The genus *Hammondia* is paraphyletic

J. T. ELLIS[1]*, D. A. MORRISON[1], S. LIDDELL[2], M. C. JENKINS[2], O. B. MOHAMMED[3], C. RYCE[1] *and* J. P. DUBEY[2]

[1] *Molecular Parasitology Unit, Department of Cell and Molecular Biology, University of Technology, Sydney, Westbourne Street, Gore Hill, NSW 2065, Australia*
[2] *Parasite Biology and Epidemiology Laboratory, Livestock and Poultry Sciences Institute, Agricultural Research Service, US Department of Agriculture, Beltsville, Maryland 20705, USA*
[3] *King Khalid Wildlife Centre, National Commission of Wildlife Conservation and Development, P.O. Box 61681, Riyadh 11575, Saudi Arabia*

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SUMMARY

The phylogenetic relationships amongst *Hammondia*, *Neospora* and *Toxoplasma* were investigated by DNA sequence comparisons of the D2/D3 domain of the large subunit ribosomal DNA and the internal transcribed spacer 1. The results obtained allow us to reject the hypothesis that *N. caninum* and *H. heydorni* are the same species and show that *Hammondia hammondi* is probably the sister taxon to *Toxoplasma gondii*.

Key words: *Hammondia* spp., *Neospora caninum*, *Toxoplasma gondii*, phylogeny.

INTRODUCTION

Among the cyst-forming coccidia, the phylogeny of *Toxoplasma*, *Neospora*, *Isospora* and *Sarcocystis* has been studied extensively and they are believed to represent a monophyletic group (Ellis *et al*. 1994, 1995; Ellis, Morrison & Johnson, 1994; Holmdahl *et al*. 1994, 1998; Ellis & Morrison, 1995; Morrison & Ellis, 1997; Tenter & Johnson, 1997). No phylogenetic studies have yet been reported that include data from *Hammondia* species.

The genus *Hammondia* currently contains 3 species: *Hammondia hammondi*, *Hammondia heydorni* and *Hammondia pardalis*. *H. hammondi* is believed to be the sister taxon to *Toxoplasma gondii* since they are genetically and antigenically closely related (Araujo, Dubey & Remington, 1984; Johnson *et al*. 1987; Ellis *et al*. 1998). *H. heydorni* and *H. pardalis*, on the other hand, have been the subject of few studies, and the literature (of any note) on the latter species is essentially limited to the description of the species (Hendricks *et al*. 1979).

Neospora caninum, because of its similarity in morphology to *T. gondii*, has frequently been misidentified (Dubey & Lindsay, 1996). Recent evidence indicates that canine and bovine strains of *Neospora* may be genetically identical (Marsh *et al*. 1995; Stenlund *et al*. 1997; Ellis *et al*. 1998) and therefore it was proposed that this genus contains only 1 species (Holmdahl *et al*. 1997).

The study described here involved the investigation of the genetic relationships between *Hammondia* spp., *N. caninum* and *T. gondii* by comparisons of large subunit (LSU) rDNA and internal transcribed spacer 1 (ITS1) sequences derived from these taxa. Previous comparisons of LSU rDNA sequences between *N. caninum* and *T. gondii* indicated the D2 domain may be phylogenetically informative (Ellis *et al*. 1998). Consequently, LSU rDNA sequences were compared from *Hammondia* spp., *N. caninum* and *T. gondii* in order to further investigate the utility of LSU rDNA for analysing the phylogenetic relationships amongst the coccidia.

The ITS1 DNAs of *N. caninum* and *T. gondii* have also been previously characterized, and they differ at approximately 20% of the nucleotides over the length of the sequence (Holmdahl & Mattson, 1996; Payne & Ellis, 1996). Therefore this locus provides an excellent marker for the differentiation of DNA from *N. caninum* and *T. gondii*. Since Homan *et al*. (1997) demonstrated that the ITS1 was highly conserved among different strains of the same species (they compared the ITS1 sequences from over 20 strains of *T. gondii* and found them to be identical), we hypothesized that a comparison of ITS1 sequences from *Hammondia* spp., *N. caninum* and *T. gondii* may also provide insight into the genetic relationships between these taxa.

MATERIALS AND METHODS

Parasites

Oocysts of *H. heydorni* were from 2 sources (described below) and the ITS1 and D2 domain of the LSU rDNA were amplified by PCR and sequenced at 2 independent laboratories (UTS and USDA).

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* Corresponding author: Department of Cell and Molecular Biology, University of Technology, Sydney, Westbourne Street, Gore Hill, NSW 2065, Australia. Tel: 0061 2 9514 4161. Fax: 0061 2 9514 4003. E-mail: j.ellis@uts.edu.au

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At USDA, *H. hammondi* oocysts were obtained from faeces of experimental cats fed tissue cysts of the H.H-34 strain. The original isolation of H.H-34 was from the faeces of a domestic cat (Riahi *et al.* 1995). Oocysts were sporulated in 2% sulphuric acid for 2 weeks at room temperature and then stored at 4 °C. *H. heydorni* oocysts were collected from a naturally infected dog (Blagburn *et al.* 1988) and stored in 2% potassium dichromate (K_2CrO_4) and were derived from the same batch used in earlier studies (Speer *et al.* 1988; Speer & Dubey, 1989). The oocysts were pelleted by centrifugation, washed 3 times with distilled water (dH_2O) to remove K_2CrO_4, treated for 10 min with 20% sodium hypochlorite, washed 5 times with dH_2O, and resuspended in sterile dH_2O.

For DNA extraction, the resuspended oocysts were pipetted dropwise into liquid nitrogen and ground to a fine powder in a sterile mortar and pestle. The extracted oocysts were resuspended in DNA extraction buffer (0·2 M Tris, pH 8·0, 0·1 M EDTA, 0·4 M NaCl) containing 1 mg/ml proteinase K and 0·1% SDS and incubated for 16 h at 50 °C. The DNA extract was treated with phenol, phenol-chloroform, chloroform and then ethanol precipitated. The DNA was pelleted by centrifugation and resuspended in sterile 0·01 M Tris, pH 8·0, 0·001 M EDTA (TE) and stored at −20 °C.

Oocysts of *H. heydorni* (to be described in detail elsewhere) were also obtained in Saudi Arabia from a red fox (*Vulpes vulpes*) that was experimentally infected with meat from a mountain gazelle (*Gazella gazella*) that contained sarcocysts in its striated muscles. Oocysts were allowed to sporulate for 3 days in a shallow layer of 2·5% K_2CrO_4 at 25 °C (Mohammed & Hussein, 1992). Oocysts were washed 5 times in dH_2O to remove the K_2CrO_4 and further purified through a discontinuous density gradient of Percoll (density 1·13 g/ml). DNA was extracted from the purified oocysts by standard procedures involving lysis in SDS, phenol/chloroform extraction and ethanol precipitation. The DNA was subject to analysis at UTS.

DNA from *H. hammondi* was also provided as a gift from Dr N. Muller (University of Berne, Switzerland).

PCR and sequence analysis of rDNA

At USDA, LSU rDNA (D2 and D3 domains) was PCR amplified, using primers CR1 (5′-CTGAAATTGCTGAAAAGGAA-3′) and CR2 (5′-CCAGCTACTAGATGGTTCGA-3′) or Tim 15 and GA1 (Ellis *et al.* 1998), by incorporating 10 μl of serial dilutions of the *H. hammondi* or *H. heydorni* DNA under standard reaction conditions. PCR products were cloned into the pCRII vector (Invitrogen) using methods supplied by the manufacturer and transfected into *Escherichia coli*. Recombinant pCRII plasmid DNA was purified and subjected to dideoxy chain termination sequencing using vector and insert-specific primers and the Sequenase sequencing kit (Stratagene, La Jolla, CA). At least 3 clones were sequenced for *H. hammondi* and *H. heydorni* with a minimum of 2 sequencing reactions for each primer. Additional clones were sequenced if there was a nucleotide discrepancy between the 3 clones.

At UTS, PCR products derived from *H. hammondi* using CR1 and CR2 or Tim 15 and GA1 were purified by a QIAquick purification column (Qiagen, USA) and sequenced by cycle sequencing with the aid of an ABI automated sequencer. A consensus sequence was produced from 6 sequencing runs (3 from each primer). The sequences of the LSU rDNA of *H. hammondi* determined independently at UTS and USDA were identical. There was insufficient genomic DNA to determine LSU rDNA sequences from the genomic DNA isolated from oocyts collected from the red fox fed gazelle meat.

The LSU rDNA sequences from *N. caninum* (NC-Liverpool strain; GenBank™ accession number AF001946), *T. gondii* (RH strain; AF07865) and *Hammondia* (AF076871, *H. hammondi*; AF076870, *H. heydorni*) were aligned using Clustal W (with default parameter options) (Thompson, Higgins & Gibson, 1994) via the Australian National Genome Information Service (ANGIS) with the corresponding sequences from *Eimeria tenella* (AF076862), *Eimeria alabamensis* (AF076861), *Frenkelia microti* (AF076864), *Frenkelia glareoli* (AF076863), *Besnoitia besnoiti* (from cattle; AF076866) and *Besnoitia* spp. (from wildebeest; AF076869).

ITS1 sequences from *Hammondia* DNA were amplified from genomic DNA using primers Tim3 and Tim11 as described (Payne & Ellis, 1996). The PCR products were generated and sequenced independently at UTS and USDA, using the procedures described above and the sequences obtained were identical. ITS1 sequences from *N. caninum* (NC-Swe-B1 strain; GenBank™ accession number AF001946), *T. gondii* (ME49 strain; L49390) and *Hammondia* (AF076857 *H. hammondi*; AF076858 *H. heydorni*) were aligned using Clustal W (with default parameter options) via ANGIS. Alignments based on secondary structure predictions of the ITS1 would be preferable for ensuring sequence homology; however, no structures have yet been predicted for coccidian ITS RNA molecules.

Analysis

Parsimony analysis was performed using the exhaustive search option in PAUP 3.1.1 (Swofford, 1993). The analysis was rooted using *Eimeria* as the outgroup.

In order to evaluate the relative magnitude of the

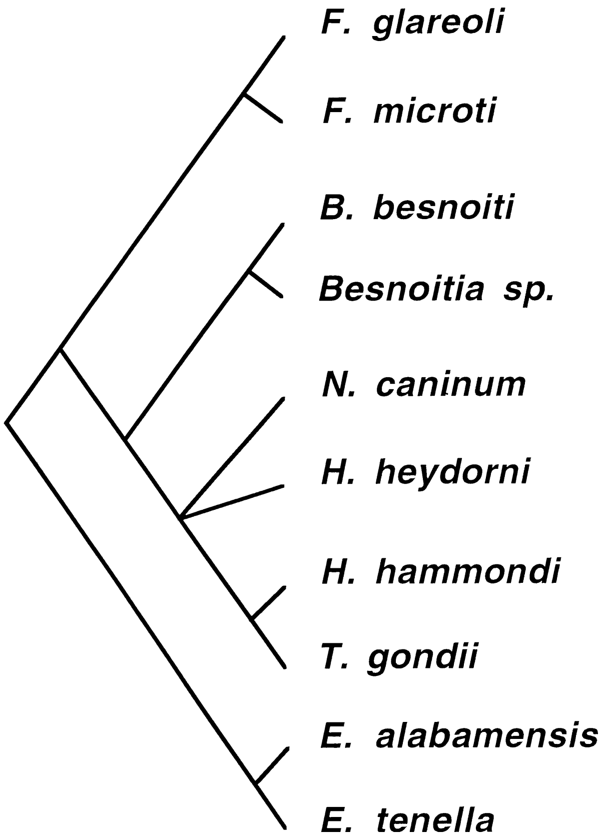

Fig. 1. Strict consensus tree derived from parsimony analysis of LSU rDNA sequence data.

phylogenetic signals in the sequence data, data from *Hammondia* spp., *N. caninum* and *T. gondii* were analysed by spectral analysis (Hendy & Penny, 1992) using the computer program Spectrum 2.0 (Charleston & Page, 1997). This analysis produces a spectrum representing the estimated branch lengths of a phylogenetic tree, with positive values for support for the branch (i.e. character-state changes that would occur on that branch) and negative values for conflict (i.e. character-state changes that contradict that branch). The estimates are produced by operating on the raw spectrum with the Hadamard transformation (Hendy & Charleston, 1993), which uses the two-state symmetrical Markov model of Cavender (1978) to correct the data for multiple character-state changes. For the sequence data, the four-state nucleotide data were mapped to two-state data representing purines and pyrimidines. The phylogenetic tree inferred was the Manhattan tree, which is the tree whose expected spectrum is the closest Manhattan distance to the spectrum obtained from the data.

RESULTS AND DISCUSSION

A Clustal W alignment of LSU rDNA sequences, which contained 637 base positions, from *Eimeria* spp., *Frenkelia* spp., *Besnoitia* spp., *Hammondia* spp., *N. caninum* and *T. gondii* was subject to parsimony analysis. Two most parsimonious trees were found with 357 steps (consistency index 0·882, homoplasy index 0·118); 13 additional trees were found within 4 steps. On both of the 2 most parsimonious trees *H. hammondi* and *T. gondii* formed a monophyletic group; the trees differed in the placement of *N. caninum* or *H. heydorni* as the sister taxon to these 2. These characteristics were shared by all the trees with 361 steps or less. A strict consensus of the 2 most parsimonious trees is shown in Fig. 1.

A summary of the nucleotide positions in the D2 and D3 domains of the LSU rDNA sequences that vary among *H. hammondi*, *H. heydorni*, *N. caninum* and *T. gondii* is shown in Table 1. There are 10 unique nucleotide differences between the sequences if one ignores gaps. The sequences of *H. heydorni*, *N. caninum*, *H. hammondi* and *T. gondii* contain 5 (positions 14, 18, 88, 112, 231), 2 (173, 177), 2 (441, 484) and 1 (17) unique character states respectively. The sequences of *H. heydorni* and *N. caninum* share 4 character states (19, 95, 185, 230); *T. gondii* and *H. hammondi* share 3 character states (96, 185, 230); *N. caninum* and *T. gondii* share 2 (50, 229) and *H. heydorni* and *H. hammondi* also share 2 (50, 229). Consequently, the most likely phylogenetic tree (no matter which inference method is used) is the one that unites *H. heydorni* with *N. caninum*, and *H. hammondi* with *T. gondii*.

The analyses presented here and elsewhere (Ellis *et al.* unpublished observations) indicate that *Besnoitia* species are the sister group to *Hammondia*,

Table 1. Summary of the variable nucleotide positions detected in the LSU rDNA among *Hammondia hammondi* (HH), *H. heydorni* (HY), *Neospora caninum* (NC) and *Toxoplasma gondii* (TG)

	Nucleotide position in the sequence alignment																		
	14	17	18	19	43	50	88	96	112	149	173	177	185	187	229	230	231	441	585
NC	T	—	T	C	T	G	T	C	C	T	C	C	C	T	C	T	T	T	T
HY	C	—	C	C	—	T	A	C	A	T	T	T	C	T	T	T	G	T	T
HH	T	—	T	T	T	T	T	T	C	—	T	T	G	—	T	C	T	C	C
TG	T	T	T	—	T	G	T	T	C	T	T	T	G	T	C	C	T	T	T

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HH     1   ACACGTCCTTATTCTTTATTAACCATCAACCTTTGAATCCCAAGCAAAAC
HY         ..........TA.TC.........C.........................
NC         ..........TA.TC..T.C....C..................A......
TG         ..................................................

HH    51   ATGAGTTTGCATCTCTCTCC-ATCGGAGAGATTTGCATTCAAGAAGCGTG
HY         .....C..............AGCGT....C.GG.............T..A
NC         .....C...T..........-T.......GGG.A................
TG         ....................-..T..........................

HH   101   ATAG-----TGCCGAAAGGT-----------ATTATTGCCTTCTTCATGT
HY         ..GC--TAC.CT...CG.A.TCGGCGATTGA.G....A............
NC         ...TACTAC.C..TGTGA..-----------TG...C.............
TG         ....-----.AT........-----------...................

HH   151   -GGATATCTTGCGCTGCTT-----------------------CCAATATT
HY         -......T...T..A.G..-------------------------------
NC         -......T...A..A....TTTTCAAGCGTTCTATTGAACGC.TG...A.
TG         T.......C..........-----------------------........

HH   201   GGAAGCCAGTGCAGATATCCGGGGGTGCACAGCGAAGGGGCTCGATTACT
HY         --.T.TAGC...T..........A...G..G.AA....A.......CT..
NC         .A...TGT.....T.........A...T..G........A....G.C...
TG         ..............G............................A...T..

HH   251   GGAAATTCGTGTCTCTGTTGGGATACTGATTTCCAGGAGTTTCTTCAGTG
HY         .......G....T..AAC.....C...C...........C.C....GAA.
NC         .......AA.......A......CTT.A.C.................A..
TG         ..................................................

HH   301   TGCATTCTTTTTTCCCACACCGTTATTTCAAACAACAAATCTGAGG-GGC
HY         ...........C.T..........T..CA..C...T.......G..GA..
NC         ............................T..............GAT-A..
TG         ..............................................-AA.

HH   351   ATTTGAGAGAGAGTGAAAGATA--CTATCTTTCTGCATTTCTCTCAGTGT
HY         ..C......GA.TCAG....CGCGA.G........T....T....TA..C
NC         G......G..-..A.......G--G.C........T.........TA.TC
TG         .....................T--G.............C......GA...

HH   401   GCTTTCAGATTGCTTCCTAAA--CTATAATGTTTATTTTAAATTTTCAGC
HY         .A.GC......AT..A.G...--.A.CG......T.C.............
NC         ...........AC..A.....AA..........-..C.............
TG         .............C.......--..........AT...............

HH   451   AATGGATGT
HY         .........
NC         .........
TG         .........
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Fig. 2. Alignment of the ITS1 DNA sequences of *Hammondia hammondi* (HH), *H. heydorni* (HY), *Neospora caninum* (NC) and *Toxoplasma gondii* (TG). Dashes represent gaps introduced into the alignment in order to maximize sequence similarity. Only the sequence of *H. hammondi* is shown along with nucleotide differences in the other sequences; dots indicate bases identical to the *H. hammondi* sequence at that position in the other sequences. Numbers refer to the base position in the sequence alignment.

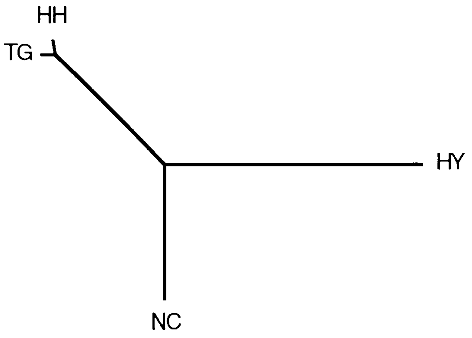

Fig. 3. Phylogenetic relationships among *Hammondia hammondi* (HH), *H. heydorni* (HY), *Neospora caninum* (NC) and *Toxoplasma gondii* (TG) as inferred from the ITS1 sequence data and the spectral analysis. The tree is an unrooted Manhattan tree. The branch lengths are proportional to the amount of inferred evolutionary change, based on purines and pyrimidines only.

Neospora and *Toxoplasma*. However, *Besnoitia* ITS1 sequences cannot be robustly aligned with those of *T. gondii* or *N. caninum* (Ellis *et al.* unpublished observations) and so in this study only unrooted trees can be analysed. A Clustal W alignment of ITS1 sequences from *H. hammondi*, *H. heydorni*, *N. caninum* and *T. gondii* is shown in Fig. 2 and the nucleotide differences between the sequences (expressed as a proportion) are shown in Table 2. The ITS1 of *T. gondii* and *H. hammondi* are most similar (they differ at less than 5% of the nucleotide positions) whereas the ITS1 of *Toxoplasma* and *H. heydorni* are the most divergent (they differ at approximately 22% of the nucleotide positions).

The spectral analysis of the ITS1 data indicates no support for a tree that unites *H. hammondi* and *H. heydorni* as a monophyletic group; there is also little support (spectral value = 0·0028) for the tree that unites *H. hammondi* and *N. caninum*, and considerable conflict (normalized spectral value = 0·1290) for this tree; and there is considerable support (0·0373) for the tree that unites *H. heydorni* and *N. caninum* with little conflict (0·0096).

The Manhattan tree derived from the ITS1 data is shown in Fig. 3. The branch lengths represent the expected number of character-state changes per site. A maximum-parsimony analysis and a maximum-likelihood analysis of the data also yield the same tree, indicating that the data are robust to the

Table 2. Proportion of nucleotide differences between the ITS1 sequences of *Hammondia hammondi*, *H. heydorni*, *Neospora caninum* and *Toxoplasma gondii*

	H. hammondi	*H. heydorni*	*N. caninum*	*T. gondii*
H. hammondi	0·000	—	—	—
H. heydorni	0·207	0·000	—	—
N. caninum	0·159	0·200	0·000	—
T. gondii	0·032	0·219	0·175	0·000

phylogenetic method used. The 2 species of *Hammondia* do not form a monophyletic group on this tree. Although the tree cannot be rooted using the ITS1 data alone (because the sequences cannot be aligned against the outgroup), the LSU rDNA sequence data for *H. hammondi*, *N. caninum* and *T. gondii* indicate that the tree should be rooted on the branch leading to *H. heydorni*.

The LSU rDNA and ITS1 sequence data, therefore, indicate that *Hammondia* as currently circumscribed is a paraphyletic group, and that this is a robust conclusion. It is likely that *H. hammondi* is the sister taxon to *T. gondii*, and so it would not be possible to make *Hammondia* a monophyletic group unless both *T. gondii* and *N. caninum* are included within the group. The close similarity between *H. hammondi* and *T. gondii* found here is consistent with previously published antigenic and genetic data (Araujo *et al*. 1984; Johnson *et al*. 1987; Ellis *et al*. 1998).

It is tempting from these results (i.e. the grouping of *N. caninum* and *H. heydorni*) to speculate that a canid may act as a definitive host in the life-cycle of *N. caninum*. This hypothesis has been recently confirmed (McAllister *et al*. 1998).

Finally, the results presented here allow us to safely reject the hypothesis put forward by Rommel (unpublished) that *N. caninum* and *H. heydorni* are the same species since they are clearly genetically distinct from each other at the rDNA.

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ADDENDUM

Recently a new *Neospora* species (*Neospora hughesi*) was isolated from the horse and the ITS1 sequenced (Marsh *et al*. 1998). A reanalysis of the data set described here (including *N. hughesi*) demonstrated *N. caninum* and *N. hughesi* to be monophyletic; and confirmed the findings that *Hammondia* is paraphyletic.

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