Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii* ✩

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Abstract

Since its first description in the late 1980s, *Neospora caninum* has been recognised as a prominent tissue cyst-forming parasite due to its ability to induce congenital disease and abortion in animals, especially cattle. It is found worldwide and is a cause of significant economic losses for the livestock industry. However, its place within the family Sarcocystidae, like that of several other taxa, remains unresolved. *Neospora caninum* shares several morphological and life cycle characters with *Hammondia heydorni*, although it is most commonly thought of as being a close relative of *Toxoplasma gondii*. This study presents information regarding the phylogenetic relationship of *N. caninum* to species currently classified into the genus *Hammondia*, as well as to two strains (RH and ME49) of *T. gondii* based on the full-length large subunit ribosomal RNA gene. Phylogenetic analyses using two alignment strategies and three different tree-building methods showed that the two species in the genus *Hammondia* are paraphyletic. *Neospora caninum* was shown to form a monophyletic clade with *H. heydorni* instead of *T. gondii*, which in turn was shown to be most closely related to *H. hammondi*. The finding that *N. caninum* and *H. heydorni* are closely related phylogenetically may aid the elucidation of currently unknown aspects of their biology and epidemiology, and suggests that *H. heydorni* should be considered in the differential diagnosis of *N. caninum* from other apicomplexan parasites. © 1999 Published by Elsevier Science Ltd on behalf of the Australian Society for Parasitology Inc. All rights reserved.

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1. Introduction

The family Sarcocystidae comprises about 200 recognised species of heteroxenous coccidia that form tissue cysts in an intermediate host. Based on phenotypic characters, the family is often subdivided into two subfamilies, the Sarcocystinae and the Toxoplasmatinae [1–4]. Traditionally, the majority of the species (i.e. more than 180) have been classified in the Sarcocystinae, which has often been further divided into two genera, *Sarcocystis* and *Frenkelia* [1, 3–9]. However, recent phylogenetic analyses based on ssrRNA and lsrRNA gene sequences showed the genus *Frenkelia* to be synonymous with *Sarcocystis* [10–12], and thus rendered the Sarcocystinae monogeneric.

By contrast, the few species classified into the Toxoplasmatinae have been placed into four different genera: *Toxoplasma* (one species), *Neospora* (two species), *Hammondia* (two species) and *Besnoitia* (six species) [3–8, 13]. In the Toxoplasmatinae, the asexual development in the intermediate host consists of two phases of endodyogeny. In the first phase, tachyzoites multiply rapidly in various host cells. In the second phase, bradyzoites multiply slowly within a tissue cyst. The bradyzoite is the only type of reproductive stage in tissue cysts of the Toxoplasmatinae and is the terminal life cycle stage in the intermediate host. If ingested by a definitive host, the bradyzoites initiate another proliferative phase (endopolygeny) in epithelial cells of the small intestine. The terminal stages of this asexual phase initiate the sexual phase of the life cycle. Gamogony and oocyst formation also take places in the epithelium of the small intestine. Unsporulated oocysts are released into the intestinal lumen and passed into the environment with the faeces. Sporogony occurs outside the host and leads to the development of disporous, tetrazoic oocysts [4, 5, 8, 14, 15].

Thus far, generic descriptions within the Toxoplasmatinae have been based only on phenotypic characters, such as host specificity, the pattern of the life cycle, the degree of heteroxeny (facultative or obligate), the mode of transmission of infectious life cycle stages, the type of host cells parasitised, and the morphology and location of the tissue cyst [1–8, 13]. However, it is often not clear what weight should be assigned to the different characters, and controversy arose about which characters to use for generic designations and which to use for subfamilial designation. For example, while species classified into the genus *Besnoitia* differ distinctly from other coccidian taxa by the unique structure of their tissue cysts and their affinity to connective tissue cells [1, 15], the genera *Toxoplasma*, *Hammondia* and *Neospora* share a broad range of phenotypic features. These similarities have led some authors [2, 16, 17] to synonymise the genus *Hammondia* with the genus *Toxoplasma*. By contrast, Frenkel [1] argued that "Any economy in generic designations simply obscures the life cycle and morphologic information which the present generic terms supply. By viewing the cyst structure and content and life cycles, several key distinctions are apparent, and their relative value as characteristics becomes clear once several species in each genus have been studied".

However, the underlying concept of biological classifications is that of evolution. Therefore, an ideal classification should be natural, i.e. it should reflect the phylogenetic relationships of the organisms included in it. The inference of phylogenetic relationships among the genera *Toxoplasma*, *Hammondia* and *Neospora* from phenotypic characters is problematic, because only few species have been described and complete life cycles are known only for *Toxoplasma gondii* and *Hammondia hammondi*. As a consequence, the characters currently used for the taxonomy of these genera are limited in their phylogenetic information content and classifications based on them are highly subjective. By contrast, molecular phylogenetic analyses based on objective data have recently provided valuable information on the phylogeny of protozoa, and have guided research on life cycles, biology and epidemiology of several parasites of medical and veterinary importance [4].

At least two species of the Toxoplasmatinae, *T. gondii* and *Neospora caninum*, are of economic importance, because they may cause abortion or congenital disease in their intermediate hosts.

Both parasites share a similar range of hosts, and an initial molecular phylogenetic analysis based on ssrRNA gene sequence comparison [18] suggested a sister relationship between them. However, *N. caninum* differs distinctly from *T. gondii* in biology and epidemiology, and it has subsequently been shown that ssrRNA gene sequences vary among virulent and avirulent strains of *T. gondii* and that the ssrRNA gene sequence of *N. caninum* falls within this level of heterogeneity [19]. Therefore, the level of divergence among ssrRNA gene sequences is too small to resolve the phylogenetic relationships of *N. caninum* to other tissue cyst-forming coccidia [4, 10, 19]. Several morphological and biological characters suggest that *N. caninum* is more closely related to *Hammondia heydorni* than it is to *T. gondii*. However, in addition to the problems associated with the inference of phylogenetic relationships from phenotypic characters described above, such comparisons of *N. caninum* and *H. heydorni* have been complicated by the incomplete knowledge of their life cycles. It was as late as last year that it was shown that *N. caninum*, like *H. heydorni*, uses a canine as a definitive host [20]. However, the endogenous development of *N. caninum* in the dog is still unknown and there is only little knowledge on the development of *H. heydorni* in intermediate hosts.

Here, we present a phylogenetic analysis based on full-length lsrRNA gene sequences of *N. caninum*, *T. gondii* and the two species of the genus *Hammondia*. The lsrRNA is about twice as long as the ssrRNA and has been shown previously to contain more phylogenetic information with respect to tissue cyst-forming coccidia [12]. Therefore, analyses based on lsrRNA should be less prone to error and provide a more reliable inference of the phylogeny of *N. caninum*.

2. Materials and methods

2.1. Parasites and genomic DNA

The Spandau strain of *Hammondia heydorni* used here was originally isolated from the faeces of a dog that had been fed oesophagi of naturally infected cattle slaughtered at the abattoir of Spandau in Berlin, Germany [21], and was maintained by alternating passages in intermediate (cattle, sheep, goats, guinea-pigs) and definitive hosts (dogs). The Munich strain of *Hammondia hammondi* was originally isolated from mice that had been inoculated with sporulated oocysts derived from the faeces of a naturally infected cat [22], and was maintained by alternating passages in mice and cats. All experimental animals were born and raised in a coccidia-free environment and were housed individually in isolation units during the course of the experiments. To expand the *Hammondia* strains for the analyses described here, mice or guinea-pigs were infected experimentally by oral inoculation of a suspension of 750 or 1000 sporulated oocysts of *H. hammondi* or *H. heydorni*, respectively. A cat was fed on muscles, brains and internal organs taken from five mice 35 days p.i. A dog was fed on muscles, brains and internal organs taken from a guinea-pig 75 days p.i. Unsporulated oocysts of *H. hammondi* or *H. heydorni* were collected from the faeces of the definitive hosts between 6 and 13 days p.i. For DNA extraction, the oocysts were enriched from faecal samples by flotation in a saturated zinc sulphate solution and further purified by discontinuous density gradient centrifugation using Percoll (Pharmacia), essentially as described previously for the purification of *Sarcocystis* bradyzoites [23]. The oocyst wall was disrupted by ultrasonication and genomic DNA was extracted from the sonicate using the QIAamp Tissue Kit, following the protocol provided by the manufacturer (Qiagen).

Genomic DNA of the Berlin strain of *Sarcocystis tenella* was extracted from bradyzoites derived from the skeletal muscles of an experimentally infected sheep, as described elsewhere [24]. The ME49 strain of *T. gondii*, which is avirulent when inoculated into mice, was grown in cell culture and genomic DNA was prepared from it as described [25].

2.2. PCR amplification, cloning and sequencing

Five overlapping fragments of the lsrRNA genes of *H. heydorni*, *H. hammondi*, *S. tenella* and *T. gondii* ME49 were amplified by PCR, as described previously [12]. These fragments encompass the lsrRNA gene from nucleotides 24–42 to nucleotides 4337–4354 (murine numbering system). The five primer pairs, PCR protocols, cloning and sequencing methods, as well as the specificity of the primers for Apicomplexa, have been reported [12].

2.3. Phylogenetic analysis

The full-length lsrRNA gene sequences of *H. heydorni*, *H. hammondi*, *S. tenella* and the *T. gondii* ME49 strain were obtained in this study. The lengths of the sequences varied from 3214 bp in *H. heydorni* to 3482 bp in *S. tenella*. In addition, the lsrRNA gene sequences of *N. caninum*, *Isospora felis*, *Eimeria tenella* and the *T. gondii* RH strain, which is virulent when inoculated into mice, were obtained from GenBank℗ (accession numbers AF001946, U85706, AF026388 and X75429, respectively).

The sequences were aligned by two alignment methods, i.e. according to their primary structure using ClustalW and according to their secondary structure using the DCSE program by De Rijk and De Wachter [26]. Both alignments were then subjected to three different tree-building methods, i.e. maximum parsimony (PAUP) [27], the distance method neighbor-joining with Kimura distance [28] and maximum likelihood (DNAml) [29]. The Kimura two-parameter distance model was used for maximum likelihood and neighbor-joining analyses, and the transition/transversion ratio for maximum likelihood was 2.000. *Eimeria tenella* was the chosen outgroup for all of the analyses.

Proportional nucleotide distance values between the different taxa were calculated from the secondary structure alignment using 'dnadist' from the PHYLIP package [J. Felsenstein, PHYLIP (Phylogeny Inference Package), University of Washington, Seattle, 1995].

3. Results

The secondary structure alignment of the full-length lsrRNA gene sequences of *N. caninum*, *H. heydorni*, *H. hammondi*, *T. gondii* ME49, *T. gondii* RH, *I. felis*, *S. tenella* and *E. tenella* contained 3478 positions, while the ClustalW alignment consisted of 3471 positions.

Using the ClustalW alignment, all of the three tree-building analyses gave a tree with the same topology, which is shown in Fig. 1. In this tree, *N. caninum* and *H. heydorni* form a monophyletic sister clade to a clade formed by *H. hammondi*, *T. gondii* ME49 and *T. gondii* RH. Maximum parsimony took 114 steps and gave consistency index and HI values of 0.944 and 0.056, respectively. The ln value for maximum likelihood was −8630.35313.

Using the secondary structure alignment, maximum likelihood and neighbor-joining analyses again gave a tree with the topology shown in Fig. 1, which showed *N. caninum* and *H. heydorni* to form a monophyletic sister clade to a clade formed by *H. hammondi*, *T. gondii* ME49 and *T. gondii* RH. Maximum parsimony gave four equally parsimonious trees, which all still supported the paraphyly of the genus *Hammondia*, and the consensus is shown in Fig. 2. Three of these four trees placed *H. hammondi* as the sister taxon to the clade including the *T. gondii* strains, while only one of the trees could not resolve the relationship between these three taxa, although it still grouped them together. The other point where the four trees did not agree is in the placement of *N. caninum* and *H. heydorni* with respect to the *H. hammondi*/*T. gondii* clade. As a result, the consensus tree in Fig. 2 shows two polytomies, one including *H. hammondi*, *T. gondii* ME49 and *T. gondii* RH, and the other including the former and *N. caninum* and *H. heydorni*. The number of steps in this analysis was 504 with consistency index and HI values of 0.941 and 0.059, respectively. Maximum likelihood had a ln value of −9404.44649.

The proportional nucleotide distance values between *N. caninum*, *H. heydorni*, *H. hammondi*, *T. gondii* ME49, *T. gondii* RH, *S. tenella*, *I. felis*

and *E. tenella* are shown in Table 1. Table 2 shows a range of morphological and life cycle characters of *N. caninum*, *H. heydorni*, *H. hammondi* and *T. gondii*.

4. Discussion

The taxonomy and classification of tissue cyst-forming coccidia have been the subject of discussion and controversy for many years. Although the tissue cyst or oocyst stages of some cyst-forming coccidia have been known for almost a century, it was only about two decades ago that heteroxenous life cycles were discovered for these parasites [4]. For example, knowledge on the life cycle of *T. gondii*, the asexual stages of which have been known since the beginning of the century, was completed only in the early 1970s. Since then, several other protozoa that had been assigned to the genus *Toxoplasma* during the first half of this century, have either been synonymised with *T. gondii*, been reclassified into other coccidian genera (such as *Atoxoplasma* or *Isospora*), or their descriptions superseded [2, 4, 30].

Until recently, *T. gondii* was generally considered as the only valid species of *Toxoplasma* [1, 3–7]. However, molecular epidemiological studies have now provided evidence that there are at least two clonal lineages within *T. gondii*, one comprising strains that are virulent in mice and another comprising strains that are
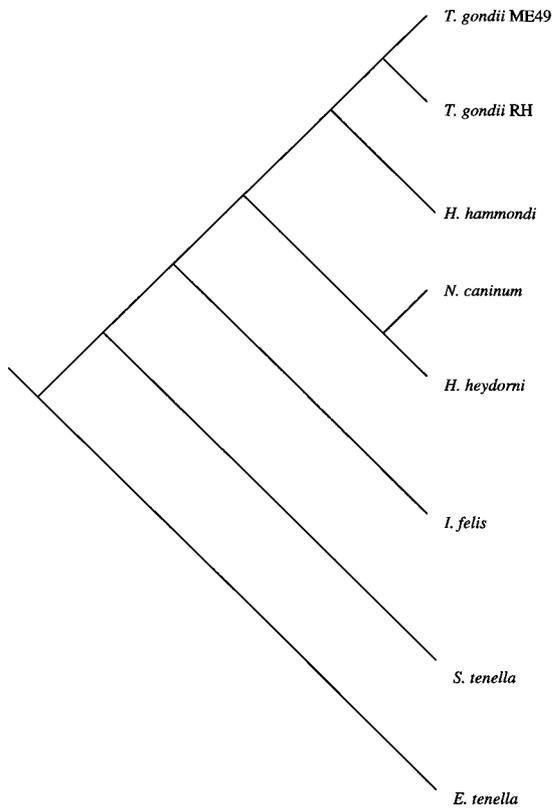

Fig. 1. Phylogenetic tree reconstructed from the ClustalW alignment of the full-length lsrRNA gene sequences by PAUP, maximum likelihood and neighbor joining, and from the DCSE secondary structure alignment by maximum likelihood and neighbor joining.
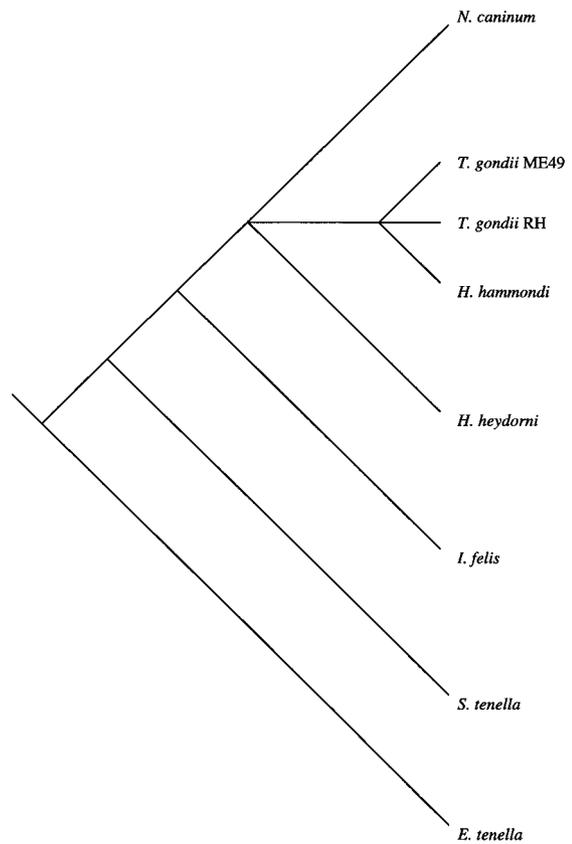

Fig. 2. Consensus tree reconstructed from the DCSE secondary structure alignment by PAUP.

Table 1
Proportional nucleotide distances among the lsrRNA gene sequences of two strains of *T. gondii* (RH and ME49), *H. hammondi*, *H. heydorni*, *N. caninum*, *I. felis*, *S. tenella* and *E. tenella*

	T. gondii (RH)	*T. gondii* (ME49)	*H. hammondi*	*H. heydorni*	*N. caninum*	*I. felis*	*S. tenella*
T. gondii (ME49)	0.0016						
H. hammondi	0.0022	0.0013					
H. heydorni	0.0069	0.0059	0.0060				
N. caninum	0.0050	0.0044	0.0050	0.0053			
I. felis	0.0824	0.0818	0.0807	0.0821	0.0830		
S. tenella	0.1440	0.1433	0.1433	0.1440	0.1431	0.1469	
E. tenella	0.1522	0.1516	0.1508	0.1525	0.1523	0.1529	0.1940

avirulent in mice [31]. This finding has raised debate on whether or not the different lineages within *T. gondii* are indicative of ongoing speciation [31].

The genus *Hammondia* was introduced in 1975 when the life cycle of its type species, *H. hammondi*, was elucidated [32]. Its differentiation from *Toxoplasma* has been based firstly on the different location of the tissue cyst, which occurs predominantly in muscles, whereas that of *T. gondii* occurs predominantly in the brain. Secondly, the life cycle of *H. hammondi* is obligately heteroxenous, whereas that of *T. gondii* is facultatively heteroxenous [32]. However, the complete life cycle is known only for *H. hammondi*, while there is still little knowledge of the life cycle of *H. heydorni*, the second species of the genus *Hammondia* [7, 14, 15, 33]. Other coccidia that had been erroneously described as life cycle stages of *Hammondia* species, such as *H. pardalis*, have later been interpreted as meronts belonging to species of *Sarcocystis* and oocysts belonging to species of *Isospora*, a case which has been well argued [34]. These species descriptions are now obsolete, so that the genus *Hammondia* currently consists of only two named species [7, 34].

Neospora is the most recent genus of tissue cyst-forming coccidia. It was introduced only in 1988 and was then differentiated from *Toxoplasma* and *Hammondia* based on ultrastructural and antigenic characters. In contrast to *T. gondii*, tachyzoites of the type species *N. caninum* have several rhoptries, and no cross-reactivity was observed between these tachyzoites and a serum derived from a *T. gondii*-infected dog in that study [13]. In addition, the tachyzoites of *N. caninum* were found to be located directly in the host cell cytoplasm without a parasitophorous vacuole. This was thought to be a distinct feature that would allow the differentiation of *N. caninum* from both *T. gondii* and *H. heydorni*, whose tachyzoites are located within a parasitophorous vacuole [13]. However, in its original description *N. caninum* tachyzoites located in host cells of naturally infected dogs were compared with *H. heydorni* tachyzoites cultured in bovine monocytes [13], because the morphology of the extraintestinal stages of *H. heydorni* in its canine host is unknown. More recent studies showed that most tachyzoites of *N. caninum* cultured in various host cells, including bovine monocytes, when examined in vitro, are in fact also located in a parasitophorous vacuole [35]. Therefore, this character can no longer be regarded as valid for a differentiation between *N. caninum* and *H. heydorni*.

By contrast, there is a similarity in ultrastructure between the tachyzoites of *N. caninum* and those of *H. heydorni*. For example, both *N. caninum* and *H. heydorni* tachyzoites have rhoptries and electron-dense bodies that are located anteriorly and posteriorly to the tachyzoite nucleus, whereas these organelles are restricted to the anterior end of the tachyzoite in *H. hammondi* and *T. gondii* [35–39].

There are many questions concerning the relationships between *N. caninum* and the species in the genera *Hammondia* and *Toxoplasma* that cannot be answered at present. It appears that several morphological and life cycle characters

Table 2
Phenotypic characters of current species of the genera Neospora, Hammondia and Toxoplasma

Character	N. caninum	H. heydorni	H. hammondi	T. gondii
Geographical distribution	Probably worldwide	Probably worldwide	Probably worldwide	Worldwide
Host range				
Definitive hosts	Dog	Canids	Felids	Felids
Intermediate hosts	Many mammals	Many mammals	Many mammals	Mammals and birds
Type of life cycle	Probably obligately heteroxenous	Probably obligately heteroxenous	Obligately heteroxenous	Facultatively heteroxenous
Type of development				
In definitive host	?	Endopolygeny and gamogony	Endopolygeny and gamogony	Endodyogeny, endopolygeny and gamogony
In environment	Sporogony	Sporogony	Sporogony	Sporogony
In intermediate host	Endodyogeny	Endodyogeny	Endodyogeny	Endodyogeny
Natural route of transmission				
Definitive to intermediate host	Via oocysts	Via oocysts	Via oocysts	Via oocysts
Intermediate to definitive host	Via tissue cysts	Via tissue cysts	Via tissue cysts	Via tissue cysts or tachyzoites
Definitive to definitive host	?	No transmission	No transmission	Via oocysts
Intermediate to intermediate host	?	?	No transmission	Via tissue cysts or tachyzoites
Vertical transmission in intermediate host	Yes	?	?	Yes
Oocyst	Disporous, tetrazoic	Disporous, tetrazoic	Disporous, tetrazoic	Disporous, tetrazoic
Size of oocyst (unsporulated; µm)	10.5–12.5×10.5–12	10–14.5×9–13	11–13×10.5–12.5	11–14×9.5–11
Size of sporocyst (µm)	7.5–9.5×5.5–6.5	5.5–9.5×4.5–6	8–11×6–7.5	8–9.5×5–6.5
Tissue cyst				
Location	CNS	Brain	Striated muscles, brain	Many tissues
Size of tissue cyst in CNS (µm)	Up to 107	At least 20	Up to 25[a]	Up to 50[b]
Thickness of tissue cyst wall (µm)	1–4	?	< 0.5	< 0.5
Septa	Absent	Absent	Absent	Absent
Size of bradyzoites (µm)	6–8×1–2	?	4–7×2	7–8×1.5
Tachyzoites in natural host				
Location	Many cell types	?	Lymphoid cells	Many cell types
Size (µm)	3–7×1–5	?	?	3.5–7×2–4
Parasitophorous vacuole	Absent or present	?	?	Present
Tachyzoites in cell culture				
Parasitophorous vacuole	Present	Present	Present	Present
Type of multiplication	Endodyogeny	Endodyogeny	Endodyogeny	Endodyogeny
Rhoptries anterior to nucleus	8–18	6–12	< 10	4–8
Rhoptries posterior to nucleus	Few	6–12	Absent	Absent
Micronemes anterior to nucleus	Many (up to 150)	?	Few	Few
Micronemes posterior to nucleus	Few	?	Absent	Absent
Micropores	Rare	?	Common	Common
Pathogenicity	Yes	?	?	Yes
Major cause of abortion	In cattle	?	?	In sheep and goats
Cross-immunity	?	?	With T. gondii	With H. hammondi

[a] Tissue cysts may be up to 25 µm in length in the brain and up to 400 µm in muscles.
[b] Tissue cysts may be up to 50 µm in length in the brain and up to 100 µm in muscles.
?, Character state is unknown.
Data listed in this table are taken from [13–15, 20, 32, 35–39, 53, 55, 56].

show more similarities between *N. caninum* and *H. heydorni* than between *N. caninum* and *T. gondii* or between the two species of *Hammondia*. However, it is not possible to directly compare any corresponding life cycle stages of these parasites in their natural hosts, because knowledge of the life cycle phase in the intermediate host is incomplete for *H. heydorni* and the life cycle phase in the definitive host is unknown for *N. caninum*. In addition, no epidemiological comparisons can be made due to a lack of diagnostic tests for the *Hammondia* species. Therefore, there are still many gaps in our knowledge of these genera.

The use of more characters, especially those of a molecular nature, are likely to add to our understanding of how much weight should be placed on the phenotypic characters described above when classifying taxa of tissue cyst-forming coccidia. Analyses based on ssrRNA gene sequences showed that *N. caninum* and *T. gondii* are much more closely related phylogenetically than had been thought from phenotypic comparisons, and it has been suggested that these two species have only recently diverged from each other and that both species may be placed into the same genus [18, 40]. To date, no ssrRNA gene sequence of a species of *Hammondia* has been available for phylogenetic analysis. However, Johnson et al. [41] could not differentiate *H. hammondi* from *T. gondii* by comparison of the GC content and restriction patterns of the ssrRNA gene. They suggested that *H. hammondi* may be either a strain of *T. gondii* or a separate species within the genus *Toxoplasma*. By contrast, isoenzyme analysis indicated distinct genetic differences between *H. hammondi* and *T. gondii* at a specific level [42], and the small level of divergence between the ssrRNA gene sequences of *T. gondii* and *N. caninum*, which falls within the level of heterogeneity observed among the ssrRNA gene sequences of different strains of *T. gondii*, suggests that ssrRNA is not a suitable character for phylogenetic analyses of these taxa [4, 19]. Clearly, there is a need for information on more phylogenetically valid characters to elucidate the relationships among the

species classified into the genera *Toxoplasma*, *Hammondia* and *Neospora*.

A recent analysis of internal transcribed spacer 1 sequences and partial lsrRNA gene sequences using maximum parsimony and spectral analysis suggested the genus *Hammondia* to be paraphyletic, and found a closer relationship between *H. hammondi* and *T. gondii* than between either of those two parasites and *H. heydorni* or *N. caninum* [43]. However, that analysis contained only few phylogenetically informative sites, and the use of a larger data set is needed to corroborate and increase the robustness of these results.

In the present study, we used phylogenetic information derived from full-length lsrRNA gene sequences of *N. caninum*, the two *Hammondia* species, and two strains that represented the virulent and avirulent lineage of *T. gondii*. To make this analysis as robust as possible, we analysed these sequences using two different alignment strategies and three different tree-building methods. The DCSE alignment is based on the secondary structure of the lsrRNA molecule, which takes into account major structural features when aligning the sequences [26]. This approach is thought to stay more faithful to comparing truly homologous characters.

All of these analyses produced trees that supported the paraphyly of the genus *Hammondia*. Specifically, *H. hammondi* and the two strains of *T. gondii* formed a monophyletic sister clade to *N. caninum* and *H. heydorni*. Five of the six trees reconstructed here suggested that the genera *Toxoplasma*, *Hammondia* and *Neospora* comprise two lineages corresponding to definitive host specificity. The first lineage comprised *H. hammondi* and the two strains of *T. gondii*, all of which are transmitted by felids, and the second lineage comprised *H. heydorni* and *N. caninum*, both of which are transmitted by canids. Only one of the trees, which was derived from a maximum parsimony analysis of the secondary structure alignment, gave a different result. This tree also showed a monophyletic relationship among the two *T. gondii* strains and *H. hammondi*, and thus supported the paraphyly of the genus *Hammondia*, but it did not resolve the relationship between the *T. gondii* species and *H. ham-*

mondi, or discriminate whether *N. caninum* or *H. heydorni* is the closest sister taxon to the former clade.

The close phylogenetic relationship between *H. hammondi* and *T. gondii* is further supported by the low genetic distances observed among their lsrRNA gene sequences. In fact, the genetic distance between the lsrRNA genes of the two *T. gondii* strains (0.0016) is about the same level as the genetic distances observed between the lsrRNA gene of either of those strains and that of *H. hammondi* (0.0013 and 0.0022). There are several phenotypic characters that also support a close relationship between *H. hammondi* and *T. gondii*. Like *T. gondii*, *H. hammondi* uses domestic and wild cats as definitive hosts and a wide range of mammals as intermediate hosts. Rats, goats and roe deer have been identified as natural intermediate hosts of *H. hammondi*, and a broad range of small rodents, as well as rabbits, pigs, goats, sheep, dogs and monkeys, have been used as experimental intermediate hosts [14, 15]. *Hammondia hammondi* tissue cysts develop in striated muscles and in the brain of the intermediate host. In addition, there are antigenic similarities between *H. hammondi* and *T. gondii* [44, 45]. A serological cross-reactivity between them has been observed for infections in several intermediate hosts, and a previous infection with *H. hammondi* can induce protective cross-immunity against *T. gondii* in mice, hamsters and goats [14, 15]. Thus, the major difference between *T. gondii* and *H. hammondi* appears to be that the life cycle of *T. gondii* is facultatively heteroxenous, whereas that of *H. hammondi* is obligately heteroxenous, i.e. there is no transmission between intermediate hosts or between definitive hosts [32]. However, it has already been suggested that the degree of heteroxeny is not a discriminating character of tissue cyst-forming coccidia at the generic level [1, 16]. For example, the closely related genus *Besnoitia* comprises species that are transmissible between intermediate hosts, and thus are facultatively heteroxenous, as well as species that are not transmissible between intermediate hosts [1].

There are also phenotypic characters that differentiate *H. hammondi* from *H. heydorni*.

Definitive hosts of *H. heydorni* are dogs, foxes and coyotes [14]. Intermediate hosts are herbivorous animals, such as cattle, sheep, goats, water buffaloes, reindeer, roe deer, moose, camels, horses and guinea-pigs [14, 33]. Definitive hosts have been found to shed oocysts of *H. heydorni* after ingestion of tissue from intermediate hosts, but not after ingestion of oocysts from other definitive hosts [46]. However, the ingestion of *H. heydorni* oocysts by dogs results in the formation of infectious stages in extraintestinal canine tissues that induce oocyst formation when being fed to other dogs [15]. Such extraintestinal stages in definitive hosts are absent in *H. hammondi*.

Thus, *H. heydorni*, like *N. caninum*, has the ability to use dogs as intermediate as well as definitive hosts, and the close phylogenetic relationship between these two parasites found here suggests that other canids, such as foxes and coyotes, should be investigated with respect to their ability to transmit *N. caninum*. The prevalence of patent *H. heydorni* infections in different dog populations varies from 0.2% in Europe to 7.4% in South America [33, 47–49]. The oocysts of *H. heydorni* are morphologically indistinguishable from those of *N. caninum* [20, 46], and this raises the question of how many of the previously reported patent infections of *H. heydorni* in dogs could have been misidentified and may in fact have been cases of dogs shedding oocysts of *N. caninum*.

In addition to the ultrastructural characters described above, there are other phenotypic characters that also support a close relationship between *H. heydorni* and *N. caninum*. Although both parasites appear to be widely distributed in many animals, levels of infection with either parasite are very low in intermediate hosts. Natural *H. heydorni* infections in intermediate hosts have been reported from Europe, Africa, Asia and the Americas [46, 47, 50, 51], but their presence usually needs to be confirmed by bioassays [52, 53]. As a consequence, little is known about the prevalence of *H. heydorni* infections in these animals.

Thus, the results obtained by the phylogenetic analyses in this study, as well as comparisons of phenotypic characters, support the hypothesis

that *N. caninum* and *H. heydorni* are more closely related to each other than either is to *T. gondii* or *H. hammondi*. This questions the validity of the current taxonomy of the genera *Toxoplasma*, *Hammondia* and *Neospora*. As described above, the genus *Hammondia* has not been generally accepted and some authors have suggested that it should be synonymised with *Toxoplasma* [16, 17]. However, this would not solve the problem of paraphyly of the current taxa in the Toxoplasmatinae, unless the genus *Neospora* was also synonymised with *Toxoplasma*. There are several phenotypic characters that may support a synonymy of these genera. For example, all three genera appear to be family specific for their definitive hosts, their life cycle phase in the definitive host (as far as it is known) is almost identical, and their oocysts are morphologically indistinguishable. In addition, they have a very low intermediate host specificity, often infecting the same range of intermediate hosts. The similarity of these phenotypic characters is supported by the low values of genetic distances (0.0013– 0.0069) among the lsrRNA genes of the species in the genera *Toxoplasma*, *Hammondia* and *Neospora* examined here, and there are only few species that have been classified into these genera.

As Levine [16] said: "There is no reason why any genus should necessarily contain only one species". However, biological classifications are in part a matter of phylogenetic relationships and in part a matter of convenience. Since its first description in 1988, almost 500 papers have been published on *Neospora*, and neosporosis is now a well-recognised disease in important livestock and domestic animals. Thus, while the phylogenetic relationships of the species studied here may call for a revised classification that synonymises the genera *Hammondia* and *Neospora* with *Toxoplasma*, convenience may call for the retention of at least two different genera, one that uses felids as definitive hosts and another that uses canids as definitive hosts.

A morphological character that has been used frequently to differentiate tissue cysts of *N. caninum* from those of *T. gondii* and *H. hammondi* is the thickness of their wall. While the latter two species have a thin tissue cyst wall ($< 1 \mu$m), the wall of mature tissue cysts of *N. caninum* is much thicker (up to 4 μm). Recently, a second species of *Neospora*, *N. hughesi*, has been described based on few morphological and molecular characters [54]. However, its complete life cycle is unknown and thus there is only limited information on this new species. Moreover, the identity and morphology of the life cycle stages of *H. heydorni* in the intermediate host is uncertain, and so far there is only one report on tissue cysts of *H. heydorni* [14, 15, 53]. However, those tissue cysts were very small, they contained only few bradyzoites and thus may not have reached maturity when they were described [53]. Therefore, further studies are required to examine whether the thickness of the tissue cyst wall or the definitive host specificity are valid characters for a taxonomic discrimination between the *T. gondii/H. hammondi* lineage and the *N. caninum/H. heydorni* lineage.

Further studies are required to investigate the definitive relationship between *H. heydorni* and *N. caninum*, and to examine whether the canid-transmitted species comprise a single or different evolutionary lineages. To adequately resolve the taxonomy of these parasites, further analyses should include a range of different isolates of *N. caninum* and *H. heydorni*, and should also include the newly described species *N. hughesi*. In the meantime, there is a great need for diagnostic methods that enable the detection and differentiation of infections with *Hammondia* species from infections with other tissue cyst-forming coccidia, such as *N. caninum*, in intermediate and definitive hosts.

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