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

David Modrý,[1,2] Jan R. Šlapeta,[1,2] Milan Jirků,[2,3] Miroslav Oborník,[2,3] Julius Lukeš[2,3] and Břetislav Koudela[1,2]

[1] Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 612 42 Brno, Czech Republic

[2,3] Institute of Parasitology, Czech Academy of Sciences[2] and and Faculty of Biology, University of South Bohemia[3], České Budějovice, Czech Republic

Author for correspondence: David Modrý. Tel: +420 5 41562979. Fax: +420 5 748841. e-mail: modryd@vfu.cz

'*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; Votypka *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

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Sequence alignments from this paper are available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).

Abbreviation: Tv/Ts ratio, transversion/transition ratio.

The GenBank accession number for the SSU rRNA sequence of *Hyaloklossia lieberkuehni* is AF298623.

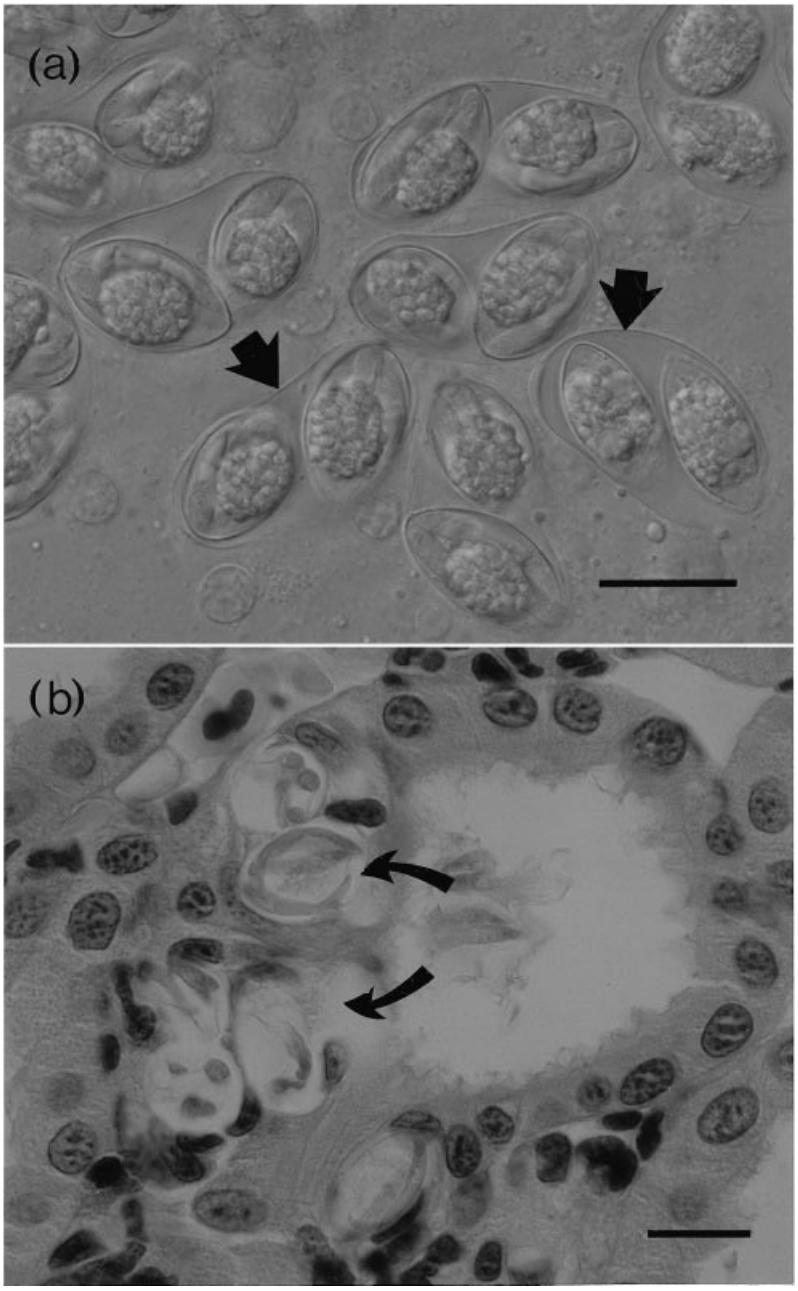

Fig. 1. (a) Nomarski interference contrast photograph of sporulated oocysts of *Hyaloklossia lieberkuehni* in squash preparation of frog kidney. Note thin, elastic and collapsible oocyst wall (arrows). (b) Histological section through tubules of infected kidney, showing several *in situ* sporulated oocysts of *H. lieberkuehni* (curved arrows). Bars, 20 μm.

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 Lanžhot, 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 rRNA gene was PCR amplified with the oligonucleotides K11 (AAAGATTAAGCCATGCA) and K12 (CAAAGGGCAGGGACGTA), 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://ijs.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/transition (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

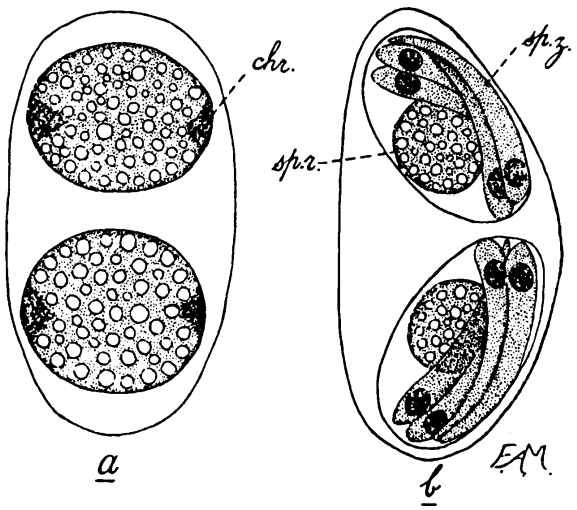

Fig. 2. The available drawings of (a) partly and (b) fully sporulated oocyst of *H. lieberkuehni*. Reprinted from Minchin (1903), after Laveran & Mesnil (1902).

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 Sarcocystinae. 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.*'

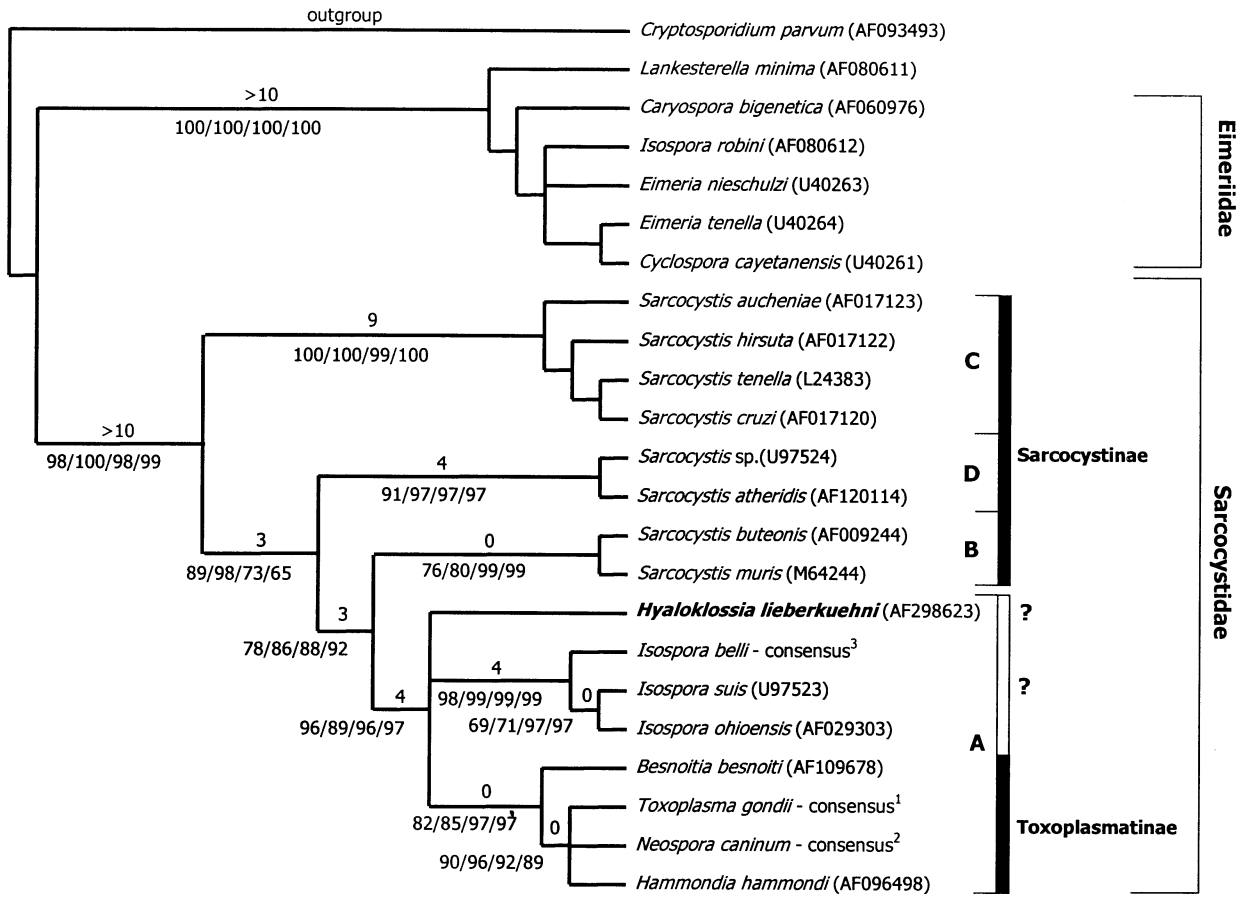

Fig. 3. Maximum-parsimony tree of the SSU rRNA sequences rooted at *Cryptosporidium parvum*; 50 % majority rule strict consensus of 30 most parsimonious trees (Tv/Ts = 1:1; 790 steps; CI = 0·71; RI = 0·78). Bootstrap values (MP Tv/Ts = 1:1; MP Tv/Ts = 1:3; distance methods LogDet and HKY85) are shown 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.

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.

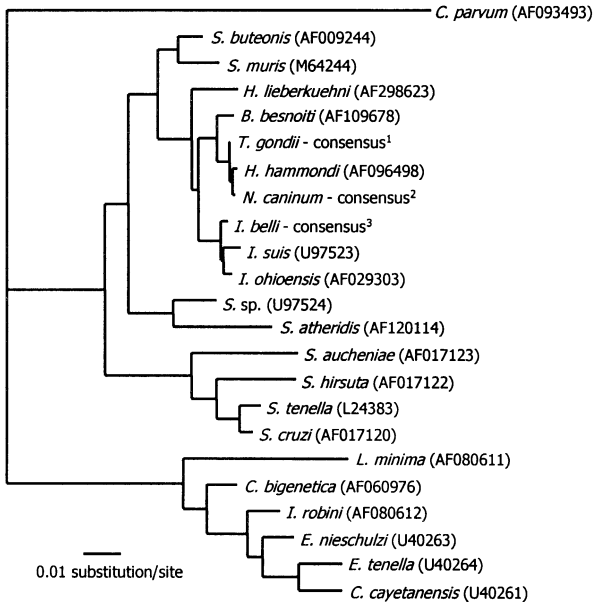

Fig. 4. Maximum-likelihood tree of the SSU rRNA sequences rooted at *Cryptosporidium parvum*. The distance scale is given under the tree. See the legend to Fig. 3 for accession numbers for consensus sequences.

Sequencing the D2 domain or the entire large-subunit rRNA gene may resolve the exact position of '*I.*' *lieberkuehni* within branch A, as has been shown previously for related species (Ellis *et al.*, 1999; Mugridge *et al.*, 1999b). The overall branching of the trees is in good agreement with the topologies published by other authors (Doležel *et al.*, 1999; Jenkins *et al.*, 1999; Holmhahl *et al.*, 1999).

Traditionally, the family Sarcocystidae is divided into two subfamilies, Sarcocystinae and Toxoplasmatinae. Recently, Jenkins *et al.* (1999) have also included mammalian *Isospora* spp. (lacking the Stieda body) into the latter subfamily, without any respect to the widely accepted definition of the Toxoplasmatinae (e.g. Frenkel *et al.*, 1987). More biological, morphological and molecular data on isosporoid coccidia are necessary to resolve the division of Sarcocystidae into the concise subfamilies.

Taxonomy

The unique combination of morphological, biological and phylogenetic feature is, in our opinion, sufficient for the separation of '*Isospora*' *lieberkuehni* on the generic level. The homoxenous life cycle distinguishes it from *Sarcocystis* and the exogenous sporulation and extraintestinal development from the mammalian *Isospora* spp. and members of the Toxoplasmatinae.

Hyaloklossia Labbé, 1896 is the oldest available synonym and should therefore be re-erected. However, the definition of the genus *Hyaloklossia* given by Labbé (1896) is insufficient and should be corrected on the basis of recent observations.

Emended genus definition of *Hyaloklossia* Labbé, 1896 (Apicomplexa: Sarcocystidae)

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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