

ORIGINAL PAPER

Molecular Phylogeny of *Besnoitia* and the Genetic Relationships Among *Besnoitia* of Cattle, Wildebeest and Goats

John T. Ellis^{a,1}, O. Joakim M. Holmdahl^a, Cheryl Ryce^a, John M. Njenga^b, Peter A.W. Harper^c, and David A. Morrison^a

^a Molecular Parasitology Unit, Department of Cell & Molecular Biology, University of Technology, Sydney, Westbourne St., Gore Hill, NSW 2065, Australia

^b Department of Clinical Studies, Faculty of Veterinary Medicine, University of Nairobi, PO Box 29053, Nairobi, Kenya

^c NSW Agriculture, PO Box 389, Goulburn, NSW 2580, Australia

Submitted August 15, 2000; Accepted October 22, 2000
Monitoring Editor: R. Iain Wilson

Knowledge on parasites of the genus *Besnoitia* is sparse, which are classified in the subfamily Toxoplasmatinae of the phylum Apicomplexa. This arrangement hypothesises that *Besnoitia* represents the sister group to species such as *Toxoplasma gondii* and *Hammondia hammondi*. In order to test this hypothesis, phylogenetic analyses of 18S ribosomal DNA (rDNA) from *Besnoitia*, *Hammondia*, *Isospora*, *Frenkelia*, *Eimeria*, *Neospora*, *Sarcocystis* and *Toxoplasma* were performed. The 18S rDNA of *Besnoitia besnoiti*, *Besnoitia jellisoni* and *Eimeria alabamensis* were amplified by PCR and sequenced. Phylogenetic analyses by parsimony and maximum-likelihood methods showed *Besnoitia* to be reproducibly the sister group to a clade containing *Hammondia*, *Neospora* and *Toxoplasma*. Furthermore, *Besnoitia* of cattle, wildebeest and goats had identical ITS1 rDNA sequences, which questions the use of the taxon *Besnoitia caprae* to describe the *Besnoitia* found in goats.

Introduction

Besnoitia are parasitic protozoa belonging to the family Sarcocystidae in the phylum Apicomplexa. Besnoitiosis in cattle (previously known as globidiosis) is caused by infections from *Besnoitia besnoiti*. The disease has distinctive symptoms (Bigalke 1981; Hofmeyr 1945; Pols 1960), and commences with fever, followed by skin lesions involving extensive thickening of the skin and associated loss in elasticity. The final stages involve loss of most of the hair and shedding of skin layers. Although mortality

associated with this disease is generally low (less than 10%), morbidity may be as high as 20%. Sterility in bulls, resulting from orchitis, may also have a significant impact on animal production. No chemotherapeutic treatment is available for this disease, although a live vaccine was developed against bovine besnoitiosis using a strain of *Besnoitia* isolated from the blue wildebeest (Bigalke et al. 1967, 1974).

Knowledge on the relationship of *Besnoitia* to other cyst-forming coccidia is sparse, although the grouping of *Besnoitia*, *Toxoplasma* and *Hammondia* into the subfamily Toxoplasmatinae is generally well accepted (Frenkel 1977; Tenter and Johnson 1997).

¹ Corresponding author;
fax 61-2-9514-4003
e-mail j.ellis@uts.edu.au

This arrangement hypothesises that *Besnoitia* represents the sister group to *Hammondia*, *Neospora* and *Toxoplasma*. In order to test this hypothesis, the phylogenetic relationships amongst 18S rDNA sequences derived from *Besnoitia*, *Hammondia*, *Iso- spora*, *Eimeria*, *Frenkelia*, *Neospora*, *Sarcocystis* and *Toxoplasma* were investigated. The 18S rDNA of *B. besnoiti*, *Besnoitia jellisoni* and *Eimeria alaba- mensis* was amplified by PCR and sequenced. The phylogenetic analyses presented here show *Besnoi- tia* to be the sister group to a clade containing *Ham- mondia*, *Neospora* and *Toxoplasma*.

A further aim of this study was to investigate how phylogenetically informative variation was distributed along the 18S rDNA of cyst-forming coc- cidia. Most of the phylogenetically informative data that supported the clade containing *Besnoitia* + *Hammondia* + *Neospora* + *Toxoplasma* were located in domain 3 encoded by the 18S rDNA.

Finally the genetic relationships amongst *Besnoi- tia* found in cattle, wildebeest and goats were inves- tigated by comparison of internal transcribed spacer (ITS) 1 rDNA sequences. The data indicate these taxa are genetically identical at this locus, and leads us to question the use of the term *Besnoitis caprae* to describe *Besnoitia* found in goats.

Results

Phylogeny

The 18S rDNA sequences of *B. besnoiti* and *B. jel- lisoni* were determined (GenBank™ accession num- bers AF109678, AF291426). The 18S rDNA se- quences from the two isolates of *B. besnoiti* (BWB and bullstrain) were identical.

Preliminary phylogenetic analyses by parsimony of a Clustal W generated alignment indicated the analyses were apparently unproblematic. *Besnoitia* was the sister group to a clade containing *T. gondii* + *H. hammondi* + *N. caninum*.

The sequence alignment used for the main phylo- genetic analyses, which defines the complete sec- ondary structure of the 18S rRNA molecule, was 1,919 characters long. The preliminary parsimony analysis using the heuristic search indicated that *Iso- spora* was polyphyletic, with *I. robini* grouping with *Eimeria* + *Cyclospora* and the other three *Iso- spora* species grouping with *Besnoitia* + *Toxoplas- ma* + *Neospora* + *Hammondia*. *Eimeria* was para- phyletic unless *Cyclospora* was also included; and *Sarcocystis* formed two groups, one of which in- cluded *Frenkelia* (i.e. *Sarcocystis* is paraphyletic).

So, the final analyses included only *I. robini* as the

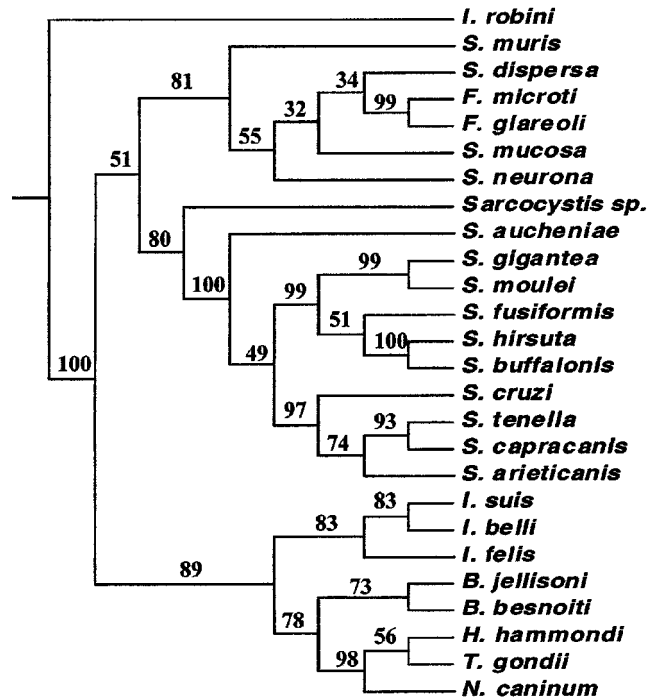


Figure 1. One of the two phylogenetic trees from the parsimony analysis of the structure alignment of the SSU rDNA, representing the relationships amongst the cyst-forming coccidia sequences using *I. robini* as the outgroup. The numbers at the nodes represent the bootstrap values (% out of 500). The maximum-likelihood analysis also produced the tree shown here.

outgroup. The parsimony analysis using the branch- and-bound search identified two equally parsimo- nious trees, which differed only in the position of *S. aucheniae*. Figure 1 shows one of these trees, with the bootstrap values obtained; the alternative tree had *S. aucheniae* as the sister taxon to the group containing *S. cruzi*. The relationships amongst the other sequences were unproblematic: *Besnoitia* was the sister group to a clade containing *T. gondii* + *H. hammondi* + *N. caninum*. *Iso- spora* was polyphyletic, and *Sarcocystis* formed two groups, one of which included *Frenkelia*. The maximum-likelihood analy- sis also produced the tree shown in Figure 1. The re- lationship of *Besnoitia* was further tested by search- ing for the optimal parsimony tree that does not have *Besnoitia* as the sister to the *T. gondii* + *H. hammondi* + *N. caninum* clade (the Bremer support index). The shortest tree had 689 instead of 686 steps, indicating considerable support for *Besnoitia* as the sister to the *T. gondii* + *H. hammondi* + *N. can- inum* clade.

The parsimony analysis of Domain 1 produced five equally parsimonious trees. These trees con-

tained most of the structure of the large *Sarcocystis* group, but neither the group containing *Frenkelia* nor the group containing *Besnoitia*. The analysis of Domain 2 produced 574 equally parsimonious trees. These trees contained most of the structure of the *Frenkelia* group, but not the large *Sarcosystis* group nor the group containing *Besnoitia*. The analysis of Domain 3 produced 639 equally parsimonious trees. These trees contained most of the structure of the *Besnoitia* + *Toxoplasma* + *Neospora* + *Isospora* group, but not either of the other two groups. So, the phylogenetic information is spread throughout the 18S rDNA sequence, but with the different Domains providing information for different parts of the phylogenetic tree.

Analysis of ITS1 Sequences

The ITS1 rDNA sequences derived from *Besnoitia* of cattle, wildebeest and goat were identical (GenBank™ accession number AF076859) and differed significantly in sequence from that derived for *B. jellisoni* ITS1 (AF076860). PCR contamination was rejected as an explanation for the identical sequences, due to the strict maintenance of aseptic technique; the use of a laminar flow cabinet for the constitution of PCR reactions, and the inclusion of appropriate negative controls in the experimental design.

Histological sections of goat fascia and adjacent adipose tissue contained numerous single and occasional clusters of large (100–400 µm) round to oval cysts of *B. caprae*. These cysts were infrequently accompanied by a mixed inflammatory cell infiltrate of neutrophils, lymphocytes and histiocytes accompanying early fibrosis, suggesting subacute mild granulomatous inflammation. The cysts had a thin outer hyalinised cyst wall, and contained an abundant and densely packed accumulation of bradyzoites. Several cysts also displayed a narrow cytoplasmic border of multiple elongated nuclei. In one section, a large arteriole contained several subintimal and intimal cysts apparently budding from the endothelium into the vessel lumen. These findings were consistent with those published previously for *B. caprae*.

Discussion

Knowledge about the phylogenetic relationships among cyst-forming coccidia, especially *Besnoitia*, is sparse. The phylogenetic analyses of 18S rDNA sequences described here have shown that *Besnoitia*, *Hammondia*, some *Isospora*, *Neospora* and

Toxoplasma form a monophyletic group and that *Besnoitia* is the sister group to the clade containing *N. caninum*, *H. hammondi* and *T. gondii*. Our results also confirm the conclusion of others that *Isospora* is polyphyletic (Carreno et al. 1998; Carreno and Barta 1999).

Historically, *Besnoitia* has been considered "Toxoplasma-like" (Dubey 1976, 1993; Kirkpatrick and Dubey 1987). For example, electron microscopy has shown that zoites of *Besnoitia* have an ultrastructure that is characteristic of cyst-forming coccidia, namely a three-layered pellicle, conoid, rhoptries, microtubules, micronemes and a polar ring (Njenga et al. 1995; Sheffield 1966; Shkap et al. 1988). Sheffield (1966) who stated that "it would be difficult to distinguish one from the other in electron micrographs" pointed out the morphological similarity of *B. jellisoni* to *T. gondii*.

There are few studies that describe the life cycle of *Besnoitia*, which appears obligately heteroxenous. Wallace and Frenkel (1975) and Frenkel (1977) demonstrated that the cat was the definitive host of *Besnoitia wallacei* and rats and mice were intermediate hosts. Smith and Frenkel (1977) showed that the cat was also the definitive host of *Besnoitia darlingi* and rodents, bats, tropical lizards and the Virginia opossum could act as intermediate hosts (Kirkpatrick and Dubey 1987). The life cycle of *B. besnoiti* has not been elucidated, and the cat does not appear to be the definitive host of this species (Diesling et al. 1988) although a range of ruminants, such as impala and blue wildebeest, act as intermediate hosts (Bigalke 1981).

The weight of evidence from parasite biology therefore indicates that the grouping of *Besnoitia*, *Hammondia*, *Toxoplasma* and *Neospora* described here (i.e. a monophyletic group sharing a common ancestor) is a reasonable conclusion. The cysts of *Besnoitia* are, however, unusual in their appearance and are quite distinctive from other cyst forming coccidia. They are large (100–500 µm), and the parasitophorous vacuole occupies most of the cell and contains numerous bradyzoites (Frenkel 1977; Sheffield 1968). It is surrounded by a multi-nucleated host-cell-derived cytoplasm, which is bounded by a thick, PAS staining, collagenous wall.

Besnoitia also differ amongst themselves in their ultrastructural and biological characteristics. For example, *Besnoitia* isolated from goats differs from *Besnoitia* of cattle in the appearance of their pellicles, micropore, orientation of the microtubules, appearance of the nucleus, appearance and development of the wall-forming bodies and the amount of lipid and amylopectin they contain (Njenga et al. 1995). Other biological differences, such as differ-

ences in their infectivity to rabbits and other laboratory animals as well as to cattle and goats, indicate that *Besnoitia* of cattle and goats are probably different biological species (Njenga et al. 1993, 1995; Ng'ang'a and Kasigazi 1994). Consequently the names *B. besnoiti* and *B. caprae* are in use to describe these taxa (Njenga et al. 1993).

The observation that *Besnoitia* from cattle, wildebeest and goat possess identical ITS1 rDNA sequences therefore requires comment. Wildebeest are recognised as an intermediate host for *B. besnoiti* (Bigalke et al. 1967), so it is not all that surprising that *Besnoitia* from wildebeest and cattle share the same ITS1 sequences. However, given that *Besnoitia* of cattle and goats differ in so many of their biological properties (Njenga et al. 1993, 1995; Ng'ang'a and Kasigazi 1994), the observation that these taxa are genetically identical at the ITS1 was surprising. Consequently, histopathological examinations were performed of the goat fascia in which the *Besnoitia* were present, and the findings were consistent with those published previously for *B. caprae* (Bwangamoi et al. 1989; Heydorn et al. 1984; Njenga et al. 1993, 1995; Ng'ang'a and Kasigazi, 1994). Therefore, we have good reason to conclude that the goat material analysed was indeed a representative sample of *B. caprae*.

The two most obvious conclusions that can be made from this study are that either *B. besnoiti* and *B. caprae* possess the same ITS1 sequence or that *B. caprae* represents a distinct population of *B. besnoiti*. Our current knowledge on *Besnoitia* is insufficient to determine which of these alternatives is correct. However, it is worth noting that virulent and avirulent populations of *T. gondii*, which differ in their biological (e.g. spectrum of disease in the mouse) and genetic (e.g. SAG2 and RAPD PCR profiles) properties (e.g. Guo and Johnson 1996; Sibley and Boothroyd 1992), possess identical ITS1 sequences (Homan et al. 1997; Payne and Ellis 1996). Holmdahl et al. (1997) also concluded that, because bovine and canine strains of *Neospora* possess identical ITS1 sequences, these isolates must also be *N. caninum*. The size and sequence content of the ITS1 is therefore recognised as a well-characterised species-specific marker amongst the coccidia (see also Barta et al. 1998; Ellis et al. 1999; Hnida and Duszynski 1999; Marsh et al. 1998, 1999) such that parasite populations sharing the same, or highly similar, ITS1 sequences are likely to be derived from the same species. If we accept these arguments, then one must reject the use of the term *B. caprae* to describe *Besnoitia* found in goats, given that it has an identical ITS1 sequence to *B. besnoiti*. Such a recommendation may not be rational in this in-

stance, because *B. caprae* and *B. besnoiti* appear as biologically distinct entities with properties that cannot be correlated with the two populations being identified as a single species. For example, *B. caprae* is not infectious to cattle or laboratory rodents whereas *B. besnoiti* is (Njenga et al. 1993, 1995; Ng'ang'a and Kasigazi 1994). Clearly, additional information is required to resolve this controversy.

Previous studies have shown those regions of rDNA which encode the helical regions of rRNA, contain most of the phylogenetically informative data present in the 18S rDNA of the cyst-forming coccidia (Ellis and Morrison 1995; Morrison and Ellis 1997). This study extends these observations and demonstrates that subsets of 18S rDNA data derived from the three major domains each independently support different parts of the phylogenetic tree obtained for the Sarcocystidae. The most logical explanation for this is that the different domains are evolving independently among the different groups which make up the cyst-forming coccidia. This hypothesis clearly requires further investigation.

Finally, various classifications have been proposed over the years for the Apicomplexa, including the cyst-forming coccidia (summarised in Ellis et al. 1998; Tenter and Johnson 1997). For example, Frenkel (1977) proposed a sub-division of the Sarcocystidae into two subfamilies: the Toxoplasmatinae (containing *Besnoitia*, *Toxoplasma* and *Hammondia*) and the Sarcocystinae (containing *Sarcocystis* and *Frenkelia*). This classification, which was based primarily on the morphological and life-cycle characteristics of the taxa concerned, hypothesises that *Besnoitia* represents the sister group to *Toxoplasma* and *Hammondia*. The analyses presented here show this to be a reasonable conclusion. In general terms, it is also worth noting that the overall relationships observed among *Frenkelia* and *Sarcocystis* (Jenkins et al. 1999; Mugridge et al. 1999) are also consistent with this classification.

At this point in time, it is not worth suggesting changes (even though they may be minor) to the classification of the cyst-forming coccidia, based on the results of rDNA analyses, for several reasons. First, there are still only a relatively small number of sequences analysed by molecular phylogeny, and it would be sensible for workers in this area to continue to add data to this arena. Second, the outcomes of analyses such as those presented here on 18S rDNA represent the evolution of one gene only, which may not reflect species evolution (Doyle 1992); that is, gene trees and species trees are not necessarily the same thing. Prior experience in other disciplines (e.g. Angiosperm Phylogeny Group

1998; Grass Phylogeny Group 2000) shows that it is necessary to look for agreement (congruence) among analyses of several loci (including protein-encoding genes or mitochondrial DNA) prior to suggesting re-classification of any group of organisms. Only in this way can we test the hypothesis that gene and organism phylogeny are correlated, since incongruence between rDNA phylogeny and other characters is quite common (e.g. Cannatella et al. 1998; Hoot et al. 1999; Martin et al. 2000; Smith and Sytsma 1994; Wingfield et al. 1994). Following analysis of other gene and protein types, the "weight-of-evidence" principle may ultimately lead to a revision in the classification of the coccidia.

Methods

Parasites

- *Besnoitia* from cattle and wildebeest. DNA of *B. besnoiti* bullstrain (from cattle) and BWB strain (from blue wildebeest) were provided by the Onderstepoort Veterinary Institute, Republic of South Africa.

- *Besnoitia* from goat. Fascia from the carcass of a chronically infected goat that was naturally infected in Kenya, containing numerous *Besnoitia* cysts, was removed and shipped to UTS. Specimens were embedded in paraffin, and 5 micron sections stained with haematoxylin and eosin. The rest of the fascia was frozen, lysed in 1% SDS, 10 mM Tris pH 8, 100 mM EDTA containing 100mg/ml of proteinase K at 56 °C, and the released DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Origin of other parasites and genomic DNAs: Culture-derived zoites of *B. jellisoni* were provided by Merck Research Laboratories, Rahway, New Jersey, USA. Genomic DNA was prepared from the zoites using the method described above. Genomic DNA from *Eimeria alabamensis* was provided by Per Thebo (Swedish University of Agricultural Sciences, Uppsala, Sweden).

PCR and sequencing of 18S ribosomal DNA: 18S rDNA was PCR amplified from genomic DNA as overlapping fragments using primers described by Holmdahl et al. (1999). The PCR fragments were purified by a QIAquick purification column (Qiagen, USA), and sequenced by cycle sequencing with the aid of an ABI automated sequencer using primers to generate the entire 18S rDNA sequence. A consensus sequence was produced from numerous sequencing runs (at least 3 from each primer).

Phylogenetic analysis: Preliminary analyses were conducted using a computer-generated align-

ment generated by Clustal W (Thompson et al. 1994) on the Australian Genome Information Service (ANGIS) with the default options for alignment. This alignment included 18S rDNA sequences from *Cyclospora* sp. (GenBank accession number U40261), *Eimeria adenoeides* (provided by Dr J. Barta), *E. alabamensis* (AF291427), *Eimeria bovis* (U77084), *Eimeria nieschulzi* (U40263), *Eimeria praecox* (U67120), *Eimeria tenella* (U40264, U67121, AF026388), *Hammondia hammondi* (AF096498), *Frenkelia microti* (AF009244), *Frenkelia glareoli* (AF009245), *Isospora belli* (U94787), *Isospora felis* (L76471), *Isospora robini* (AF080612), *Isospora suis* (U97523), *Neospora caninum* (L24380, U03069, U16159, U17346), *Sarcocystis arieticanis* (L24382), *Sarcocystis aucheniae* (AF017123), *Sarcocystis buf-falonis* (AF017121), *Sarcocystis capracanis* (L76472), *Sarcocystis cruzi* (AF006472, AF017120), *Sarcocystis dispersa* (AF120115), *Sarcocystis fusiformis* (U03071), *Sarcocystis gigantea* (L24384), *Sarcocystis hirsuta* (AF006469, AF006475, AF017122), *Sarcocystis moulei* (L76473), *Sarcocystis mucosa* (AF109679), *Sarcocystis muris* (M64244), *Sarcocystis neurona* (U07812, U33149), *Sarcocystis* sp. (U97524), *Sarcocystis tenella* (L24383) and *Toxoplasma gondii* (M97703, U12138, L37415, X68523, X75453, X75429, X75430, U00458, U03070, L24381, X65508). The alignment was analysed using the simple heuristic search option in PAUP 3.1.1 (Swofford 1993).

Further analyses were conducted using a sequence alignment based on that described by Van de Peer et al. (1997) which defines the complete secondary structure of the 18S rRNA molecule. This original alignment is available from the SSU rRNA database (<http://www-rrna.uia.ac.be>) maintained by R. De Wachter (Departement Biochemie, Universiteit Antwerpen) and included 18S rDNA sequences from the above named taxa. The alignment was manually edited in order to remove minor inconsistencies between taxa; consensus sequences were then derived using the MacClade 3.06 computer program (Maddison and Maddison 1992) for those taxa where there were several sequences available. Standard IUPAC codes were used for those nucleotide positions which contained more than one possible character state in the consensus sequence. The final alignment is available at <http://www.science.uts.edu.au/sasb/alignments.html>, contains 34 sequences and is 1,919 characters long including gaps. Parsimony analyses were performed using a) the heuristic search option with 100 random starts in PAUP 3.1.1 (Swofford 1993) with *Eimeria*, *I. robini* and *Cyclospora* specified as the outgroup; b) the branch-&-bound option with *I. robini* as the out-

group. Bootstrap analysis was performed using 500 bootstraps. Maximum-likelihood (ML) analyses were performed using DNAML in PHYLIP 3.5c (Felsenstein 1995) with the Kimura 2-parameter model, global rearrangements and 10 random starts.

The structural sequence alignment was also partitioned into three data sets containing Domains 1, 2 and 3 of the rRNA secondary structure (Gutell et al. 1994; van der Peer et al. 1998). The Domain 1 data set was 706 characters long, and is here defined as spanning Helices 1 through to 21 (as defined by van der Peer et al., 1998); Domain 2 was 574 characters long, and contained nucleotides of Helix 22 through to 31; and Domain 3 was 639 characters long, and spanned Helices 2 through to 50. The alignments were analysed by heuristic parsimony as described above.

PCR and Sequencing of ITS1: ITS1 sequences from *Besnoitia* of cattle, wildebeest and goat, and *B. jellisoni*, were amplified from genomic DNA using primers Tim3 and Tim11 as described (Payne and Ellis 1996; Ellis et al. 1999). These primers are known to PCR amplify DNA from *N. caninum*, *Hammondia* spp. and *T. gondii*, but not from any other organisms tested including DNA from higher eukaryotic organisms (Ellis et al. 1999; Payne and Ellis 1996). The PCR products obtained were purified by a QIAquick purification column (Qiagen, USA) and sequenced by cycle sequencing with the aid of an ABI automated sequencer. The ITS1 sequences were aligned using Clustal W (with default parameter options).

Acknowledgements

We thank Dr T. De Waal (Onderstepoort, RSA) for providing the DNA from *B. besnoiti*; Merck Research Laboratories for *B. jellisoni*; Professor R. De Wachter (Antwerpen, Belgium) for the 18S rDNA sequence alignment. OJM was on leave at UTS from the Department of Parasitology, National Veterinary Institute and Swedish University of Agricultural Sciences, Uppsala, Sweden.

References

- Angiosperm Phylogeny Group** (1998) An ordinal classification of flowering plants. *Ann Missouri Bot Gard* **85**: 531–553
- Barta JR, Coles BA, Schito ML, Fernando MA, Martin A, Danforth HD** (1998) Analysis of infraspecific variation among five strains of *Eimeria maxima* from North America. *Int J Parasitol* **28**: 485–492
- Bigalke RD** (1981) Besnoitiosis and globidiosis. *Curr Top Vet Med Anim Sci* **6**: 429–442
- Bigalke RD, Schoeman JH, McCully RM** (1974) Immunisation against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of *Besnoitia besnoiti* grown in cell cultures. 1. Studies on rabbits. *Onderstepoort J Vet Res* **41**: 1–6
- Bigalke RD, Van Niekerk JW, Basson PA, McCully RM** (1967) Studies on the relationship between *Besnoitia* of blue wildebeest and impala, and *Besnoitia besnoiti* of cattle. *Onderstepoort J Vet Res* **34**: 7–28
- Bwangamoi O, Carles AB, Wandera JG** (1989) An epidemic of besnoitiosis in goats in Kenya. *Vet Rec* **125**: 461
- Cannatella DC, Hillis DM, Chippindale PT, Weigt L, Rand AS, Ryan MJ** (1998) Phylogeny of frogs of the *Physalaemus pustulosus* species group, with an examination of data incongruence. *Syst Biol* **47**: 311–335
- Carreno RA, Barta JR** (1999) An eimeriid origin of isosporoid coccidia with stieda bodies as shown by phylogenetic analysis of small subunit ribosomal RNA gene sequences. *J Parasitol* **85**: 77–83
- Carreno RA, Schnitzler BE, Jeffries AC, Tenter AM, Johnson AM, Barta JR** (1998) Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isospora* is most closely related to *Toxoplasma* and *Neospora*. *J Euk Microbiol* **45**: 184–188
- Diesling L, Heydorn AO, Matuschka FR, Bauer C, Pipano E, De Waal DT, Pothgieter FT** (1988) *Besnoitia besnoiti*: studies on the definitive host and experimental infections in cattle. *Parasitol Res* **75**: 114–117
- Doyle JJ** (1992) Gene trees and species trees: molecular systematics as one-character taxonomy. *Syst Bot* **17**: 144–163
- Dubey JP** (1976) A review of *Sarcocystis* of domestic animals and of other coccidia of cats and dogs. *J Am Vet Med Assoc* **169**: 1061–1078
- Dubey JP** (1993) Intestinal protozoa infections. *Vet Clin North Am: Small Animal Practice* **23**: 37–55
- Ellis JT, Morrison DA** (1995) Effects of sequence alignment on the phylogeny of *Sarcocystis* deduced from 18S rDNA sequences. *Parasitol Res* **81**: 696–699
- Ellis JT, Morrison DA, Jeffries AC** (1998) The Phylum Apicomplexa: an Update on the Molecular Phylogeny. In Coombs GH, Vickerman K, Sleigh MA, Warren A (eds) *Evolutionary Relationships Among Protozoa*. Kluwer, Dordrecht, pp 255–274
- Ellis JT, Morrison DA, Liddell S, Jenkins MC, Mohammed OB, Ryce C, Dubey JP** (1999) The genus *Hammondia* is paraphyletic. *Parasitology* **188**: 347–557
- Felsenstein J** (1995) PHYLIP (Phylogenetic inference package). University of Washington, Seattle

- Frenkel JK** (1977) *Besnoitia wallacei* of cats and rodents: with a reclassification of other cyst-forming isosporoid coccidia. *J Parasitol* **63**: 611–628
- Grass Phylogeny Working Group** (2000) A Phylogeny of the Grass Family (Poaceae), as Inferred from Eight Character Sets. In Jacobs SWL, Everett J (eds) *Grasses: Systematics and Evolution*. CSIRO Publishing, Melbourne, pp 3–7
- Guo Z-G, Johnson AM** (1996) DNA polymorphisms associated with murine virulence of *Toxoplasma gondii* identified by RAPD PCR. *Curr Top Microbiol Immunol* **219**: 17–26
- Gutell RR, Larsen N, Woese CR** (1994) Lessons from an evolving rRNA – 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* **58**: 10–26
- Heydorn AO, Senaud J, Mehlhorn H, Heinonen R** (1984) *Besnoitia* sp. from goats in Kenya. *Z Parasitenkd* **70**: 709–713
- Hnida JA, Duszynski DW** (1999) Using the ITS1 region of the ribosomal gene complex to clarify the taxonomy and systematics of some *Eimeria* species of murid rodents. *Parasitology* **119**: 349–357
- Hofmeyr CFB** (1945) Globidiosis in cattle. *J S Afr Vet Med Assoc* **16**: 102–109
- Holmdahl OJM, Morrison DA, Ellis JT, Huong LTT** (1999) Evolution of ruminant *Sarcocystis* (Sporozoa) parasites based on small subunit rDNAs sequences. *Mol Phylogenet Evol* **11**: 27–37
- Holmdahl OJM, Björkman C, Stenlund S, Uggla A, Dubey JP** (1997) Bovine *Neospora* and *Neospora caninum*: One and the same. *Parasitology Today* **13**: 40–41
- Homan WL, Limper L, Verlaan M, Borst A, Vercammen M, van Knapen F** (1997) Comparison of the internal transcribed spacer, ITS1, from *Toxoplasma gondii* isolates and *Neospora caninum*. *Parasitol Res* **83**: 285–289
- Hoot SB, Magallon S, Crane PR** (1999) Phylogeny of basal eudicots based on three molecular data sets: atpB, rbcL, and 18S nuclear ribosomal DNA sequences. *Ann Missouri Bot Gard* **86**: 1–32
- Jenkins MC, Ellis JT, Liddell S, Ryce C, Munday BL, Morrison DA, Dubey JP** (1999) The relationship of *Hammondia hammondi* and *Sarcocystis mucosa* to other heteroxenous cyst-forming coccidia as inferred by phylogenetic analysis of the 18S SSU ribosomal DNA sequence. *Parasitology* **119**: 135–142
- Kirkpatrick CE, Dubey JP** (1987) Enteric coccidial infections. *Isospora, Sarcocystis, Cryptosporidium, Besnoitia* and *Hammondia*. *Vet Clin North Am: Small Animal Practice* **17**: 1405–1420
- Maddison WP, Maddison DR** (1992) *MacClade: Analysis of Phylogeny and Character Evolution*. Sinauer Associates, Sunderland
- Marsh AE, Barr BC, Packham AE, Conrad PA** (1998) Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). *J Parasitol* **84**: 983–991
- Marsh AE, Barr BC, Tell L, Bowman DD, Conrad PA, Ketcherside C, Green T** (1999) Comparison of the internal transcribed spacer, ITS-1, from *Sarcocystis falcatula* isolates and *Sarcocystis neurona*. *J Parasitol* **85**: 750–757
- Martin P, Kaygorodova I, Sherbakov DY, Verheyen E** (2000) Rapidly evolving lineages impede the resolution of phylogenetic relationships among *Clitellata* (Annelida). *Mol Phylogenet Evol* **15**: 355–368
- Morrison DA, Ellis JT** (1997) Effects of nucleotide sequence alignment on phylogeny estimation – a case study of 18S rDNAs of Apicomplexa. *Mol Biol Evol* **14**: 428–441
- Mugridge NB, Morrison DA, Johnson AM, Luton K, Dubey JP, Votycka J, Tenter AM** (1999) Phylogenetic relationships of the genus *Frenkelia*: a review of its history and new knowledge gained from comparison of large subunit ribosomal RNA gene sequences. *Int J Parasitol* **29**: 957–972
- Njenga JM, Bwangamoi O, Kangethe EK, Mugera GM, Mutiga ER** (1995) Comparative ultrastructural studies on *Besnoitia besnoiti* and *Besnoitia caprae*. *Vet Res Comm* **19**: 295–308
- Njenga JM, Bwangamoi O, Mutiga ER, Kangethe EK, Mugera GM** (1993) Preliminary findings from an experimental study of caprine besnoitiosis in Kenya. *Vet Res Comm* **17**: 203–208
- Ng'ang'a CJ, Kasigazi S** (1994) Caprine besnoitiosis: studies on the experimental intermediate hosts and the role of the domestic cat in transmission. *Vet Parasitol* **52**: 207–210
- Payne S, Ellis J** (1996) Detection of *Neospora caninum* DNA by the polymerase chain reaction. *Int J Parasitol* **26**: 347–351
- Pols J** (1960) Studies on bovine besnoitiosis with special reference to the aetiology. *Onderstepoort J Vet Res* **28**: 265–356
- Shkap V, Yakobson BA, Pipano E** (1988) Transmission and scanning electron microscopy of *Besnoitia besnoiti*. *Int J Parasitol* **18**: 761–766
- Sheffield HG** (1966) Electron microscope study of the proliferative form of *Besnoitia jellisoni*. *J Parasitol* **52**: 583–594
- Sheffield HG** (1968) Observations on the fine structure of the “cyst stage” of *Besnoitia jellisoni*. *J Protozool* **15**: 685–693
- Sibley LD, Boothroyd JC** (1992) Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature* **359**: 82–85
- Smith DD, Frenkel JK** (1977) *Besnoitia darlingi* (Protozoa, Toxoplasmatidae); cyclic transmission by cats. *J Parasitol* **63**: 1066–1071

Smith JF, Sytsma KJ (1994) Molecules and morphology – congruence of data in *Columnnea* (Gesneriaceae). *Plant Syst Evol* **193**: 37–52

Swofford DL (1993) PAUP (Phylogenetic Analysis Using Parsimony). Smithsonian Institution, Washington

Tenter AM, Johnson AM (1997) Phylogeny of the tissue cyst-forming coccidia. *Adv Parasitol* **39**: 69–139

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680

Van de Peer Y, Van der Broeck I, De Rijk P, De Wachter R (1998) Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res* **26**: 179–182

Wallace GD, Frenkel JK (1975) *Besnoitia* species (Protozoa, Sporozoa, Toxoplasmatidae): Recognition of cyclic transmission by cats. *Science* **188**: 369–371

Wingfield BD, Grant WS, Wolfaardt JF, Wingfield MJ (1994) Ribosomal RNA sequence phylogeny is not congruent with ascospore morphology among species in *Ceratocystis* sensu stricto. *Mol Biol Evol* **11**: 376–383