The genus *Hammondia* is paraphyletic

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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 investi-
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 (K₂CrO₇) 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 (dH₂O) to remove K₂CrO₇, treated for 10 min with 20% sodium hypochlorite, washed 5 times with dH₂O, and resuspended in sterile dH₂O.

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% K₂CrO₇ at 25°C (Mohammed & Hussein, 1992). Oocysts were washed 5 times in dH₂O to remove the K₂CrO₇ 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'-CTGAAA-TTGTGAAAAAGGA-3') and CR2 (5'-CCAGC-TACTAGATGTTTCGA-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 deoxy 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 oocysts 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
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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,
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.

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

<table>
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<th></th>
<th>H. hammondi</th>
<th>H. heydorni</th>
<th>N. caninum</th>
<th>T. gondii</th>
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<td>0.219</td>
<td>0.175</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**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
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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.

REFERENCES


