Analysis of the ITS region and partial ssu and lsu rRNA genes of *Blastocystis* and *Proteromonas lacertae*

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SUMMARY

Blastocystis is a common single-celled enteric parasite found in a large variety of hosts. Recent molecular analysis supports the concept that this eukaryotic organism is a stramenopile most closely related to *Proteromonas lacertae*, a parasite of reptiles. In this study, the internal transcribed spacer region, partial small subunit rRNA and large subunit rRNA genes from 7 *Blastocystis* isolates (5 human, 1 pig and 1 sheep), and a *Proteromonas lacertae* isolate were amplified by PCR, cloned and sequenced. *Blastocystis* was found to be a typical eukaryote with both ITS1 and ITS2 regions present. Phylogenetic analysis based on the entire PCR amplicon revealed that the *Blastocystis* isolates did not segregate according to host or geographic origin. The highest sequence identities with the conserved *Blastocystis* 5·8S rDNA sequence were with the stramenopiles *Fibrocapsa japonica*, *Chattonella marina*, *Cylindrotheca closterium* and *Hyphochytrium catenoides*. The most parsimonious tree based on the 5·8S rDNA sequence from *P. lacertae*, 11 other stramenopiles, 2 fungi, 3 algae and 3 alveolates showed *Blastocystis* positioned within the stramenopiles, with *P. lacertae* as its closest relative. This work therefore supports the hypothesis that *Blastocystis* is most closely related to *P. lacertae*, and that it should be regarded as an unusual stramenopile.

Key words: *Blastocystis*, *Proteromonas lacertae*, ITS, 5·8S, ssu rRNA, lsu rRNA, stramenopile, molecular phylogeny.

INTRODUCTION

Blastocystis is a poorly understood extracellular, single-celled eukaryotic organism frequently found in the gastrointestinal tract of humans and other mammalian hosts (Zierdt, 1991; Stenzel & Boreham, 1996). Currently, *Blastocystis hominis* is the scientific name used for all human isolates (Boreham & Stenzel, 1998; Yoshikawa *et al*. 1998; Tan, Singh & Yap, 2002). Controversy exists as to whether different species/strains/genotypes of *Blastocystis* are pathogenic to humans (reviewed by Tan *et al*. 2002). The parasite has also been found in birds, reptiles and arthropods (Abe *et al*. 2002; reviewed by Tan *et al*. 2002). In addition, several recent publications have concluded that many isolates are zoonotic or have zoonotic potential (Arisue, Hashimoto & Yoshikawa, 2003; Noël *et al*. 2003, 2005; Abe, 2004; Yoshikawa *et al*. 2004).

Multiple studies have shown considerable heterogeneity among *Blastocystis* isolates from a wide range of hosts and geographical locations utilizing a variety of morphological, molecular and biochemical techniques (reviewed by Tan *et al*. 2002; Arisue *et al*. 2003; Noël *et al*. 2003, 2005). Molecular data predominantly utilizing the small subunit ribosomal (ssu rRNA) gene, have phylogenetically positioned *Blastocystis* in the Kingdom Chromista within the stramenopiles (Silberman *et al*. 1996; Van de Peer *et al*. 2000; Arisue *et al*. 2002; Noël *et al*. 2003). Stramenopiles (also referred to as heterokonts) comprise species that either possess tripartite tubular hairs (also known as tripartite 'retronemes', Cavalier-Smith, 1998), or species that have been derived from such organisms (Patterson, 1989; Cavalier-Smith, 1998). Based on the ssu rRNA gene sequence, the stramenopile *Proteromonas lacertae* has been found to be the closest relative to *Blastocystis* to date (Silberman *et al*. 1996; Arisue *et al*. 2002; Noël *et al*. 2003). However, other phylogenetic studies using molecular data have placed *Blastocystis* within the sarcomastigotes (Ho *et al*. 2000), or have been unable to resolve the organism's phylogenetic positioning (Johnson *et al*. 1989; Nakamura *et al*. 1996).

The internal transcribed spacer (ITS) region of ribosomal DNA, defined as the unit containing the ITS1 spacer, 5·8S rRNA gene and ITS2 spacer, has not been investigated in *Blastocystis* or *P. lacertae*. The ITS1 and ITS2 have been shown to vary considerably in length and sequence and have been useful for discriminating interspecific and intraspecific genetic variation from a wide variety of organisms, including bacteria, plants and vertebrates (Allard & Honeycutt, 1991; Baldwin, 1992; McLaughlin *et al*. 1993). In particular, the ITS regions have been shown to be useful for strain and species identification in eukaryotic parasites (Collins & Allsopp, 1999; Pélandakis, Serre & Pernin, 2000;

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Table 1. *Blastocystis* isolates from culture used for molecular analysis

Isolate	Host	ATCC	Origin
Nand II	Human	50177	Maryland, USA
Otz	Human	50588	Maryland, USA
Jns	Human	50589	Maryland, USA
Jdr	Human	50587	Maryland, USA
Rmb 1069	Human	N/A	Papua New Guinea
P 45	Pig	N/A	Texas, USA
Sh 779	Sheep	N/A	Texas, USA

Som, Asam & Bhattacharya, 2000; Oliver *et al*. 2001). The aim of this study was to sequence the ITS region from 7 *Blastocystis* isolates and 1 *P. lacertae* isolate, and to investigate the extent of sequence heterogeneity among the *Blastocystis* isolates, and between *Blastocystis* and *P. lacertae*.

MATERIALS AND METHODS

Parasite isolate origin and culture

The 7 *Blastocystis* isolates were obtained from various sources (Table 1). The 4 human axenic isolates (Nand II, Otz, Jns and Jdr) were purchased commercially (ATCC, Manassas, VA, USA). The xenic human isolate (Rmb 1069) was collected from Papua New Guinea during a parasite survey (Ashford & Atkinson, 1992), and the 2 xenic *Blastocystis* animal isolates (Sh 779 and P 45) originated from Texas, USA. All *Blastocystis* isolates were grown in culture as previously described (Hoevers *et al*. 2000; Snowden *et al*. 2000). The *Proteromonas lacertae* isolate (ATCC 30270; Manassas, VA, USA) originated from a sand lizard (Prague, Czech Republic, 1973), and organisms were maintained in culture following recommended protocols. *Blastocystis* and *P. lacertae* organisms from culture were concentrated by centrifugation and purified by density-gradient centrifugation to decrease bacterial concentrations in xenic isolates if necessary (Hoevers *et al*. 2000). The gDNA extraction methodology for all isolates was achieved as previously described (Hoevers *et al*. 2000).

Polymerase chain reaction (*PCR*)

The forward primer BL-ITSF (5′-CGCACCTA CCGATTGAATG-3′) was designed in a highly conserved area, 157 bases from the 3′ end of the ssu rRNA gene based on available *Blastocystis* ssu rRNA gene sequences in GenBank (NCBI, http://www.ncbi.nlm.nih.gov/). This primer was used for PCR amplification of all *Blastocystis* and *P. lacertae* isolates. The previously described primer ITS4 was used as a reverse primer for PCR amplification of *Blastocystis* isolates (White *et al*. 1990). The reverse primer used for *P. lacertae* PCR amplification, BL28SR (5′-CAATTCAGCAGGTCTTCTTAC-3′), was designed based on the conserved sequence found within the partial lsu rRNA gene of all *Blastocystis* isolates from this current study. All primers were synthesized by Genosys Biotechnologies (The Woodlands, TX, USA).

Amplification by PCR was achieved with the GeneAmp kit (GeneAmp PCR Core Reagents, N808-0009; Perkin-Elmer Cetus, Roche Molecular Systems, NJ, USA) following the manufacturer's instructions using 50 μl reaction volumes and 50 ng genomic DNA. The PCR reactions were run in a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA) using the following temperature profile: hot start 95 °C, 1 min; denaturing 93 °C, 1 min; annealing 52 °C, 1 min; extension 72 °C, 1 min. After 30 cycles, a final extension step was added for 20 min at 72 °C. Amplicons were detected on 1% agarose gels, and samples purified using spin columns (Bio-Rad, Hercules, CA, USA).

Cloning and sequencing

The purified PCR amplicons were cloned using the pCR2.1-TOPO vector (TOPO TA cloning kit, InVitrogen, Carlsbad, CA, USA), and transformants were selected from each isolate and screened by PCR following the manufacturer's instructions. Positive transformants were grown overnight in Miller's broth with ampicillin (Sigma, St Louis, MO, USA), and plasmid DNA was purified using a QIAPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA). Recombinant plasmids were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in ABI Prism Models 373a or 377 automated sequencers with version 3.2 or 3.3 software. Three clones of each PCR product from at least 2 separate PCR reactions were sequenced in both directions using the vector primers T7 and M13R.

Sequence analysis

All sequences (excluding vector and primer sites) were subjected to advanced BLAST searches (Altschul *et al*. 1990) in the GenBank database to confirm isolates as *Blastocystis* or *P. lacertae*. *Blastocystis* sequences were compared using Genestream software (Pearson, 1997), and all resulting sequences were aligned using the software program Sequencer (version 3.1.2., Gene Codes Corp., Ann Arbor, MI, USA), and later refined manually.

Phylogenetic analyses using maximum parsimony (MP) and maximum likelihood (ML) methods were performed using PAUP* 4.0b10 software (Swofford, 1996). Gaps were treated as missing, and support for

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Table 2. Variations in nucleotide length* and GC% content (in parenthesis) of the sequenced genes with boundaries putatively identified among the 7 *Blastocystis* and 1 *Proteromonas lacertae* isolates

Isolate	ITS PCR amplicon	ssu rRNA (partial)	ITS 1	5·8S rRNA	ITS2	Accession number
Nand II	553 (43·8)	129 (41·8)	96 (46·9)	135 (42·2)	138 (44·9)	AY125913
Otz	585 (41·0)	129 (41·8)	129 (49·6)	135 (42·2)	137 (42·3)	AY125914
Jns	550 (43·1)	129 (41·8)	94 (45·7)	135 (42·2)	137 (43·1)	AY125915
Jdr	565 (43·2)	129 (41·8)	108 (47·2)	135 (42·2)	138 (42·0)	AY125916
Rmb 1069	568 (42·8)	130 (38·5)	111 (45·9)	135 (42·2)	137 (44·5)	AY125917
P 45	553 (42·3)	129 (34·9)	96 (47·9)	135 (42·2)	138 (44·9)	AY125918
Sh 779	553 (44·1)	129 (42·6)	96 (47·9)	135 (42·2)	138 (44·9)	AY125919
P. lacertae	654 (26·8)	142* (31·0)	160 (26·3)	154 (37·7)	165 (10·9)	AY224080

* Including primer sites.

the most parsimonious tree(s) was tested by bootstrapping (Felsenstein, 1985). In addition, the phylogenetic signal was tested using the permutation-tail-probability (PTP) test (Faith, 1991). For ML-based analysis several DNA evolution models were tested to select the best fitting parameters (Posada & Crandall, 1998), and the resulting trees were compared to those obtained by MP.

RESULTS

PCR amplicons and BLAST searches

Three clones were successfully produced and sequenced for each *Blastocystis* or *P. lacertae* isolate. The PCR amplicon for all *Blastocystis* isolates ranged from 550 to 585 bp in length, and that of *P. lacertae* was 654 bp in length (Table 2). All 3 cloned sequences from each isolate showed 100% sequence identity and therefore each isolate is represented by a single consensus sequence rather than sequences from each of the three clones. The BLAST searches confirmed each sequence as *Blastocystis* or *P. lacertae* based on known sequences at the 3′ end ssu rRNA gene. Sequences excluding primer sites were deposited in the GenBank database (Table 2).

Sequence comparisons and alignment

Using the GenBank BLAST results and comparisons to designated regions according to Katiyar, Visvesvara & Edlind (1995), a putative determination of the 8 consensus *Blastocystis* and *P. lacertae* ITS1, 5·8S, ITS2 and 5′ lsu rRNA boundaries were made (Fig. 1). Sequence similarities among the *Blastocystis* isolates varied greatly depending on which regions were analysed. Overall, the sequences were found to be AT rich regardless of gene region or isolate or genus (Table 2).

Using Genestream software, the entire ITS sequence (excluding primer binding sites) was compared among the *Blastocystis* isolates, resulting in sequence similarities ranging from 96·5% to 85·7% (data not shown). The ITS1 and ITS2 *Blastocystis* regions varied both in length (94–111 bp and 137–138 bp, respectively) and sequence (45·7–49·6 and 42–44·9 GC % content, respectively) (Table 2, Fig. 1). Reliable alignment using the *P. lacertae* ITS 1 and ITS 2 regions was not possible with the *Blastocystis* sequences, and thus only the partial ssu rRNA, 5·8S and partial lsu rDNA sequence alignments are shown (Fig. 1). No significant sequence similarities were observed between *Blastocystis* or *P. lacertae* ITS1 or ITS2 sequences alone and any GenBank entries.

The 5·8S (135 bp) and partial lsu rRNA (55 bp) *Blastocystis* gene sequences were identical for all 7 isolates (Table 2, Fig. 1). When conducting standard BLAST searches with these sequences, the highest consistent sequence identities (up to 92%) were found with the 5·8S rRNA gene sequences of the stramenopiles *Hyphochytrium catenoides* (X80346, X80345) *Fibrocapsa japonica* (AF112991), *Chattonella marina* (AF137074), *Cylindrotheca closterium* (AF289049) and the fungus, *Acaulospora denticulata* (AJ239115). The 5·8S region of *P. lacertae* was longer (154 bp) than that of *Blastocystis* and showed a 94% sequence similarity to *Blastocystis*. Excluding the *Blastocystis* sequences, the highest sequence similarities (90%) of the *P. lacertae* 5·8S rDNA sequence were found using BLAST to be with fungi of the genera *Glomus* and *Archaeospora*.

The partial ssu rRNA gene varied in GC content among the *Blastocystis* isolates from 34·9% (P 45) to 41·8% (Nand II, Otz, Jns and Jdr) (Table 2). The 4 axenic human isolates obtained from ATCC were 100% similar in their partial ssu rRNA gene sequence, whereas the human Rmb 1069, and the animal P 45 and Sh 779 differed from these by at least 18 nucleotides (Fig. 1). As expected, within the partial ssu rRNA gene, the greatest variation among all *Blastocystis* and *P. lacertae* isolates was found in the V9 region (Fig. 1) as inferred from a combination of sources (Nickrent & Sargent, 1991; http://rrna.uia.ac.be/rrna-cgi/query-cgi.tcl).

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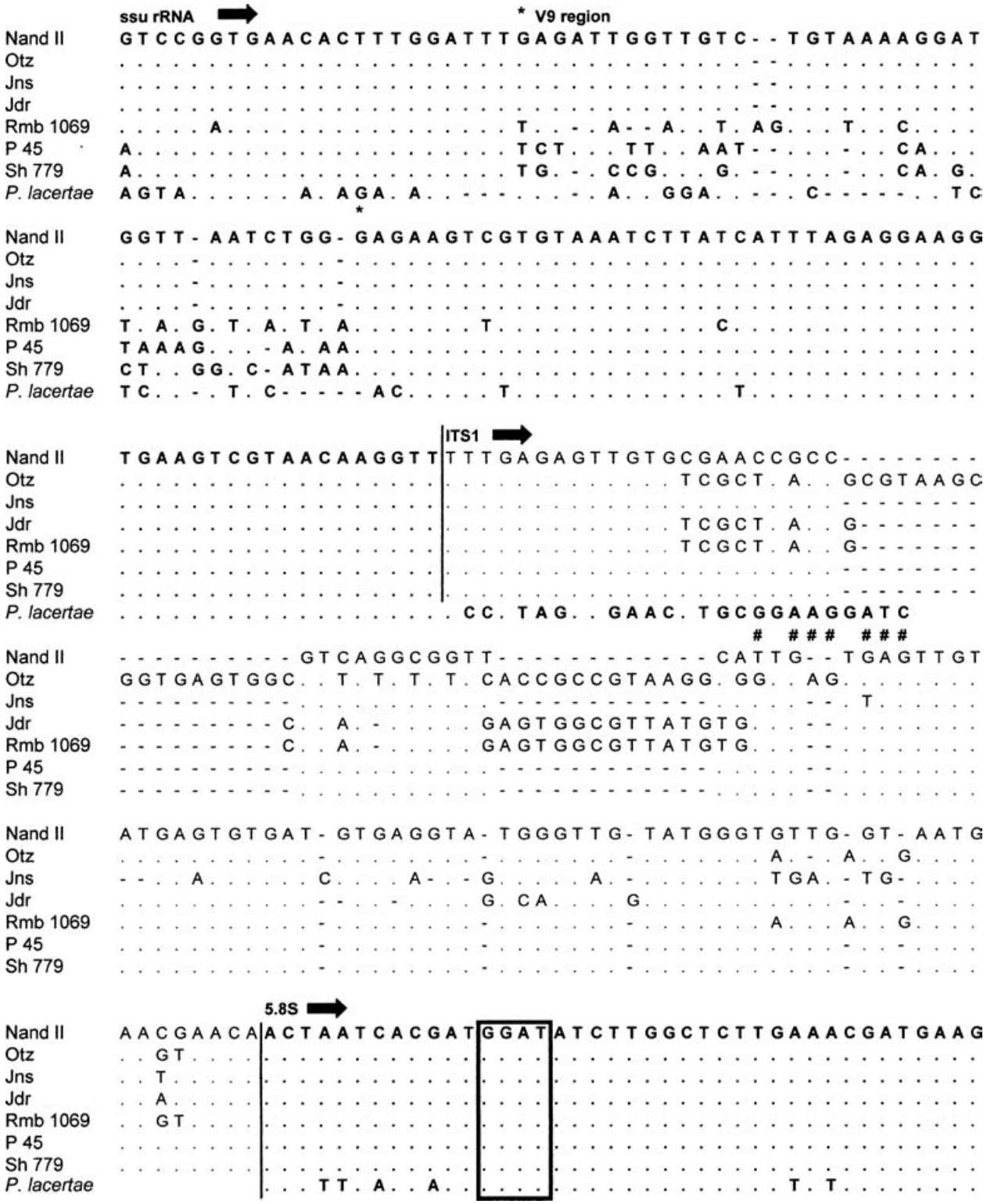

Fig. 1. (*cont.*)

Phylogenetic analyses

Phylogenetic analysis of the partial ssu, partial lsu and 5·8 rDNA including all *Blastocystis* isolates and the *P. lacertae* isolate produced a single tree using MP (stable to successive approximation character weighting, branch and bound search, PTP value = <1/100), in which 2 groups were produced (Fig. 2A, sequence alignment not shown). Nodes supported by bootstrap values <75 were collapsed, and once again, the results obtained by ML analyses supported the relationships and topology found by MP (GTR Model). The human isolate, Rmb 1069, and the pig and sheep isolates were found to have the closest relationships with *P. lacertae* (Fig. 2A).

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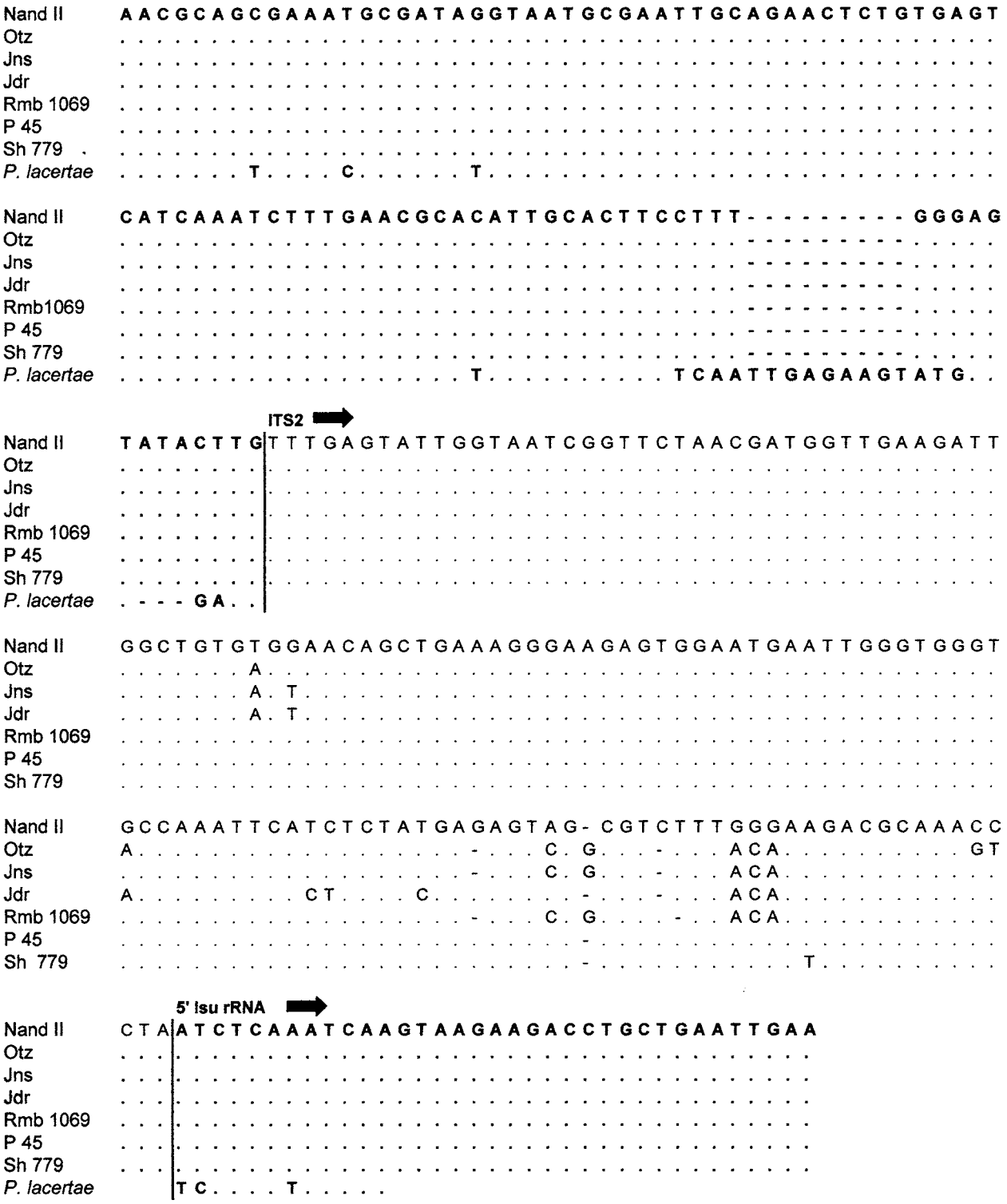

Fig. 1. Alignment of the internal spacer region of 7 *Blastocystis* isolates (Nand II, Otz, Jns, Jdr, Rmb 1069, P 45 and Sh 779) with flanking partial ssu and lsu rDNA excluding primer sites. Boundaries were putatively identified and the 5·8S region, partial ssu and lsu rDNA sequences from *Proteromonas lacertae* are also shown. The partial ssu, lsu rDNA and 5·8S regions are shown in bold. (-) indicates a gap, (.) indicates that the same base occurs as that shown for Nand II. The boxed area in the 5·8S region represents the conserved eukaryotic GGAT motive. The boundaries of the variable region V9 are indicated by asterisks in the partial ssu rDNA region, and the #'s indicate the 7 nucleotides which differ from the *P. lacertae* GenBank ssu rRNA gene sequence.

The ITS 1 regions from the *Blastocystis* isolates were shown to have a strong bias towards unambiguous transversion events compared to base transitions (ti/tv ratio <1) (Fig. 1). This evidence of saturation and the difficulty of accurately aligning the *Blastocystis* ITS1 region sequences led us to construct phylogenetic trees excluding this region. There were 397 genetically informative differences

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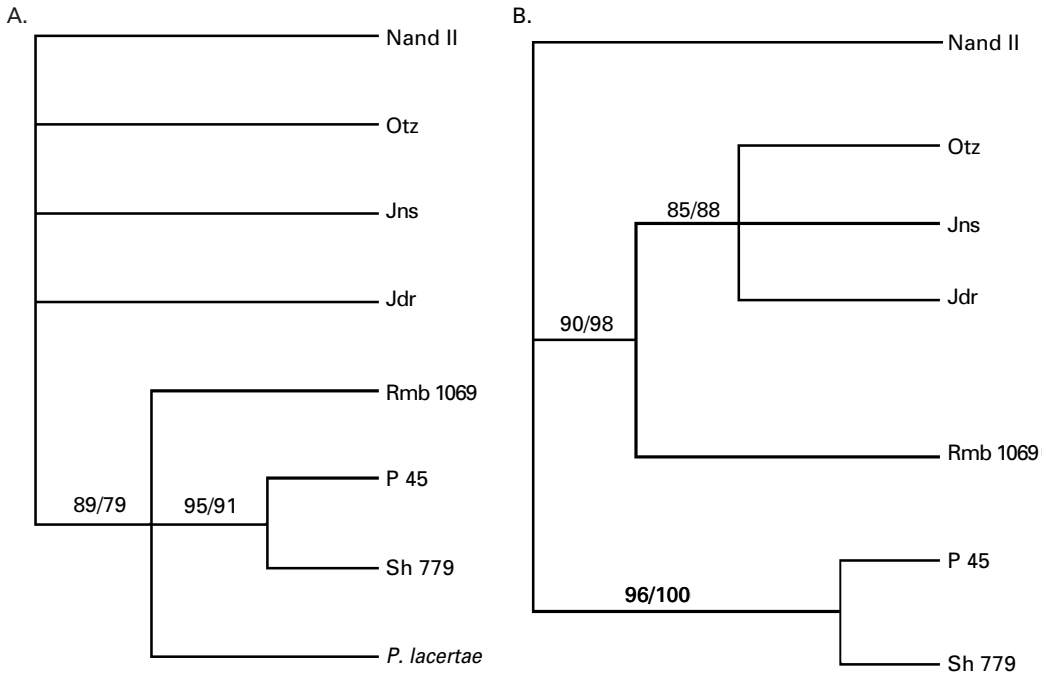

Fig. 2. (A) Unrooted phylogenetic tree with *Blastocystis* and *Proteromonas lacertae* isolates, excluding the ITS 1, ITS 2 regions and missing sequence from *P. lacertae*. This is the most parsimonious tree using maximum parsimony obtained by branch and bound in PAUP* 4.0b10 and stable to successive approximation character weighting. Numerical values above nodes represent bootstrap values (100 reps) first using maximum parsimony, and those obtained by maximum likelihood respectively. Tree length = 71·50, CI = 0·9685, RI = 0·8200, PTP value = < 1/100) (B) Unrooted phylogenetic tree for data set of *Blastocystis* isolates using maximum parsimony-no ITS1. This is the most parsimonious tree obtained by branch and bound in PAUP* 4.0b10 and stable to successive approximation character weighting. Numerical values above nodes represent bootstrap values (100 reps) using maximum likelihood and those obtained by maximum parsimony respectively. Tree length = 44 steps; CI = 0·9607, RI = 0·8871, PTP value = < 1/100.

among the isolates, and only 1 tree was produced stable to successive approximation character re-weighting using MP (branch and bound search). The PTP analysis (100 replications) gave the probability of finding this tree of 44 steps with randomized data as <1/100.

Figure 2B shows the single most parsimonious unrooted tree obtained with high bootstrap values (88–100), for the 7 *Blastocystis* isolates using MP. It depicts close relationships between the 2 animal isolates, P 45 and Sh 779 and the 3 human isolates Otz, Jns and Jdr. The human isolates did not form a single clade or a monophyletic group. The high bootstrap values correlated to high unambigious substitution values (data not shown). Using ML analysis (HKY85 model), with the same dataset, the bootstrap values remained high (85–96) and the topology remained the same (Fig. 2B).

The 5·8S rRNA genes from a variety of eukaryotic organisms available in GenBank were aligned with that of *Blastocystis* and *P. lacertae*. Figure 3 shows a rooted representative phylogenetic tree of their relationships (ML, HKY85 + G + I model, heuristic search). Within the dataset of 21 taxa, there were 116 informative characters and the bootstrap values supporting these relationships ranged from 50 to 90. The *Blastocystis* and *P. lacertae* form a single clade within a monophyletic group made up of stramenopiles, green algae, and fungi.

DISCUSSION

For the first time, the ITS region and a portion of the lsu rRNA gene have been amplified, cloned and sequenced from *Blastocystis*. In addition, new sequence data from 6 isolates (human Jdr, Jns, Otz, Rmb 1069, 1 pig and 1 sheep) from the 3′ end of the ssu rRNA gene were generated. The study also resulted in new ITS1, 5·8S, and incomplete lsu rDNA sequence from the *P. lacertae* isolate. Variation was observed in PCR amplicon length and sequence among the *Blastocystis* isolates. The ITS alignment of the *Blastocystis* ITS1, 5·8S rRNA and ITS2 gene sequences with several stramenopiles and fungi made it possible to putatively predict the start and end of each region in *Blastocystis*. *Blastocystis* was found to be a typical eukaryote since all 3 gene regions (ITS1, 5·8S and ITS2) were present in its rRNA genes. Unsurprisingly, the 5·8S rRNA gene was found to be highly conserved among the *Blastocystis* isolates, and the conserved GGAT motif found in all eukaryotic 5·8S rRNA sequences was present (Katiyar *et al*. 1995).

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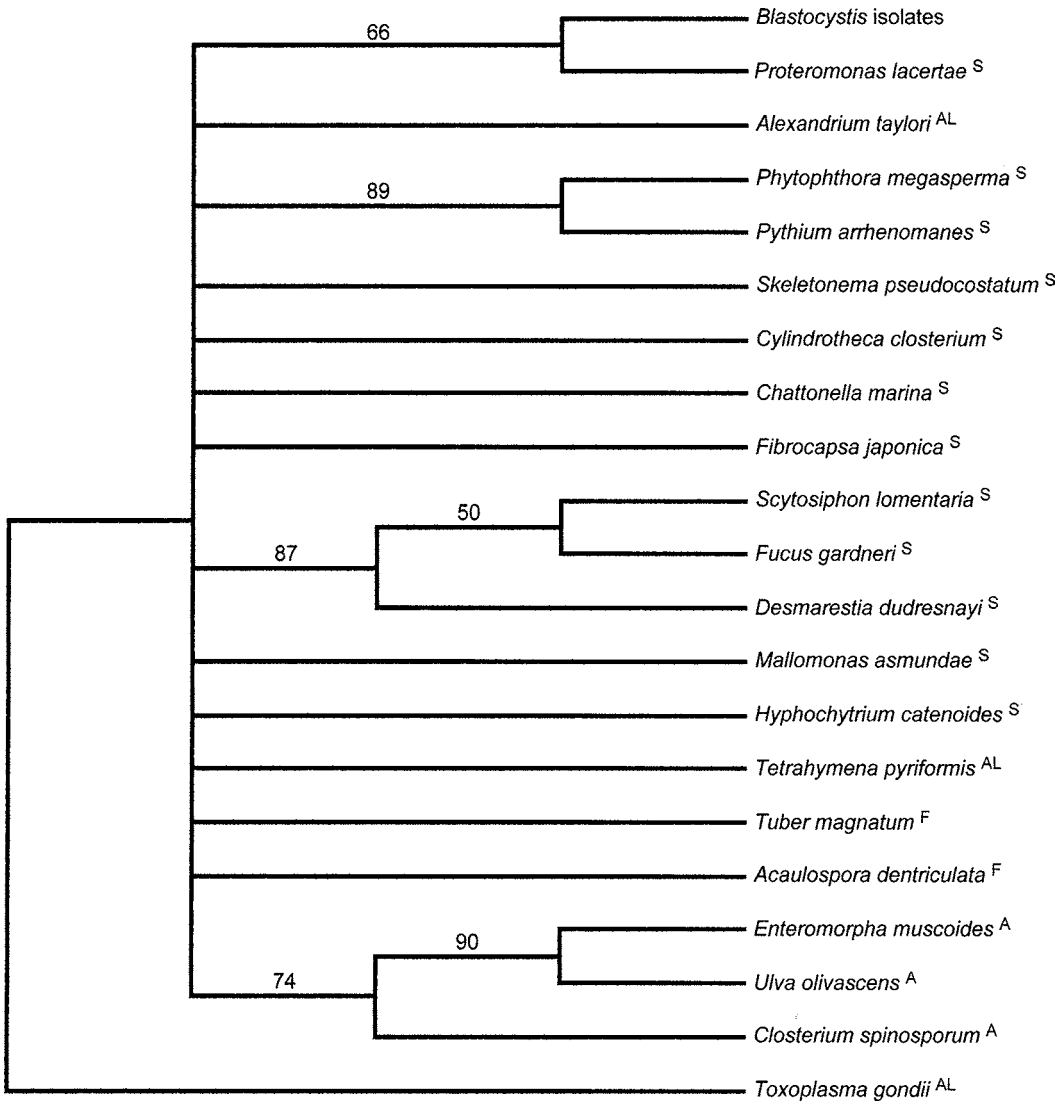

Fig. 3. Rooted phylogenetic tree for *Blastocystis*, *Proteromonas lacertae*, and other selected stramenopiles (^{S}), green algae (^{A}), fungi (^{F}), and alveolates (^{AL}) based on their 5·8S rDNA sequence. The corresponding GenBank Accession numbers are as follows: *Toxoplasma gondii* L255635, *Closterium spinosporum* AF419989, *Ulva olivascens* AJ234322, *Enteromorpha muscoides* AJ234307, *Acaulospora dentriculata* AJ239115, *Tuber magnatum* AJ586308, *Tetrahymena pyriformis* X01533, *Hyphochytrium catenoides* X80346, *Mallomonas asmundae* AF409122, *Desmarestia dudresnayi* AJ439832, *Fucus gardneri* AF102941, *Scytosiphon lomentaria* D16558, *Fibrocapsa japonica* AF112991, *Chattonella marina* AF137074, *Cylindrotheca closterium* AF289049, *Skeletonema pseudocostatum* Y11511, *Pythium arrhenomanes* AJ233444, *Phytophthora megasperma* AY423298, *Alexandrium taylori* AJ291785. This depicted tree was obtained in PAUP* 4.0b10 using maximum likelihood (heuristic search) with settings corresponding to the HKY85 + G + I model. The above nodes represent bootstrap values (100 reps).

The molecular heterogeneity previously reported in several studies of the ssu rDNA was also demonstrated in the ITS regions of our 7 *Blastocystis* isolates since no sequences were identical (reviewed by Tan *et al*. 2002; Arisue *et al*. 2003; Noël *et al*. 2003, 2005; Thathaisong *et al*. 2003). In various publications, analysis of the ssu rRNA genes of more than 40 isolates from human and other mammalian, reptilian and avian hosts has resulted in a variety of suggested subgroupings defined as 'genotypes, clades, clusters, ribodemes or subtypes'. The sequence variation in the ITS region of the *Blastocystis* isolates in this report did not segregate according to host or on isolate geographic origin. This is a similar finding to that of Hoevers *et al*. (2000) and Snowden *et al*. (2000) where isolates were compared by RFLP of the ssu rRNA from multiple human and animal hosts from various geographical locations.

There were no nucleotide differences found among the replicates of cloned *Blastocystis* sequences of the ITS region. In comparison, ssu rRNA gene sequence differences among *Blastocystis* clones are reported by both Arisue *et al*. (2003), and Noël *et al*. (2003). However, it is unknown at this point in time if *Blastocystis* have more than 1 copy of the rRNA gene. In order to overcome this potential problem of mixed

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isolates and to assess rRNA gene copy number, this situation clearly needs to be addressed.

Several publications have used the terminology 'zoonotic' as a designation in various *Blastocystis* studies (Abe, Wu & Yoshikawa, 2003; Noël *et al*. 2003, 2005; Thathaisong *et al*. 2003; Abe, 2004; Yoshikawa *et al*. 2004). Although many human isolates have been found to have similar ssu rRNA gene sequences to isolates originating from various animals, no human isolate has yet been found with the exact sequence as an animal isolate. In addition, no clinical evidence has shown direct transmission of parasites from animals to humans (or visa versa). The small number of experimental infections in rodent models with *Blastocystis* human or swine isolates have shown limited success (Pakandl, 1992; Moe *et al*. 1997). Therefore, we feel that the use of the terminology 'zoonotic isolates' is an overinterpretation of existing data. Further biological and epidemiological studies are needed to confirm or refute the hypothesis that some strains or species of *Blastocystis* show a true zoonotic potential.

The stramenopile, *P. lacertae* (Heterokonta, Slopainia, Proteromnadidae) was selected for further molecular characterization in this study because of the close similarity between ssu rRNA genes of that organism and *Blastocystis* (Leipe *et al*. 1996; Silberman *et al*. 1996; Noël *et al*. 2003). *P. lacertae* is a flagellated enteric parasite found worldwide in reptiles (Barnard & Upton, 1994). The ATCC isolate used in this study originated from the sand lizard, *Lacerta agilis agilis*. The organism has a direct life-cycle with 2 morphological forms, the trophozoites found in the host's large intestine, and the infective cysts which are expelled in the feces. These life-cycle characteristics are somewhat similar to what is known or suspected for *Blastocystis*.

Initial ITS amplification of the *P. lacertae* isolate with primers BL-ITSF and ITS4 was not successful, and therefore the primer BL28SR was designed and used instead. The ITS regions of *P. lacertae* showed close homology with the same regions in the new *Blastocystis* sequences, reinforcing the close taxonomic relationship between these 2 genera. Only one sequence for *Proteromonas* is available in GenBank (Accession number U37108). Our sequencing identified 7 nucleotide differences in the ssu rDNA 3′ end when compared to the GenBank *P. lacertae* sequence. These differences correspond to the primer site used by Leipe *et al*. (1996), and therefore the GenBank sequence is likely to have incorrect nucleotides incorporated from their initial PCR. Both *P. lacertae* cultures originated from the same isolate source, ATCC 30270.

The fact that the highest sequence identities using BLAST of the *Blastocystis* 5·8S rRNA gene sequences were with stramenopiles strengthens the previous reports that place the taxonomic classification of *Blastocystis* within the stramenopiles (Kingdom Chromista) (Silberman *et al*. 1996; Van de Peer *et al*. 2000; Arisue *et al*. 2002; Noël *et al*. 2003). The stramenopiles are a diverse group of organisms made up of algae, fungi and heterotrophs (Van der Auwera & de Wachter, 1998). Interestingly, the 4 stramenopiles that aligned best using BLAST with the *Blastocystis* isolate sequences (excluding *P. lacertae*) are considered heterokont algae (*Fibrocapsa japonica*, *Chattonella marina*, and *Cylindrotheca closterium*) and heterokont fungi (*Hyphochytrium catenoides*). Unfortunately, available stramenopile molecular data remain comparatively limited to ssu rDNA sequences, greatly restricting the phylogenetic analyses that can be conducted, and the confidence of the concluding results based on other portions of ribosomal RNA genes. As more stramenopile genes of different isolates and genera are sequenced and submitted to GenBank, these relationships may resolve, and a clearer understanding of the taxonomic positioning of *Blastocystis* will result.

Ideally when comparing *Blastocystis* isolates, a combination of different genes and morphological characters should be utilized and phylogenetic results compared. The only study to have achieved this, at least at the molecular level, has been that of Arisue *et al*. 2002, comparing the ssu rRNA, ATPase B subunit, EF-2, HSP70c, v-ATPB gene sequences of a *Blastocystis* isolate (strain HE87-1) to other eukaryotes. Based on their ssu rRNA, HSP70c, and a combination of all the datasets, *Blastocystis* clearly fit within the stramenopile group. However, *P. lacertae* was only utilized in their ssu rRNA gene comparison, and the phylogenetic analyses utilizing the EF-2, ATPase B subunit, and v-ATPB genes gave different conclusions. In addition, like other studies based on phylogenetic analyses, the models and options choosen within the software program will influence the results obtained. This makes comparing phylogenetic trees from multiple studies using *Blastocystis* isolates difficult. Furthermore, because of the great heterogeneity within this genus, numerous isolates and many clones will have to be compared to help resolve the species issue.

If *Blastocystis* is indeed a stramenopile, it would be the only stramenopile found as an enteric parasite in humans to date (Silberman *et al*. 1996). In addition, its morphology would be atypical of this group, as *Blastocystis* does not have tripartite hairs that are found in all known stramenopiles, including *P. lacertae* (Cavalier-Smith, 1998). Also, morphological characteristics such as the absence of flagella clearly differentiate it from *P. lacertae*, thus, closer relatives may yet be discovered. Unfortunately, due to the heterogeneity of *Blastocystis* morphologically, a phylogenetic analysis based on morphology is a challenge.

In summary, this study showed that the 5·8S rDNA is not phylogenetically informative at the

genus level and, as expected, is highly conserved among *Blastocystis* isolates. The 7 Blastocystis isolates showed great genetic heterogeneity in sequence within the ITS1 and ITS2 regions. Our phylogenetic analysis supports the hypothesis that *Blastocystis* is a stramenopile, and is a close relative of *P. lacertae*.

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