Close sequence identity between ribosomal DNA episomes of the non-pathogenic *Entamoeba dispar* and pathogenic *Entamoeba histolytica*

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Entamoeba dispar and *Entamoeba histolytica* are now recognized as two distinct species – the former being nonpathogenic to humans. We had earlier studied the organization of ribosomal RNA genes in *E. histolytica*. Here we report the analysis of ribosomal RNA genes in *E. dispar*. The rRNA genes of *E. dispar*, like their counterpart in *E. histolytica* are located on a circular rDNA molecule. From restriction map analysis, the size of *E. dispar* rDNA circle was estimated to be 24·4 kb. The size was also confirmed by linearizing the circle with *Bsa*HI, and by limited DNAseI digestion. The restriction map of the *E. dispar* rDNA circle showed close similarity to EhR1, the rDNA circle of *E. histolytica* strain HM-1:IMSS which has two rDNA units per circle. The various families of short tandem repeats found in the upstream and downstream intergenic spacers (IGS) of EhR1 were also present in *E. dispar*. Partial sequencing of the cloned fragments of *E. dispar* rDNA and comparison with EhR1 revealed only 2·6% to 3·8% sequence divergence in the IGS. The region Tr and the adjoining *Pvu*I repeats in the IGS of EhR1, which are missing in those *E. histolytica* strains that have one rDNA unit per circle, were present in the *E. dispar* rDNA circle. Such close similarity in the overall organization and sequence of the IGS of rDNAs of two different species is uncommon. In fact the spacer sequences were only slightly more divergent than the 18S rRNA gene sequence which differs by 1·6% in the two species. The most divergent sequence between *E. histolytica* and *E. dispar* was the internal transcribed spacer, ITS2. Therefore, it was concluded that probes derived from the ITS1 and ITS2 sequences would be more reliable and reproducible than probes from the IGS regions used earlier for identifying these species.

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1. Introduction

Amebiasis is caused by infection with the intestinal pathogen *Entamoeba histolytica*. The parasite is morphologically indistinguishable from the nonpathogenic species *Entamoeba dispar*, and for almost fifty years the two were classified together as a single species, *E. histolytica*. Extensive epidemiological studies (Britten *et al* 1997; Haque *et al* 1998), nucleotide sequence analysis (Mirelman *et al* 1997), analysis of cysteine proteinase gene family (Bruchhaus *et al* 1996), differences in the activities of the pore forming peptides (Nickel *et al* 1999), antigenic differences in the lectin (Petri *et al* 1990), and phylogenetic studies based on riboprinting (Clark and Diamond 1997) have led to the re-classification of these two organisms as distinct species. Data from epidemiological studies shows that about 9% of asymptomatic individuals in endemic areas are infected with *E. dispar* and about 1% are infected with *E. histolytica* (Jackson and Ravdin 1996). There are no reported cases of invasive amebiasis amongst individuals harbouring *E. dispar*, while about 10% of asymptomatic carriers of *E. histolytica* eventually come down with disease. A comparative functional genomic analysis of the two species should provide clues to the key processes that trigger the switch from a commensal to invasive state in amoebic infection.

The ribosomal RNA genes in *E. histolytica* are known to be located on extrachromosomal circular molecules

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(Bhattacharya *et al* 1998). In our analysis of sequence organization of the rDNA circle of *E. histolytica* strains we found two kinds of arrangement. In some strains, there is one rDNA transcription unit per circle while in others there are two rDNA units organized as inverted repeats. In the latter, the intergenic spacers (IGS) upstream of the two rDNA units are not identical in sequence. The spacer sequences upstream of the rightward rDNA unit are invariably present in strains with a single rDNA unit per circle, while the spacer sequences found upstream of the leftward rDNA unit are missing in these strains. Probes from the upstream and downstream spacers of *E. histolytica* rDNA failed to hybridize with the corresponding regions in *E. moshkovskii* rDNA, and partial sequence analysis of the latter showed no similarity with the *E. histolytica* spacer sequences (Sehgal *et al* 1994). Thus, spacers show distinct inter- and intra-specific patterns.

IGS sequences are important since they contain regulatory elements that control transcription and replication of rRNA genes. Being more divergent than the rRNA coding sequences, they are also indicators of evolutionary relatedness between species. Here we report our analysis of the rDNA circle of *E. dispar* which shows a complete conservation of spacer sequences when compared with *E. histolytica*. This remarkable sequence similarity lends support to the notion that *E. histolytica* and *E. dispar* are sibling species that have diverged relatively recently.

2. Materials and methods

2.1 *Culture conditions, isolation of DNA and Southern hybridization*

Xenic cultures of *E. dispar* strain CDC 0784 were maintained with associated bacterial flora and axenic culture of *E. histolytica* strain HM-1:IMSS were maintained in TYI-S-33 medium at 36°C (Diamond 1978, 1982). Erythromycin (0·13 mg per ml) was added to the medium before inoculating *E. dispar*. Mid-log phase cultures (grown for 96 h) were used for isolation of DNA. Cells were harvested by centrifuging at 275 *g* for 8 min at 4°C and washed twice with phosphate buffered saline to remove the bacterial population associated with amoebae. Total genomic DNA was purified by the phenol-chloroform method as described for *E. histolytica* (Bhattacharya *et al* 1989). DNA (3–4 μg) was digested with indicated restriction enzymes and separated by electrophoresis in 0·8% (w/v) agarose gels (unless indicated otherwise). DNA was transferred onto nylon membrane (Genescreen plus) for Southern hybridization. Appropriate DNA fragments were generated from cloned rDNA molecules after digestion with suitable restriction enzymes. Fragments so generated were used as probes after radiolabelling the DNA by the random priming method (Feinberg and Vogelstein 1983). Southern blots were hybridized at 65°C, overnight in a solution containing 1% SDS, 1 M NaCl and 3×10^5 cpm ml^{-1} of DNA probe. Blots were washed with 2 × SSC at 37°C followed by three changes of 2 × SSC and 1% SDS at 65°C and a last wash with 0·1 × SSC, before being autoradiographed.

2.2 *Cloning and sequencing of genomic DNA fragments*

Total *E. dispar* genomic DNA and plasmid vector pBluescript II (KS^+) (Stratagene) DNA were digested with *Eco*RI and/or *Hin*dIII. Ligated DNA was transformed into *Escherichia coli* DH5*α* cells and selected in the presence of IPTG and X-Gal following the protocol described (Sambrook *et al* 1989). The identity of clones was confirmed by Southern hybridization of cloned DNA with appropriate probes. DNA sequencing was performed by dideoxy chain termination method (Sanger *et al* 1977) using either universal or internal primers. Nucleotide sequence analysis was carried out using GCG package. The BLAST email server was used to search for homologues in the nucleic acid database. Sequence of parts of intergenic spacer of rDNA determined in this study (see figure 2) have been submitted to the EMBL database. The accession numbers are AJ306923, AJ306924, AJ306925, AJ306926 and AJ306827.

2.3 *RNA isolation and Northern hybridization*

Total RNA was essentially extracted and purified by the method described (Chomczynski and Sacchi 1987). It involved a single step RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. RNA was fractionated by agarose-formaldehyde gel electrophoresis (Sambrook *et al* 1989). The gel running buffer consisted of 20 mM MOPS, 8 mM sodium acetate and 100 mM EDTA prepared in DEPC treated water, and 2·2 M formaldehyde, pH > 4·0. The RNA samples were denatured and electrophoresed through 1% (w/v) agarose gel for 4 h at 1·5 V/cm. The transfer for Northern blots was carried out on nylon membrane in 20 × SSC for 12–15 h, followed by rinsing with 2 × SSC, and baking at 80°C in vacuum for 2 h. Hybridization and washing conditions were the same as those for Southern analysis.

2.4 *PCR amplification*

Oligos were designed from the region of maximum mismatch in the 18S rRNA and ITS-2. The following primers

were used to differentiate *E. histolytica* from *E. dispar*. *E. histolytica* specific primers were:

Eh 1 5′-AGAGAAGCATTGTTTCTAGATCTG-3′ (18S)
Eh 2 5′-TTAATTATTAGACAAAGCCT-3′ (18S)
Eh 3 5′-TTATTGGTCTGGTCTGTC-3′ (ITS-2)

E. dispar specific primers were:

Ed 1 5′-GAAGAAACATTGTTTCTAAATCCA-3′ (18S)
Ed 2 5′-CTACCTATTAGACATAGCCT-3′ (18S)
Ed 3 5′-TTTATTAACTCACTTATA-3′ (ITS-2)

Amplification conditions were: denaturation at 94°C for 1 min, annealing at 45°C for *E. histolytica* specific primers and 40°C for *E. dispar* specific primers, followed by extension at 72°C for 1 min. The amplification was carried out for 30 cycles in a DNA Thermal cycler (MJ Research, USA).

3. Results

3.1 *The rDNA episome of* E. dispar

The rRNA genes of *E. histolytica* are known to be located on circular episomes (Bhattacharya *et al* 1989). The aim of the present study was to understand the organization of rRNA genes in *E. dispar*, a closely related nonpathogenic species. To this end, total genomic DNA of *E. dispar* was digested with restriction enzymes and Southern blots were hybridized with probes derived from the rDNA circle of *E. histolytica*. These probes spanned the entire rDNA circle, that is, the rRNA coding region, the upstream intergenic spacer and the downstream intergenic spacer. When a restriction map was constructed from the data, it was clear that the rRNA genes in *E. dispar* are also located on a circular molecule (figure 1). The size of the circle was calculated to be 24·4 kb from the restriction map. The size was confirmed by electrophoretic

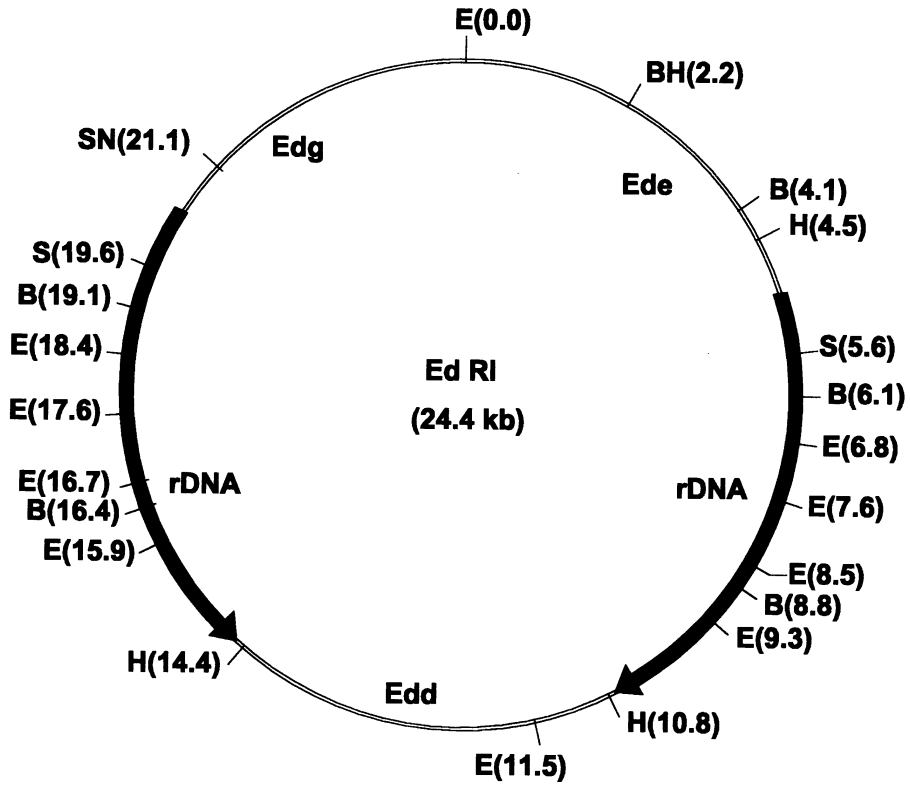

Figure 1. Organization of the rDNA circle of *E. dispar*. The map was derived by Southern blot analysis of *E. dispar* DNA digested with various enzymes and probed with DNA fragments of the *E. histolytica* rDNA circle (HMe, HMd, HMg, HMa and HMb, Som *et al* 2000). Restriction enzyme sites indicated on the circle are *Eco*RI (E), *Hin*dIII (H), *Bgl*II (B), *Sac*II (S), *Bsa*HI (BH), *Sna*BI (SN). The *Eco*RI site at 12 o'clock position corresponds to nucleotide No. 1. Starting from this, the restriction sites have been numbered (in kb) in clockwise direction. Solid arrows indicate the two rDNA inverted repeats. Ede, Edd and Edg are *Eco*RI fragments that contain the intergenic spacers.

analysis of *E. dispar* rDNA digested with *Bsa*HI (which cuts the circle once) and with *Sac*II (which cuts the circle twice). The important features that emerged from the restriction map were as follows. Firstly, the size and organization of the *E. dispar* rDNA circle (named EdR1) was very similar to the rDNA circle of *E. histolytica* strain HM-1:IMSS (EhR1) which was earlier reported from our laboratory (Sehgal *et al* 1994). The rRNA coding regions (rDNA in figure 1) were organized as inverted repeats in both EdR1 and EhR1. Each inverted repeat contains a complete rRNA transcription unit coding for the 18S-, 5·8S- and 28S-rRNAs. Secondly, the organization of restriction enzyme sites in EdR1 and EhR1 was almost identical, not only in the rDNA region but also in

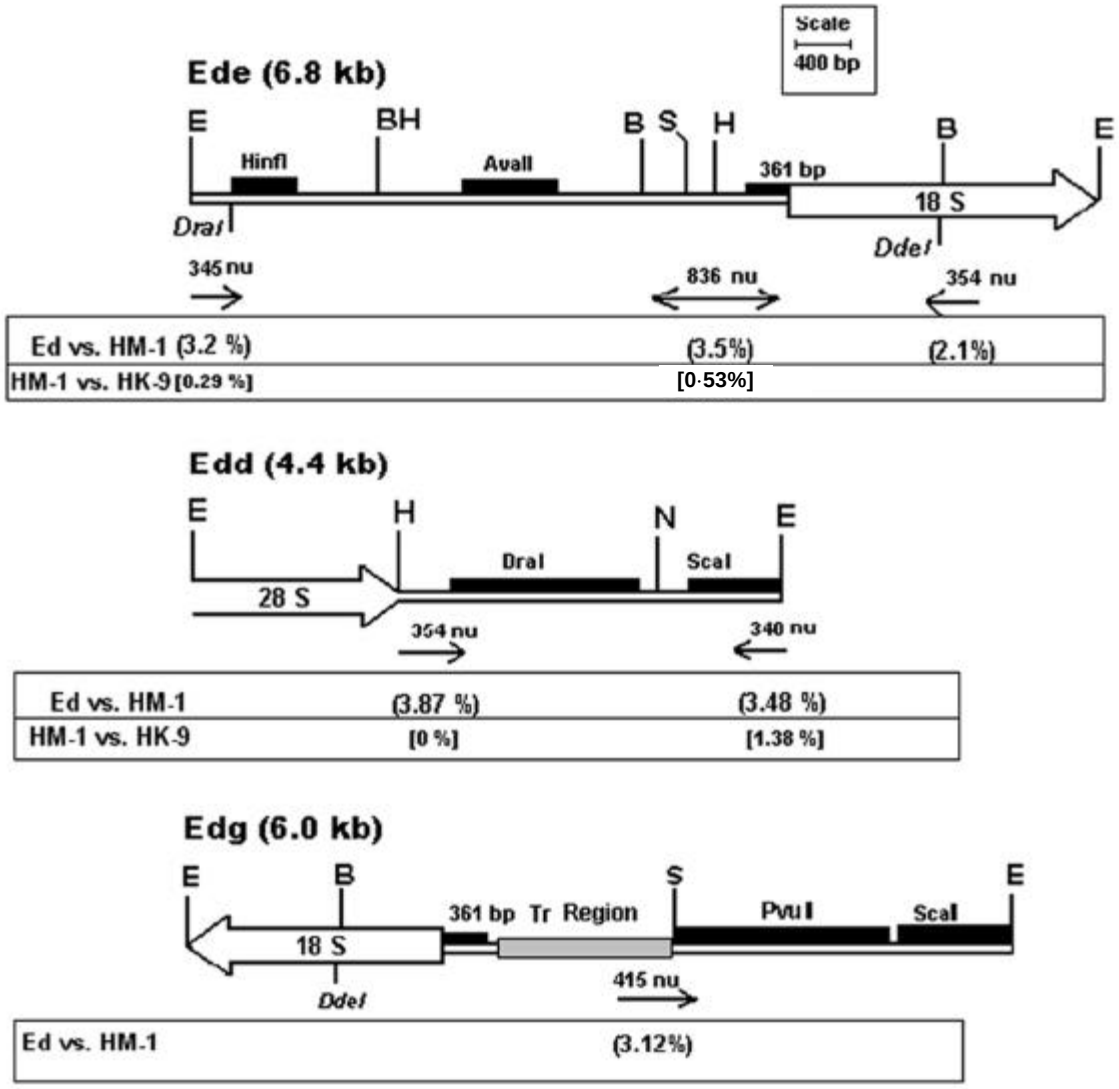

Figure 2. Analyses of cloned fragments of *E. dispar* rDNA by restriction enzyme digestion and partial sequencing. *Eco*RI fragments Ede, Edd and Edg of *E. dispar* rDNA were cloned in the *Eco*RI site of plasmid vector pBS KS$^+$. Restriction enzymes used to obtain maps of the cloned DNAs were E-*Eco*RI, BH-*Bsa*HI, B-*Bgl*II, S-*Sca*I, N-*Nde*I. Arrows below each map indicate the location of regions sequenced and the direction of single-strand sequencing. The length of sequenced stretch is given (in nu) on top of the arrow. Percent variation in the *E. dispar* versus *E. histolytica* strain HM-1:IMSS sequence, and *E. histolytica* strain HM-1:IMSS versus strain HK-9 sequence (where available) is given below each arrow in the box. Thick, open arrows denote the rRNA coding regions and the direction of transcription. The filled boxes labelled with restriction enzymes denote various families of short tandem repeats in which the indicated restriction enzyme has a unique site. The 361 bp region immediately upstream of rDNA in Ede and Edg is identical for both units. The Tr region in Edg is known to be transcribed. Restriction enzyme sites (DraI and DdeI) that have arisen in *E. dispar* but are absent in *E. histolytica* are shown in italics.

the upstream and downstream IGSs (contained in fragments marked Edg, Ede and Edd in figure 1). The latter observation was interesting since an earlier study with the rDNA circle of *E. moshkvskii* had shown a complete lack of sequence homology in the IGSs when compared with *E. histolytica* (Ramachandran 1993). Thirdly, EdR1 contained the IGS in Edg, which is upstream of the leftward rDNA unit. The equivalent of this spacer is found in the *E. histolytica* strain HM-1:IMSS, but is absent in the rDNA circles of several other *E. histolytica* strains (HK-9, Rahman) (Ramachandran 1993). In these latter *E. histolytica* strains the rDNA episome contains only the rightward rDNA unit per circle, and the leftward rDNA unit along with its upstream IGS (the Edg equivalent) is missing. Therefore, its presence in *E. dispar* is significant.

3.2 *Restriction enzyme– and nucleotide sequence – analysis of the EdR1 IGSs*

We decided to determine the extent of sequence divergence between EdR1 and EhR1 by comparing the sequences, especially of the IGSs of the two molecules. The *Eco*RI fragments that contained the spacers of EdR1 (Ede, 6·8 kb; Edd, 4·4 kb; Edg, 6·0 kb) were used for the analysis. Their restriction maps are detailed in figure 2. Each fragment contains, in addition to the spacer, a part of the rRNA-coding region (18S in Ede and Edg, and 28S in Edd). The corresponding spacers of EhR1 have been shown to contain families of short tandem repeats named after the restriction enzymes that have unique sites in each family (Sehgal *et al* 1994). Restriction enzyme analysis of Ede, Edd and Edg clones showed the same repeat families to be also present in *E. dispar*. Thus by partial digestion it was determined that, Ede contained two *Hin*fI- and five *Ava*II-repeats; Edd contained six to eight *Dra*I- and seven *Sca*I-repeats; and Edg contained eleven *Pvu*I- and six *Sca*I-repeats.

For sequence comparison, selected regions of Ede, Edd and Edg were sequenced. These regions are denoted by arrows in figure 2. The extent of sequence divergence between *E. dispar* and *E. histolytica* strain HM-1:IMSS was found to range from 2·8% to 3·8%. As a comparison,

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E.d.  ACTATAAACGATGTCAACCAAGGATTGGATGAAATTCAGATGTACAAAGATGAAGAAAGC 60
lab   ACTATAAACGATGTCAACCAAGGATTGGATGAAATTCAGATGTACAAAGATGAAGAAAGC 60
E.h   ACTATAAACGATGTCAACCAAGGATTGGATGAAATTCAGATGTACAAAGATAGAGAA-GC 59
      ************************************************  **** **

E.d.  ATTGTTTCTAAATCCTAAGTATATCAATACTACCTTGTTCAGAACTTAAAGAGAAATCTT120
lab   ATTGTTTCTAAATCCTAAGTATATCAATACTACCTTGTTCAGAACTTAAAGAGAAATCTT120
E.h   ATTGTTTCTAGATC-TGAGTATATCAATATTACCTTGTTCAGAACTTAAAGAGAAATCTT118
      ********** *** * ************ ******************************

E.d.  GAGTTTATGGACTTCAGGGGGAGTATGGTCACAAGGCTGAAACTTAAAGGAATTGACGGA180
lab   GAGTTTATGGACTTCAGGGGGAGTATGGTCACAAGGCTGAAACTTAAAGGAATTGACGGA180
E.h   GAGTTTATGGACTTCAGGGGGAGTATGGTCACAAGGCTGAAACTTAAAGGAATTGACGGA178
      ************************************************************

E.d   AGGGCACACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAA240
lab   AGGGCACACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAA240
E.h   AGGGCACACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAA238
      ************************************************************

E.d   GACCGAACAGTAGAAGGAATGACAGATTAAGAGTTCTTTCATGATTTATTGGGTAGTGGT300
lab   GACCGAACAGTAGAAGGAATGACAGATTAAGAGTTCTTTCATGATTTATTGGGTAGTGGT300
E.h   GACCGAACAGTAGAAGGAATGACAGATTAAGAGTTCTTTCATGATTTATTGGGTAGTGGT298
      ************************************************************

E.d   GCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGA 357
lab   GCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGA 357
E.h   GCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCAGGTTAATTCCGGTAACGAACGA 355
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Figure 3. Clustal W (1·82) multiple sequence alignment of a part of 18S region between published *E. dispar* sequence (E.d), *E. dispar* strain cultivated in our laboratory (lab) and *E. histolytica* (E.h) sequences. Identical nucleotides are marked by asterisk. The gaps show the variation between *E. dispar* and *E. histolytica* sequences.

the variation between *E. histolytica* strains HM-1:IMSS and HK-9 was determined in some of these regions. It was between 0·29% to 0·53%. We also sequenced a part of the 18S-coding region of *E. dispar*. The sequence perfectly matched with the reported sequence of *E. dispar* 18S rDNA (Novati *et al* 1996), and showed 2·1% divergence from the corresponding *E. histolytica* sequence (figure 3).

3.3 *Authenticity of* E. dispar *used in the analysis*

The striking similarity between EdR1 and EhR1 raised a doubt as to the possibility of inadvertent contamination

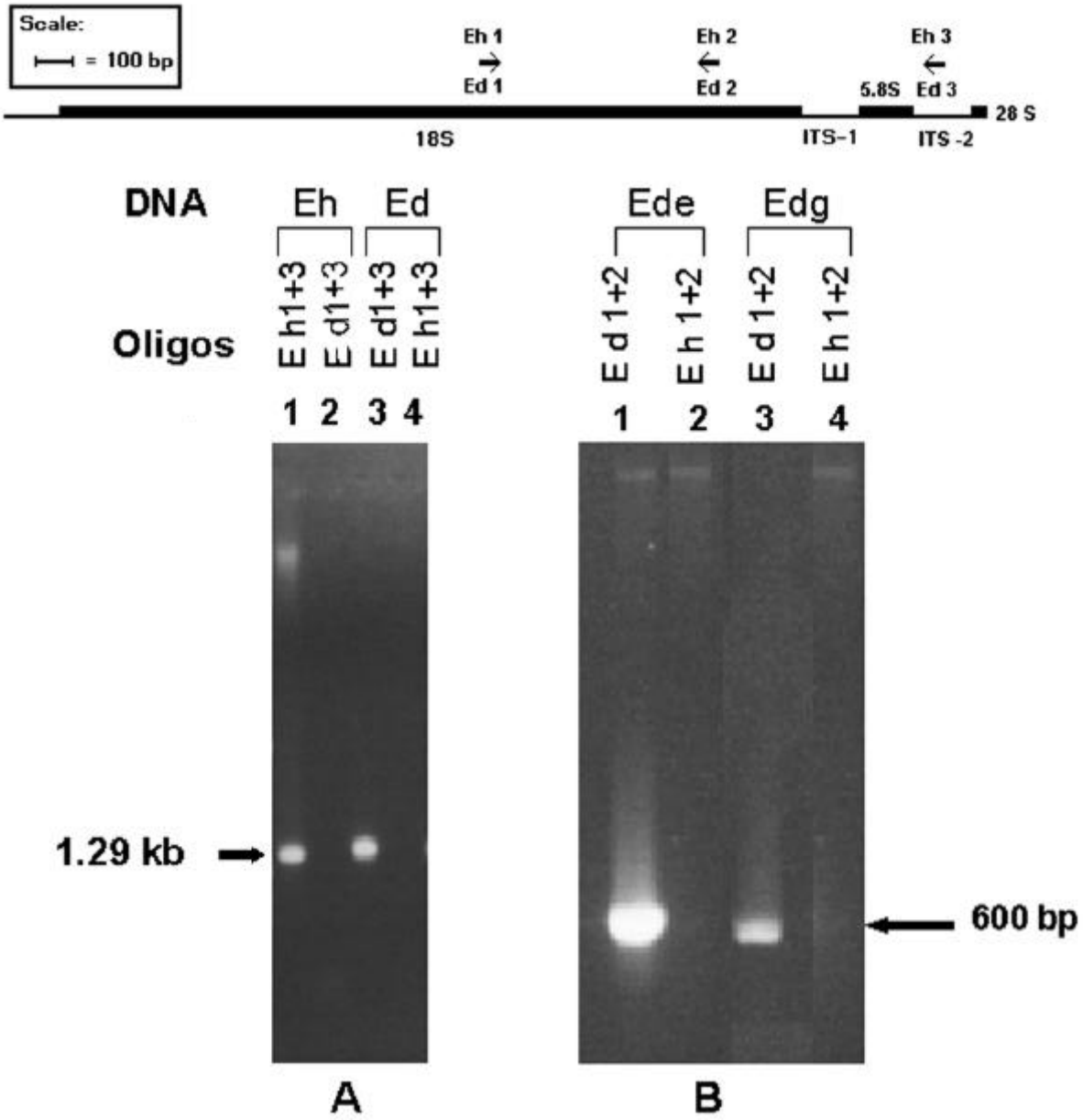

Figure 4. PCR amplification using species-specific primer pairs. The location of primers (Eh1, 2, 3 and Ed1, 2, 3) in the 18S rDNA and ITS-2 is shown in the top panel. **(A)** PCR amplification of total genomic DNA of *E. histolytica* (lanes 1 and 2) and *E. dispar* (lanes 3 and 4) with *E. histolytica*-specific primer pairs – Eh1 and Eh3 (lanes 1 and 4) and *E. dispar*-specific primer pairs – Ed1 and Ed3 (lanes 2 and 3). **(B)** PCR amplification of cloned *Eco*RI fragments of *E. dispar* rDNA (see figure 1) – Ede (lanes 1 and 2) and Edg (lanes 3 and 4) with *E. dispar*-specific primer pairs – Ed1 and Ed2 (lanes 1 and 3) and *E. histolytica*-specific primer pairs – Eh1 and Eh2 (lanes 2 and 4). Conditions for PCR amplification are described in § 2. Amplified products were separated by electrophoresis through 1% agarose gels for 6 h at 0·8 V/cm. Sizes of amplified fragments are indicated.

with *E. histolytica* DNA. This is not likely, since the *E. histolytica* HM-1:IMSS cells being grown in our laboratory for the past four years have a shorter rDNA circle called EhR2 (Ghosh *et al* 2001). This circle is only 14 kb in size and lacks the leftward rDNA unit and the Hmg upstream region. In addition, the sequence of 18S rDNA from our *E. dispar* sample matched exactly with the reported *E. dispar* sequence (figure 3). To directly demonstrate the authenticity of the *E. dispar* DNA used, PCR amplification was carried out with species-specific primers. The sequence of 18S rDNA and ITS1 and 2 of *E. dispar* is known (Novati *et al* 1996; Som *et al* 2000). Primers were designed from regions of maximum sequence divergence between *E. histolytica* and *E. dispar*. Primer sequences are given in § 2 and their location in 18S rDNA and ITS-2 is shown in figure 4. The primer pair (1 + 3) in which one primer was derived from 18S rDNA and the second from ITS-2, amplified the expected 1·29 kb fragment when the *E. histolytica*-specific primer was used with *E. histolytica* DNA but not with *E. dispar* DNA. Similarly, the *E. dispar*-specific primer pair amplified the 1·29 kb fragment only from *E. dispar* DNA (figure 4A). Since the *E. dispar* DNA used in this study showed absolutely no amplification with the *E. histolytica* primer pair (figure 4A, lane 4), the possibility of any contamination was ruled out. The cloned *Eco*RI fragments of *E. dispar* rDNA (Ede and Edg) were also tested for amplification with *E. histolytica*-specific and *E. dispar*-specific primer pairs derived from 18S rDNA (primer pairs 1 + 2). Both fragments amplified the expected 600 bp band only with the *E. dispar*-specific primer pairs (figure 4B). Thus, the cloned fragments used for sequence analysis of IGS were *E. dispar*-specific.

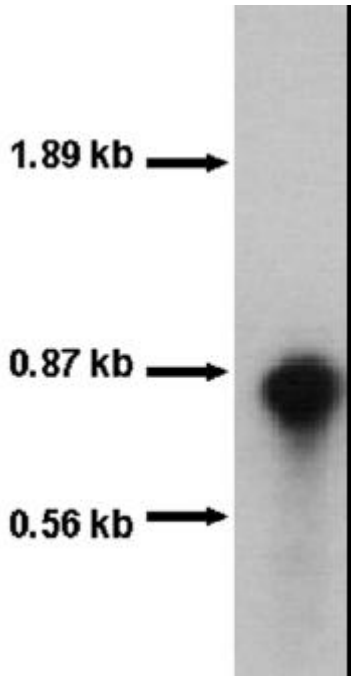

Figure 5. Northern blot analysis of *E. dispar* RNA with Tr specific probe. The electrophoresis conditions are described in § 2. The blot was hybridized with Tr probe, a deleted subfragment generated from HMg fragment of EhR1 by deletion (Som *et al* 2000). The region equivalent to HMg in the *E. dispar* rDNA circle is labelled Edg (figures 1 and 2). The molecular weight markers are indicated by arrows.

3.4 *The upstream IGS in Edg is transcribed in* E. dispar

Results from the *E. histolytica* rDNA circle had shown that, apart from the rRNAs themselves, only one other transcript could be detected from EhR1. This was transcribed from a region called Tr in the Hmg upstream spacer of EhR1 (Sehgal *et al* 1994). We wanted to see whether Edg, the equivalent upstream spacer in EdR1, was also transcribed. Northern blots of *E. dispar* RNA were hybridized with the Tr probe from EhR1 which is located between the *Pvu*I repeats and 361 bp region upstream of 18S rDNA (this region is marked in Edg, figure 2). The probe hybridized with a broad band of average size 0·75 kb (figure 5), which is similar to the result earlier reported for *E. histolytica*.

4. Discussion

The sequence divergence of IGS and ITS regions of rRNA genes have been used for species identification and phylogenetic analysis (Dover *et al* 1993). Closely related species differ in the exact sequence of the spacers, although overall sequence organization is conserved. We were interested to compare the IGS sequences of rRNA genes of *E. histolytica* and *E. dispar* to estimate the closeness of these sibling species. The data presented here suggests that not only are the spacer sequences organized in a similar fashion in the two species, the sequences themselves are very similar. A total of 2·5 kb of sequence was determined from various parts of the spacer region (figure 2) and the sequence differences ranged from 2·8% to 3·87%.

The extremely high level of identity between the *E. histolytica* and *E. dispar* IGS sequence over its entire length is unusual, even for closely related species. Such identity has not been reported so far in any other system, whether protozoa (Dietrich *et al* 1993; Uliana *et al* 1996), plant (Rogers and Bendich 1987), lower animal (Chen *et al* 2000) or human (Gonzalez and Sylvester 2001). By contrast, the 18S rDNA sequence which is normally very conserved, has diverged by as much as 1·6% between the two species. Our data would suggest that the constraints

on sequence divergence in the rDNA of Entamoeba are significantly different from those operating in other organisms, whereby relatively substantial changes are permitted in the 18S rRNA gene sequences while the repeat families in the IGS regions are well conserved. By analogy with other systems, the IGS sub-repeats are known to contain the transcriptional promoter, terminator and enhancer elements, besides the origin of rDNA replication. One may therefore conclude that the basic transcription and replication machinery, at least of rDNA, has diverged little between *E. dispar* and *E. histolytica*.

Since the secondary structure folding pattern of the *E. histolytica* 18S rDNA has been worked out (Ramachandran 1993), it is possible for us to locate nucleotide changes observed in *E. dispar* 18S rDNA with respect to *E. histolytica*. It was found that of a total of 31 nucleotide changes observed, only 5 were in constant regions and the rest were in variable regions of 18S rDNA. Half of the changes were compensatory, or the change did not affect hydrogen bonding. Only one-third of the changes were in loop regions, and these were mostly A to G or G to A transitions. Therefore, the 1·6% sequence divergence of 18S rDNA between the two species may result in very limited functional divergence of the two rRNAs. A similar assessment cannot be made of the changes observed in the IGS region of the two species unless a functional analysis of the various repeat- and non repeat-segments of the IGS is carried out.

The differences in the 18S rRNA gene sequences between *E. histolytica* and *E. dispar* have been used for species identification by riboprinting (Clark and Diamond 1997). In this work we show that specific primers from the divergent regions in the 18S gene can differentially amplify DNA from the cognate species. While probes from the 18S gene are expected to be reliable and reproducible, one may get misleading results with probes from the IGS, for example, the P145 repeat (*Pvu*I repeat in EhR1 as reported by Sehgal *et al* 1994) was considered to be *E. histolytica*-specific (Samuelson *et al* 1989). Here we show that this repeat is found in *E. dispar* also (Edg, figure 2). On the other hand, this repeat is absent in some strains of *E. histolytica* (e.g. HK-9 and Rahman) and may also be lost from the same strain maintained under laboratory conditions (Ghosh *et al* 2001). Therefore, until further information is obtained regarding the stability of repeats in the IGS, this region should be avoided for species identification.

Acknowledgements

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