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ORIGINAL PAPER

Molecular biological comparison of different *Besnoitia* species and stages from different countries

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Abstract *Besnoitia besnoiti* tissue cysts from a recent outbreak in cattle in Germany were characterized with respect to their internal transcribed spacer regions 1, 2, and 18S rDNA gene sequences. These results were compared with own sequences of an Israelian isolate of *B. besnoiti* and of *Besnoitia jellisoni* cystozoites stored for years in liquid nitrogen. Furthermore, material was studied that was obtained from white mice (Balb/C) that had been successfully infected by intraperitoneal infection of fresh cystozoites from the German outbreak. All results were then compared and discussed with respect to databank sequences of other *Besnoitia* species. Comprehensive phylogenetic studies of *B. besnoiti* isolates from Germany revealed almost identical sequence alignments when compared to previously sequenced *B. besnoiti* isolates from Israel and Spain. More importantly, phylogenetic analysis revealed two distant clusters of *Besnoitia* species: the first one includes *Besnoitia akodoni*, *Besnoitia darlingi*, and *Besnoitia oryctofelisi*, while the second cluster includes *B. besnoiti*, *Besnoitia bennetti*, *Besnoitia tarandi*, and the *Besnoitia* species of rodents (*B. jellisoni*). The also *B. jellisoni* named species of the GenBank (AF 076860) must be another one, since our strain derives directly from Frenkel. These findings give strong hints that *B. besnoiti* has a cycle between rodents and a predator and that cattle and other are only accidental hosts.

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Introduction

In the literature, different species of *Besnoitia* have been described to produce tissue cysts in mammals such as several ruminants, donkeys, rodents, rabbits, or opossums (e.g., review Mehlhorn et al. 2009). However, their life cycle is not known in several cases, while in others, only scarce data are available, although the transmission in the life cycles had been described in *Besnoitia akondoni*, *Besnoitia darlingi*, and *Besnoitia orytofelisi* (see review Mehlhorn et al. 2009). *Besnoitia besnoiti* from cattle was originally described by Besnoit and Robin (1912) in France but became confirmed to occur in Israel, Southern Europe, Kasachstan, and South Korea, too (Heydorn et al. 1984; Cortes et al. 2006a, b, 2007; Buchev et al. 2007; Bak et al. 1968). Other species apparently occur in the USA in a variety of hosts (Dubey et al. 2002, 2003; Dubey and Lindsay 2003). When studying the morphology of the cyst stages, it turned out that *Besnoitia* cysts represent a swollen host cell with hypertrophied nuclei which contain a parasitophorous vacuole with thousands of cyst merozoites

(cystozoites) that reproduce by endodyogeny. When looking at their fine structure, the differences between the cysts within different hosts are rather low (D'Haese et al. 1977; Sénaud and Mehlhorn 1978; Heydorn et al. 1984; Ayroud et al. 1995; Dubey and Lindsay 2003; Dubey et al. 2003, 2004, 2005a, b; Mehlhorn 2008; Mehlhorn et al. 1974, 2009), while there are many light microscopic papers (Bilgalke 1981; Bilgalke and Schoeman 1967; Davies et al. 1936; Diesing et al. 1988; Njenga et al. 1999; Nobel et al. 1977; Peteshev and Polomoshnov 1976; Scholtyseck et al. 1973; Shkap et al. 1995). Since the life cycle of *B. besnoiti* and its pathways of transmission are not known and since it occurred recently in the federal state of Bavaria, Germany (rather far away from its sites in Southern Europe; Mehlhorn et al. 2009; Gollnick et al. 2009), it seems important to compare known species at the level of molecular biological data in order to collect data which might help to elucidate pathways of transmission. Thus, the present study compares the internal transcribed spacer (ITS), ITS1 and ITS2, obtained from recent cyst material from Bavaria with that of *B. besnoiti* from Israel and *Besnoitia jellisoni* stages obtained from the rodent strain isolated by Frenkel in the year 1953 and later given to the institutes of Heydorn and Mehlhorn, where it was kept on jirds and white mice by subcutaneous or intraperitoneal injection.

Material and methods

Material

Three different sources of *Besnoitia* material were used. First, cysts originated from two ethanol-fixed pieces of infected skin of two animals: "skin I" originated from a bull, "skin II" from a calf which both derived from fresh biopsy material of the recent German outbreak of besnoitiosis (Mehlhorn et al. 2009). Second, cultured stages of *B. besnoiti* and cyst merozoites obtained from cysts of *B. jellisoni* were used. Both had been stored in liquid nitrogene in the Berlin Institute of Veterinary Parasitology. The Berlin *B. besnoiti* stages originated from Israel, while the cyst stages of *B. jellisoni* were a gift of Prof. Dr. Frenkel representing the original isolates from the year 1953. Finally, we infected intraperitoneally white mice (Balb/C) with living cyst stages from the recent outbreak in Bavaria and prepared after 5 months blood, skin, and muscle tissue for PCR probes.

Methods

For extracting genomic DNA from the skin samples, a little piece was cut off and dried in a speedvag to remove the ethanol. Then, 180 µl buffer ATL of the QIAmp DNA Mini Kit (Qiagen, Hilden Germany) were added.

The cystozoites of the cell cultures and the cystozoites from cysts were spun down (10 min, 15,000 rpm Eppendorf centrifuge rotor F241.5), the supernatant was removed, and the pellets were resolved in each 180 µl buffer ATL. To the fresh mouse samples (blood, skin, muscles, all between 30 and 50 mg), 180 µl of buffer ATL were added. After adding 20 µl Proteinase K to each sample, they were incubated at 56°C overnight. The following DNA extraction was performed according to the manufacturer`s instructions. DNA of the samples of blood, skin, and muscles of the infected white mouse was extracted according to the instructions of the QIAmp DNA Mini Kit.

The ITS1 fragment of *Besnoitia* was amplified by PCR using the primers:

BesITS1F: TGACATTTAATAACAATCAACCCTT (Cortes et al. 2007),
BesITS1R: GGTTTGTATTAACCAATCCGTGA (Cortes et al. 2007).

The ITS2 region was amplified with the primers

ITS3: GCATCGATGAAGAACGCAGC (White et al. 1990),
ITS4: TCCTCCGCTTATTGATATGC (White et al. 1990).

For PCR, 1 µl of template DNA was mixed with 1 µl (10 µM) of each primer, 7 µl water, and 10 µl mastermix (Mastermix Y, Peqlab, Erlangen, Germany). The final 20-µl sample was covered with two to three drops of mineral oil (Sigma M5904, St. Louis, MO, USA). The PCR program started with a 15-min denaturation step at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 50°C, and 2 min polymerization at 72°C. The program ended with a final polymerization step of 20 min at 72°C.

PCR products were cleaned with the Nucleospin Extract II Kit (Macherey & Nagel, Dormagen, Germany) and sequenced by Seqlab (Göttingen, Germany).

For comparison, the following *Besnoitia* sequences were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Only the parts of the sequences corresponding to the fragments amplified, being the used primer pairs, were considered in the following calculations. The Program "ClustalX" (Jeanmougin et al. 1998) was used to calculate neighbor-joining trees (bootstrap condition: random number generator seed 111, 1000 bootstrap trials).

Accession numbers of the sequences from GenBank:

B. besnoiti Israel DQ227420
B. besnoiti Portugal AY833646
B. besnoiti Spain 1 DQ227419
B. besnoiti Spain 2 DQ227418
Besnoitia akodoni AY545987
Besnoitia bennetti AY665399
Besnoitia darlingi AF489696

B. jellisoni AF 076860
Besnoitia oryctofelisi AY182000
Besnoitia tarandi AY665400
Sarcocystis felis AY190082
Eimeria lancasterensis EU302674

Results

ITS1 results

The phylogram based on the ITS1 sequences shows that the DNA isolated from the skin samples fits very good with all other *B. besnoiti* ITS1 sequences, indicating that the cattle in Germany was indeed infected with *B. besnoiti*. Furthermore, the sequences of *B. bennetti*, our *B. jellisoni* (with the exception of *B. jellisoni* AF 976869 from GenBank) and *B tarandi* are placed in the *B. besnoiti* clade in a way that prohibits molecular differentiation of these species based on the ITS1. The three remaining species (*B. akondi*, *B. darlingi*, *B. oryctofelisi* plus the *B. jellisoni* isolate) form a clade of their own (Fig. 1). The DNA results from the *B. besnoiti*-infected mice were coincident with the original cyst material of the Bavarian outbreak indicating that the experimental/transmission of cystozoites from cattle to mice was successful and that they can apparently persist for long, although new cysts had not been seen.

The phylogram is based on the analysis of the ITS1 sequences. The sequences obtained in the present study derived from the following materials: (a) laboratory strain of *B. besnoiti* isolated in Israel, (b) *B. jellisoni* from Berlin (cystozoites given by Frenkel), (c) skins I and II are isolated from the 2009 Bavarian outbreak. All other sequences are from GenBank. *S. felis* was used as outgroup.

ITS2 results

Unfortunately, the used primers were inappropriate to amplify the ITS2 of *B. jellisoni* and skin I. Our ITS2 sequences from the Israelian strain and the skin II sample form an own clade with the *B. besnoiti* database sequences, indicating close relationship (Fig. 2)

The phylogram is based on the analysis of the ITS2 sequences. The sequences sequenced here are the sequences "Israel" (laboratory strain of *B. besnoiti* isolated in Israel); skin II are *Besnoitia* sequences isolated from the skin samples. All other sequences are from GenBank. *Besnoitia* is always abbreviated with *B.*; *E. lancasterensis* is used as outgroup.

Results of the SSU sequences

Unfortunately, we only succeeded in amplifying the SSU sequence of our Israelian strain, which can be seen at GenBank. Our sequences can be seen at GenBank with the accession numbers FN257462–FN257465, FN435987–FN435988, and FN435991.

Discussion

Although the collection of sequence data and their analysis with appropriate software are rather simple processes, interpretation of the resulting data in classical taxonomic units is still difficult. This is complicated by the fact that there is even no definition for the basic taxonomic unit—the species—without weak points. So, it is impossible to determine a species on basis of molecular data derived only from one gene, especially as there are no hints, in which degree of diversity is needed at the species border. In very detailed examinations of the relationships of butterflies, no correlation between the established species and their genetic diversity was found. On the other hand, the genetic diversity of different African cichlid species is in the same range as it is found in different populations of North American fish of one species. So, up to now, the status of a species must be deduced from the analysis of molecular, morphological, biochemical, and/or ecological data all together (Sperling 2003; Carvalho and Hause 1999).

In our attempt to identify *Besnoitia* in cattle samples, we used the sequences of three different parts of the eukaryotic rDNA cluster. In eukaryotes, the genes for the 18S rRNA, the 5.8S RNA and 28S RNA are arranged after each other on the DNA strand, separated by short DNA pieces called ITS1 and ITS2. To produce RNA for the ribosomes, the complete cluster including the ITS1 and ITS2 is transcribed in one huge transcription product, then the rRNA is cut off and used in the ribosome formation. The ITS1 and ITS2 are simply degraded. As there is no known selective pressure on the sequence of the ITS1 and ITS2, they are highly variable and show differences even between different populations of one species and closely related species (Hwang and Kim 1999). The third sequence is a variable portion of the 18S rDNA, which was already useful in the differentiation of some *Cryptosporidium* species (Elsheikha et al. 2005a, b).

Our most significant results are based on the analysis of the ITS1 sequences, because there were several sequences in GenBank which can be compared with each other and with our new sequenced samples. All our sequences are grouped together with all other *B. besnoiti* sequences and with the sequences from *B. jellisoni*, *B. bennetti*, and *B. tarandi.* In detail, nearly all sequences were identical; only in two sequences, a single nucleotide was different. Our strain of *B. jellisoni* is identical with the original described one of Frenkel. Thus, it might be concluded that *B. jellisoni* AF 076860 derives from another species of *Besnoitia* and

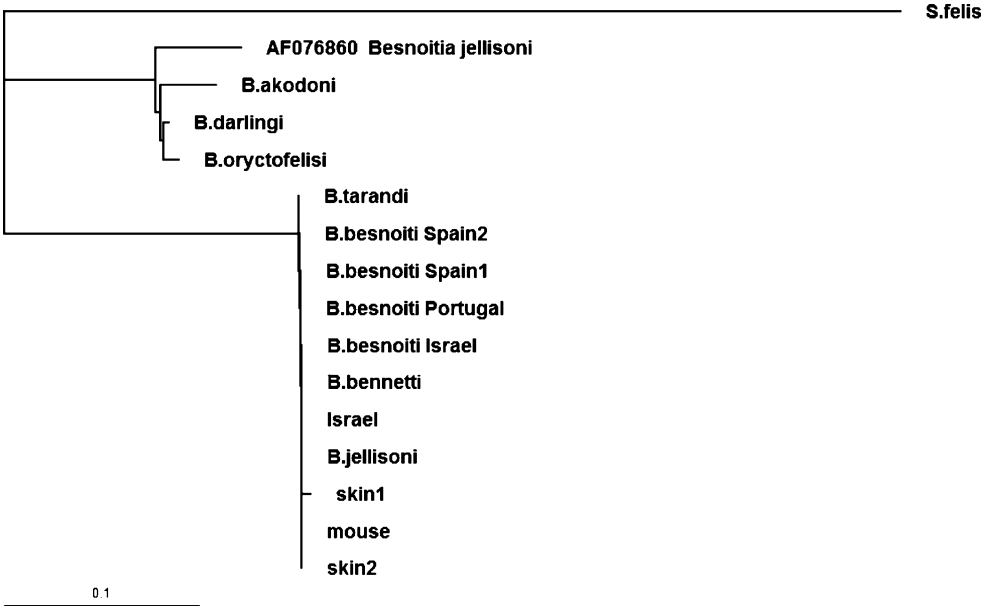

Fig. 1 Phylogram based on the ITS1 sequences

thus is grouped together with *B. darling*, *B. akondoni*, and *B. oryctofelis* in the ITS1 phylogram. As mentioned above, there is no rule to determine a species by differences in their sequences, but only one different nucleotide will surely be insufficient to determine different species, especially as we used a highly variable piece of DNA. So, in any way, our ITS-1 findings are a strong hint that the samples from Bavaria contained parasites of the genus *Besnoitia*. Whether they belong to *B. besnoiti* or to another species in this genus can only finally be decided after consideration of other data.

But at least, the data available for the ITS2 showed the close relationship of the *Besnoitia* isolated in Bavaria with the species in the clade identified by the examination based on the ITS1 sequences. Examinations based on ITS2 and SSU sequences are hampered by the rare data for these sequences. Finally, we do not know why we did not succeed in amplifying the ITS2 of *B. jellisoni*. We used a primer pair developed originally for molds, but they are useful for almost all eukaryotes (White et al. 1990). But "almost all" means "not all," and as a single nucleotide mismatching at the 3′-end of a primer may lead to complete loss of function of the primer, we had perhaps the following situation, that there is a single nucleotide exchange at the 5′-end of the region, where one of the primers should hybridize. This is, however, no reason to determine a species border, but this might be sufficient to lead to a total loss of the function of the primer.

When interpreting the results of the recent sequencing study, it seems rather clear that there are two clusters of "species." Since our species named *B. jellisoni* derives definitively from that described by Frenkel (since we got it as a gift), the other species *B. jellisoni* is not identical to ours but represents another one. This finding gives the chance to

Fig. 2 Phylogram based on the ITS2 sequences

speculate on the up-to-now unknown life cycle of *B. besnoiti*. Perhaps, cattle and other ruminants are not obligatory intermediate hosts but become only occasionally infected when feeding oocysts/sporocysts excreted by predators that normally catch mice and similar rodents. The latter would be the typical intermediate hosts of *Besnoitia*, which—as it is often the case in very long existing life cycles—do not show severe or even clearly visible signs of disease. Such a transmission concept would be supported by our findings that mice being experimentally infected with *Besnoitia* cyst stages remain PCR-positive for months. Perhaps, cystozoites or even a small number of newly formed cysts remain infectious for months.

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