Blastocystis elongation factor-1α: genomic organization, taxonomy and phylogenetic relationships

L. C. HO[1], A. ARMUGAM[2], K. JEYASEELAN[2], E. H. YAP[1] *and* M. SINGH[1]*

[1] *Department of Microbiology*
[2] *Department of Biochemistry, Faculty of Medicine, National University of Singapore, 5 Science Drive 2, Singapore 117597*

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SUMMARY

The elongation factor-1 alpha (EF-1α) is a highly conserved ubiquitous protein that is involved in translation and is desirable for use in phylogenetic studies on *Blastocystis*, an enigmatic intestinal parasite with a contentious taxonomic position. In the present study, a PCR product (BEα) that codes for a major part of the coding region of the EF-1α protein was amplified. Genome walking experiments together with cloning were implemented to elucidate the 5′ and 3′ ends of the EF-1α gene of the human isolate, *Blastocystis hominis* C. The genomic organization and the potential transcription factor binding sites of the 5′ end of *B. hominis* C EF-1α were identified. A comparative study on the deduced amino acid sequences of BEα of 13 *Blastocystis* isolates from various hosts was done to evaluate the phylogenetic relationship among the species†. A phylogenetic reconstruction analysis with other eukaryotic EF-1α sequences was carried out to trace the phylogenetic position of *Blastocystis* among eukaryotic organisms.

Key words: *Blastocystis*, elongation factor-1 α, genomic organization, phylogeny, taxonomy.

INTRODUCTION

Blastocystis spp. are intestinal parasites found in a wide variety of animal hosts including humans (Teow *et al*. 1992; Stenzel & Boreham, 1996; Chen *et al*. 1997*a*). *Blastocystis hominis* was first described by Brumpt (1912) as a yeast. Subsequent morphological studies indicated that it is more likely to be a protozoan in the Subphylum Sarcodina (Zierdt, 1973, 1988). Molecular phylogenetic studies based on the comparison of small subunit ribosomal RNA (SrRNA) sequences suggested that *B. hominis* is unrelated to yeasts and could be placed on an outer group of the clade that links ciliates and apicomplexans (Johnson *et al*. 1989). More recently, Silberman *et al*. (1996) sequenced the complete *Blastocystis* SrRNA genes and placed *Blastocystis* within the stramenopiles. Nakamura *et al*. (1996) analysed the amino acid sequence of *B. hominis* translation elongation factor-1 alpha (EF-1α) and from a phylogenetic reconstruction analysis, using a maximum likelihood method of protein phylogeny, suggested that *B. hominis* should not be included within the fungal lineages and that its divergence is earlier than that of *Trypanosoma*, *Euglena*, *Dictyostelium*, and other higher eukaryotes. To date, the taxonomic home of *Blastocystis* is still uncertain.

Many attempts have been employed to understand the molecular biology of this enigmatic parasite. Upcroft *et al*. (1989) by field-inversion gel electrophoresis (FIGE) showed that the chromosomal karyotypes of *B. hominis* and *Saccharomyces cerevisiae* are similar in number and distribution. Our previous study using pulsed-field gel electrophoresis (PFGE) on isolates of *B. hominis* showed otherwise (Ho *et al*. 1994). Animal isolates of *Blastocystis* showed distinctly different karyotypic patterns by PFGE (Singh *et al*. 1996; Chen *et al*. 1997*b*). DNA polymorphism among strains of *Blastocystis* species was also revealed by the polymerase chain reaction technique (Yoshikawa *et al*. 1996, 1998).

In this study, we examined the nucleotide sequences of the elongation factor-1 alpha (EF-1α) gene of *Blastocystis* and the genetic relatedness among different isolates of the parasite from humans and animals. The EF-1α gene is highly conserved and is suitable as a phylogenetic marker for eukaryotes (Roger *et al*. 1999). Primers were designed to amplify the EF-1α gene from DNA of different *Blastocystis* species. Genome walking, cloning and cycle sequencing were implemented and the complete EF-1α gene of *B. hominis* including the 5′ and 3′ flanking regions has been elucidated. Sequences of the 965 bp fragment of EF-1α gene (BEα) from 13 isolates were aligned and analysed. Phylogenetic relationships were determined among the isolates and other eukaryotic organisms and the taxonomic position of *Blastocystis* deduced.

* Corresponding author: Department of Microbiology, Faculty of Medicine, National University of Singapore, 5 Science Drive, Singapore 117597. Tel: +65 8743277. Fax: +65 7766872. E-mail: micmulki@nus.edu.sg

† The nucleotide sequence data reported in this paper are available in the GenBank database with the accession numbers as shown in Table 1.

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MATERIALS AND METHODS

Source of Blastocystis

Axenic cultures of 13 *Blastocystis* isolates (Table 1) from humans, rats and reptiles (Singh *et al*. 1996; Chen *et al*. 1999; Ng & Tan 1999) were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% horse serum as described previously (Ho *et al*. 1993).

Genomic DNA isolation

Cultures were harvested by centrifugation and washed with sterile saline. The pellets were lysed in Tris–SE buffer (10 mM Tris–HCl buffer, pH 8·0, 75 mM NaCl, 25 mM EDTA, pH 8·0) containing 1% SDS and Proteinase K (Sigma) and incubated at 55 °C overnight. Total nucleic acids were extracted following the salt–chloroform method described by Mullenbach, Lagoda & Welter (1989). The DNA preparations were treated with RNAse A at 37 °C for 30 min and kept at −20 °C until use.

PCR amplification of genomic DNA (*BE*α)

The DNA samples from the 13 isolates of *Blastocystis* were amplified with standard PCR amplification conditions of 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min using the Perkin-Elmer DNA Thermal Cycler 480 (Jeyaseelan *et al*. 1998). Oligonucleotide primers, synthesized by NUMI (National University Medical Institutes, Singapore) based on the EF-1α mRNA gene sequence, D64080 (Nakamura *et al*. 1996) were used. The sense and antisense primers used were labelled as BlaxF and BlaxR respectively, and for a nested PCR amplification, BlaiF and BlaiR (Table 2). All the primers were deduced using the Primer-Select program from DNASTAR, Inc. (Madison, WT). The genomic PCR products (BEα) were analysed on agarose gels. The appropriate bands were excised from the gel and purified through the Qiagen silica matrix spin column (QIAGEN Gel Extraction kit, Qiagen) for further experiments.

Direct sequencing

The ABI PRISM Dye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase, FS (Perkin–Elmer) was used to process the purified DNA for sequencing. DNA sequencing was performed using Automated DNA sequencer (Model 373A, Applied Biosystems Inc., USA) following the procedure used previously by Armugam *et al*. (1997).

Genome walking through 5′ and 3′ flanking regions

The DNA of *B. hominis* isolate C was used for further analysis of the unknown genomic DNA sequences adjacent to the known EF-1α gene sequence of *Blastocystis*. To construct pools of uncloned, adaptor-ligated genomic DNA fragments, the genomic DNA was digested with restriction enzymes *Dra*I, *Stu*I, *Eco*RV, *Sca*I and *Pvu*II and ligated with the Genome Walker Adaptor (Universal Genome Walker; Clontech, USA). The adapter-ligated genomic DNA fragments also known as Genome Walker libraries, were used as templates in DNA walking experiments (Clontech, USA). To map the 5′ and 3′ end of the EF-1α gene, the adapter primer 1 (AP1), 5′-GTAATACGACTCACTATA-GGGC-3′ and the gene-specific primers BlagR and BlaiF (Table 2) were used for the 5′ and 3′ end extension respectively.

The amplification technique used was the 'touchdown' PCR (Don *et al*. 1991; Roux 1995), which involved a single annealing/extension temperature that was several degrees higher than the melting temperature of the primers during the initial PCR cycles. This temperature was later reduced to the primers' melting temperature for the remaining PCR cycles. The cycling parameters used to map the 5′ end of the gene were 7 cycles of 25 sec at 94 °C and 3 min at 70 °C (touchdown) and 32 cycles of 25 sec at 94 °C and 3 min at 55 °C followed by 7 min at 67 °C after the final cycle. As for the 3′ end region, the cycling parameters used were 7 cycles of 25 sec at 94 °C and 3 min at 72 °C and 32 cycles of 25 sec at 94 °C and 3 min at 67 °C followed by a final extension of 67 °C for 7 min after the last cycle. The genomic PCR products of the desired size were purified through a Qiagen spin column.

Cloning and analyses of the 5′ and 3′ flanking region

The purified DNA fragments flanking the 5′ and 3′ ends of the *B. hominis* C BEα gene were ligated into pT-Adv vectors and transformed into *Escherichia coli* strain TOP 10F′ (AdvaTAge PCR cloning, Clontech, USA). The recombinants were selected on LB-Amp (50 μg/ml) plate supplemented with X-Gal and IPTG according to the protocol of Sambrook, Fritsch & Maniatis (1989). Positive colonies were checked for correct fragment size inserts by digesting with *Eco*RI restriction enzyme. Using M13 forward and reverse primers, the 3′ and 5′ end selected clones were sequenced. The sequences were subsequently sequenced on both strands with sense and antisense primers BlawF and BlawR for the 3′ end mapping, and Blag1F/2F and Blag2R/3R/4R for extension of the 5′ end.

The sequences of the DNA fragments obtained through amplification with the various designed primers were aligned and joined to give the full EF-1α gene. The ends of the fragments were overlapped to ensure that the entire gene was screened. Sequence analysis of the gene was done. Regulatory sequences identified as binding sites for DNA binding proteins

Table 1. Isolates of *Blastocystis* and their hosts

Host	Isolate	Sequence reference	GenBank accession no.
Human	*Blastocystis hominis* B	B	AF090737
	Blastocystis hominis C	C	AF223379
	Blastocystis hominis E	E	AF091356
	Blastocystis hominis G	G	AF091357
	Blastocystis hominis H	H	AF091358
	Blastocystis hominis S	S	AF091359
Rats			
Wistar	*Blastocystis ratti* WR1	W	AF091363
	Blastocystis ratti WR2	R	AF091364
Sprague–Dawley	*Blastocystis ratti* S1	D	AF091365
Reptiles			
Iguana lizard	*Blastocystis cycluri*	I	AF091362
	Blastocystis sp. Isolate R44	U	AF223380
Python	*Blastocystis pythoni*	P	AF091360
Sea snake	*Blastocystis lapemi*	L	AF091361

Table 2. Primers used to amplify EF-1α gene fragments of *Blastocystis*

Primer	Sequences 5′–3′
BlaxF	GGTCACCGTGACTTCATAAAG
BlaxR	AGCGAAACGACCAAGAGG
BlaiF	AACATGCCCTGGTACAAGGG
BlaiR	GGATCGTTCTTGGCATCT
BlawF	GTCTGTGAAGGACATTCACCGTG
BlawR	GTTTGTGCCGTTCATCTGTAGA
BlagR	CCCTTGTACCAGGGCATGTTMGCAGARCGC
Blag1F	CGGTTGTACGTTTGATGCC
Blag2R	GGCCTGAGAGTACCGGTG
Blag2F	GCAAGGAAGGCGCAATCTCT
Blag3R	GACTTGCCAGAATCGACGTGTCC
Blag4R	AAGCCAGTCGTTTCATTTTACCT

were searched using the transcription factors search program (TRANSFAC; Heinemeyer *et al*. 1998).

Amino acid sequence alignment and analysis

Sequence analyses were carried out using DNASTAR (USA) and DNASIS v2.5 (Hitachi Software Engineering) programs. The DNASIS multiple alignment (Higgins & Sharp, 1988) and editing functions arrange sequences hierarchically for taxonomic purposes. The amino acid and nucleotide sequences corresponding to the open reading frames of *Blastocystis* EF-1α were aligned and compared. Nucleotide sequences reported in this paper have been assigned GenBank accession numbers as shown in Table 1. The genetic relatedness among the *Blastocystis* species was established. The nucleotide sequences corresponding to the complete coding region of the EF-1α gene of *B. hominis* C were aligned with sequences of the same alpha subunit in other species in the animal kingdom. In total 21 sequences of eukaryotic EF-1α gene were selected for this study from the GenBank database. The amino acid sequences corresponding to the BEα gene of *Blastocystis* were compared. A phylogenetic relationship was built among the eukaryotes using the DNASIS program. Alignments are constructed by progressively aligning sequences according to the branching order in an initial phylogenetic tree. The program takes as input a dendrogram produced by applying UPGMA method to a matrix of similarity scores for all the aligned sequences.

RESULTS

Amplification of elongation factor-1 alpha gene from Blastocystis *isolates*

A pair of oligonucleotide primers BlaxF and BlaxR, based on the elongation factor-1 alpha (EF-1α) mRNA of *B. hominis* strain HE87-1 (BLAEF1A-D64080) was designed. This set of primers was able to amplify a fragment of 962 bp (BEα) from all the DNAs isolated from *Blastocystis* spp. A second set of

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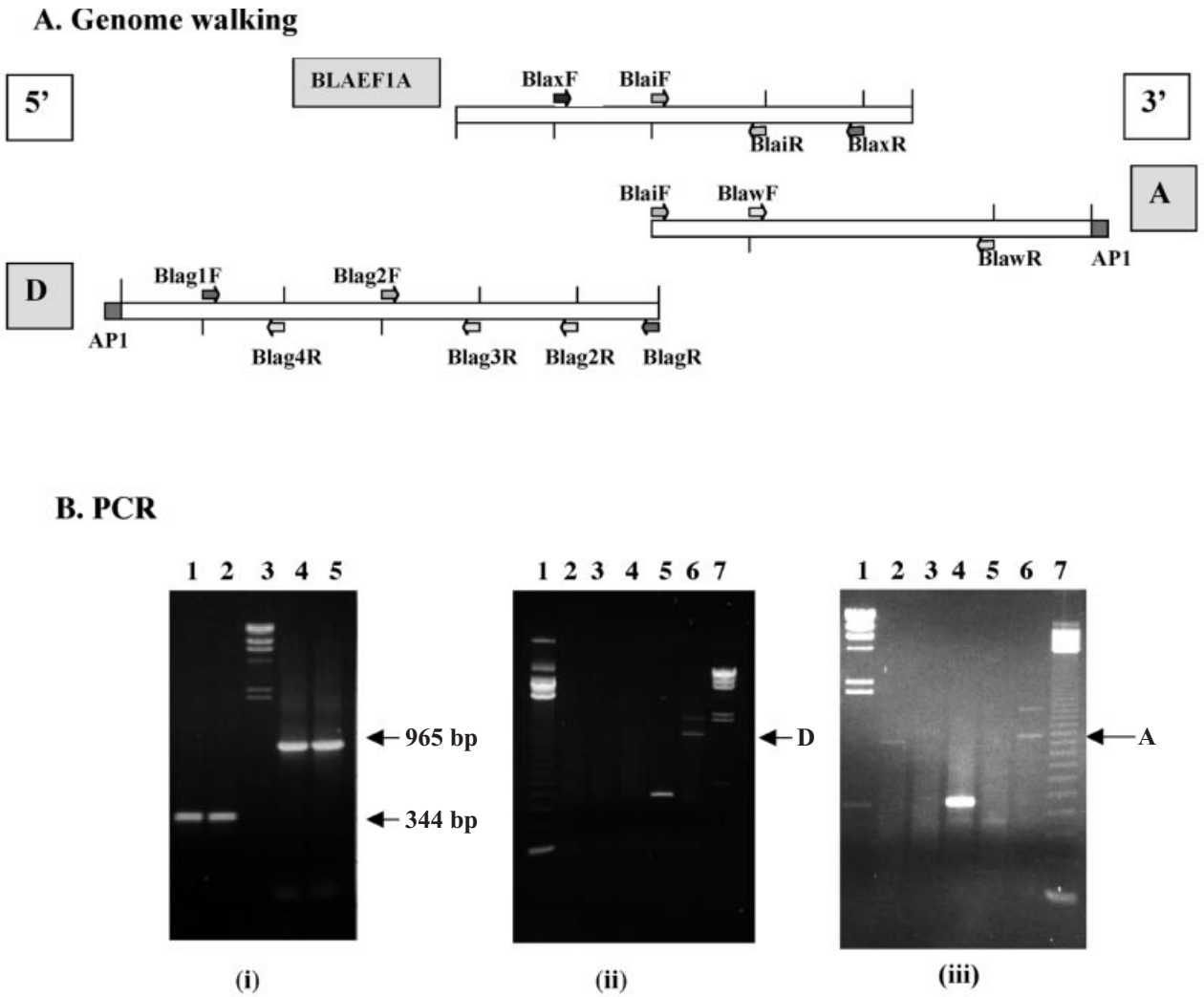

Fig. 1. Strategies for PCR. (A) Schematic illustration of the strategies for the amplification of genomic DNA and genome walking to map the 5′ and 3′ ends of the EF-1α gene of *Blastocystis*. The locations of primers (sense→ and antisense←) and their respective base number relative to the complete coding regions of the EF-1α gene of *Blastocystis* are indicated. Gene-specific primer (GSP) BlagR and adapter primer (AP1) were used to amplify adapter-ligated libraries to map the 5′ end of the BEα gene: fragment D, an amplification product from the *Pvu*II library was selected and sequenced. For the 3′ end extension, GSP BlaiF and AP1 were used to amplify fragment A from the *Pvu*II library which was then cloned and sequenced. (B) Agarose gel electrophoresis of PCR products generated from the genomic PCR and genome walking PCR to elucidate the complete nucleotide sequence of the *Blastocystis* EF-1α gene. (i) Amplification of partial EF-1α gene using genomic PCR: Lanes 1 and 2: nested PCR products of 344 bp using primers BlaiF & BlaiR Lane 3: λ phage *Hin*dIII digest size marker. Lanes 4 and 5: PCR products of 965 bp using primers BlaxF & BlaxR. (ii) Amplification of the 5′ end of the EF-1α gene: Lane 1: 123 bp ladder of DNA size marker. Lanes 2–6: Genome walking PCR products from *Dra*I, *Stu*I, *Eco*RV, *Sca*I and *Pvu*II adapter-ligated DNA libraries, respectively, using primers BlagR and AP1. Lane 7: λ phage *Hin*dIII digest size marker. (iii) Amplification of the 3′ end of the EF-1α gene: Lane 1: λ phage *Hin*dIII digest size marker. Lanes 2–6: Genome walking PCR products from *Dra*I, *Stu*I, *Eco*RV, *Sca*I and *Pvu*II adapter-ligated DNA libraries, respectively, using primers BlaiF and AP1. Lane 7: 123 bp ladder of DNA size marker. A and D are the *Pvu*II fragments (Fig. 1A) used for genome walking.

primers, BlaiF and BlaiR developed for nested PCR gave a 344 bp product. Amplification with these primer pairs gave a strong, single and reproducible band of the desired size to all the isolates of *Blastocystis* in each case (Fig. 1B i) except for isolate U which gave additional 3 bands of sizes 1·4, 1·9 and 2·4 kb. The sequences of these primers are shown in Table 2 and their respective locations within the EF-1α gene are also shown in Fig. 1A.

Cloning of the 5′ and 3′ ends of B. hominis *C EF-1α gene*

Gene-specific primers and adaptor-primer 1 (AP1) were used to amplify adaptor-ligated libraries to map the 5′ and 3′ end of the EF-1α gene of *B. hominis* C. A fragment size of at least 1 kb was chosen from the libraries to ensure that the genome walking would cover further than the N and C terminals of the gene.

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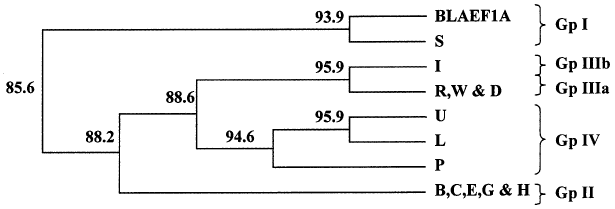

Fig. 2. Phylogenetic tree of *Blastocystis* species derived from the amino acid sequences of a major part of the coding region of the EF-1α gene. A dendrogram constructed for phylogenetic analysis with DNASIS software package is shown. The abbreviations used for sequence references are the same as in Table 1.

The nucleotide (nt) sequence of the 3′ end of the EF-1α gene was elucidated by subcloning and sequencing the 1·2 kb PCR fragment (A) from the *Pvu*II library (lane 6, Fig. 1B iii) generated using AP1, the adapter primer and gene specific primer, BlaiF (Fig. 1A). Likewise, the 5′ end of the *Blastocystis* EF-1α gene was elucidated by subcloning and sequencing the 1·4 kb genome walking PCR fragment (D) from the *Pvu*II library (lane 6, Fig. 1B ii) generated using AP1 and BlagR, as sense and antisense primers respectively (Fig. 1A).

Analysis of EF-1α gene of B. hominis *C*

Sequencing and analysis of the PCR products from the above experiments on both strands using appropriate primers gave the complete gene sequence of the EF-1α gene of *B. hominis* C (GenBank Accession no. AF223379). The 5′ flanking region of the EF-1α gene of *B. hominis* C showed many potential transcription factors (TFs) and their binding sites. Among the TFs found was a CCAAT enhancer binding protein CEBPb (nt 587) and a CCAAT element further upstream (nt 304). There were 3 possible sites of TATA boxes at positions 431, 451 and 643. Other interesting elements present in the 5′ region of the gene were the HSF (nt 400), USF (nt 339), STATx (nt 156) and the GATA boxes (nt 54 and 135) from a list of many other TFs found.

Analysis of the amino acid sequences of elongation factor-1 alpha from Blastocystis *spp.*

The DNA sequences of the 965 bp PCR products of the different isolates labelled as shown in Table 1 (sequence reference) were translated and the amino acids (a.a.) corresponding to each of the sequences were deduced. The 290-amino acid segments encoded by the coding region excluding the N and C terminal regions were aligned for all the 13 isolates. To understand the relationships among the *Blastocystis* spp, a phylogenetic tree was drawn using DNASIS program (Fig. 2). The 13 isolates have been found to cluster as 4 main groups: Group I – human isolates S and HE87-1 (BLAEF1A); Group II – human isolates B, C, E, G and H; Group IIIa – rat isolates R, W and D; Group IIIb – reptilian isolate I; Group IV – reptilian isolates P, U and L.

The BEα of *B. cycluri* (isolate I) exhibiting high homology with that of the rat isolates remained as a separate group (IIIb) from the other reptilian isolates in Group IV. The human isolates were split into 2 different groups with isolate S in Group I and the other isolates in Group II. The BLAEF1A which has been used as a reference was placed under Group I since it showed a high homology to isolate S.

Generally, homology between all the isolates was high. Amino acid sites where variable residues were found have been highlighted in Fig. 3. The human isolates, *B. hominis* B, C, E, G and H showed identical a.a. sequences except for isolate S (S). Interestingly, there was high homology between the sequences of S and BLAEF1A when alignment was made, differing only at a.a. sites (S/BLAEF1A), 195 (K/N), 200 (K/E), 285 (M/Q) and 1 less a.a. for BLAEF1A at site 222 (F/-).

A comparison of the amino acid sequences of the *Blastocystis ratti* isolates R and W (R and W) from the Wistar rats and isolate D (D) from Sprague Dawley rats gave perfect homology. Similar amino acids were seen in the sequences for *B. ratti* isolates and that of the reptilian *Blastocystis* but were different from that of *B. hominis* at a.a. sites (*B. ratti* and reptilian *Blastocystis*/*B. hominis*) 64 (T/A,N), 116 (A/N), 161 (V/T), 169 (S/T), 198 (A/S) and 243 (L/M,V) (Fig. 3).

Of the 4 reptilian isolates, 2 were obtained from snakes and the other 2 were from lizards. The a.a. sequences of *B. cycluri* and *B. ratti* had surprisingly similar sequences with differences only in a.a. positions 57, 98 and 217. The a.a. sequences of *B. ratti* were exclusively similar to that of *B. cycluri* isolate at a.a. positions 185 (V), 194 (I), 223 (E), 240 (T), 259 (T), 261 (I) and 289 (T). There were also several sites where the amino acids were unique only to that isolate. These positions are shown in bold italics in Fig. 3.

Phylogenetic relationship of EF-1α gene of Blastocystis

With the amino acid sequences of the BEα segment of the EF-1α gene of *Blastocystis*, groupings which correlated well with their speciation were obtained. A phylogenetic tree was built based on the same alpha subunit of other eukaryotes (Fig. 4). Comparison of these tree topologies suggested that relationships among the 13 isolates of *Blastocystis* were clustered together.

DISCUSSION

The amino acid composition of EF-1α is free from the drastic variation of genome base composition and

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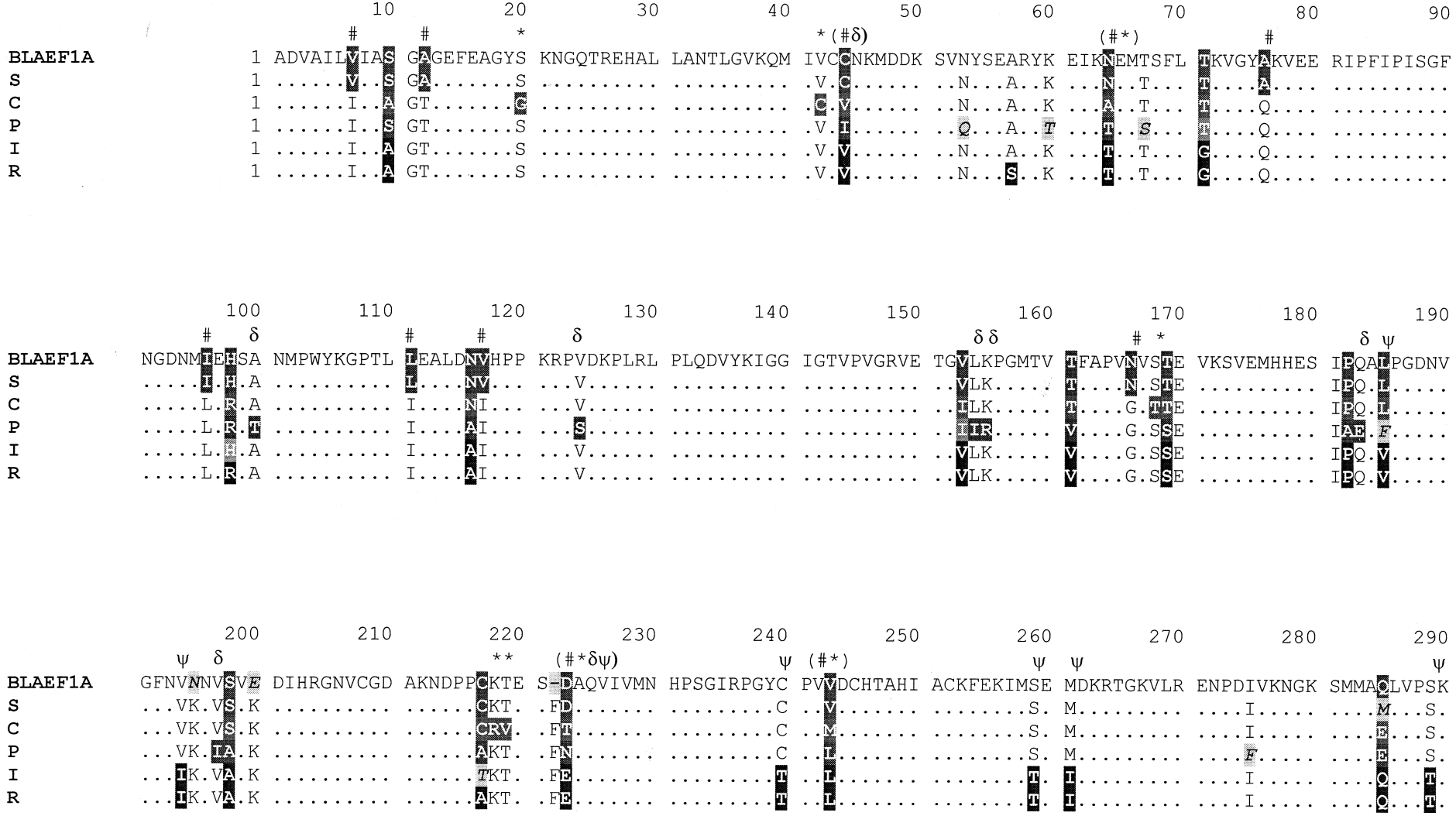

Fig. 3. Comparison of the 290 amino acids in BEα gene of *Blastocystis* spp. The derived amino acid sequences of represented isolates of *Blastocystis* from each group were aligned using the DNASIS program. Positions with variable residues are highlighted. Sites where the amino acids are different from all the others are marked with bold italics and highlighted. Sites where a specific group was seen different from the other groups are indicated by the respective symbols: Gp I with #; Gp II with ★; Gp III with ψ and Gp IV with δ. The BLAEF1A sequence was used as a reference.

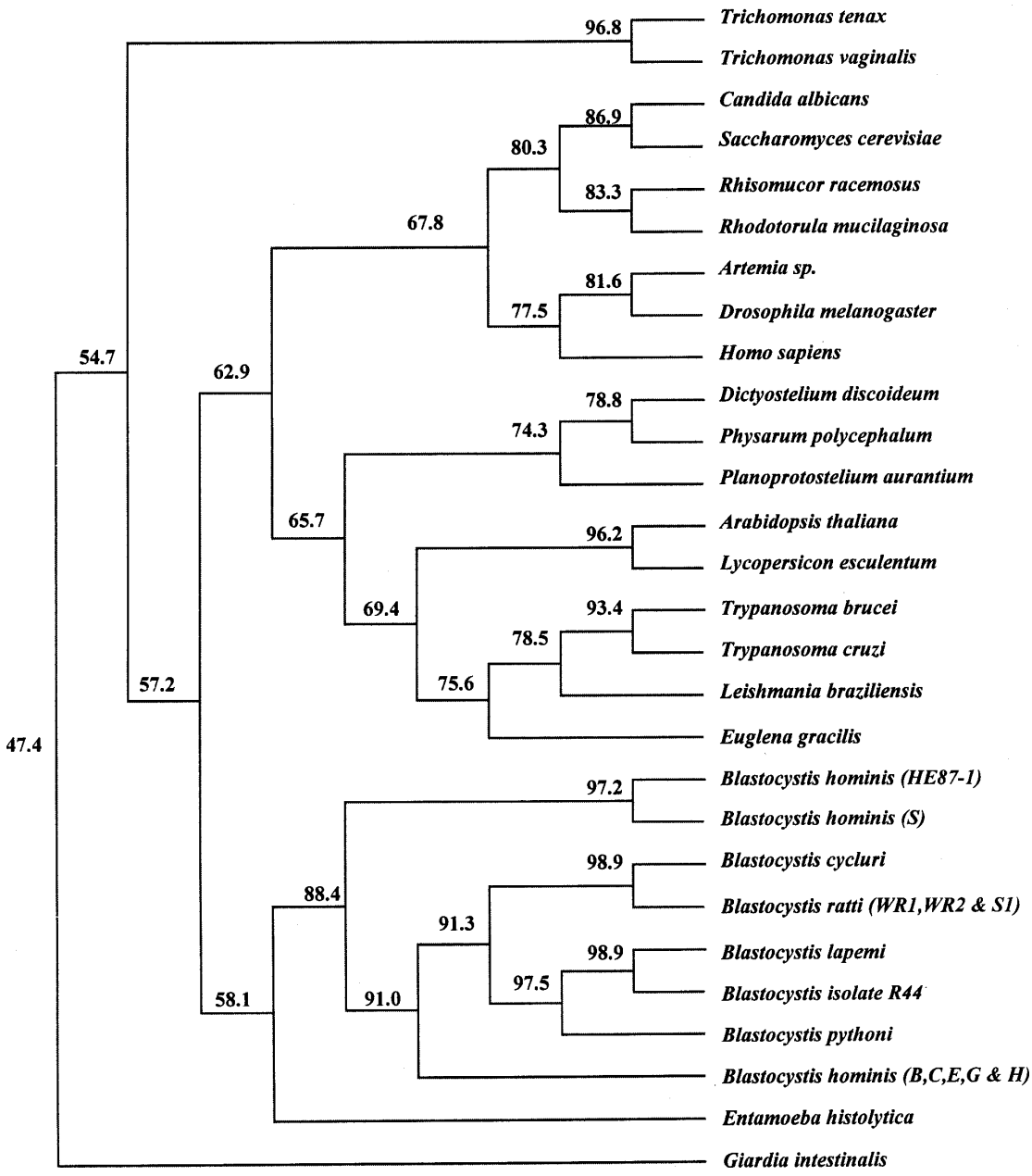

Fig. 4. EF-1α tree of eukaryotes. A eukaryote tree based on molecular properties of the proteins encoded by the amino acid sequences of the EF-1α gene corresponding to the BEα segment in *Blastocystis*. Sequences from representative organisms from the different phyla of the plant and animal kingdom were selected from the GenBank: Animalia – *Homo sapiens* (accession nos. NM001402), *Drosophila melanogaster* (X06869), *Artemia* sp. (X03349); Viridiplantae – *Lycopersicon esculentum* (X53043), *Arabidopsis thaliana* (X16430); Fungi – *Saccharomyces cerevisiae* (X00779), *Candida albicans* (M29934), *Rhodotorula mucilaginosa* (AF016239), *Rhizomucor racemosus* (J02605); Slime mold – *Planoprotostelium aurantium* (AF016240), *Physarum polycephalum* (AF016243); *Dictyostelium discoideum* (X55972); *Blastocystis hominis* (D64080, also see Table 1); Protozoa – *Leishmania braziliensis* (U72244), *Trypanosoma brucei* (U10562), *Trypanosoma cruzi* (L76077), *Euglena gracilis* (X168479), *Entamoeba histolytica* (M92073), *Trichomonas tenax* (D78479), *Trichomonas vaginalis* (AF058282), *Giardia intestinalis* (D14342). The tree was constructed using the UPGMA method in the DNASIS program. The calculated matching percentages were indicated as numbers at each branch point of the dendrogram (Higgins & Sharp, 1988).

hence phylogenetic relationships among protozoa by using these conservative proteins are highly desirable to understand the early evolution of eukaryotic cells (Hashimoto *et al*. 1995), Roger *et al*. (1999) have also shown the EF-1α to be useful as a phylogenetic marker for eukaryotes. Based on the EF-1α mRNA sequence (BLAEF1A, Nakamura *et al*. 1996), we have prepared a pair of sense and antisense primers (BlaxF and BlaxR) which amplified a PCR product of 965 bp (BEα) that was consistently reproducible with the PCR products of all the 13 isolates of *Blastocystis* species. A nested PCR was also per-

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formed to give a 344 bp product and the authenticity of the amplification thus checked. It was found that the DNA sequences of BEα cover a major part of the coding region for the EF-1α gene of *Blastocystis* making it ideal for use in studying genetic relatedness among *Blastocystis* spp. Apart from *B. hominis* we have been able to sequence the 965 bp fragment (BEα) of the EF-1α gene of *Blastocystis* from rats and reptiles. The DNA sequences are given the GenBank accession numbers. This is the first time that such sequences have been obtained from a variety of animal *Blastocystis* spp.

The complete coding region of *B. hominis* isolate C with its 5′ and 3′ flanking region was elucidated through genome walking and cloning experiments. Cycle sequencing with designed primers on the 1·2 kb fragment A gave an extension of 450 bp for the 3′ end and the 1·4 kb fragment D, an extension of 785 bp upstream from the start codon of the EF-1α gene. We present here for the first time some potential regulatory protein binding sites that flanked the EF-1α gene of *Blastocystis*. The BLAEF1A sequences represent the EF-1α mRNA of *B. hominis* strain HE87-1 (Nakamura *et al*. 1996). When alignment of BLAEF1A sequences was made with that of *B. hominis* C, which was amplified from DNA, there were no indications of the presence of any intron within the EF-1α gene of *Blastocystis*. An alignment with the sequences of the mRNA for EF-1α gene of *B. hominis* C should be done in order to confirm the presence of introns.

In separate studies done by Ho *et al*. (1994) and Yoshikawa *et al*. (1998), genetic similarities of the 5 *B. hominis* isolates B, C, E, G and H was observed. The human isolate S of *B. hominis* surprisingly gave a karyotypic pattern different from other *B. hominis* isolates (unpublished data). Not surprisingly, the amino acid sequence homology between the *B. hominis* isolates, except for isolate S, were 100 %. *Blastocystis hominis* S was hence placed into a different group (Gp I) in our speciation study. The human isolates B, C, E, G and H were obtained from Singapore whilst the S isolate was from Pakistan, suggesting existence of different strains of *B. hominis*. Interestingly, the BEα sequence of isolate S had high homology to the Japanese isolate (HE87-1). A chicken isolate (CK86-1) in Japan had shared PCR bands with the human isolate HE87-1, and Yoshikawa *et al*. (1996) suggested the existence of zoonotic strains of *Blastocystis*.

Nakamura *et al*. (1996) presented an EF-1α tree of eukaryotes where deduced amino acid sequences of 1 isolate of *B. hominis* (HE87-1) EF-1α were aligned with those from 14 eukaryotic and 3 archaebacterial EF-1α. They were unable to resolve the taxonomic position of *Blastocystis* with statistical significance. Roger *et al*. (1999) were silent as to where *B. hominis* should be positioned. Currently only 2 complete sequences of *Blastocystis* EF-1α gene (BLAEF1A and *B. hominis* C) are available for studies. Since the EF-1α is a highly conserved protein, an unambiguous alignment could be constructed excluding both the N and C terminal regions. Thus, the BEα segment of the EF-1α gene of *Blastocystis* has been used not only for studying the genetic relatedness among the species but also the relationships with other eukaryotes in the plant and animal kingdom. The 13 positions of *Blastocystis* EF-1α gene used for comparison clustered in the same group. Within the same group many amino acid substitutions were seen among the *Blastocystis* isolates. This analysis suggests that *Blastocystis* is a highly evolved organism in its molecular properties probably due to mutational substitutions to suit the various environmental conditions of the different species. The parasite diverged within the same group and the isolates can be clearly seen to be in the same genus. The position of *Blastocystis* species, was found to be branching off together with *Entamoeba*. Like *Entamoeba histolytica*, *Blastocystis* is an intestinal parasite that has its habitat in the anaerobic environment of the caecum. Zierdt's findings in 1988 of motile feeding pseudopodia like that of the amoeba was one of the considerations to reclassify *B. hominis* from Sporozoa to Sarcodina, (Order, Amoebida). Our study suggests a genetic link besides the morphological link between *E. histolytica* and *Blastocystis*.

A recent phylogenetic reconstruction based on 16S-like rDNA had demonstrated that an amoeba, *Hyperamoeba*, is the sister taxon to the plasmodial slime mold *Physarum polycephalum* (Zaman *et al*. 1999). Based on mitochondrial characteristics, *Blastocystis*, like the slime molds, possesses mitochondria with tubular cristae, unlike *Entamoeba* which is amitochondriate. Cavalier-Smith (1998) presented a revised six-kingdom system of life and interestingly, he grouped Mycetozoa and Archamoebae under a new Subphylum Conosa within the Phylum Amoebozoa in the Infrakingdom Sarcomastigota which typically includes sarcodines, flagellates, or amoeboflagellates. Our results on the EF-1α gene sequences of *Blastocystis* suggest that the taxonomic position of *Blastocystis* should be in the Subphylum Conosa and whether it is in a new Infraphylum in this group needs to be determined.

In conclusion, our present study on the EF-1α gene of *Blastocystis*, reveals the complete sequences of the coding region and its 5′ and 3′ flanking regions of *B. hominis* C. Potential regulatory protein binding sites at the 5′ region of the gene have been shown. Further studies on RNAs of *B. hominis* C like primer extension analysis could be carried out to determine the transcription initiation sites of the EF-1α gene. The BEα gene segment of EF-1α has proven to be useful for speciation study. Our present study suggests that *Blastocystis* may be placed under the Subphylum Conosa. More positions of entamoeba,

mycetozoan and even stramenopile EF-1α when available would phylogenetically place *Blastocystis* more precisely. Phylogenies based upon comparisons of sequences of transcription factor; DNA-dependent RNA polymerase, ATPase, tubulin and heat shock genes have been done for many other organisms. Whether these would be useful for phylogenetic taxonomic studies in *Blastocystis* remains to be seen.

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