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Dog shedding oocysts of *Neospora caninum*: PCR diagnosis and molecular phylogenetic approach☆

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Abstract

Results of molecular determination of a dog isolate of *Neospora caninum* in the Czech Republic are presented. Colorless bisporocystic oocysts measuring 10–13 μm × 10–11 μm were recovered from feces and used for DNA isolation. A diagnostic PCR procedure using previously described molecular methods was performed to determine the species. The *N. caninum* species-specific primers based on the Nc5 region produced a positive result, while primers specific for *Hammondia heydorni* rDNA internal transcribed spacer 1 (ITS1) was negative. Sequencing and phylogenetic comparison of ITS1 rDNA and the D2 domain of the large subunit rDNA (D2 LSU) determined our isolate to be *N. caninum*. Phylogenetic analysis of closely related genera *Toxoplasma*, *Neospora* and *Hammondia* based on ITS1 and D2 LSU robustly distinguished three clades: (i) *Toxoplasma gondii* + *Hammondia hammondi*, (ii) *N. caninum* + *Neospora hughesi*, and (iii) *H. heydorni*. Based on phylogenetic relationships we propose three acceptable suggestions to solve the problem of taxonomy of these genera.
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Keywords: *Neospora*; *Hammondia*; *Toxoplasma*; Coccidia; Species-specific PCR; Identification; Diagnosis

☆ Nucleotide sequence data reported are available in the GenBank data base under the accession numbers AF432123–AF432126, AF487893.

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1. Introduction

Dogs are hosts to three groups of *Isospora*-like coccidian parasites that differ in size and shape of the oocyst. Oocysts of *Isospora* spp. form two groups: large (25–35 μm) spindle-shaped oocysts belonging to *Isospora canis*, while intermediate oocysts (17–23 μm) belong to the *Isospora ohioensis*-group (Lindsay et al., 1997). Members of this group are clinically important for causing diarrhea in puppies. A third group with small (10–13 μm) oocysts is comprised of two members of the subfamily Toxoplasmatinae, namely the clinically important *Neospora caninum* and the non-pathogenic *Hammondia heydorni*. Oocysts of these species are morphologically indistinguishable and the coprological diagnosis in definitive hosts is thus difficult or even impossible. Similar problem with coprological diagnosis has been encountered in cats shedding oocysts of *Toxoplasma gondii* and/or *Hammondia hammondi* (Dubey, 1993).

In contrast to the plethora of papers dealing with *N. caninum*, and the cumulating knowledge about this parasite, *H. heydorni* is still poorly understood (Dubey, 1999; Hemphill and Gottstein, 2000; Schock et al., 2001). A recent report about *Hammondia* sp. from foxes brought some new questions on this taxon (Schares et al., 2002). Whether there are more species within the *H. heydorni* clade has to be further determined, but the fox isolate represents a relevant candidate for a new species. The epidemiology of neosporosis as a clinical disease, as well as its possible conspecificity with *H. heydorni*, and generic affiliation of both taxa are still unsettled issues, being a subject to a lively discussion (Mehlhorn and Heydorn, 2000; Heydorn and Mehlhorn, 2002; Dubey et al., 2002a).

The aim of this report is to summarize our knowledge about the diagnostic approaches for distinguishing *N. caninum* and *H. heydorni* oocysts in the feces of dog and to describe differential PCR procedure for the diagnosis of the *N. caninum*/*H. heydorni* oocysts in the clinical material. Furthermore, the taxonomy of the genera *Toxoplasma*, *Neospora* and *Hammondia* is discussed in the frame of presented phylogenetic reconstruction including the new *N. caninum* and *H. heydorni* isolates.

2. Materials and methods

2.1. Examination of feces

In October 2000, a fresh fecal sample from a 1-year old German Shepherd from Central Bohemia, Czech Republic, was examined using flotation in modified Sheather's sugar solution (s.g. 1.30). For morphometrical comparison, 35 randomly selected oocysts were measured with a calibrated ocular micrometer using an Olympus BX 60 microscope equipped with Nomarski interference contrast optics. Oocysts were further purified by centrifugation, counted with a haemocytometer, and stored in 2.5% (w/v) potassium dichromate at 4 °C for further use.

2.2. DNA isolation

Oocysts were repeatedly washed in PBS and homogenized by grinding with 0.5 mm glass beads (Sigma) for 30 min with an occasional dip in liquid nitrogen. Total DNA was isolated

from the oocysts using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions, producing a final volume of 200 μl.

2.3. Diagnostic PCRs

Diagnostic PCR with the JS4–JS5 primer pair, targeting the species-specific region of the internal transcribed spacer 1 rDNA (ITS1 rDNA) of *H. heydorni* was performed as described previously (Šlapeta et al., 2002). All PCR reactions were performed in a 25 μl volume containing 5 μl of sample DNA, 10 mM dNTPs, 2.5 mM $MgCl_2$, 2.5 μl of 10× PCR Mg-free buffer, 1 U of *Taq* polymerase (Promega), and 10 pmol of each primer. PCR was performed in an Eppendorf thermal cycler (Eppendorf Mastercycler Personal) with conditions as follows: initial denaturation at 94 °C for 5 min, was followed by 35 cycles at 95 °C for 1 min, 65 °C for 1 min, and 74 °C for 1.5 min, with a final extension at 74 °C for 10 min.

We have used the *N. caninum* species-specific primer pair Np21plus–Np6plus which anneals into the Nc5 region, described by Müller et al. (1996). The addition of uracyl DNA glycosylase present in the original protocol was omitted. The following conditions were used: initial denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, 63 °C for 1 min, and 74 °C for 3.5 min, and a final extension at 74 °C for 10 min. This method has been successfully used to detect the *N. caninum* tissue cysts as well as sporozoites. The sensitivity threshold of the *N. caninum* and *H. heydorni*-specific protocols was a single zoite and 10 oocysts, respectively. *N. caninum* NC-1 and *H. heydorni* CZ-1 served as positive controls, and PCR reactions without DNA served as negative control.

2.4. Amplification and sequence analysis of ITS1 and D2 LSU rDNA

Amplification of ITS1 rDNA and the D2 domain (D2) of the large subunit rDNA (LSU rDNA) was performed using the JV2(REV)-TIM11 and CR1–CR2 primer pairs, respectively (Payne and Ellis, 1996; Ellis et al., 1998). JV2(REV) is a reverse complement to JV2, annealing to the conserved 5′-end of the small subunit rDNA (Šlapeta et al., 2002). PCR was performed in Eppendorf thermal cycler under the following conditions: initial denaturation at 94 °C for 5 min, amplification by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 74 °C for 2 min, and a final extension at 74 °C for 10 min. Amplicons were run on 1.5% agarose gel in the presence of ethidium bromide, gel-purified (Qiagen), and cloned using the TOPO-TA cloning kit (Invitrogen). Three separate clones were sequenced from both strands on automatic sequencing machine (Beckman).

The D2 LSU rDNA was further amplified from previously identified *H. heydorni* isolates CZ-1, CZ-2 and CZ-3 (Šlapeta et al., 2002). Oocysts of CZ-1, CZ-2 and CZ-3 isolates were originally recovered from dog feces in the Czech Republic in a parasitological survey in 1999. Amplification, cloning and sequencing of D2 LSU rDNA was performed as described.

Sequences assembled using the program SeqMan II (DNAStar Inc., Madison, WI, USA) were submitted to the BLAST search at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). For detailed analysis all closely related sequences were aligned using Clustal X (Thompson et al., 1997) and analyzed using the program MEGA2.1 (S. Kumar, K. Tamura, I.B. Jakobsen, M. Nei, 2001, MEGA2: Molecular Evolutionary Genetics Analysis software,

Arizona State University, Tempe, AZ, USA). Sequence alignments are available upon request or are at ftp://vfu.cz/slapeta/alignments/cz4.

3. Results

3.1. Microscopic examination

Oocysts obtained from the dog's feces were colorless, almost spherical, 11.5 (10–13) × 10.8 (10–11) μm, with a thin (<1 μm) single-layered oocyst wall. They contained two broadly oval tetrazoic sporocysts, 9.7 (9–10) × 6.6 (6–7) μm in size. A total of 10^6 oocysts were recovered using the sugar-concentration technique. This isolate is further referred to as CZ-4.

3.2. Diagnostic PCR for N. caninum and H. heydorni

Approximately 3×10^3 oocysts of CZ-4 were used for total DNA isolation. The DNA was first subjected to PCR with the JS4-JS5 primers. This reaction, which is diagnostic for *H. heydorni*, was negative for CZ-4. However, another PCR reaction performed with primers Np6plus–Np21plus yielded a ∼350 bp long abundant product. The amplicon is characteristic for the presence of the *N. caninum* DNA. Importantly, an additional band of ∼370 bp, that was significantly less abundant, was also amplified. The intensity and size of both bands was identical in the CZ-4 and the *N. caninum* (NC-1) isolates (Fig. 1).

3.3. Sequence analysis of ITS1 and D2 LSU rDNA

When the 441 bp long ITS1 rDNA sequence of CZ-4 was subjected to a BLAST search, it gave the best score with *N. caninum*. The total of 22 sequences of related ITS1 rDNAs from the genera *Toxoplasma*, *Neospora* and *Hammondia* was aligned for a detailed comparison. The alignment consisted of 459 residues, out of which 204 residues were variable. The obtained CZ-4 sequence was identical with the sequences AF038860–AF038861, AF249968–AF249970 and AF029702, and differed in only three residues from the sequences U16159–U16160, all of which are assigned to *N. caninum*. The ITS1 rDNA sequences of the *Neospora hughesi* isolate from horses (AF038859 and AF249967) differ from CZ-4 in 7 and 10 residues, respectively (Table 1). In phylogenetic reconstruction, CZ-4 clearly groups with the *N. caninum*-clade (Fig. 2a).

We have also cloned and sequenced the D2 domain of LSU rDNA of CZ-4, which comprised of 582 bp. As expected, the BLAST search gave the best score with the *N. caninum* sequences. For a comparative analysis, sequencing was extended to the isolates of *H. heydorni* CZ-1, CZ-2 and CZ-3. All of these isolates were, based on their ITS rDNA sequence, determined as *H. heydorni* (Šlapeta et al., 2002). A total of 20 D2 LSU rDNA sequences of *Toxoplasma*/*Neospora*/*Hammondia* spp. were aligned into a 587 residues-long alignment which contains only 30 variable sites. The 2D LSU rDNA sequence of CZ-4 was identical with the *N. caninum* AF249971–AF249972 sequences, while it differed in three residues from *N. caninum* AF001946. The D2 LSU rDNA sequences of the *H. heydorni* isolates

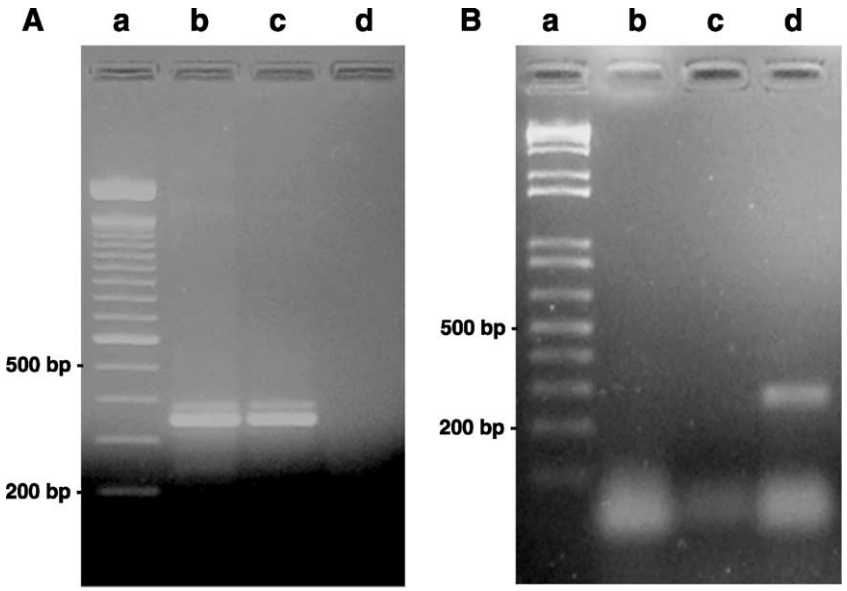

Fig. 1. (A) Amplification with the Np6p–Np21p primer pair specific for *N. caninum*. Note that with both the CZ-4 isolate and the NC-1 isolate, used as a positive *N. caninum* control, identical bands (∼ 350 and 370 bp) were obtained. *H. heydorni* gave a negative result. (B) Amplification with the JS4–JS5 primer pair specific for *H. heydorni*. Note that the only amplification band (∼280 bp) produced was with the *H. heydorni* positive control. Lanes: (a) 100 bp DNA ladder; (b) *N. caninum* NC-1 (control DNA); (c) isolate CZ-4; (d) *H. heydorni* (control DNA).

CZ-1, CZ-2 and CZ-3 were identical. When compared with the other *H. heydorni* LSU rDNA sequences available in the GenBank, there is either one (AF076870) or no difference (AF159240, AF096502). Importantly, the CZ-1, CZ-2 and CZ-3 sequences differed in nine residues from the CZ-4 sequence. Phylogenetic analysis based on these sequences clearly

Table 1
Summary of a pair-wise comparison of the ITS1 and D2 LSU rDNA sequences of *Toxoplasma*, *Neospora* and *Hammondia*[a]

	T. gondii-clade	*N. caninum*-clade	*H. heydorni*-clade
ITS1 rDNA			
T. gondii-clade	0–17	∼25%	∼30%
N. caninum-clade	121–110	0–13	35%
H. heydorni-clade	133–147	149–167	2–9
D2 LSU rDNA			
T. gondii-clade	0–8	∼1.8%	∼2.0%
N. caninum-clade	6–14	0–3	∼1.7%
H. heydorni-clade	6–16	6–18	0–5

[a] Distances in lower left are number of differences (including gaps), and in upper right are distances using the Kimura-2 parameter (in %). *T. gondii*-clade includes *H. hammondi*; *N. caninum*-clade includes the CZ-4 isolate and *N. hughesi*; *H. heydorni*-clade includes the *Hammondia* sp. (FOX2000) isolate.

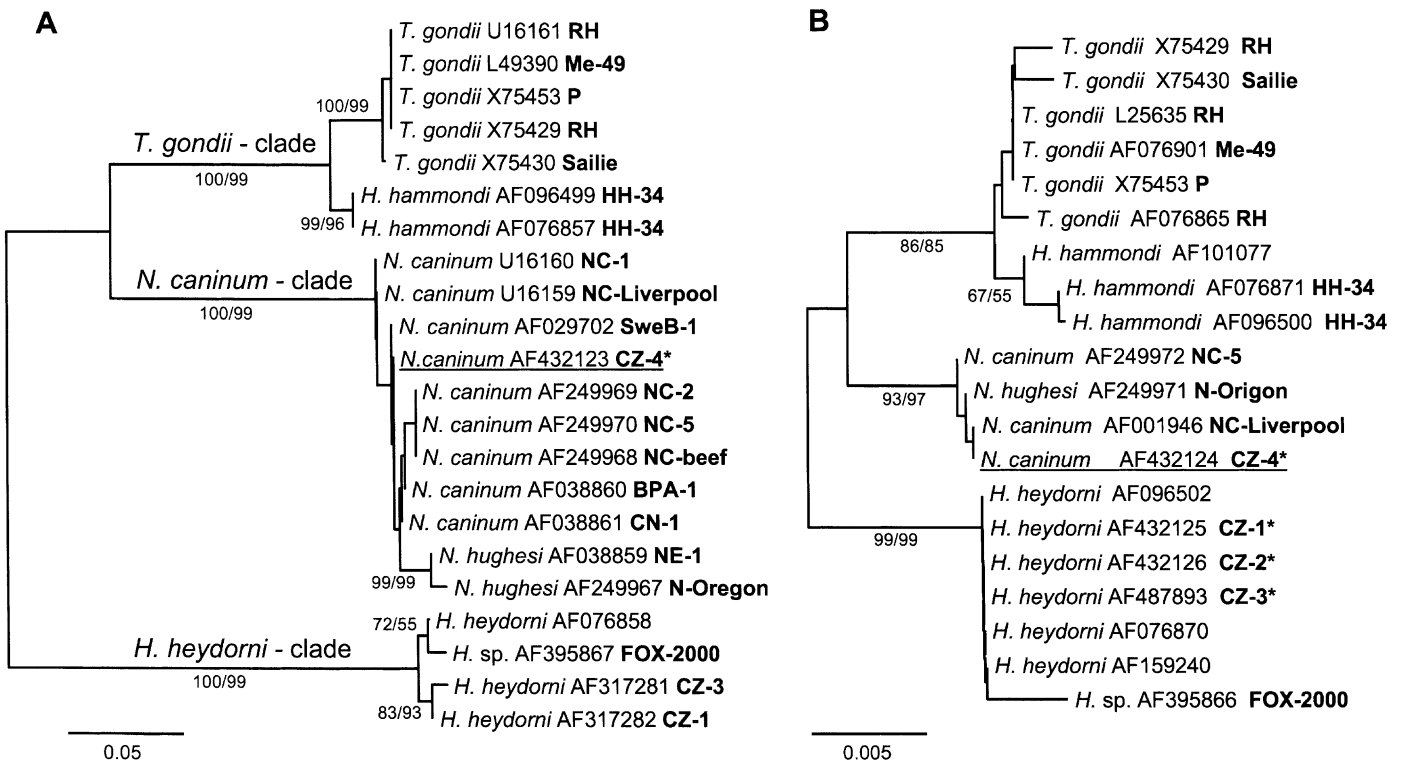

Fig. 2. Phylogram of the relationships between coccidians belonging to the subfamily Toxoplasmatinae with regard to the position of CZ-4 (underlined). The unrooted trees were constructed using the minimum evolution method, Kimura 2-parameter. Numbers at the branches represent bootstrap support using minimum evolution with 1000 replicates/maximum parsimony with 500 replicates. If available, designation of a strain/isolate is given in bold. Sequences obtained within this study are marked with an asterisk (*). (A) Tree constructed with the ITS1 rDNA sequences. Note a robust division into three distinct clades. (B) Tree constructed with the D2 LSU rDNA sequences. Note the congruence with the ITS1 rDNA-based tree, even when D2 LSU rDNA contains much less variable sites. The tree scales are 10:1. In both trees, the CZ-4 isolate clearly clusters with the *N. caninum*-clade.

groups CZ-4 in the *N. caninum*-clade, while the affiliation of the CZ-1, CZ-2 and CZ-3 isolates with the *H. heydorni*-clade is strongly supported (Fig. 2b).

3.4. Phylogenetic relationships within Toxoplasma, Neospora and Hammondia

Analysis of both genetic markers recovered three distinct clades (the *T. gondii*-, the *N. caninum*-, and the *H. heydorni*-clade). Pair-wise differences between these clades are significant (Table 1), while each of them represents a rather homogeneous assembly (Table 1; diagonal cells). To further explore relationship of the above mentioned clades, we rooted our phylogenetic trees on the closest known sister taxon - the cyst-forming coccidium of the genus *Besnoitia* (Ellis et al., 2000). Although ITS1 rDNA possesses enough variables within *Toxoplasma*/*Neospora*/*Hammondia* (204), due to high divergence, the closest sister taxa (*Besnoitia* spp.) cannot be robustly aligned (Ellis et al., 1999). The D2 LSU rDNA alignment, in which the three genera differ in 30 variables only, was extended by the addition of *B. besnoiti* AF076900 and *B. jellisoni* AF076868 as outgroups. In such a tree, the *N. caninum*- and *H. heydorni*-clades are monophyletic with *T. gondii* representing a sister clade (data not shown). However, monophyly of the *N. caninum*/*H. heydorni* clade was supported only by low (<50%) bootstrap values.

4. Discussion

The morphology of oocysts, combined with the results of a diagnostic PCR reaction, and the sequence analysis clearly demonstrate that the CZ-4 isolate is conspecific with the organism referred to as *N. caninum*. Although dogs were experimentally shown to act as definitive hosts of *N. caninum*, the first report of oocysts being shed by a naturally infected dog was reported only recently from Argentina (Basso et al., 2001). As far as we are aware, herein we describe the first case of the *N. caninum* oocysts recovered from dog's feces outside the New World. Available diagnostic tests enabled us to quickly and unambiguously determine the isolate. The importance of positive and negative controls in diagnostic PCR cannot be overstated, and at the same time possible mixed infections have to be considered.

In order to confirm the results obtained by PCR, we have sequenced ITS1 and D2 LSU rDNA of *N. caninum* and D2 LSU rDNA of *H. heydorni*. Both regions have been successfully used to distinguish between closely related species of *Hammondia*, *Neospora* and *Toxoplasma*, as well as among other coccidian genera (Ellis et al., 1998, 1999; Mugridge et al., 2000; Dubey et al., 2001). Analyses of all up to date available sequences of these loci clearly recognize three distinct clades, namely the *N. caninum*-clade (*N. caninum* and *N. hughesi*), the *H. heydorni*-clade (*H. heydorni*) and the *T. gondii*-clade (*T. gondii* and *H. hammondi*). Both loci were informative enough to affiliate our isolates with one of the clades, however, for detailed species detection the ITS1 rDNA is preferable due to its higher variability. Even closely related *N. caninum* and *N. hughesi* (Dubey et al., 2001) differ in the ITS1 rDNA sequence. In contrast, D2 LSU rDNA is too conservative to distinguish between both species and in cases like this cannot be suggested as a marker of choice.

When *Besnoitia* spp., the closest known sister cyst-forming coccidia of veterinary concern (Leighton and Gajadhar, 2001), was used to root our phylogenetic tree, a monophyletic clade of *N. caninum*, *N. hughesi* and *H. heydorni* was recovered. However, using the D2 LSU rDNA domain only, the cladogenesis within these clades was not robust. This is not surprising since D2 LSU rDNA is highly conserved and contains only 30 variables within genera *Toxoplasma*, *Neospora* and *Hammondia*. As pointed out by Mugridge et al. (2000), it is important to use the full-length LSU rDNA for phylogenetic analysis of Sarcocystidae, since the use of partial sequences has its limitations. Nevertheless, our results obtained with D2 LSU rDNA are congruent with the analyses of the full length gene, in which the monophyly of *N. caninum* and *H. heydorni* was supported as well (Mugridge et al., 2000). Importantly, these results render the genus *Hammondia* paraphyletic (Ellis et al., 1999; Mugridge et al., 1999, 2000). Moreover using D2 LSU rDNA, we were able to extend our analysis to the isolates defined previously as *H. heydorni* (Šlapeta et al., 2002), and confirm their affiliation with the *H. heydorni*-clade.

Some inconsistencies with the identity of *N. caninum* were found in recent literature. Heydorn and Mehlhorn (2002) reviewed the available literature about *N. caninum* and *H. heydorni* and concluded that *N. caninum* represents a *nomen nudum*. In contrast, Dubey et al. (2002a) arrived to an opposite conclusion, declaring *N. caninum* and *H. heydorni* separate species. Interestingly, Schares et al. (2001a,b) recently described a series of experiments with the *N. caninum*/*H. heydorni* ("Berlin 1996") isolate and has brought new data to the distinctness of both species (see also Müller et al., 2001; Schares et al., 2002). However, due to the lack of clear bioassays the origin of isolates described in the above-mentioned papers must therefore be taken with caution. The available PCR-assay should be considered prior experiments with oocysts reflecting morphology of *N. caninum*/*H. heydorni* to identify the agent and to rule out multi-infection.

Leaving aside the controversy and focusing on available molecular diagnostic and DNA sequence techniques, *N. caninum* and *H. heydorni* are clearly distinguishable taxa. Published reports that dealt with ITS1 and LSU rDNA sequences of *N. caninum* and *H. heydorni*, and PCR diagnostic techniques found significant differences (Ellis et al., 1999; Mugridge et al., 1999; Hill et al., 2001; Šlapeta et al., 2002). Molecular data presented herein furthermore support these differences.

In contrast to species which are, with some peculiarities specific to parasites, believed to represent biological entities, the genus is a more or less formal classification unit (Kunz, 2002). Depending on their group of interest, taxonomists use various concepts of a genus. The cyst-forming coccidia are considered to form a monophyletic group consisting of two sister clades (Dubey, 1993; Mugridge et al., 2000). Generic boundaries used for the classification within these two clades should be as equal as possible. When comparing both groups, however, one easily realizes that this is not the case, since different taxonomic concepts have in general been adopted for each group. Despite several attempts to use important differences in the life cycle for classification, the enormously diversified coccidia within the Sarcocystinae are still included into a single genus (Odening, 1998). In contrast, very closely related organisms within the Toxoplasmatinae are ranked to different genera *Toxoplasma*, *Hammondia* and *Neospora*. Based on molecular phylogenetic analysis (Ellis et al., 1999; Mugridge et al., 1999, 2000) at least three plausible solutions for the problem are in concordance with the International Code of Zoological Nomenclature (ICZN, 2000):

1. To synonymize the genera *Hammondia* and *Neospora* with the genus *Toxoplasma* and, importantly, retain all currently recognized species (*gondii*, *hammondi*, *heydorni*, *caninum*, *hughesi*) until more data is available.
2. To respect available phylogenetic reconstruction which supports three distinct clades [(*Toxoplasma* and *H. hammondi*) (*Neospora*) (*H. heydorni*)]. Thus, three individual clades would be classified as genera *Toxoplasma* (*T. gondii* and *H. hammondi*), *Neospora* (*N. caninum*, *N. hughesi*), and curiously, new genus would have to be erected to accommodate *H. heydorni*, since the generic name *Hammondia* (typified by *H. hammondi*) falls in synonymy with *Toxoplasma*.
3. Last, to stress the trend of co-evolution with the canine and feline definitive hosts and strictly adhere to phylogenetic reconstruction, [(*H. heydorni* and *Neospora*) (*Toxoplasma* and *H. hammondi*)]. This relationship is weakly supported by the D2 LSU rDNA- and the full-length LSU rDNA-based analyses. However, the co-evolution with definitive host is a feature rather common within Sarcocystidae (e.g. Holmdahl et al., 1999; Doležel et al., 1999). If a similar concept is applied here, *H. hammondi* would be treated as a member of the feline genus *Toxoplasma*, while *H. heydorni* would be transferred into the canine genus *Neospora*.

5. Conclusion

Despite the very similar morphology of oocysts, the combined results of a diagnostic PCR can distinguish between *N. caninum* and *H. heydorni* oocysts. Application of these techniques on the dog isolate CZ-4 demonstrated conspecificity with *N. caninum*. The epidemiology of *N. caninum* is still unresolved and future diagnosis of dog isolates that resemble *Neospora*/*Hammondia* description may lead to new conclusions and re-evaluation of the related coccidia.

Added note: In a recent article, Dubey et al. (2002b) reviewed the status of *N. caninum* and re-described the genus and the species. In this publication, the ITS1 rDNA is cited as a valuable species-specific marker that distinguishes *N. caninum* from *H. heydorni*. This is in agreement with our CZ-4 isolate belonging to *N. caninum*. However, the three plausible solutions reported for the problem with genera *Neospora, Hammondia* and *Toxoplasma* remain valid.

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References

Basso, W., Venturini, L., Venturini, M.C., Hill, D.E., Kwok, O.C., Shen, S.K., Dubey, J.P., 2001. First isolation of *Neospora caninum* from the feces of a naturally infected dog. J. Parasitol. 87, 612–618.

Doležel, D., Koudela, B., Jirků, M., Hypša, V., Oborník, M., Votýpka, J., Modrý, D., Šlapeta, J.R., Lukeš, J., 1999. Phylogenetic analysis of *Sarcocystis* sp. and *Sarcocystis dispersa* supports the co-evolution of sarcocysts with the final hosts. Int. J. Parasitol. 29, 795–798.

Dubey, J.P., 1993. *Toxoplasma*, *Neospora*, *Sarcocystis* and other tissue cyst-forming coccidia of humans and animals. In: Kreier, J.P. (Ed.), Parasitic Protozoa, vol. 6, 2nd ed. Academic Press, New York, pp. 1–131.

Dubey, J.P., 1999. Neosporosis-the first decade of research. Int. J. Parasitol. 29, 1485–1488.

Dubey, J.P., Liddell, S., Mattson, D., Speer, C.A., Howe, D.K., Jenkins, M.C., 2001. Characterization of the Oregon isolate of *Neospora hughesi* from a horse. J. Parasitol. 87, 345–353.

Dubey, J.P., Hill, D.E., Lindsay, D.S., Jenkins, M.C., Uggla, A., Speer, C.A., 2002a. *Neospora caninum* and *Hammondia heydorni* are separate species. Trends Parasitol. 18, 66–69.

Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkås, I., Björkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modrý, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J.L., Lindsay, D.S., 2002b. Redescription of *Neospora caninum* and its differentiation from related coccidia. Int. J. Parasitol. 32, 929–946.

Ellis, J.T., Amoyal, G., Ryce, C., Harper, P.A., Clough, K.A., Homan, W.L., Brindley, P.J., 1998. Comparison of the large subunit ribosomal DNA of *Neospora* and *Toxoplasma* and development of a new genetic marker for their differentiation based on the D2 domain. Mol. Cell. Probes 12, 1–13.

Ellis, J.T., Morrison, D.A., Liddell, S., Jenkins, M.C., Mohammed, O.B., Ryce, C., Dubey, J.P., 1999. The genus *Hammondia* is paraphyletic. Parasitology 118, 357–362.

Ellis, J.T., Holmdahl, O.J., Ryce, C., Njenga, J.M., Harper, P.A., Morrison, D.A., 2000. Molecular phylogeny of *Besnoitia* and the genetic relationships among *Besnoitia* of cattle, wildbeest and goats. Protist 151, 329–336.

Hemphill, A., Gottstein, B., 2000. A European perspective on *Neospora caninum*. Int. J. Parasitol. 30, 877–924.

Heydorn, A.O., Mehlhorn, H., 2002. *Neospora caninum* is an invalid species name: an evaluation of facts and statements. Parasitol. Res. 88, 175–184.

Hill, D.E., Liddell, S., Jenkins, M.C., Dubey, J.P., 2001. Specific detection of *Neospora caninum* oocysts in fecal samples from experimentally-infected dogs using the polymerase chain reaction. J. Parasitol. 87, 395–398.

Holmdahl, O.J., Morrison, D.A., Ellis, J.T., Huong, L.T., 1999. Evolution of ruminant *Sarcocystis*(Sporozoa) parasites based on small subunit rDNA sequences. Mol. Phylogenet. Evol. 11, 27–37.

ICZN, 2000. International Code of Zoological Nomenclature, 4th ed. International Trust for Zoological Nomenclature, London. 306 pp.

Kunz, W., 2002. When is a parasite species a species? Trends Parasitol. 18, 121–124.

Leighton, F.A., Gajadhar, A.A., 2001. *Besnoitia* spp. and besnoitiosis. In: Samuel, W.M., Pybus, M.J., Kocan, A.A. (Eds.), Parasitic Diseases of Wild Mammals, 2nd ed. Mason Publishing/The Veterinary Press, London, pp. 468–478.

Lindsay, D.S., Dubey, J.P., Blackburn, B.L., 1997. Biology of *Isospora* spp. from humans, nonhuman primatesand domestic animals. Clin. Microbiol. Rev. 10, 19–34.

Mehlhorn, H., Heydorn, A.O., 2000. *Neospora caninum*: is it really different from *Hammondia heydorni*or is it a strain of *Toxoplasma gondii*? An opinion. Parasitol. Res. 86, 169–178.

Mugridge, N.B., Morrison, D.A., Heckeroth, A.R., Johnson, A.M., Tenter, A.M., 1999. 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*. Int. J. Parasitol. 29, 1545–1556.

Mugridge, N.B., Morrison, D.A., Heckeroth, A.R., Tenter, A.M., Johnson, A.M., 2000. Effects of sequence alignment and structural domains of ribosomal DNA on phylogeny reconstruction for the protozoan family Sarcocystidae. Mol. Biol. Evol. 17, 1842–1853.

Müller, N., Zimmermann, V., Hentrich, B., Gottstein, B., 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. J. Clin. Microbiol. 34, 2850–2852.

Müller, N., Sager, H., Hemphill, A., Mehlhorn, H., Heydorn, A.O., Gottstein, B., 2001. Comparative molecular investigation of Nc5-PCR amplicons from *Neospora caninum* NC-1 and *Hammondia heydorni*—Berlin-1996. Parasitol. Res. 87, 883–885.

Odening, K., 1998. The present state of species-systematics in *Sarcocystis* Lankester, 1882 (Protista, Sporozoa, Coccidia). Syst. Parasitol. 41, 209–233.

Payne, S., Ellis, J., 1996. Detection of *Neospora caninum* DNA by the polymerase chain reaction. Int. J. Parasitol. 26, 347–351.

Schares, G., Heydorn, A.O., Cüppers, A., Conraths, F.J., Mehlhorn, H., 2001a. Cyclic transmission of *Neospora caninum*: serologic findings in dogs shedding oocysts. Parasitol. Res. 87, 873–877.

Schares, G., Heydorn, A.O., Cüppers, A., Conraths, F.J., Mehlhorn, H., 2001b. *Hammondia heydorni*-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. Parasitol. Res. 87, 808–816.

Schares, G., Heydorn, A.O., Cüppers, A., Mehlhorn, H., Geue, L., Peters, M., Conraths, F.J., 2002. In contrast to dogs, red foxes (*Vulpes vulpes*) did not shed *Neospora caninum* upon feeding of intermediate host tissues. Parasitol. Res. 88, 44–52.

Schock, A., Innes, E.A., Yamane, I., Latham, S.M., Wastling, J.M., 2001. Genetic and biological diversity among isolates of *Neospora caninum*. Parasitology 123, 13–23.

Šlapeta, J.R., Koudela, B., Votýpka, J., Modrý, D., Hořejš, R., Lukeš, J., 2002. Coprodiagnosis of *Hammondia heydorni* in dogs by PCR based amplification of ITS 1 rRNA: differentiation from morphologically indistinguishable oocysts of *Neospora caninum*. Vet. J. 163, 147–154.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4876–4882.