Sequence heterogeneity of the small subunit ribosomal RNA genes among *Blastocystis* isolates

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SUMMARY

Genes encoding small subunit ribosomal RNA (SSUrRNA) of 16 *Blastocystis* isolates from humans and other animals were amplified by the polymerase chain reaction, and the corresponding fragments were cloned and sequenced. Alignment of these sequences with the previously reported ones indicated the presence of 7 different sequence patterns in the highly variable regions of the small subunit ribosomal RNA. Phylogenetic reconstruction analysis using *Proteromonas lacertae* as the outgroup clearly demonstrated that the 7 groups with the different sequence patterns are separated to form independent clades, 5 of which consisted of the *Blastocystis* isolates from both humans (*B. hominis*) and other animals. The presence of 3 higher order clades was also clearly supported in the phylogenetic tree. However, a relationship among the 4 groups including these 3 higher order clades was not settled with statistical confidence. The remarkable heterogeneity of small subunit ribosomal RNAs among different *Blastocystis* isolates found in this study confirmed, with sequence-based evidence, that these organisms are genetically highly divergent in spite of their morphological identity. The highly variable small subunit ribosomal RNA regions among the distinct groups will provide useful information for the development of group-specific diagnostic primers.

Key words: *Blastocystis*, genetic diversity, phylogeny, small subunit ribosomal RNA.

INTRODUCTION

Blastocystis is an anaerobic intestinal parasite found in a wide range of vertebrate hosts including humans. It has a number of unique biological features, such as a large membrane-bound central vacuole taking up as much as 95% of the cell, multiple nuclei, and cytochrome-free mitochondria (Zierdt, 1991; Boreham & Stenzel, 1993; Stenzel & Boreham, 1996). *Blastocystis* shows a variety of cell forms during its life-cycle, but no flagellated forms have ever been identified. There are indications that its anaerobic mitochondria are functional, although the mitochondria lack many enzymatic activities in the energy-producing pathway (Zierdt, 1991).

The small subunit ribosomal RNA (SSUrRNA) phylogeny demonstrated that *Blastocystis* belongs to stramenopiles (Silberman *et al*. 1996), a diverse heterogeneous group of mostly unicellular algae and protists that include brown algae, diatoms, oomycetes and several other lineages (Patterson, 1989). The closest relative of *Blastocystis* in the stramenopiles is *Proteromonas lacertae*, a commensal in the hindgut of lizards and amphibia (Leipe *et al*. 1996). Recently, the monophyletic origin of *B. hominis* with stramenopiles in the SSUrRNA tree was reconfirmed by a tree of cytosolic-type heat shock protein 70 (Arisue *et al*. 2002). However, phylogeny of translation elongation factor 1α (EF-1α) has not yet supported this finding (Nakamura *et al*. 1996; Arisue *et al*. 2002).

Blastocystis isolates from humans have generally been described as *Blastocystis hominis*, while those from other animal hosts have been given their species names on the basis of the host of origin, *in vitro* culture characteristics, and/or karyotypic patterns (Belova & Kostenko, 1990; Belova, 1991, 1992; Teow *et al*. 1991; Singh *et al*. 1996; Chen *et al*. 1997; Krylov & Belova, 1997). However, all isolates reported so far, irrespective of whether they are from humans or other animals, have been morphologically indistinguishable in spite of their extensive genetic diversity (Müller, 1994; Mansour *et al*. 1995; Yoshikawa *et al*. 1996, 1998, 2000; Böhm-Gloning, Knobloch & Walderich, 1997; Clark, 1997; König & Müller, 1997; Ho *et al*. 2000; Hoevers *et al*. 2000; Snowden *et al*. 2000; Ho, Jeyaseelan & Singh, 2001; Kaneda *et al*. 2001). The role of this organism in disease is still uncertain, although there are many reports both implying and denying it as a pathogen (Stenzel & Boreham, 1996). Genetic diversity among different *Blastocystis* isolates could be related significantly to the uncertainty in the role of *Blastocystis* in disease (Clark, 1997).

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In this work, we sequenced SSUrRNA genes of 16 *Blastocystis* isolates from humans and other animals in order to estimate quantitatively the genetic diversity of *Blastocystis* by the use of sequence data. Alignment and phylogenetic reconstruction analyses using all the SSUrRNA sequence data currently available clearly revealed that these sequences could be divided into 7 distinct groups.

MATERIALS AND METHODS

Blastocystis *isolates and DNA extraction*

Sixteen *Blastocystis* isolates from 8 humans, 2 chickens, 2 quails, 2 pigs, 1 monkey and 1 rat were used in this work (Table 1). These isolates, except for the B strain of *B. hominis*, were cultured in diphasic agar-slant medium as described previously (Yoshikawa *et al*. 1996). Genomic DNAs of *Blastocystis* isolates were extracted using a DNAzol reagent (Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) according to the manufacturer's protocol. Genomic DNA of the B strain of *B. hominis* was obtained from Dr M. Singh, The National University of Singapore.

Cloning and sequencing of the SSUrRNA genes

A pair of sense (5′-GCTTATCTGGTTGATCCTGCCAGTAGT-3′) and anti-sense (5′-TGATCCTTCCGCAGGTTCACCTA-3′) primers (Yoshikawa *et al*. 2000) was used to amplify almost the entire SSUrRNA genes of *Blastocystis*. Using the pair of primers and the genomic DNA of each isolate as template with standard PCR conditions, a 1·6 kb product was amplified, purified, and cloned into a pT 7 plasmid (Novagen, Madison, WI, USA). Nucleotide sequence was determined by a double-stranded dideoxy sequencing method using an ABI Genetic Analyzer PRISM Model 310 (Perkin-Elmer Cetus, Norwalk, CT, USA) with a Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Cetus, Norwalk, CT, USA). If multiple clones were available from a single isolate, these clones (up to 5) were sequenced. All clones in this study were sequenced with both strands by primer walking.

Sequence alignment and phylogenetic reconstruction analysis

The sequences obtained in this work were aligned manually with the previously published SSUrRNA sequences of *Blastocystis* and of *Proteromonas lacertae*, according to the secondary structure-based SSUrRNA alignment constructed by Van de Peer *et al*. (http://rrna.uia.ac.be/) (Van de Peer & De Wachter, 1997). Unambiguously aligned 1378 positions were selected and used for calculation of pairwise sequence identity (or distance) and for phylogenetic reconstruction analysis.

The maximum likelihood (ML) method was used to infer phylogenetic relationships among sequences. The analysis was performed with the NUCML program in the MOLPHY (version 2.3) program package (Adachi & Hasegawa, 1996). The HKY85 model of nucleotide substitutions (Hasegawa, Kishino & Yano, 1985) was assumed in the analysis, because the model better approximated the data set than did more simple models. The transition/transversion ratio for the HKY85 model was estimated from the data set under analysis. Since the number of operational taxonomic units (OTUs) to be analysed was large, we selected the best tree with the highest log-likelihood value only among the alternative trees that were produced by the heuristic search methods. The local rearrangement option (-R) and the quick-add OTU option (-q -n2000) of the NUCML program were used for the heuristic search. Several different trees including the neighbour joining (NJ) tree were used as initial trees in the analysis with the -R option. Bootstrap proportion (BP) for each node of the best tree was calculated by applying the resampling estimated log-likelihood (RELL) method (Kishino, Miyata & Hasegawa, 1990) with 10 000 resamplings to the alternative trees mentioned above.

RESULTS AND DISCUSSION

Comparison of the Blastocystis *SSUrRNA sequences*

Each of the sequences obtained in this work showed the highest similarity with SSUrRNA sequences of the other *Blastocystis* isolates reported so far (Silberman *et al*. 1996; Arisue *et al*. 2002). Sequence heterogeneity was found among different clones of several isolates (Table 1). The number of positions with difference among different clones was shown for each isolate. Clones from the isolate QQ98-4 showed the highest sequence heterogeneity. Pairwise differences for unambiguously aligned 1378 positions ranged between 0% and 7% among all pairs of the 40 sequences of *Blastocystis* isolates including the ones that have already been published in the database (data not shown). The maximum value of the difference, 7·0%, was found between the 2 isolates, HJ97-2 (Clone 'a' or 'b') and HT98-1. In order to compare the pairwise difference values among distantly related *Blastocystis* isolates with those among other eukaryotic taxa, slightly fewer positions (1297 positions) were selected from the alignment including fungi and green plants and were used for calculation. The difference between the isolates HJ97-2 (Clone 'a' or 'b') and HV93-1 (Clone 'b'), HV93-13 (Clone 'a'), HJ96A-26 (Clone 'b') or HT98-1 was 5·2%, and it was the largest among all the pairs within *Blastocystis*. It was comparable with the differences between *Saccharomyces cerevisiae* and *Pneumocystis carinii* (4·2%) and between *Zea mays* and *Chlorella kessleri* (5·8%). Even if the comparison was restricted

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Table 1. *Blastocystis* isolates and their SSUrRNA sequences used in this work

(For the strain QQ98-4, the number of different positions among clones was counted only within the regions excluding gaps. Since the CK86-1 and NIH-1295-1 had been identified as zoonotic strains (Yoshikawa *et al*. 1996; Clark, 1997), they were designated as *B. hominis*.)

Isolates	Species	Hosts	Countries	References	Clones with different sequences	Numbers of different positions	Accession numbers
HV93-13	*B. hominis*	Human	Japan	Yoshikawa *et al*. (1998)	a,b	2	AB070986, AB091233
HJ96-1	*B. hominis*	Human	Japan	Present study		—	AB070987
HJ96A-26	*B. hominis*	Human	Japan	Yoshikawa *et al*. (2000)	a,b,c	3	AB070988, AB091234-5
HJ96A-29	*B. hominis*	Human	Japan	Yoshikawa *et al*. (2000)		—	AB070989
HJ96AS-1	*B. hominis*	Human	Japan	Yoshikawa *et al*. (2000)	a,b,c,d	15	AB070990, AB091236-8
HJ97-2	*B. hominis*	Human	Japan	Present study	a,b	11	AB070991, AB091239
HT98-1	*B. hominis*	Human	Thailand	Present study		—	AB070992
B	*B. hominis*	Human	Singapore	Ho *et al*. (1994)		—	AF408427
CK86-1	*B. hominis*	Chicken	Japan	Yoshikawa *et al*. (1996)	a,b,c	9	AB070993, AB091240-1
CK92-4	*Blastocystis* sp.	Chicken	Japan	Present study	a,b	5	AB070994, AB091242
QQ93-3	*Blastocystis* sp.	Japanese quail	Japan	Yoshikawa *et al*. (1998)	a,b	2	AB070995, AB091243
QQ98-4	*Blastocystis* sp.	Japanese quail	Japan	Present study	a,b,c,d,e	36	AB070996, AB091244-7
JM92-2	*Blastocystis* sp.	Japanese monkey	Japan	Yoshikawa *et al*. (1998)		—	AB070997
SY94-3	*Blastocystis* sp.	Pig	Japan	Yoshikawa *et al*. (1998)	a,b	1	AB070998, AB091248
SY94-7	*Blastocystis* sp.	Pig	Japan	Present study	a,b,c	6	AB070999, AB091249-50
RN94-9	*Blastocystis* sp.	Brown Norway rat	Japan	Yoshikawa *et al*. (1998)	a,b	2	AB071000, AB091251
Nand II	*B. hominis*	Human	USA	ATCC 50177		—	U51151
HE87-1	*B. hominis*	Human	Japan	Yoshikawa *et al*. (1996)	a,b	6	AB023499, AB023578
NIH-1295-1	*B. hominis*	Guinea-pig	USA	ATCC 50578		—	U51152
—	*Blastocystis* sp.	Guinea-pig	USA	Leipe *et al*. (1996)		—	U26177

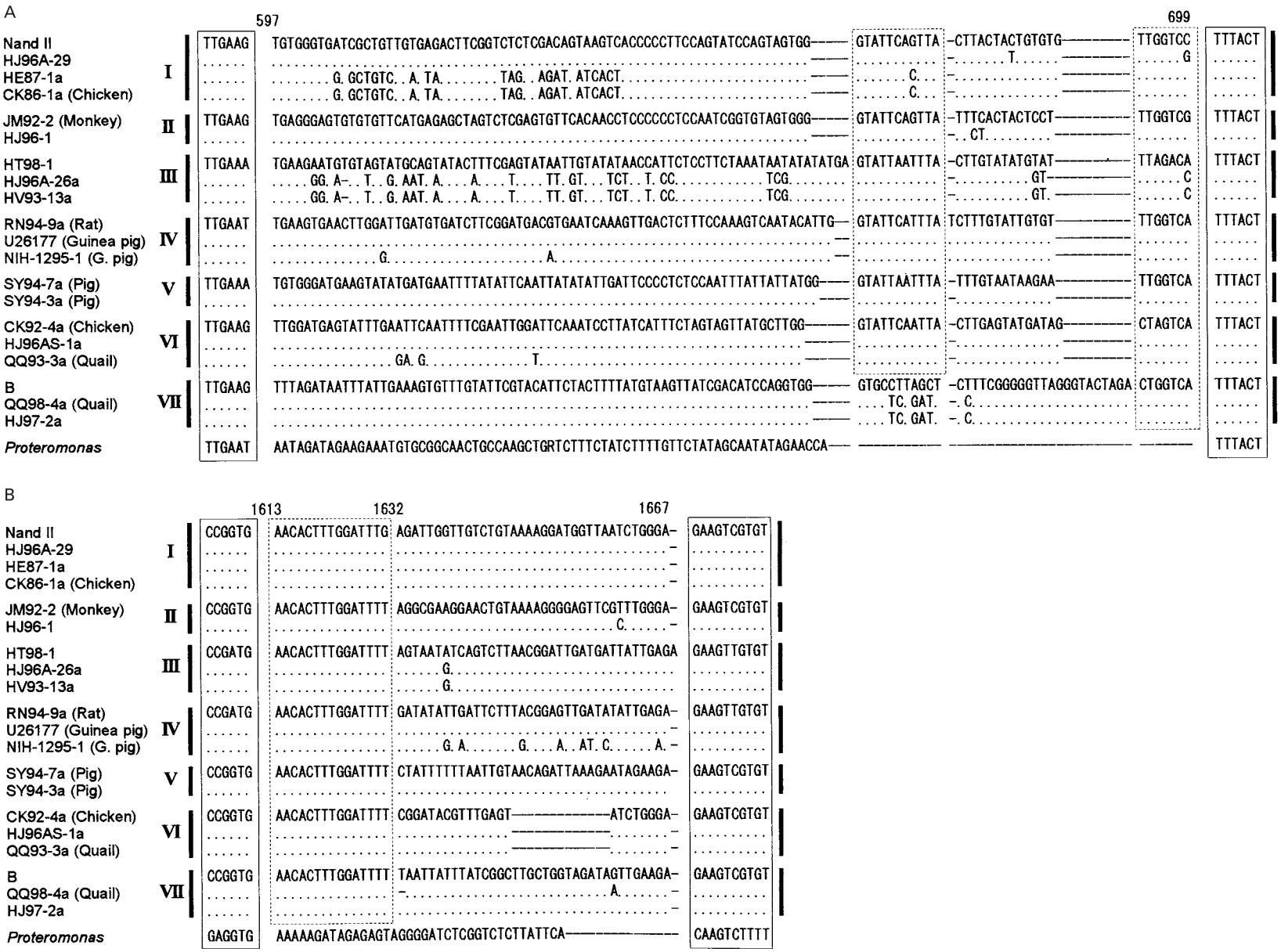

Fig. 1. For legend see opposite page.

only to the 629 positions from the universal core region (Van de Peer *et al*. 2000) that should be very conservative, the maximum difference among the *Blastocystis* isolates was shown to be 3·6% between B, CK92-4 (Clone 'b'), or HJ96AS-1 (Clone 'c' or 'd') and HT98-1 or HV93-13 (Clone 'a' or 'b'). The value was also comparable with the differences between *S. cerevisiae* and *P. carinii* (3·2%) and between *Z. mays* and *C. kessleri* (3·8%). Judging from these values, the *Blastocystis* isolates with a remarkable pairwise difference in the SSUrRNA gene could be regarded as different species. If the high genetic diversity existed within an intra-species level, the evolutionary rate of *Blastocystis* SSUrRNA gene might be extremely accelerated.

The alignment demonstrated clearly that the major part of the SSUrRNA sequences are conserved among all different *Blastocystis* isolates currently available. However, several regions were found to be divergent among these isolates. Two regions shown by partial alignments in Fig. 1 were especially remarkable in sequence heterogeneity. These were located within the highly variable regions of the putative SSUrRNA secondary structure elements, corresponding respectively to the helices E23 (Fig. 1A) and 49 (Fig. 1B) of the *Homo sapiens* sequence (Van de Peer *et al*. 2000). Based on the global alignment including these 2 regions, we tentatively divided the SSUrRNA sequences into 7 groups, I through VII, as shown in Fig. 1. Since these regions were highly variable among the distinct groups, specific diagnostic primers for each group could be developed based on the sequence information of these regions.

Phylogeny of the Blastocystis *SSUrRNA sequences*

The phylogenetic tree of the SSUrRNA sequences of *Blastocystis* isolates was inferred by the ML method using the sequence of *Proteromonas lacertae* as the outgroup for rooting the tree. The transition/transversion ratio estimated from the data set was 2·545. The best tree (Fig. 2) revealed the presence of 7 different monophyletic clades, I through VII, each of these with 100% bootstrap support. No clear resolution was detected for the branching order within each of the 7 clades. Estimated internal branch lengths within each clade were extremely short. Pairwise distances of the sequences among each clade were estimated to be less than 0·01 substitutions/site. These clades corresponded exactly to the groups, I through VII, in the partial alignments in Fig. 1, indicating that the sequence patterns of the variable regions correlated well with the phylogenetic tree-based grouping, which was inferred by the positions excluding the variable regions. Among the 7 groups, 3 close relationships, I and II, III and IV, and VI and VII, were reconstructed with 100% bootstrap support. However, the branching order among the 4 groups including these 3 higher order clades, I/II, III/IV, V and VI/VII was not clearly resolved, and thus the earliest branch of the *Blastocystis* clade was uncertain. Although there remains uncertainty in the SSUrRNA tree in Fig. 2, the tree far better resolved the relationship among different *Blastocystis* isolates than did the EF-1α tree previously reported (Ho *et al*. 2000). This is probably because the amount of amino acid replacement in the EF-1α data set was not enough for resolving them. In order to obtain more clear resolution on the uncertain part of the tree in Fig. 2, further sequence data will be needed especially from genes that have faster evolutionary rates than that of the SSUrRNA.

All of the heterogeneous sequences from each single isolate were located within an independent clade (I, III, IV, V, VI or VII) with no exception and the sequence variation among different clones for each isolate is not so remarkable (Fig. 2), suggesting that the heterogeneity might be due to the presence of multi-copy genes of SSUrRNA in the *Blastocystis* genome. However, we cannot entirely exclude a possibility that mixed infection caused the heterogeneity. It will be necessary to explore to what extent the sequence heterogeneity exists among different copy genes of the *Blastocystis* SSUrRNA in order to clearly settle this issue.

Based on the restriction fragment length polymorphism (RFLP) analysis of SSUrRNA (riboprinting), Clark (1997) inferred a phylogenetic tree among 7 distinct riboprint patterns (ribodemes) of *B. hominis*. Based on the examination of the 40 SSUrRNA sequences, searching for restriction sites of the enzymes that Clark (1997) used, we found that *B. hominis* (Nand II), *Blastocystis* sp. (JM92-2), *B. hominis* (HJ96A-26a, HJ96A-26b, and HV93-13b),

Fig. 1. Alignments of the highly variable regions of the SSUrRNA sequences of *Blastocystis* isolates and *Proteromonas lacertae*. Partial alignments (A) and (B) correspond to the putative secondary structure elements of the *Homo sapiens* sequence, E23 and 49, respectively. Only names of the isolates (or clones) are shown for *Blastocystis* sequences. When the isolates are from animals except for humans, the host names are shown in parentheses. When different clones from a single isolate are available, only one sequence (clone 'a') is shown. I through VII are distinct groups with different sequence patterns. Complete nucleotide sequences are shown only for the isolates on the top of each group and for *Proteromonas*. For each group, dot (.) denotes a base that is the same as the one in the upper line, while a base different from the one in the upper line is shown by its character state. Hyphen (-) denotes a gap. Signatures conserved between *Blastocystis* and *Proteromonas* are shown by boxes with solid lines, and those among most of the distinct groups of *Blastocystis* isolates by boxes with dotted lines. Residual numbers of the SSUrRNA of the Nand II strain are indicated over the alignments.

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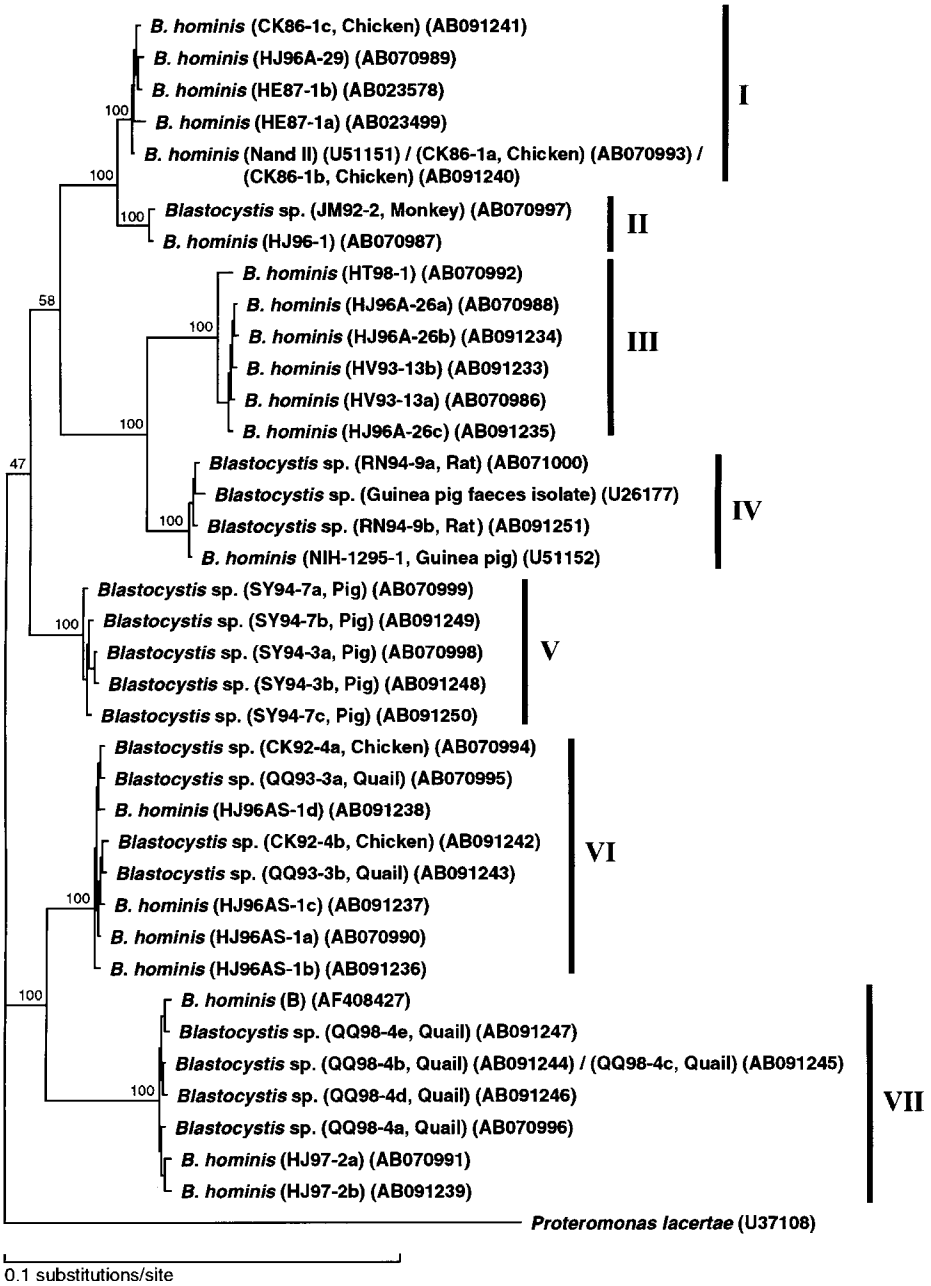

Fig. 2. Phylogenetic tree of the SSUrRNA sequences of *Blastocystis* isolates with *Proteromonas lacertae* as the outgroup. The best tree finally selected by the maximum likelihood analysis is shown. Bootstrap proportions (%) are attached to the internal branches except for the very short ones within each group. Names of the isolates (clones) and accession numbers in the database are shown in parentheses. The horizontal length of each branch is proportional to the estimated number of substitutions. In total 1378 unambiguously aligned positions were selected and used. Alignment is available from the authors on request.

and *B. hominis* (NIH-1295-1) correspond to the ribodemes, RD1, RD6, RD2, and RD3, respectively. No ribodeme patterns of Clark (1997) were found to correspond to the other isolates, suggesting that more ribodemes would be needed for describing the diversity of *Blastocystis* isolates used in this work. The same sequences provide exactly the same ribodemes but, in contrast, the same ribodemes do not always guarantee that their sequences are the same. Therefore, we cannot simply compare the tree in Fig. 2 based on the sequence data with Clark's (1997) RFLP-based tree. However, the location in our tree of the 6 sequences mentioned above was comparable with that in Clark's (1997) tree.

Using a different approach, the random amplified polymorphic DNA (RAPD) analysis, 4 subtypes of *B. hominis* were also proposed by Yoshikawa *et al.* (1998, 2000). In their RAPD analysis, diagnostic primer sets SB82 and SB83 were developed on the basis of the target sequence of the Nand II strain (Yoshikawa *et al.* 1998). Both of these primer sets amplified specific bands for the isolates, Nand II,

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CK86-1, and HE87-1 (Yoshikawa *et al*. 1998), while the SB83 amplified minor bands also for the isolate, HJ96-A29 (Yoshikawa *et al*. 2000). Neither the SB82 nor the SB83 amplified any bands for the isolates, JM92-2 and HJ96-1, and for other isolates. These findings are comparable with the present SSUrRNA sequence-based grouping. The group I almost corresponds to the subtype 1 in the RAPD analysis. On the other hand, the groups III, VI, and VII contain the isolates belonging to the subtypes 3, 4, and 2, respectively.

The sequences of the 1378 positions were exactly the same between the Nand II strain of *B. hominis* and the clone CK86-1a or CK86-1b of the isolate CK86-1 from chicken, which was already identified as a zoonotic strain (Yoshikawa *et al*. 1996, 1998). Although no sequence information was available, 3 human isolates had riboprints (RD3) identical to that of the guinea-pig isolate (NIH-1295-1) that belongs to the group IV in the present analysis (Clark, 1997). The finding indicates that the group IV also contains human isolates with zoonotic origin. At present, the groups I, II, IV, VI, and VII comprised the isolates both from humans (*B. hominis*) and from other animals. The relationships among hosts were not in agreement with the genuine phylogeny of the animals in any part of the tree. As demonstrated in Figs 1 and 2, very closely related SSUrRNA sequences coexist both in humans and in other animals, strongly suggesting that *Blastocystis* is not host-specific but may be cross-infective among various hosts.

Extensive genetic diversity of Blastocystis

Immunological analyses (Müller, 1994; König & Müller, 1997), isoenzyme analyses (Mansour *et al*. 1995), RAPD analyses (Yoshikawa *et al*. 1996, 1998, 2000), and RFLP analyses of the SSUrRNA (riboprinting) (Böhm-Gloning *et al*. 1997; Clark, 1997; Hoevers *et al*. 2000; Snowden *et al*. 2000; Yoshikawa *et al*. 2000; Kaneda *et al*. 2001) and of EF-1α (Ho *et al*. 2001) have shown that extensive genetic diversity exists among *Blastocystis* isolates from humans and other animals. In this work, we have also demonstrated the presence of remarkable genetic diversity based on the sequence comparison of the SSUrRNA genes. The present analysis could quantitatively evaluate the genetic distances among different *Blastocystis* isolates. These lines of evidence imply that the extensive genetic diversity is a major cause of the uncertainty of the role of *Blastocystis* in disease. It is probable that a restricted number of isolates are responsible for the pathogenicity of *Blastocystis*. Re-examination of the role of *Blastocystis* in disease would be needed to take such genetic diversity into consideration on the basis of the phylogenetic framework.

Blastocystis isolates have been found in various vertebrate hosts, including rodents, pigs, cows, birds, reptiles, frogs, and some of these have been regarded as zoonotic strains by the evidence of genotypic homology to the human isolates (*B. hominis*) (Yoshikawa *et al*. 1996, 1998; Clark, 1997; Snowden *et al*. 2000; Abe *et al*. 2002). The present SSUrRNA analysis also suggested the probable zoonotic origin of several isolates (Fig. 2). As the zoonotic potential of *Blastocystis* has been gradually confirmed, the general method for species designation of *Blastocystis* becomes problematic. For example, *Blastocystis* isolates from birds were named on the basis of the host origin and *in vitro* culture characteristics (Belova & Kostenko, 1990; Belova, 1991, 1992). The isolates from reptiles were named in the similar way with additional information on the karyotypic patterns (Teow *et al*. 1991; Singh *et al*. 1996). In addition, 2 isolates, *B. lemuri* and *B. ratti*, from mammalian hosts, except for humans, were also given host oriented names (Chen *et al*. 1997; Krylov & Belova, 1997). Although we do not have enough information currently on the genetic diversity of these isolates from various animal hosts, it is likely that several of these are genetically identical to, or very closely related to, the various isolates from humans (*B. hominis*), as suggested by the cross-infective nature of *Blastocystis*. If such zoonotic potential was usually the case, the host name-based identification system of new species would produce a confusing situation in the near future. As far as *Blastocystis* isolates cannot morphologically be distinguishable, classification of these isolates should be carried out using phylogenetic information. Sequencing analysis of various isolates with different genotypes for SSUrRNA and other genes can further provide information on phylogenetic relationships and evolution of *Blastocystis* spp. A standardized method of species identification should be settled based on the information in the future.

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