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Entamoeba dispar: Genetic Diversity of Iranian Isolates Based on Serine-Rich *Entamoeba dispar* Protein Gene

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Abstract: The nucleotide sequences of Serine-Rich *Entamoeba histolytica* Protein (SREHP) gene have already exhibited stable and significant polymorphism in the gene studies. Serine-rich protein is also present and polymorphic in *Entamoeba dispar* which called SREDP. The polymorphism of the Serine-Rich *Entamoeba dispar* Protein (SREDP) gene among 8 isolates obtained from Iranian cyst carriers were analyzed by a nested PCR-RFLP followed by sequencing of the PCR products. From those isolates, six distinct DNA patterns were observed after PCR-RFLP of the nested PCR, whereas sequencing showed 8 different patterns among the isolates. The results demonstrate an extensive genetic variability among Iranian *E. dispar* isolates. The repeat-containing region of the SREDP was found extensively polymorphic in size, number and order of repeat units. Genetic diversity of Iranian *E. dispar* isolates based on the SREDP was more polymorphic in comparison of Serine-Rich *Entamoeba histolytica* Protein (SREHP) of the *E. histolytica* isolates as well as were different from a few known SREDP genes.

Key words: *Entamoeba dispar*, SREDP, genetic diversity

INTRODUCTION

Based on morphology and structure, *Entamoeba dispar* is very similar to *Entamoeba histolytica* (WHO/PAHO/UNESCO, 1997). Although, there is some evidence that *E. dispar* is able to produce intestinal lesions in animals (Espinosa Cantellano *et al*., 1997) and epithelial cell monolayer *in vitro* (Espinosa-Cantellano *et al*., 1998), the general agreements say that *E. dispar* is a non-pathogenic parasite. These realities suggest that the non-pathogenicity of some strains of this species is doubtful and more investigations are required (Pinheiro *et al*., 2005).

The first nucleotide sequences have been shown the stable and significant polymorphism in the Strain Specific Gene (SSG) and the Serine-Rich *E. histolytica* Protein (SREHP) gene. SSG is absent from *E. dispar* and all other species as far as is known (Clark, 2006) but SREHP is also present and polymorphic in *E. dispar* and called SREDP (Mai and Samuelson, 1998). Ayeh-Kumi *et al*. (2001) studied clinical samples from Bangladesh; they used a nested PCR of the SREHP gene coupled with restriction digestion. Twenty five genotypes have been found among 42 intestinal isolates and 9 genotypes among the isolates from 12 ALA samples. In Clark and Diamond (1993) study, 16 different genotypes were observed among 18 isolates of *E. histolytica* from diverse geographical locations using the combined results of restriction digestion of SREHP and amplification of the SSG locus. Ghosh reported 5 different SREDP sequencing patterns from 5 isolates and showed high genetic variation among the *E. dispar* in compare with *E. histolytica* (Ghosh *et al*., 2000). Haghighi *et al*. (2003) investigated 79 isolates of *E. histolytica*, mostly from

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Japan and Thailand by sequencing of four loci (two tRNA-linked STR loci, Chitinase and SREHP); however, it was failed to find an association between the parasite genotype and the outcome of infection. Cloning and characterization of SREHP gene from an Iranian *E. histolytica* isolate was also studied (Rasti *et al*., 2006), but no genetic variation was reported.

In the present study, the genetic variability of *E. dispar* strains based on the SREDP gene locus was analyzed using RFLP-nested PCR and sequencing of the obtained genes.

MATERIALS AND METHODS

Stool specimens: Eight *E. dispar* strains were previously isolated from three different climatic regions including central, northern and southern regions of Iran (Hooshyar *et al*., 2004; Kobayashi *et al*., 2005), which were analyzed in this study from June 2005 to May 2006. The *Entamoeba* strains were originally detected microscopically and differentiated from *E. histolytica* by PCR. The cultivated isolates in Robinson medium were transferred to Japan and maintained in liquid nitrogen in National Institute of Infection Diseases (NIID), Tokyo, Japan.

Extraction of genomic DNA and PCR identification: The DNA was extracted for PCR amplification in this study after recovery and re-cultivation of the frozen isolates in Robinson medium (Robinson, 1968). Total genomic DNA from trophozoites was purified using QIAamp DNA stool mini-kit in NIID (QIAGEN, Tokyo, Japan) according to the manufacturer's directions. The purified DNA was transferred to Iran and maintained in -20°C until used.

To confirm the presence of *E. dispar* and for exclusion of *E. histolytica* from study, PCR with two sets of species-specific primers from locus D-A gene was performed (Hsp1 and Hsp2 for *E. histolytica* and Dsp1 and Dsp2 for *E. dispar*) (Zaki and Clark, 2001; Zaki *et al*., 2002; Ali *et al*., 2005). PCR amplification consisted of 35 cycles of 45 sec at 93°C, 30 sec at the primer-dependent annealing temperature and 60 sec at 72°C with a final extension of 5 min at 72°C. The PCR products were electrophoresed in 1.2% agarose gel (Fermentas, No. R0491) containing ethidium bromide (Sambrook and Russell, 2001), was photographed under UV light (UVI doc Deluxe GAS 9000, England).

Nested SREDP gene PCR amplification: A set of primers [SREHP-BamH1 (S) (5` GAGGATCCATGTTC GCA TTTTTATTGT 3`) and SRD-Bam (AS) (5`GAGGATCCTT AGAAGACAATTGCCA3`)] which were designed based on SREDP gene (Ghosh *et al*., 2000) were used for initial PCR, followed by a second set of primers [Bam-SRED (S) (5`TTTATTGGATCCACTACT GCAACTA3`) and Bam-SRED(AS)(5`AAAGCAGGATCCATAATGAATGG AC3`)].

External PCR was carried out in a 50 µL reaction mixture containing 200 ng of DNA, 2 µM of each external primer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP and 0.2 µL of Taq DNA polymerase (5 units μL^{-1}) with the following cycling parameters: (1) pre-denaturation at 94°C for 5 min (2) 35 cycles of denaturing at 93°C for 45 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec and (3) final extension at 72°C for 10 min.

Internal PCR carried out in a 50 µL reaction mixture containing 1 µL of the first PCR product, 2 µM of each internal primer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP and 0.3 µL of Taq DNA polymerase (5 units μL^{-1}) in the same external cycling parameters with the exception of annealing at 64°C for 30 sec. The amplified fragment of SREDP gene was almost 680 bp. PCR product was purified by Q-Biogen Gene clean II Kit (BIO101, Lajolla, Calif).

AluI digestion of the nested SREDP PCR products: The PCR products were additionally analyzed by digestion with the restriction endonuclease AluI (Gibco BRL, Life Technologies). Seven microliter of SREDP PCR product was digested for 2 h at 37°C according to the manufacturer's recommended procedure. Digested PCR products were electrophoresed in 2% agarose gel, in TBE buffer, ethidium bromide solution 10 mg mL^{-1}, at 100 V for 1 h. The gel was visualized under UV light and photographed (Sambrook and Russell, 2001).

Sequence analysis: SREDP PCR products were directly sequenced using an Applied Biosystems (ABI) Terminator Cycle Sequencing Ready Reaction kit (BigDye ® Terminator V3.1 Cycle Sequencing Kit) on an ABI Analyzer. The obtained sequences were manually edited and aligned using Lasergene software (version 6.00).

RESULTS AND DISCUSSION

Eight *E. dispar* isolates were analyzed in this study. The polymerase chain reaction using Dsp1+Dsp2 reconfirmed that all the samples were *E. dispar*. The nested SREDP PCR fragments from the 8 Iranian strains showed slightly polymorphism of size among the isolates (Fig. 1). In the other hand to better understand the nature of the polymorphism observed by gel electrophoresis,

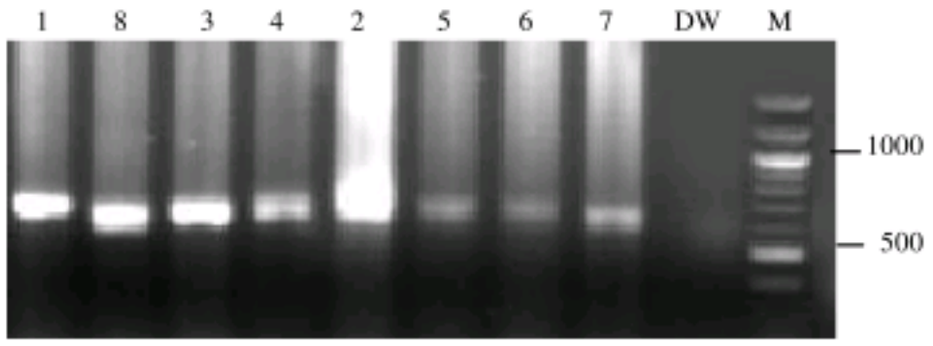

Fig. 1: Agarose gel electrophoresis of locus SREDP from Iranian *E. dispar* isolates. Lane 1 to 8: PCR fragment of the 8 isolates. Number of the lanes is in order to size of the sequencing results in Fig. 2, Lane DW: Negative control with distilled water. Lane M: 100 bp DNA ladder

the resultant nucleotide sequences of SREDP from all 8 isolates were used to investigate genetic diversity of *E. dispar* isolates. Sequencing of SREDP, showed 8 new genotypes with a highly polymorphic repeat-containing region of 20 to 24 distinct repeat units (Fig. 2). Sequencing revealed the appearance of the characteristic 648-720 bp band in 8 isolates of *E. dispar*, in which 684 bp fragments were found in 4 (50%) of the samples. The repeat-containing region of SREDP was found to be extensively polymorphic in size, species, number and order to repeat units among the isolates. Figure 2 shows sequencing analysis of the SREDP gene from HHR 1 IR strain (accession No. AB253476) and schematic diagram for genetic diversity of the gene repeat units. Nucleotide sequences of the *E. dispar* isolates were submitted to the GenBank/DDBJ/EMBL and are available for public access under the accession No. AB253475-AB253482. We also verified the AluI RFLP analysis of SREDP from all 8 isolates. Six distinct DNA patterns were observed after AluI digestion of nested PCR products. We conducted a computational RFLP analysis by AluI digestion of the SREDP locus based on the nucleotide sequences of the SREDP loci (Fig. 3). Although, all 8 genotypes showed distinct computational RFLP pattern, but the isolates with database accession number AB253476 and AB253479 as well as AB253478 and AB253481 showed very close patterns not possible to differentiate in agarose gel electrophoresis.

Studies show that *E. dispar* is perhaps 10 times more prevalent than *E. histolytica* worldwide, but local prevalence may vary meaningfully (Ali *et al*., 2005). Serine-Rich *E. histolytica* Protein (SREHP) gene is a surface protein also present and polymorphic in *E. dispar* and called SREDP (Mai and Samuelson, 1998). Although in serine-rich *E. histolytica* protein gene, polymorphism in lengths, types and numbers of internal repeat units were previously reported by Clark and Diamond (1993), Ghosh *et al*. (2000) and Haghighi *et al*. (2002, 2003), but genetic variation was studied just in 5 isolates in the SREDP gene (Ghosh *et al*., 2000). The present study is an initial experience of characterizing and sequencing of SREDP gene among Iranian *E. dispar* isolates in Iran. SREDP gene sequences contain conserved regions and internal tandem dodecapeptides and octapeptides repeat units like SREHP gene sequences (Haghighi *et al*., 2002). However, the patterns and sizes of amplified fragments corresponding to SREDP in *E. dispar* showed highly variable completely different from SREHP in *E. histolytica* strains (Rasti *et al*., 2006; Haghighi *et al*., 2002, 2003). A significant polymorphism among *E. histolytica* isolates collected from a wide geographic range was shown in previous study (Ghosh *et al*., 2000; Clark and Diamond, 1993).

PCR-RFLP of SREDP gene indicated 6 different patterns of 8 *E. dispar* strains which are emphasized by nucleotide sequencing of 8 different patterns. Recently in Bangladesh and Georgia, an extensive genetic diversity of *E. histolytica* was also observed by analysis of the SREHP gene by PCR amplification and AluI digestion (Simonishvili *et al*., 2005; Ayeh-Kumi *et al*., 2001). The serine-rich gene of *E. histolytica* was investigated by nested PCR amplification and restriction enzyme fragment length polymorphism in 26 clinical isolates of Turkey. After AluI digestion of nested PCR products, 12 different DNA patterns were observed which was demonstrated a comprehensive genetic variability of Turkish *E. histolytica* clinical isolates (Tanyuksel *et al*., 2008). In another study, 13 different profiles were generated from 23 *E. histolytica* isolates from Cameroon, Zimbabwe and South Africa, while 20 others were generated from 38 *E. histolytica* PCR positive stool samples from South Africa with RFLP-PCR (Samie *et al*., 2008).

Haghighi *et al*. (2003) found the most noteworthy and extensive variations among the SREHP locus. They demonstrated that *E. histolytica* had a highly complex genetic structure independent of geographic location, but failed to find an association between the parasite genotype and the outcome of infection.

Ayeh-Kumi *et al*. (2001) examined the combinations of the nested PCR results and the AluI digestion of the PCR products. That examination yielded 25 distinct DNA banding patterns among the 42 stool isolates and an additional 9 distinct patterns among the 12 liver abscess isolates (Ayeh-Kumi *et al*., 2001). The results here demonstrate an extensive genetic variability among SREDP gene of 8 Iranian *E. dispar* isolates. From those isolates six distinct DNA patterns were observed after

ATATTGACCA AGAGGTAAAA GATACTAAGA TTTACAGTGT ATTTTTAAAG AACGAAGCTA
GTCCTGAAAA ACTTGAAGAA AATAAAGAAG ATGAAGAAAA AGAAAAAAGT AGTTCATCAA
ACACAGGTGA TGAACCAGAC AATAATTCAG AAGCAAAATC AGATGATGAA CCAGAAGCAA
GTTCAAACGA TAAGTCAGAT GATAAAGTAG AAGCAAGTTC AAGTGATAAA CCAGAAGCAA
GTTCAAGTGA TAAGCCAGAT AATAAACCAG AAGCAAGTTC AAGTGATAAA CCAGAAGAAA
GTTCAAGTGA TAAACCAGAA GCAAGCTCAA GTGATAAGCC AGAAGCAAGT TCAAGTGATA
AACCAGATAA TAAACCAGAA GAAAGTTCAA GTGATAAGCC AGATAATAAA CCAGAAGAAA
GTTCAAGTGA TAAGCCAGAT AATAAACCAG AAGCAAGTTC AAGTGATAAG CCAGATAATA
AACCAGAAGC AAGTTCAAGT GATAAGCCAG ATAATAAACC AGAAGCAAGT TCAAGTGATA
AACCAGATAA TAAACCAGAA GCAAGTTCAA GTGATAAACC AGATAATAAA CAGAAGCAA
GCTCAGCTAC TAATTCAAAT GATAATTCAG AAAGTAGTTC AGATGATCAT AATAACCTTG
ATGCA

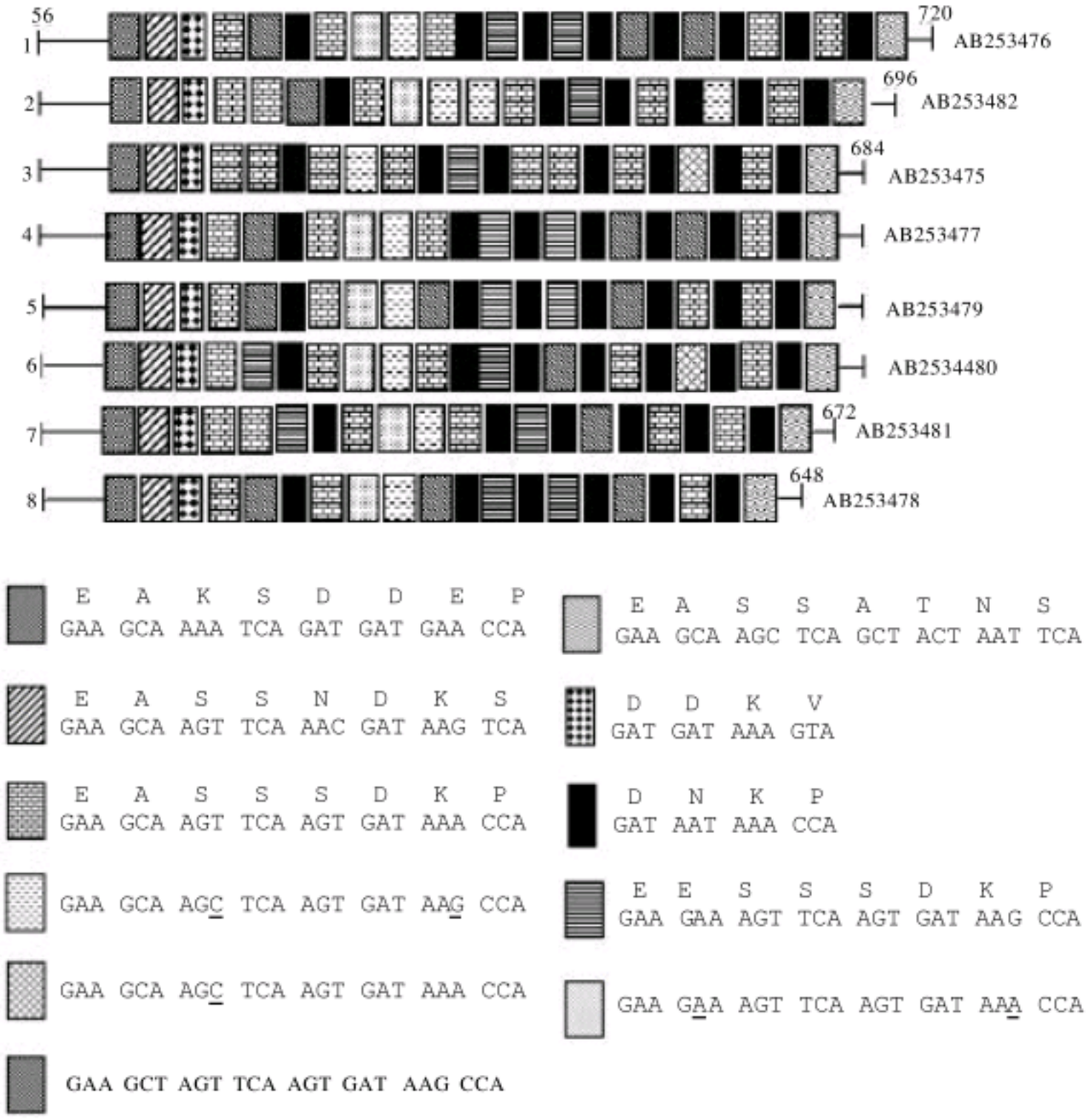

Fig. 2: Sequence and schematic representation of the polymorphisms in novel genotypes of SREDP in 8 Iranian *E. dispar* isolates. Sequencing and the underline repeat-containing region of SREDP in the sequence derived from sample HHR 1 IR (Accession No. AB253476). The sequence No. 3 (accession No. AB253475) was used as a standard strain of *E. dispar* (Kobayashi *et al* ., 2005). The sequences of each of the 8 variants of the 24 and 12 nucleotide repeats are shown with a corresponding shaded block. The conserved non-repeated regions are shown as a single line. Genotypes of the 8 isolates and their related accession numbers are shown to the left and right of the schematics, respectively. The nucleotide and deduced amino acid sequences of tetra- and octapeptide repeats are shown above

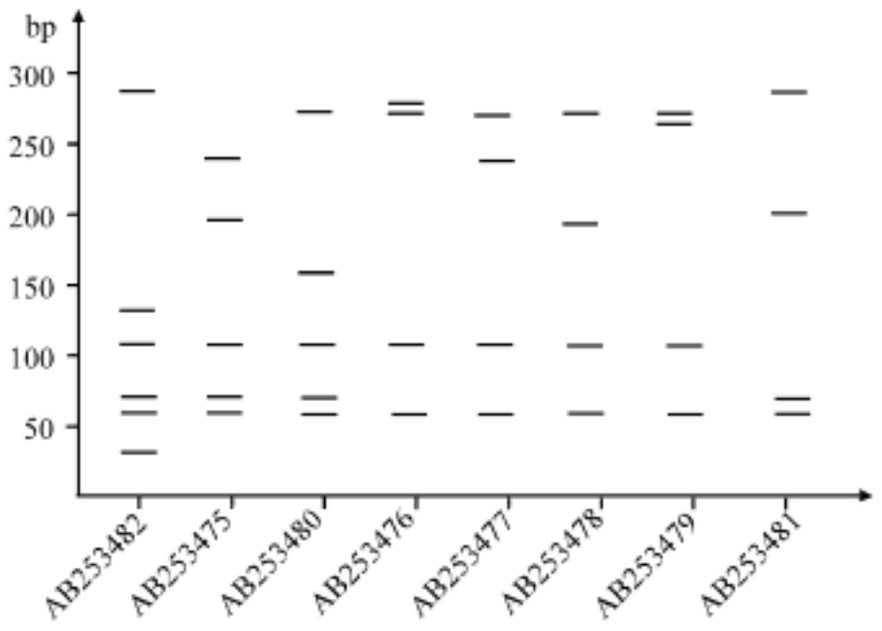

Fig. 3: Schematic profile of AluI digests of the 8 novel genotypes. The isolates AB253476 and AB253479 as well as AB253478 and AB25381 showed very close patterns. Note that the individual genotype is designated for each DNA fragment and the database accession number of the isolates

PCR-RFLP of the nested PCR, whereas sequencing showed 8 different patterns among the isolates with a highly polymorphic repeat-containing region of 20 to 24 distinct repeat units. Genetic diversity of Iranian *E. dispar* isolates based on the SREDP gene showed 8 novel *E. dispar* genotypes which were distinct from only 5 known SREDP genes (Ghosh *et al*., 2000).

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