Phylogenetic position of a renal coccidium of the European green frogs, ‘Isospora’ lieberkuehni Labbé, 1894 (Apicomplexa: Sarcocystidae) and its taxonomic implications

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‘Isospora’ lieberkuehni, an unusual isosporoid renal coccidium that parasitizes the European water frog was isolated from the edible frog, Rana kl. esculenta, in the Czech Republic. Sequencing of the small-subunit (SSU) rRNA gene showed that it belongs to the family Sarcocystidae, being closely related to a clade comprising members of the subfamily Toxoplasmatinae. The position within Sarcocystidae correlates with the mode of excystation via collapsible plates as postulated by previous authors. Phylogenetic, morphological and biological differences between ‘Isospora’ lieberkuehni and the other Stieda-body-lacking members of the genus Isospora justify separation of this coccidium on a generic level. Hyaloklossia Labbé, 1896 is the oldest available synonym and is herein re-erected. The original definition of the genus Hyaloklossia is emended based on recent observations.

Keywords: Isospora lieberkuehni, Hyaloklossia, Sarcocystidae, Coccidia, phylogenetic analysis

INTRODUCTION

The families Eimeriidae and Sarcocystidae of the Apicomplexa represent a highly diversified group of intracellular parasites of vertebrate and invertebrate hosts. The traditional classification of these parasites based on the number of sporocysts per oocyst was repeatedly questioned and its more or less artificial character was discussed (e.g. Box et al., 1980; Frenkel, 1977). The growing amount of molecular data on various species of coccidia enables us to draw a more accurate picture of the phylogeny of these parasites. The SSU rRNA gene has been shown to provide good phylogenetic resolution at the generic and/or specific levels and, based on its sequences, the phylogenetic positions of several members of the medically important families Eimeriidae and Sarcocystidae (mostly from domestic animals and man) were determined and repeatedly used in diagnostics and taxonomy (Barta et al., 1997; Eberhard et al., 1999; Ellis et al., 1998; Votyka et al., 1998). The assemblage of coccidia sharing the ‘two sporocyst, each with four sporozoites’ type of oocyst represents the taxonomically most complex and probably artificial group. It comprises medically important genera Besnoitia, Hammondia, Isospora, Neospora, Sarcocystis and Toxoplasma, and its phylogeny and taxonomy attracted much attention in recent years (Doležel et al., 1999; Ellis et al., 1998; Jenkins et al., 1999; Johnson, 1998; Mehlhorn & Heydorn, 2000; Mugridge et al., 1999a, b; Tenter & Johnson, 1997).

Recently, the genus Isospora was found to be polyphyletic, consisting of two distantly related clades. The Isospora species that contain the Stieda and substieda bodies and are parasites of birds showed close affinity to the Eimeriidae, while the Isospora species from mammalian hosts, having sutures in the sporocyst wall and lacking the Stieda and substieda bodies, grouped together within branch A (sensu Doležel et al., 1999) of...
Sarcocystidae (Carreno & Barta, 1999). However, the diversity of parasites belonging to the genus Isospora, as it is presently defined, is probably higher and requires more attention.

The renal coccidium of water frogs of the genus *Rana* reported first by Lieberkühn in 1854 represents a species with several unique features. It was described by Labbé under the name *Klossia lieberkuehni* and, 2 years later, the same author re-evaluated its status and erected the genus *Hyaloklossia* to accommodate this unusual species (Lieberkühn, 1854; Labbé, 1894, 1896). However, later authors made *Hyaloklossia* synonymous with *Diplospora* Labbé, 1893 (Laveran & Mesnil, 1902; Minchin, 1903) and later with *Isospora* Schneider, 1881 (Nöller, 1923; Doflein & Reichenow, 1929). The combination of direct life cycle, extra-intestinal development, *in situ* sporulation and unusual oocyst morphology distinguish *Isospora* lieberkuehni from other members of the genus *Isospora* as well as from members of the Sarcocystidae. Here we present a phylogenetic analysis of this parasite and discuss its taxonomic implications.

**METHODS**

**Organism.** *Isospora* lieberkuehni was isolated from the edible frog, *Rana* kl. *esculenta* Linnaeus, 1758, collected in Lanzhot, Southern Moravia, Czech Republic (48° 43’ N, 16° 58’ E), in May 1999 and during the spring of 2000. Frogs were dissected and infected kidneys were examined by using native squash preparations and processed for histology.
Oocysts were examined, measured and photographed using Nomarski interference contrast optics. Heavily infected kidney tissue was preserved in absolute ethanol.

**DNA extraction, PCR and sequencing.** Total cell DNA from ‘I.’ lieberkuehni was isolated as described previously (Votypka et al., 1998). The SSU rDNA gene was PCR amplified with the oligonucleotides K11 (AAAGATTAGGCCATGCA) and K12 (CAAGGGCCAGGGAGTCGA), which anneal to the conserved 5′ and 3′-end regions of the gene. Conditions were as follows: initial denaturation 95 °C for 4 min followed by 30 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 10 min. The amplicon was purified on 0.75% agarose gels, gel-isolated and cloned using the TOPO TA Cloning version E (Invitrogen). Both strands were sequenced on an automated sequencer using BigDye DNA Sequencing Kit (Perkin-Elmer).

**Phylogenetic analysis.** Based on taxonomic position, biology and morphology, a set of coccidian species was selected to cover the biodiversity of the Sarcocystidae and Eimeriidae; Cryptosporidium parvum or members of the Eimeriidae (data not shown) were used as outgroup(s). Only reliable SSU rRNA sequences as of November 2000 were obtained from the GenBank/EMBL/DDJB databases and were adopted in this phylogenetic analysis. The SSU rRNA genes were aligned together with the newly sequenced ‘I.’ lieberkuehni using the program CLUSTAL x (Thompson et al., 1997). The dataset was analysed with a variety of alignment parameters and varying numbers of sequences. The stability was tested with different numbers of representatives of the groups. Alignment was corrected by eye and ambiguously aligned regions were excluded. Since we were primarily interested in the position of ‘I.’ lieberkuehni, the 5′ and 3′ regions not available in our sequence were removed from the alignment. Sequence alignments were analysed with the program package PAUP*, Version 4.0b4 (Swofford, 1998) and are available as supplementary data in IJSEM Online at (http://ijsem.sgmjournals.org/). Phylogenetic relationships were reconstructed using the distance, parsimony and maximum-likelihood methods. The distance method was performed by using heuristic search with the ME objective setting, LogDet and HKY85 matrices. Parsimony and maximum-likelihood analyses were performed using heuristic settings with gaps treated as missing data and transversion/transversion (Tv/Ts) ratios of 1:1 and 1:3. Maximum-likelihood was performed using the heuristic search and a substitution type 6 (GTR model), and tested also by the Puzzle algorithm. Bootstrap analysis (200 replicates for maximum-likelihood, 1000 replicates for maximum-parsimony and distance method) and the Bremer decay indexes (number of extra steps for a clade not to be unequivocally supported) were established.

**RESULTS AND DISCUSSION**

**Morphology and biology**

The endogenous stages of ‘I.’ lieberkuehni were found in 25% (n = 16) of frogs of different age groups captured in April and May, while no infection (n = 18) was detected in July. The only stages observed were mature gamonts and oocysts located in the epithelium of renal tubules (Fig. 1b). In situ sporulated oocysts of ‘I.’ lieberkuehni were bisporocystic, elongated, with very thin elastic oocyst wall, 35–45 × 20–25 μm, each with two sporocysts. Sporocysts were broadly spindle-shaped, 25–30 × 14–16 μm, lacking the Stieda and substieda bodies. Sporozoites (four in each sporocyst) were elongated, banana-shaped, 17–21 × 3–4 μm in size. The sporocyst residuum appeared as a spherical to subspherical cluster of irregular granules (Fig. 1a). Many sporocysts liberated from ruptured oocysts were observed in the renal tubules. The morphology, localization of the endogenous stages and the host of the studied coccidium fit well with the original description of Klossia lieberkuehni, as well as with the observations of ‘I.’ lieberkuehni by later authors (Kazubski & Grabda-Kazubska, 1973; Labbé, 1894; Laveran & Mesnil, 1902) (Fig. 2). Observed seasonal occurrence is in agreement with an earlier report by Nöller (1923). Simple experiments described by this author also proved the direct life cycle, clearly distinguishing this parasite from members of the Sarcozystinae. It is probable that the endogenous development of this coccidium depends on the seasonal breeding of water frogs. The development in renal tubules and consequent release of oocysts culminate during spring when the breeding activity of frogs peaks and the oocysts are discharged into the aquatic environment represent a source of infection for the developing tadpoles. However, further research is required to settle this issue.

Generally, there are two modes of excystation of the Isospora species and the excystation structures seem to represent an important feature for classification (Box et al., 1980). Whereas some species do excyst by a collapse of plates, in other species excystation occurs by an escape of sporozoites through an opening in the sporocyst wall plugged by the Stieda body (Speer et al., 1973). Carreno & Barta (1999) recently demonstrated a strong correlation between the mode of excystation and phylogeny. The phylogenetic position of ‘I.’
Fig. 3. Maximum-parsimony tree of the SSU rRNA sequences rooted at Cryptosporidium parvum; 50% majority rule strict consensus of 30 most parsimonious trees (Tv/Ts = 1:1; 790 steps; CI = 0.71; RI = 0.78). Bootstrap values (MP Tv/Ts = 1:1; MP Tv/Ts = 1:3; distance methods LogDet and HKY85) are shown below and the Bremer’s indices above the line. The generally accepted taxonomic classification is indicated to the right. Consensus sequences: 1 Toxoplasma gondii, from L37415, U12138, L24381, X65508, U03070, U00458, M97703, X68523, X75453, X75429 and X75430; 2 Neospora caninum, from L23380, U03069, U16159 and U17346; and 3 Isospora belli, from U94787 and AF10693.

*I. lieberkuehni* within the Sarcocystidae correlates well with the absence of the Stieda and substieda bodies in its sporocysts.

**Phylogenetic analysis**

The phylogenetic position of *I. lieberkuehni* was evaluated by analysing the SSU rRNA gene. From oocysts isolated from the frog’s kidney, an almost full-length (1564 bp long) region of the SSU rRNA was sequenced. The analysis was based on a final alignment (1730 bp long) consisting of 23 taxa, from which ambiguously aligned areas (113 bp) were removed.

The studied isolate clusters within Sarcocystidae in a major group (branch A *sensu* Doležel et al., 1999) that comprises medically important parasites of the subfamily Toxoplasmatinae. This branch contains three clades supported by high bootstrap values (Fig. 3): (i) the *Isospora* species lacking the Stieda body (all parasites of mammals); (ii) the ‘heteroxenous’ coccidia (*Neospora/Hammondia/Toxoplasma/Besnoitia*); (iii) *I. lieberkuehni*. All methods used strongly support monophyly of branch A and clades (i) and (ii) (Fig. 3), however, the position of *I. lieberkuehni* within branch A is unstable. Both matrices used in the distance method placed the studied species as an ancestral representative of branch A, however, with a low bootstrap support (59%; 61%). Certain degree of instability of the *I. lieberkuehni* clade is manifested in the maximum-likelihood tree (−ln = 6771-68832), where it was placed an ancestral representative of branch A (50%) (Fig. 4). Moreover, the Puzzle algorithm further supported this branching order (83%) (data not shown). In the maximum-parsimony analysis (234 and 202 informative and non-informative sites, respectively; Tv/Ts = 1:1; 30 most parsimonious trees; 790 steps; CI = 0.71; HI = 0.78) our isolate branched off on the basis of branch A (49%), while a change of Tv/Ts ratio to 1:3 (six most parsimonious trees; 1406 steps; CI = 0.73; HI = 0.80) resulted in its placement as a sister taxon to the *Isospora* clade (45%). Taken together, these results show a ‘trichotomy’, caused by a lack of resolution within the above-mentioned clades within branch A.
Phylogeny and taxonomy of ‘Isospora lieberkuehni’

Oocysts bisporocystic, with a thin, elastic, relatively fragile oocyst wall; sporocysts tetrazoic; sporocyst wall composed of plates joined by sutures; Stieda and substieda bodies absent. Life cycle is homoxenous, endogenous development extraintestinal; sporulation of oocysts typically endogenous (in situ). Type species: *Hyaloklossia lieberkuehni* (Labbé, 1894). Type host: *Rana* kl. esculenta L.

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