Molecular phylogenetic analysis in *Hammondia*-like organisms based on partial Hsp70 coding sequences

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SUMMARY

The 70 kDa heat-shock protein (Hsp70) sequences are considered one of the most conserved proteins in all domains of life from Archaea to eukaryotes. *Hammondia heydorni*, *H. hammondi*, *Toxoplasma gondii*, *Neospora hughesi* and *N. caninum* (*Hammondia*-like organisms) are closely related tissue cyst-forming coccidians that belong to the subfamily Toxoplasmatinae. The phylogenetic reconstruction using cytoplasmic Hsp70 coding genes of *Hammondia*-like organisms revealed the genetic sequences of *T. gondii*, *Neospora* spp. and *H. heydorni* to possess similar levels of evolutionary distance. In addition, at least 2 distinct genetic groups could be recognized among the *H. heydorni* isolates. Such results are in agreement with those obtained with internal transcribed spacer-1 rDNA (ITS-1) sequences. In order to compare the nucleotide diversity among different taxonomic levels within Apicomplexa, Hsp70 coding sequences of the following apicomplexan organisms were included in this study: *Cryptosporidium*, *Theileria*, *Babesia*, *Plasmodium* and *Cyclospora*. Such analysis revealed the *Hammondia*-like organism to be the lowest divergent group when compared to other groups within the phylum Apicomplexa. In conclusion, the Hsp70 coding sequences proved to be a valuable genetic marker for phylogenetic reconstruction and may constitute a good candidate to be used with other genes for species phylogeny within this group of organisms.

Key words: Apicomplexa, coccidian, Toxoplasmatinae, heat-shock protein, ITS-1, phylogeny.

INTRODUCTION

Hammondia heydorni, *Hammondia hammondi*, *Toxoplasma gondii*, *Neospora hughesi* and *Neospora caninum* are closely related tissue cyst-forming coccidians with a 2-host life-cycle. *Hammondia heydorni* and *N. caninum* use canids as definitive hosts whereas felids are the definitive hosts for *T. gondii* and *H. hammondi* (Frenkel and Dubey, 1975; McAllister *et al.* 1998; Lindsay *et al.* 1999, 2001; Gondim *et al.* 2004). Because the oocyst stage of these coccidians is morphologically similar, fecal diagnosis is difficult and there is intense debate about their taxonomy and phylogenetic relationships (Ellis *et al.* 1999; Dubey *et al.* 2002; Heydorn and Mehlhorn, 2002).

Comparison of large subunit rDNA and internal transcribed spacer-1 rDNA (ITS-1) sequences derived from *H. heydorni*, *H. hammondi*, *T. gondii* and *N. caninum* revealed that the genus *Hammondia* is paraphyletic (Ellis *et al.* 1999). Characterization of alpha-tubulin gene sequences from *T. gondii*, *N. caninum* and *H. heydorni* shows these genes to posses a similar level of DNA sequence similarity amongst each other (Siverajah *et al.* 2003). In addition, comparison among introns of the alpha-tubulin gene of different isolates of *Hammondia* oocysts from canids showed them to be genetically different (Abel *et al.* 2006). Based on the sequence data from ITS-1 and 2 *H. heydorni*-specific loci, Sreekumar *et al.* (2004) identified 2 distinct groups among the *H. heydorni* isolates. In spite of the fact that the nucleotide diversity was low, in both cases the authors suggested that probably more than 1 species exist within the taxon known as *H. heydorni*.

The Hsp70 comprise a set of chaperones that assist a large variety of protein folding processes in almost all cellular compartments. They all consist of the same working parts: a highly conserved NH2-terminal ATPase domain of 44 kDa and a COOH-terminal region of 25 kDa, divided into a conserved substrate binding domain of 15 kDa and a less-conserved immediate COOH-terminal domain of 10 kDa (Bukau and Horwich, 1998). The Hsp70

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proteins are essential and highly conserved across prokaryotes and eukaryotes. In fact they are one of the most conserved proteins known to date that is found in all biota (Gupta and Golding, 1993).

Although Hsp70 is a powerful marker for evolutionary studies of a number of organisms including eukaryotes and prokaryotes (Gupta, 1998; Budin and Philippe, 1998; Karlin and Brocchieri, 1998; Sulaiman *et al*. 2000; Fast *et al*. 2002; Slapeta and Keithly, 2004), evolutionary studies in Apicomplexa using this marker have only been done with *Cryptosporidium*. The sequence data for the Hsp70 coding genes have provided a useful locus for investigating phylogenetic relationships within this genus (Sulaiman *et al*. 2000; Zhu *et al*. 2000; Xiao *et al*. 2002).

Morrison *et al*. (2004) in a comprehensive and consistent phylogeny of the coccidia using the 18S ribosomal coding gene have suggested the analysis using protein coding sequences for phylogenetic purposes in order to produce a species phylogeny of this group of organisms. Their review stimulated the present study of Hsp70 coding genes for evolutionary studies within *Hammondia*-like organisms, highlighting the genetic diversity among *H. heydorni* isolates.

MATERIALS AND METHODS

Parasites

Oocysts of *T. gondii* and *H. hammondi* were obtained from feces of naturally infected cats, whereas oocysts of *N. caninum* and *H. heydorni* were obtained from feces of naturally infected dogs. Canine and feline feces were examined for oocysts by a conventional flotation method, using sucrose solution. Floated material was transferred to a slide and examined by light microscopy. When 10–12 μm sized oocysts were observed, the slide was washed with 1 ml of TE buffer (10 mM Tris-HCl, pH 8·0; 1 mM EDTA, pH 8·0) in sterile Petri dishes. The oocysts were then transferred to 1·5 ml microtubes and washed twice with TE buffer. After the last wash, the supernatant was discarded and the pellet was resuspended in 500 μl of lysis buffer (10 mM Tris-HCl, pH 8·0; 25 mM EDTA, pH 8·0; 100 mM NaCl, 1% SDS). The oocyst suspension was submitted to 3 freeze-thaw cycles and then proteinase K was added at a concentration of 10 μg/ml. The suspension was incubated at 37 °C. After overnight incubation the DNA was extracted using a mixture of phenol-chlorophorm, isoamyl-alcohol (25 : 24 : 1) and ethanol precipitated as described elsewhere (Sambrook *et al*. 1989). DNA of *N. hughesi* was obtained from tachyzoites of the Oregon strain (Dubey *et al*. 2001) grown on Vero cells and was kindly supplied by Dr L. F. P. Gondim, Federal University of Bahia, Brazil.

PCR

The nucleotide sequence of the Hsp70 coding gene of *T. gondii* (U85648) was retrieved from GenBank and submitted to the BLAST search (blastn) in the *N. caninum* database available in the website of The Institute of Genomic Research (www.tigr.org). A single tentative consensus (TC) sequence of *N. caninum* was found. Consensus sequences of conserved segments identified within the Hsp70 coding sequence of *T. gondii* and the TC sequence of *N. caninum* were used to design 2 primers flanking 1103 bp (HSPF: 5′CAG TCG GAC ATG AAG CAT TGG C3′ and HSPR: 5′ATC GCA CGC TCA CCT TCG TAC AC3′ as sense and reverse primers, respectively). The Hsp70 coding sequence of *T. gondii* is 2001 nucleotides long (stop codon not included). The primer HSPF anneals from the position 250 to 271, whereas the primer HSPR hybridize from the nucleotide 1330 to 1352. The PCR-Hsp70 cycling conditions used were 94 °C for 3 min, followed by 40 cycles of 94 °C for 50 sec, 56 °C for 50 sec and 72 °C for 50 sec. The PCR was finished with a final extension of 72 °C for 5 min. The primers, dNTPs and $MgCl_2$ were used at a final concentration of 0·5 μM, 200 μM and 1·5 mM, respectively. *Taq* DNA polymerase platinum (Invitrogen, Carlsbad, CA) was used at a final concentration of 1·25 Units/50 μl. Five microlitres of the buffer supplied with the enzyme and 5 μl of template DNA were added to the PCR mixture. A second PCR (PCR-ITS1) was carried out employing primers directed to the common toxoplasmatiid 18S and 5·8S rRNA genes. The amplification was performed in 50 μl of final solution as previously described (Rodrigues *et al*. 2004) but using primers JS4 (Slapeta *et al*. 2002*a*) and CT2b (5′TTG CGC GAG CCA AGA CAT C3′, this study). The primers JS4 and CT2b flank about 500 bp within 18S, ITS-1 and 5·8S sequences of the members of the subfamily toxoplasmatinae. The PCR-ITS1 products were directly sequenced and the sequences were submitted to the BLAST search (blastn, www.ncbi.nlm.nig.gov/BLAST) in order to identify the species of each parasite.

Sequencing and sequence alignment

After elution from agarose gel by using a clean-up system (GFX, GE Healthcare, Buckinghamshire, UK) the amplicons of PCR-Hsp70 were sequenced in both directions using the ABI chemistry (ABI PRISM, Foster City, CA) with the sense and reverse primers. A third sequencing primer (HSPFII: 5′GAA CGT CCT CAT CTT CGA CAT GG3′, from the position 582 to 604 within the Hsp70 encoding gene of *T. gondii*) internal to the fragment of 1103 bp was designed in order to sequence the internal segment of the amplicon. Each strand was sequenced at least 4 times in order to increase the

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reliability of the results. The sequences were assembled and the contig formed with the phred-base calling and the phrap-assembly tool available in the suite Codoncode Aligner v.1.5.2 (CodonCode Corp. Dedham, MA, USA). The assemblies were recovered with each residue score equal or greater than 20. The amplicons of PCR-ITS1 were sequenced exactly as above but using the oligonucleotides JS4 and CT2b as sequencing primers. The nucleotide sequences were aligned by using the software ClustalX v.1.83 (Thompson *et al*. 1997).

Phylogenetic analysis

The aligned sequences of each allele of *Hammondia*-like organisms were used as input to the program PAUP* v.4.0b10 (Sinauer Associates, Inc.). Phylogenies were reconstructed with maximum likelihood (ML) and maximum parsimony (MP). For the ML analyses the evolutionary model of nucleotide substitution was calculated by using the software Modeltest v.3.7 (Posada and Crandall, 1998) using the hierarchical likelihood ratio tests (hLRTs). The trees of MP analysis were searched with the branch and bound search and the characters were considered unordered. The trees reconstructed with MP were bootstrapped with heuristic search and 1000 replicates whereas the data obtained with ML were bootstrapped with 100 replicates. The final trees were drawn using TreeView version 1.6.6 (Page, 1996). In the Hsp70 phylogeny, sequences of *Cyclospora* and *Eimeria* were used to serve as outgroup.

Nucleotide diversity within Apicomplexa

Hsp70 coding sequences of one representative of each species of *Hammondia*-like organisms and several other homologue sequences available in the GenBank databases were used in this study (Table 1). The sequences from GenBank were obtained by using the translating BLAST tool (tblastx, www.ncbi.nlm.nig.gov/BLAST) and the coding sequence of Hsp70 of *T. gondii* (U85648) as query. Nucleotide sequences from the following members of Apicomplexa were retrieved: *Babesia*, *Cyclospora*, *Cryptosporidium*, *Plasmodium* and *Theileria*. This search did not find any sequence from *Sarcocystis*. The nucleotide diversity within different groups of sequences was computed with DnaSP v.4.10 (Rozas *et al*. 2003).

RESULTS

We sequenced 951 nucleotides of the Hsp70 genes (corresponding to 317 aminoacids) and the complete ITS-1 rDNA sequences from 14 *H. heydorni* isolates, 2 *H. hammondi* isolates, 3 *N. caninum* isolates, 6 *T. gondii* isolates and 1 *N. hughesi* isolate. In spite of the fact that we used uncloned field isolates, no DNA polymorphism was observed in any of the chromatogram files.

Multiple sequence alignments of the Hsp70 genes revealed 6 alleles of *H. heydorni*, 1 allele of *H. hammondi*, 1 allele of *N. caninum*, 1 allele of *N. hughesi* and 2 alleles of *T. gondii* (Table 2). No gap was encountered. The 2 alleles of *T. gondii* differ by 2 synonymous substitutions. Only 2 synonymous substitutions are responsible for the difference between the alleles of *N. hughesi* and *N. caninum*.

The alignment of the ITS-1 rDNA sequences revealed 5 alleles of *H. heydorni*, 1 allele of *H. hammondi*, 2 alleles of *N. caninum*, 1 allele of *N. hughesi* and 2 alleles of *T. gondii* (Table 2). The 2 ITS-1 alleles of *N. caninum* differ by 1 replacement. Only 1 transition contributes to the difference between the 2 alleles of *T. gondii*. The ITS-1 alleles of *N. caninum* and *N. hughesi* differ by 7 or 8 nucleotides and 1 dinucleotide indel.

The number of nucleotide differences between each pair of alleles from *H. heydorni* sequences is shown in Tables 3 and 4. The differences between the alleles of *H. hammondi* and the homologous sequences of *T. gondii* strain RH (U85648 and X75429) are 24 and 14 residues at the Hsp70 and ITS-1 loci, respectively.

The Hsp70 predicted amino acid sequences of *T. gondii* and *H. hammondi* are identical. The Hsp70 predicted sequences of *N. caninum*, *N. hughesi* and *H. heydorni* have 100% of identity. The difference between Hsp70 sequences of *N. caninum*/*N. hughesi*/*H. heydorni* and *T. gondii*/*H. hammondi* is only 1 conservative substitution (Asp→Glu).

For the ML analyses, the selected model of nucleotide substitution assumed equal base frequencies, equal transversion frequencies, variable transition frequencies and Gamma distributed rate variation among sites (this model is named TrNef+G) (Tamura and Nei, 1993). In Fig. 1 the phylogenetic trees show 2 well-supported clades grouping the *H. heydorni*. One of these clades is formed by the Hsp70 alleles I and II. The other clade is compound by the alleles IV, V and VI. The position of allele III is uncertain.

The branching order within the *Hammondia*-like organisms is not well resolved. *Neospora caninum*, *N. hughesi*, *H. hammondi* and *T. gondii* branch together to the exclusion of *Hammondia heydorni* in the ML and MP analyses but with low statistic support (Fig. 1, panels A and B).

The 6 Hsp70 alleles and 5 ITS-1 alleles revealed the existence of 8 haplotypes among the isolates of *H. heydorni* which were separated into 3 genetic groups, named A, B and C (Table 2).

Multiple sequence alignments of the Hsp70 genes of the organisms presented in Table 1 did not reveal any gap within the sequences. The average number

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Table 1. Taxons used in the nucleotide diversity analysis and average number of nucleotide differences per site between two sequences (Pi) in each group of partial Hsp70 coding sequences

Parasite	GeneBank[1]	Pi	S.D.[2]
Plasmodium			
Plasmodium falciparum	M19753.1	0·147	0·026
Plasmodium vivax	DQ316787.1		
Plasmodium yoelii	XM_721661.1		
Plasmodium cynomolgi	M90978.1		
Plasmodium berghei	XM_674208.1		
Cryptosporidium			
Cryptosporidium baileyi	AJ310880.1	0·146	0·018
Cryptosporidium andersoni	AY954893.1		
Cryptosporidium parvum	AJ310881.1		
Cryptosporidium serpentis	AF221541.1		
Cryptosporidium muris	AB089286.1		
Cryptosporidium wrairi	AF221536.1		
Cryptosporidium hominis	XM_661662.1		
Cryptosporidium meleagridis	AF402280.1		
Cryptosporidium felis	AF221538.1		
Cryptosporidium canis	AY120920.1		
Babesia			
Babesia gibsoni	AB083515.1	0·116	0·009
Babesia bovis	AF107118.1		
Babesia odocoilei	AB248740.1		
Babesia divergens	AB248739.1		
Babesia canis canis	AB248734.1		
Babesia canis vogeli	AB248733.1		
Babesia canis rossi	AB248738.1		
Babesia ovis	AB248741.1		
Babesia caballi	AB248742.1		
Theileria			
Theileria sergenti	D12692.1	0·197	0·032
Theileria cervi	AB248748.1		
Babesia equi	AB248743.1		
Theileria annulata	J04653.1		
Theileria parva	XM_759624.1		
Cyclospora			
Cyclospora colobi	DQ062460.1	0·094	0·029
Cyclospora cayetanensis	AY240875.3		
Cyclospora cercopitheci	DQ062461.1		
Hammondia-like			
Toxoplasma gondii (strain RH)	U85648.1	0·092	0·018
Hammondia hammondi (isolate300)	DQ997596		
Neospora caninum (isolate AL)	DQ997594		
Neospora hughesi (strain Oregon)	DQ997595		
Hammondia heydorni (isolate BR)	DQ997579		

[1] Accession number in GenBank (www.ncbi.nlm.nih.gov).
[2] S.D., Standard deviation of Pi.

of nucleotide differences per site between 2 sequences (Pi) in each group of sequences was calculated and the values can be found in Table 1. Although the *Hammondia*-like group is predominantly compound by distinct genera, this group presented the lowest value of nucleotide diversity (0·092). The highest nucleotide diversity was encountered in the group formed by the Theilerias (0·197).

DISCUSSION

Although widely used for inferring phylogenies of a number of organisms, the Hsp70 coding sequences should be used with caution for the evolutionary studies of Apicomplexa. The first codons of the 5′terminal region and the 3′terminal region encoding a 10 kDa putative substrate-stabilization domain (Bukau and Horwich, 1998) produced poor alignments as many gaps have to be assumed. The 3′terminal region of Hsp70 coding sequences of some parasites is known to code for repeat units that vary in the number of copies (Sheppard *et al*. 1989; Sharma, 1992; Lyons and Johnson, 1998). The number of such repeats varies even among sequences from the same species (Lyons and Johnson, 1998). To overcome this problem we analysed a fragment

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Table 2. Hsp70 and ITS-1 alleles from *Hammondia heydorni*, *Toxoplasma gondii*, *Neospora caninum*, *Neospora hughesi* and *Hammondia hammondi*

Order[1]	Isolate	Origin	HSP70[2]	ITS-1[3]	gbHSP70[4]	gbITS-1[5]	Genetic group[6]
1	HheV2 (Virginia-1)	USA	I	I	DQ997572	DQ997598	A
2	Hhe47	Brazil	I	I	DQ997573	DQ997599	A
3	Hhe147	Brazil	I	I	DQ997574	DQ997600	A
4	Hhe376	Brazil	I	I	DQ997575	DQ997601	A
5	HheV1 (Manhattan-1)	USA	II	I	DQ997576	DQ997602	A
6	HheV3 (Arg33)	Argentina	II	II	DQ997577	DQ997603	A
7	HheV5 (Brazil-1)	Brazil	III	III	DQ997578	DQ997604	C
8	HheBR	Brazil	IV	IV	DQ997579	DQ997605	B
9	HheRHN	Brazil	IV	IV	DQ997580	DQ997606	B
10	HheV7 (Arg32)	Argentina	IV	IV	DQ997581	DQ997607	B
11	HheV8 (Arg52)	Argentina	IV	IV	DQ997582	DQ997608	B
13	HheOTO	Brazil	V	IV	DQ997584	DQ997610	B
14	Hhe95	Brazil	V	V	DQ997583	DQ997611	B
12	HheV9 (Arg29)	Argentina	VI	IV	DQ997585	DQ997609	B
15	Tgo64	Brazil	I	I	DQ997586	DQ997612	ND
16	TgoAN	Brazil	I	II	DQ997587	DQ997613	ND
17	TgoH2	Brazil	II	II	DQ997588	DQ997614	ND
18	TgoP1	Brazil	I	II	DQ997589	DQ997615	ND
19	Tgo115	Brazil	I	II	DQ997590	DQ997616	ND
20	Tgo12_06	Brazil	I	II	DQ997591	DQ997617	ND
21	NcaPR	Brazil	I	I	DQ997592	DQ997618	ND
22	Nca10_06	Brazil	I	I	DQ997593	DQ997619	ND
23	NcaAL	Brazil	I	II	DQ997594	DQ997620	ND
24	Nhu (Oregon1)	USA	I	I	DQ997595	DQ997621	ND
25	Hha300	Brazil	I	I	DQ997596	DQ997622	ND
26	Hha305	Brazil	I	I	DQ997597	DQ997623	ND

[1] Isolates of *Hammondia heydorni* (1–14); isolates of *Toxoplasma gondii* (15–20); isolates of *Neospora caninum* (21–23); isolate of *Neospora hughesi* (24); isolates of *Hammondia hammondi* (25–26).
[2] Alleles of Hsp70 (each different allele is numbered with roman characters).
[3] Alleles of ITS-1 (each different allele is numbered with roman characters).
[4] GenBank Accession numbers of Hsp70 coding sequences.
[5] GenBank Accession numbers of ITS-1 sequences.
[6] Genetic groups of *H. heydorni* isolates, ND: genetic groups not determined for other species than *H. heydorni*.

Table 3. Number of nucleotide differences between each pair of ITS-1 alleles from *Hammondia heydorni*

ITS-1 alleles	I	II	III	IV	V
I					
II	1				
III	6	7			
IV	5	6	1		
V	4	5	2	1	

Table 4. Number of nucleotide differences between each pair of Hsp70 alleles from *Hammondia heydorni*

Hsp70 alleles	I	II	III	IV	V	VI
I						
II	1					
III	8	9				
IV	9	10	5			
V	10	11	6	1		
VI	8	9	4	1	2	

that starts after and ends before those regions and spans 951 nucleotides within part of the conserved ATPase and substrate-binding site domains.

Considering the infection by coccidians, there is the possibility that the oocysts were from animals infected with multiple species or multiple lineages of a single species. However, this possibility was ruled out because no DNA polymorphisms were observed in any of the sequence files, suggesting that there were no indications for mixed infections.

Considering the Hsp70 gene phylogeny of the *Hammondia*-like organisms, at least 2 distinct genetic lineages could be recognized within the clade formed by the *H. heydorni* species. A third lineage may exist if one considers the differences between the allele III and the alleles IV, V and VI. In the ML tree, the allele III is grouped together with alleles IV, V and VI while MP tree separated the allele III from the other alleles. In both cases, the position of allele III

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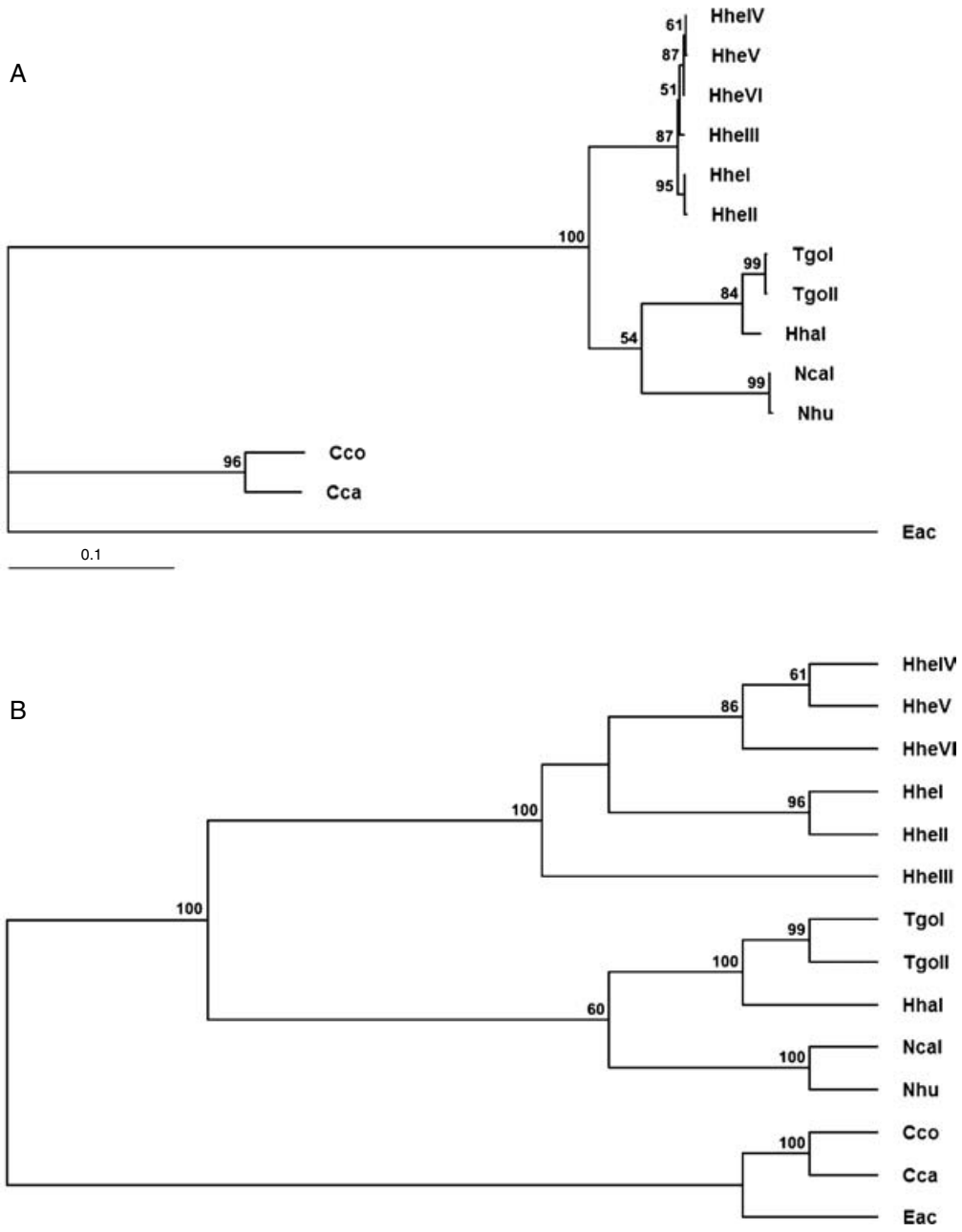

Fig. 1. Phylogenetic relationships among the *Hammondia*-like organisms inferred from the alignment of the Hsp70 coding employing Maximum Likelihood (ML) and Maximum Parsimony (MP) analysis (panels A and B, respectively). The trees are rooted with *Cyclospora colobi* (Cco, DQ062460.1), *C. cayetanensis* (Cca, AY240875.3) and *Eimeria acervulina* (Eac, Z26134.1). The numbers indicate the number of times that the branches are supported after 100 (ML analyses) and 1000 (MP analyses) bootstrap replicates. The branch lengths in the ML tree are proportional to the amount of evolutionary changes. Only 1 MP tree with 607 steps was found.

has low statistic support. The 6 Hsp70 alleles and 5 ITS-1 alleles revealed the existence of 8 haplotypes among the isolates of *H. heydorni*. In fact, a notable similarity of the haplotypes at each of the 2 loci was observed and the isolates could be separated into 3 genetic groups named A, B and C. 'Recombinants' are found only within each distinct genetic group. Obviously, these conjectures are valid if one considers that the population structure of *H. heydorni* is clonal. The clonality of such a population remains to be elucidated.

Based on 2 *H. heydorni* specific loci (HhAP07 and HhAP10) and ITS-1 rDNA sequences, Sreekumar *et al.* (2004) also found at least 2 distinct genotypes among their isolates of *H. heydorni*. Some of the isolates analysed by these authors were also evaluated here and in both cases such isolates were clustered identically. The isolate HheV5, named Brazil-1 by Sreekumar *et al.* (2004), was the only taxon classified into genetic group C because it proved to be quite divergent from those of groups A and B at Hsp70 loci.

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The nucleotide diversity existing between the 2 species of *Neospora* at Hsp70 loci is lower than that observed at ITS-1. The differences between the 2 lineages of *H. heydorni* at ITS-1 loci level are comparable to that existing between *N. caninum* and *N. hughesi*. But at Hsp70 loci, the nucleotide diversity among *H. heydorni* lineages is much higher than that existing between the 2 species of *Neospora*. Thus, if *N. caninum* and *N. hughesi* are considered different species, the 2 genetically distinct lineages of *H. heydorni* would be separate species of a single genus, based on genetic criteria.

The phylogenetic analyses presented here indicated that *T. gondii* and *H. hammondi* are sister taxons, rendering the genus *Hammondia* paraphyletic, thus confirming previous observations (Ellis *et al*. 1999). The comparison of Hsp70 partial coding sequences from *H. heydorni*, *T. gondii* and *N. caninum* revealed these genes to possess a similar level of evolutionary distance. In fact, the placement of *Neospora* spp. as sister group of *T. gondii*/*H. hammondi* remains difficult to demonstrate as the Hsp70 gene trees did not resolve the relationships between the major *Hammondia*-like groups with confidence. Ellis *et al*. (1999) and Mugridge *et al*. (2000) faced the same problem when they reconstructed the Toxoplasmatinae phylogeny using the large subunit rDNA sequences. This lack of resolution may be explained by the hypothesis that these groups emerged as a result of radiation, which may be explained as a burst process of multiple simultaneous speciation events (Hoelzer and Melnick, 1994). However, the robustness of such a hypothesis would increase when more genes are analysed.

The study of the nucleotide diversity of Hsp70 coding sequences within some groups of Apicomplexa revealed the *Hammondia*-like group as one of the less divergent groups. The low variability existing among *Hammondia*-like organisms has been already described with 18S and ITS-1 rDNA genetic sequences (Ellis *et al*. 1999; Jenkins *et al*. 1999; Mugridge *et al*. 1999, Slapeta *et al*. 2002*b*; Sreekumar *et al*. 2004; Morrison *et al*. 2004).

Based on phylogenetic analysis, 3 plausible solutions for the nomenclature of the *Hammondia*-like organisms were outlined by Slapeta *et al*. (2002*b*). While it is now evident that the majority of researchers agree that the *Hammondia*-like organisms are formed by at least 5 species, the question that remains is how many genera need to be kept. The mounting evidence from nucleotide diversity and phylogenetic analysis of Hsp70 locus supports the first scenario, which would retain only a single genus. The phylogenetic analysis of Hsp70 coding genes also supports the view that 3 distinct clades would be classified as genera *Toxoplasma* (*T. gondii* and *H. hammondi*), *Neospora* (*N. caninum* and *N. hughesi*), and a new genus to accommodate the 2 variants of *H. heydorni* (the second scenario). According to our data, the third scenario outlined by Slapeta *et al*. (2002*b*) i.e. grouping the *Hammondia*-like organisms based on the host (feline isolates are *Toxoplasma* and canine isolates are *Neospora*) should be discarded.

Similar to rRNA, the Hsp70 is present in all species and performs an essential function within the cell. Thus, comparative genealogies using both markers are desirable in order to infer the true species phylogenies and produce a robust taxonomy, not only for *Hammondia*-like organisms but probably for any taxonomic level.

The choice of Hsp70 coding sequences for phylogenetic reconstruction of this group of organisms presented some advantages. Firstly, it allows the inclusion of outgroups with high confidence and lower risk to the long-branch attraction artifact due to the low distance of the ingroup taxa to outgroup. An important criterion for the reliability of a marker is its sensitivity to the long-branch attraction artifact which is decreased when the distance to the outgroup decreases (Felsenstein, 1978) and the use of an appropriate outgroup is needed because this determines the root of the trees. We used the Hsp70 genetic sequence from *Cyclospora* spp. and *E. acervulina* as outgroup because they are the most closely related of the *Hammondia*-like organisms with Hsp70 coding sequences available in GenBank.

Secondly, because of the similar size of this gene in various species and its high degree of conservation, a reliable alignment from several species was readily obtained. It is noteworthy to stress that no gaps where observed in the alignment produced with the Hsp70 homologous fragments of the apicomplexans, hence phylogenies using such data require minimal or no *a priori* assumptions. As stated before, the alignment of homologous positions in a set of sequences is the starting point in phylogenetic analyses from which all inferences are derived (Gupta, 1998). Finally, the alignment of Hsp70 sequences from closely related species shows many conserved regions that may serve as anchorage site for consensus primers, which can be successfully used for PCR amplification of a wide range of organisms. With aligned sequences of *N. caninum* and *T. gondii* we were able to design consensus primers capable of amplifying Hsp70 genetic sequences of *H. hammondi*, *H. heydorni* and *N. hughesi*.

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