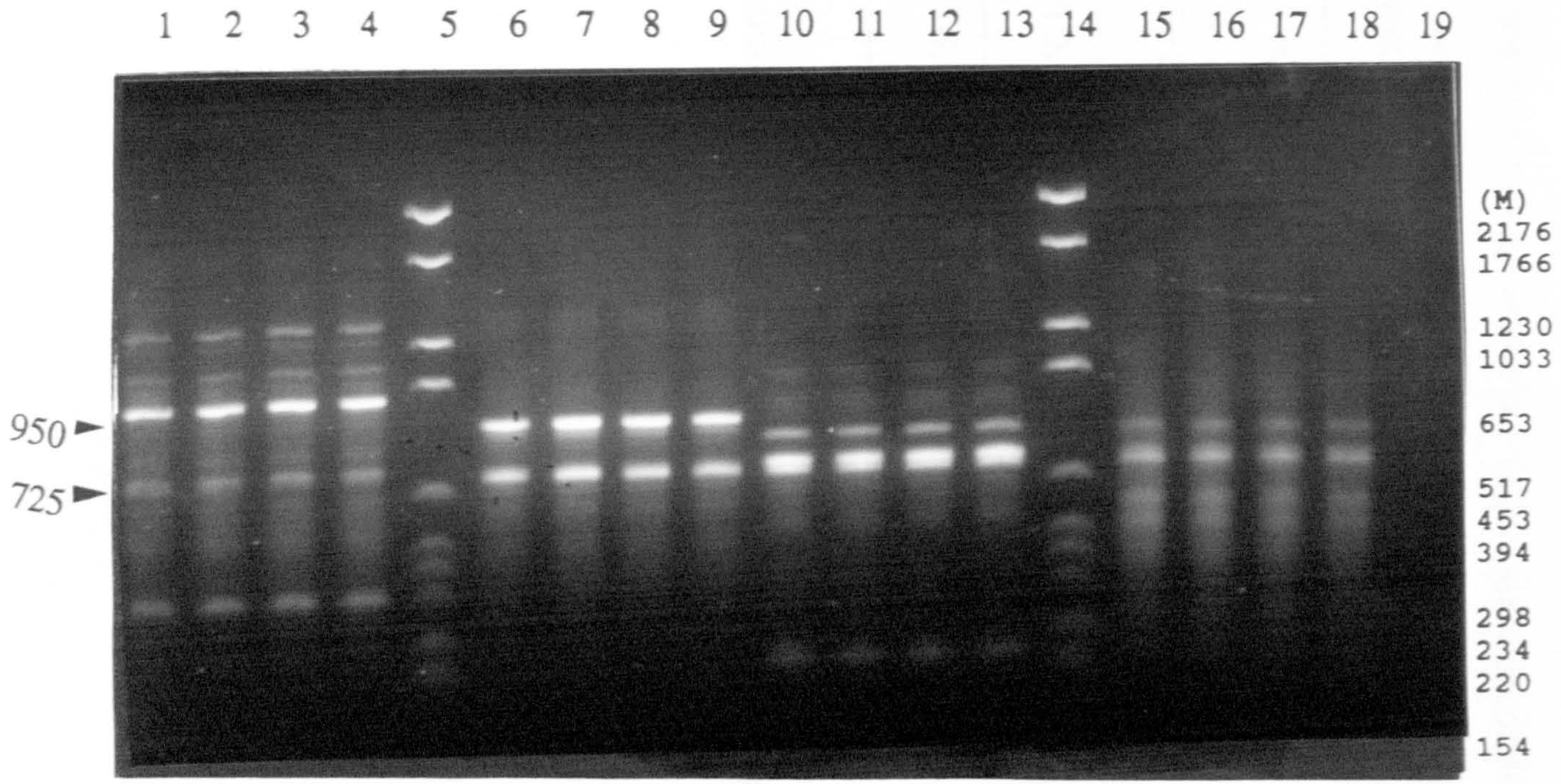


Table 7.2-- The main products of AB4 primer in *An. subpictus* populations

Population	Population Specific Pattern
Coastal	1380, 1115, 950*, 700, 383
Inland 1	1380, 875, 700
Inland 2	810, 725, 700, 530, 250
Inland 3	810, 725, 700, 580, 520, 250

* underline fragments : bands with higher intensity in each population

Fig. 7.3- RAPD primer AB4 reamplification of a 720bp common band in *An. subpictus* populations from Sri-Lanka: coastal (1-4), inland1 (5,7), inland2 (8-9), inland3 (10-11); marker (6)

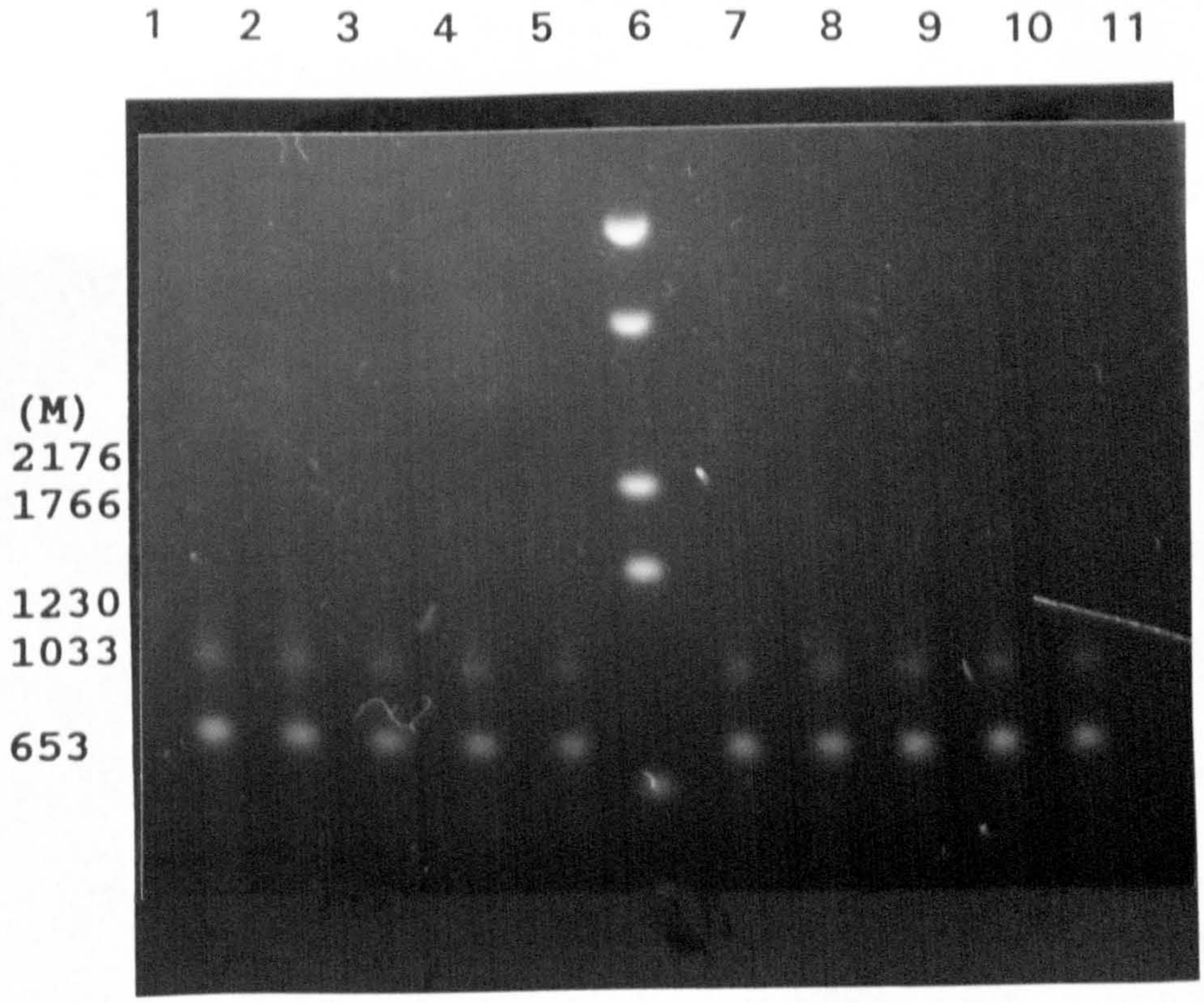
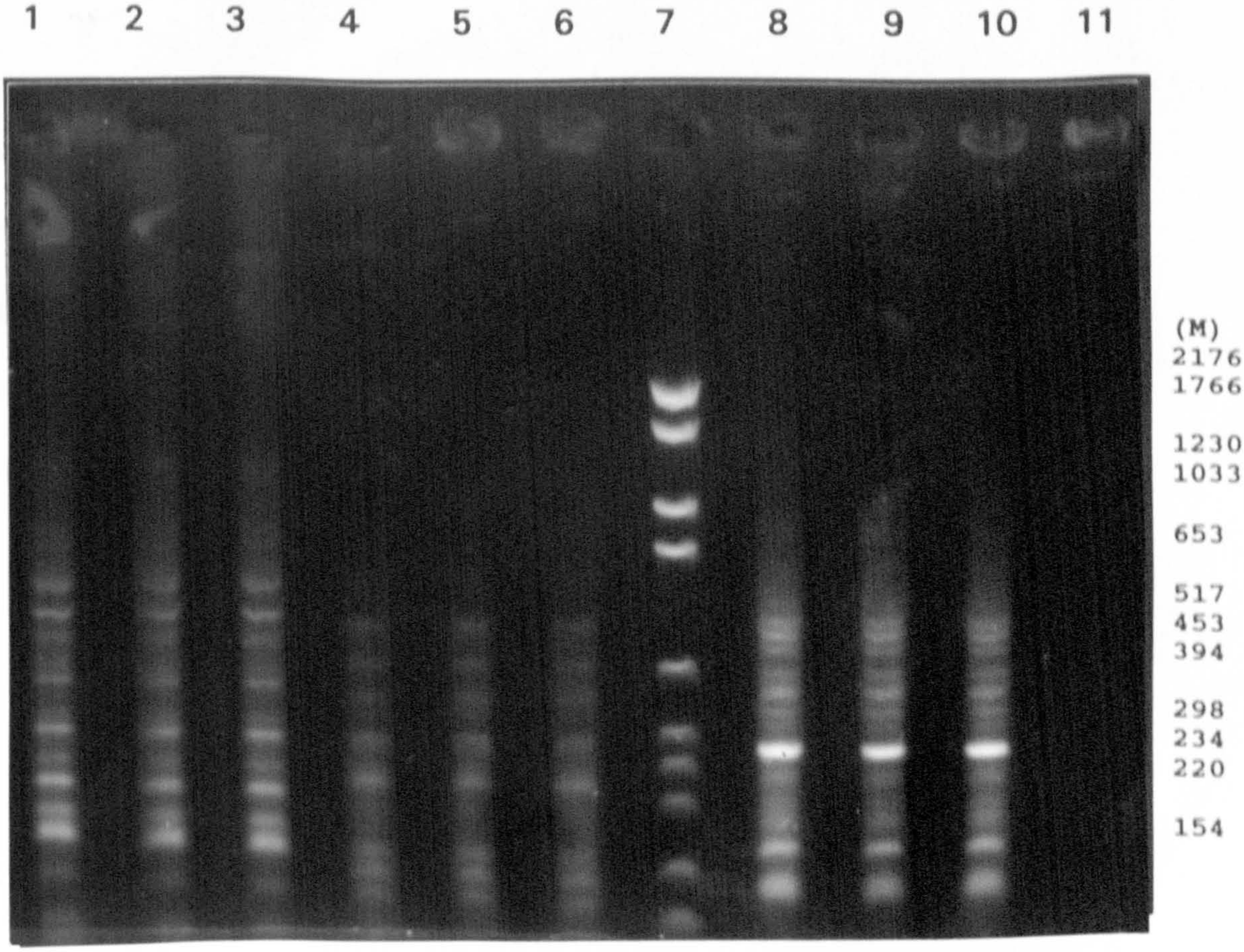


Fig. 7.4- RAPD fingerprints with primer UBC-308 in *An. subpictus* populations from Sri Lanka: inland1 (1-3), coastal (4-6), inland2 (8-9), inland3 (10); marker (7); -ve (11). A 478bp band in inland2 &inland3, a 420bp in coastal and a 380bp in inland1 specimens are population-specific fragments.



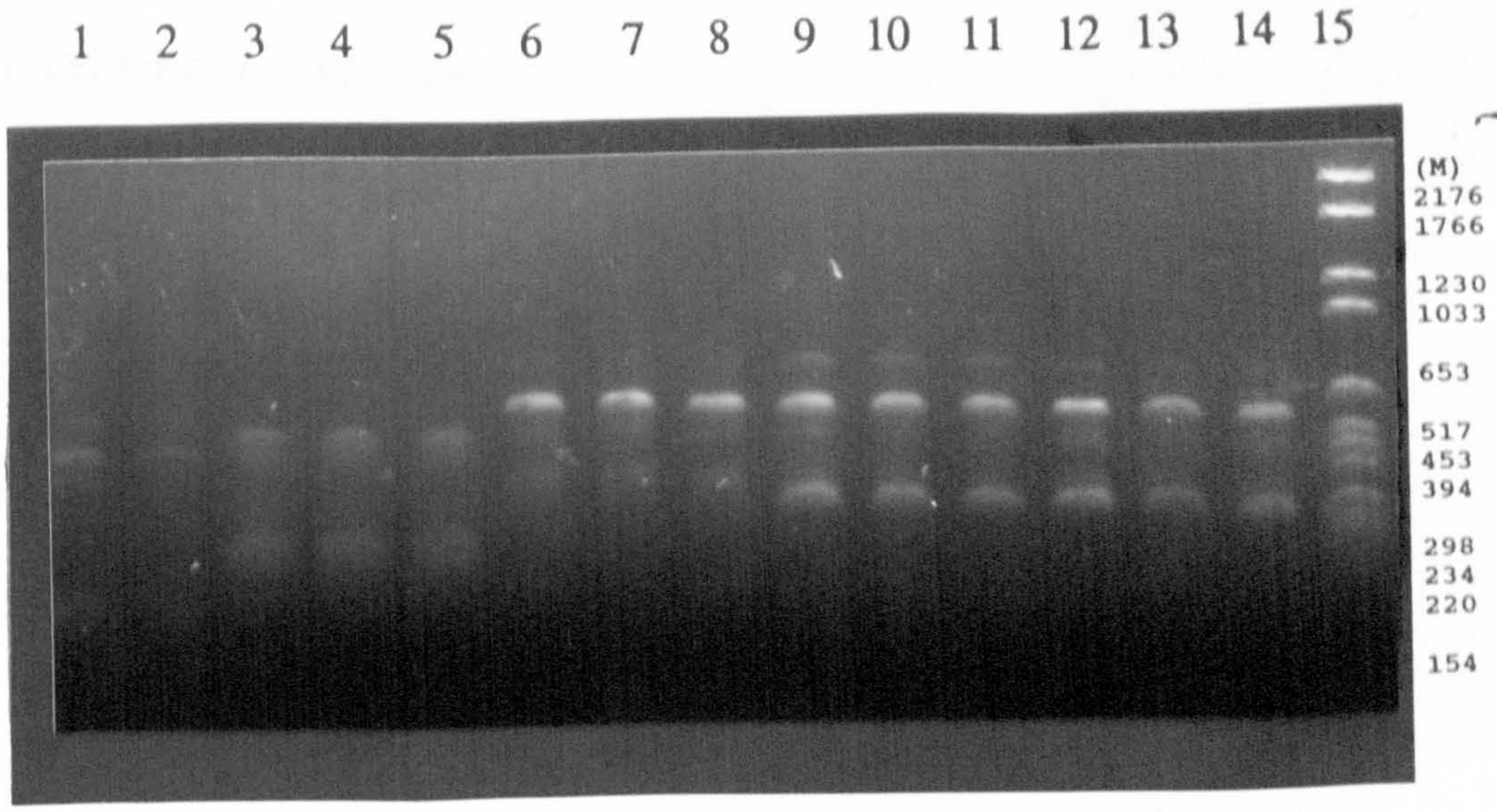
UBC-353

Products of this primer in 45 cycles of 39°C annealing (Prog 2), also differentiated coastal and inland 1 populations of *An. subpictus* but the pattern of inland 2 and inland 3 are identical (data not shown).

AB19

In a two step annealing (45 and 36°C- Prog 3B) this RAPD primer has shown 4 population-specific patterns with a maximum of two main bands for each population (although inland2 showed two different patterns in its specimens): a 510bp band in coastal, 538bp in inland 1, 580, 270bp bands in inland 3. One group in Inland 2 has an identical pattern with inland 3 but other group has a 400bp specific band (Fig. 7.5).

Fig. 7.5- RAPD-AB19 pattern produced in a two step annealing (45 and 36°C) program with *An. subpictus* coastal and inland populations from Sri-Lanka: coastal (1-2), inland1 (3-5), inland2 (6-10), inland3 (11-14); marker (15).



7.4.2 SSR PCR

GT1

$(GT)_7$ATCC repeat primer produced three distinct fingerprints with no shared bands for coastal, Inland1 and inland2 (Fig. 7.6).

7.4.3 RAPD + SSR

Fingerprints have been produced through amplification by mixing a RAPD primer (AB19) and a SSR primer (GT2) with *An. subpictus* populations in a two steps annealing program. The result revealed a new pattern in each population, with 3 groups detectable (Fig. 7.7).

Fig. 7.6- SSR primer (GT_{17}ATCC fingerprints in a two step annealing (50 and 36°C) program in *An. subpictus* populations from Sri-Lanka: coastal (1-2), inland1 (3-4), inland2 (5-6); marker (8); -ve (7). The three populations have not any common band. However, a 570bp fragment is shared between coastal and inland1 specimens.

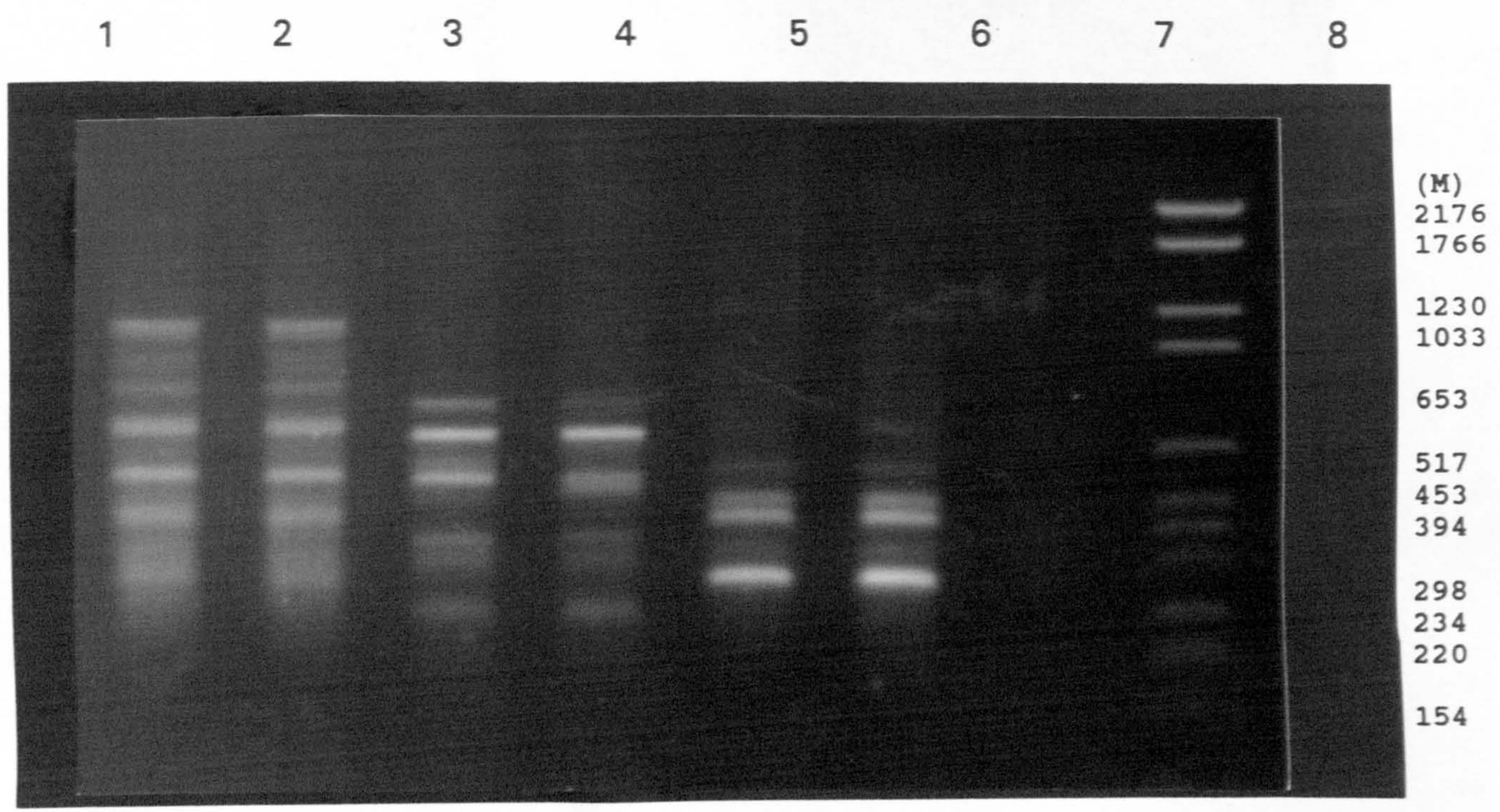
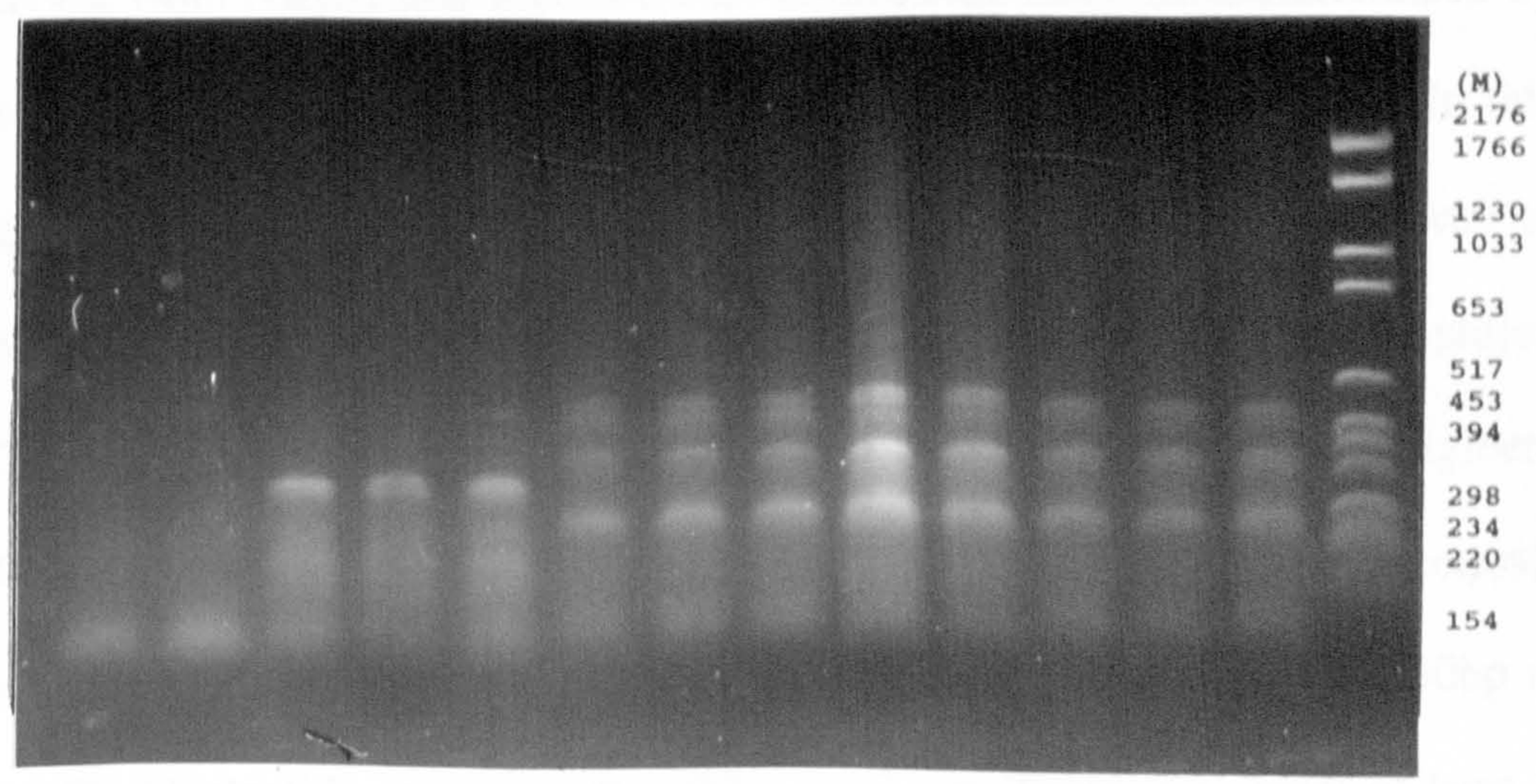


Fig. 7.7- Fingerprints of *An. subpictus* populations collected from Sri-Lanka, produced by mixing a RAPD primer (AB19) and a SSR primer, $(GT)_7TGTA$ in a two step annealing (45 and 36°C) program. Coastal (1-2), inland1 (3-5), inland2 (6-10), inland3 (11-13); marker (14).



7.4.4 rDNA-ITS2

RAPD and SSR primers'results showed that morphologically identified *An. subpictus* mosquitoes collected from coastal and inland areas in Sri-Lanka could be differentiated by their population-specific patterns to at least three genetically different populations. In this case, a sequence-based technique which amplifies a conserved region is the best way to evaluate and compare RAPD results. The rDNA-ITS2 region has been selected and amplified by 5.8S and 28S conserved primers (Fig. 7.8). The total size of amplified fragment comprising 144bp of 5.8s, the ITS2 region and 43bp from the 28s gene is 667bp in coastal, 662bp in inland 1, 756bp in inland 2, 757bp in inland 3.1 and inland 3.2, and 750bp in inland3.3 (Fig. 7.9). Nucleotide sequence data (Fig. 7.10 and Table 7.3) from at least 3 individuals from each group showed ITS2 is 476bp in coastal, 473 in inland 1, 564 in inland 2 and inland 3.1, and 561bp in inland 3.2. (Table 3, Fig. 10). So the *An. subpictus* populations fall into two groups, first, individuals from the coastal area and inland 1, and another group contains inland 2 and inland 3 populations (Fig. 7.11). The GC content of ITS2 in these populations range between 54.4 in inland2 to 58.2 in inland31 (Table 4) which is similar to GC% of *An. fluviatilis* (54.7) and *An. culicifacies* (59). Sequence differences in *An. subpictus* species complex (with and without scoring the gaps) is summarized in tables 5 and 6 which revealed that coastal and inland 1 fall into one group and inland 2 plus the two different variants of inland3 in to another group.

Fig. 7.8- Diagram of a single rDNA unit, the location and direction of primers used and the region of rDNA amplified and sequenced.

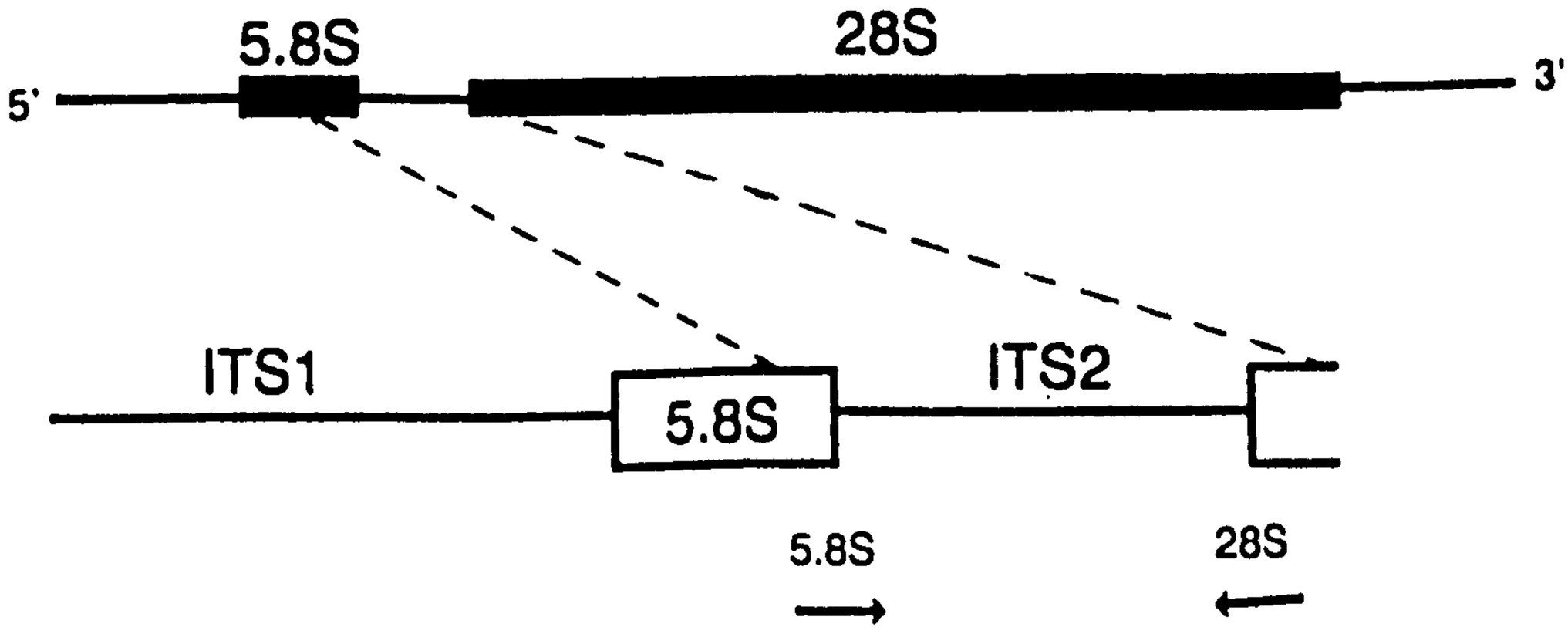
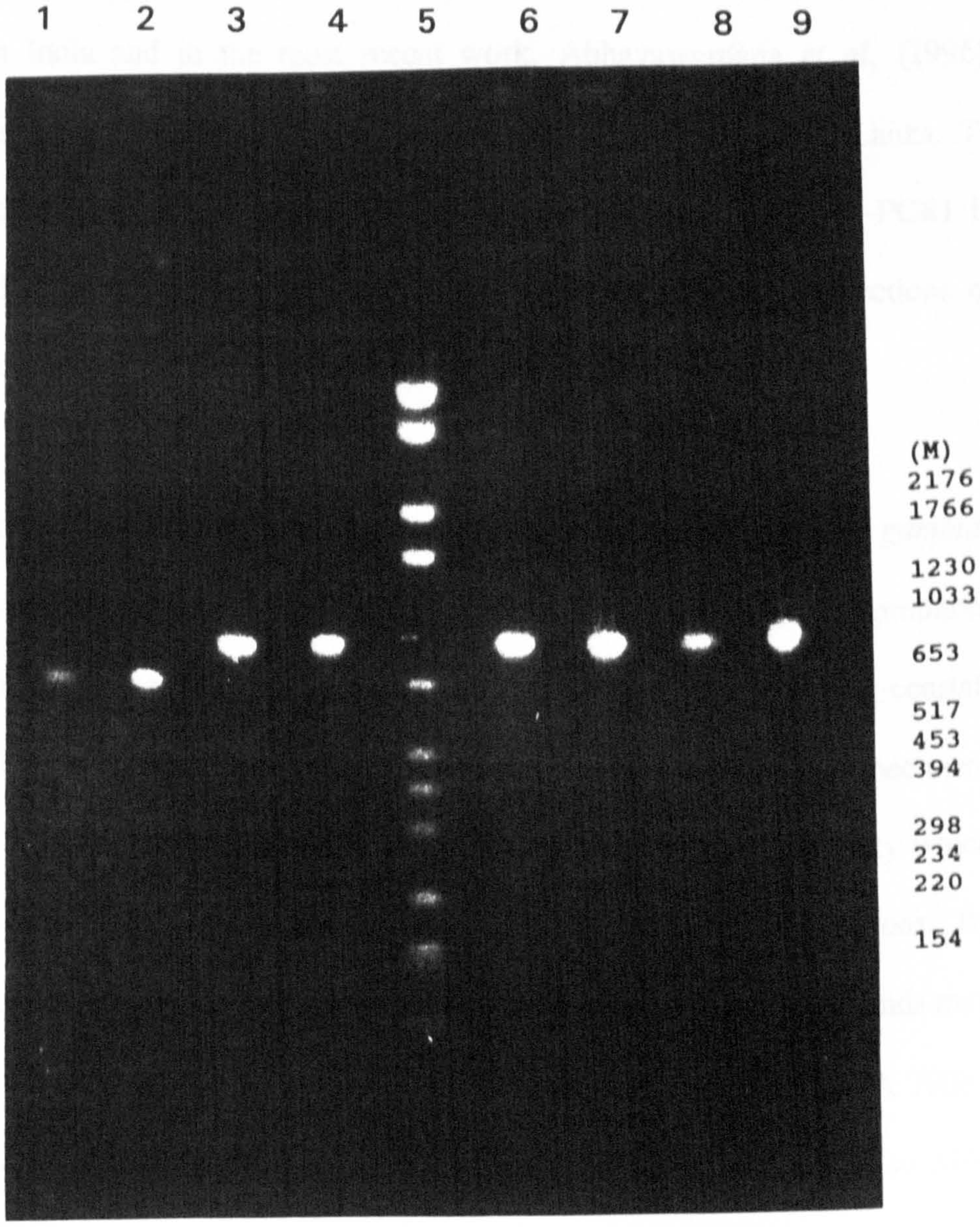


Fig. 7.9- rDNA-ITS2 specific primers' products in *An. subpictus* populations: coastal (1), inland1 (2), inland2 (3-4), inland3 (6-9); marker (5). Amplified fragments contain 147bp of 5.8s, ITS2, and 44bp from 28s region. Total size of amplified fragment is about 665bp in coastal and inland1 specimens, and 755bp in inland2 and inland3.



7.5 Discussion

Anopheles subpictus is the most abundant anopheline in most parts of the Indian subcontinent (Rao 1984). Based on population genetics interpreted from chromosomal evidence, Suguna *et al.* (1994) showed that *Anopheles subpictus* comprises at least four biological species in India and in the most recent work, Abhayawardana *et al.* (1996), reported the occurrence of two sibling species of *Anopheles subpictus* in Sri Lanka. To evaluate the application and ability of PCR-based techniques (mainly RAPD-PCR) in epidemiological studies compared to cytogenetics, we examined different collections of coastal and inland populations of this species complex from Sri Lanka.

RAPD has proved useful in finding inter and intra-specific variation in *An. gambiae* and *An. culicifacies* species complexes (Ch. 4 and 5). Of the RAPD primers, AB4 produced the most clear and specific patterns and detected at least three different populations; coastal, inland 1, inland2 & 3, although there are some differences between inland 2 and 3 specimens (Fig. 7.2 and Table 7.2). In addition, another feature of RAPD, the band intensity which appears greater in some fragments, seems to be consistent across all populations. By changing the amplification conditions to a higher annealing temperature, the faint bands may disappear and with a simpler pattern it is possible to find and characterize bands. A 700bp species-specific common band has been found in all examined specimens and may be suitable for further characterization.

Other RAPD primers also revealed variation between these populations with either more (UBC-308) or fewer products (UBC-353, AB19) but still three or four types of variants were detectable within *An. subpictus* collections.

SSR primers have been applied in this study because it is well established that loci containing tandem simple repeat sequences are a rich source of informative markers (Epplen *et al.* 1991, Queller *et al.* 1993). SSR primers, like RAPDs have been used one at a time to amplify genomic segments flanked by inversely oriented, closely spaced repeated sequences (Zietkiewicz *et al.* 1994). The result obtained by using SSR primer, $(GT)_7$ATCC, also confirmed variation between *An. subpictus* specimens but surprisingly with no shared band in inland and coastal populations, perhaps because the 3' anchored sequence avoid priming from within a $(GT)_n$ element and provides the specificity that reduces the number of targeted genomic loci to those matching the 3'-terminal residues.

The application of a RAPD together with a repeat primer may improve the detection of variation within populations by producing both dominant (RAPD) and co-dominant markers (SSR) with a high degree of allelic polymorphism (Wu *et al.* 1994, Matioli *et al.* 1995).

ITS2:

the rDNA-ITS region is relatively free of structural and functional constraints, which allows it to evolve relatively rapidly. Moreover, the effects of molecular drive and concerted evolution generally act to minimize the degree of intra-specific variation (Tang *et al.* 1996; Hillis & Dixon 1991; Hillis and Davis 1986; Schlotterer *et al.* 1994). However, the degree of intra-specific variation in the ITS appears to vary from species to species, for example significant variation in the ITS has been identified in studies of various mosquitoes (Wesson *et al.* 1993).

In order to compare with RAPD and SSR-PCR, and to determine whether nuclear rDNA sequences provide a useful means for assessing the structure of populations of *An. subpictus*, sequence analysis of rDNA-ITS2 region has been amplified. ITS2 regions from representative *Anopheles* species in the subgenera *Callia*, *Anopheles* and *Nyssorhynchus* have been examined (Porter & Collins 1991; Paskewitz *et al.*; Fritz *et al.* 1994).

The results of this study revealed the possibility of new members within the *An. subpictus* complex since (1) the size of ITS2 varies between different populations from inland and coastal areas (473-564bp- Table 7.3) and (2) degree of sequence variation (8-13%, Table 7.5, 7.6) is greater than that reported in the *Cx. pipiens* species complex (3%) (Severini *et al.* 1996). However, extreme variability of ITS2 among individual ticks has been observed (Rich *et al.* 1997).

A phylogenetic tree based on the ITS2 sequence clustered coastal and inland 1 in one group and inland2 and 3 in another group with *An. fluviatilis*, an out-group, far from these two groups.

The results of this study using RAPD, SSR fingerprints and ITS2 sequence is in concordance with previous cytogenetic studies and has detected at least two species: individuals from coastal area and inland 1 are considered to be species B whereas inland 2 and 3 specimens probably correspond to species A.

Table 7.3- Size, Initiation and ending sequences of ITS2 in An. subpictus populations and other species. Coastal (C), Inland1 (I1),Inland2 (I2), Inland3 (I3)

SPECIES	SIZE	BEGINING	END
An. gambiae	426	CCTACTAATTACCAAAGT	TTATACCACGTAGGCCT
An. culi	372	TTGAGTGCCTACCAAAT	CCTTACCAGTAGCCT
An. subC	476	CTACTAGGTACTTCGAT	TTATACTGGCCGTAGGCCT
An. subI1	473	CTACTAGGTACTTCGAT	CACAATATCCAGTAGGCCT
An. subI2	564	CTACTAGAGTACTGAGA	ACCACACTCCAGTTAGGCCT
An. subI31	564	CTACTAGAGTACTGACA	ACCACACTCCAGTTAGGCCT
An. subI32	564	CTACTAGGTACTGAAAT	ACCACACTCCAGTTAGGCCT
An. fluvi	375	CTACCAATTCCTTGTTA	TACCTTTACAAGTAGGCCT
An. quadm	299	GTGCCCATATTTGCA-CAA	GGAGCACATGGGCCTCAA
Cx. quinq	330	CCTATATTTATCTATTCAA	ATAAACCCCC---ATGTAG
Cx. pip.cx		GGTCATCC--ACACACCGG	TACCCCCTAAATTTAAGCAT
Ixodes dami	680	ATCATATACAAGAGAGGAG	ACAAAATAAGATTC-GAATTG

Table 7.4- GC% of ITS2 region in different species

Species	GC%
An. subpictus (coastal)	56.1
An. subpictus (inland1)	57.1
An. subpictus (inland2)	54.4
An. subpictus (inland3-1)	58.2
An. subpictus (inland3-2)	55.3
An. gambiae	55
An. culicifacies	59
An. fluviatilis	54.7
Ae. aegypti	50
Culex spp.	60.5
Drosophila	18
Xenopus	88

Table 7.5- -sequence differences in An. subpictus species complex populations (without gaps)

	coastal	inland 1	inland 2	inland 3(1)	inland3(2)
coastal		5/468	31/366	29/366	30/366
inland 1	5/468		29/366	27/366	32/366
inland 2	31/366	29/366		12/563	14/564
inland 3(1)	29/366	27/366	12/563		11/564
inland 3(2)	30/366	32/366	16/564	14/564	

Table 7.6- - sequence differences in An. subpictus species complex populations (witht gaps)

	coastal	inland 1	inland 2	inland 3(1)	inland3(2)
coastal		10/477	48/366	48/366	50/366
inland 1	10/477		49/366	46/366	52/366
inland 2	48/366	49/366		14/563	16/564
inland 3(1)	48/366	46/366	14/563		15/564
inland 3(2)	50/366	52/366	16/564	15/564	

Fig. 7.10- CLUSTAL V multiple sequence alignment in 3 individuals from each population of *An. subpictus*: coastal, inland1, inland2, and inland3. The whole sequence of ITS2, position of 5.8s, 28s primers are bold and specified.

```
                                5.8S primer
                            <------------------>
COASTAL1        ATCACTCGGCTCGTGGATCGTTGAAGACGCAACTGAACTCGCGCGTCAAG
COASTAL2        ATCACTCGGCTCGTGGATCGTTGAAGACGCAACTGAACTCGCGCGTCAAG
COASTAL3        ATCACTCGGCTCGTGGATCGTTGAAGACGCAACTGAACTCGCGCGTCAAG
INLAND11        ATCACTCGGCTCGTGGATCGTTGAAGACGCAACTGAACTCGCGCGTCAAG
INLAND12        ATCACTCGGCTCGTGGATCGTTGAAGACGCAACTGAACTCGCGCGTCAAG
INLAND13        ATCACTCGGCTCGTGGATCGTTGAAGACGCAACTGAACTCGCGCGTCAAG
INLAND21        ATCACTCGGCTCGTGGATCGTTGAAGACGCAGCTGAAAT-GCGCGTCA-G
INLAND22        ATCACTCGGCTCGTGGATCGTTGAAGACGCAGCTGAAAT-GCGCGTCA-G
INLAND23        ATCACTCGGCTCGTGGATCGTTGAAGACGCAGCTGAAAT-GCGCGTCA-G
INLAND31        ATCACTCGGCTCGTGGATCGTTGAATACGCAACTGAGAT-GCGCGTCATA
INLAND32        ATCACTCGGCTCGTGGATCGTTGAATACGCAACTGAGAT-GCGCGTCATA
INLAND33        ATCACTCGGCTCGTGGATCGTTGAAGACGCAGCTTAAAT-GCGCGTCA-G
                ************************** ***** ** *  * ********

COASTAL1        AATGTGAACTGCAGGACACATG-AACACCGATA-AGTTGAAC-GCATATG
COASTAL2        AATGTGAACTGCAGGACACATG-AACACCGATA-AGTTGAAC-GCATATG
COASTAL3        AATGTGAACTGCAGGACACATG-AACACCGATA-AGTTGAAC-GCATATG
INLAND11        AATGTGAACTGCAGGACACATG-AACACCGATA-AGTTGAAC-GCATATG
INLAND12        AATGTGAACTGCAGGACACATG-AACACCGATA-AGTTGAAC-GCATATG
INLAND13        AATGTGAACTGCAGGACACATG-AACACCGATA-AGTTGAAC-GCATATG
INLAND21        AATGTGAACTGCAGGACACATGAAACACCGATA-AGTTGAAC-GCATATG
INLAND22        AATGTGAACTGCAGGACACATGAAACACCGATA-AGTTGAAC-GCATATG
INLAND23        AATGTGAACTGCAGGACACATGAAACACCGATA-AGTTGAAC-GCATATG
INLAND31        AATGTGAACTGCAGGACACATGCAACACCGATATAGTTGAACTGCATATG
INLAND32        AATGTGAACTGCAGGACACATGCAACACCGATATAGTTGAACTGCATATG
INLAND33        AATGTGAACTGCAGGACACATGAA-CACCGATA-AGTTGAAC-GCATATG
                ************************ * ******** ******** *******

COASTAL1        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
COASTAL2        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
COASTAL3        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND11        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND12        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND13        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND21        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND22        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND23        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND31        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND32        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
INLAND33        GCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC
                ************************************************

                ITS2------>
COASTAL1        TAG-GTACTTCGATTTTCCTATAATTAGACTACAGACGGGCGCCACTAAT
COASTAL2        TAG-GTACTTCGATTTTCCTATAATTAGACTACAGACGGGCGCCACTAAT
COASTAL3        TAG-GTACTTCGATTTTCCTATAATTAGACTACAGACGGGCGCCACTAAT
INLAND11        TAG-GTACTTCGATTTTCCTATAATTAGACTACAGACGGGCGCCACTAAT
INLAND12        TAG-GTACTTCGATTTTCCTATAATTAGACTACAGACGGGCGCCACTAAT
INLAND13        TAG-GTACTTCGATTTTCCTATAATTAGACTACAGACGGGCGCCACTAAT
INLAND21        TAGAGTACTGAGAAATTCCTATAACTTGACTACAGACGGCGCCCACAAAC
INLAND22        TAGAGTACTGAGAAATTCCTATAACTTGACTACAGACGGCGCCCACAAAC
INLAND23        TAGAGTACTGAGAAATTCCTATAACTTGACTACAGACGGCGCCCACAAAC
INLAND31        TAGAGTACTGACAAATTCCTATAACTTGACTACTGACGGCGCC-ACTAAC
INLAND32        TAGAGTACTGACAAATTCCTATAACTTGACTACTGACGGCGCC-ACTAAC
INLAND33        TAG-GTACTGAAATATTCCTATAACTTGACTACAGACGGCGCC-ACAAAC
                *** *****   *  ********* * ****** *****   * ** **
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COASTAL1      GGGCTGACGGGTTATCCGTCG-TCTGGCGTGCGACTGTGCAGCATGGCGT
COASTAL2      GGGCTGACGGGTTATCCGTCG-TCTGGCGTGCGACTGTGCAGCATGGCGT
COASTAL3      GGGCTGACGGGTTATCCGTCG-TCTGGCGTGCGACTGTGCAGCATGGCGT
INLAND11      GGGCTGACGGGTTATCCGTCG-TCTGGCGTGCGACTGTGCAGCATGGCGT
INLAND12      GGGCTGACGGGTTATCCGTCG-TCTGGCGTGCGACTGTGCAGCATGGCGT
INLAND13      GGGCTGACGGGTTATCCGTCG-TCTGGCGTGCGACTGTGCAGCATGGCGT
INLAND21      GGGCTGACGGGCCATCCGTCG-TCCGGCGTGCGACTGTGCAGCATGGCGT
INLAND22      GGGCTGACGGGCCATCCGTCG-TCCGGCGTGCGACTGTGCAGCATGGCGT
INLAND23      GGGCTGACGGGCCATCCGTCG-TCCGGCGTGCGACTGTGCAGCATGGCGT
INLAND31      GGGCTGACGGGCCATCCGTCTCTCCGGCGTGCGACTGTGCAACATGGCCT
INLAND32      GGGCTGACGGGCCATCCGTCTCTCCGGCGTGCGACTGTGCAACATGGCCT
INLAND33      GGGCTGACGGGCCATCCGTCG-TCCGGCGTGCGACTGTGCAACATGGCGT
              ********** ******* ** ****************** ****** *

COASTAL1      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGATACTCTGTTT
COASTAL2      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGATACTCTGTTT
COASTAL3      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGATACTCTGTTT
INLAND11      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGATACTCTGTTT
INLAND12      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGATACTCTGTTT
INLAND13      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGATACTCTGTTT
INLAND21      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGACACT--GTTT
INLAND22      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGACACT--GTTT
INLAND23      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGACACT--GTTT
INLAND31      GCTCGGGTCTCGGCGTGGACCCTAGGGCGCTGAAAGTGGACACT--GTTT
INLAND32      GCTCGGGTCTCGGCGTGGACCCTAGGGCGCTGAAAGTGGACACT--GTTT
INLAND33      GCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGACACT--GTTT
              ************************** ****************** ***  ****

COASTAL1      GAGCGGCACCTTTGCGTGTGCTCTCCTAAGTGTCGACGTATGGTGAGGGT
COASTAL2      GAGCGGCACCTTTGCGTGTGCTCTCCTAAGTGTCGACGTATGGTGAGGGT
COASTAL3      GAGCGGCACCTTTGCGTGTGCTCTCCTAAGTGTCGACGTATGGTGAGGGT
INLAND11      GAGCGGCACCTTTGCGTGTGCTCTCCTAAGTGTCGACGTATGGTGAGGGT
INLAND12      GAGCGGCACCTTTGCGTGTGCTCTCCTAAGTGTCGACGTATGGTGAGGGT
INLAND13      GAGCGGCACCTTTGCGTGTGCTCTCCTAAGTGTCGACGTATGGTGAGGGT
INLAND21      G-GCGGCACCTGCGCGTGTGCTCTC---ACTGTTGACGTATGGTGAGGGT
INLAND22      G-GCGGCACCTGCGCGTGTGCTCTC---ACTGTTGACGTATGGTGAGGGT
INLAND23      G-GCGGCACCTGCGCGTGTGCTCTC---ACTGTTGACGTATGGTGAGGGT
INLAND31      G-GCGGCACCTGCGCGTGTGCTCTC---ATTGTTGACGTATGGTGAGGGT
INLAND32      G-GCGGCACCTGCGCGTGTGCTCTC---ATTGTTGACGTATGGTGAGGGT
INLAND33      G-GCGGCACCTGCGCGTGTGCTCTC---AGTGTTGACGTATGGTGAGGGT
              * ********* ************   * *** ****************

COASTAL1      ATTGTCAAGCCGCACGGTGCGACAACACAAGCGTACTGTCGAGTTTGGTG
COASTAL2      ATTGTCAAGCCGCACGGTGCGACAACACAAGCGTACTGTCGAGTTTGGTG
COASTAL3      ATTGTCAAGCCGCACGGTGCGACAACACAAGCGTACTGTCGAGTTTGGTG
INLAND11      AGTGTCAAGCCGCACGGTGCGACAACACAAGCGTACTGTCGAGTTTGGTG
INLAND12      AGTGTCAAGCCGCACGGTGCGACAACACAAGCGTACTGTCGAGTTTGGTG
INLAND13      AGTGTCAAGCCGCACGGTGCGACAACACAAGCGTACTGTCGAGTTTGGTG
INLAND21      ATTGTCAAATCGCACGGTTCGACAACA--AGCGTACCGTCGAGTTTGGTG
INLAND22      ATTGTCAAATCGCACGGTTCGACAACA--AGCGTACCGTCGAGTTTGGTG
INLAND23      ATTGTCAAATCGCACGGTTCGACAACA--AGCGTACCGTCGAGTTTGGTG
INLAND31      ATTGTCAAATCGCACGGTTCGACAACA--AGCGTACCGTCGAGTTTGGTG
INLAND32      ATTGTCAAATCGCACGGTTCGACAACA--AGCGTACCGTCGAGTTTGGTG
INLAND33      ATTGTCAAATCGCACGGTTCGACAACA--AGCGTACCGTCGAGTTTGGTG
              * ******  ******** ********  ******* **************

COASTAL1      CAATCGGATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACAATCG
COASTAL2      CAATCGGATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACAATCG
COASTAL3      CAATCGGATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACAATCG
INLAND11      CAATCGGATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACACTCG
INLAND12      CAATCGGATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACACTCG
INLAND13      CAATCGGATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACACTCG
INLAND21      CAATCGGATGCCTACTACCATGGGCGGAGCCGGGGTGCATTCAACACTCG
INLAND22      CAATCGGATGCCTACTACCATGGGCGGAGCCGGGGTGCATTCAACACTCG
INLAND23      CAATCGGATGCCTACTACCATGGGCGGAGCCGGGGTGCATTCAACACTCG
INLAND31      CAATCGGATGCCTACTACCATGGGCGGAGCCGGCGTGCATTCAACACTCG
INLAND32      CAATCGGATGCCTACTACCATGGGCGGAGCCGGCGTGCATTCAACACTCG
INLAND33      CAATCGGATGCCTACTACCATGGGCGGAGCCGGCGTGCATTCAACACTCG
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COASTAL1        ACGTG--TGCGTC--CTG--TATCAACCGGAT-------GCCAAC-----
COASTAL2        ACGTG--TGCGTC--CTG--TATCAACCGGAT-------GCCAAC-----
COASTAL3        ACGTG--TGCGTC--CTG--TATCAACCGGAT-------GCCAAC-----
INLAND11        ACGTG--TGCGTC--CTG--TATCAACCGGAT-------GCCAAC-----
INLAND12        ACGTG--TGCGTC--CTG--TATCAACCGGAT-------GCCAAC-----
INLAND13        ACGTG--TGCGTC--CTG--TATCAACCGGAT-------GCCAAC-----
INLAND21        ACGT-CTTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAAA
INLAND22        ACGT-CTTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAAA
INLAND23        ACGT-CTTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAAA
INLAND31        ACGTTCCTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAAA
INLAND32        ACGTTCCTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAAA
INLAND33        ACGT-CCTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAGA
                ****   **  **  *  *  *  ****  * *       ***  *

COASTAL1        ----------------------------------------TGCTG----TCA-
COASTAL2        ----------------------------------------TGCTG----TCA-
COASTAL3        ----------------------------------------TGCTG----TCA-
INLAND11        ----------------------------------------TGCTG----TCA-
INLAND12        ----------------------------------------TGCTG----TCA-
INLAND13        ----------------------------------------TGCTG----TCA-
INLAND21        CAGGACACTTGAATCAACCTTGGTGGTACACCCCACATGTTGGTGGTCAA
INLAND22        CAGGACACTTGAATCAACCTTGGTGGTACACCCCACATGTTGGTGGTCAA
INLAND23        CAGGACACTTGAATCAACCTTGGTGGTACACCCCACATGTTGGTGGTCAA
INLAND31        CAGGACACT-GAATCGATCTTGGTGGTACAACCCACATGTTGGTGGTCAA
INLAND32        CAGGACACT-GAATCGATCTTGGTGGTACAACCCACATGTTGGTGGTCAA
INLAND33        CAGGACACT-GAATCGATCTTGGTGGTACACCC-ACATGTTGGTTGTCAA
                                                  ** **    ***

COASTAL1        GTTGGTGGTGT------CCGCGC--AAACA-------------GGACGC-
COASTAL2        GTTGGTGGTGT------CCGCGC--AAACA-------------GGACGC-
COASTAL3        GTTGGTGGTGT------CCGCGC--AAACA-------------GGACGC-
INLAND11        GTTGGTGGTGT------CGGCGC--AGACA-------------GGACGC-
INLAND12        GTTGGTGGTGT------CGGCGC--AGACA-------------GGACGC-
INLAND13        GTTGGTGGTGT------CGGCGC--AGACA-------------GGACGC-
INLAND21        GTTG-TGGTGTAACTATCGGAGTCAAAACATGATGGCGGCGATGGACGCC
INLAND22        GTTG-TGGTGTAACTATCGGAGTCAAAACATGATGGCGGCGATGGACGCC
INLAND23        GTTG-TGGTGTAACTATCGGAGTCAAAACATGATGGCGGCGATGGACGCC
INLAND31        GTTG-TGGTGTAACTATCGGAGTCAAAACATGATGGCGGCGATGGACGCC
INLAND32        GTTG-TGGTGTAACTATCGGAGTCAAAACATGATGGCGGCGATGGACGCC
INLAND33        GTTGGTGGTCTAACTATCGGAGTCAAAACATGATGGCGGCGATGGACGCC
                **** **** *       * * *   * ***                ******

COASTAL1        ---GCGCGTACGCTTGA-GTC---------GTGTAACGCGTG----CGAC
COASTAL2        ---GCGCGTACGCTTGA-GTC---------GTGTAACGCGTG----CGAC
COASTAL3        ---GCGCGTACGCTTGA-GTC---------GTGTAACGCGTG----CGAC
INLAND11        ---GCGCGTACGCTTGA-GTC---------GTGTAACGCGTG----CGAC
INLAND12        ---GCGCGTACGCTTGA-GTC---------GTGTAACGCGTG----CGAC
INLAND13        ---GCGCGTACGCTTGA-GTC---------GTGTAACGCGTG----CGAC
INLAND21        ACXGCACTCACCCCCTACGTCCCTCGCTGAGTGTATCGTGTGTTATCCAT
INLAND22        ACXGCACTCACCCCCTACGTCCCTCGCTGAGTGTATCGTGTGTTATCCAT
INLAND23        ACXGCACTCACCCCCTACGTCCCTCGCTGAGTGTATCGTGTGTTATCCAT
INLAND31        ACXGCACTCACCCCCTACGTCCCTCGCTGAGTGTATCGTGTGTTATCCAT
INLAND32        ACXGCACTCACCCCCTACGTCCCTCGCTGAGTGTATCGTGTGTTATCCAT
INLAND33        ACXGCACTCACCCCCTACGTCCCTCGCTGAGTGTATCGTGTGTTATCCAT
                   ** *  ** *   * ***           ***** ** ***    * *

COASTAL1        CCATACACGTACCTGCTTGAGCTATGCGTTGCGAGTTGGAGAGTTGCCAG
COASTAL2        CCATACACGTACCTGCTTGAGCTATGCGTTGCGAGTTGGAGAGTTGCCAG
COASTAL3        CCATACACGTACCTGCTTGAGCTATGCGTTGCGAGTTGGAGAGTTGCCAG
INLAND11        CCATACACGTACCTGCTTGAGCTATGCGTTGCGAGTTGGAGAGTTGCCAG
INLAND12        CCATACACGTACCTGCTTGAGCTATGCGTTGCGAGTTGGAGAGTTGCCAG
INLAND13        CCATACACGTACCTGCTTGAGCTATGCGTTGCGAGTTGGAGAGTTGCCAG
INLAND21        CCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGAT-GAGAG
INLAND22        CCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGAT-GAGAG
INLAND23        CCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGAT-GAGAG
INLAND31        CCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGAT-GAGAG
INLAND32        CCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGAT-GAGAG
INLAND33        CCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGAT-GAGAG
                ******** ****** ******* *******          *** * * **
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COASTAL1        ACGGAGGAAATACTACAATTACTGGCCGTAGGCCTTCAAGTGAATGT-TG
COASTAL2        ACGGAGGAAATACTACAATTACTGGCCGTAGGCCTTCAAGTGAATGT-TG
COASTAL3        ACGGAGGAAATACTACAATTACTGGCCGTAGGCCTTCAAGTGAATGT-TG
INLAND11        ACGGAGGAAATACCACAAT-ATC--CAGTAGGCCTC--AATGA-TGTGTG
INLAND12        ACGGAGGAAATACCACAAT-ATC--CAGTAGGCCTC--AATGA-TGTGTG
INLAND13        ACGGAGGAAATACCACAAT-ATC--CAGTAGGCCTC--AATGA-TGTGTG
INLAND21        TTGCCAAACACACAGACCACACTCCAGTTAGGCCTTCAAAGTGATAGTTG
INLAND22        TTGCCAAACACACAGACCACACTCCAGTTAGGCCTTCAAAGTGATAGTTG
INLAND23        TTGCCAAACACACAGACCACACTCCAGTTAGGCCTTCAAAGTGATAGTTG
INLAND31        TTGCCAAACACACAGACCACACTCCAGTTAGGCCTC--AAGTGATAGTTG
INLAND32        TTGCCAAACACACAGACCACACTCCAGTTAGGCCTC--AAGTGATAGTTG
INLAND33        TTGCCAAACACACAGACCACACTCCAGTTAGGCCTC--AAGTGATAGTTG
                 *    * * **        *        *******   *      *   **
                                  <----ITS2

                                28S primer
                              ---------------------->
COASTAL1            ACTACCCCCTAAATTTAAGCAT
COASTAL2            ACTACCCCCTAAATTTAAGCAT
COASTAL3            ACTACCCCCTAAATTTAAGCAT
INLAND11            ACTACCCCCTAAATTTAAGCAT
INLAND12            ACTACCCCCTAAATTTAAGCAT
INLAND13            ACTACCCCCTAAATTTAAGCAT
INLAND21            ACTACCCCCTAAATTTAAGCAT
INLAND22            ACTACCCCCTAAATTTAAGCAT
INLAND23            ACTACCCCCTAAATTTAAGCAT
INLAND31            ACTACCCCCTAAATTTAAGCAT
INLAND32            ACTACCCCCTAAATTTAAGCAT
INLAND33            ACTACCCCCTAAATTTAAGCAT
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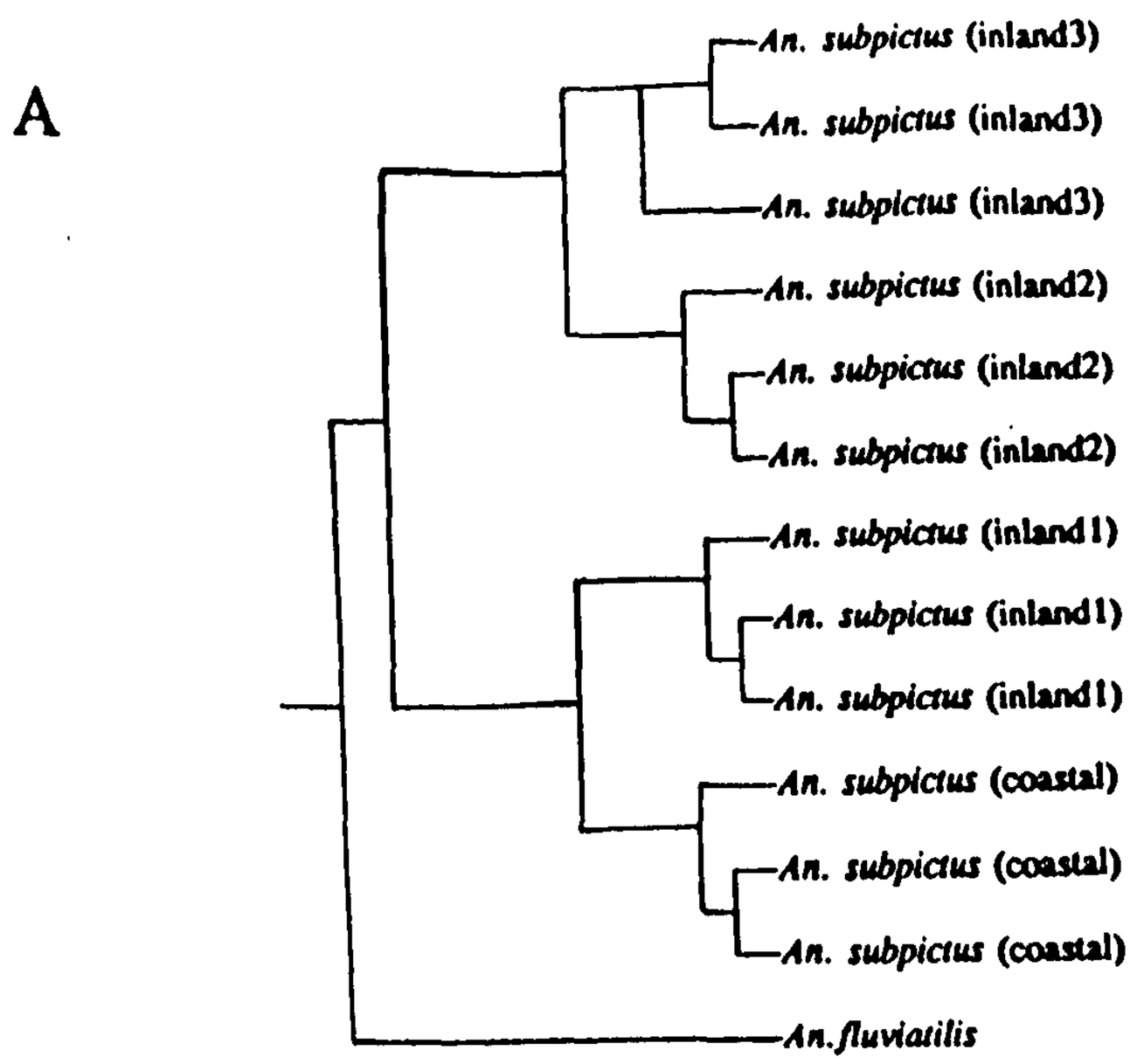
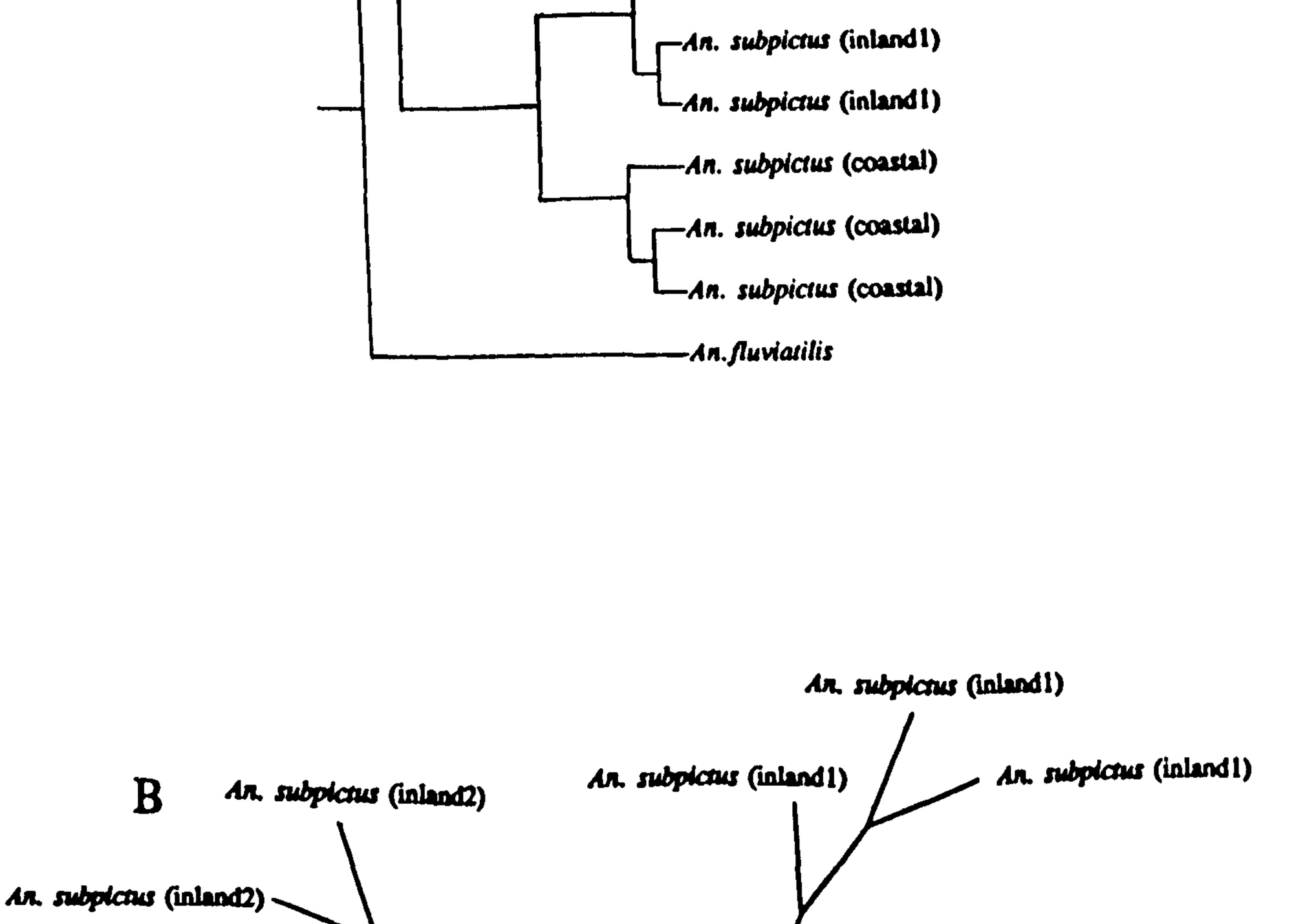
Fig. 7.11- Phylogenetic tree based on amplified fragment sequence of *An. subpictus* populations and An. fluviatilis as out-group, using A) DNAPARS and B) DRAWTREE in PHYLIP program.





CHAPTER EIGHT

GENETIC VARIATION IN

ANOPHELES STEPHENSI

8.1 ABSTRACT

Anopheles stephensi Liston 1901, a major malaria vector in Middle East and Indian sub-continent is not currently recognized as species complex, but there are three described forms in this species known as urban, rural and intermediate. RAPD and SSR primers have been applied to address the issues of genetic variation in field collected specimens and laboratory strains, differences between urban and rural populations and the inheritance of RAPDs in F1 progenies of *An. stephensi* laboratory strains.

The results showed that RAPD can readily differentiate seven laboratory strains. Individuals collected from Iran and Tadjikestan have more similar patterns than those produced in Pakistan specimens.

Experiments with urban and rural populations of *An. stephensi* revealed simple population-specific markers in each group. Although this result is promising but it needs further investigation in order to find their relatedness to three reported races in India.

The findings of the progeny experiment, showed the power of RAPD by

amplification of consistently reproducible patterns in parents and three life stages of progenies, which is useful to address the relatedness of laboratory crosses. This study confirmed the inheritance of RAPD bands in a dominant fashion, although the percentage of non-parental bands is higher than previously reported.

8.2 INTRODUCTION

An. stephensi Liston, 1901, is the major urban vector of malaria in India, Pakistan, Iran, and Iraq. It has also been reported from Afghanistan, China, Myanmar, Nepal, Taiwan, and Thailand is known as a polytypic species. Earlier, Sweet & Rao (1937) classified it into two varieties on the basis of ridges on egg float. These were named *An. stephensi stephensi* or type form and *An. stephensi mysorensis*. The type form is mainly urban whereas mysorensis is rural. The study of inversion polymorphism contributed to a better interpretation of bionomical variations in *An. stephensi*. Especially observations of Coluzzi *et al.* (1973) indicated that chromosomal variants might be associated with differences between rural and urban populations of this species described by Sweet & Rao (1937). Aslamkhan (1973) reported sex chromosomes and later it was in 1979 that Sharma *et al.* isolated and genetically analyzed two new mutant, *red-eye* and *greenish brown-larvae* in *An. stephensi*. They suggested red-eye as the first sex-linked mutant in *An. stephensi*.

Following the work of Coluzzi's group in Rome, Subbarao (1985) reported polytene chromosome inversion contrasts between rural and urban populations of *An. stephensi*. However, more interestingly, in 1992, Suguna reported the occurrence of two different types of Y chromosomes in morphologically similar *An. stephensi*, associated with contrasted malaria vector status in two urban areas, where larvae of this species were collected from the wells in two cities of South India.

Cuticular hydrocarbons comparison of four *An. stephensi* strains originated

from former USSR, India, Pakistan and Iraq indicated that the four groups were distinct and that, on average, 78% of the population could be separated on the basis of the quantities of some of the cuticular hydrocarbons (Anyanwu *et al.* 1993).

In 1995, Setty, *et al.* isolated a new larval colour mutant, brown larva (b), from the Bangalore, India strain of *Anopheles stephensi* Liston. The gene b is an autosomal recessive with uniform expression and complete penetrance. They conducted extensive crosses to establish allelism between brown larva (b) and green larva (g) reported previously in *An. stephensi* from our laboratory. The wild-type is dominant to green larva, which, in turn, is dominant to brown larva. These larval colour mutants belong to an allelic series.

Recent genetic studies have revealed three races, i.e. *An. stephensi stephensi*, *An. stephensi mysorensis* and *An. stephensi* intermediate (Nagpal & sharma 1995). This species in urban areas predominantly breeds in wells, overhead or ground-level water tanks, cisterns, tanks,coolers, roof gutters and other artificial containers. Scanty breeding is also reported in recent years from rice-fields, and polluted and brakhish water habitats. Roberts (1996) reported that Larvae of *An. stephensi* Liston and *An. culicifacies* Giles survived best in fresh water, but some *An. stephensi* were able to tolerate up to 50% sea water and the females had a similar ovipositional preference for fresh water.

Larvae are shade lovers. Adults rest in houses, cattlesheds and barracks. They feed predominantly on cattle in rural areas and human in urban areas (Nagpal & Sharma 1995).

This mosquito is resistant to DDT, HCH and Malathion in many areas. In larvae of *An. stephensi*, DDT resistance of 30-40 fold, involving no cross-resistance to pyrethroids, showed fully dominant monofactorial inheritance (Malcolm 1990).

An. stephensi main identification characters:

1 Apical and subapical pale bands equal and separated by a dark band.

2 Plapi with speckling.

3 foreleg tarsomeres without broad bands.

4 legs with speckling.

5 Thorax with broad scales.

8.3 MATERIALS AND METHODS

8.3.1 Mosquitoes

DNA has been amplified from dried specimens from the field and museum collections of *An. stephensi* from urban and rural areas in Iran, collected by Dr. H. Ladonni and other staff in Tehran Medical Sciences University, or provided by Prof. Zaim. Dr Mark Rowland kindly sent some specimens which have been collected during an ecological study in Pakistan. other specimens came from seven laboratory strains of *An. stephensi* from Iran (BAN, TEH), Iraq (IRQ), Dubai (APR, LPR), India (IND-S, BEECH) all reared in LSTM insectary, one strain from Tadzhikestan (probably originally from India) and a strain homozygous for the red-eye mutant of sent by Prof. C. Curtis (LSTMH). Fig.3.1 and Fig.8.1 show the origin of specimens applied in this study.

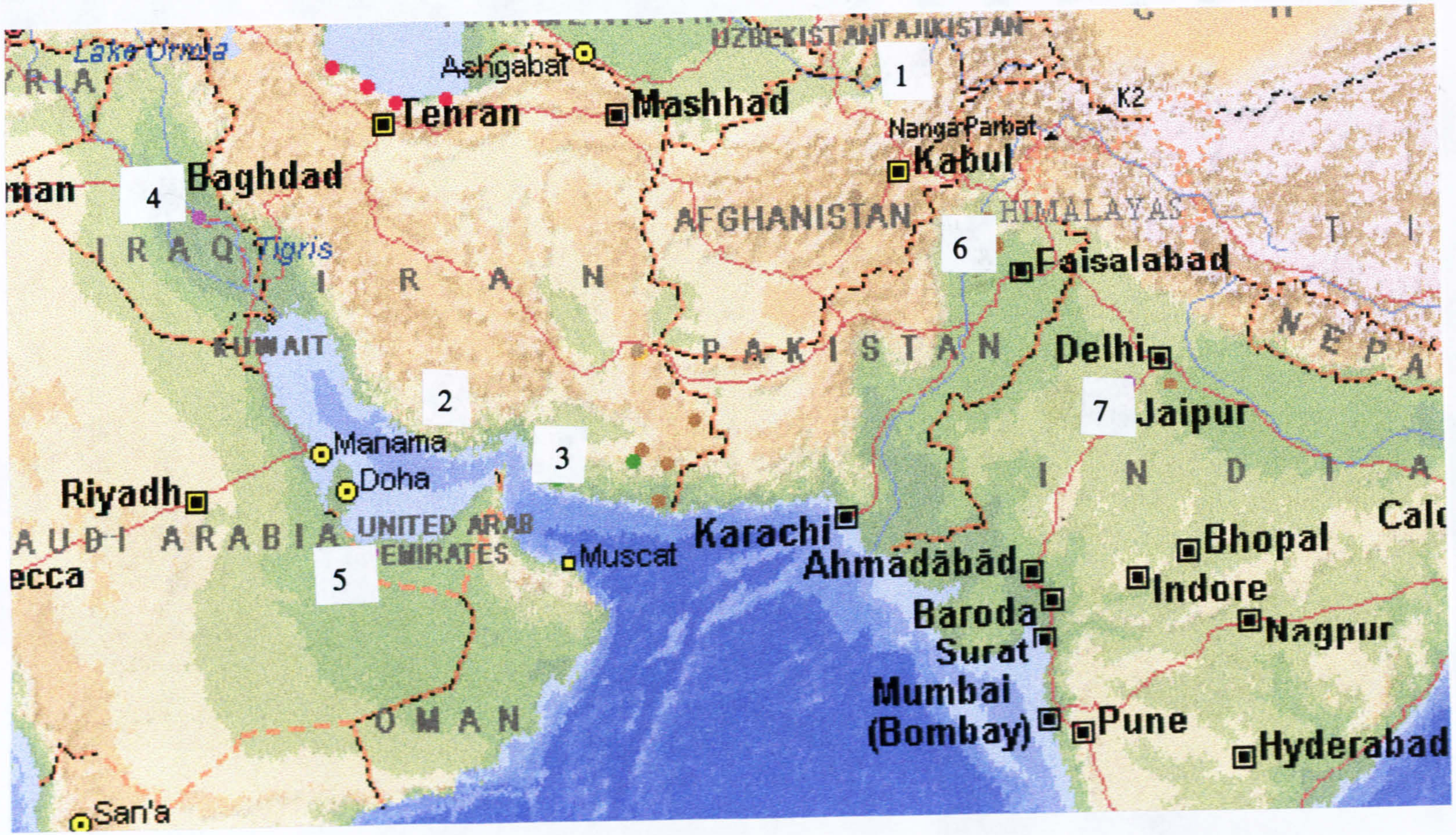
8.3.2 Genomic DNA extraction

Both a phenol extraction method (Ballinger-Crabtree 1992) and the method described by Collins *et al.* (1987) have been used to extract DNA from individual mosquitoes. Air-dried pellets of DNA were subsequently resuspended in 100 μl of double distilled H_2O or TE buffer and stored at 4°C.

8.3.3 Primers :

17 RAPD primer from the UBC (University of British Columbia, Canada) and

Fig. 8.1- Origin of *An. stephensi* specimens have been used in this study:
Tadjikestan (1), Iran (2,3), Iraq (4), Dubai (5), Pakistan (6), India (7)



two SSR primer were used. These had been found useful in our laboratory in studying other anopheline species complexes. Table 7.1 lists the names, sequences and GC content of the primers for which results are presented.

8.3.4 DNA amplification and PCR products gel electrophoresis

All RAPD reactions were performed in a total volume of 25 μl. Each mixture contained 2.5 μl of 10X reaction buffer, 2 mM $MgCl_2$, 10-100 ng of primer, 0.001% gelatin, 0.1 mM each of dATP, dTTP, dCTP, and dGTP, 0.5 unit of promega Taq polymerase, and sterile double-distilled water to 25 μl. Reactions were overlaid with 50 ul of mineral oil and amplified in a Hybaid Omnigene thermal cycler.

The RAPD programme used was :(1) one cycle of 94°C for 5 min; (2) 15 cycles of 94°C for 30 sec, 36°C for 30 sec, 72°C for 30 sec; (3) 20 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec; (4) one cycle of 72°C for 7 min (Prog 6, Ch.3). Following PCR, 10 μl of amplified DNA mixed with Ficol/orange G loading buffer and electrophoresed in 6% polyacrylamide (acrylamide gel for runing the PCR products and silver stain procedure is as mentioned in Ch.3) or 1% agarose (Appligen) in TBE buffer containing ethidium bromide at 1.5 v/cm for 16 hours (large gel) or 5 v/cm for two hours (mini gel). A mixture of BglI and HinfII digest of pBR328 (Boehringer Mannheim) was used as molecular weight marker for PCR product size determination (molecular weights 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154 bp). Gels visualised under UV light and photographed with black and white Polaroid 55 film or Ilford FP4 film with a

Table 8.1 selected primers and their sequences used in the study of genetic variation in *An. stephensi*

PRIMER	5'.....SEQUENCE.....3'	GC%
M13	GTAAAACGACGGCCAGT	53
OPA1	CAGGCCCTTC	70
OPA8	GTGACGTAGG	60
UBC301	CGGTGGCGAA	70
UBC302	GCGGGAGACC	80
UBC303	CGGCCCACGT	80
UBC304	AGTCCTCGCC	70
UBC306	GTCCTCGTAG	60
UBC319	GTGGCCGCGC	90
UBC345	GCGTGACCCG	80
UBC353	TGGGCTCGCT	70
AB1	GTTTCGCTCC	60
AB4	GGACTGGAGT	60
AB11	GTAGACCCGT	60
AB13	TTCCCCCGCT	70
AB15	GGAGGGTGTT	60
AB19	ACCCCCGAAG	70
X18	$(GT)_7TGTA$	44
X19	$(AGT)_5GCCA$	42

Polaroid MP4 land camera. .

8.3.5 Isoenzyme analysis:

Starch and acrylamide gels were prepared as described in material and method (Ch.3). The following enzymes were used: ODH, EST, PGM, HEX, SOD, LDH (details in Ch.3), with EST being visualised on starch gels and the others on acrylamide gels.

8.3.6 Crossing experiment

This experiment carried out by two strains of *An. stephensi* originated from India (IND-S) and Dubai (LPR). IND-s was a susceptible strain and LPR has been selected for resistant to insecticides. Although forced mating has been applied, the results of this part of study are based on natural mating between a female IND-S and a male LPR which their F1 progeny in larval, pupal and adult stages have been collected and after DNA extraction were used for amplification with RAPD and SSR primers. The same method has been used for crossing experiment with IND-S (female parent) and red-eye (male parent).

8.4 RESULT

8.4.1 Isoenzyme:

Four loci have been detected based on *EST* enzyme activity in 5 laboratory stocks: BAN, LPR, APR, IRQ, and TEH on starch gel (Fig. 8.2). Four loci have been found in these strains and one out of four loci has shown two allelic forms as faster mobility in BAN and TEH (originated from Iran) than in *An. stephensi* strains from Iraq and Dubai, while other loci have same patterns in all five strains.

ODH enzyme produced three identical loci which allow the differentiation of *An. stephensi* strains from Iraq, Dubai, and Iran (Fig. 8.3).

Results with isoenzyme proved useful in differentiation of laboratory strains, but its main limitation is the need for using live mosquitoes or those kept in -70°C. With *LDH* enzyme in the progeny of a cross between IND-S and LPR strains both parental bands were seen as shown in below:

Female parent	Male parent	larva (F1)	Adult (F1)
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8.4.2 RAPD-PCR: Fingerprints of seven laboratory strains:

In order to look for evidence of intra-specific variation within seven laboratory stocks of *An. stephensi*, over 20 primers from different commercially available series (OPA, UBC, AB01) have been tested.

Fig. 8.2- EST enzyme activity in 5 strains of *An. stephensi*:
BAN (1,5), LPR (2,9), IRQ (6), APR (7), TEH (8), lanes 3-4
are *An. gambiae* s.s. strains (16CSS and KWA).

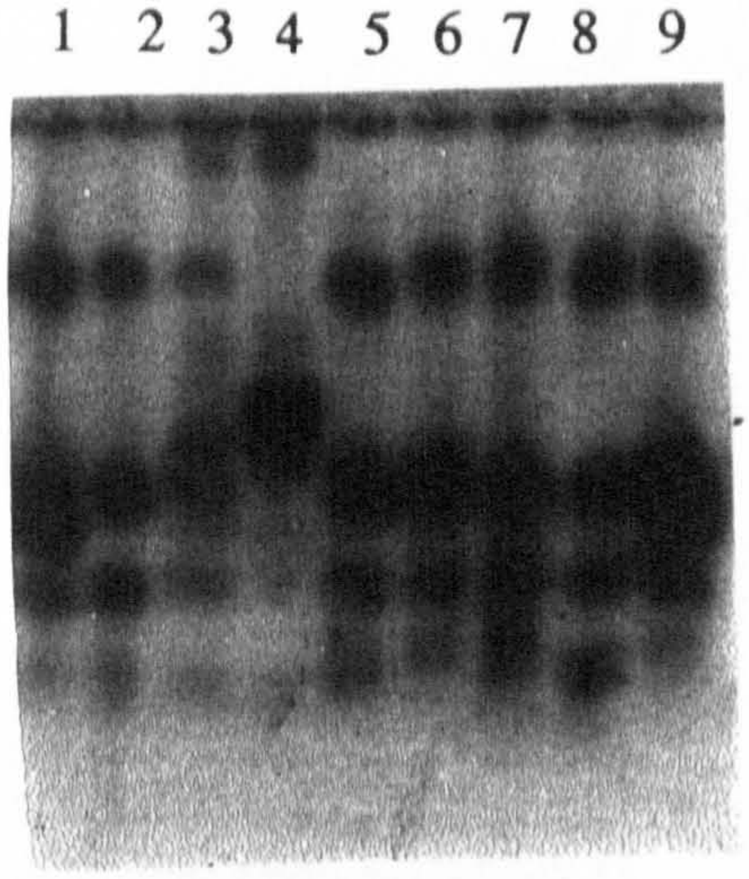
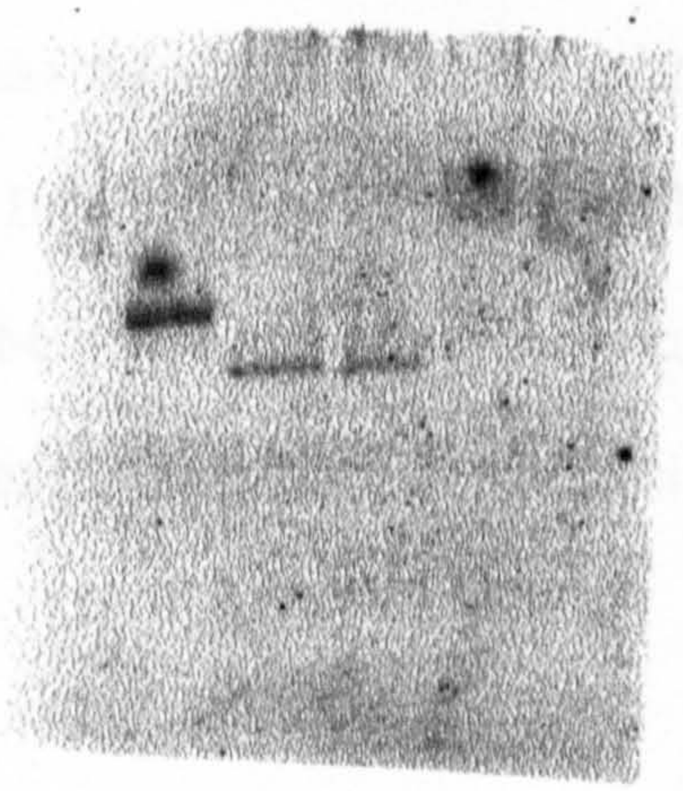


Fig. 8.3- *odh* enzyme activity in 5 strains of *An. stephensi*: DUBAI (1), IRQ (2), *An. gambiae* strains 16CSS & KWA (3-4), BAN (5), TEH(6).

1 2 3 4 5 6



AB11

Of these primers, AB11 produced differentiation patterns for BAN, TEH, LPR, APR, IRQ, IND-S, and BEECH (Fig. 8.4). Even in strains originated from the same country, although there are some common bands, other site-specific fragments are detectable. For example, LPR and APR which originated from Dubai have eight common bands, but LPR has four and APR one specific fragment. Variation is greater in the strains from Iran examined (BAN and TEH) with seven bands not shared. IND-S and BEECH both of which originate from India, share two main 1570 and 730bp fragments whereas a 800bp fragment is specific for IND-S.

UBC-303

Another RAPD primer, UBC-303, readily differentiates laboratory stocks of *An. stephensi* (Fig. 8.5) with a 320 bp common band, and the following main bands scored in each strain:

TEH: 320, 580, 730

IRQ: 320, 410, 710

APR: 320, 410, 680, 730

BEECH: 320, 420, 730

IND-S: 320, 420, 580, 730

When 303 primer products were run on an acrylamide gel and silver stained, better resolution of bands is detectable in the range of 230-450bp (Fig. 8.6). Again strain specific differences can be seen.

The other RAPD primers examined fall into two groups based on their products: 1)

Fig. 4- RAPD-6-AB11 amlification of DNA extracted from seven laboratory colonies originated from Middle East and Indian sub-continent: Ban (1-2), TEH (3-5), LPR (6-7), APR (8-10), IRQ (12-14), IND-S (15-17), BEECH (18-20), Marker (11). Populationhas-specific patterns allow their differentiation even between those originated from the same country.

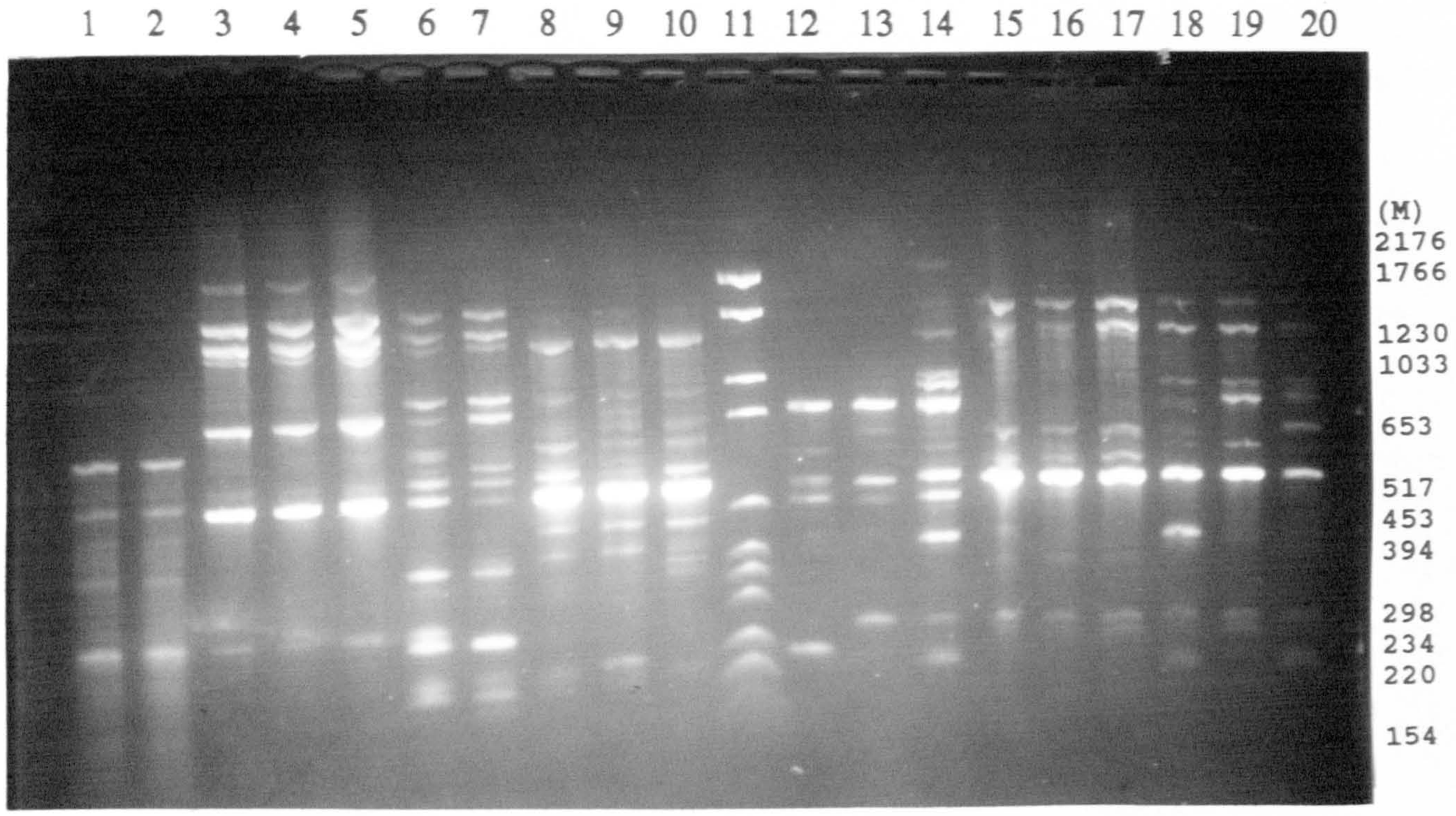


Fig. 8.5- RAPD-1-303 products in *An. stephensi* strains in a two steps annealing (36, 50°C) program. TEH (1-2), IRQ (3-4), APR (5-6), BEECH (7-8), IND-S (9-10); Marker (12); -ve (11). This primer differentiate five examined strains with population-specific bands. A 320bp fragment is present in all specimens indicate with arrow.

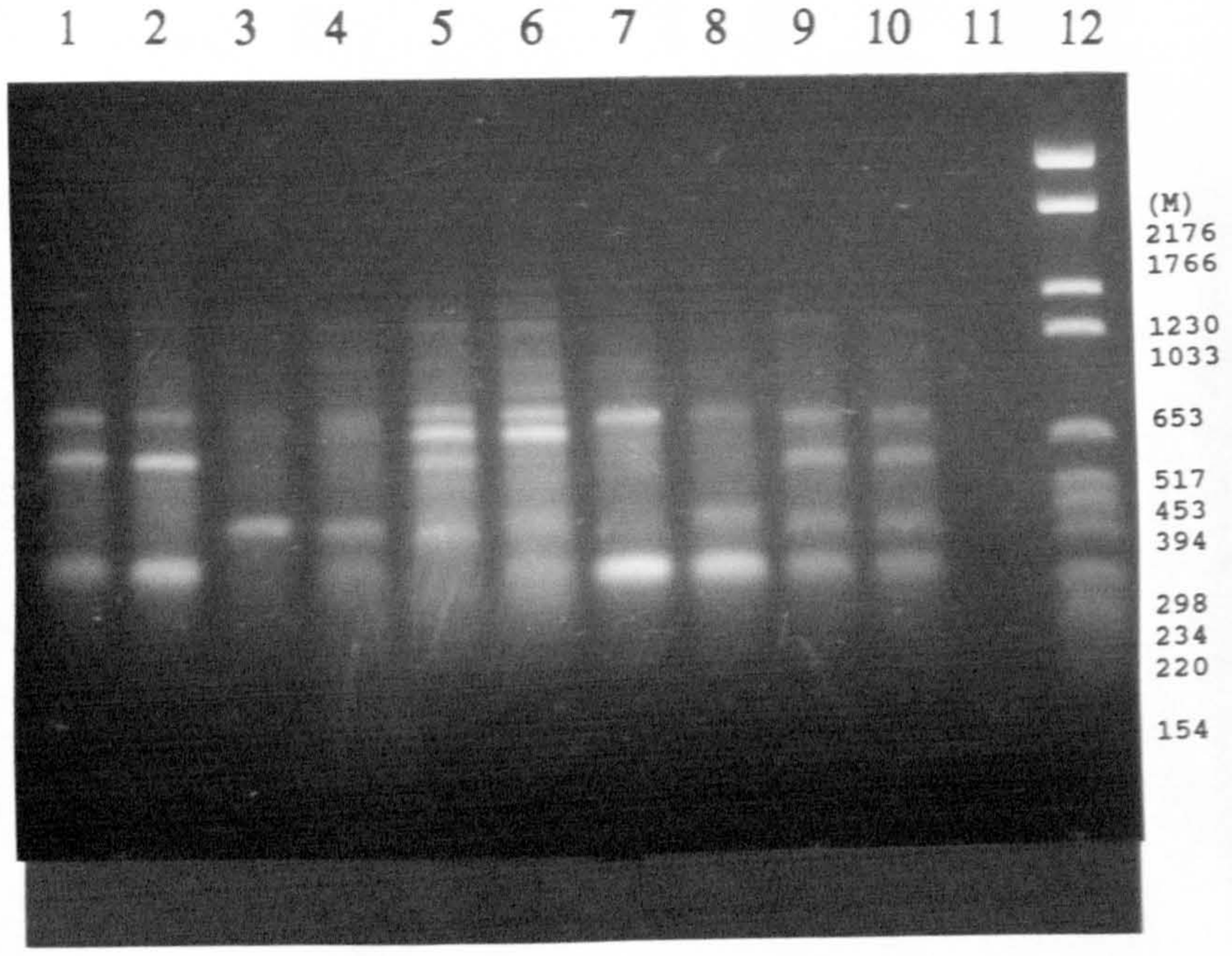
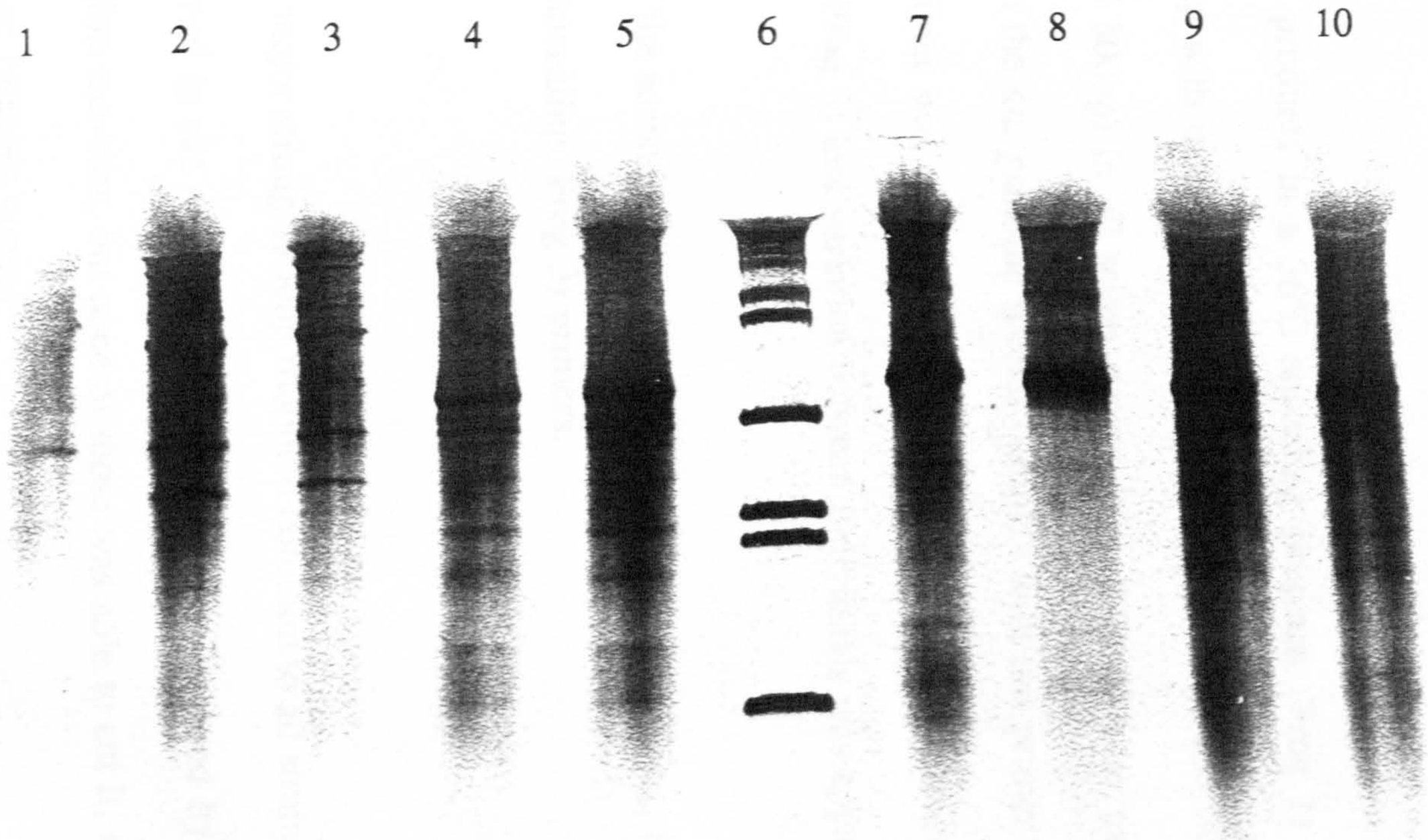


Fig.6- RAPD-6-303 products in *An. stephensi* strains in a two steps annealing (36, 50°C) program run on a 6% acrylamide gel. IRQ (1), APR (2-3), TEH (4-5), BEECH (7-8), IND-S (9-10); Marker (6)



those primers which showed extensive variation between individuals of each strain and, 2) primers which produced common patterns in different strains.

M13

The M13 primer products in a 36°C annealing program (Prog 1) are an example of the first group with more variation in individual patterns and two main common bands (450 and 800bp) in all seven strains (Fig. 8.7). Similar extensive variation has been seen in the *An. gambiae* species complex with this primer (Ch.4). However, when M13 primer was used in a program with two different annealing temperatures (50, 36°C- Prog 3) less variation between individuals was apparent.

Two examples of the second group are: AB13 (in 36°C annealing, Prog 1), and OPA1 (in 50, 36°C annealing, Prog 3) primers.

AB13

AB13 produced a major strong 1230bp fragment common to all strains of *An. stephensi* (Fig. 8.8). I tried to cut AB13 primer 1230bp common band by EcoRI, DraI and HindIII restriction enzymes, but none of those was able to cut it, although these selected enzymes are frequent cutters.

OPA1

The OPA1 primer showed two main common bands (285 and 1040bp) in these strains (Fig. 8.9) and some other faint fragments.

Fig. 8.7- RAPD-6-M13 amplification of DNA extracted from seven laboratory colonies originating from Middle East and Indian sub-continent: Teh (1-4), LPR (5-7), APR (8-10), IRQ (12-13), BEECH (14-15), IND-S (16-18), BNA(19-20), Marker (11). This primer showed more variation within individuals of each population. However, all strains share two 450 and 800bp fragments.

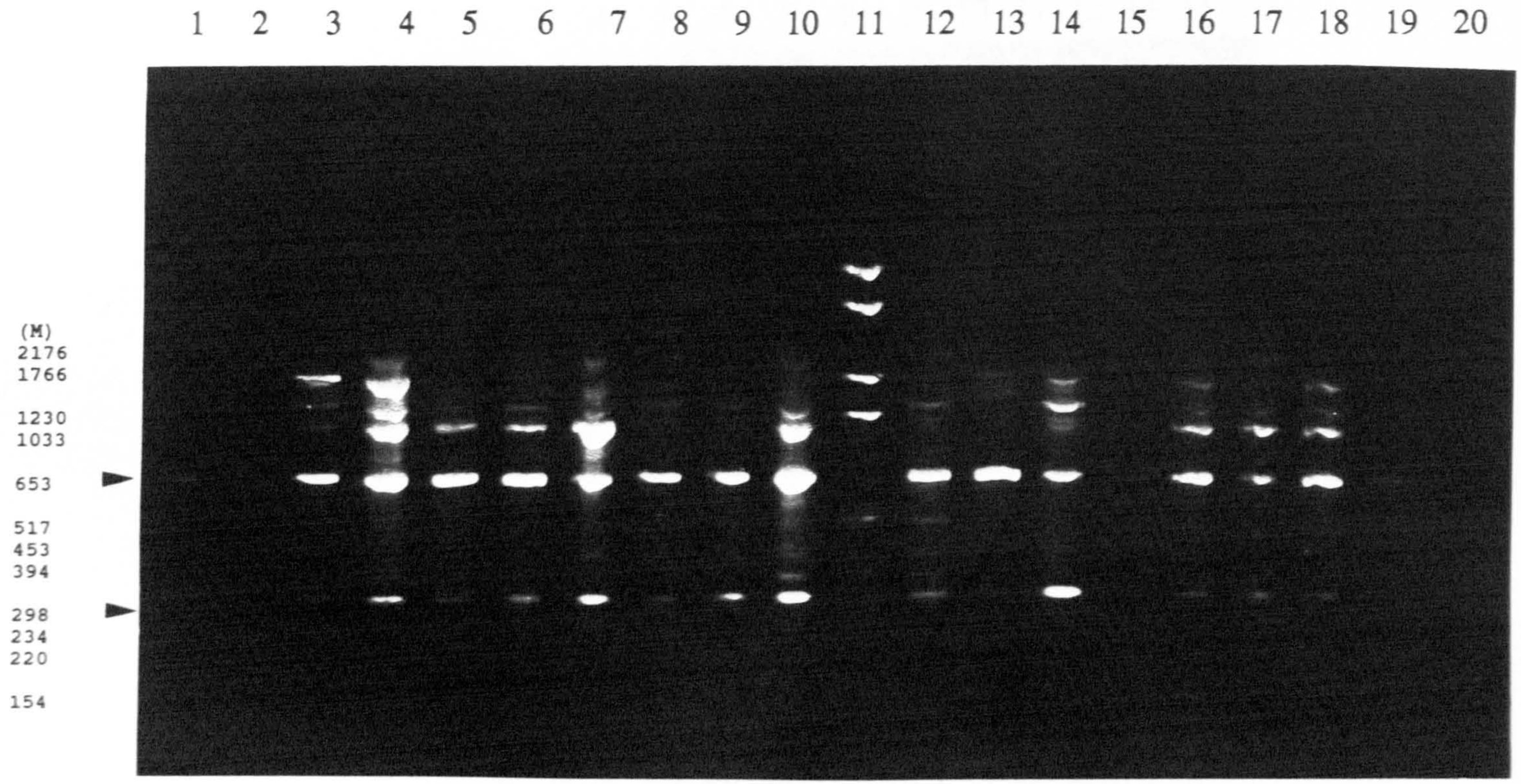


Fig. 8.8- RAPD-6-AB13 primer products in *An. stephensi* strains in a two steps annealing (36, 50°C) program: BAN (1), TEH (2), LPR (3,4), APR (5,6), IRQ (8-9), IND-S (10-11), BEECH (12-13); Marker (7); -ve (14). This primer produce a major 1230bp band in all strains.

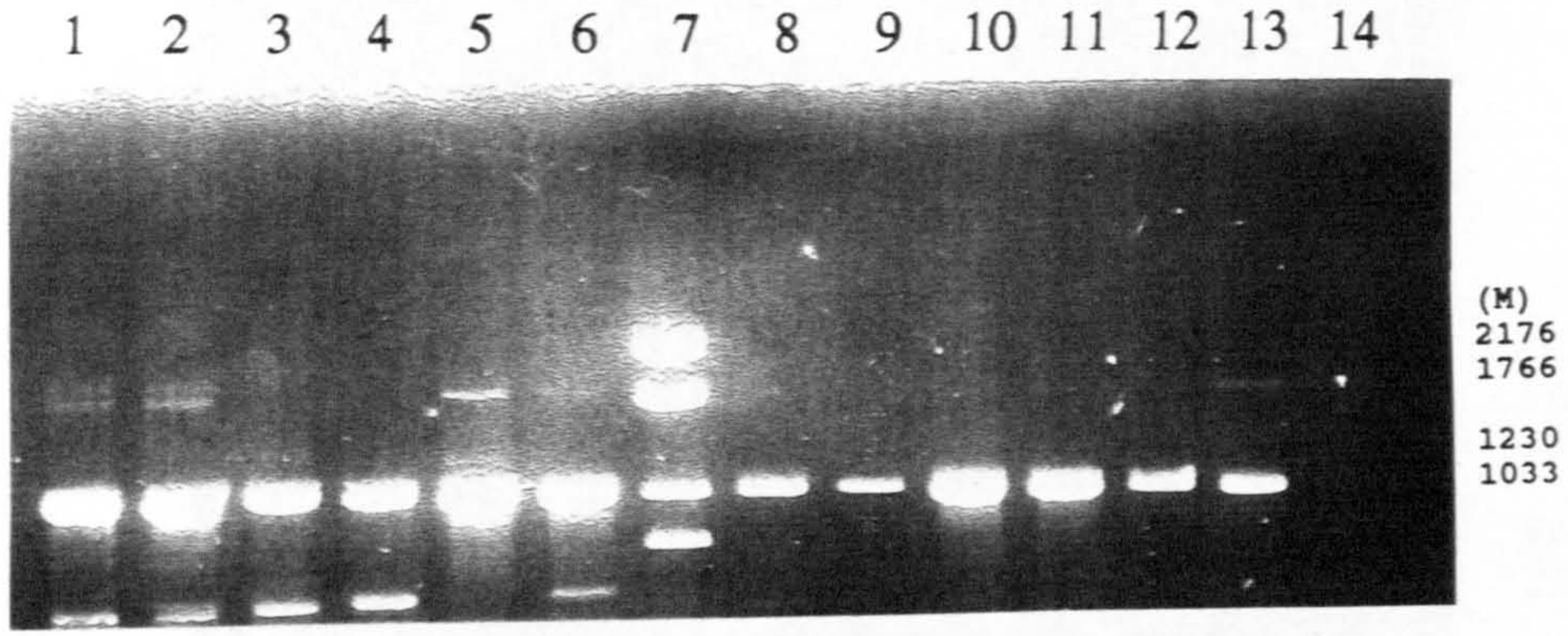
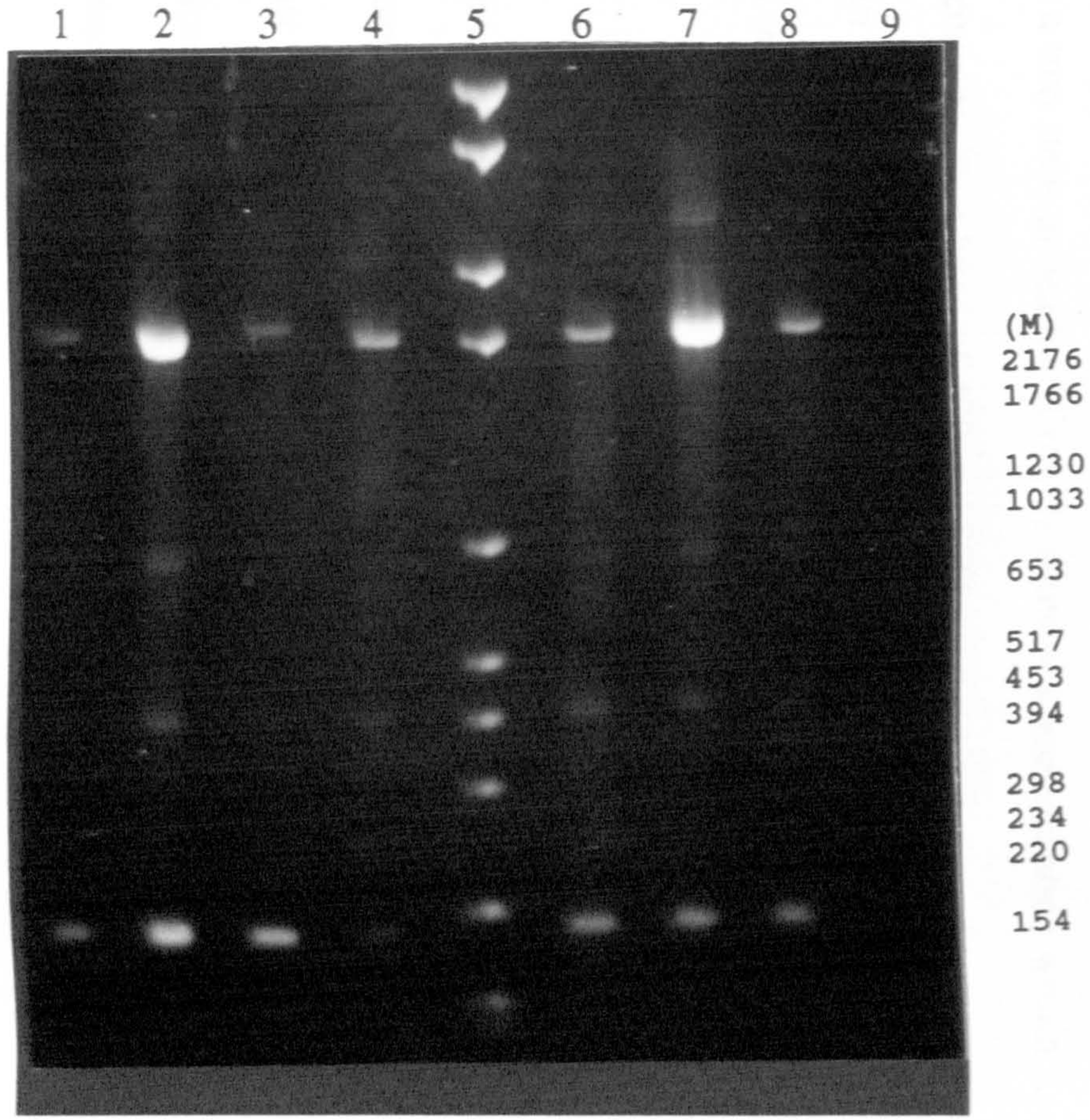


Fig. 8.9- RAPD-1- primer OPA1 products in *An. stephensi* strains in a two steps annealing (36, 50°C) program. BAN (1), TEH (2), LPR (3), APR (4,6), BEECH (7)), IND-S (8); Marker (5); -ve (9). All strains share two 285 and 1040bp common bands.



8.4.3 RAPD-PCR with *An. stephensi* field materials:

DNA extracted from dried field specimens collected in Iran, Pakistan and Tadzhikestan have been tested with RAPD primers.

M13F

This primer in a two steps annealing (Prog 3) showed some variation within specimens from each country. Two band of 400 and 800bp are common to the three countries. *An. stephensi* from Tadzhikestan have other shared bands with either Iran or Pakistan specimens (Fig not included).

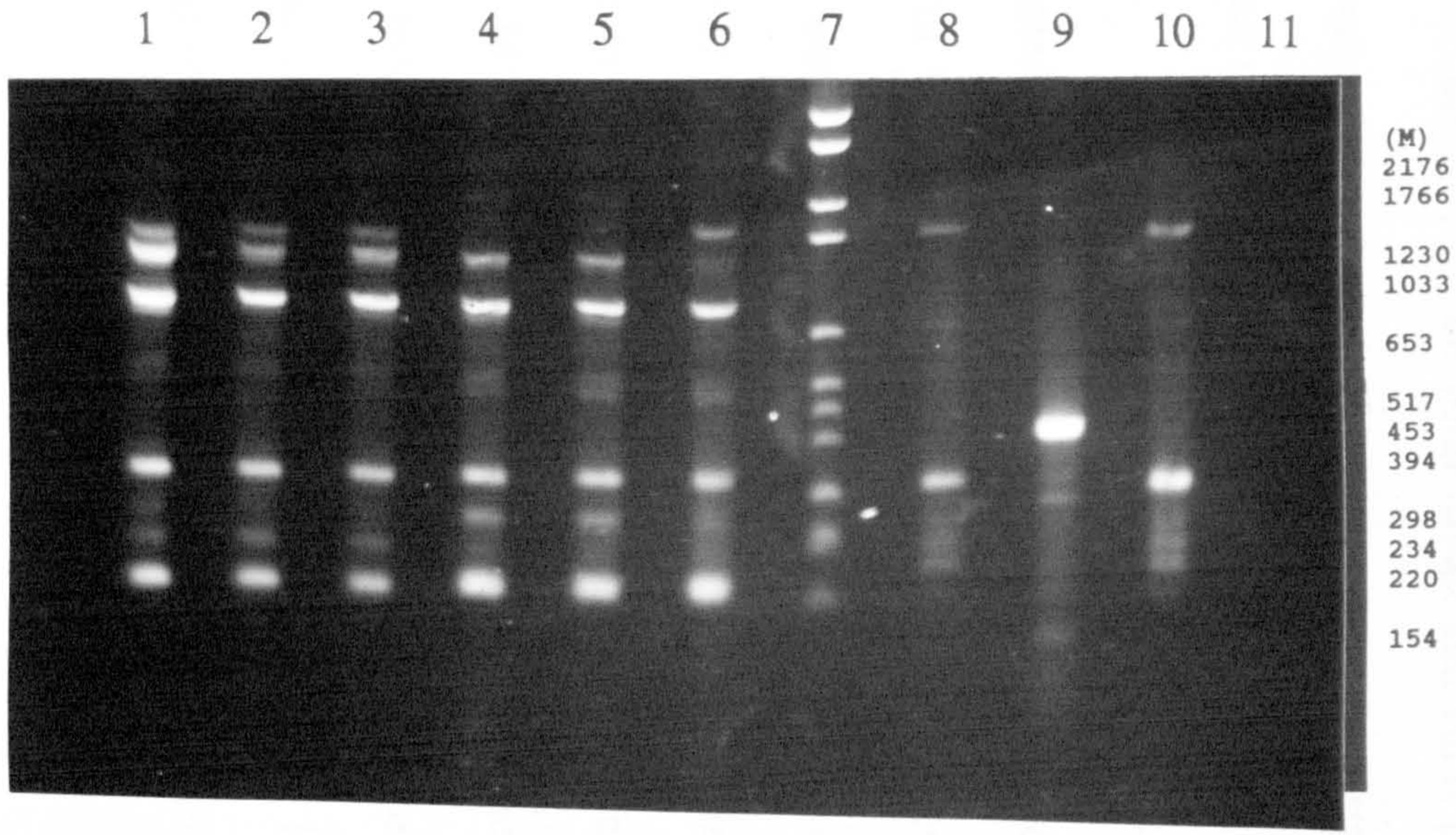
AB11

PCR amplification with the AB11 primer revealed that *An. stephensi* from the three countries share two bands of 427and 1060bp but surprisingly, mosquitoes from Tadzhikestan were most similar to those from Iran (Fig. 8.10). One specimen from Pakistan (Lane 9) produced a 420bp fragment which is absent in all other examined individuals from field and laboratory populations of *An. stephensi*.

8.4.4 RAPD fingerprints in *An. stephensi* from urban and rural areas in Iran

In this part of the study RAPD-PCR has been used to examine the classification of *An. stephensi* into two varieties *An. stephensi stephensi* or type form which is mainly urban and *An. stephensi mysorensis* or rural form (Sweet & Rao, 1937; Rao *et al.* 1938). Although Subbarao *et al.* (1987) reported a new category

Fig. 8.10- RAPD-3- primer AB11 products in *An. stephensi* from three countries in a two steps annealing (36, 50°C) program: Tadshikestan (1-3), Iran (4-6), Pakistan (8-10; Marker (7); -ve (11). All individuals share two 427 and 1060bp fragments. However, mosquitoes from Tadshikestan are more similar to Iran population.



designated as "intermediate" and based on their observation all three forms,i.e. type-form, intermediate, and mysorensis were in semi-urban areas while only intermediate and mysorensis were seen in rural areas.

For this purpose, samples drawn from urban and rural populations of *An. stephensi* from Iran were collected by staff of Kazeroon field station in Kazeroon, Fars province have been used.

It should be noted that *An. stephensi* is the primary vector of malaria in Hormozgan Province of Iran (Manouchehri *et al.* 1992). However, in Sistan and Baluchistan, where it was once one of the most important vectors, *An. stephensi* responded well to indoor residual spraying (Manouchehri & Janbakhsh 1977). This mosquito is resistance to DDT, dieldrin and malathion in different parts of Iran (Mofidi et al. 1958, Mofidi & Samimi 1960, and Manouchehri *et al.* 1976). However, In past and recent years almost all researches have been focused on *An. stephensi* ecology, specially its response to insecticides. There is no report of its population genetics and systematics despite morphological identification.

AB4

After receipt of the specimens, DNA was extracted from the dried individuals using AB4 primer sequence, which had successfully amplified genomic DNA from other *Anopheles* species (*An. culicifacies*, *An. gambiae*) revealed a very distinct pattern in each population. Rural specimens had a 930bp specific band while

mosquitoes collected in urban area had two specific bands of 240 and 810bp. Both populations shared a 710bp band (Fig. 8.11).

UBC-353

Eight other primers from UBC and AB series have been used with DNA from these two populations but only UBC-353 primer produced a distinctive pattern. With this primer, rural specimens showed two 1260 and 2650bp bands with urban population. However two fragments of 1060 and 1400bp size (Fig. 8.12) are specific to urban samples.

Fig. 8.11- RAPD-Prog 1 amplification of primer AB4 in *An. stephensi* urban and rural populations from Iran: Rural (2-4), Urban (5-7); Marker (1), -Ve (8). Rural populations have a 930bp specific band and urban-specific bands are two 240 and 810bp fragments.

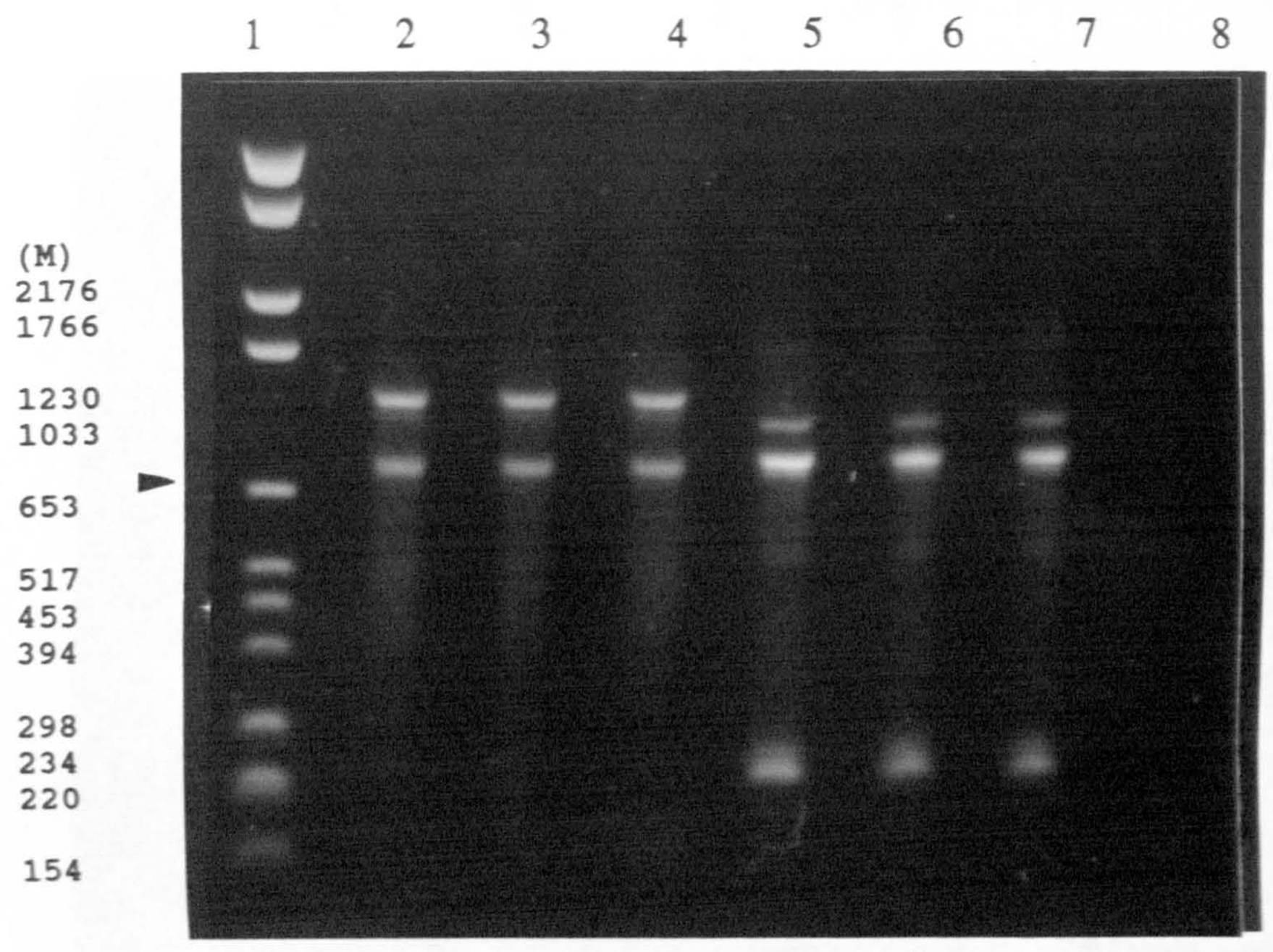
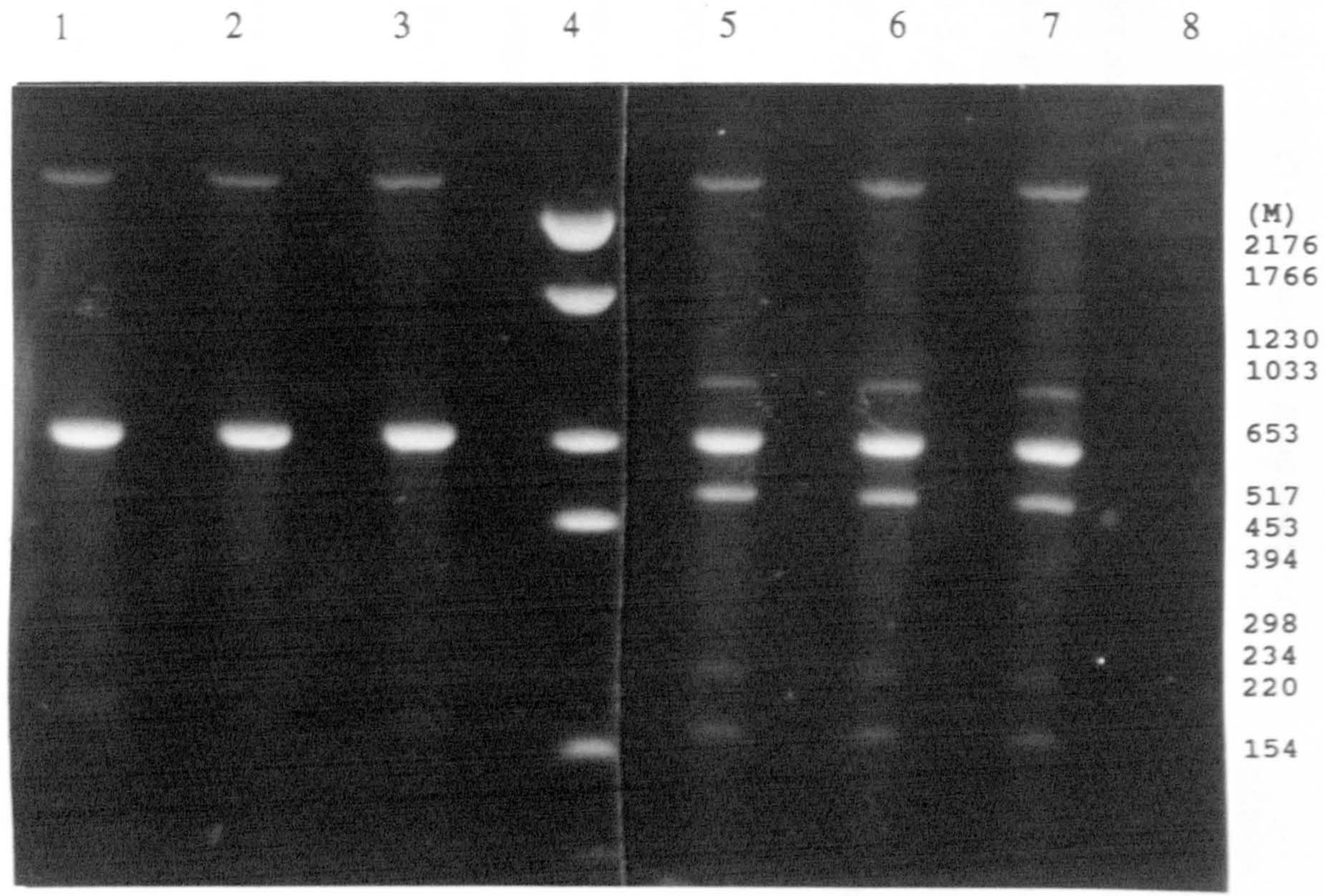


Fig. 8.12- RAPD (Prog 3) UBC-353 products in *An. stephensi* urban and rural populations from Iran: Rural (1-3), Urban (5-7); Marker (4). Urban-specific bands are two 1060 and 1400bp fragments while two populations share two bands in 1260 and 2650 bp size.



8.4.5 Determination of paternity in *An.stephensi*

Crossing experiment and RAPD markers in progenies

An. stephensi is a well adapted species in laboratory for breeding and subsequent experiments such as crossing, insecticide resistance, insect immunology, development of malaria parasite cycle in vector, etc. For these reasons, two strains of *An.stephensi* (IND-S and LPR) originated from India and Dubai (see Ch.3 for more details) and their F1 progenies (larvae, pupae, and adults) have been used to determine the:

1)- inheritance of RAPD bands

2)- percentage of non-parental bands

3)- differences in life stages pattern

4)- reproducibility of RAPDs

Although the eggs have also been screened, because of their small size and the probability of carrying more than one egg in each extraction tube and subsequent contamination, this life stage was excluded from the paternity study. The results of 12 primers are summarized below.

AB11

This primer in Prog 1, showed the inheritance of bands from parents to progenies with some anomaly in larvae that did not amplify fragments of higher than 450bp in size. Female parent has a 720bp which is not present in male and progenies

and larvae showed a non-parental band of 430bp. The red-eye strain which is homozygous for this mutant, could be readily differentiated from LPR, IND-s and their progenies by a 650bp band (Fig. 8.13).

AB11 in Prog 8 revealed a different pattern, with a main band of 750bp which is not present in male parent and two larvae. In contrast, female parent, a larva and a adult progeny have not some high molecular weight bands (Fig. 8.14).

In crossing experiment between female IND-S and red-eye as male parent, AB11 primer showed that the reproducibility and inheritance of bands could be achieved in other strains. three fragments of 170, 260 and 750bp are the main bands (Fig. 8.15).

AB15

With this primer, pupae and adults pattern is almost similar to parents, but like AB11 primer, larvae did not show high molecular weight bands, perhaps due to a stage specific pattern. The number of non-parental bands are 3, 2, and 2 in larvae, pupae and adults, especially a 435bp which is present only in larvae (Fig. 8.16).

AB19

AB19 primer products in two different concentrations of primer (70 and 20ng) showed the same common bands in two amplifications. The inheritance of bands and their brightness is similar in parents and progenies. In experiment with 20ng of primer, red-eye strain has two specific bands of 600 and 1050bp which can be use in differentiation of this strain. Some parental bands which did not present in

Fig. 8.13- RAPD-1-AB11 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) and red-eye strain: Larvae (1-3), Pupae (4-6), Adults (7-9), F (10), M (11); red-eye (13-19); Marker (12); -ve (20). Rang of main fragments in cross experiment is 175-540. A 440bp present only in larvae. F has a specific 710bp which did not amplified in progeny.

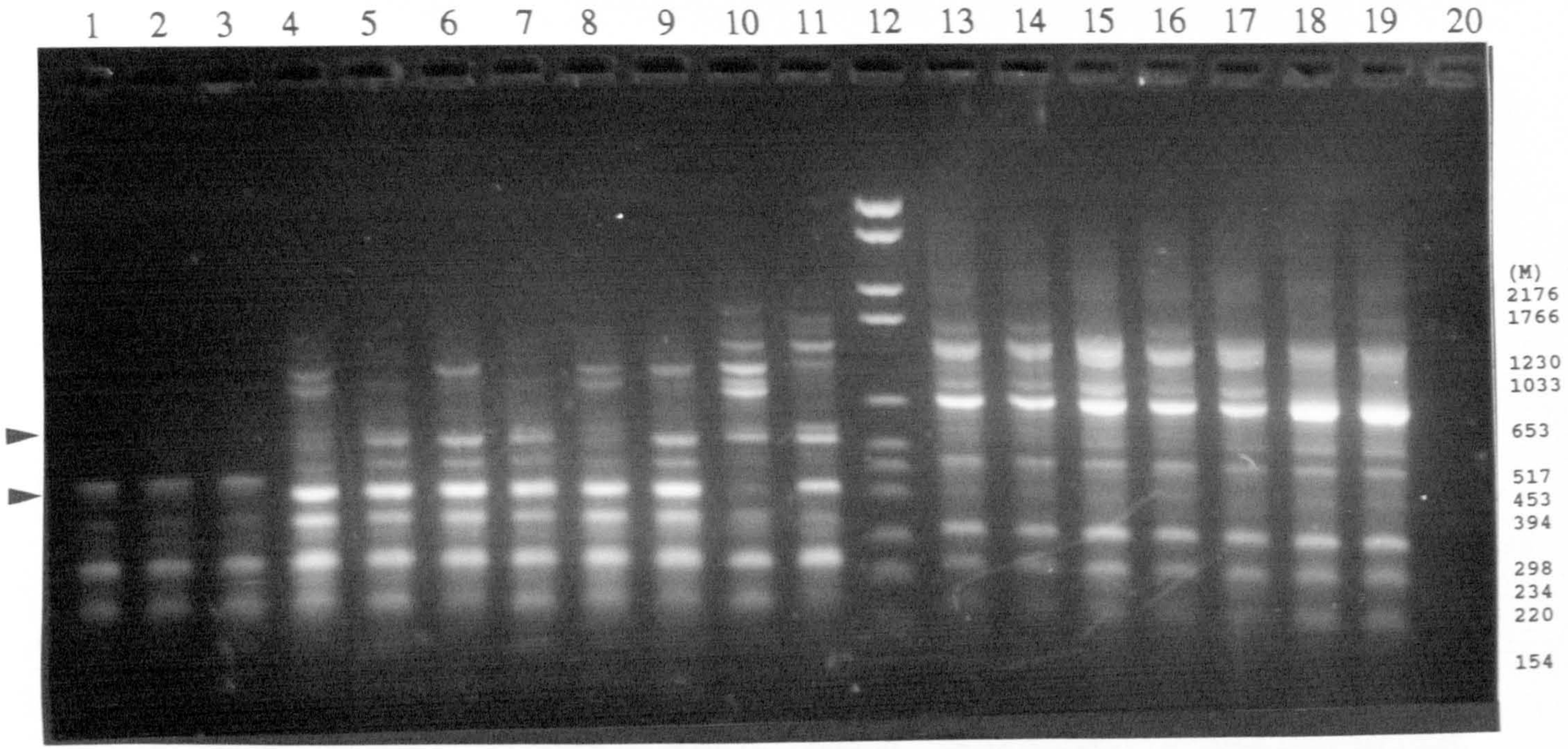


Fig.14- RAPD-8-AB11 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M): Larvae (2-6), Pupae (7-10), Adults (12-17), F (18), M (19); Marker (1,11); -ve (20). Range of main fragments in cross experiment is 270-750. two non-parental bands present only in larvae and one in pupae and adult.

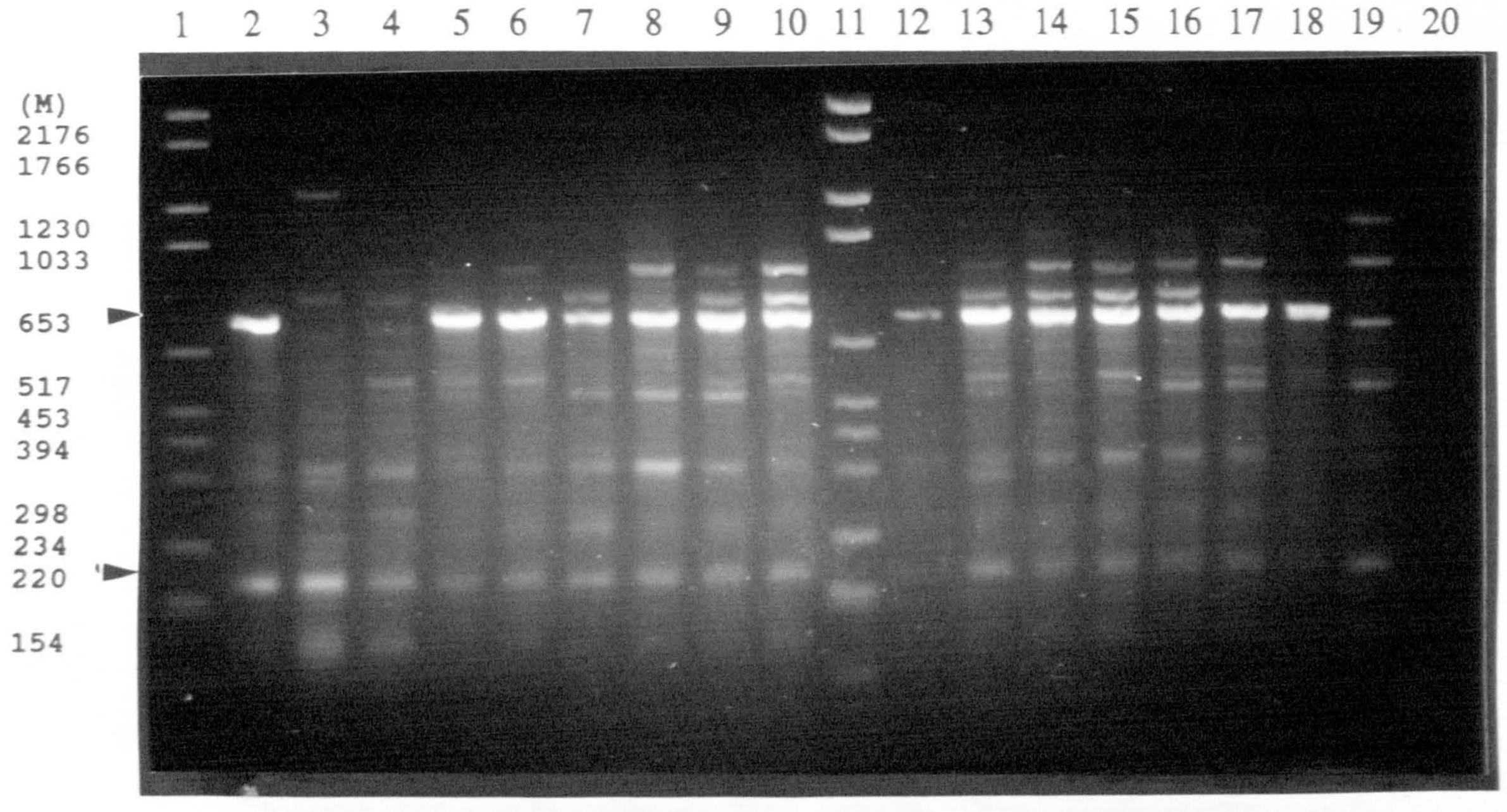


Fig. 8.15- RAPD-AB11 primer products (Prog 2) in *An. stephensi* cross experiment between IND-S (F) X red-eye (M): Larvae (1-2), pupae (3-4), Adult (5-6), F (7), M (8); Marker (9). Lanes 10 is another red eye as control

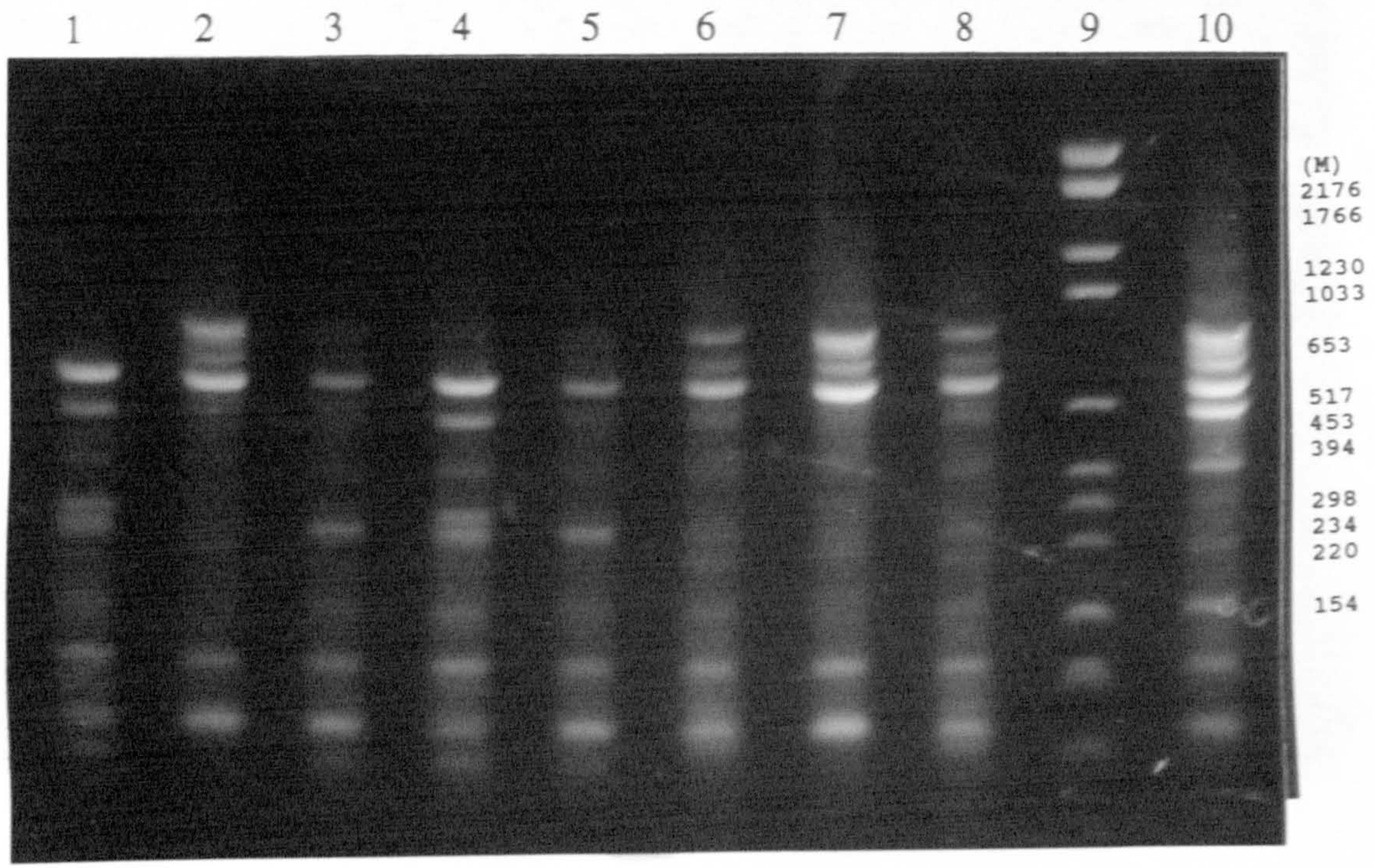
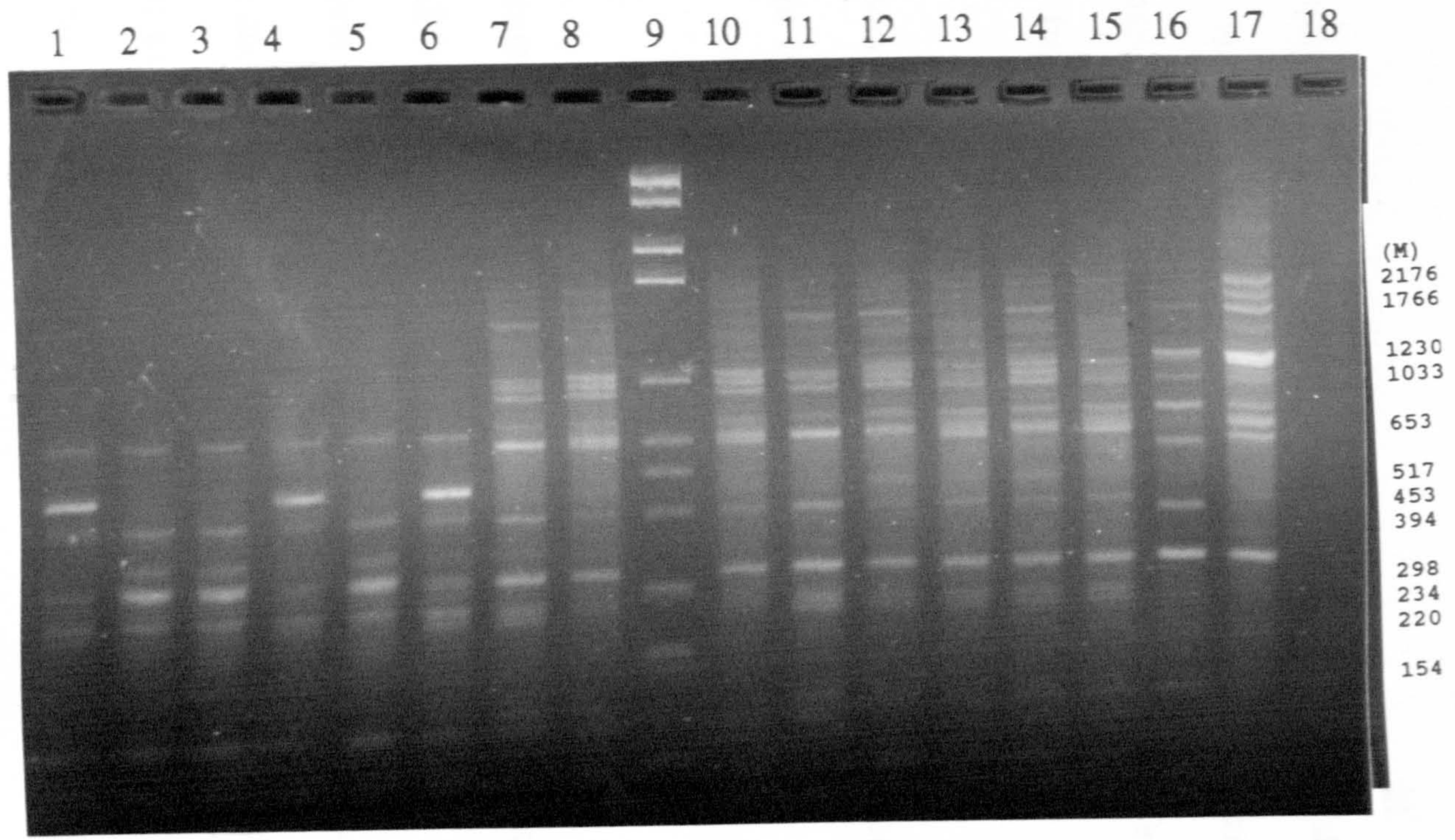


Fig. 8.16- RAPD-Prog 1-AB15 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) and their progeny: Larvae (1-6), Pupae (7-8,10), Adults (11-15), F (16), M (17); Marker (9); -ve (18). Range of main fragments is 280-669 Three non parental bands in larvae and two in pupae and adult progeny are detectable.



amplification with less primer, have appeared in conditions with highe concentration (Fig. 8.17 and 8.18)

UBC-301

This primer amplified a band in 270bp size which is present in male parent and some individuals in three life stages, but not in female parent and other progenies. The brightness of two higher bands are similar in parents and adult progenies, while these fragments do not present in larvae (Fig. 8.19).

UBC-302

Complete reproducibility of parental bands in three life stages is demonstrated (Fig. 8.20). The size of Products range between 575-1360bp.

UBC-304

The best presentation of inheritance of parental bands to progenies and reproducibility of RAPDs in all specimens and in different life stages has been achieved by amplification with primer UBC-306 (Fig. 8.22). The presence of a 385bp fragment in progenies and not in parents confirmed that non-parental bands are not the artifact of crossing experiment. It also proved the presence of some bands that inherited to progenies by one of the parents (male or female), which did not present in another parent. For example a 370 bp band only amplified in male parent and most progeny but not in female parent. On the other hand, a 410bp fragment present only in female parent and some progenies in three life stages but not in male parent and the positive control which is another male in LPR strain.

Fig. 8.17- RAPD-(Prog 1)-AB19 (70 ng) products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strain: Pupae (3-5), Larvae (6-8), Adults (9-12), F (13), M (14); red-eye (13-18); Marker (2,15); -ve (16). Range of main fragments in cross experiment is 260-1780bp.

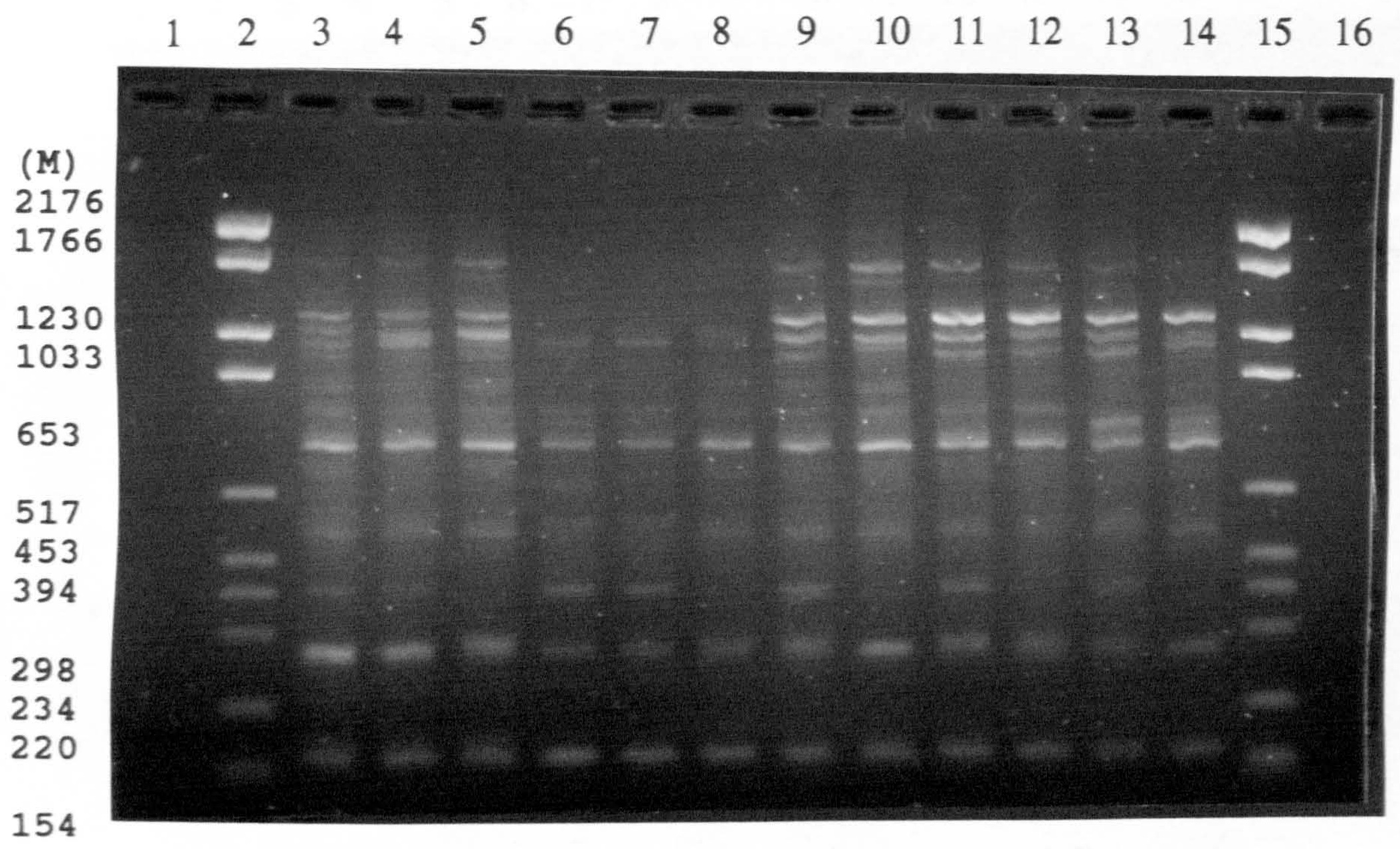


Fig. 8.18- RAPD-(Prog 3)6-AB19 (20 ng) products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) and red-eye strain: Larvae (1-3), Pupae (4-6), Adults (7-9), F (10), M (11); red-eye (13-18); Marker (12); -ve (19). Rang of main fragments in cross experiment is 250-1440bp. Two main bands in 250 and 380bp size are characteristic for all individuals, whereas red-eye specimens have two specific 600 and 1050bp bands.

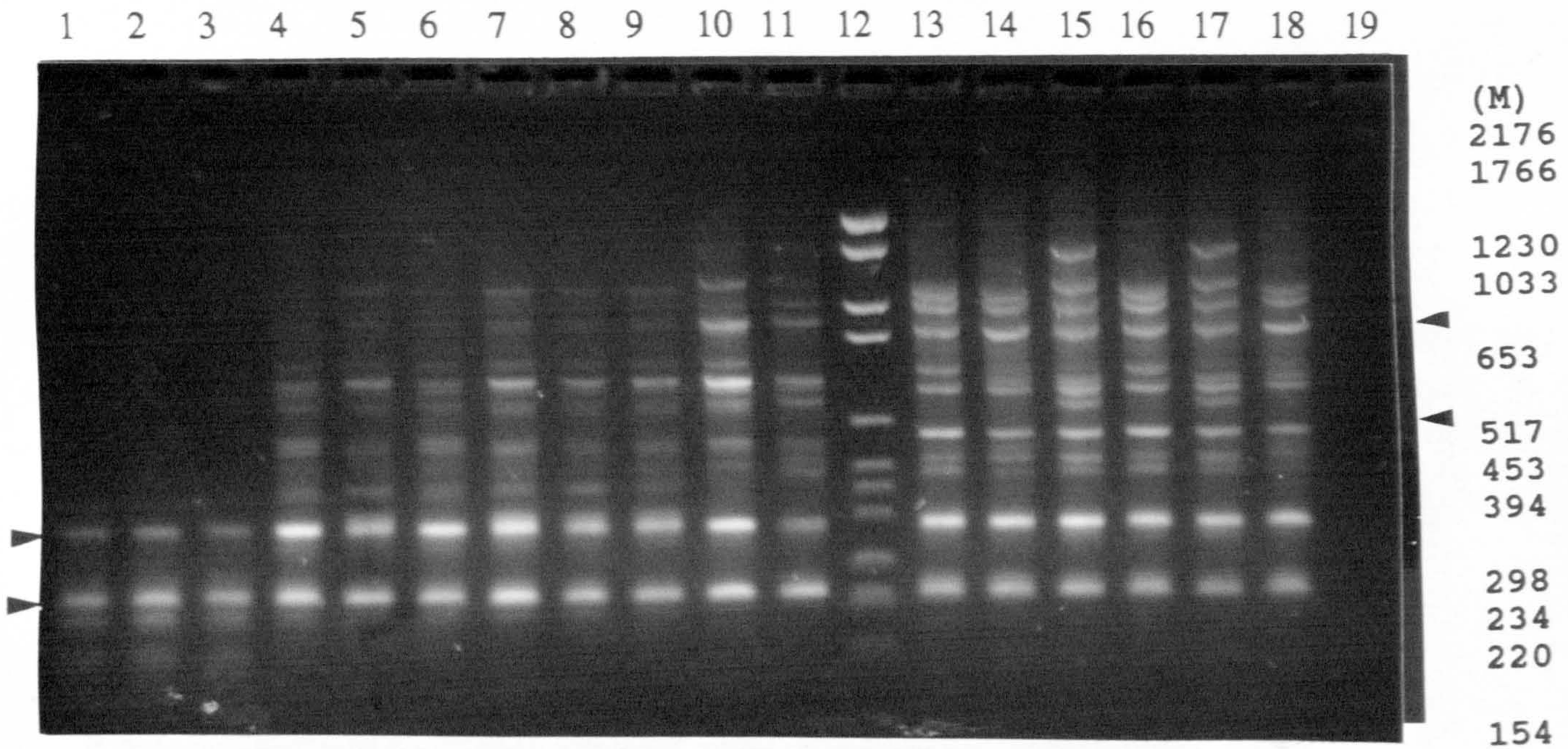


Fig. 8.19- RAPD-1-UBC-301 products in *An. stephensi* cross experiment IND-S (F) X red-eye (M). Larvae (1-2), Pupae (3-4), Adult (5-8), F (9), M (10); Marker (11). Two fragments in 159 and 325bp only present in progeny. Range of main fragments is 270-1450bp.

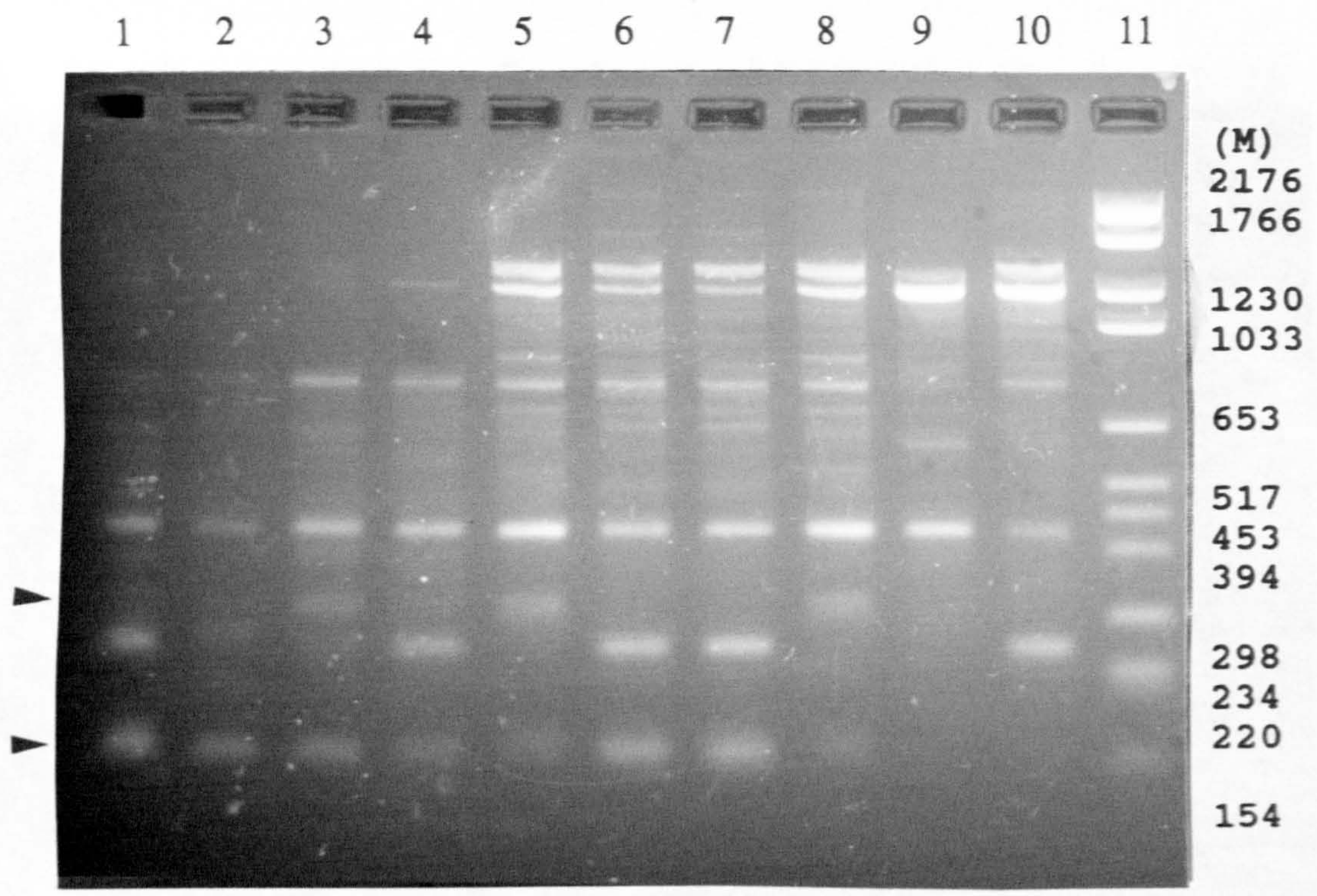


Fig. 8.20- RAPD-1-UBC-302 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strain: Larvae (1-6), Pupae (7-9), Adults (11-13), F (14), M (15); Marker (10); -ve (16). Rang of main fragments in cross experiment is 575-1360bp. A 770bp present only in progeny but not in parents.

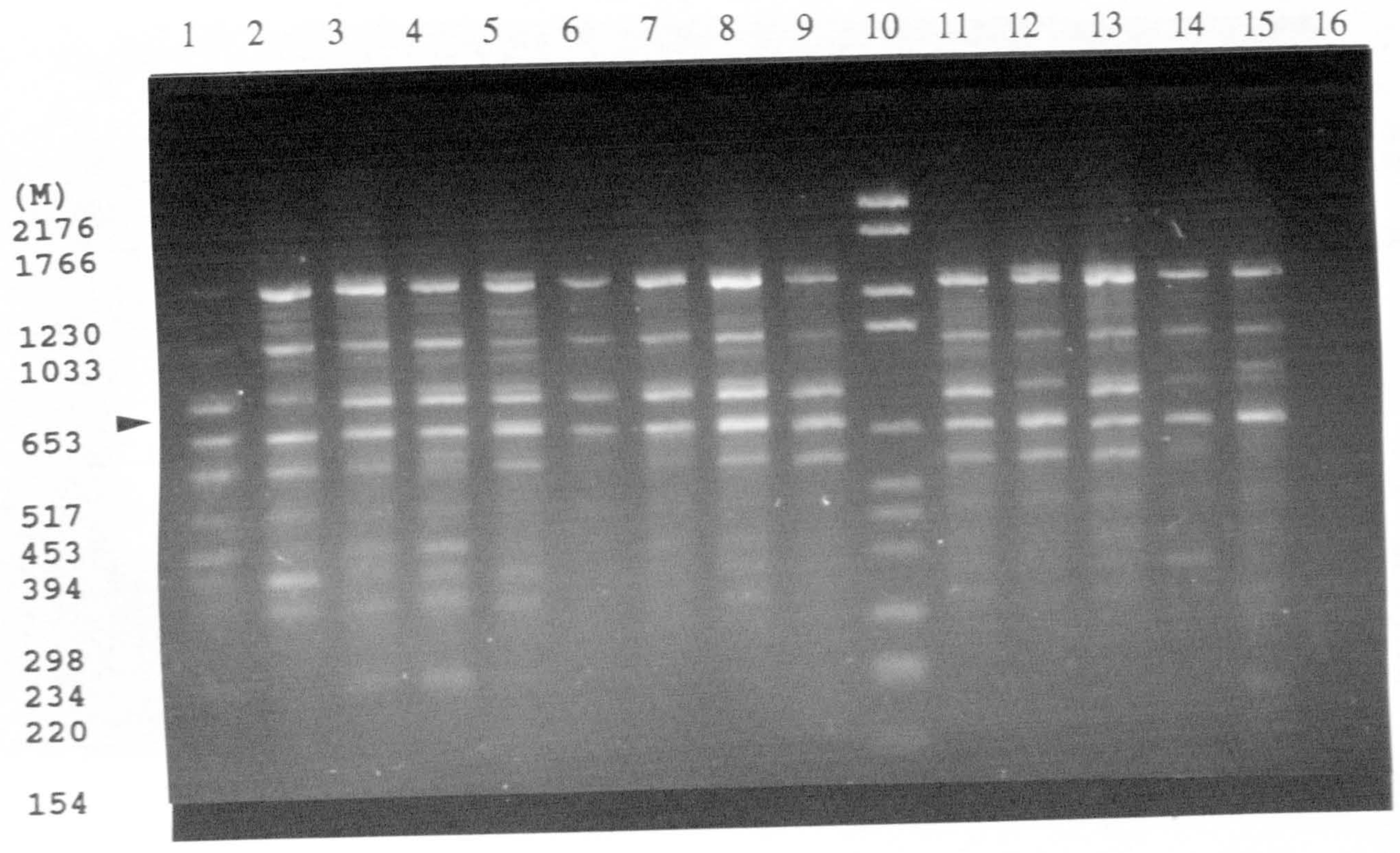
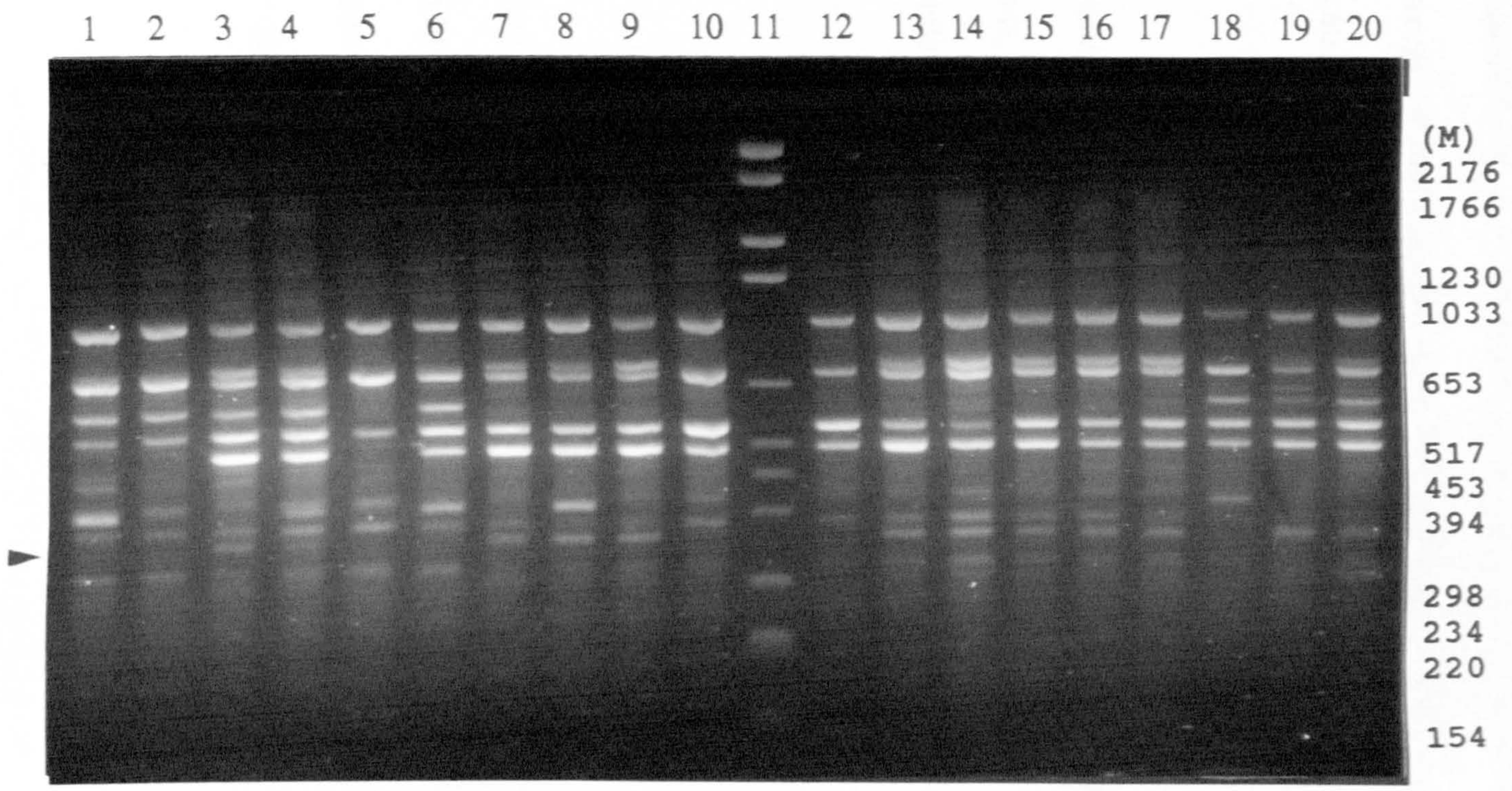


Fig. 8.21- RAPD-6-UBC-304 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strain: Larvae (1-10), Pupae (12-14), Adults (15-17), F (18), M (19) Male (LPR) as +control (20); Marker (11). Range of main fragments in cross experiment is 480-890bp. A 385bp present only in progeny but not in parents.



UBC-306

This primer showed an extensive variation in larval pattern by absence of some parental bands especially those of high molecular weight, non-parental bands even in individual of larvae. However, pupae and adults inherited all parental bands (Fig. 8.22). The main amplified band are in the range of 270-1450bp.

UBC-319

This primer consistently amplified the parental bands in progenies. Regarding the brightness of bands, the same intensity of a band of 277bp in parents was observed in larvae, while the other 365bp showed less intensity in larvae and other stages (Fig. 8.23).

OPA8

All parental bands have been amplified by primer OPA8 in progenies. These products in parents and progenies demonstrate the high degree of reproducibility of RAPD markers. It is interesting that a fragment of 800bp present only in parents and did not amplified in progenies (Fig. 8.24)

UBC-353

This RAPD primer amplified all parental bands in larvae, pupae and adults of F1 progeny. The main bands are in the range of 340-570 with a 380bp non-parental band present in most individuals (Fig. 8.25B).

Fig. 8.22- RAPD-6-UBC-306 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strain: Larvae (2-5), Pupae (7-9), Adults (10-15), F (16), M (17); Marker (6,18); -ve (1). Rang of main fragments in cross experiment is 270-1450bp. The most variable pattern and more non-parental bands have been detected by this primer.

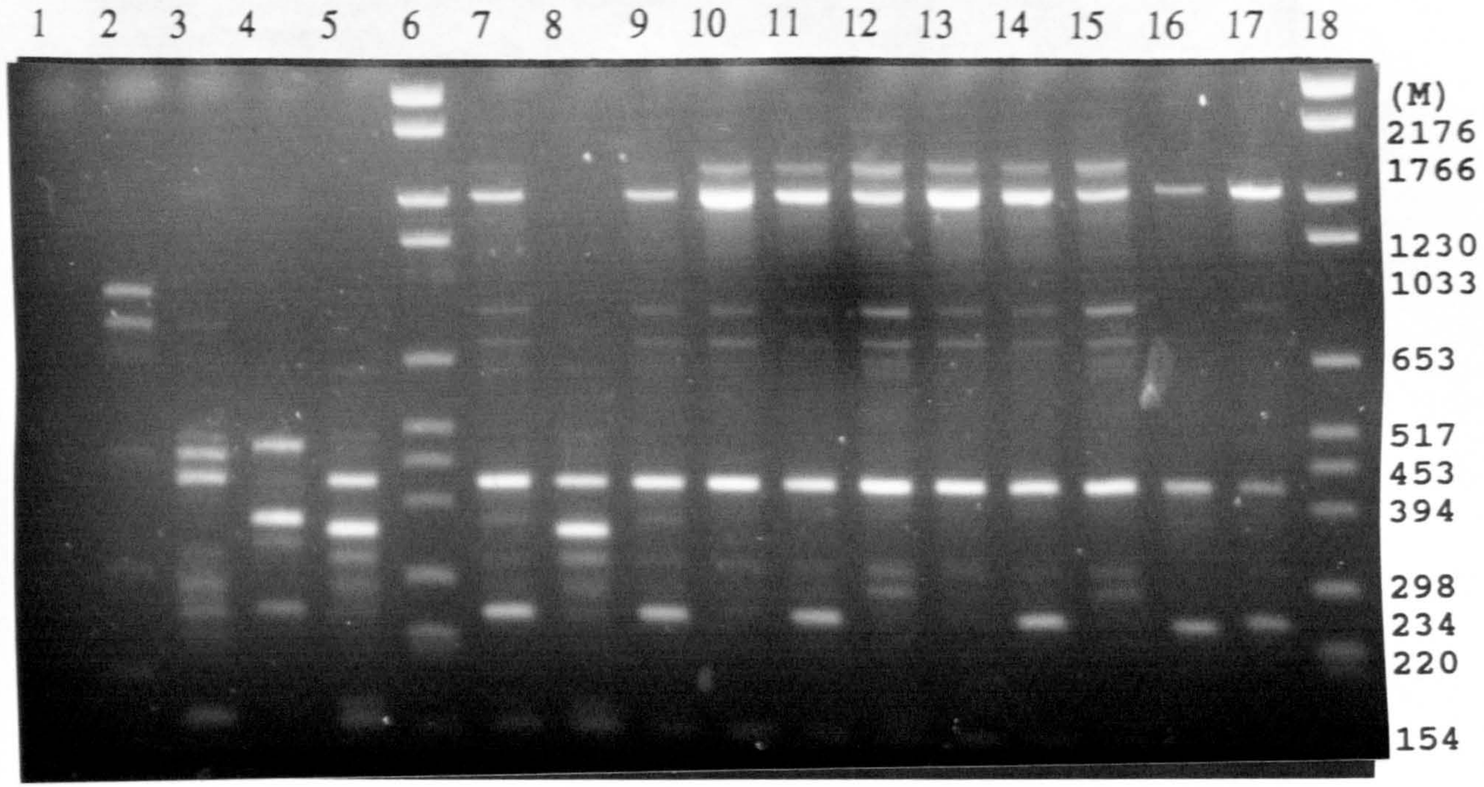


Fig. 8.23- RAPD(Prog 1) UBC-319 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strain: Larvae (1-4), Pupae (5-7), Adults (8-12), F (14), M (15); Marker (13); -ve (16). Rang of main fragments in cross experiment is 277-780bp. This primer did not produce any non-parental bands in progeny.

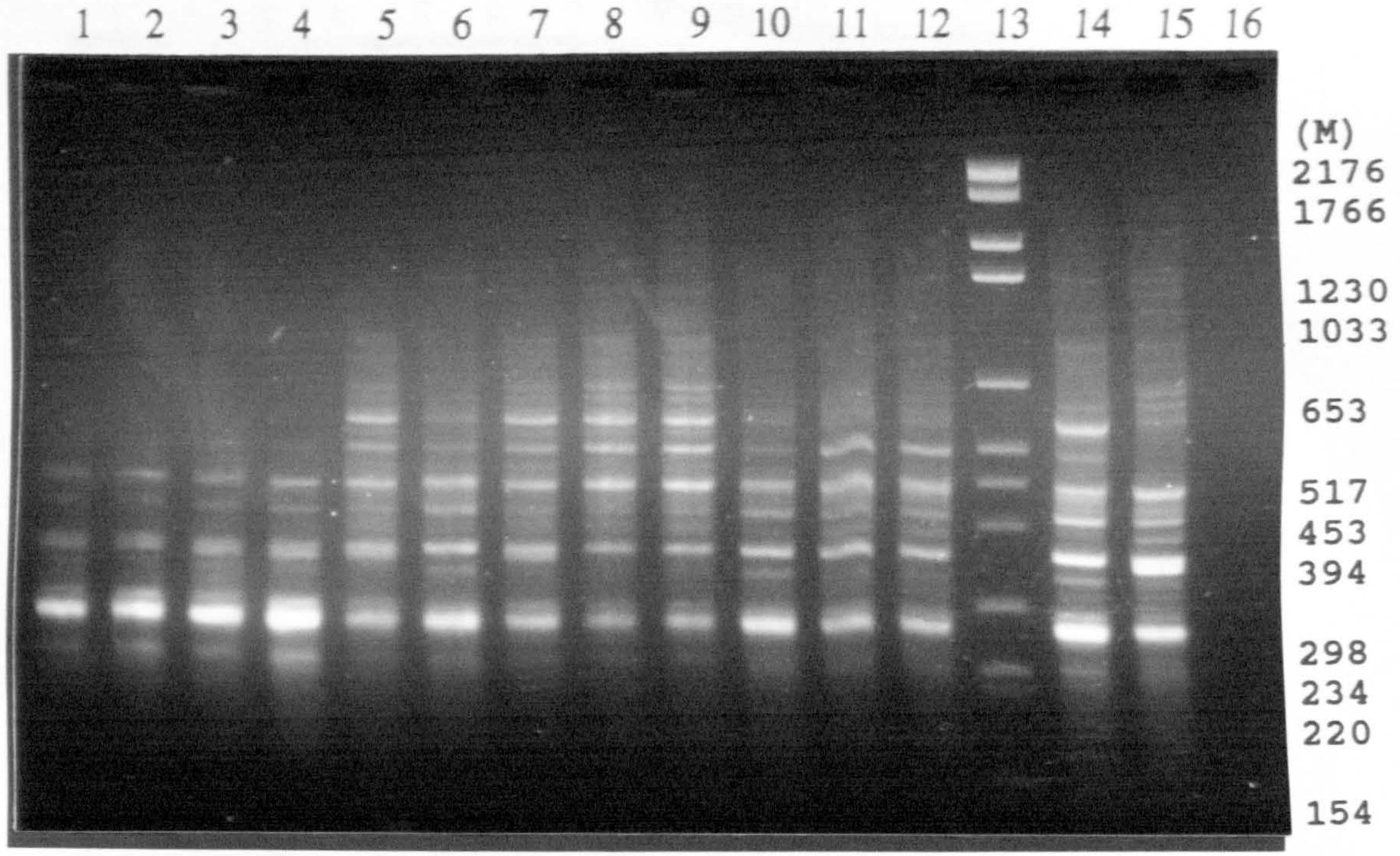


Fig. 8.24 RAPD-Prog 1-OPA8 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strains: Larvae (1-3), Pupae (4-6), Adults (7-9), F (10), M (11); marker (12). Range of main fragments in cross experiment is 425700bp. No non-parental band has been detected with this primer in progeny

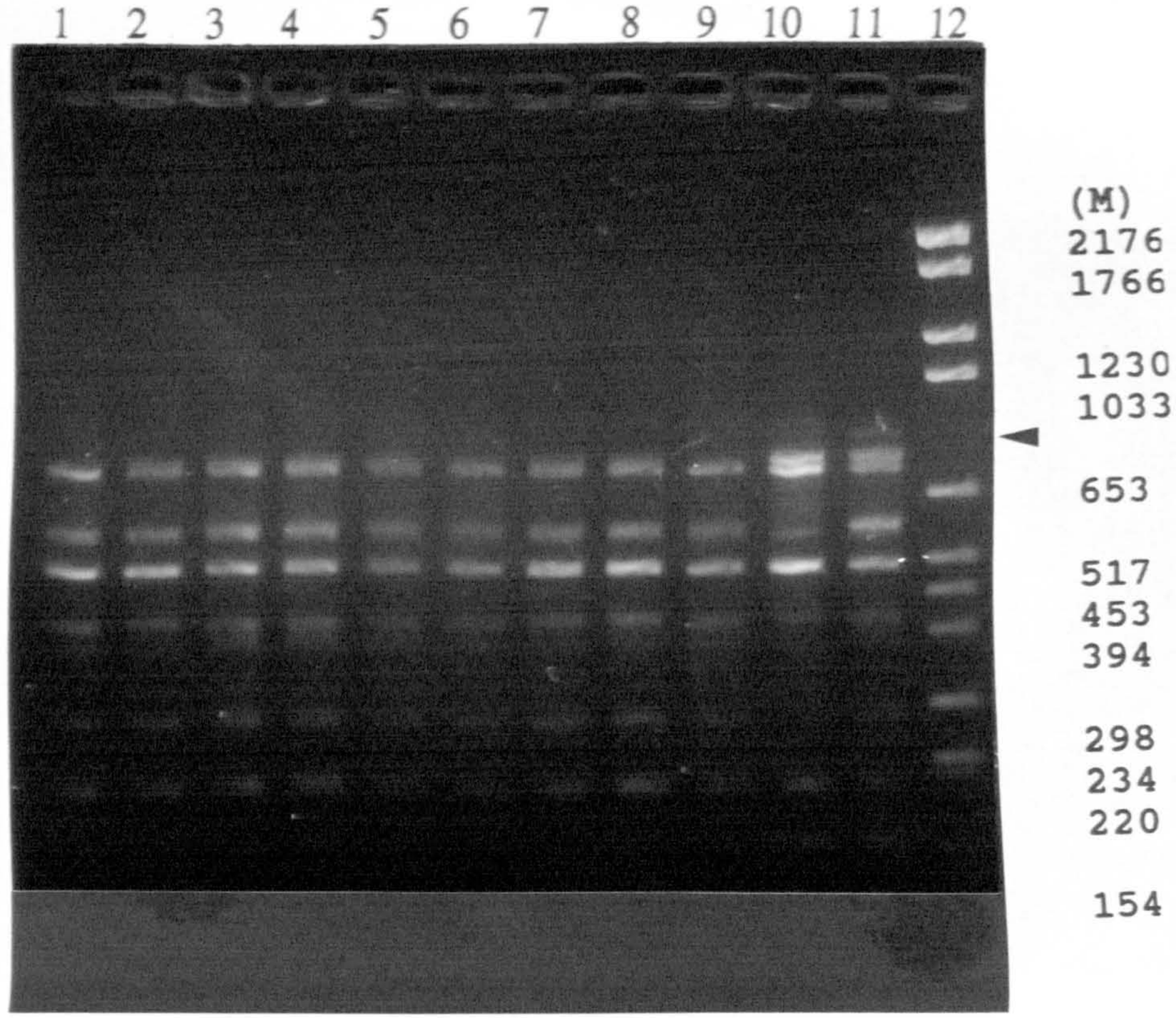
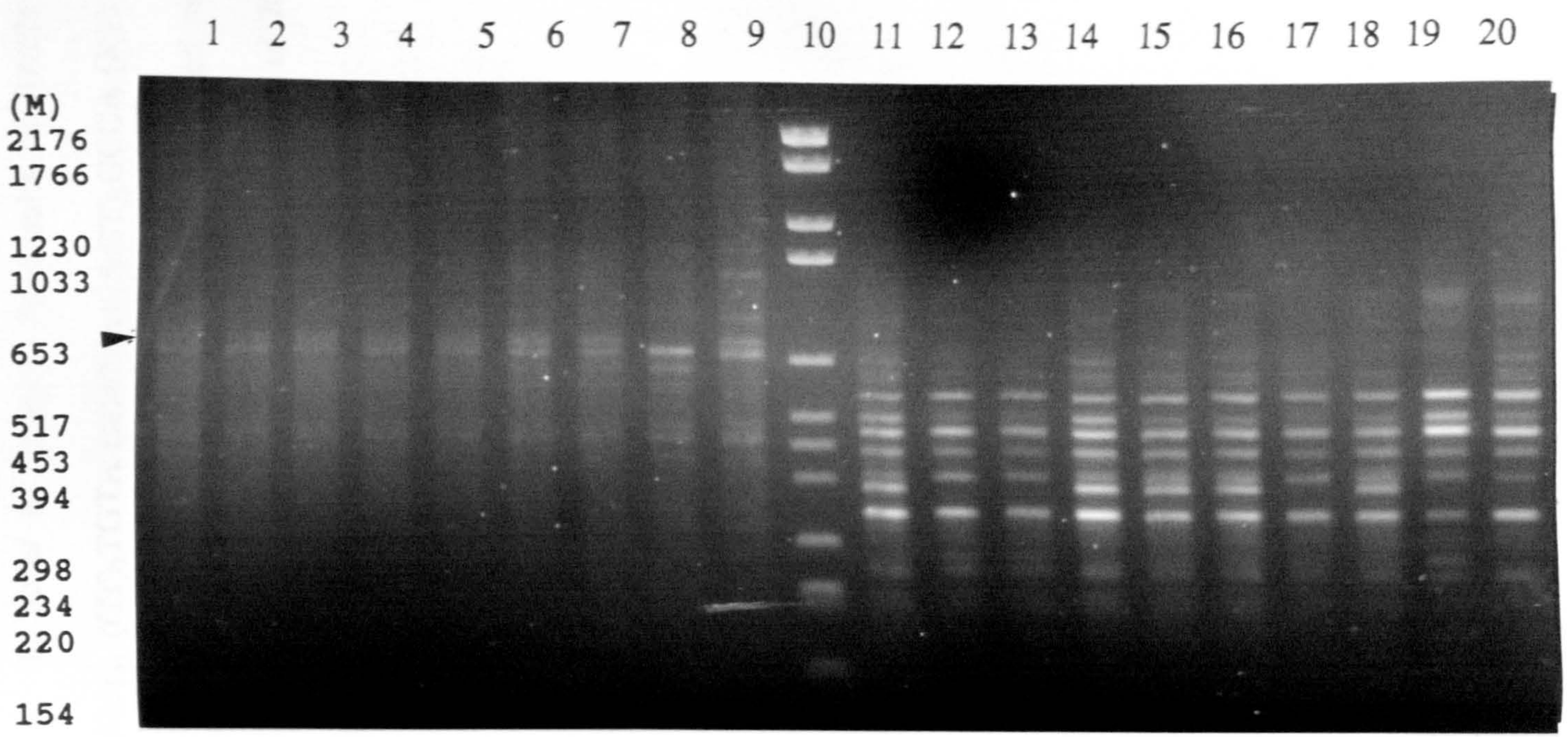


Fig. 8.25A, 25B- RAPD-6-X18 (left panel) and UBC-353 (right panel) products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strain:
X18 (left panel): Larvae (1-2), Pupae (3-4), Adults (5-7), F (8), M (9); Marker (10). Rang of main fragments in cross experiment 460-710bp. This primer also did not produce any non-parental bands in progeny.
UBC-353 (right panel): Larvae (11-12), Pupae (13-14), Adults (15-18), F (19), M (20); Marker (10). Range of main fragments in cross experiment 340-570bp. This primer produced two non-parental bands in each life stage of progeny.

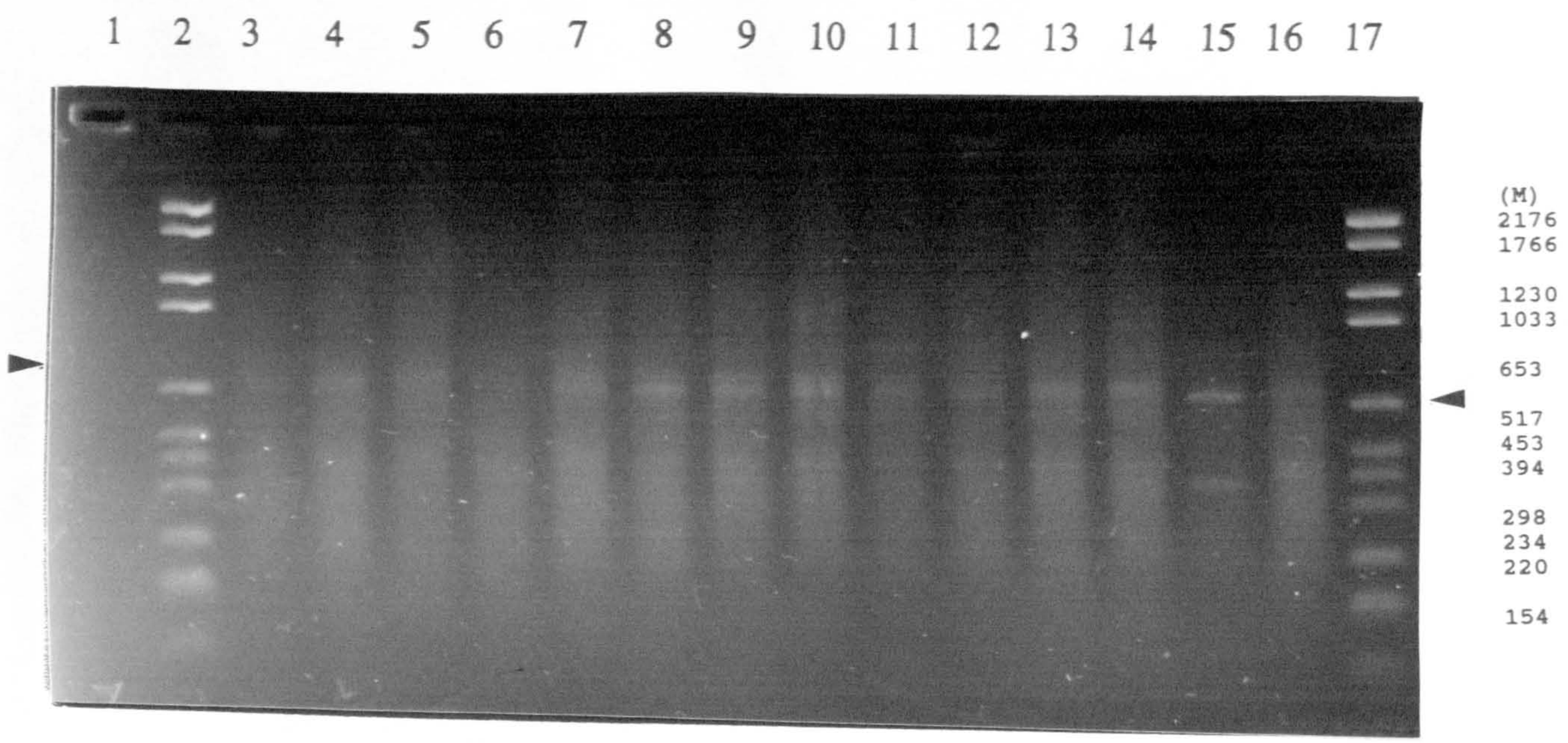


SSR PCR.

Crossing experiment and SSR markers in progenies

In order to compare the results of RAPD with other markers, two SSR primers, $(GT)_7TGTA$ (X18) and $(AGT)_5GCCA$ (X19) have been applied with IND-S, LPR and their progenies. Both primers amplified parental bands in progenies (Fig. 8.25A, and Fig. 8.26). In X18 products a band of 700bp present in male parent and progenies but not in female parent.

Fig. 8.26 - RAPD-6-X19 products in *An. stephensi* cross IND-S (female parent-F) X LPR (male parent-M) strains: Larvae (3-6), Pupae (7-9), Adults (10-14), F (15), M (16); Marker (2,17); -ve (1). Rang of main fragments in cross experiment is 425-700bp. No non-parental bans has been detected with this primer in progeny.



8.5. DISCUSSION

An.stephensi liston 1901 is known to be a single but polytypic species. Although *An.stephensi* has an enormous geographical range, from the arabian peninsula in the west to China in the east, there is no direct evidence that it constitutes anything other than a single species.

In this part of project, genetic variation in *Anopheles stephensi* has been studied in laboratory and field materials, urban and rural populations, and in F1 progenies of two laboratory strains, using RAPD and SSR primers. There are no published data on PCR in general and especially RAPD identification of *An. stephensi* that could serve for comparison of the results of this study. Therefore ecological and cytogenetic data have been used for evaluation of RAPD results.

8.5.1 Intra-specific variation in Laboratory strains

AB11 and UBC303 can differentiate the Laboratory strains originated from Iraq, Iran, Dubai and India which provide basic data for further evaluation of site specific variation within this species. However, there are other primers such as M13 which produce more variation within each strain. On the other hand, primers AB13 and OPA1 produced almost unique patterns as common bands to all strains which seems useful for further characterization.

8.5.2 Variation in *An.stephensi* specimens from three countries

Preliminary results with field specimens revealed high similarity in RAPD patterns of Iran and Tadzhikestan, while Pakistan specimens showed a different pattern. AB11 amplified a strong fragment of 420bp only in one individual from Pakistan. One possible explanation is the presence of a commensal or pathogens in this mosquito. However, it seems unlikely that other individuals from same package and with same collection and preservation would not also be infected and hence show this band. Another possibility is contamination during extraction, although is possible but has minor effect. A more logical explanation may be arise that this sample was of mixed DNA (a part of an other species stocked and carried with that individual into the extraction buffer), because that band was not detected in other examined *An. stephensi*. However, genetic variation based on dynamics of gene flow between local population should not be ignored.

8.5.3 Differences in rural and urban populations

An.stephensi collected from two urban (Kazeroon) and two rural area in Fars province were used to define their variation and its correlation with the ecological and cytogenetic differences of these two populations described by Sweet & Rao (1937), Rao *et al.* (1938), and Subbarao *et al.* (1987). The result of this part of the study using RAPD primers AB4 and UBC-353 differentiated two populations by simple patterns while no variation within population detected. These data could act as the basis for evaluation of other urban and rural populations, especially cytogenetically identified specimens.

In india the vectorial capacity of the malaria vector *An.stephensi* differs between urban and rural populations. In some respect *An.stephensi* would appear to be analogous to *Aedes aegypti*, another urban mosquito that has an even more extensive range. It might be hypothesised that *An.stephensi* originated as a rural mosquito, represented by *mysorensis*. On the other hand, *stephensi stephensi*, a form that was particularly adapted to an urban, man-made environment has spread and assisted by human population movement, beyond the range of the original rural form.

However, although there are no apparent pre- or postcopulatory barriers between these populations, urban populations are highly polymorphic in their polytene chromosomes, whereas rural populations are almost monomorphic (Coluzzi *et al.* 1985).

In the man-adapted *An.gambiae* complex, *An.gambiae* and *An.arabiensis*, research using microsatellite loci and mtDNA, has provided evidence of extensive gene flow between populations separated by considerable distances, 6000 km in the case of *An.gambiae* and 2000 km in the case of *An.arabiensis* (Lehmann 1996a, 1996b, 1997; Donnely *et al.*, submitted). The gene flow values (N_M) quoted by these authors would be more than sufficient to prevent the emergence of reproductively isolated forms.

In the analogy with *Ae. aegypti*, *An.stephensi stephensi* would be the equivalent of the man-adapted form of *Ae. aegypti queenslandensis*, and *An.stephensi*

mysorensis similar to feral populations, *Ae. aegypti formosus* found in Africa, south of the Sahara which is mainly zoophilic.

8.5.4 Paternity experiment and inheritance of RAPD markers

Extensive series of crosses that have taken place in many laboratories emerged no evidence on reproductive isolation of *An.stephensi* populations (in contrast to *An.gambiae* species complex cross experiments which revealed the species composition).

In this part of the study, genomic DNA from two identified strains of *An. stephensi*, IND-S (female parent) and LPR (male parent) and their F1 progenies in different stages (eggs, larvae, pupae, and adults) were screened for polymorphism with random and simple sequence repeat primers. The range of size of products from these primers varies between 175-1780bp (Table 8.2), although primers AB11 (Fig. 8.13), AB15 (Fig. 8.16), AB19 (Fig. 8.18) and UBC 319 (Gif. 8.23) did not amplify some high molecular weight bands in larvae.

Parents and resulting progeny were scored for the presence and absence of individual bands. All polymorphic and monomorphic bands have been scored and although allelism of bands and homology of bands of the same size in parents and progenies must be ascertained by southern blotting or segregation analysis, but it is not practical due to the number of primers examined and those homologous amplification products. No significant differences have been found in rapdem of

Table 8.2 · RAPD and SSR primers fingerprints in *An. stephensi* strains; INDS(female parent) X LPR (male parent) cross and their progeny

PRIMER	N. of Bands in Progeny & Parents					Range of Bands (bp)	Range of Main Bands (bp)
	Larvae	Pupae	Adults	Female	Male		
UBC-301	8(2*)	7(2)	12(4)	6	6	160-1450	270-1450
UBC-302	16(0)	13(0)	14(0)	11	12	205-1360	575-1360
UBC-304	19(1)	16(1)	17(1)	8	12	300-1530	480-890
UBC-306	17(6)	13(6)	12(6)	4	7	160-1700	270-1450
UBC-319	8(0)	11(0)	13(0)	16	16	225-1020	277-780
UBC-353	11(2)	12(2)	14(2)	10	12	230-900	340-570
AB15	11(3)	14(2)	14(2)	12	14	130-980	280-669
AB11(prog.8)	15(2)	12(1)	11(1)	8	10	200-1040	270-750
AB11(prog.6)	7(1)	12(0)	11(0)	13	11	175-1200	175-540
AB11*	16(2)	15(1)	15(1)	12	13	165-990	175-900
AB19(70ng)	17(2)	11(1)	14(1)	11	11	250-1780	250-1780
AB19(20ng)	5(2)	14(3)	11(3)	13	13	150-1840	250-1440
X18	3(0)	3(0)	5(0)	9	8	425-1070	460-710
X19	4(0)	4(0)	4(0)	2	4	425-1070	425-700
OPA8	9(1)	9(1)	8(1)	8	8	166-800	500-800
Total bands	166(24)	166(20)	175(22)	143	157		
non-parental %	14.45	12.04	12.57				

(*): non-parental band AB11*: AB11 primer products in INDS X Red Eyes cross

different life stages, and it confirmed that genomic DNA does not differ between life stages.

The total number of bands produced by 12 primer is 166 in larvae and pupae, and 175 in adult progeny, while in female and male parents is 143 and 157 respectively. The majority of polymorphism for band presence/absence as well as for band brightness were inherited as dominant markers that are either homozygous or heterozygous for an amplifiable allele.

However, the percentage of non-parental bands in larvae, pupae, and adult progeny is 14.45, 12.04, and 12.57% which is higher than those reported in other species of insects, plants and primates (Ayliffe *et al.* 1994, Scott *et al.* 1992, Reidy *et al.* 1992, Hunt & Page 1992). The reasons for amplification of non-parental bands probably are:

1)- natural genetic assortment

2)- point mutation

3)- amplification of co-dominant markers from the same locus

4)- insertion or deletion in subjected sequences

5)- Artifacts

Artifacts may drive from:

1- non-specific priming or

2- from heteroduplex formation between related amplification product or

3- contamination

but in repeated crossing experiments, individuals from different extractions, even after

treatment with RNA-ase, showed the same patterns.

These proportion of RAPD markers which are not inherited in a dominant fashion are suitable to address issues of paternity and relatedness of individuals in genetically uncharacterized species. Results of this section is summarized in Table 8.2 and figures 8.13-8.26. But in a short review on each primer's fingerprint in parents and progenies, it is obvious that the RAPD and those primers carrying Simple sequence repeats, produced a variety of common and specific (in parents or progeny) bands which are useful in defining the levels of variations within species and finding the borders of taxonomic levels in members of *An.stephensi* which has a wide rang of distribution from middle East to China. These patterns also, could be use to evaluate the mechanisms involved in population isolation and their diversity.

There are some parental bands which have not amplified in F1 progeny (i.e. a 800bp in parents with OPA8, or AB19 products that a 890bp band present in parents but not in progeny). Others present in one or two stages of offspring's life cycle but not in all stages. For example UBC302 amplified a 830bp band in male parent which do not present in female parent and progeny. On the other hand a 790bp present only in progeny and was not amplified from either parental DNA by UBC 302 primer.

Another feature of RAPD amplification is the brightness of bands which in most primers, they inherited in same degree but there is sometimes a reverse

appearance , i.e. in primer UBC302, a 560bp band in progeny is very strong but in parents seems too faint. Also in each life-stage with primers AB11, AB15, AB19, UBC-301, UBC-306 and UBC-319, there are identical groups, differ in the number of bands and their brightness, perhaps due to their stage specific patterns.

The overall results of this study confirmed the power of RAPD in defining the genetic variation of *An.stephensi* populations. It is concluded that basic findings in different part of study especially the paternity experiment could be useful for evaluating laboratory crosses like the trials on sterile mosquito project in India (Jayarman, 1997).

CHAPTER NINE

ANOPHELES MACULIPENNIS SPECIES COMPLEX FROM IRAN AND FORMER SOVIET UNION (FSU)

9.1 ABSTRACT

RAPD, SSR and ITS2 sequences have been used to determine genetic variation and to resolve the species composition of field caught of *An. maculipennis s.l.*, collected during ecological and cytological studies from Iran and Russia.

Those primers of random and simple repeats, allow the differentiation of *An.maculipennis* s.s from *An. sacharovi* in Iran specimens, and *An. beklemishevi* from two forms of *An. messeae* from Russia. ITS2 sequence in Iran populations of *An.maculipennis* s.l. found three groups, perhaps corresponding to *An. sacharovi*, *An. messea*, and *An. atroparvous*. As it is the first study on molecular systematics of *An.maculipennis s.l.* from Iran, further investigation is needed for better understanding of the species composition in this species complex.

9.2 INTRODUCTION

9.2.1 Background

An. maculipennis was the first recorded example of a sibling species complex among malaria vectors. The presence of various morphological and physiological races with differences in vectorial importance and recognition of the 'phenomenon of anophelism without malaria' (Hackett, 1937) led subsequently to more detailed studies of the polytene chromosomes as a means of characterising the genetics of theses 'forms' (Frizzi, 1947; Stegni & Kabanova, 1976).

These studies ultimately resulted in the recognition of 8 species of the *An. maculipennis* Meigen, *An. atroparvus* Van Thiel, *An. labranchiae* Falleroni, *An. messeae* Falleroni, *An. sacharovi* Favre, *An. subalpinus* Hackett & Lewis, *An. melanoon* Hackett and *An. beklemishevi* Stegnii & Kabanova. A subsequent revision by White (1978) resulted in the addition of *An. martinius* Shingarev and *An. sicaulti* Roubaud to the complex. A further 5 or 6 species are known from the Nearctic region (White 1978).

Of the above species, three were formerly the most important vectors of epidemic malaria in europe, *An. atroparvus*, *An. labranchiae* and *An. sacharovi*. The latter species is the main vector in Turkey and, together with *An. superpictus* and *An. pulcherrimus*, the most important vectors of malaria in the former soviet union, although *An. messeae* has been implicated in the resurgence of malaria in Russia and

the Ukeaine (Nikolaeva, personal communication).

The situation with respect to the *An.maculipennis* complex in Iran is less clear, but it appears that several members of the complex may occur there (see below).

Although studies of polytene chromosomes in larvae has been the main tool for studying the *An.maculipennis* complex, studies of isoenzyme (Bullini, 1973) and cuticular hydrocarbons (Phillips, *et al.*, 1990) have also been made. However, none of these techniques is very well suited to detailed studies of the behaviour and vectorial capacity of adult mosquitoes. Some preliminary DNA sequencing studies of the ITS2 region of rDNA have shown that there are likely to be species-specific molecular markers for some of the palaearctic species of the complex (Adeniran and Townson, personal communication) but there is no published work describing the use of molecular markers for these species.

This chapter describes some preliminary studies of the *An.maculipennis* complex from Iran and Siberia in which RAPD and ITS2 is used to study the population genetic structure.

9.2.2 Field studies in Iran and Russia

During 1987-1989, an investigation on the ecology of *An. maculipennis s.l.* as the main malaria vector in the southern coastal region of the Caspian sea in Northern Iran, showed that this species is active from May to September with a peak in July. These mosquitoes breed readily in rice-fields, spring and clean standing waters and adult mosquitoes have been found mainly in animal shelters (95%).

Insecticide susceptibility tests carried out during this study showed that this species is susceptible to dieldrin, malathion, deltamethrin and resistant to DDT. Collections of *An. maculipennis* on human and animal baits showed that the biting on humans started at 19.00 hrs with a peak between 20.00-21.00 hrs. The bitting pattern on animal bait (cattle) was more or less the same as human bait. Application of an Enzyme-Linked Immunosorbent Assay (ELISA) in this study did not detect human blood in mosquitoes collected from indoor resting places but previous studies have shown that the anthropophilic index for this species in northern Iran is 1.7-4.9% (Djadid & Manouchehri 1994).

Dissection of mosquitoes revealed that hibernation in this species starts in October and a complete fat body could be seen in February. The following mosquito larvae have been found in *An. maculipennis* breeding places: *An. hyrcanus*, *An. claviger*, *Cx. pipiens*, *Cx. mimeticus*, *Cx. tritaeniorhynchus*, *Ae. vexans*, *Culiseta subochrea*, *Uranotaenia unguiculata*. Adults of *An. algeriensis* and *An. hyrcanus*,

have also been found in resting places of *An. maculipennis* (Djadid & Manouchehri 1994).

On the other hand, *An. sacharovi*, *An. superpictus*, and *An. pulcherrimus* are the most important vectors of malaria amongst the 13-15 species of anophelines recorded in the territories of the FSU, while the others, *An. maculipennis*, *An. martinius*, *An. atroparvus*, and *An. messeae* appear to be less effective . However, *An. messeae* is the main vector of malaria in Russia and Ukraine and is reported to be extending its range and increasing in population density in many areas. However, recently ELISA showed the human biting index of the local rural population of *An. messeae* is relatively low (3.9%). Nevertheless, the vectorial capacity appeared to be dangerously high due to its high population density (Nikolaeva *et al.* 1996).

Gordeev & Sibataev (1996) studied chromosome polymorphism and morphological variation in central and peripheral populations of *An. messeae* from Siberia, Kyrgyzstan, and southern Kazakistan. As with many species with chromosome polymorphism, the level of inversion polymorphism decreases towards the boundary of its range.

9.2.3 Aim

Following an approach from Dr. Mikhail Gordeev from Research Institute of Biology and Biophysics, Tomsk State University, Russia, who is working on ecological and cytological aspect of malaria vectors in FSU especially *An. maculipennis* species complex, this study was carried out to investigate and compare the level of polymorphism between and within populations of *An. beklemishevi*, *An. messeae* (northern and southern inversions) and *An. maculipennis s.l.* from Iran.

9.3. MATERIAL AND METHODS

9.3.1 Mosquito & their origin:

Dr. Mikhail Gordeev from Research Institute of Biology and Biophysics, Tomsk State University, Russia, kindly sent 90 larvae of *An. beklemishevi* and *An. messeae* without salivary glands preserved in alcohol following fixation in Carnoy's. The karyotypes of these larvae and their sex and species composition have been determined by Dr. Gordeev (Table 9.1). All these samples were from one breeding place in Teguldet village (about 200 km to the east of Tomsk, West Siberia in 1995. Dried, pinned *An. maculipennis s.l.* from Iran were collected by Djadid from Guilan (Ansali) and Mazandran (Ramsar, Babol, Sari) provinces in Iran during 1988-89 (see Fig 3.1).

9.3.2 DNA preparation

DNA was prepared by and phenol extracted after the method of Ballinger-Crabtree *et al.* 1992).

9.3.3 Primer

6 primers from AB01 series and three SSR primer have been used for this study, but only two of those, AB19 (ACCCCCGAAG) and X19 [(AGT)$_5$GCCA] produced amplification products that were discriminative. For ITS2 primers sequences see Ch.3.

Table 9.1- List of karyotypes in *An. messeae* and *An. beklemishevi* from Russia

$XL_{00(01,11)}2R_{00}3R_{00(01,11)}3L_{00(01,11)}$ - "southern" chromosomal combinations in An. messeae;
$\mathbf{XL_{11(12,22)}2R_{11(01)}3R_{11(01,00)}3L_{00(01,11)}}$ - "northern' karyotypes in An. messeae;
m - male; f - female;
An. beklemishevi XL_{01} - heterozygotes in An. beklemishevi (we did not determine homozygotes).

9.3.4 PCR amplification and product analysis

PCR amplification and agarose gel electrophoresis was carried out as described in chapter 3.

9.3.5 Data analysis

ITS2 sequence of *An.maculipennis* s.l. from Iran were compared to the unpublished sequence data from Russian members of *An.maculipennis* (Townson and Adeniran) by CLUSTAL V.

9.4 RESULTS

9.4.1 SSR primer

X19, an AGT repeat, differentiated *An. beklemishevi* from *An. messeae* ('northern' and 'southern' inversions) and *An. maculipennis s.l.* from Iran (Fig. 9.1). The number of amplified bands ranged between 3-5 and in the size between 235-800bp. Table 9.2 contains the main fragments size in three species.

An. messeae northern and southern inversions could be distinguished by a 800bp band which is specific to the southern population. In *An. beklemishevi* 445,485bp double bands were present in all individuals examined, which shows the homogeneity of this population. *An. maculipennis s.l.* from Iran shared the 445bp band with *An. beklemishevi* but has another 640bp specific band (not included in Fig. 1) in specimens collected from two sites about 700km apart.

9.4.2 RAPD primers

AB19 primer

This primer produced a single 700bp product in *An. beklemishevi* , five bands in *An. messeae* northern and southern karyotypes with two main bands (255,320bp), and three main bands of 400, 500, and 565bp in *An. maculipennis s.l.* from Iran. Distinct species-specific patterns allow the differentiation of three species, but as it has shown in Fig. 2 there is no consistent difference between northern and southern populations of *An. messeae* and the two Iran's populations of *An. maculipennis s.l.*.

Fig. 9.1- SSR X19 primer product in *An. messeae* (northern: 1-2, 6-10, southern: 3-5) and *An. beklemishevi* (12-17) from Russia; Marker (11); -ve control (18).

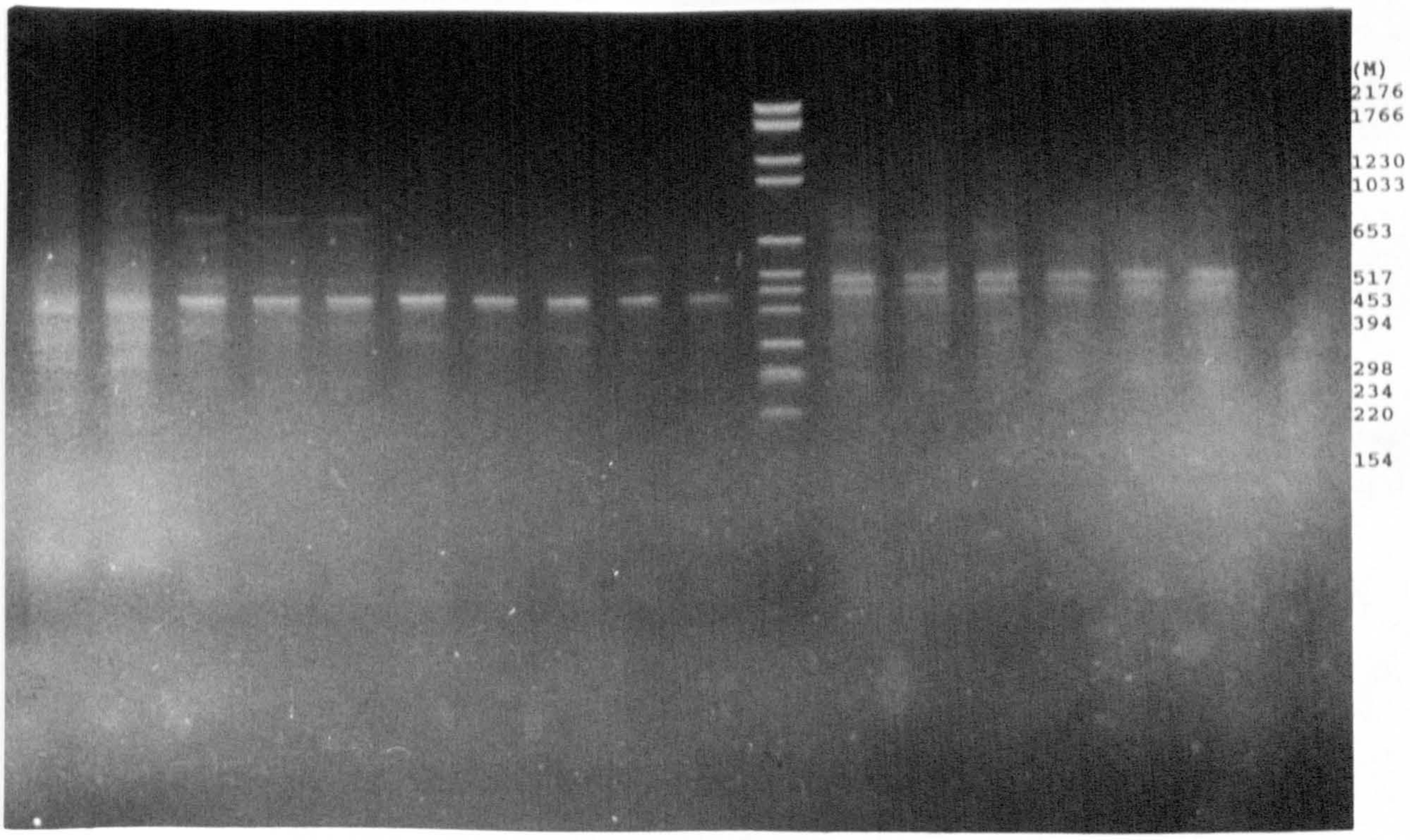
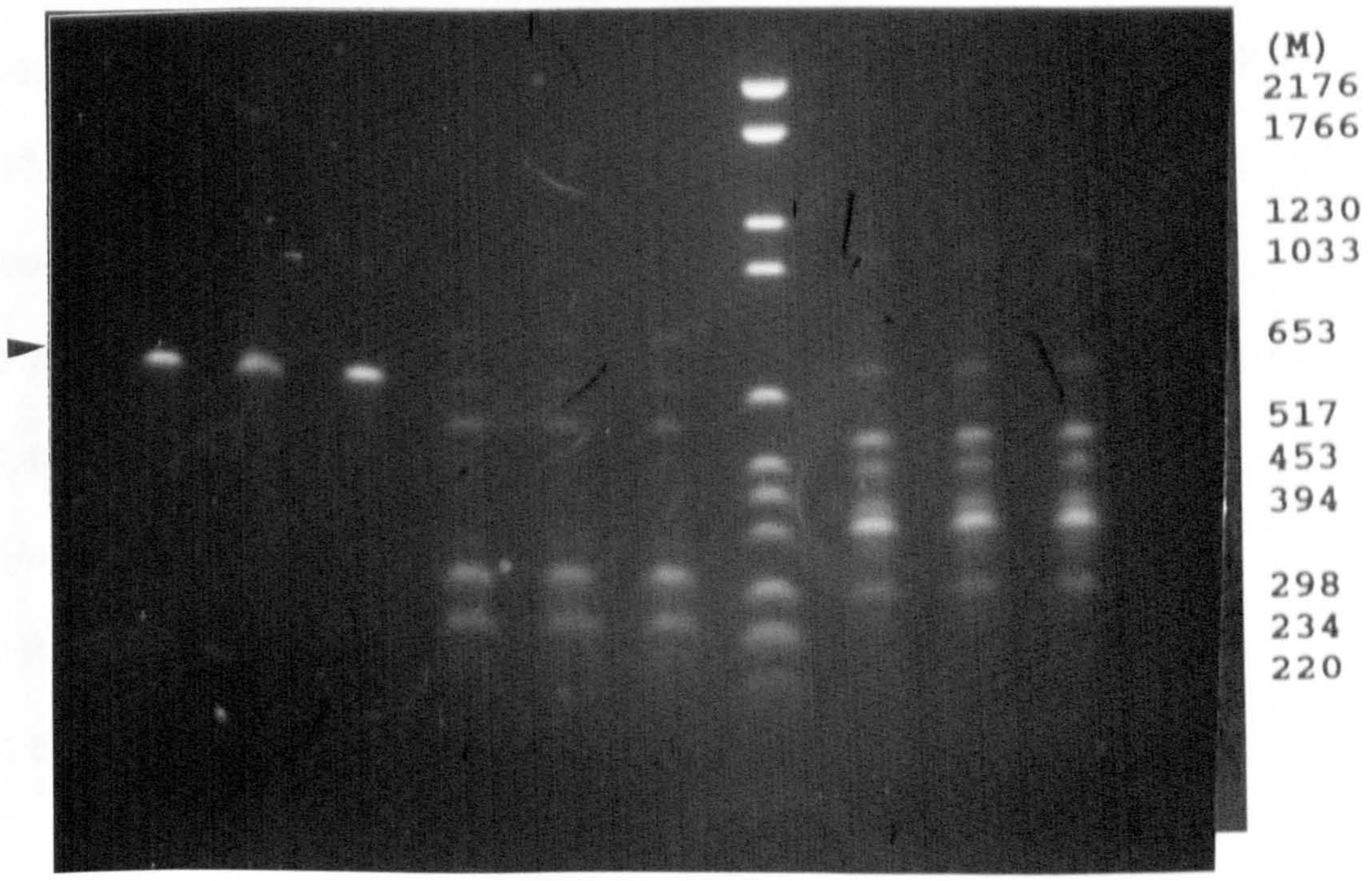


Table 9.2- Species-specific pattern in each member of *An. maculipennis* species complex. *An. messeae* produced three different patterns within northern population. *An. beklemishevi* showed a double bands pattern which is consistent in all individuals examined.

SPECIES	MAIN FRAGMENTS SIZE
An. beklemishevi	445, 485, 680, 760 bp
An. messeae (Northern1)	408, 430 bp
An. messeae (Norhern2)	417, 430 bp
An. messeae (Northern3)	417, 430, 590 bp
An. messeae (Southern)	417, 430, 800 bp
An. maculipennis	445, 640 bp

Fig. 9.2- RAPD AB19 primer product in *An. beklemishevi* (1-3) and *An. messeae* (Northern (4,5), southern (6)) from Russia, and *An. maculipennis* from Iran (8-10), Marker (7); -ve control (11).



M13 primer

RAPD amplification of this primer also produced different patterns in *An. maculipennis s.l.* and *An. sacharovi* from iran (Fig. 9.3).

9.4.3 rDNA ITS2 PCR

In order to determine the sequence variation within this species complex from Iran, the ITS2 region have been amplified (Fig. 9.4) and compared to those data from *An. atroparvous* and *An. messea* (Fig. 9.5 and 9.6). Using PHYLIP program, two phylogenetic tree based on ITS2 sequence were constructed (Fig. 9.7). Both trees showed that *An. maculipennis* s.l. comprises three different types of sequences.

Iran 1 from Anzali in south western area of Caspian sea corresponds to An. messea, Iran 2 from Ramsar is similar to An. sacharovi from Iran, and Iran 3 from Babol (Mazandran) corresponds to An. atroparvous

Fig. 9.3- RAPD primer M13 Amplification products of malaria vectors from Iran: *An. stephensi* (1-3), *An. maculipennis s.l. (4-5)*, *An. sacharovi (6)*, marker (7), *An. dthali* (8)

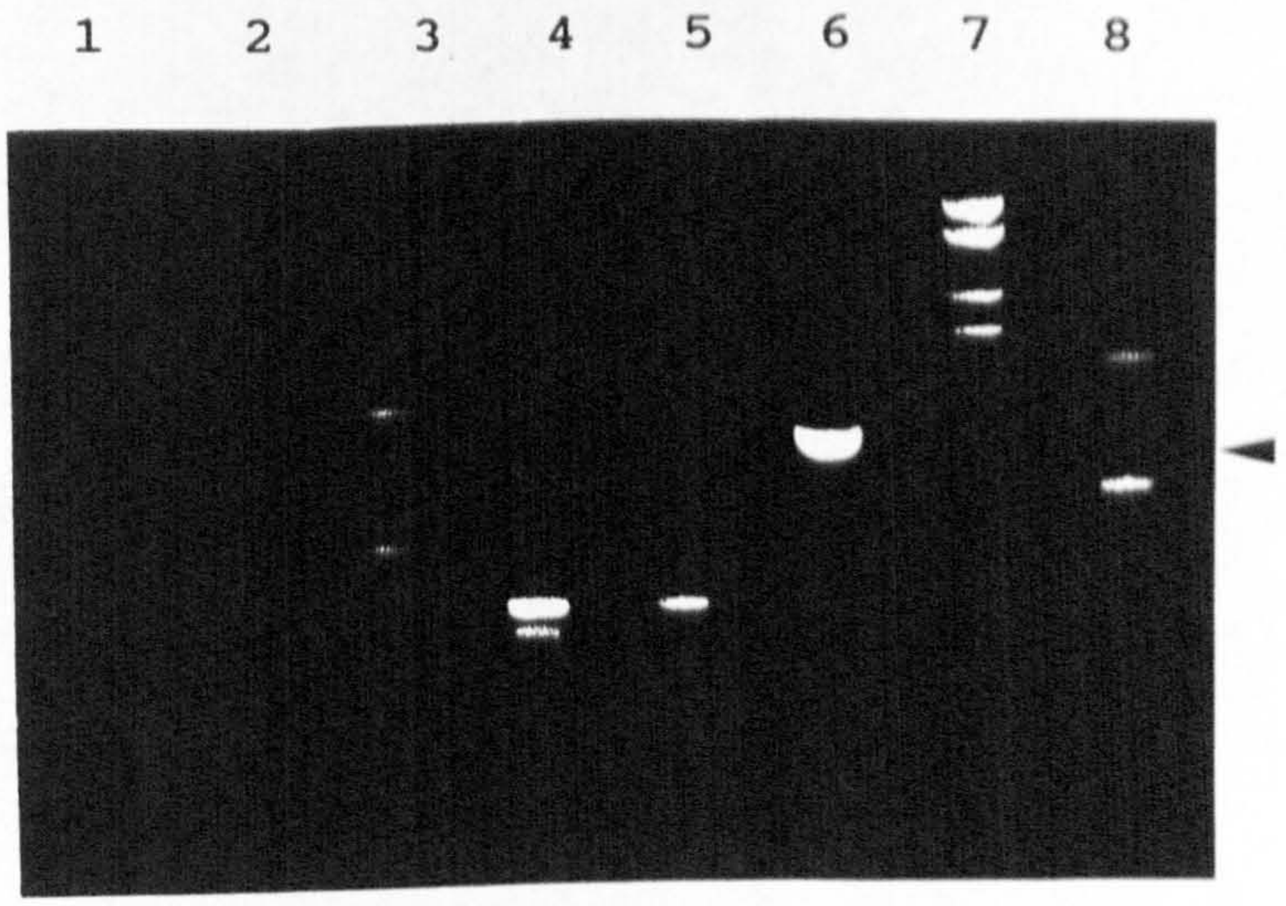
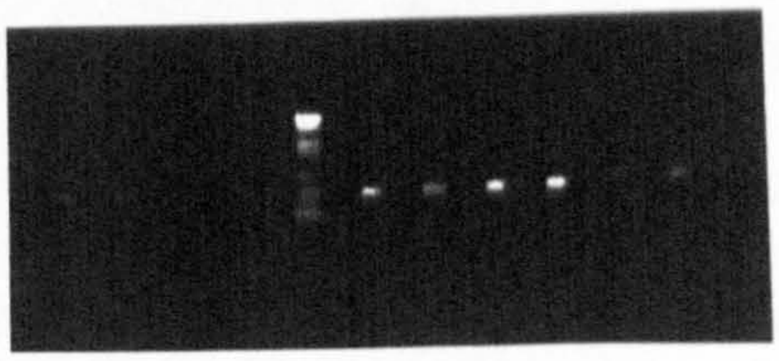


Fig. 9.4- ITS2 amplification products in *An. maculipennis s.l.* (1-4, 6-9), and *An. sacharovi* (10-11); marker(5)



9.5 DISCUSSION

The main objectives of this study was the detection of differences between members of *An. maculipennis* species complex from Iran and Russia. Both SSR and RAPD primers can differentiate three species. SSR primer X19 has the ability to distinct northern and southern populations of *An. messeae*, and also showed different patterns within northern specimens. The simple pattern of each species (one or two main bands) with this primer is an advantage.

The results with RAPD primer AB19 are also encouraging since it produced a single band in *An. beklemishevi* and could also be used in differentiation of three species. Based on egg ridge pattern, Faghih *et al.* (1969) and Manouchehri *et al.* (1976) claimed that four members of *An. maculipennis* species complex are present in northern Iran: *An. maculipennis* s.s. (typicus) in Ramsar (Mazandran province), *An. subalpinus* in Sari, Babolsar, Chalous in Mazandran , and Astanch in Guilan provinces, and *An. melanoon* in Astaneh, with *An. subalpinus* and *An. melanoon* sympatric in Astaneh on the border of the two provinces. *An. sacharovi* is present in all parts of these two provinces in northern Iran. Results of this study did not show any differences among *An. maculipennis* s.l. populations from Iran but M13 detected differences between *maculipennis s.s.* and *sacharovi*.

ITS2 region has been amplified in order to determine sequence variation in *An.maculipennis s.l.* specimens collected from four areas in southern part of Caspian

sea. The results confirmed those sequence correspond to *An. sacharovi*, *An. messeae* and *An. atroparvous*. Although the preliminary results with RAPD, SSR and ITS2 are promising and can serve as first report on molecular systematics of members of this species complex in Iran, but a better understanding of species-specific characters and intra-specific variation within this complex requires that more specimens to be examined. Nevertheless these preliminary results form a useful basis for future more detailed work.

Fig. 9.5 - CLUSTAL V multiple sequence alignment in *An. messeae* (seqmest) from Russia and *An. maculipennis* species complex (seq9mac, seq5mac, seq13mac) from Iran.

```
seqmest         GCTAAATGCGCGTCACAATGTGAACTGCAGGACACATGAA-CACCGATAA
seq9mac         GCTAAATGCGCGTCACACTGTGAACTGCAGGACACATGAA-CACCGATAA
seq5mac         GCTAAATGCGCGTC-CAATGTGAACTGCAGGACACATGAAACACCGATAA
seq13mac        GCTAAATGCGCGTCACAATGTGAACTGCAGGACACATGAA-CACCGATAA
                ********************** ************************* *********

seqmest         GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
seq9mac         GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
seq5mac         GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
seq13mac        GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
                ************************************************

seqmest         GAGTGCCCATATTTGACCC--ATTCAAGTCAAACTACGT--ACCTCCGTG
seq9mac         GAGTGCCTATATTTGACCC--A-----GGTCAAACTACGT--ACCTCCGGG
seq5mac         GAGTGCCCATATTTGATCATAACCCAAGCCAAACGGCGT--ACCTCACCG
seq13mac        GAGTGCCTATTTTTGACCATCA--GAAGTCAAACTACGTCGGCGGCGCCG
                ******* ** ***** *   *      * *****  ***   *  *  *

seqmest         TACGTGCAT-GATGATGAAAGA-GTTTGGA---ACACCTTCCTT-CTCTT
seq9mac         TACGTGCAT-GATGATGAAAGA-GTTTGGA---ACACCATCCTT-CTCTT
seq5mac         TACGTGGA--GTTGATGAAAGG-GTCTGGA--TACGCCATCCTTTCTCTT
seq13mac        TACGTGCATAGATGATGAAAGATGGTGGGACGTAAAACATCCCATCTCTT
                ****** *  * *********  *   ***   *   * ***   *****

seqmest         GCATTGAA-AGCGCAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTG
seq9mac         GCATTGAA-AACGCAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTG
seq5mac         GCATCGAA-GTCGTAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTG
seq13mac        GCATTGAATACCGTAGTGTGTAACA-CCCAGGGCTTCAACTTGCAAAGTG
                **** ***   ** ** ***** ** ***  ** ******************

seqmest         GCCATGGGGCTGACACCTCACCACCATCAGCGTGC-TGTGTAGCGTGTTC
seq9mac         GGCATGGGGCTGACACCTCACCACCATCAGCGTGC-TGTGTATCGTGTTC
seq5mac         GCCATGGGGCTGACACCTCACCACCATCAGCGTGC-TGTGTATCGTGTTC
seq13mac        ACCATGGGGCCAACACTTCACCGCCATCT-TGTGCATGTGTAGTGTGTGC
                  ********  **** ***** *****   **** ******  **** *

seqmest         GGCCCAGTAAGGTCATCGTGAGGCGTCACCTAACGG-GGAAGCACAC-AC
seq9mac         GGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGG-GGAGGCACAC-AC
seq5mac         GGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGG-AGAAGCACCA-GC
seq13mac        GGCCTAGCTTGGTTAACGTGAGGCGA-ACCCAACGGAGGAAGCACAATAC
                **** **   *** * *********  *** *****  ** ****    *

seqmest         TGTTGCGCGTATCTCGTGGTTCTAACCCAACCATAGCAGCAGAGGTACAA
seq9mac         TGTTGCGCGTATCTCATGGTT---ACCCAACCATAGCAGCAAAGATACAA
seq5mac         TGCTGCGTGTATCTCATGGTTACC-CCCAACCATAGCAGCAGAGATACAA
seq13mac        AACTGCGCGTATCTCATGGTTCTAACCCAACCATAGCAACAGAGATACAA
                  **** ******* *****     ************** ** ** *****

seqmest         GACCAGCTCCTAGCGGCGGGAGCTCATGGGCCTCAAATAATGTGTGAGTA
seq9mac         CACCGGCTCCTAGT------AGCCCATGGGCCTCAAATAATGTGTGACTA
seq5mac         GACCAGCTCCTAGCAGCGGGAGCTCATGGGTCTCAAATAATGTGAGACTA
seq13mac        AACCAGCTCCTAGCTACGGGAGTACATAGGCCTCAAATAATGTGAGACTA
                 *** ********         **   *** ** ************** ** **

seqmest         CCCCCTAAATTTAAGCATA
seq9mac         CCCCCTAAATTTAAGC-TA
seq5mac         CCCCCTAAATT--AGC-TA
seq13mac        CCCCCTAAATTTAAGC-TA
                ***********  *** **
```

Fig. 9.6- CLUSTAL V multiple sequence alignment in *An. atroparvous* (SEQATT) from Russia and *An. maculipennis* species complex (seq9mac, seq5mac, seq13mac) from Iran

```
SEQATT      GCTAAATGCGCGTCACAATGTGAACTGCAGGACACATGAA-CACCGATAA
seq9mac     GCTAAATGCGCGTCACACTGTGAACTGCAGGACACATGAA-CACCGATAA
seq5mac     GCTAAATGCGCGTC-CAATGTGAACTGCAGGACACATGAAACACCGATAA
seq13mac    GCTAAATGCGCGTCACAATGTGAACTGCAGGACACATGAA-CACCGATAA
            ********** ** ********************** *********

SEQATT      GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
seq9mac     GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
seq5mac     GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
seq13mac    GTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTACACATTTTT
            ************************************************

SEQATT      GAGTGCCCATATTTGATCATAACCCAAGCCAAACGGCGT--ACCTCACCG
seq9mac     GAGTGCCTATATTTG------ACCCAGGTCAAACTACGT--ACCTCCGGG
seq5mac     GAGTGCCCATATTTGATCATAACCCAAGCCAAACGGCGT--ACCTCACCG
seq13mac    GAGTGCCTATTTTTGACCATCA--GAAGTCAAACTACGTCGGCGGCGCCG
            ******* ** ****       *   * * *****  ***   *  *  *

SEQATT      TACGTGGA--GTTGATGAAAGG-GTCTGGA--TACGCCATCCTTTCTCTT
seq9mac     TACGTGCAT-GATGATGAAAGA-GTTTGGA---ACACCATCCTT-CTCTT
seq5mac     TACGTGGA--GTTGATGAAAGG-GTCTGGA--TACGCCATCCTTTCTCTT
seq13mac    TACGTGCATAGATGATGAAAGATGGTGGGACGTAAAACATCCCATCTCTT
            ****** *  * *********  *   ***   *   *****   *****

SEQATT      GCATCGAA-GTCGTAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTG
seq9mac     GCATTGAA-AACGCAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTG
seq5mac     GCATCGAA-GTCGTAGCGTGTAGCAACCCCAGGTTTCAACTTGCAAAGTG
seq13mac    GCATTGAATACCGTAGTGTGTAACA-CCCAGGGCTTCAACTTGCAAAGTG
            **** ***   ** ** ***** ** ***  ** ****************

SEQATT      GCCATGGGGCTGACACCTCACCACCATCAGCGTGC-TGTGTAGCGTGTTC
seq9mac     GGCATGGGGCTGACACCTCACCACCATCAGCGTGC-TGTGTATCGTGTTC
seq5mac     GCCATGGGGCTGACACCTCACCACCATCAGCGTGC-TGTGTATCGTGTTC
seq13mac    ACCATGGGGCCAACACTTCACCGCCATCT-TGTGCATGTGTAGTGTGTGC
              ********  **** ***** *****   **** ******  **** *

SEQATT      GGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGGAG-AAGCACCA-GC
seq9mac     GGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGGGG-AGGCACAC-AC
seq5mac     GGCCCAGTTCGGTCATCGTGAGGCGTTACCTAACGGAG-AAGCACCA-GC
seq13mac    GGCCTAGCTTGGTTAACGTGAGGCGA-ACCCAACGGAGGAAGCACAATAC
            **** ** * *** * *********  *** ***** * * ****    *

SEQATT      TGCTGCGTGTATCTCATGGTTACC-CCCAACCATAGCAGCAGAGATACAA
seq9mac     TGTTGCGCGTATCTCATGGTTACC-C--AACCATAGCAGCAAAGATACAA
seq5mac     TGCTGCGTGTATCTCATGGTTACC-CCCAACCATAGCAGCAGAGATACAA
seq13mac    AACTGCGCGTATCTCATGGTTCTAACCCAACCATAGCAACAGAGATACAA
               **** **************     *   ********** ** ********

SEQATT      GACCAGCTCCTAGCAGCGGGAGCTCATGGGTCTCAAATAATGTGAGAGTA
seq9mac     CACCGGCTCCTAGTAGC------CCATGGGCCTCAAATAATGTGTGACTA
seq5mac     GACCAGCTCCTAGCAGCGGGAGCTCATGGGTCTCAAATAATGTGAGACTA
seq13mac    AACCAGCTCCTAGCTACGGGAGTACATAGGCCTCAAATAATGTGAGACTA
             *** ********    *         *** ** ************** ** **

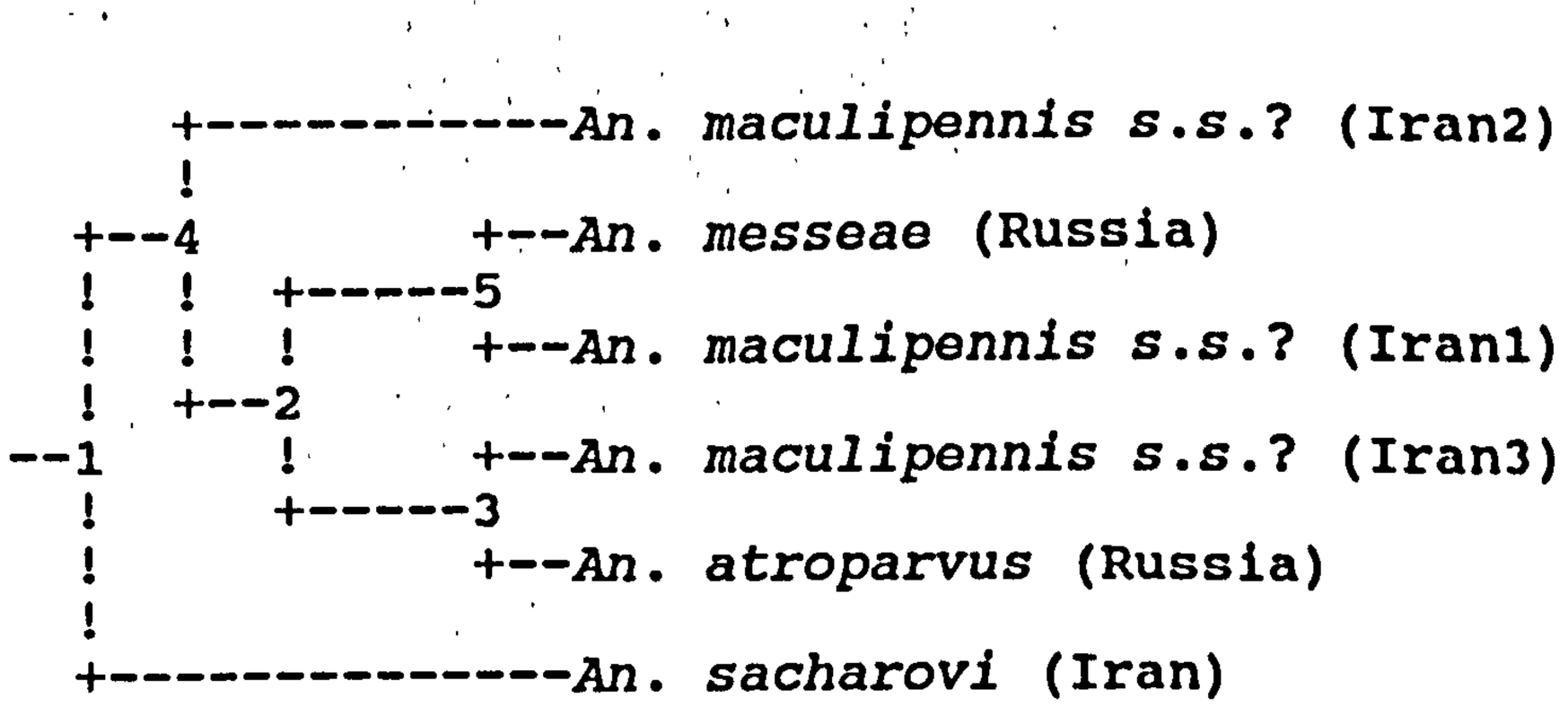
SEQATT      CCCCCTAAATTTAAGCATA
seq9mac     CCCCCTAAATTTAAGC-TA
seq5mac     CCCCCTAAATT--AGC-TA
seq13mac    CCCCCTAAATTTAAGC-TA
            ***********   *** **
```

Fig. 9.7- Phylogenetic tree based on ITS2 sequence in *An. maculipennis* species complex from Iran and Russia by two methods:

A: DNA parsimony algorithm, version 3.54c in PHYLIP

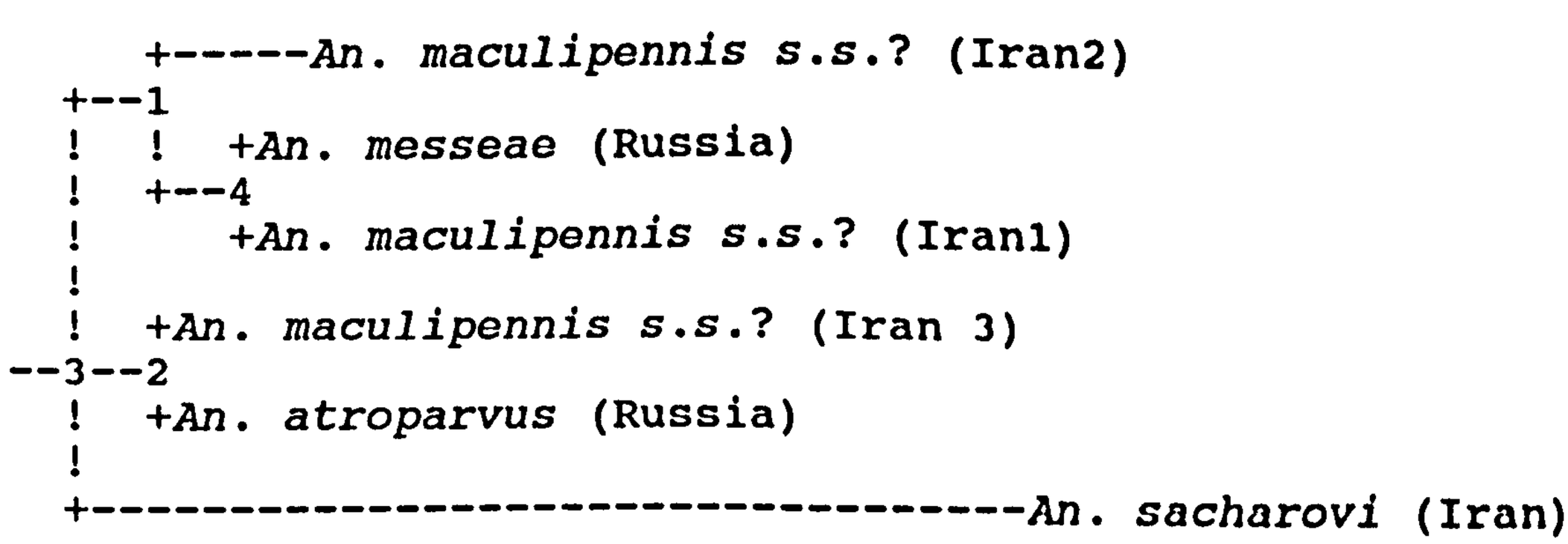
One most parsimonious tree found:

requires a total of 362.000



B: Fitch-Margoliash method version 3.54c in PHYLIP

examined 38 trees



CHAPTER TEN

GENERAL DISCUSSION AND CONCLUSION

10.1 MOLECULAR SYSTEMATICS OF SPECIES COMPLEXES

Systematics is primarily a science concerned with the diversity of organisms and the relationships between them (Simpson, 1961). As pointed out by Mayr and Ashlock (1991) it is a concept that embraces diversity and all biological interactions among organisms and this explains why such a broad area of common interest has developed between systematics, evolutionary biology, ecology and behavioral biology. The advent of molecular biological tools, and particularly the use of PCR, has revolutionised systematics, leading to a whole new area of science, termed Molecular Systematics.

Although the tools are new, at its heart this science is concerned with many of the same issues that dominated old systematics (Mayr, 1969). Thus a major concern in species level systematics (perhaps what Mayr and Ashlock (1991) called microtaxonomy), is to determine, by comparison, what the unique properties are of individual species, to discover what properties species might share with each other and what the biological causes are for these differences or shared characters. Finally it concerns itself with variation within and between species.

Nevertheless, although the questions themselves are scarcely new, so powerful are these techniques that biologists can revisit important areas at the interface with systematics, particularly those concerning the ecology of organisms and their population genetics. This revival is illustrated by the enormous interest in molecular ecology as shown by the success of the relatively new scientific journal, Molecular Ecology, which devotes itself to precisely this interface and which, according to SCI journal Citation Report, is already more heavily cited than any of the standard journals in either ecology or entomology.

There are powerful reasons for developing the molecular systematics of disease vectors, many of them related to improving our knowledge of disease epidemiology or applying new, molecular based methods of control (Curtis and Townson, 1998). Furthermore, molecular systematics can help us explain otherwise puzzling differences in vectorial capacity and response to control.

Apart from these essentially practical reasons, the application of molecular techniques to the systematics of disease vectors also contributes to the fundamental sciences on which it draws. Thus studies of the systematics of anopheline mosquitoes can contribute to a better understanding of the evolutionary forces that shape species and even of the speciation process itself. The apparently recent evolutionary history of the *An. gambiae* complex provides a particular opportunity of observing evolution in action. Additionally new insights into the forces that determine population structure have come from molecular systematic studies directed at microsatellite loci in the

complex (Lehmann et al., 1996b, 1997).

In *Anopheles* sibling species complexes, the most important issue to emerge is the difference in biology, ecology and response to control among the constituent species. It has long been accepted that vector control, either by reducing the infected vectors' expectation of life or reducing the vector-human contact rate is the most effective, appropriate and realistic method in malaria control. However, it seems that these measures can not rely on traditional tools alone and as Collins and Besansky (1995) commented in referring to Spielman *et al.* (1993), it is time for 'turning deep into the laboratory'. Major progress in the application of technology to systematic problems is expected in two directions: (1) increase in analytical power per unit effort, and (2) simplification in technology, and ultimately reduction in expense. The introduction of RAPD fingerprinting is a substantial contribution toward the second direction (Hadrys *et al.*, 1992) although its analytical power is improved by a better understanding of the nature of RAPD patterns.

Phil Hedrick (1992) suggested that answers to puzzles in population biology would develop rapidly through developing RAPD-PCR and its application in different organisms.

10.2 THE CURRENT STUDY

The results of the present study using a range of different DNA-based techniques, should be seen as a part of our efforts to understand the complexity of the malaria vector genome. The current studies have been largely designed to apply and develop RAPD and other related PCR-based techniques to provide more data and hence insight into the population genetics of species complexes.

10.2.1 Species specific markers

The concordance of the RAPD results obtained in this study with other available genetic and chromosomal data from these species complexes, provides strong support for the application of RAPD and related techniques in molecular systematics to species complexes. RAPD seems a particularly sensitive technique for the study of population structure in different geographic populations.

RAPD markers have been found within a wide range of specimens, mainly field collected, *An. gambiae*, *An. culicifacies*, *An. maculipennis*, *An. fluviatilis*, *An. subpictus* species complexes and in *An. stephensi*. These markers can be used for species diagnosis, detection of genetic variation within species and sub-species or varieties, and in progeny studies.

In *An. gambiae*, for example, although a molecular diagnostic method based on rDNA-IGS sequence has been described by Scott *et al.* (1993), the demonstration in this thesis that four RAPD primers can differentiate all six members of the

complex suggests that RAPD is especially likely to be useful where gene-specific primers are not available, the situation at present with most malaria vectors. Elsewhere in this study RAPD has been shown to be of value in the differentiation of species A and B in *An. culicifacies*; *An. messeae* and *An. beklemieshevi* in *An. maculipennis* complex and sibling species of the *An. subpictus* complex.

10.2.2 Intra-specific markers

Intra-specific variation is an important issue in the study of populations. The results with *An. gambiae*, *An. culicifacies*, *An. supbictus*, and *An. fluviatilis* revealed reliable markers which can be use for identification of new members within a complex or provide basic evidence for subsequent investigations by sequence-based techniques. Primary results with RAPD primers showed an unusual RAPD pattern in populations of *An. culicifacies* from Iran and further investigation by ITS2 primers demonstrated the presence of a related variant form, outside the *An. culicifacies* complex. In *An. subpictus* also, the presence of at least two species have been confirmed by both RAPD and ITS2. Generating RAPD pattern for progeny in *An. stephensi*, and *An. gambiae* demonstrated the reproducibility of RAPD.

All the available data demonstrate that there is a vast reservoir of as yet unknown nuclear DNA polymorphism in malaria vectors and RAPD can help detect some of this hidden genomic variation. In this thesis, other techniques such as rDNA PCR (ITS2 and IGS), SSR-PCR and DNA probes have been compared with RAPD results. These comparisons reveal that RAPD will be valuable in studies involving

molecular taxonomy, especially with species complexes. It is also an appropriate choice in epidemiological studies where an estimate of the genetic relatedness of different geographic populations of a species will be an important tool in studies of vector movement (as demonstrated within *An. culicifacies* populations from Iran and Sri-Lanka).

The use of RAPD and other PCR-based techniques in this study provides a picture of the population structure of malaria vectors, in which drift and selection are the major causes of differentiation. Furthermore, the fidelity of the RAPD-PCR patterns and their reproducibility over widely separated localities indicates that the genetic differences between and within members of each species complex are not simply artifacts of isolation by distance. Hence it can explain that sometimes the amount of variation among individuals of a species was much greater than that detected among different species of a complex, perhaps due to local diversity or differences in the amount of localized repetitive sequences (i.e. *An. arabiensis* populations in *An. gambiae* complex).

Favia *et al.* (1994a) reported that of all RAPD markers detected, only about 10% can be useful for the generation of strain-specific identifiers. They concluded that the combination of the presence or absence of distinct bands, with fingerprints using completely different primers and methods, can, after a relatively short period of standardization, provide an ideal method for cryptic species identification (Wilkerson *et al.*, 1993).

Ballinger-Crabtree *et al.* (1992), working on *Aedes aegypti* sub-species and populations, revealed that RAPD fragments may be 99.1% diagnostic for subspecies. In their study amplification products and statistical analysis of the population frequencies of 16 of the RAPD fragments generated were required to differentiate between 11 populations using estimation of genetic distances. In addition, the similarity index method was used to determine the relatedness of un-known populations to known populations (Ballinger-Crabtree *et al.*, 1992).

Variation of amplification products and banding patterns between closely related individuals or species in RAPD is either due to polymorphisms in the sequence recognized by the primer, which results in the absence of an amplification product, or by length polymorphisms between the primer sequences, which gives rise to amplified fragments of different lengths.

Because linkage between different arbitrary priming sequences is extremely unlikely, the number of independent polymorphic markers analyzed can be rapidly increased by pooling markers revealed by several primers. The amplification of monomorphic RAPD markers may be kept to a minimum by choosing the right primer-template combination, and any monomorphic markers may be removed from the analysis to decrease background band sharing (Hadrys *et al.*, 1992).

The variation detected by random sequences may represent non-coding sequences, and as different parts of the genome evolve at different rates (Kimura,

1983; Nei, 1987), it is possible that one or several regions amplified by PCR evolve at a higher rate compared with for example the enzymatic loci (Baruffi *et al.*, 1995; Begun & Aquadro, 1993). Thus the power of RAPD to detect genetic variation may in part be explained in terms of the higher evolutionary rates of the DNA regions which RAPD primers amplify, especially if they contain micro- and/or minisatellites. However, Favia *et al.* (1994b) believe that RAPD polymorphisms are due to other reasons than mere differences in microsatellite-like repeats.

Another possible explanation for the greater variability detected by RAPD-PCR is that RAPD-PCR preferentially amplifies DNA from highly variable regions. Although drift may contribute to the decreasing variation at the anonymous DNA sequences (RAPD), directional selection may have reinforced the action of drift in the reduction of variability.

Because RAPDs are PCR-based, analysis requires only minute amounts of sample material, in our hands, even less than 1/1000th of the DNA that can be obtained from a single anopheline mosquito. Therefore, RAPDs can be used in conjunction with other DNA studies, and it is possible to perform RAPD and chromosomal or allozyme analysis on the same individual.

10.2.3 RAPD characterization

In this study, further characterization of some fragments of interest have been achieved by using the RAPD products as probes (in *An. culicifacies* species complex)

or cloning and sequencing RAPD fragments (*An. bwambae* and *An. culicifacies*) in the hope of providing more data on the nature of RAPD bands. Surprisingly in both species a common homologous sequence was found, perhaps because of the presence of regions homologous to AB11 primer sequence in both fragments (see Ch. 4 for details). These regions also contain some repeats and showed apparent homology with published sequences which needs further investigation.

Williams *et al.* (1990), working with Glycine soya and G. max, cloned 11 RAPD-PCR amplified fragments and used these as probes of total digested genomic DNA in a southern analysis. Only five of these fragments were found to be repetitive, indicating that not all amplified regions are repetitive.

Favia *et al.* (1994a) detected the repetitive nature of the RAPD markers using dot blot hybridization but not by in situ hybridization to polytene chromosomes. However, the laboratory of Coluzzi that G. Favia's work was carried out has extensively mapped RAPD loci. On average, one polymorphic locus for every primer has been identified (Favia *et al.*, 1994b).

Generally, the proportion of monomorphic bands exceeds that of polymorphic ones in laboratory strains as well as in the wild populations. RAPD analysis using four primers on the 11 populations of *Glycine max* allows the definition of 176 fragments, only two of which have been found to be monomorphic. However, bands reported as monomorphic may hide genetic heterogeneity, because of the dominance

of RAPD markers (Williams *et al.*, 1990; Baruffi *et al.*, 1995)

The mean value of polymorphism detected by PCR is probably an underestimate because of the dominance of RAPD markers (Haymer & McInnis, 1994). In comparison to multilocus enzyme electrophoresis, RAPD analysis reveals a surprisingly high degree of polymorphism in *C. capitata* and exceeds about seven fold the number of biochemical loci analyzed (176 vs. 26). In addition, the degree of polymorphism revealed by these RAPD markers is extensive: in the wild populations, the mean proportion of polymorphic DNA fragments is 0.66 whereas that for polymorphic biochemical loci is 0.38 (Haymer *et al.*, 1992).

10.2.4 Phylogeny based on RAPD, SSR and ITS2 data

Phylogenetic trees were constructed based on both RAPD, SSR markers (presence and absence of bands) and/or ITS2 sequences of *An.gambiae*, *An.culicifacies*, *An.fluviatilis*, *An.subpictus* and *An.maculipennis*. Each species appeared to be correctly placed in the trees. In *An. gambiae*, by scoring all RAPD fragments in six members of complex, and calculating various distance indices, the two most important species *An.gambiae s.s.* and *An. arabiensis* appeared as sister taxa, which is in concordance with phylogenetic trees based on rDNA and mtDNA.

However, it is well known that the phylogenetic trees reconstructed from different genes for the same sets of organisms are often different (e.g. Goodman *et al.* ,1982; Hedges, 1994). This is true even with mitochondrial DNA (mtDNA), where all genes are inherited together without recombination and there is no confusion

of orthologous and paralogous genes (e.g. Cao *et al.*, 1994a & 1994b; Simon *et al.*, 1994; Honeycutt *et al.*, 1995). These differences may be caused by sampling error of nucleotides or codons, different patterns of nucleotide or amino acid substitutions, etc., but in most cases it is difficult to know which of the reconstructed trees is the correct one because the true tree is unknown (Russo *et al.*, 1996). It is possible that some genes are more suitable for reconstructing a phylogenetic tree than others, but it is usually difficult to know which gene is the best (Russo *et al.*, 1996).

On the other hand Wilkerson *et al.* (1995) suggest that because the taxa within *An. albitarsis* were distinguished by strictly correlated, fixed markers rather than allele frequency differences, statistical analysis were not necessary to support the hypothesis of reproductive isolation. Besides, those taxa recognized by RAPD were sympatric at many localities and therefore considered to be reproductively isolated groups and represent distinct species.

10.3 REPRODUCIBILITY OF RAPD

One of the criticisms that has been at RAPD PCR concerns its reproducibility. The results of this study show that using an appropriate DNA extraction method and optimized conditions, reproducibility is not a problem with RAPD amplification. The main concern about reproducibility is the quality and quantity of extracted DNA. A wide variety of DNA extraction techniques have been used in other organisms and often these can be applied in insects without any particular problem. However, for restriction enzyme digestion, library construction and as reported in this study for

RAPD-PCR and related techniques, it is necessary to remove most of the protein, RNA and a further purification by phenol extraction is recommended.

1.3.1 DNA optimization

In epidemiological studies involving population genetics, which may require the processing of large numbers of DNA preparations at the same time, using as crude a sample as possible, will reduce the labour, time and hence costs of the study. For PCR, a small amount of DNA prepared in this way for example using boiling water extraction or homogenization and one step centrifugation does not affect normal PCR amplification, nor inhibit Taq activity. However, these kind of DNA preparations are not suitable for further analysis due to rapid degradation or changes in the secondary structure of the DNA, a problem mentioned in other's works (e.g. Black *et al.*, 1992). In this study, in which most specimens were collected in the field, the following procedures have been applied whenever possible.

1- On arrival, the first step was the correct identification of all individual specimens by available morphological keys although the correct identification of all mosquito samples used in this study have often been confirmed by those collaborators who kindly collected and/or sent specimens. For identification of members of the *An.gambiae* complex the diagnostic rDNA-PCR of Scott *et al* (1993) was employed, and in *An.subpictus* species complex identification was made by cytogenetic examination.

2- Prior to extraction, specimens have been left for at least half an hour in double distilled water in order to remove any external contaminants (small parts or scales from other specimens, ectoparasites, etc.) or to allow diffusion of the previous preservation solutions, and increasing the permeability of insect tissue to extraction buffer. However, some specimens on arrival were found to be covered with fungi due to bad preservation conditions (high humidity, insufficient preservative, etc.); these were discarded and never used.

3- single specimen of all life stages (eggs, larvae, pupae, adult) produced a sufficient amount of DNA for RAPD. However, working with eggs especially needs care and caution because of their small size and the possibility of contamination.

4- DNA was successfully isolated from fresh mosquitoes in all stages, alcohol preserved adult and larvae, lactophenol preserved larvae, and dried pinned museum specimens all were successful, but with some variation in the quantity and quality of the final DNA and of the amplified products. However, these problems can be minimised by using a standardized phenol extraction method or by adding a phenol extraction step to other methods. Phenol removes proteins which have remained bound to the DNA. This is an important result of this work, as it enables a wide variety of samples, preserved in different ways, to be analyzed with confidence. Reducing the amount of DNA in a PCR reaction helps, by dilution, to reduce the interference that may act as PCR inhibitors. Furthermore, applying one or two positive control from other species in each series of extractions, and comparing those

patterns with previously amplified products, is an indication of correct amplification.

However, minor differences in amplified patterns (e.g. amplifying non-specific bands, etc.) as a result of using different extraction methods is a matter of concern in all kinds of PCR technology. However, the presence of the main specific pattern always with higher intensity will provide the right identification/differentiation.

5- Thoroughly grinding is essential, especially in RAPD studies. Specimens can be ground to powder over liquid nitrogen, crushed in ice-cold homogenization or extraction buffer, or even without the need for ice, at room temperature (in Mini-Prep extraction).

6- The incubation time and temperature depends on the extraction method and insect size. However, a shorter incubation time needs a higher incubation temperature and vice versa.

In this study incubation times of 20, 30, 45, 60, 120 minutes and overnight have been used at 90, 75, 65 and 50°C although for routine extraction, an overnight incubation yields better results.

7- On removing the supernatant after first centrifugation, a few μl of the supernatant have been left in order to avoid contamination with an inhibitor such as SDS.

8- Adding potassium acetate or sodium acetate at an appropriate concentration and leaving overnight in the refrigerator allows better precipitation of the protein/detergent complex.

9- More DNA was recovered when, after adding ethanol, the tubes were left for 15 min at -20°C, at least half an hour on ice, or overnight in refrigerator.

10- After the final centrifugation, a dark pellet (yellow to black) is visible which is due to co-purification of mosquitoes' pigments with nucleic acids. However, often the pellet is not visible, although after running the resuspended pellets on agarose gel or on petri dish the presence and the quality of DNA can be determined.

The above procedures allowed the amplification of a reproducible and consistent pattern in all experimental work with different species.

Black *et al.* (1992) by comparing RAPD patterns among aphid daughters from a single mother found that bands in each lane are identical in mobility and intensity, indicating the reproducibility of the technique. But later, DNA from the same individuals showed a change in the number of bands . The change was apparently due to degradation of template DNA during freezing and thawing on consecutive days. This instability was also due to the manner in which the DNA was extracted (Black, 1993). However, he concluded that AP-PCR patterns are likely to be irreproducible in dried or otherwise preserved specimens where template degradation has been extensive.

10.3.2 Primers

It has been shown that if the RAPD amplification is repeated two or more times, the majority of markers are clearly reproducible and scorable (Hadrys *et al.*, 1992; Williams *et al.*, 1990; Arnold *et al.*, 1991; Hu & Quiros, 1991; Klein-Lankhorst *et al.*, 1991). Favia *et al.* (1994b) found that about 30% of primers used had to be discarded because of the high frequency of inconsistent results that they produced.

Primers are also very important in reproducibility of RAPD and their sequences can vary in length (Williams *et al.*, 1990; Welsh & McClelland, 1990), but in practice, 10-mer primers have been adapted as something of a universal standard. However, primers of lengths other than ten nucleotides (ranging from nine to sixteen) have been used by Chapco *et al.* (1992) and Perring *et al.* (1993). Innis & Gelfand (1990) described the basic role in designing PCR primers such as average G+C contents and the avoidance of internal palindromic sequences, self-compatible ends or runs of three or more identical nucleotides at 3' ends. The G+C and purine contents in RAPD primers is in the range of 60-80% with a tendency to have increasing representation of C residues towards the 3' end. These two features will provide more efficient amplification because G and C nucleotides form a more stable pairing configuration in double-strand DNA, and because the fidelity of pairing at the 3' end seems to be an important factor in promoting successful amplification in any PCR (Innis & Gelfand, 1909).

In doing RAPD-PCR, the initial choice of a primer is a major variable

determining what and how much real genetic variation will be identified. The observation that single substitutions, specially in the 3' ends of the primer, can change amplified banding patterns (Williams *et al.*, 1993) implies that annealing in RAPD must be precise (Black, 1993). It is the primer itself (along with the annealing temperature) that determines which regions of the genome will be amplified by the polymerase chain reaction (Haymer, 1994). Although other aspects of this method including the actual PCR cycling profile parameters such as denaturation time and extension time can influence the results, if all other things are equal, minor differences in these parameters do not result in substantial changes to sequences being amplified (Yu & Pauls, 1992).

Primer size will determine the degree of specificity in genome scanning. It may be expected that primers of short length will amplify an unreasonably large number of sequences and that larger primers will amplify too few sequences to be routinely informative. Beyond a certain primer size (15-mer) increasing primer length may also increase non-specific primer annealing, consequently increasing the probability of random non-reproducible amplification patterns. All studies using standard RAPD conditions (fragment separation on agarose gels) have found 10-bp primers to be an efficacious size. A G+C content of the primer similar to the G+C content of the analyzed genome will maximize the frequency of binding sites and hence amplification products.

10.3.3 Cycling parameters

Specially annealing temperatures and ramp times (the time taken to lower or raise the temperature between two steps), also affect banding patterns (Roherdanz *et al.*, 1993; Ellsworth *et al.*, 1993).

10.3.4 Band appearance

When the RAPD products have been amplified, in analyzing its pattern, band appearance should be included in any interpretation. By loading different amounts of the amplified product on a gel and running the gel for a longer time at a low voltage, Pillay & Kenny (1995) have observed that band brightness may be due to the presence of double bands that usually appears as single bands on the gel. They described additional amplification products appearing as faint bands, probably as a result of non-specific priming or artifacts of PCR. However, in the subsequent paragraphs they accepted that a faint band after hybridization to the similar-sized radiolabelled fragment, was a legitimate product of amplification, with strong homology to the probe. On the other hand, another labelled band revealed the presence of a fragment which has not been detected on ethidium bromide-stained agarose gel, and showed polymorphism for fragment length with a higher molecular weight band.

10.3.5 Co-migrating bands

Another important appearance of RAPD bands on gels is the presence of co-migrating bands. An assumption of the use of the RAPD technique is that amplified fragments are unique, i.e. that the procedure does not amplify two distinct fragments

which co-migrate on gels because of similar size. Co-migration in the RAPD technique is easily detected by eluting individual PCR products from gels and reprobing the products via southern analysis (Hadrys *et al.*, 1992). Alternatively, polyacrylamide gel electrophoresis may be used to increase the resolution of band separation.

Favia *et al.* (1994) defined as useful only those bands that were prominent in a given fingerprint of one strain and where no bands migrating with an apparent length of -+ 100 bp were present in the comparison strain. They claimed that the electrophoretic system employed could not detect differences of less than about ten base pairs.

Smith *et al.* (1994) by analysis of RAPD patterns among *Xanthomonas campestris* strains showed that fragments of equal mobility were not necessarily homologous and similarity in RAPD patterns among species do not reflect evolutionary relationships. In contrast, this study proved strong sequence homology of co-migrating bands in *An. culicifacies* species A and B (see Ch.5, AB11 primer results). Furthermore reamplification of co-migrating bands in *An. subpictus* (Ch. 7, AB4 product) in different populations revealed the homology of these fragments. However, in *An.gambiae* species complex, a 330bp band common to different species of the complex on agarose gel, showed different size in acrylamide gel and silver staining.

10.3.6 Amplification artifact and contamination

Artifactual variation and contamination in PCR products is another problem common to all kinds of PCR, including RAPD. The presence of artifactual variation in RAPD banding patterns exemplifies the importance of spectrophotometric analysis to standardize the DNA concentrations across all individuals and demonstrates the need for consistency between separate amplifications. However, contaminating RNA in typical applications involving multiple samples may hinder the accurate determination of DNA concentration and may cause artifacts. On the other hand it is not applicable in a routine population study which needs field friendly procedure, to reduce the whole analysis time from DNA extraction to scoring the bands on gel.

Further, the nature of the artifact may vary as a function of the base composition of a selected primer. Each nucleotide primer may, therefore, require optimization with respect to the amplification conditions, concentration of primer relative to the template, and the magnesium concentration.

The discrimination of true polymorphism from artifactual variation is critical, particularly when amplification products are scored as present or absent without regard to the mode of inheritance (Ellsworth, 1993). Some RAPD fragments may be ambiguous and not easy to score (Williams *et al.*, 1990). These unclear and non-reproducible fragments, which may drive from non-specific priming or from heteroduplex formation between related amplification products (or other secondary structure artefact, which can prevent normal amplification patterns) are not useful as

genetic markers.

Regarding contamination, all samples used in this study have been identified to species before DNA extraction. In order to avoid contamination during amplification always one negative and one positive control have been included. However, where contamination was detected in a negative control (depending on the amplification condition sometimes due to contaminated Taq polymerase or ddH_2O), all products of that amplification have been discarded and not included in the analysis.

We can exclude the possibility that the similarity of banding patterns in laboratory strains and their progeny (Ch.8, *An.stephensi*) results from contamination, given that certain biochemical and morphological markers allow us to recognize these strains.

However, the term random in RAPD may be somewhat misleading in that the only random component is the initial choice of a primer for the PCR. Amplification is constrained to occur only in regions of the genome where a particular DNA sequence and its inverse (actually the reverse complement) are found within a window of size ranges, usually between 0.1 and 3kb apart (Haymer, 1994)(Fig. 2.2). Regions of the genome containing closely spaced inverted terminal repeats would meet this requirement (Black *et al.*, 1992).

Typically, these are repetitive sequences localized in the telomeres and centromeres of chromosomes, the same regions identified in genetic fingerprinting techniques by Jeffreys *et al.* (1985a, 1985b). Black (1993) has described an excellent general overview of the application and potential pitfalls in the use of RAPD method.

10.4 ADVANTAGES AND DISADVANTAGES OF RAPD

The conventional PCR which exponentially amplifies DNA fragments *in vitro*, is very powerful but it needs prior sequence information on targeted DNA, to create and design specific primers and this limits the applicability of the method to few organisms or highly conserved regions in genomic DNA. In order to overcome this handicap specially in analyzing highly variable regions which could be used for identification/differentiation of species or even below the species level (strains, varieties, isolates, etc.), RAPD-PCR has been introduced by Williams *et al.* (1990) which like other kind of PCR-based techniques has its advantages and also disadvantages. Its advantages are including:

1- A greater amount of genetic variation is detected than with allozyme electrophoresis.

2- Of greater importance to entomologists, RAPD and AP-PCR permit genetic variation to be detected in small arthropods.

3- The technique is easily learned and applied

4- If random mating is assumed, RAPDs can still be used in estimating genetic distance among populations.

5- The large number of bands amplified in individuals, permits their use in molecular ecology, genetic fingerprinting, species diagnostics and differentiation of cryptic species.

6- they do not require cloning or DNA sequence information for primer design.

7- They do not employ radioactive markers and

8- Genetic polymorphisms can be visualized within 24 h from extraction of genomic DNA.

9- Suitability for work on anonymous genomes

10- Applicability to problems where only limited quantities of DNA are available

11- Requires the least in technology, labour and expenses. The average expense for one RAPD fingerprint can be as low as US$2.00;in contrast the cost of producing one individual DNA fingerprint by Southern hybridization can be very substantial (Weatherhead & Montgomerie, 1991).

12- RAPDs are detected more easily than RFLPs even in analyses of genomes with high levels of heterozygosity (Williams *et al.*, 1990; Carlson *et al.*, 1990; Hu & Quiros, 1991).

The disadvantages are:

1- They require stringent standardization before they can be used reliably and consistently. It is sensitive to reaction conditions like 1) shape of the temperature profile, which is a property of the thermal cycler and must be standardized 2) type of polymerase used and 3) Mg^{2+} concentration, and slight changes in the conditions may affect the reproducibility of amplification products and influence banding patterns (number, size and intensities of bands)(Williams *et al.*, 1990; Arnold *et al.*, 1991; Carlson *et al.*, 1991; Klein-Lankhorst *et al.*, 1991). Although the results of this work showed that these changes can be used in favour of generating new markers (specially by using less DNA and using two step annealing program).

2- The optimal concentration of template DNA per reaction may vary substantially from typical conditions (1-10 ng per reaction) depending on the primer-template condition used.

3- A reliable method for DNA extraction that yields stable template must be developed (as described in section 10.3). 4- Most RAPD polymorphisms segregate as dominant markers in that homozygote (two copies of an amplifiable region) are

indistinguishable from heterozygote (one copy) and which are inherited in a Mendelian fashion (Williams *et al.*, 1990; Carlson *et al.*, 1991; Martin *et al.*, 1991; Welsh *et al.*, 1991b, 1991c). However, this dose not limit their application in studies of the breeding structure of natural populations and in mapping (Dimopoulos *et al.*, 1996).

10.5 SUMMARY

In a recent review of the species concept in blood-sucking insect vectors, Lane (1997) has discussed some of the difficulties with the biological species concept. He also reviews some alternative concepts. Lane points out that the criteria that systematists use in practice are somewhat different from those that test adherence to the concept. Nevertheless, as Townson points out (in preparation), "...in anopheline sibling species complexes the criteria and methods most commonly employed in the early stage of studies may be considered as directly testing hypotheses inherent in the biological species concept. Thus such work often involves looking for evidence of heterozygote deficiency in polytene chromosome inversions or allozymes, or carrying out crosses to determine the level of sterility in the F1 progeny. The approach with these methods differs appreciably from that adopted with the more conventional morphological characters used by systematists, in that there is a more precisely defined genetic component and adherence to fairly rigid, objective criteria, notwithstanding the absence of an agreed minimum threshold for gene flow, below which we can conclude species exist. All this is not to say that the concept of species as a group of organisms sharing a common mate recognition system, attributable to Paterson (1985), is without merit, but its use as a yardstick is, with current techniques, impracticable."

In the same review, Lane (1997) discusses sibling species and points out that in cases were active speciation is taking place, it may be impossible to determine

which of the taxa recognized are species in an unambiguous way. This is clearly true with the ecophenotypes described in West African populations of *An.gambiae* by Coluzzi and his co-workers (see Chapter 2, and 4). It may also be relevant to the molecularly differentiable forms of *An.culicifacies* species B described in this thesis and under investigation by others in Liverpool (see Chapter 5).

The results of this project in different chapters with different species have attempted to summarize the progress made in overcoming its disadvantages and bearing this in mind, if RAPD is applied properly, it will provide the right direction in molecular systematics especially in population study of *Anopheles* species complexes. These findings begin to address the mechanism of specific and intra-specific variation in malaria vectors revealed by RAPD and related techniques. How conserved are the mechanisms of reproducibility of RAPD and how they could be applied to insects in general?

There appears to be a great deal of variation in the populations examined in this study which it would be interesting to follow up by characterization with more RAPD fragments. In most of those species, RAPD have been applied for the first time. The accuracy of the data has been examined and confirmed by comparing with other techniques which we recommend for future studies.

Finally, as the subject of all efforts in molecular systematics studies with malaria vectors is to provide better understanding of different aspects of their

genetics, and ecology and biological differences, it would be useful if a wide range of specimens from different species mainly from the field could be examined. These will provide more reliable data for designing and running any control measure because sometimes we forget 'the natural populations are those which live in nature'.

CHAPTER ELEVEN

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