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A Model for Taxonomic Work on Homoxenous Coccidia: Redescription, Host Specificity, and Molecular Phylogeny of *Eimeria ranae* Dobell, 1909, with a Review of Anuran-Host *Eimeria* (Apicomplexa: Eimeriorina)

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ABSTRACT. We attempt to extend knowledge of anuran *Eimeria*, and to provide a model for a complex approach to studies on coccidia. New host and geographic records of coccidia in European Anura are provided. In the second part, *Eimeria ranae* Dobell, 1909 is redescribed from European terrestrial frogs of the genus *Rana* based on light microscopic and ultrastructural data on both exogenous and endogenous developmental stages, host specificity, and molecular phylogenetic data. Results of experimental transmissions show for the first time that the host specificity of *E. ranae* is restricted to the genus *Rana* and that isolates from tadpoles and adults are conspecific. Disappearance of infection during metamorphosis was confirmed experimentally, suggesting that infections in adults result from reinfections. Poikilotherm-host *Eimeria* species possessing a Stieda body (SB) are for the first time included in a molecular phylogenetic analysis. *Eimeria ranae* and *Eimeria arnyi* from a colubrid snake form together a well-supported clade, basal to other SB-bearing coccidia. The other analysed reptile–host eimerians, *Eimeria tropidura* and *Choleoeimeria* sp., which possess bivalved sporocysts and lack a SB, represent a distinct basal lineage of the eimeriid clade. The third part of the article reviews anuran-host *Eimeria*. Three distinct oocyst morphotypes, apparently correlating with the character of endogenous development, are recognized and characterized among anuran eimeriids.

Key Words. Amphibia, Anura, experimental infections, metamorphosis, morphotypes, SSU rDNA sequence, tadpoles, ultrastructure.

THE order Eucoccidiorida, commonly known as the coccidia, is taxonomically the most diverse order within the subclass Coccidiasina, and includes numerous families and genera with unclear phylogenetic relationships. Most taxonomic studies on coccidia are limited to inadequate species descriptions based on the morphology of exogenous stages (oocysts), and there is a lack of information on the life cycles and biology of most species. Estimations of coccidian diversity suggest that even in the most studied hosts, such as rodents, only about 8% of the expected total of coccidian species is known (Tenter et al. 2002). Traditional generic classification of homoxenous eimeriorinid coccidia, Eimeriidae sensu lato (s.l.), is based primarily on quantitative phenotypic characters, namely the number of sporocysts and sporozoites within the oocyst (Upton 2000). However, the incongruity of this classification with molecular phylogenetic analyses results in current taxonomic confusion.

The genus *Eimeria* Schneider, 1875 comprises homoxenous coccidia possessing four dizoic sporocysts within the oocyst. With more than 1,300 described species (Duszynski, Couch, and Upton 2000), the genus belongs to one of the most speciose eukaryotic genera, and may serve as a model for studies on coccidian evolution and host–parasite coevolution. As in other coccidian genera, only a minority of species has been described, most of them improperly. One of the ways to fill these gaps in our knowledge might be to shift our attention from the established models (i.e. avian- and mammalian-host coccidia) to hitherto neglected genera considered synonyms of *Eimeria* (see p. 331 in Upton 2000 for list), as well as to eimerians of neglected hosts. The incorporation of such ''missing links'' represents a sound approach to elucidate eimeriid as well as general coccidian taxonomy and phylogeny (Kopečná et al. 2006).

Among coccidia, species infecting poikilotherm hosts are not well studied. Despite numerous species descriptions (Duszynski et al. 2000), studies focused on their life cycles and biology are rare. Among the named *Eimeria* spp., about 500 species (38%) are reported to parasitize poikilotherm hosts. However, only a single species, *Eimeria tropitura* Aquino-Shuster, Duszynski, and Snell, 1990 from tropidurid lizards has been included in phylogenetic analyses (Morrison et al. 2004). As shown in a recent review by Duszynski, Bolek, and Upton (2007), the least explored vertebrate-host coccidia are those parasitizing amphibians. For example, only 18 species of *Eimeria* have been described from anurans. As shown by other studies (Duszynski et al. 2007 and references therein), the relative scarcity of known anuran *Eimeria* is caused by a lack of information rather than low diversity.

The present study is focused on *Eimeria ranae* Dobell, 1909, an improperly described species from the European frog, *Rana temporaria* L. based on single measurements of an oocyst and a sporocyst without providing morphometrical variability and information on endogenous development. We attempt to extend our knowledge of the inadequately known anuran *Eimeria*, and to provide a model for a complex approach to focused studies on single coccidian species. Our study is composed of three parts: (1) we summarize new host and distribution records of European anuran coccidia; (2) we redescribe *E. ranae* using morphological features, experimentally evaluate its host specificity, and provide a phylogenetic analysis based on small subunit (SSU) ribosomal DNA (rDNA) sequences; and (3) we include a taxonomic review of anuran-host *Eimeria* to provide stimulus for future studies.

MATERIALS AND METHODS

Collection, handling, and examination of hosts. During 2001–2005, a total of 3,703 larvae and 499 adults representing seven anuran species were examined: *Rana dalmatina* Fitzinger—1,270 tadpoles/92 adults; *R. temporaria* L.—1,421/201; *Pelophylax* kl. *esculentus* (L.) (formerly *Rana* kl. *esculenta*)—100/61; *Bufo bufo* (L.)—865/105, *Pseudepidalea viridis* (Laurenti) (formerly *Bufo viridis*)—0/2; *Bombina variegata* L.—0/30; *Hyla arborea* L.—47/8 (see ''Localities'' and Table 1 for details). We consistently use recently established amphibian nomenclature (Frost 2007).

Adult anurans were collected either in the breeding ponds during the spring spawning, or in the vicinity of the breeding sites during their terrestrial phenological phase. In the lab, the frogs were individually kept at room temperature. Upon defecation, feces from individual animals were homogenized and sieved. Then,

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Table 1. Results of coprological examination of anurans collected in the Czech Republic.

Host species (*n*—totals)	Coccidian taxon	Devel. st.: Locality prevalence/n
Rana temporaria	*Eimeria ranae*[a]	T: Loc1 15%/667, Loc2 27%/754 Ad: Loc1 33%/48, Loc2 27%/135, Loc8 44%/18
	Goussia sp.[a]	T: Loc1 ?/667, Loc2 ?/754
Rana dalmatina[a]	*Eimeria ranae*[a]	T: Loc1 17%/1,270 Ad: Loc1 73%/80, Loc5 50%/12
	Goussia sp.[a]	T: Loc1 ?/1,270
Pelophylax kl. *esculentus*	*Eimeria prevoti*[a]	Ad: Loc7 33%/15
	Hyaloklossia lieberkuehni	Ad: Loc2 33%/30, Loc6 38%/16
	Goussia sp.[a]	T: Loc4 ?/50, Loc6 ?/50
Bufo bufo[a]	*Goussia* sp.[a]	T: Loc1 ?/420, Loc2 ?/396, Loc3 ?/49
	No coccidia detected	Ad: Loc1 0%/48, Loc2 0%/57
Pseudepidalea viridis	*Isospora brumpti*[a]	Ad: Loc2 50%/2
Bombina variagata	No coccidia detected	Ad: Loc2 0%/30
Hyla arborea	No coccidia detected	T: Loc1 0%/47
		Ad: Loc2 0%/8

[a]New host and geographic records; ?, uncertain—*Goussia* prevalence values obtained by coprological examination are unreliable due to intermittent oocyst shedding.

Ad, infections in adults; Loc, locality; *n*, sample size; T, infections in tadpoles.

1/4–1/3 of each fecal sample was examined by a flotation method using sucrose solution (s.g. 1.3); the remaining feces was used for experimental infections. Most animals were released at the original locality within 1 wk after collection. Selected frogs (9 *R. temporaria* and 10 *R. dalmatina*) shedding the highest numbers of oocysts were euthanized by pithing and processed for parasitological examination as described elsewhere (Jirků and Modrý 2006a). In addition, colon contents of roadkills (21 *R. dalmatina*, 33 *R. temporaria*, 37 *B. bufo*) were collected and examined at all localities to increase sample sizes.

Tadpoles were collected at the same breeding sites as adults, placed individually into 100-ml vials filled with dechlorinated tap water, transported to the lab, and kept in open vials for 24 h at room temperature exposed to daylight. Fecal debris from each vial was collected after ~24 h by Pasteur pipette, homogenized, and examined by flotation. Selected tadpoles were pithed and dissected in a 10% (v/v) buffered formalin bath. Various tissues were examined in fresh preparations with the gastrointestinal tract and liver processed for histology. The remaining tadpoles were released at the original locality within 1 wk after collection at a place from which they could not return to the original population.

Localities. Extensive studies were conducted at two principal localities, with amphibians from several other localities in the Czech Republic being examined. Locality (Loc) 1. Zaječí (Zayetchee) potok, vicinity of Brno, 16°36′23″E, 49°14′15″N, 303 m above sea level (a.s.l.): *R. dalmatina, R. temporaria*, *H. arborea*, and *B. bufo*. Loc 2. Raduň, Zámecký rybník, vicinity of Opava, 49°53′23″N, 17°56′38″E, 301 m a.s.l.: *R. temporaria*, *P.* kl. *esculentus*, *H. arborea*, *B. bufo*, *P. viridis*, and *B. variegata*. Additional frogs were collected at the following localities: Loc 3. Babí doly, vicinity of Brno, 16°36′12″E, 49°17′22″N, 390 m a.s.l.: *B. bufo*; Loc 4. Šnejdlík Pond, vicinity of České Budějovice, 14°25′04″E, 49°00′20″N, 380 m a.s.l.: *P* kl. *esculentus*; Loc 5. Růženčin lom, Brno-Hády, 16°40′22.69″E, 49°13′0.78″N, 355 m a.s.l.: *R. dalmatina*; Loc 6. Lanžhot, vicinity of Břeclav, 16°58′04″E, 48°42′38″N, 160 m a.s.l.: *P* kl. *esculentus*; Loc 7. Chomoutov, vicinity of Olomouc, 17°14′21″E, 49°38′51″N, 215 m a.s.l.: *P* kl. *esculentus*; Loc 8. Jedovnice, vicinity of Brno, 16°46′31″E, 49°20′01″N, 484 m a.s.l: *R. temporaria*.

Microscopy. Squash preparations of various viscera, oocysts concentrated by flotation, and histological sections were examined by light microscopy using an Olympus AX 70 microscope (Osaka, Japan) equipped with Nomarski interference-contrast optics. For histology, tissues of dissected frogs were fixed in 10% (v/v) formalin, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin, and mounted in Canada balsam. Measurements were obtained using a calibrated ocular micrometer on at least 30 individuals of each developmental stage.

For transmission electron microscopy (TEM), tissues were fixed overnight with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4 °C, postfixed for 2 h at 4 °C in 1% (w/v) osmium tetroxide, dehydrated through an ethanol series and embedded in Durcupan via acetone. Semithin (400 nm) sections were stained with toluidine blue. Ultrathin sections were double stained with uranyl acetate and lead citrate and viewed in a Jeol 1010 TEM (Tokyo, Japan).

Experimental amphibian transmissions. For all experiments, isolates of *E. ranae* from adult frogs and tadpoles from Loc 1 were used. Sieved fecal samples containing oocysts of *E. ranae* were pooled and kept in dechlorinated tap water in 0.5-L containers without preservatives to avoid intoxication of experimental animals. Every 3 days, a fecal debris/oocyst suspension was stirred and sedimented (1 h). Then, water was removed and the tanks were refilled with fresh water. The fecal debris/oocyst suspension was used for infection experiments. Infectious material originating from adult frogs and tadpoles was always used separately. Selected samples were stored in potassium dichromate for monitoring of long-term survival of oocysts.

Experimental animals were kept at ~20 °C in a separate facility for amphibians with artificial illumination simulating actual outdoor photoperiod. Coccidia-free tadpoles were raised from eggs collected from Loc 1 and 2. Tadpoles of each species were kept together in aerated dechlorinated tap water and fed ad libitum with a universal granulated fish food Lon Mix (Aqua Tropic Lonský, Prague, Czech Republic) and chopped lettuce until the start of experimental trials. Post-metamorphic juveniles and adults were kept in plastic vivaria containing wet coco substrate and plastic shelters, fed ad libitum with wingless *Drosophila melanogaster* and *Gryllus assimilis* supplemented with Reptivite (Zoo Med Laboratories Inc., San Luis Obispo, CA). *Xenopus laevis* and *Pleurodeles waltl* were obtained from laboratory colonies of the University of Veterinary Pharmaceutical Sciences, Brno, Czech Republic.

In each experiment, a group of 50 tadpoles was used. After a day of starvation, each experimental group was placed into a 4-L tank and fed with a mixture of fecal debris containing oocysts and granulated fish food. After 12-h exposure, the tadpoles were relocated into coccidia free 25-L tanks. As a test of the infectivity of oocysts, 50 tadpoles of either *R. dalmatina* or *R. temporaria*, each in three separate experiments, were exposed to oocysts originating from wild conspecific tadpoles. Using this method of exposure, it was impossible to estimate infectious doses, which are therefore not provided. Tanks with 200–300 tadpoles of *R. dalmatina*, *R. temporaria*, *P.* kl. *esculentus*, *B. bufo*, *X. laevis*, and 200 larvae of *P. waltl* were kept until metamorphosis as negative controls to ensure that the experimental animals were coccidia-free at the outset of the trials. All fecal sediments from tanks with tadpoles were removed using a rubber hose, concentrated by repeated sedimentation, homogenized, and examined by flotation every second

Table **2.** Cross-transmission experiments: no. of experimental trials resulting in infections/no. of experimental trials conducted.

Experimental animals	Origin of infectious material			
	Tadpoles *Rana dalmatina*	Adult *Rana dalmatina*	Tadpoles *Rana temporaria*	Adult *Rana temporaria*
Rana dalmatina	2/3	1/3	2/3	2/2
Rana temporaria	3/4	2/3	3/3	1/3
Pelophylax kl. *esculentus*	0/4	—	0/2	—
Bufo bufo	0/4	—	0/2	—
Hyla arborea	0/1	—	—	—
Xenopus laevis tadpoles	0/4	—	0/2	—
Xenopus laevis adults	0/8 adults	—	—	—
Pleurodeles waltl larvae	0/1	—	0/1	—
Pleurodeles waltl adults	0/4 adults	—	—	—

Each trial involved 50 experimental tadpoles, except for *H. arborea* and *P. waltl* when 20 individuals were used in each trial.

day in the case of experimental tadpoles and once weekly in tadpoles used as negative controls.

In order to test the host specificity of *E. ranae*, tadpoles of seven different anuran species were exposed to oocysts of *E. ranae* originating from naturally infected adults or tadpoles of *R. temproraria* and *R. dalmatina* (Table 2). In addition, eight adult *X. laevis* and four adult *P. waltl* were orally inoculated with 0.25–0.50 mL of fecal debris/oocyst suspension originating from *R. dalmatina*.

To assess the fate of infection during and after the metamorphosis, 20 experimentally infected tadpoles of *R. dalmatina* were kept beyond metamorphosis. Frogs were dissected and processed for histology at 2-wk intervals for the first 2 mo, at 4-wk intervals for the third and fourth month, and at 12-wk intervals until 15 mo of age. All feces were continuously collected from vivaria housing metamorphosed frogs and examined by flotation.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing. Total DNA of *E. ranae* was isolated from the mashed intestine of a tadpole of its type host, *R. temporaria* (from Loc. 1), which was heavily infected with merogonic and gamogonic stages, as described elsewhere (Maslov et al. 1996). The SSU rDNA was amplified using universal eukaryotic primers (Medlin et al. 1988). For PCR, the program was 30 cycles of 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min. Polymerase chain reaction products were gel-purified, cloned into the TOPO TA vector (Invitrogen, Carlsbad, CA), and sequenced. An almost complete 1, 787-bp long nucleotide sequence of the SSU rDNA of *E. ranae* was deposited in GenBank™ under the Accession number EU717219.

Phylogenetic analysis. The newly obtained sequence for the nuclear SSU rRNA gene from *E. ranae* was identified using nucleotide BLAST at NCBI (Altschul et al. 1990, 1997). The sequence was aligned together with relevant publicly available homologues using CLUSTALX (Thompson et al. 1997); alignment was manually checked and gaps and ambiguously aligned regions were excluded from analysis. The Modeltest 3.7 program (Posada and Crandall 1998) was used to specify the appropriate model for nucleotide substitutions for the particular dataset (TN93). Phylogenetic trees were constructed using maximum parsimony (PAUP*; Swofford 2000) and maximum likelihood methods (PhyML; Guidon and Gasquel 2003). Maximum likelihood tree was computed using the TN93 model with discrete γ distribution in four categories; the proportion of invariant sites (0.296), γ shape parameter (0.509), TS/TV ratio for purines (2.281) and pyrimidines (4.424) were estimated from the dataset. The Bayesian tree was computed using MrBayes with priors, chain number, and temperature set to default. The SSU rDNA sequences representing all eimerian lineages were included in our analysis; *Eimeria reichenowi* and *Eimeria gruis* were excluded due to their highly unstable position in our analyses.

GenBank™ sequences included in the SSU rDNA analysis were the following: *Atoxoplasma* sp. AY331571, *Caryospora bigenetica* AF060975, *Choleoeimeria* sp. AY043207, *Cyclospora cayetanensis* AF111183, *Cyclospora colobi* AF111186, *Cyclospora papionis* AF111187, *Eimeria acervulina* U67115, *Eimeria alabamensis* AF291427, *Eimeria albigulae* AF307880, *Eimeria arnyi* AY613853, *Eimeria bovis* U77084, *Eimeria catronensis* AF324213, *Eimeria chaetopidi* AF339489, *Eimeria chobotarii* AF324214, *Eimeria dipodomysis* AF339490, *Eimeria falciformis* AF080614, *Eimeria langebartelii* AF311640, *Eimeria maxima* U67117, *Eimeria mitis* U40262, *Eimeria mivati* U76748, *Eimeria necatrix* U67119, *Eimeria nieschulzi* U40263, *Eimeria peromysci* AF339492, *Eimeria pilarensis* AF324215, *Eimeria praecox* U67120, *E. ranae* EU717219, *Eimeria reedi* AF311642, *Eimeria separata* AF311643, *Eimeria telekii* AF246717, *Eimeria tenella* U40264, *Eimeria tropidura* AF324217, Intranuclear coccidium AY728896, *Isospora robini* AF080612, *Lankesterella minima* AF080611; Sarcocystidae: *Besnoitia bennetti* AY665399, *Besnoitia besnoiti* AF109678, *Besnoitia jellisonii* AF291426, *Cystoisospora timoni* AY279205, *Cystoisospora ohioensis* AY618555, *Hammondia hammondi* AF096498, *Hyaloklossia lueberkuehni* AF298623, *Isospora beli* AF106935, *Isospora felis* L76471, *Isospora orlovi* AY365026, *Neospora caninum* U17346, *Sarcocystis gallotiae* AY015112, *Sarcocystis muris* M34846, *Sarcocystis neurona* U07812, *Sarcocystis rodentifelis* AY015111, *Toxoplasma gondii* U12138; others: *Adelina bambarooniae* AF494059, *Adelina bambarooniae* AF494058, *Adelina dimidiata* DQ096835, *Adelina grylli* DQ096836, *Babesia motasi* AY533147, *Babesia orientalis* AY596279, *Cytauxzoon felis* AY679105, *Goussia janae* AY043206, *Hepatozoon americanum* AF176836, *Hepatozoon catesbianae* AF130361, *Hepatozoon* sp. AF297085, *Hepatozoon* sp. AB181504, *Theileria annulata* AY524666, *Theileria bufeli* AY661513, *Theileria sergenti* AY661515.

RESULTS

New records of anuran coccidian. Five coccidian species were found in *R. dalmatina*, *R. temporaria*, *P.* kl. *esculentus*, *P. viridis*, and *B. bufo*. *Hyla arborea*, and *B. variegata* were negative (Table 1).

Of all seven amphibian species examined, *E. ranae* was recorded only in tadpoles and adults of the terrestrial ranids *R. dalmatina* and *R. temporaria*. We believe, our isolates are conspecific with *E. ranae* sensu Dobell, (1909), and provide its redescription using current standards.

Redescription of *Eimeria ranae* Dobell, 1909. Early sporogonic stages were represented by sporonts composed of rough granules (Fig. 1), while pyramidal stages (Fig. 2–4) and oocysts containing four blastomeres and oocyst residuum (Fig. 5) represented late sporogony. Fully sporulated oocysts (Fig. 6–10) were variable in both shape and size, broadly elliptical to spherical with length–width ratio (L/W) 1.1 (range 1.0–1.2), measuring 19.5 (17.0–21.0) × 17.9 (16.0–21.0) μm with fine, smooth, and colorless wall (Fig. 1). The oocyst residuum was spherical to subspherical, 7–9 μm in diameter, and composed of a compact mass of granules of relatively uniform size (1.0–1.5 μm in diameter), often with spherical vacuole (3–4 μm in diameter). A micropyle and polar granule were absent. Sporocysts were dizoic, 11.1

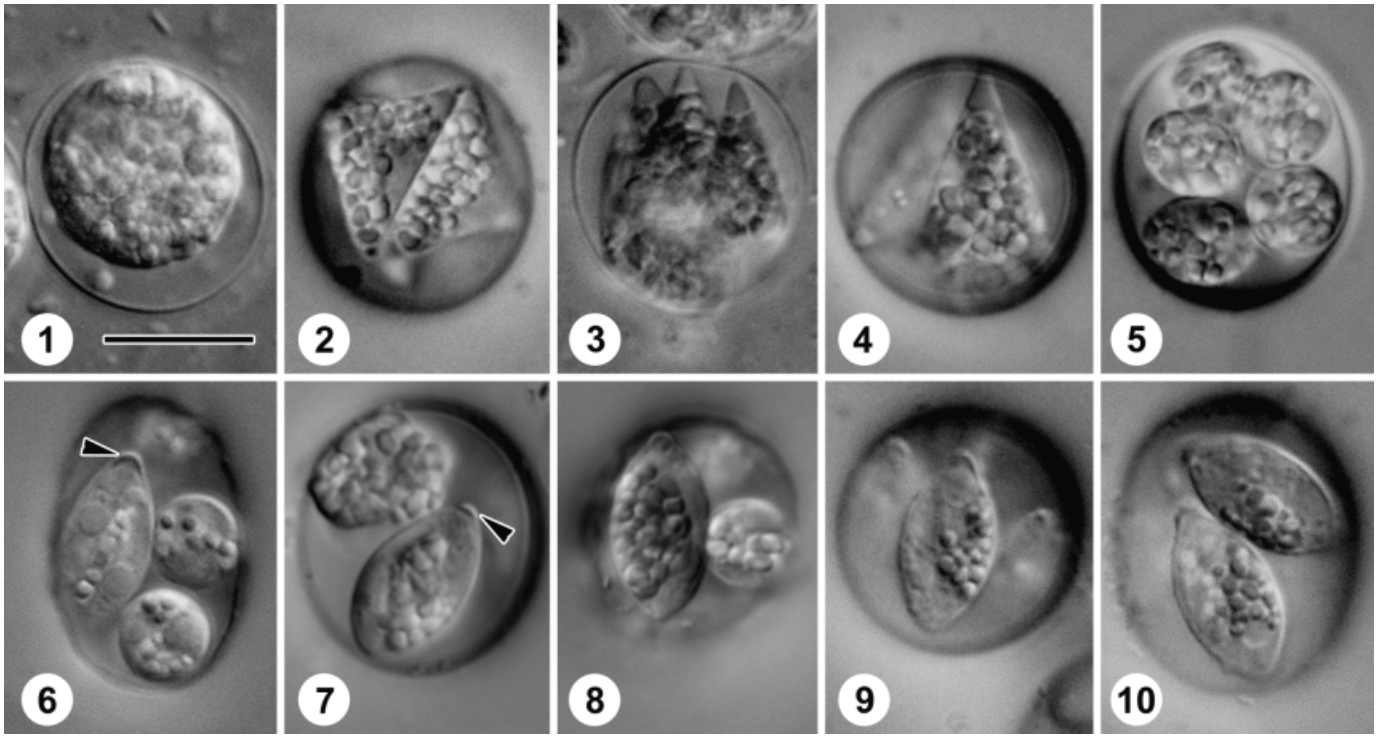

Fig. **1–10.** Sporogonic stages of *Eimeria ranae*, Nomarski interference contrast. **1.** Sporont. Note the fine oocyst wall in optical section. **2–4.** Pyramidal sporogonic stages. **5.** Oocyst containing blastomeres. **6–10.** Mature, fully sporulated oocysts. Note shape and size variability of both oocysts and sporocysts and distinct Stieda bodies (arrowheads). All to same scale; scale bar = 10 μm.

(10.0–13.0) × 7.0 (6.0–8.0) μm, with a prominent Stieda body (SB), 1.5–2.0 μm wide, 0.5–1.0 μm high, slightly hollowed on the inner side (Fig. 6). Sporocyst shape varied even within a single oocyst, from broadly elliptical to navicular (Fig. 6–11). The sporocyst pole bearing the SB was often somewhat tapered (Fig. 11). Finely granulated sporozoites possessed a centrally located nucleus or refractile body (2.5–3.5 μm in diameter). The sporocyst residuum was usually compact, elliptical, measuring 4.0–5.5 × 3.0–4.0 μm. It was rarely scattered among sporozoites, and composed of spherical to elliptical granules of variable size (0.5–2.0 μm in diameter) (Fig. 6–10).

Endogenous development. Endogenous stages are located extranuclearly in the cytoplasm of enterocytes, usually in the region above the host cell nucleus. In adult hosts, the endogenous development was confined to the small intestine, while in tadpoles, the entire intestine was parasitized. As a rule, weak to moderate infections were typical for adult frogs (data not shown), in which scattered developmental stages were encountered in histological sections. Heavy infections were often observed in tadpoles, where the stages often formed dense aggregations (Fig. 12, 15). In such areas, normally elliptical stages became deformed, presumably as a result of lack of space. Compared with gamogonic stages, meronts were rarely observed in histological sections. Mature meronts of variable size (9.0–17.0 × 7.0–17.0 μm) contained over 20 merozoites (4.0–5.0 × 1.5 μm) per section (Fig. 12, 13). Mature microgamonts (14.0–20.0 × 8.0–14.0 μm) were irregular in shape, and contained spirally arranged microgametes (Fig. 12, 14, 15). Spherical to elliptical macrogamonts (16.0–22.0 × 8.0–20.0 μm) were the most numerous stages, possessing a large, usually excentrically positioned nucleus (4.0 μm in diam.) with distinct micronucleus and moderately stained granules (Fig. 12, 15). Unsporulated oocysts of relatively uniform size (15.0–17.0 × 14.0–16.0 μm) were frequently observed within the sectioned enterocytes. Occasionally, oocysts were found located in the apical part of the host cell, just below the microvilli, sometimes bulging out into the gut lumen (Fig. 16). Oocysts showing signs of sporulation were not observed in histological sections or TEM preparations.

Pathology. Despite heavy infections in tadpoles, no inflammatory response was observed in affected tissues, but moderate histopathological changes were associated with aggregations of endogenous stages in tadpoles. In such cases, most of the volume of epithelial cells in large portions of the affected epithelia was occupied by endogenous stages (Fig. 12, 15). Despite the obvious pathological process, experimental tadpoles that shed comparable quantities of oocysts as the histologically examined ones, showed no mortality or morbidity (compared with uninfected controls) and successfully completed the metamorphosis. No histopathological changes were observed in infected adults (data not shown).

Ultrastructure. Only gamonts in various stages of development and unsporulated oocysts were observed in ultrathin sections. Immature macrogamonts (7.5–12.0 × 7.5–9.0 μm) were characterized by the presence of extensive endoplasmic reticulum forming a thick layer on the periphery of the cell (Fig. 17, 18). The cytoplasm contained a prominent nucleus with a distinct nucleolus, large amylopectin granules, peripherally located, elongated to dumbbell-shaped mitochondria, and lipid inclusions. Advanced macrogamonts were characterized by less extensive endoplasmic reticulum and the appearance of small, globular, dense granules (Fig. 19). In mature macrogamonts and zygotes (11.0–18.0 × 8.0–15.0 μm) the endoplasmic reticulum and small, globular, dense granules were replaced by the wall-forming body-like granules, and numerous sub-membranous vesicles containing irregular osmiophilic material, which seemed to communicate with the cytoplasmic membrane by means of a narrow pore (Fig. 20, 21). The wall-forming body-like granules, similar in shape and size to the lipid inclusions, had a different density and possessed a narrow halo. These granules apparently originate from the globular dense

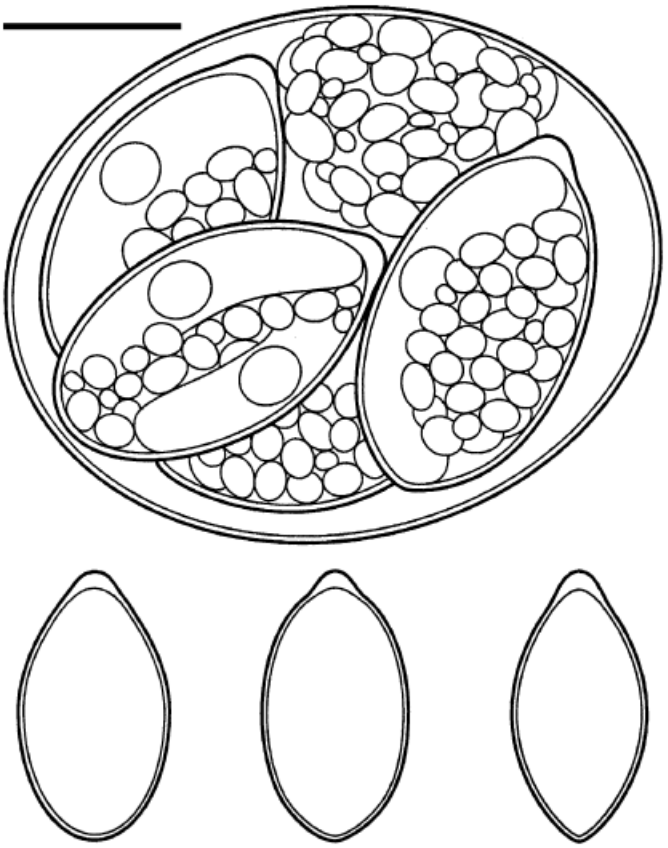

Fig. **11.** Composite line drawing of *Eimeria ranae* oocyst; Scale bar = 5 μm. Below, drawings (not to scale) of sporocysts show variability of sporocyst shape (left to right): elliptical with slightly pointed anterior pole and rounded posterior pole (cf. Fig. 7), broadly elliptical with pointed posterior pole (cf. Fig. 8), and navicular with pointed both poles (cf. Fig. 9.).

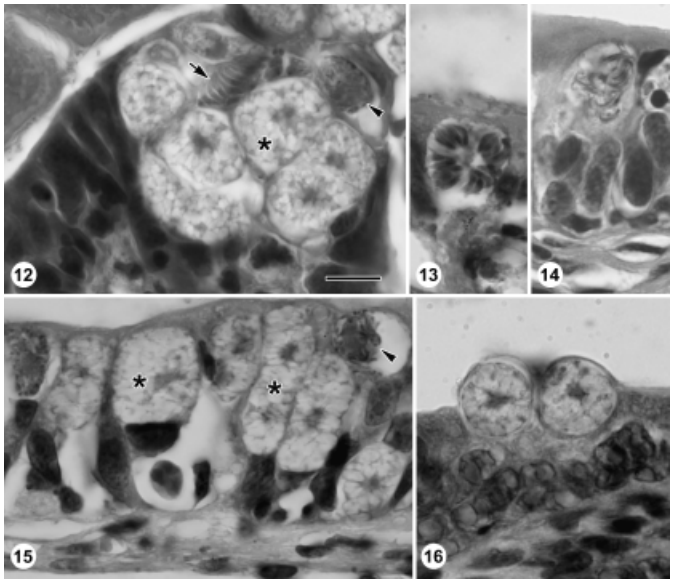

Fig. **12–16.** Endogenous developmental stages of *Eimeria ranae*. Histological sections, H&E. **12.** Aggregation of macrogamonts (*), microgamonts (arrowhead), and single meront (arrow) within the intestinal epithelium of a tadpole. The object at the upper left corner is *Opalina ranarum*. **13.** Mature meront. **14.** Mature microgamont. **15.** Aggregation of gamogonic stages (symbols as in Fig. 12). **16.** Unsporulated oocysts still enclosed by the host cell membrane protruding into the intestinal lumen. All to same scale; Scale bar = 10 μm. H&E, hematoxylin and eosin.

granules confined to immature macrogamonts. The space between the mature macrogamont and the membrane of parasitophorous vacuole was filled with an amorphous substance and droplets resembling the amylopectin granules (Fig. 20).

Immature microgamonts (Fig. 22) were characterized by peripherally arranged nuclei adjoined by centrioles, their endoplasmic reticulum, small scattered amylopectin granules, and lipid inclusions. Mature microgamonts with differentiated microgametes were loaded with clusters of fine amylopectin granules within the residual cytoplasm (Fig. 23, 24).

The cytoplasm of oocysts differed from that of macrogamonts by the absence of the wall-forming body-like granules and submembranous vesicles containing irregular osmiophilic material. The fine structure of oocysts, encircled by a thin bilayered oocyst wall, was improperly preserved, probably due to the low permeability of the oocyst wall for the fixatives (Fig. 25).

Sporulation. In fecal samples from adults examined immediately after defecation, as well as in colon contents of dissected frogs, up to 20% of oocysts were in various stages of sporulation, ranging from dividing sporonts to fully sporulated oocysts. This phenomenon was less pronounced in feces of tadpoles in which the oocysts showing signs of sporulation were only rarely observed. Sporulation of oocysts stored in water or potassium dichromate was markedly asynchronous. In all samples, ~20–100% of oocysts remained unsporulated regardless of the length of the incubation. After 3–4 wk of storage in water or potassium dichromate, free sporocysts were observed in storage medium, suggesting that the thin wall of some oocysts disintegrated. Morphologically intact oocysts/sporocysts were present in samples for up to 7 mo of storage.

In histological sections, only unsporulated oocysts were observed within both epithelial cells and intestinal lumen of tadpoles, whereas in adults, partly sporulated oocysts containing sporoblasts were occasionally observed within the intestinal lumen.

Experimental amphibian transmissions. During the experiments on tadpoles of *R. dalmatina* and *R. temporaria*, we confirmed both the intraspecific and cross-specific infections (Table 2). Also, the inter-stadial transmissions of *E. ranae* from both tadpoles and adults of *R. temporaria* to the tadpoles of *R. dalmatina* and vice versa were successful. These results confirmed the conspecificity of isolates of *E. ranae* originating from tadpoles and adults of the two *Rana* spp. Eight of 24 (33%) of the experiments involving receptive hosts of the genus *Rana* (including one of six positive control experiments) did not result in infections suggesting limited viability of infectious material.

We failed to infect tadpoles or adults of *P.* kl. *esculentus*, *H. arborea*, *B. bufo*, *X. laevis*, and *P. waltl* in which both flotation and histological examination failed to reveal developmental stages of *E. ranae*. All negative control tadpoles remained negative.

In all trials, the tadpoles of *R. dalmatina* and *R. temporaria* experimentally exposed to *E. ranae* started to shed oocysts 18–22 days post-infection (= prepatent period). The length of the patent period was not recorded, as the shedding of oocysts was terminated by metamorphosis. The longest period, for which an experimentally infected tadpole expelled oocysts before metamorphosis was 12 days.

Both coprological and histological examinations of juvenile *R. dalmatina*, experimentally infected as tadpoles were consistently negative up to 15 mo post-metamorphosis.

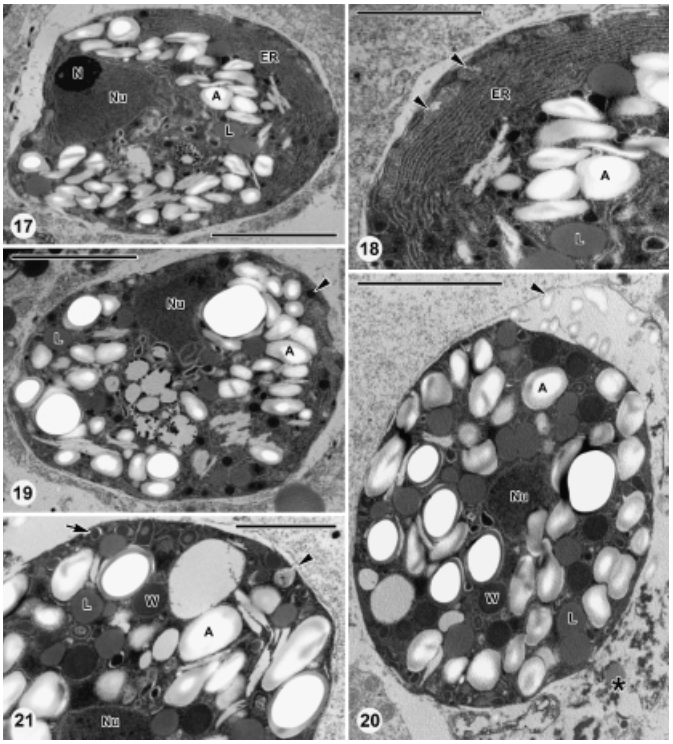

Fig. **17–21.** Ultrastructure of macrogamonts of *Eimeria ranae* in various stages of development. TEM. **17.** Immature macrogamont. **18.** Enlarged periphery of young macrogamont showing typical thick layer of endoplasmic reticulum and mitochondria (arrowheads). **19.** Advanced macrogamont. Note numerous globular dense granules (arrowhead). **20.** Mature macrogamont. Note amorphous substance (*) and amylopectin-like granules (arrowheads) within the parasitophorous vacuole. **21.** Enlarged periphery of mature macrogamont showing sub-membranous vesicles containing osmiophilic material (arrow) seemingly communicating with cell surface (arrowhead). Nu, nucleus; N, nucleolus; A, amylopectin granules; L, lipid inclusions; ER, endoplasmic reticulum; W, wall-forming body-like granules; TEM, transmission electron microscopy. Scale bars: Fig. 17, 19–21 = 4 μm; Fig. 18 = 2 μm.

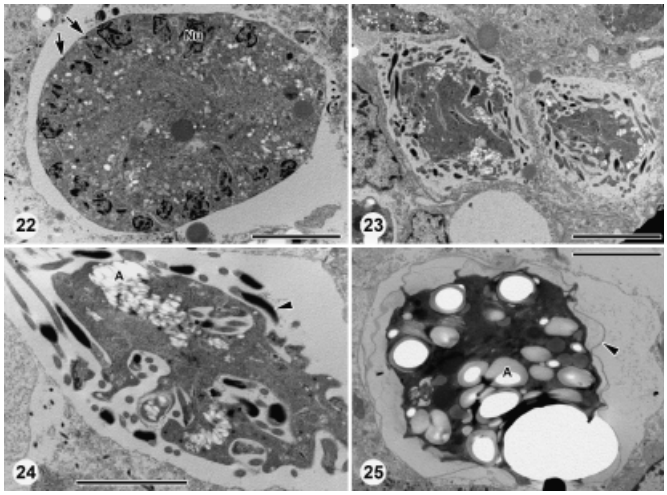

Fig. **22–25.** Ultrastructure of macrogamonts and unsporulated oocyst of *Eimeria ranae*. TEM. **22.** Immature microgamont with visible centrioles (arrows) and typical multiple nuclei distributed at its periphery. **23.** Mature microgamonts. **24.** Detail of residual cytoplasm of mature microgamont surrounded by microgametes (arrowhead). **25.** Unsporulated oocyst. Note absence of the halo-encircled wall-forming body-like granules visible in macrogamont at the Fig. 21. Arrowhead shows oocyst wall. Nu, nucleus; A, amylopectin granules. Scale bars: Fig. 22 = 2 μm; 23–25 = 4 μm. TEM, transmission electron microscopy.

Phylogenetic analysis. Comparision of available sequences in GenBank™ revealed that the sequences of *E. ranae* and the most closely related *E. arnyi* were 97% identical. From 1,647 characters, 944 characters were constant, and 459 were parsimony informative. In our analysis (Fig. 26), the three main coccidian clades—eimeriid, sarcocystid, and adeleid, were recognized. All analyses showed that within the Eimeriidae s.l. (the sister clade of the sarcocystid lineage), those from poikilotherm hosts (unspecified intranuclear coccidium, *Choleoeimeria* sp., *E. tropidura*, *E. arnyi*, and *E. ranae*) formed the most basal branches. The only exceptions from this pattern were the poikilotherm-host *Lankesterella minima* and *Caryospora bigenetica*, which showed unstable position(s) in different analyses. *Eimeria tropidura* clustered together with *Choleoeimeria* sp. and formed a well-supported lineage, which is sister to the clade comprising all SB-bearing eimeriid coccidia. Within this main eimeriid coccidian lineage, *E. ranae* and *E. arnyi* formed a basal lineage supported by high bootstrap values (100/100).

DISCUSSION

The bias in our knowledge, and perspectives on a conceptual approach to coccidian classification and taxonomy were thoroughly discussed by Tenter et al. (2002). Herein, we present a multifaceted study of the anuran coccidium *E. ranae*, in which we have addressed morphology of all developmental stages, host specificity, and phylogeny.

Host specificity of anuran *Eimeria*. Eimeriid coccidia are considered to be highly host-specific protistan parasites, which fact is reflected in their amazing diversity. So far, host specificity is one of the major pillars of the taxonomy of coccidia (Hnida and Duszynski 1999; Joyner 1982; Long and Joyner 1984). Thus, many authors restricted their differential diagnoses to groups of *Eimeria* species affecting only the same host genus or family. Experimental studies on host specificity of members of the genus *Eimeria* are fragmentary and it is questionable how deeply the descriptions of coccidia and their taxonomy are distorted because of lack of information about host specificity. However, experimental evidence from the most studied host group, rodents, shows that host specificity at the generic level is common among rodent-host *Eimeria* (Hnida and Duszynski 1999; Levine and Ivens 1988), while only a small number of species has been shown to be either host species-specific, or crossing generic, or even familial borders.

We assessed host specificity of *E. ranae* on the levels of host species and developmental stage. This approach revealed interesting traits that correlate with the complex life cycle of amphibians. In wild anurans *E. ranae* was found only in *R. temporaria* and *R. dalmatina*. A successful experimental cross-infection of *R. temporaria* tadpoles with oocysts from both tadpoles and adults of *R. dalmatina* and vice versa confirmed the conspecificity of *E. ranae* isolates from the two *Rana* spp., as well as conspecificity of isolates from tadpoles and adults. On the contrary, exposure of the semi-aquatic ranid *P.* kl. *esculentus* to the oocysts of *E. ranae* did not result in an infection. Importantly, we did not record *E. ranae* in *P.* kl. *esculentus* at localities where it occurs sympatrically with *R. temporaria*.

Currently, 44 species are recognized within the genus *Rana*. Among these, 11 species form a monophyletic Western Palearctic clade, within which *R. temporaria* and *R. dalmatina* represent the most distantly related taxa (Veith, Kosuch, and Vences 2003).

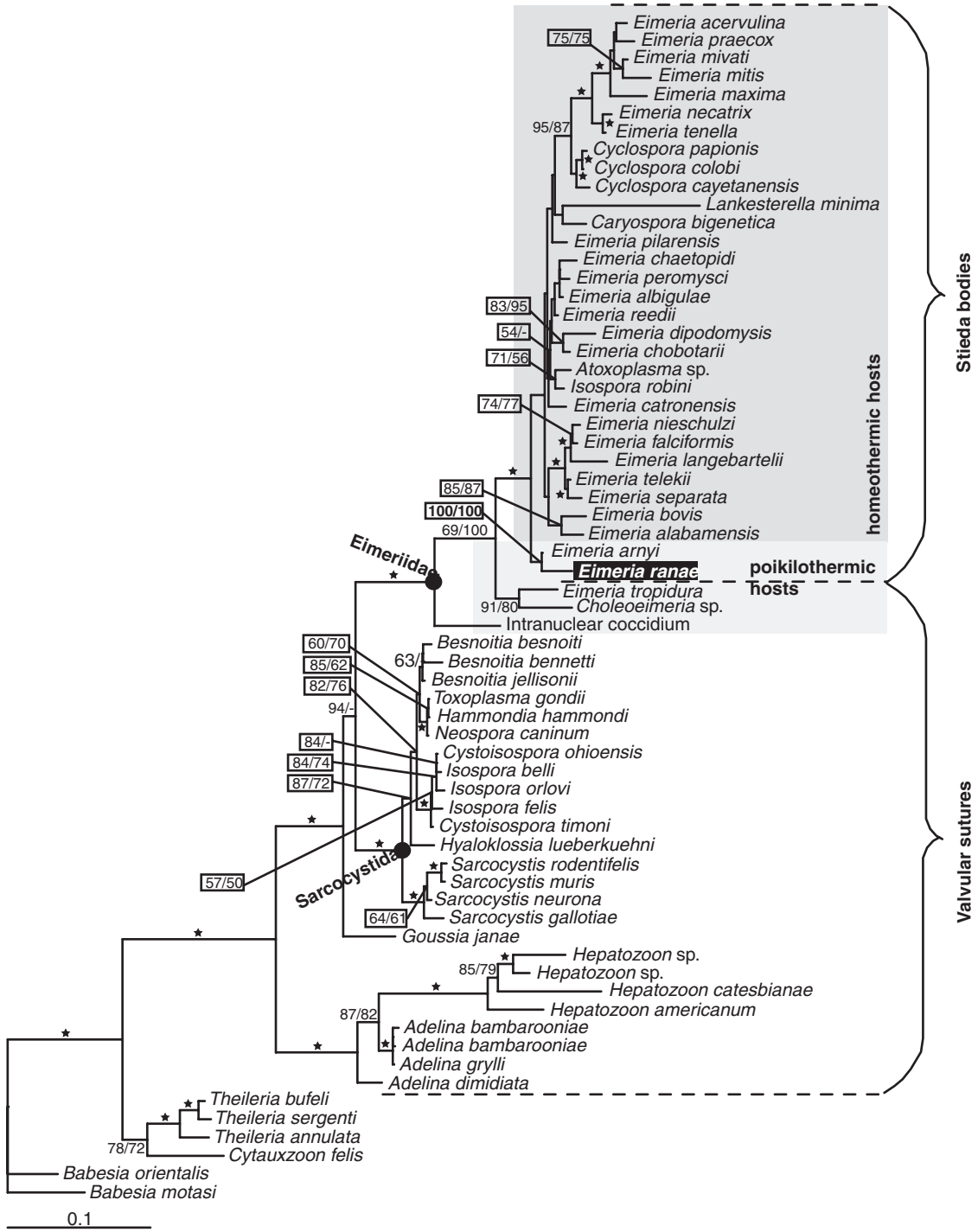

Fig. **26.** Bayesian phylogenetic tree as inferred from partial sequences of the small subunit (SSU) rDNA. Numbers above branches indicate Bayesian posterior probability/maximum likelihood boostraps/maximum parsimony bootstraps. Black stars show nodes supported by pp equal to 1.00 and both bootstraps over 90%. Brackets indicate character of excystation structures.

Infectivity of *E. ranae* for the two distant host species suggests its possible infectivity for the West Palearctic *Rana* spp. in general. Therefore, on the level of host-taxon, the specificity of *E. ranae* seems to be restricted to terrestrial ranids of the genus *Rana*, and its records from *P.* kl. *esculentus* (Dobell, 1909; Kazubski and Grabda-Kazubska 1974) probably represent an erroneous determination or predation-associated pseudoparasitism, rather than geographic variation in the host spectrum. The erroneous species determination is likely since Dobell (1909) noted that only oocysts from *R. temporaria* were studied in detail. The host specificity restricted to the genus rather than species of host might be common in anuran *Eimeria* spp., as reflected in records of *E. algonquini* and *E. kermiti* in four sympatric ranids *Lithobates* spp. in Canada and *E. streckeri* in two species of the genus

Pseudacris (Hylidae) in USA (Bolek, Janovy, and Irizarry-Rovira 2003; Chen and Desser 1989; Upton and McAllister 1988).

Fate of infection after metamorphosis. The infection in tadpoles by an *Eimeria* is unprecedented, since the only coccidians known to date to parasitize tadpoles were members of the genus *Goussia*, *Isospora cogginsi*, and *Hyaloklossia lieberkuehni* (Bolek et al. 2003; Molnár 1995; Nöller 1920; Paperna, Ogara, and Schein 1997). Our experimental infections confirmed that oocysts from adults are infective to tadpoles of the same species. Available field data on *I. cogginsi* and experimental observations on *H. lieberkuehni* suggest that more coccidian genera show such a pattern (Bolek et al. 2003; Nöller 1923).

Coprological examination of feces expelled by frogs experimentally infected as tadpoles shows that the infection disappears during the host's metamorphosis and the infections in adult anurans result from re-infections rather than from previous infections of tadpoles. The only anuran-host coccidian known to withstand tadpole metamorphosis is the renal coccidium *H. lieberkuehni*, in which Nöller (1923) experimentally confirmed continuity of the infection from tadpoles to the emerged frogs. Among other anuran coccidia, disappearance of infection during metamorphosis has been repeatedly observed in *Goussia* spp. parasitizing tadpoles, suggesting that this phenomenon might be a common trait of intestinal coccidian infections of tadpoles (Duszynski et al, 2007; Jirků and Modrý 2006b; Molnár 1995; Nöller 1920; Paperna et al. 1997). Metamorphosis-related modification of the gastro-intestinal tract in anuran tadpoles is a complex and abrupt process during which a complete exchange of the intestinal epithelium is followed by a significant shortening of the intestine (Ishizuya-Oka and Ueda 1996; Pretty, Naitoh, and Wassersug 1995). In larvae of caudate amphibia, only minor metamorphosis-related modifications of the gastro-intestinal tract occur, and the infection loss is likely to be absent or less pronounced. The assumption that the infection loss is a trait specific for infections in anuran tadpoles is congruent with observation of continuous shedding of *Eimeria ambystomae* oocysts before, during, and after metamorphosis in the tiger salamander, *Ambystoma tigrinum* (Bolek et al. 2003).

Phylogenetic affinities of *Eimeria ranae*. Together with *E. arnyi* from snake, *E. ranae* forms a lineage supported by high bootstrap values that is basal to the clade comprising other SB-bearing eimeriids, such as avian- and mammalian-host *Eimeria*, *Cyclospora* spp., and avian-host *Isospora*. *Eimeria ranae* and *E. arnyi* not only constitute a well-supported lineage despite their geographic and host-taxonomic distance, but also their oocyst/sporocyst morphology is similar, characterized by the presence of an oocyst residuum and distinct SB (Upton and Oppert 1991). In congruence with other studies (e.g. Morrison et al. 2004), our results further support paraphyly of the genus *Eimeria* and monophyly of the SB-bearing coccidians, regardless of their current generic affiliation. In summary, the phylogenetic pattern within Eimeriidae s.l. reflects the character of excystation structures (i.e. SB vs. bivalved sporocysts with suture), rather than the host taxon and/or phenotypic features traditionally used for generic classification, in particular the number of sporocysts and sporozoites per oocyst (Upton 2000).

Diversity of anuran *Eimeria*. Anurans, represented by ~5,500 extant species in 395 genera and 45 families, constitute a vast majority (~88%) of extant species of amphibians (Frost 2007). However, only ~4,500 specimens representing ~1% (~65/5,500) of anuran species in 7% (30/395) of the genera and 31% (14/45) of the families have ever been examined for coccidia. From these studies, there has been description of 30 named species of coccidians, which have been classified into four genera within the two families Eimeriidae and Sarcocystidae (excluding seven spp. and two genera of the family Lankesterellidae). These figures contrast with corresponding numbers for the best-studied host group, the rodents for which 15% (300/2015) species, 34% (150/443) genera, and 52% (15/29) families have been examined so far. This has resulted in >500 named coccidian species classified into about 10 genera within the families Eimeriidae and Sarcocystidae (Tenter et al. 2002).

Out of 18 nominal *Eimeria* spp. parasitizing anuran hosts (Table 3), 10 originate from the Holarctic region (five from North America, five from Europe), three from the Afrotropic region, three from the Oriental region (the Indian subcontinent), and two from the Neotropic region. This account shows that areas of the highest anuran diversity, the Neotropic, Afrotropic, Oriental, and Australian realms and Madagascar (Duellman and Trueb 1994) remain virtually unexplored.

To summarize taxonomic information on the individual species we used reviews of amphibian coccidia by Upton and McAllister (1988), Duszynski et al. (2007) as well as original descriptions (references in Table 3). Our review reflects a recently recognized significance of the excystation structures and character of endogenous development for the classification of *Eimeria* s.l. (Jirků et al. 2002; Paperna and Landsberg 1989). Based on oocyst morphology, we distinguish three morphotypes among the anuran-host *Eimeria* spp., and show their apparent correlation with the character of endogenous development (Table 3).

Morphotype 1 (seven spp.) is characterized by the presence of an oocyst residuum and distinct SB. When compared with reptile- and homeiotherm-host *Eimeria*, the SB is relatively small (especially in *E. streckeri*), but it is always clearly discernible. All four species of this morphotype, for which data on endogenous development are available, are localized extranuclearly in enterocytes. This morphotype resembles *Eimeria* spp. occurring in squamate reptiles, birds, and mammals, and most probably represents typical SB-bearing *Eimeria*, as suggested by our phylogenetic analysis of *E. ranae*.

Morphotype 2 (five spp.) is characterized by the absence of an oocyst residuum and the presence of a barely discernible SB. All three species of Morphotype 2, for which data on endogenous development are available, parasitize nuclei of enterocytes. It is not clear, whether the SB of these species is homologous to the SB of other eimerians, and ultrastructural data are needed to resolve this issue.

Morphotype 3 (two spp.) is characterized by the absence of an oocyst residuum, absence of a SB, very small oocysts (diam. <12 μm), and endogenous sporulation. Extranuclear localization in enterocytes was described in both species placed into this morphotype. These morphological features closely resemble those of the genus *Goussia* Labbé, 1896. In fact, the only reason why species of Morphotype 3 cannot be assigned to the genus *Goussia* is the lack of information on the presence or absence of a suture in the sporocyst wall.

Miscellaneous species. There are two species among anuran-host eimerians that could be assigned to one of the above characterized morphotypes, but differ in certain features. First, *E. algonquini* would fit to Morphotype 1, but it possesses unique banana-shaped sporocysts, in which a SB was not observed originally (Chen and Desser 1989). The second species, *Eimeria bufomarini*, fits to Morphotype 3, due to lack of an oocyst residuum, small oocysts (maximum diam. 10 μm), extranuclear localization of its endogenous stages, and endogenous sporulation. However, a barely visible SB was mentioned in the original description (Paperna and Lainson 1995). In addition, fragility of the oocyst wall is frequently referred to as a typical feature of the anuran-host coccidia (Upton and McAllister 1988). While this feature is common also among fish-, caudate amphibian-, and some chelonian-host coccidia, it clearly reflects affinity of hosts to aquatic and/or humid (micro)habitats and most likely lacks any taxonomic significance.

Table 3. Morphotypes of Anuran-host *Eimeria* spp. (i.e. tetrasporocystic coccidia) based on excystation and endogenous development characteristics.

Species	Host(s)	Oocyst shape Oocyst size L/W (if specified by author)	PG Loc	Sporocyst shape Sporocyst size Character of Stieda Body	Localities	Relevant references
Morphotype 1—oocyst residuum present, clearly discernible Stieda body, endogenous development extranuclear						
Eimeria cyanophlyctis Chakravarty and Kar (1952)	*Euphlyctis cyanophlyctis* (formerly *Rana cyanophlyctis*)	Oval (elliptical) 15.4–19.8 × 15.4–17.6	– EN	More or less spindle-shaped 11.0 × 4.4–6.6 ?[a]	Vicinity of Calcutta, India	Chakravarty and Kar (1952)
Eimeria kermiti Chen and Desser (1989	*Lithobates* spp.: *L. catesbeianus**; *L. clamitans*; *L. septentrionalis*; *L. sylvaticus* (formerly *Rana* spp.)	Elliptical 25.1 (24.7–26.6) × 19.5 (17.6–20.1)	+ ?	Elliptical 9.9 (9.3–10.4) × 6.6 (6.0–7.1) SB small, clearly discernible	Pee Wee Lake, Algonquin Park, Ontario, Canada	Chen and Desser (1989)
Eimeria leptodactyli Carini (1931)	*Leptodactylus ocellatus*	Elliptical 23 × 17	– ?	Elliptical with one pole tapered 9 × 6.5 SB present on tapered pole	South America	Carini (1931)
Eimeria prevoti (Laveran and Mesnil 1902) Syn: *Paracoccidium prevoti, Eimeria prevunti* (lapsus)	*Pelophylax* kl. *esculentus* (formerly *Rana* kl. *esculenta*)	Spherical to ovoidal 16.5 (15.3–16.8) × 12.8 (12.2–13.7) * 20–22 × 12–15	– EN	Elliptical, disintegrating in time? SB distinct	Paris, France*; Normandie, France; Chomutov, Czech Republic	Boulard (1975), Doflein (1909), Laveran and Mesnil (1902), this study
Eimeria ranae Dobell (1909) Syn: *Coccidium ranae* (nomen nudum)	*Rana temporaria**; *Rana dalmatina*; *Pelophylax* kl. *esculentus* ?	Eliptical to spherical 19.5 (17.0–21.0) × 17.9 (16.0–21.0) L/W 1.1 (1.0–1.2) * ~ ovoid, 18–22 in diameter	– EN	Elliptical to navicular 11.1 (10.0–13.0) × 7.0 (6.0–8.0) *oval, 14 × 7 SB distinct	Cambridge, England; Munich, Germany; Central Poland; Czech Republic*	Dobell (1909), Kazubski and Grabda-Kazubska (1974), this study
Eimeria streckeri Upton et McAllister (1988)	*Pseudacris streckeri streckeri**; *Pseudacris triseriata triseriata*	Spherical 18.8 (16.8–21.5) × 18.7 (16.8–20.8) L/W 1.0 (1.0–1.1)	–[b] ?	Ovoid 11.1 (9.6–12.8) × 7.7 (7.2–8.8) SB small but clearly discernible	Dallas County, TX, USA*; Pawnee Lake, Lancester County, Nebrasca, USA	Upton and McAllister (1988), Bolek et al. (2003)
Eimeria terraepokotorum Jirků and Modrý (2006a)	*Hoplobatrachus occipitalis*	Elliptical to ovoidal 20.2 (18.0–24.5) × 16.0 (13.5–18.5) L/W 1.3 (1.1–1.4)	– EN	Elliptical 9.8 (8.5–11.5) × 7.2 (6.0–8.0) SB distinct	Nginyang, Rift Walley Province, Kenya	Jirků and Modrý (2006a)
Morphotype 2—oocyst residuum absent, barely discernible Stieda body, endogenous development intranuclear						
Eimeria fitchi McAllister, Upton, Trauth and Bursey (1995)	*Lithobates sylvaticus* (formerly *Rana sylvatica*)	Ovoidal 21.9 (20.0–24.0) × 14.3 (13.2–15.2) L/W 1.5 (1.3–1.7)	+[c] ?	Ovoidal 10.9 (9.8–11.2) × 7.4 (7.0–8.0) SB barely discernible	6 km SW of Melbourne, off State Hwy 9, Izard County, Arkansas, USA	McAllister et al. (1995)
Eimeria flexuosa Upton and McAllister (1988)	*Pseudacris streckeri streckeri*	Irregular, elastic(?) oocyst wall 17.0 (15.2–19.2)	+ ?	Ovoid 10.3 (9.6–12.0) × 7.3 (6.4–8.0) SB barely discernible	Dallas County, TX, USA	Upton and McAllister (1988)
Eimeria fragilis Jirků and Modrý (2005)	*Chiromantis kelleri* (*C. petersii kelleri* in description)	Elliptical 18.5 (17.0–19.5) × 15.2 (14.5–16.0) L/W 1.2 (1.1–1.3)	– IN	Broad navicular , disintegrating 10.6 (9.5–12.0) × 6.8 (6.0–7.0) SB barely discernible	Kula Mawe, Eastern Province, Kenya	Jirků and Modrý (2005)

Table **3.** (Continued).

Species	Host(s)	Oocyst shape Oocyst size L/W (if specified by author)	PG Loc	Sporocyst shape Sporocyst size Character of Stieda Body	Localities	Relevant references
Eimeria mazzai Yakimoff and Gousseff (1934) Syn: *Eimeria transcaucasica*	*Bufo bufo*	Spherical 16–18	– ?	Ovoid 6–8 × 4 ?	Zurnabad, Azerbaijan	Yakimoff and Gousseff (1934)
Eimeria ranarum (Labbé, 1894) Syn: *Acystis parasitica* (in part), *Caryphagus ranarum*, *Coccidium ranarum*, *Karyophagus ranarum*	*Pelophylax* kl. *esculentus*	Ovoidal 18–20 × 12–16	– IN	Elliptical 7 × 4 ?	France*; Poland	Doflein (1909)
Eimeria wambaensis Jirků and Modrý (2005)	*Hyperolius viridiflavus*	Elliptical to ovoidal 17.0 (15.0–18.5) × 13.0 (11.0–14.0) L/W 1.3 (1.1–1.6)	– IN[d]	Broadly navicular 8.7 (8.0–10.5) × 6.0 (5.5–7.0) SB barely discernible	Wamba, Rift Walley Province, Kenya	Jirků and Modrý (2005)
Morphotype 3–oocyst diameter <12 μm, oocyst residuum absent, sporocysts apparently devoid of Stieda body, endogenous sporulation, endogenous development extranuclear						
Eimeria himalayana[e] Ray and Misra (1943)	*Duttaphrynus himalayanus* (formerly *Bufo himalayanus*)	Broadely elliptical, very fine oocyst wall 7.0–10.0	– EN	Spindle-shaped, disintegrating 5.0 × 2.8	Mukteswar-Kumaun, Uttar Pradesh, India	Ray and Misra (1943), Upton and McAllister (1988)
Eimeria laminata Ray (1935)	*Duttaphrynus melanostictus* (formerly *Bufo melanostictus*)	Spherical 8–11	– EN	Spindle-shaped 4.6–5.8 × 3	Calcutta, India	Ray (1935)
Miscellaneous species						
Eimeria algonquini Chen and Desser (1989) (Susp. morphotype 1)	*Lithobates* spp.: *L. catesbeianus**; *L. clamitans*; *L. septentrionalis*; *L. sylvaticus* (formerly *Rana* spp.)	Spherical, OR present 15.8 (14.5–16.1)	– ?	Bananna-shaped 19.5 (18.7–20.4) × 4.2 (3.8–4.6) SB absent?	Lake Sasajewun, Algonquin Park, Ontario, Canada	Chen and Desser (1989)
Eimeria bufomarini Paperna and Lainson (1995) (Susp. morphotype 3)	*Rhinella marina* (formerly *Bufo marinus*)	Spherical to subspherical, OR absent 9.2 (8.7–10.0) × 9.0 (8.7–10.0) L/W 1.0 (1.0–1.1)	– EN	Elliptical, 1.7 (1.6–1.7) 6.3 (6.0–6.5) × 3.7 (3.7–4.0) SB barely discernible	Salvaterra, Marajo Island, Pará, Brazil; Belém, Pará, Brasil	Paperna and Lainson (1995)
Species inquirenda—only single measurement of immature oocyst/sporocyst available						
Eimeria belawini Yakimoff (1930)	*Hyla arborea*	Spherical, 12.2 in diameter OR absent	? ?	unsporulated, 4.4 in diameter	Piatigorsk, Caucasus Mts., Russia	Yakimoff (1930)

EN, extranuclear; IN, intranuclear; Loc, localization within host cells; L/W, length/width ratio; OR, oocyst residuum; PG, polar granule; SB, Stieda body; +, present; –, absent; ?, unknown. Asterisk (*) indicates type host, type locality and data from original description. All species for which data are available complete endogenous development within epithelial cells of small intestine (except *Eimeria wambaensis*, see footnote). Measurements are in μm. Synonymy is after Upton and McAllister (1988)

[a]Stieda body is not mentioned in description, but one pole of sporocyst is described as more tapering than the other.

[b]Authors pointed out, that one PG may be found rarely.

[c]Typical free PGs absent, but authors described one to three fragments adhering surface of sporocysts.

[d]Endogenous development occurs in both small and large intestine.

[e]Upton & McAllister (1988) qualified original spelling ''*himalayanum*'' to be a lapsus and corrected to ''*himalayana*''; *Eimeria himalayani* Ray et Misra, 1943 of Paperna and Lainson (1995), lapsus.

Laveran and Mesnil (1902) proposed a new genus *Paracoccidium* to accommodate *E. prevoti*. The reason for this arrangement was the unusual mode of sporogony, during which sporocysts disintegrate soon after the formation of sporozoites, so that the sporozoites lie freely within the oocyst. A similar phenomenon was observed also in the anuran-host *E. fragilis* and *Eimeria himalayana* (Jirků and Modrý 2005, Ray and Misra 1943) and several reptile–host coccidia (Asmundsson, Duszynski, and Campbell 2006; Paperna and Landsberg 1989). Although relevance of the proposed generic name was discussed by Paperna and Lainson (1995), it is doubtful that the sporocyst disintegration in otherwise morphologically, biologically, and probably phylogenetically distant coccidia is of taxonomic significance and *Paracoccidium* should therefore be treated as a junior synonym of *Eimeria*. Finally, we propose *Eimeria belawini* to be regarded as a species inquirenda, because its description based on immature oocysts is insufficient (Yakimoff 1930).

From the preceding discussion it is obvious that the anuran–host *Eimeria* represent morphologically, and probably phylogenetically, a heterogenous group of apicomplexans. Importantly, our analysis shows that together with a thorough description of oocyst morphology (Duszynski and Wilber 1997), information on localization and basic morphology of endogenous developmental stages should become mandatory for new coccidian species descriptions wherever possible. The new species descriptions should also include a molecular phylogenetic analysis or material for DNA isolation should be deposited together with type material.

Oocyst morphology of *Eimeria ranae*. Oocysts of *E. ranae* isolates studied by us match the original oocyst description in all but one feature: namely, the presence of a ''knob-like eminence at either end of sporocyst'' (Dobell, 1909). We believe that the Dobell's ''knob-like eminences'' represent the pointed posterior sporocyst poles and misidentified SBs and not a SB and a para-SB as interpreted by Duszynski et al. (2007). Dobell, (1909) highlighted the navicular shape of the sporocysts of *E. ranae*, which we also observed, but this sporocyst shape variant was not more common than other shapes and we do not regard it as a diagnostic feature. The collapsing oocyst wall mentioned by Dobell (1909) was not directly observed by us, possibly because the wall occasionally disintegrates completely during flotation, but the presence of free sporocysts in stored fecal samples confirms the occurrence of this phenomenon in our isolates.

Wall-forming body-like structures. Macrogamonts of coccidia from fish and amphibian hosts generally lack typical wall-forming bodies, and no organelles involved in oocyst wall formation have been identified with certainity (Paperna 1995; Paperna and Lainson 1995; Paperna et al. 1997). In *E. ranae*, we were able to follow, at the ultrastructural level, a complete sequence of macrogamont development up to the formation of oocysts. The most remarkable feature was the emergence of dense wall-forming body-like granules in advanced macrogamonts. These were absent in young macrogamonts, and disappeared from oocysts, possibly as a consequence of their participation in oocyst wall formation. Generally, the distinctiveness of the wall-forming bodies in coccidian macrogamonts seems to be positively correlated with the thickness of the oocyst wall, which explains their lesser prominence in amphibian and fish coccidians, which typically have a very thin oocyst wall.

Exogenous sporulation within host. Partly to fully sporulated oocysts were regularly observed in fresh feces from adults. In agreement with Duszynski et al. (2007), we explain this phenomenon by retention of feces within the intestine, which is common in adult anurans. On the other hand, the infrequent presence of partly sporulated oocysts in fresh feces from tadpoles is congruent with the habit of continuous defecation by tadpoles. In addition, examination of a few hundreds of histological sections of infected intestines revealed only sporonts within epithelial cells in both tadpoles and adults, whereas oocysts showing signs of sporulation were observed in the intestinal lumen of adults. Thus, *E. ranae* should be regarded as having exogenous sporulation, beginning after release of oocysts into the host intestinal lumen.

Methodological considerations. We used the flotation method using sucrose (Sheather's) solution for coprological examinations throughout the study. This method has been criticized because the thin-walled oocysts of amphibian coccidia rapidly disintegrate in hypertonic flotation solutions and wet mounts were recommended as a method of choice for amphibian coccidia by Upton and McAllister (1988). These authors also noted that the chances of false negatives are high in wet mounts, which is in agreement with our observations. However, flotation is a cheap method providing reliable detection and easy purification and concentration of oocysts, even if these are present in small numbers. The thin-walled oocysts tend to collapse within 15 min. However, this time is sufficient for examination. In addition, the flotation method facilitates photodocumentation by eliminating artefacts from preparations and by reducing Brownian motion.

Although ideal for diagnostics, the flotation method is useless for concentrating the oocysts of *E. ranae* for storage and for experiments, as they invariably disintegrate once exposed to flotation solution. Repeated sedimentation of fecal samples in water proved to be the most suitable method of concentration and storage of these oocysts. Importantly, water must be changed every 2 days to reduce the development of undesirable microflora. This method turned out to be suitable for the storage of the infectious material for up to 4 months. Regardless of the storage medium, 20–100% of oocysts failed to complete sporulation in individual samples, resulting in low numbers of infectious oocysts available for the experiments. Oocysts of *E. ranae* probably require specific conditions for sporulation, which we failed to simulate, and this fact might explain the negative results of one of the positive controls in our experiments.

TAXONOMIC SUMMARY

Phylum Apicomplexa
Order Eucoccidiorida
Suborder Eimeriorina
Family Eimeriidae
Eimeria ranae Dobell, 1909

Diagnosis. Typical anuran *Eimeria* with fine oocyst wall; oocysts variable in both shape and size, broadly elliptical to spherical, 19.5 (17.0–21.0) × 17.9 (16.0–21.0) μm; oocyst residuum usually compact, often with vacuole. Micropyle and polar granule absent. Sporocysts dizoic, 11.1 (10.0–13.0) × 7.0 (6.0–8.0) μm, with a prominent SB, slightly hollowed on the inner side. Sporocyst shape variable, from broadly elliptical to navicular. The sporocyst pole bearing the SB often slightly tapered. Sporozoites possess a centrally located nucleus or refractile body. Sporocyst residuum usually compact, rarely scattered among sporozoites. Endogenous stages are located extranuclearly in the cytoplasm of enterocytes, usually in the region above the host cell nucleus.

Type host. *Rana temporaria* L. (Anura: Ranidae).

Synonymy. *Coccidium ranae* Dobell, 1908 (nomen nudum)

Other hosts. *Rana dalmatina* Fitzinger in Bonaparte (Anura: Ranidae) (this study). Records in *P.* kl. *esculentus* (L.) (Anura: Ranidae) (Dobell, 1909; Kazubski and Grabda-Kazubska 1974) warrant re-evaluation. Apparently restricted to hosts of the genus *Rana*.

Type locality. Zaječí (Zayetchee) potok, vicinity of Brno, 16°36′23″E, 49°14′15″N, Czech Republic.

Other localities. Cambridge, England and Munich, Germany (Dobell, 1909); Konin and Gopło, Poland (Kazubski and Grabda-Kazubska 1974), Raduň, Růženčin lom and Jedovnice, Czech Republic (this study, see ''Materials and Methods'').

Site of infection. Epithelial cells of the small intestine of adult frogs, whole intestine of tadpoles; extranuclear.

Prevalence. Approximately 15% of adult *R. temporaria* examined by Dobell, (1909). In our study, the infection was recorded in 30% ($n = 201$) of adult *R. temporaria*, 70% ($n = 92$) of adult *R. dalmatina*, 21% ($n = 1,421$) of all examined *R. temporaria* tadpoles, and 17% ($n = 1,270$) of all examined *R. dalmatina* tadpoles (these totals do not reflect temporal prevalence changes which will be analysed in separate study).

Neotype material. Histological sections of *R. temporaria* tadpole with infected intestine; infected tadpoles of *R. temporaria* and *R. dalmatina* from the type locality in absolute ethanol, digital micrographs, and neosymbiotype (sensu Frey et al. 1992) *R. temporaria* specimen with liver tissue sample in absolute ethanol—deposited at the type parasitological collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, No. IPASCR Prot. Coll.: P-1. No type material by Dobell, (1909) exists. Nucleotide sequence of the SSU rDNA is deposited in GenBank™, Accession no. EU717219.

Remarks. Other anuran eimerians possessing oocyst residuum can be clearly distinguished from *E. ranae* on the basis of oocyst morphology. *Eimeria algonquini* has distinctly different (banana shaped) sporocysts; *E. prevoti* possesses distinctly smaller oocysts and smaller, often disintegrating sporocysts; for *Eimeria leptodactyli* scanty oocyst residuum often composed of granules arranged (?) in rosettes is characteristic; *Eimeria kermiti* differs in the presence of polar granule(s); *Eimeria streckeri* differs by very fine SB and wider sporocysts; *Eimeria cyanophlyctis* possesses scanty sporocyst residuum and narrower sporocysts; *Eimeria terraepokotorum* posseses elongated oocysts resulting in a high mean length/width ratio never reaching values below 1.1 (common in *E. ranae*), presence of refractile bodies within sporozoites, and absence of vacuole within the oocyst residuum (see Table 3 for comparision of measurements of *Eimeria* spp. mentioned above).

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