

New species of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa) from amphibian host: morphology, biology and phylogeny

Miloslav Jirků^{1,2}, Andrea Valigurová³, Břetislav Koudela^{1,2}, Jaroslav Křížek², David Modrý^{1,2} and Jan Šlapeta⁴

¹Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, 612 42 Brno, Czech Republic;

²Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic;

³Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic;

⁴Faculty of Veterinary Science, University of Sydney, McMaster Building – B14, New South Wales 2006, Australia

Key words: *Cryptosporidium fragile*, new species, frog, *Duttaphrynus melanostictus*, ultrastructure, host specificity, phylogeny, quarantine, global amphibian decline

Abstract. *Cryptosporidium fragile* sp. n. (Apicomplexa) is described from black-spined toads, *Duttaphrynus melanostictus* (Schneider) (Amphibia, Anura, Bufonidae) from the Malay Peninsula. The parasitized animals were directly imported from Malaysia and harboured *C. fragile* at the time of arrival. Oocysts were subspherical to elliptical with irregular contour in optical section, measuring 6.2 (5.5–7.0) × 5.5 (5.0–6.5) μm. Oocyst wall was smooth and colourless in light microscopy. The endogenous development of *C. fragile* in the stomach of black-spined toad was analysed in detail using light and electron microscopy. Cryptosporidian developmental stages were confined to the surface of gastric epithelial cells. In transmission experiments, *C. fragile* has not been infective for one fish species, four amphibian species, one species of reptile and SCID mice. Full length small subunit rRNA gene sequence was obtained. Phylogenetic reconstruction revealed distinct status of *C. fragile* within the clade of species with gastric localisation including *Cryptosporidium muris* Tyzzer, 1907, *Cryptosporidium serpentis* Levine, 1980 and *Cryptosporidium andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blagburn, 2000. Described characteristics differentiate *C. fragile* from the currently recognized *Cryptosporidium* species. Our experience with the description of *C. fragile* has led us to revise the recommended criteria for an introduction of a new *Cryptosporidium* species name. *C. fragile* is the first species described and named from an amphibian host. Its prevalence of 83% (15/18) in black-spined toads within the 3 months after importation calls for strict quarantine measures and import regulation for lower vertebrates.

The genus *Cryptosporidium* was erected by Ernest E. Tyzzer for a peculiar parasite affecting mouse stomach, *Cryptosporidium muris* Tyzzer, 1907 (Tyzzer 1907). Later he recognized a distinct species *Cryptosporidium parvum* Tyzzer, 1912 affecting mouse intestine (Tyzzer 1912). In the following years, multiple new cryptosporidian species were described, infecting majority of vertebrates. Initially, cryptosporidia were considered of a doubtful clinical significance, but with increasing knowledge the disease, cryptosporidiosis, was recognized as a major concern in immunocompromised and even healthy hosts (Fayer et al. 2000, Chen et al. 2002, Thompson et al. 2005).

Currently, there are 21 recognized species within the genus *Cryptosporidium* in fish, reptiles, birds and mammals (Šlapeta 2008). Seven of these affect host's stomach – *C. muris* Tyzzer, 1907 in rodents, *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blagburn, 2000 in cattle, *C. serpentis* Levine, 1980 in snakes and lizards, *C. galli* Pavlásek, 1999 in birds, and three species in fish stomach – *C. cichlidis* (Paperna et Vilenkin, 1996), *C. reichenbachlinkei* (Paperna et Vilenkin, 1996) and *C. molnari* Alvarez-Pellitero et Sitjà-Bobadilla, 2002 (Paperna and Vilenkin 1996, Tilley et

al. 1990, Lindsay et al. 2000, Alvarez-Pellitero and Sitjà-Bobadilla 2002, Ryan et al. 2003a).

Here we report on a cryptosporidian infection in toads directly imported to the Czech Republic from Malaysia for pet trade. We summarise morphological characteristics, results of transmission experiments and molecular data to describe this isolate as a new *Cryptosporidium* species, representing the first characterized species from an amphibian host.

MATERIALS AND METHODS

Origin of the parasites and host animals. In total, 18 adult black-spined toads, *Duttaphrynus* (syn. *Bufo*) *melanostictus* (Schneider) (Amphibia, Bufonidae) were obtained via import of pet animals from the Malay Peninsula in April 2006 (n = 9) and May 2006 (n = 9) in accordance with the Czech Republic regulations. Toads arrived in plastic boxes with moist moss, 3–4 individuals per box, and were quarantined in the premises of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (UVPB). The animals were housed individually in 8-litre glass vivaria with wet coco substrate, and fed 2–3 times weekly with crickets (*Gryllus assimilis*) supplemented with Reptivite (ZooMed Laboratories Inc).

Parasitological examination. All faeces expelled by individual toads were monitored for the presence of parasites using flotation (sugar solution, s.g. 1.3) from the day of import up to 90 days after arrival. Animals shedding oocysts were euthanised by pithing. Fresh squash preparations of various viscera, gastrointestinal smears, histological preparations and oocysts concentrated by flotation were examined by light microscopy using an Olympus AX 70 microscope equipped with Nomarski interference-contrast optics (NIC). For histology, the tissues of three animals were fixed in 10% buffered formalin or AFA (Alcohol-Formalin-Acetic Acid), embedded in Histoplast II and 6 μm sections were stained with haematoxylin-eosin (H&E). For transmission electron microscopy (TEM), the tissues of two animals were fixed overnight at 4°C in 3% glutaraldehyde in 0.2 M phosphate buffer and processed as described previously (Valigurová et al. 2008). Ultrathin sections were double-stained with uranyl acetate and lead citrate and viewed in a JEOL 1010. For scanning electron microscopy (SEM), the tissues of three animals were fixed overnight at 4°C in 3% glutaraldehyde in cacodylate buffer and processed according to Valigurová et al. (2008). Samples were examined using a JEOL JSM-7401F, field emission scanning electron microscope capable of high resolution of up to 1.0 nm.

Experimental host transmissions. Sieved faecal samples containing *Cryptosporidium* oocysts were pooled and kept in dechlorinated tap water in 0.5-litre containers without preservatives to avoid intoxication of experimental animals. Every two days, the suspension was stirred, sedimented (1 h), water removed and the tanks were re-filled with fresh water. The sedimented debris containing oocysts (<7 days old) was used for experimental inoculations. Before each trial, the presence of morphologically intact oocysts in infectious material was confirmed using flotation and oocysts quantification in the Bürker's chamber. Animal work has been approved by the Animal Ethics Committee, UVPB. Experimental animals were inoculated orally, except tadpoles and fish fed (after one day starvation) with a mixture of granulated fish food and the sedimented debris containing oocysts (see Table 1). Experimental animals were kept in separate facility at 20–23°C and artificial 12/12 h day/night cycle (UVPB). All faeces were daily collected from experimental enclosures and examined by flotation up to 8 weeks post inoculation (p.i.). After 8 weeks

p.i., randomly selected animals (15/20 *Poecilia reticulata*, 2/2 adult *Bufo bufo*, 20/40 *B. bufo* tadpoles, 2/2 adult *Rana temporaria*, 2/2 adult *Litoria caerulea*, 5/5 adult *Xenopus laevis*, 15/20 *X. laevis* tadpoles, 1/1 *Pantherophis guttatus* and 2/2 SCID mice) were examined histologically.

Molecular characterisation and phylogenetic analysis.

Mashed scraping of stomach mucosa containing microscopically confirmed cryptosporidia was used for the DNA isolation. Preparation in 200 μl normal saline solution was extracted using DNA Mini Kit (Qiagen). DNA was eluted in 100 μl of distilled water and stored at –20°C.

The full-length small subunit rRNA gene (SSU rDNA) was amplified using 5'-AAC CTG GTT GAT CCT GCC AGT AGT C and 5'-TGA TCC TTC TGC AGG TTC ACC TAC G primers (Xiao et al. 1999). PCR conditions consisted of 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 120 s; including an initial denaturation at 94°C for 5 min and a final extension at 72°C for 10 min. PCRs were carried out in 50 μl , containing 5 μl of DNA eluate, 100 pmoles of each primer, 1 U of *Taq* DNA polymerase in PCR buffer (TaKaRa). Negative and positive controls were included in all PCR runs. PCR reaction was electrophoresed and visualised with ethidium bromide. Amplicons were cloned into pCR2.1 vector according to manufacturer's instructions (TA TOPO Cloning Kit, Invitrogen). Full inserts were bidirectionally sequenced (Biotech, Germany) from 6 random clones. The SSU rDNA are available in the GenBank™ under the accession numbers EU162751–EU162754.

Nucleotide sequences were searched (blastn) against a local database of available *Cryptosporidium* spp. sequences, comprising 622 SSU rDNAs from GenBank™ (as of 28/8/2007). New sequences were manually added to the curated alignment of sequences comprising named species, major genotypes and the nearest neighbours (available upon request from authors). The alignments were analysed using a maximum likelihood (ML) phylogenetic approach PhyML 2.4.4 (Guindon and Gascuel 2003) that requires a model of nucleotide evolution selected using standard AIC in ModelTest 3.6 (Posada and Crandall 1998). Maximum parsimony (MP) and Minimum evolution (ME) were calculated in MEGA4 (Tamura et al. 2007).

Table 1. Experimental host transmissions of *Cryptosporidium fragile* sp. n.

Experimental host	Host stage (age)	No. infected (control)	Dose (oocysts)	Result	Host origin
FISH					
<i>Poecilia reticulata</i>	Adults (>1 year)	20 (10)	10 ³ –10 ⁴	Neg.	PC
AMPHIBIANS					
<i>Bufo bufo</i>	Adults (>3 years)	2 (2)	10 ⁴	Neg.	W
	Tadpoles	40 (40)	unknown	Neg.	W
<i>Rana temporaria</i>	Adults (>3 years)	2 (2)	10 ⁴	Neg.	W
<i>Litoria caerulea</i>	Adults (>3 years)	2 (1)	10 ⁴	Neg.	PI
<i>Xenopus laevis</i>	Adults (>2 years)	5 (5)	10 ⁴	Neg.	LC
	Tadpoles	20 (20)	unknown	Neg.	LC
REPTILES					
<i>Pantherophis guttatus</i>	Adult (2 years)	1 (1)	10 ⁴	Neg.	PC
MAMMALS					
<i>Mus musculus</i> SCID	Subadults (12 weeks)	2 (2)	10 ⁴	Neg.	LC

LC – laboratory colony (*Xenopus laevis* from the University of Veterinary and Pharmaceutical Sciences Brno and SCID mice from the Charles Rivers Laboratories); PC – pet trade, captive-bred; PI – pet trade, import from the wild; W – wild-caught in Brno, Czech Republic.

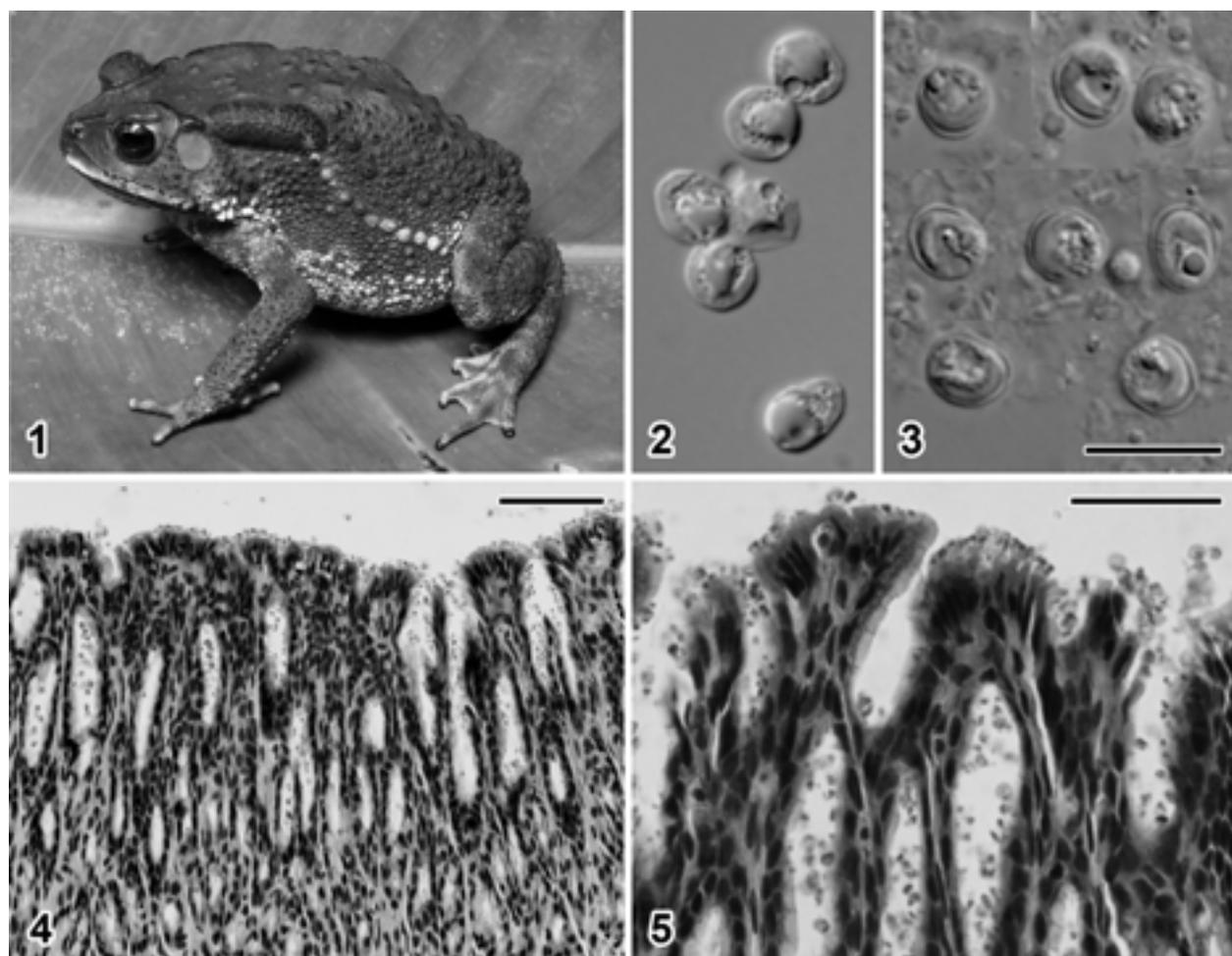
RESULTS

Parasitological examination of black-spined toads, *Duttaphrynus melanostictus* (Fig. 1) from the Malay Peninsula, revealed that 83% (15/18) passed oocysts of *Cryptosporidium*. Morphological, experimental and molecular analyses showed that the isolate represents a new species, the description of which follows.

Cryptosporidium fragile sp. n. Figs. 2–35

Oocyst morphology. Oocysts (Figs. 2, 3, 6) obtained by flotation method (Fig. 2) from fresh faeces were irregular in shape, subspherical to elliptical; usually with apparently deformed/uneven contour in optical section; $6.2 (5.5–7.0) \times 5.5 (5.0–6.5) \mu\text{m}$; length/width 1.13 (1.00–1.30) ($n = 50$); oocyst wall smooth, colourless in light microscopy. To confirm that the oocyst shape variability is not an artefact, oocysts were examined in native preparations from freshly euthanised animals. Oo-

cysts observed in squash preparations of gastric mucosa (Fig. 3) showed the same degree of the shape variability; $6.5 (6.0–7.5) \times 5.8 (5.0–6.5) \mu\text{m}$; length/width 1.13 (1.00–1.36) ($n = 50$). Oocyst residuum was composed of a mass of fine granules $0.5 \mu\text{m}$ in diameter, and a spherical globule $1.5–2.0 \mu\text{m}$ in diameter. A large vacuolated area occupied a significant part of the oocyst residuum in all oocysts. Sporulation was endogenous; oocysts were fully sporulated in fresh faeces and gut content. Four vermiform sporozoites lay parallel along one side of oocyst, tightly enclosing oocyst residuum. Neither nucleus nor refractile bodies were observed within sporozoites using light microscopy. Noteworthy, oocysts were extremely sensitive to hypertonic conditions, crumpling immediately when exposed to flotation solutions. The fragility of oocysts was further confirmed by SEM examination of oocysts concentrated by flotation (not shown). Oocysts spontaneously disintegrated after 4 weeks of storage in water at 4°C , 10°C and 20°C .



Figs. 1–5. Type host and light microscopy of *Cryptosporidium fragile* sp. n. **Fig. 1.** Black-spined toad, *Duttaphrynus melanostictus*, the type host. Specimen from the Malay Peninsula, snout-vent length 73 mm. **Fig. 2.** Oocysts from faeces, flotation (NIC). **Fig. 3.** Composite micrograph of oocysts in squash preparation of the gastric mucosa (NIC). **Figs. 4, 5.** Stomach of infected toad in longitudinal histological sections (H&E). Scale bars: Figs. 2, 3 (same scale) = $10 \mu\text{m}$; Fig. 4 = $100 \mu\text{m}$; Fig. 5 = $50 \mu\text{m}$.

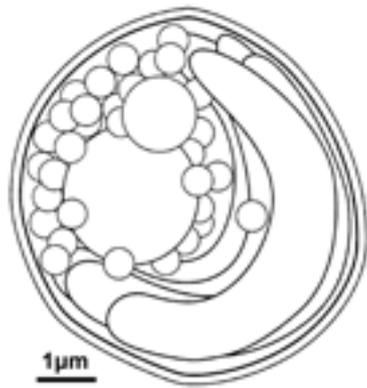


Fig. 6. Composite line drawing of the oocyst of *Cryptosporidium fragile* sp. n. showing features recognizable using light microscopy. Oocyst wall is shown in optical section.

Prevalence and patent period. Out of 18 toads examined, four (22%) passed oocysts at the time of importation. Subsequently, another 11 out of 14 remaining toads started to shed oocysts 6–35 days after arrival. A single oocyst-shedding event was recorded in two animals (24 and 35 days after arrival). The rest of the animals shed oocysts for 22–46 days, however the oocyst shedding was mostly intermittent with 7–25 days long recess periods with negative coprological results. Continuous oocyst shedding without recesses was observed in a single animal and lasted for 31 days. In total, 15 (83%) toads shed oocysts and three toads (17%) remained negative for cryptosporidia during the 90 days of the study. The infections spontaneously disappeared 24–51 days after arrival as indicated by negative coprological results of individual animals and negative results of histology in two animals one week after last oocyst shedding event.

Pathology. At arrival, the toads showed signs of weight loss, weakness and/or apathy that subsided together with eventual spontaneous disappearance of infection in all individual toads. Histopathological examination of the affected gastric tissues showed mild mucosal thickening (Figs. 4, 5) with no further signs of acute or chronic gastritis.

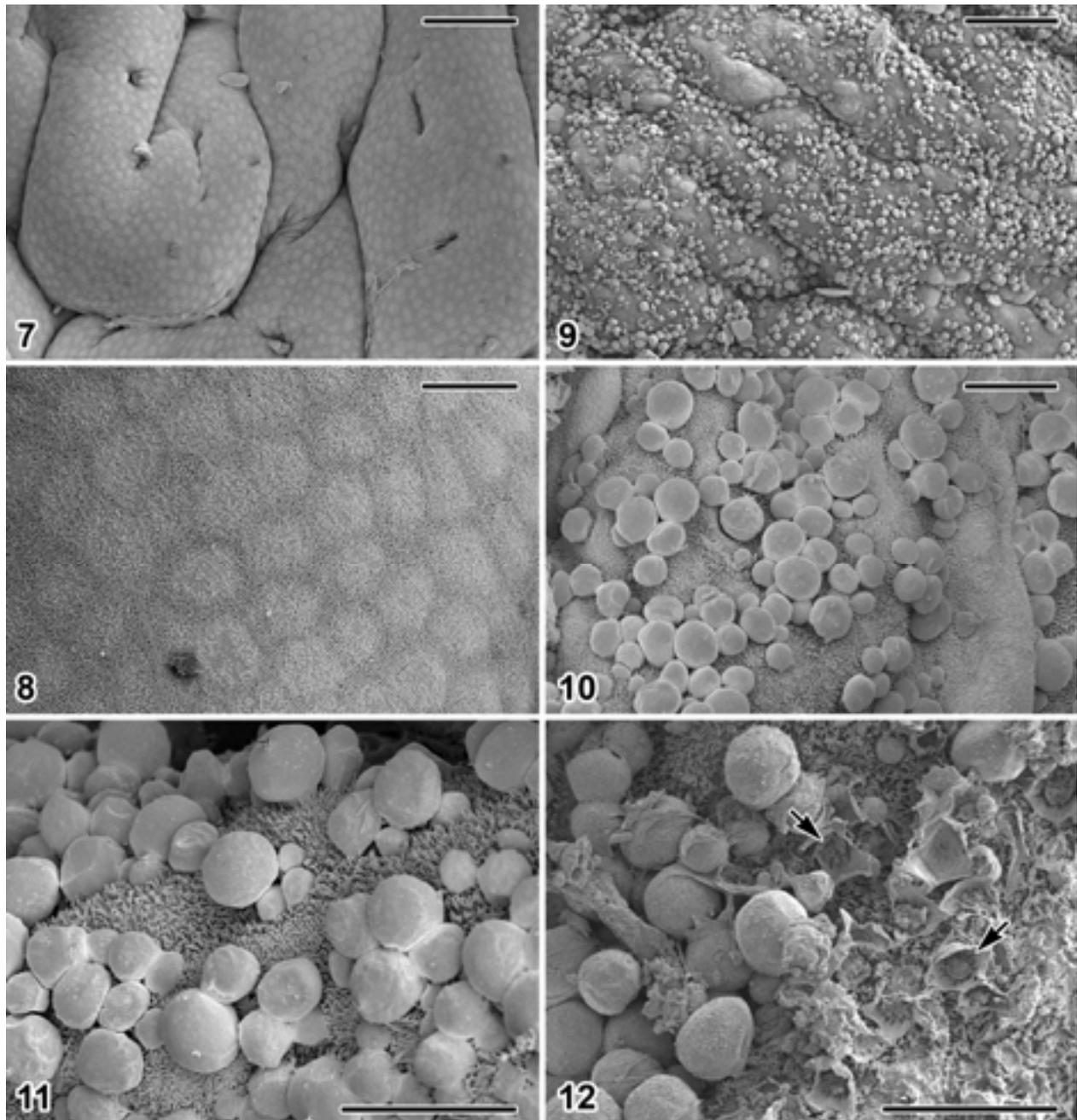
The mucosal surface of the stomach, examined by SEM, revealed severe infection in animals shedding oocysts and unmasked additional pathology. Stomach of *C. fragile*-affected toads exhibited an irregular surface with foci of swollen cells protruding into the lumen (Figs. 9, 10), contrasting with the smooth surface of the stomach mucosa of uninfected animals (Figs. 7, 8). Swollen cells, not directly parasitized by *C. fragile*, had rounded appearance, and were bulbously protuberant with less distinct intercellular borders when compared with unaffected stomach (compare Figs. 7, 8 and 9–12).

Site of infection. Histological examination of gastrointestinal tract of toads shedding *C. fragile* oocysts revealed the presence of cryptosporidian developmental

stages confined to the surface of gastric epithelial cells (Figs. 4, 5). No developmental stages were found in other examined tissues. The parasites were located within parasitophorous sac(s) at the microvillous surface of the epithelium, evenly distributed throughout both the luminal surface of gastric epithelium and the crypts of gastric glands (Figs. 4, 5, 9–13).

Endogenous development and ultrastructure. All developmental stages, except for invading zoites and early trophozoites, were observed in TEM and exhibited typical apicomplexan ultrastructural characteristics (e.g. three-layered pellicle; zoites with apical complexes). Trophozoites and successive stages were enveloped by the parasitophorous sac (Figs. 10–14) possessing distinct radial folds on outer surface of its base (Figs. 17, 22). Typical multilayered dense band was present within affected host cell, separating its unmodified part from the rest modified by the parasitophorous sac formation (Fig. 14). The most distinct layer of dense band was apparently indented (Fig. 21). The feeder organelle consisted of numerous longitudinally oriented fine membranous lamellae (Figs. 14, 19). The top of the feeder organelle was separated from filamentous projection by a seemingly two-layered dense line (Fig. 16). Trophozoites variable in shape, contained nucleus with a prominent nucleolus (Figs. 13, 14). Meronts possessed a large residual body and merozoites, which were short and plump while budding (Fig. 14). The anterior region of merozoites was occupied by rhoptries and numerous micronemes (Fig. 15). Macrogamonts, the most commonly observed developmental stages, filling almost the entire space of parasitophorous sac, possessed a large nucleus with a prominent single nucleolus, several dense bodies and amylopectin granules (Fig. 16). Microgamonts possessed a large residual body surrounded by bullet-like microgametes with a flattened anterior end and elongate compact nucleus (Figs. 17, 18). Microgametes lacked flagella. Mature oocysts showed a large oocyst residuum composed of numerous amylopectin granules and few, relatively large, lipid vacuoles. Four curved sporozoites containing a large nucleus and distinct micronemes lay on one side of each oocyst (Figs. 19, 20). Papular structures were present on the inner surface of oocyst wall (Fig. 19). Various developmental stages, still enclosed within parasitophorous sac, were often found detached from the epithelial surface (Fig. 19).

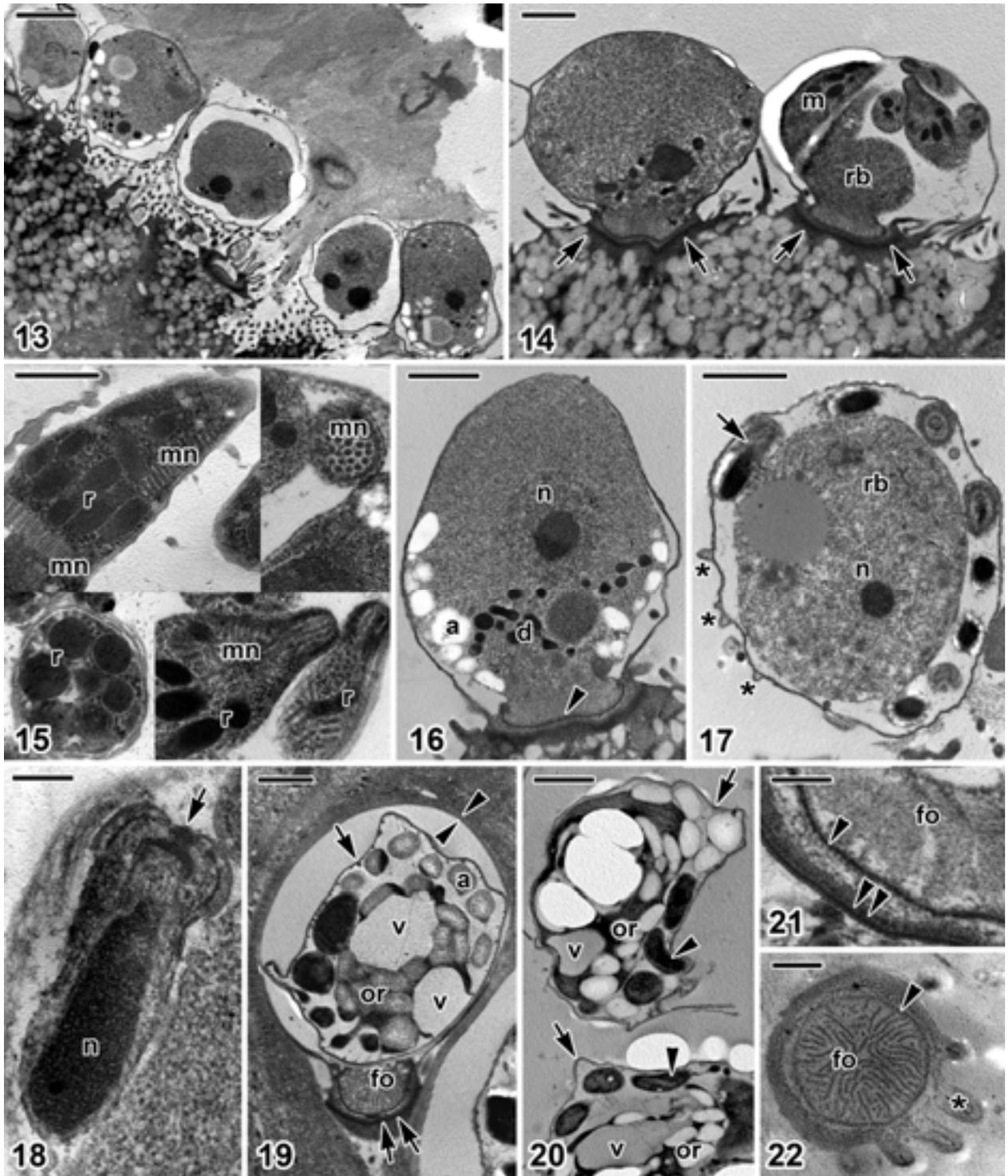
In SEM, free zoites were occasionally observed on the gastric luminal surface (Fig. 23). The youngest observed stages were invading zoites partly enveloped by rising membrane protrusions of the host cell. After formation of parasitophorous sac, bell-shaped trophozoites developed (Fig. 24) and these became rounded (Fig. 25), eventually growing into mature stages (Fig. 26). Young undifferentiated trophozoites were often surrounded by elongated microvilli, which sometimes secondarily (probably due to processing) adhered to the



Figs. 7–12. Comparison between gastric epithelium of non-infected and infected toad of *Duttaphrynus melanostictus*. **Figs. 7, 8.** Surface of gastric epithelium of non-infected toad. **Figs. 9, 10.** Surface of infected gastric epithelium covered by numerous developmental stages of *Cryptosporidium fragile* sp. n. **Fig. 11.** Surface of gastric epithelium during acute cryptosporidiosis. **Fig 12.** Surface of gastric epithelium during post-acute cryptosporidiosis. Note the presence of numerous ruptured parasitophorous sacs (arrows). Scale bars: Figs. 7, 9 = 50 μ m; Figs. 8, 10–12 = 10 μ m.

outer surface of parasitophorous sac (Fig. 24). Mature trophozoites and successive stages were completely enveloped by the parasitophorous sac with distinct, structurally uniform, radial folds that were evenly distributed around its base (Figs. 26–28). Some of completely enveloped parasites were located on a long stem of the host cell origin, the surface of which sometimes

possessed microvilli (Figs. 27, 28). Meronts produced merozoites, which were short and plump while budding (Fig. 29) and more elongate and slender when fully matured (Fig. 30). Maturing oocysts were enveloped by parasitophorous sac and showed marked superficial ornamentation (Fig. 31) and a typical semicircular longitudinal suture in the oocyst wall (Fig. 32). Numerous



Figs. 13–22. Transmission electron micrographs of *Cryptosporidium fragile* sp. n. **Fig. 13.** Gastric epithelium covered by trophozoites and macrogamonts. **Fig. 14.** Immature macrogamont and meront. Note the distinct dense bands (arrows). **Fig. 15.** Internal details of merozoites, composite figure. **Fig. 16.** Macrogamont; dense line (arrowhead). **Fig. 17.** Microgamont with microgametes (arrow). **Fig. 18.** Microgamete; apical adhesive zone (arrow). **Fig. 19.** Oocyst within detached parasitophorous sac; oocyst wall and papular structures on its inner surface (arrow); parasitophorous sac (arrowheads); dense band area (double arrow). **Fig. 20.** Released oocysts in gastric lumen; sporozoites (arrowheads) with distinct micronemes; oocyst wall (arrows). **Fig. 21.** Detail of host-parasite interface; dense line (arrowhead); dense band (double arrowhead). **Fig. 22.** Feeder organelle in cross-section; dense line (arrowhead). *Abbreviations:* a – amylopectin granules; d – dense bodies; fo – feeder organelle; m – merozoite; mn – micronemes; n – nucleus; or – oocyst residuum; r – rhoptries; rb – residual body; v – vacuole; * – radial folds of parasitophorous sac. Scale bars: Figs. 13, 19 = 2 μ m; Figs. 14, 16, 17, 20 = 1 μ m; Figs. 15, 22 = 500 nm; Fig. 18 = 200 nm; Fig. 21 = 250 nm.

developmental stages were found detached from the epithelial surface (Fig. 33), showing the base of parasitophorous sac with distinct radial folds inserted under the button-like area of dense band (Figs. 27, 28, 33). Ruptured, empty parasitophorous sacs were often present on the epithelial surface, revealing their bases with the feeder organelles encircled by Y-shaped membrane junctions (annular rings) (Figs. 34, 35).

Experimental host transmissions. None of the experimental animals showed clinical signs of infection and no oocysts were passed in their faeces up to 8 weeks p.i. (Table 1). No endogenous stages were seen during the histological and fresh examinations of gastrointestinal tissue samples from animals killed 8 weeks p.i.

Molecular characterisation and phylogenetic analysis. We sequenced a complete *C. fragile* SSU rDNA sequences. Four obtained sequences, each 1,748 bp long, differed from each other at 7 single nucleotide positions (4 × A>G, 2 × T>C, 1 × G>A). Pairwise distance with available sequences in GenBank™ revealed that the intestinal *C. parvum* group of sequences was 90% identical while the pairwise distance with the gastric *C. muris* / *C. andersoni* group of sequences was 93%. Amplification of the protein-coding genes using published primers for COWP and actin (Spano et al. 1997, Xiao et al. 2000, Sulaiman et al. 2002) repeatedly failed to produce a cryptosporidian amplicon.

Phylogenetic reconstruction (Fig. 36) revealed the affinity of *C. fragile* with the gastric clade including *C. muris*, *C. andersoni*, *C. serpentis* and *C. galli*. Sequence of *C. galli* and genotypes from star tortoise *Geochelone elegans*, leopard gecko *Eublepharis macularius* and Eurasian woodcock *Scolopax rusticola* belong to the gastric clade but have less than 50% sequence coverage of the full length SSU rDNA and were excluded from the analysis where only complete sequences were included (Fig. 36, inset). ME and MP phylogenetic reconstruction revealed monophyly of the gastric clade. ML did not support the monophyly of gastric sequences, lining them up at the base of the intestinal species. Sequences of *C. fragile* formed a distinct clade within the gastric genotypes and named species. The basal position of *C. fragile* in the gastric clade was supported by low bootstrap support.

DISCUSSION

The species identification of apicomplexan parasites is traditionally based on the host specificity, the site of infection, and the morphology of life-cycle stages including oocysts. In an attempt to better characterize and describe *C. fragile* we have re-evaluated the current practice for naming new *Cryptosporidium* species.

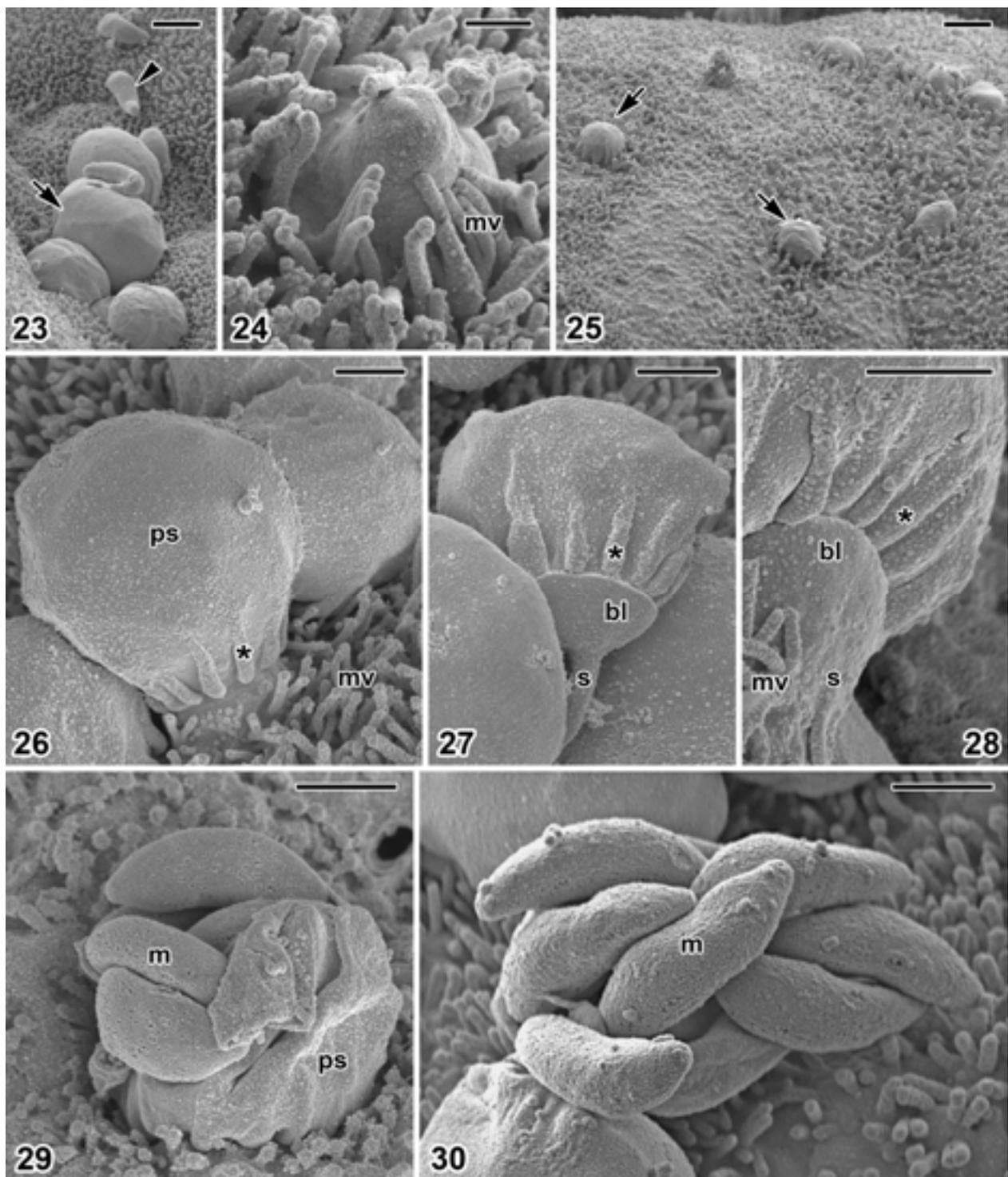
The criteria for naming a new species in the genus *Cryptosporidium*

Naming a new species is nomenclatural act that is covered by certain rules and practices. In case of the

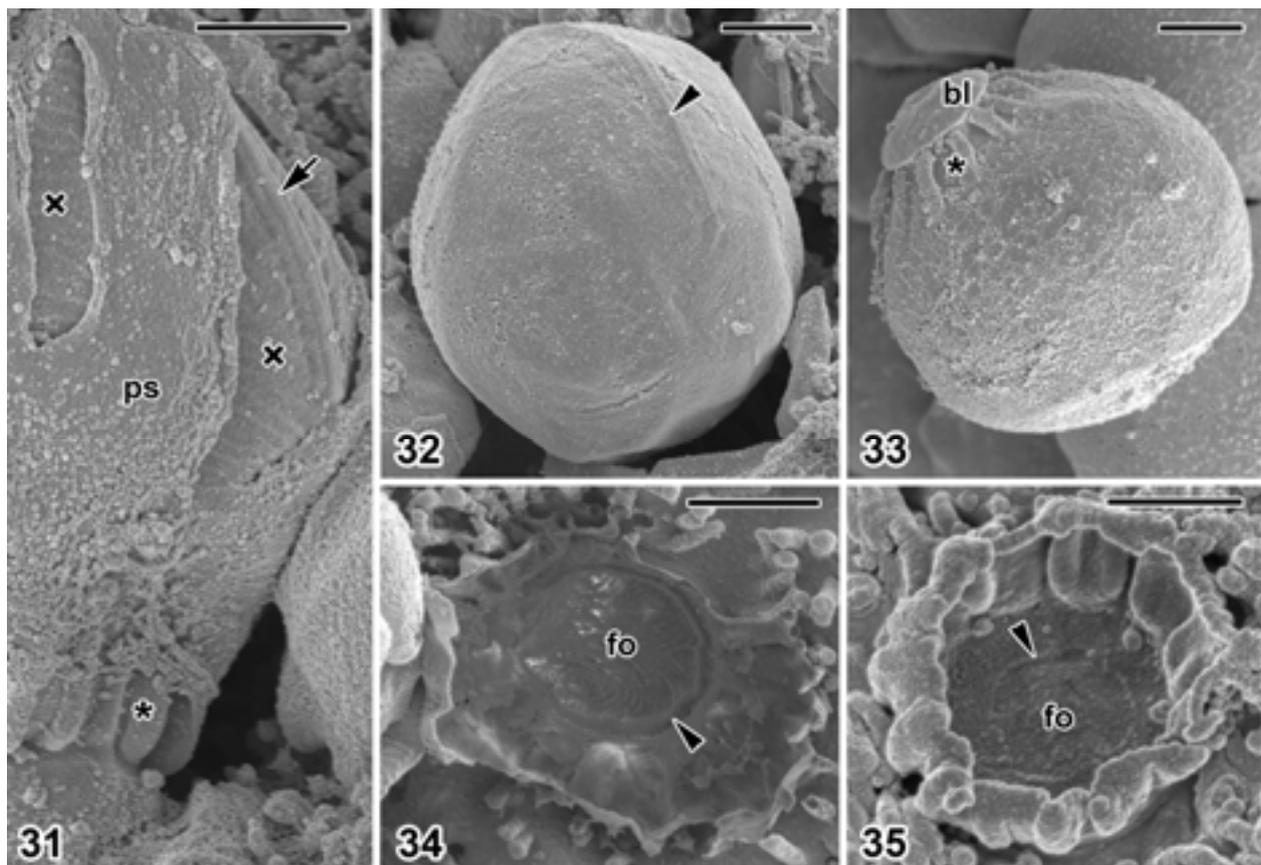
majority of parasitic protozoa including *Cryptosporidium* spp., it is the International Code of Zoological Nomenclature (ICZN). The stability and longevity of a taxon stands on its holotype, the name bearing specimen used for its description. The type is the specimen(s) illustrated/photographed or described (Article 72.5.6). On the other hand, the fact that the specimen illustrated no longer exists, or cannot be traced, does not invalidate the type designation (Article 73.1.4). The lack of reference material coupled with lack of recognizable morphological differences among named *Cryptosporidium* spp. have been a major drawback in resolving some of the nomenclatural issues. A consensus on what are the minimal criteria for description of a new species of *Cryptosporidium* has been previously summarised into four recommended requirements: (i) morphometric studies of oocysts; (ii) genetic characterisations; (iii) demonstration of natural and, whenever feasible, at least some experimental host specificity; and (iv) compliance with ICZN (Egyed et al. 2003, Xiao et al. 2004a).

While we agree, that the oocyst morphology is important, it must not be the only morphological trait investigated for a new species designation. Demonstration of development within the host and ultrastructural analyses should be mandatory information in all descriptive works. While describing *C. fragile* we employed an array of ultrastructural techniques, to better characterize this new species. Besides morphology, the biological characteristics play an essential role in establishing a new species. Localisation, host specificity and pathology associated with an individual host will remain to play a vital role in the species identification. After justifying the mandatory morphological and biological description, an attempt to characterize the isolate genetically at least on a single molecular marker should be made. If such an attempt is considered, complete gene sequences are preferable or even mandatory. Finally yet importantly, it is the differential diagnosis where authors must include the statement how to distinguish the organism from already named species. Singly genetic distinction from already known species should not merit value of differential diagnosis in the species description.

Our experience with the description of *C. fragile* has led us to review and revise the recommended criteria for an introduction of a new *Cryptosporidium* species name. We summarise the recommended criteria as follows, in compliance with the ICZN. A robust description of (i) morphology of exogenous stages (oocysts) and the morphological demonstration of the developmental stages, accompanied by a deposition of preserved infected tissues for further morphological analyses and (ii) biological characterisation. These should lead to (iii) a differential diagnosis that should, if possible, be supported by (iv) genetic characterisation accompanied by a deposition of sequences and material for further DNA characterisation. Deposited material should be made available



Figs. 23–30. Scanning electron micrographs of *Cryptosporidium fragile* sp. n. **Fig. 23.** Unidentified developmental stages (arrow) and released motile zoites (arrowhead). **Fig. 24.** Young, bell-shaped trophozoite. **Fig. 25.** Surface of gastric epithelium with several rounded trophozoites (arrows). **Fig. 26.** Unidentified developmental stages. **Figs. 27, 28.** Developmental stages with stems. **Fig. 29.** Immature meront with budding merozoites and ruptured parasitophorous sac. **Fig. 30.** Mature meront with slender merozoites. *Abbreviations:* bl – button-like dense band area; m – merozoite; mv – microvilli; ps – parasitophorous sac; s – stem; * – radial folds of parasitophorous sac. Scale bars: Figs. 23, 25 = 2 μ m; Fig. 24 = 500 nm; Figs. 26–30 = 1 μ m.



Figs. 31–35. Scanning electron micrographs of *Cryptosporidium fragile* sp. n. **Fig. 31.** Oocyst enveloped by ruptured parasitophorous sac; oocyst wall suture (arrow). Note the oocyst wall surface ornamentation (x). **Fig. 32.** Oocyst enveloped by parasitophorous sac showing protruding oocyst wall suture (arrowhead). **Fig. 33.** Detached parasitophorous sac containing unidentified developmental stage showing the button-like dense band area and radial folds of parasitophorous sac. **Fig. 34.** Base of recently ruptured parasitophorous sac with well preserved lamellae of the feeder organelle; Y-shaped membrane junction (arrowhead). **Fig. 35.** Base of ruptured parasitophorous sac revealing signs of microvillar surface regeneration as reflected by barely discernible feeder organelle and Y-shaped membrane junction (arrowhead) remnants. *Abbreviations:* bl – button-like dense band area; fo – feeder organelle; ps – parasitophorous sac; * – radial folds of parasitophorous sac. All scale bars = 1 μ m.

via a museum or other academic institution. The above-mentioned criteria were followed in the description of *C. fragile* and an effort has been made to fulfil all these requirements.

***Cryptosporidium fragile*: species parasitizing amphibian stomach**

Oocyst morphology, shape and size in particular, are traits differentiating the intestinal and stomach species, as originally pointed out by Tyzzer (1912). The oocysts of intestinal species are rounded, measuring 4–6 μ m in diameter, while those of gastric species are larger and elliptical, reaching 7–9 μ m. The subspherical to elliptical shape of *C. fragile* already indicates its affinity with the gastric species. The oocysts of *C. fragile* are smaller compared with *C. muris*, *C. andersoni* or *C. galli*, but comparable in size with *C. serpentis* (Table 2). The shape variability and the irregular shape of the oocysts (Figs. 2, 3, 6) represent other traits not recorded previously in *Cryptosporidium* spp. The surface ornamenta-

tion of the oocyst wall of *C. fragile* (Fig. 31) differs from smooth to finely wrinkled oocyst wall of *C. parvum* (Reduker et al. 1985) and smooth oocyst wall of *C. muris* (Valigurová et al. 2008). The fragility of the oocysts is a noteworthy character not even reported for the fish species (Alvarez-Pellitero and Sitjà-Bobadilla 2002).

The gastric cells parasitized by *C. fragile* occasionally formed what we call in this study the stem-like structures (Figs. 27, 28). This feature was previously observed in ileum of guinea pig infected with *C. wrairi* (Vetterling et al. 1971), suggesting that it might be common but overlooked in other cryptosporidia. The bell-shaped early trophozoites of *C. fragile* observed in our SEM preparations (Fig. 24) are comparable with those observed in TEM micrographs in cryptosporidia from fish and reptiles (Ostrovská and Paperna 1990, Paperna and Vilenkin 1996). The dense band below the feeder organelle in *C. fragile* was thick and less clearly

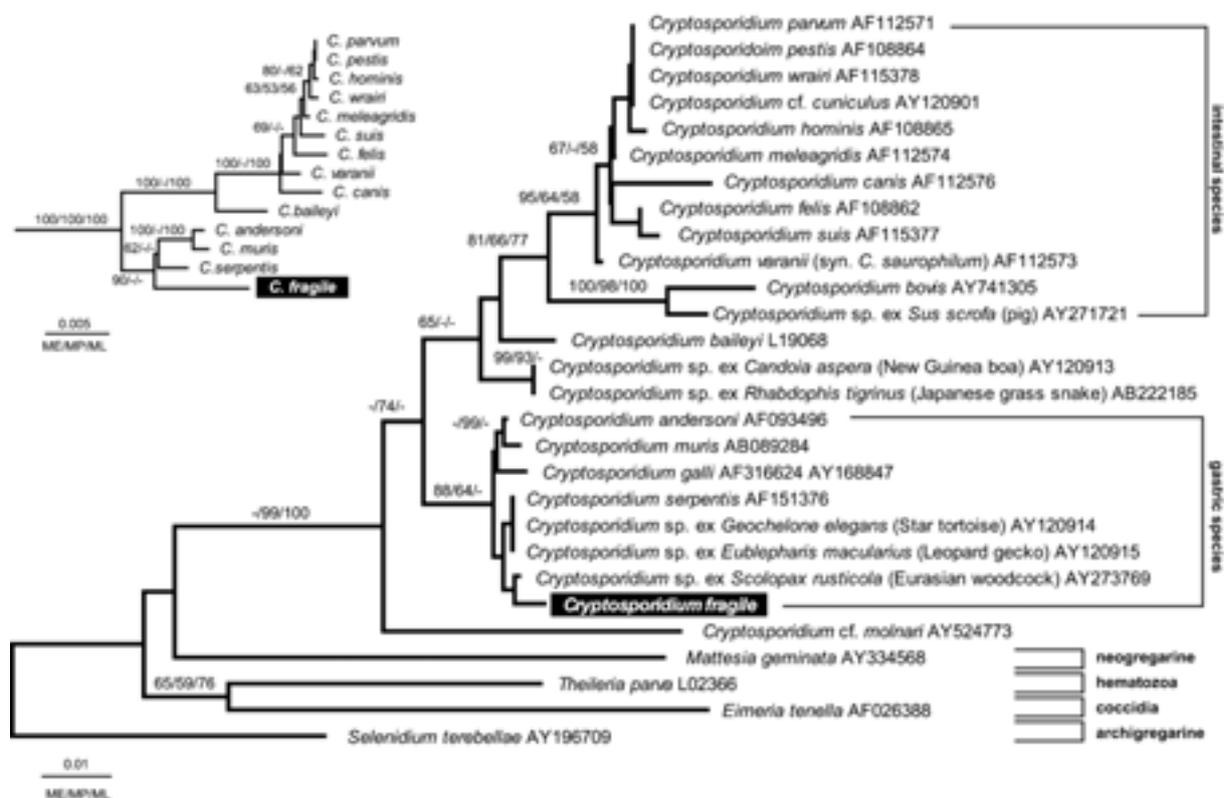


Fig. 36. Phylogenetic reconstruction of *Cryptosporidium* species for the SSU rDNA. Reconstruction using available sequences from named *Cryptosporidium* species and selected genotypes with partial coverage and dataset of complete SSU rDNA sequences only (inset). The phylogenetic tree was inferred using the Minimum Evolution method. The bootstrap support values (>50%) for Minimum Evolution (1,000 replicates) / Maximum Parsimony (500 replicates) / Maximum Likelihood (100 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. Minimum Evolution and Maximum Parsimony were calculated in MEGA4. Maximum Likelihood was calculated in PhyML 2.4.4 using GTR+G+I model.

differentiated into distinct layers compared to majority of the other cryptosporidian species; however, higher magnification revealed several layers, of which one was more distinct and finely indented as reported previously in *C. muris* and *C. wrairi* (Vetterling et al. 1971, Uni et al. 1987, Valigurová et al. 2007). Parasitophorous sac of *C. fragile* invariably possesses distinct, structurally uniform, radial folds evenly distributed around its base. The absence of these folds or their structural and spatial irregularity together with the presence of distinct and bulky filamentous projection in *C. muris* (weakly developed in *C. fragile*), make these two species clearly distinguishable from each other at the ultrastructural level (Valigurová et al. 2008).

Despite mentioned differences, the ultrastructure of *C. fragile* developmental stages was comparable to other cryptosporidian species. Unfortunately, many of the observed characters are insufficiently characterized in other *Cryptosporidium* spp., hampering the significance in species identification. Nevertheless, we find morphological traits correlating with individual host groups. Evident similarities occur within *Cryptosporidium* species from (i) fish, (ii) mammals and (iii) reptiles.

In the fish-affecting species, the surface of parasitophorous sac is covered by so-called rudimentary microvilli (Paperna and Vilenkin 1996). These structures, together with the localisation of the oocysts deep within the gastric mucosa, were used to erect the genus *Piscicryptosporidium* for the fish species (Paperna and Vilenkin 1996), which is currently considered a synonym of *Cryptosporidium* (Alvarez-Pellitero and Sitjà-Bobadilla 2002, Alvarez-Pellitero et al. 2004). We did not detect these structures in the amphibian gastric mucosa affected by *C. fragile*. In general, fish cryptosporidia exhibit well developed massive lamellae of the feeder organelle and inconspicuous filamentous projection (Paperna and Vilenkin 1996, Alvarez-Pellitero and Sitjà-Bobadilla 2002, Alvarez-Pellitero et al. 2004). Similarly, *C. muris* and *C. andersoni*, gastric species from mammalian hosts, seemingly exhibit common features, i.e. the shape and bulkiness of the filamentous projection (Uni et al. 1987, Masuno et al. 2006, Valigurová et al. 2007). On the other hand, the button-shaped feeder organelle with fine lamellae is present in *C. fragile* and both gastric as well as intestinal species from reptiles (Brownstein et al. 1977, Koudela and Modrý 1998). The

Table 2. Summary of biological and morphological characteristics of named gastric *Cryptosporidium* species (*C. baileyi* and *C. hominis* are included for comparative purposes).

Name	Oocysts size (μm)	Localisation in the host	Type host / host range	Reference
<i>C. fragile</i> sp. n.	6.2 (5.5–7.0) \times 5.5 (5.0–6.5)	Stomach	Toad / *	This study
<i>C. muris</i>	8.4 (7.5–9.8) \times 6.3 (5.5–7.0)	Stomach	Mouse / rodents	Lindsay et al. 2000
<i>C. andersoni</i>	7.4 (6.0–8.1) \times 5.5 (5.0–6.5)	Stomach	Cattle / *	Lindsay et al. 2000
<i>C. serpentis</i>	6.2 (5.6–6.6) \times 5.3 (4.8–5.6)	Stomach	Corn snake / reptiles	Upton et al. 1989, Graczyk et al. 1998
<i>C. galli</i>	6.6 (5.9–6.7) \times 5.8 (4.9–6.1)	Stomach	Chicken / birds	Ryan et al. 2003a
<i>C. molnari</i>	8.25 (8.0–8.5) \times 6.3 (6.2–6.4)	Stomach [†]	Sea bream / sea fish	Alvarez-Pellitero and Sitjà-Bobadilla 2002
<i>C. baileyi</i>	4.72 (3.23–5.45) \times 4.47 (3.02–5.04)	Bursa Fabricii and cloaca	Chicken / birds	Current et al. 1986
<i>C. hominis</i>	6.2 (6.3–5.6) \times 4.6 (4.8–4.5)	Intestine	Human / *	Morgan-Ryan et al. 2002

[†]*Cryptosporidium molnari* is rarely found also in intestine; *the species is either host specific or there is no comprehensive information available; oocyst sizes are derived from the referenced publications.

lamellae in these cryptosporidia seem to be fragile and often become disrupted during the specimen processing. Obviously, the morphology of both feeder organelle and the whole attachment site differ considerably between cryptosporidia found in different hosts and might be of some taxonomic significance.

Amphibians are rarely reported to be parasitized with *Cryptosporidium* spp. (Wright and Whitaker 2001, Cranfield and Graczyk 2006). Correspondingly, amphibians were refractory to experimental infection with characterized *Cryptosporidium* species (Graczyk et al. 1996, 1998). The zoonotic AUCP-1 strain of *C. parvum* ‘bovine genotype’, now recognized as *C. pestis* (Šlapeta 2006), was experimentally inoculated into African clawed frogs (*Xenopus laevis*) and poison-dart frogs (*Dendrobates auratus*), but experimental animals remained negative (Graczyk et al. 1996). Similarly, the inoculation of adult African clawed frogs (*X. laevis*) and tadpoles and adults of wood frogs (*Rana sylvatica*) by *C. serpentis* did not result in infections (Graczyk et al. 1998). In our cross-transmission experiments, we selected such hosts as to rule out the conspecificity of the studied isolate with some of the already described *Cryptosporidium* spp. Negative results of experiments support our conclusion that *C. fragile* is a separate species. Although the SCID mice are known to be a suitable model for intestinal and gastric cryptosporidiosis (Mead et al. 1991, Lindsay et al. 2000), they remained negative after inoculation with *C. fragile* indicating its inability to affect mammalian hosts. We further evaluated the host specificity of *C. fragile* in an array of frogs and tadpoles. None of the inoculated amphibians was found to be a susceptible host. Because we could not rule out the latent cryptosporidiosis in available *Cryptosporidium*-negative black-spined toads, we did not attempt to inoculate them as a positive control. However, the inoculum was freshly prepared and presence of high numbers of morphologically intact oocysts was confirmed before each trial. Although experiment using an outbred or wild animal is biased by the immunological status of the host, our findings suggest *C. fragile* to be host specific.

Phylogenetic analysis based on SSU rDNA has supported the fact that *C. fragile* falls in the monophyletic clade comprising the gastric species, using ME and MP phylogenetic methods. ML did not support the monophyly of gastric SSU rDNA sequences lining them up at the base of the intestinal species due to a poor reliability of the alignment of SSU rDNA, as previously discussed by Morrison (2006). Unfortunately, only partial SSU rDNA are available for novel genotypes as well as *C. galli* (Ryan et al. 2003a, b, 2004, Xiao et al. 2004b). The partial nature of these sequences rendered the position of *C. fragile* inaccurate within the gastric species and genotypes. Nonetheless, the sequence of *C. fragile* is distinct from any of the sequences available in databases, supporting the description of the new species.

Amphibian cryptosporidiosis: fact or fiction

Occurrence of *Cryptosporidium* oocysts in the faeces of a captive mice-fed ornate horned frog, *Ceratophrys ornata* (Crawshaw and Mehren 1987) is probably the first report related to amphibians. Another two reports deal with natural and experimental cryptosporidiosis in giant toads, *Rhinella marina* (previously *Bufo marinus*) (Arcay and Bruzal 1993, Arcay et al. 1995). While these reports provide noteworthy data about cryptosporidiosis in *R. marina*, the identity of *Cryptosporidium* isolate used in the experiments is unclear and the cryptosporidiosis in *R. marina* thus needs further investigation and re-evaluation.

The only well described cryptosporidiosis in frogs is a case of proliferative gastritis in a single laboratory-reared African clawed frog (*X. laevis*) associated with finding of *Cryptosporidium* sp. in the gastric mucosa (Green et al. 2003). The authors have demonstrated developmental stages using histological techniques as well as TEM; moreover the oocysts were recovered from the sediment in the aquarium. The affected frog had been laboratory-reared by a commercial producer and kept in a laboratory facility in the USA with ~2,000 individuals, but no other frog in the facility was affected. The identity of this *Cryptosporidium* sp. remains elusive, but it is unlikely that it was any of the known species. Cryptosporidia are considered to be host specific within either

fish, reptiles, birds or mammals. The only exception is *C. meleagridis* infecting both mammals and birds (Akiyoshi et al. 2003). Conspicuity of *X. laevis*-isolate with *C. fragile* is unlikely because the morphological characteristics well differentiate these two cryptosporidia. The button-shaped feeder organelle is apparently more compact with an obvious Y-shaped membrane junction and the dense band is more distinct in *C. fragile* [compare our Figs. 14, 19 with fig. 2A in Green et al. (2003)]. Moreover, neither the fragility nor the irregular shape of *C. fragile* oocysts was noticed by Green et al. (2003).

The prevalence of *C. fragile* in toads in Malaysia remains unknown. Similarly, we do not know whether *Duttaphrynus melanostictus* is the true natural host or an accidental host that acquired the infection in the exporter facilities. Generally, quarantine measures during the importation/exportation of live exotic amphibians and fish are weak or nil and need careful revision. Strict implementation of quarantine and further regulations are necessary. Our experience with *C. fragile* imported from Malaysia suggests that quarantine measures for live amphibians are insufficient both in the exporting as well as the importing country. We recommend the examination of imported amphibians for the presence of *Cryptosporidium* spp. as an obligatory part of implemented measures.

Further research is needed to elucidate the conditions under which an amphibian becomes a susceptible host and the extent of cryptosporidiosis in amphibians. Such information are of special significance in the context of the global amphibian declines, frequently associated with emerging infectious diseases that cause mass die-offs and extinctions of naïve amphibian populations (Daszak et al. 2003, Stuart et al. 2004, Skerratt et al. 2007).

Taxonomic summary

Cryptosporidium fragile sp. n.

Type host: Black-spined toad *Duttaphrynus melanostictus* (Schneider, 1799) (Anura: Bufonidae).

Type locality: Malay Peninsula; exact locality unknown.

Site of infection: Gastric mucosal cells.

Prevalence: 22% (4/18) and 83% (15/18) *D. melanostictus* infected at the time of import and within 90 days of the study, respectively.

Type material/hapantotype: Histological sections of infected stomach, gold-coated infected stomach tissue, infected stomach in absolute ethanol, digital photo-

micrographs on CD, and symbiotype *D. melanostictus* specimen with liver tissue sample in absolute ethanol – deposited at the type parasitological collection of the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice, No. IP ASCR Prot. Coll.: P-3.

DNA sequences: SSU rDNA submitted to GenBank™ under the accession numbers EU162751–EU162754.

Etymology: The specific epithet “*fragile*” (Latin, adj., meaning fragile, easily broken) refers to the extraordinary sensitivity of oocysts to hypertonic conditions and early disintegration of oocysts in water.

Remarks. Up to date there are seven *Cryptosporidium* spp. described to parasitize primarily stomachs of vertebrates; *C. muris* and *C. andersoni* in mammals, *C. serpentis* in reptiles, *C. galli* in birds, and *C. cichlidis*, *C. reichenbachklinkei* and *C. molnari* in fish. *Cryptosporidium fragile* is the first species described from an amphibian host. Oocyst size of *C. fragile* is similar to *C. serpentis*, but its oocysts differ in marked shape variability and irregularity. In addition, we could not experimentally infect the corn snake (*Pantherophis guttatus*) with *C. fragile*. Oocysts of cryptosporidia from fish are smaller, while mammalian and avian cryptosporidia are larger. Ultrastructurally, oocysts of *C. fragile* possess a surface ornamentation not recorded in other *Cryptosporidium* spp. *Cryptosporidium fragile* differs from all ultrastructurally characterized species in the weakly developed filamentous projection coupled with the extremely fine lamellae of the button-shaped feeder organelle and the thick dense band. Furthermore, the structurally uniform radial folds evenly distributed around the base of parasitophorous sac, which are inserted under the button-like area of the dense band, differentiate *C. fragile* from other *Cryptosporidium* spp. possessing irregular folds or lacking these folds.

Acknowledgements. This study was supported by grants of the Grant Agency of the Czech Republic Nos. 524/03/H133 and 524/05/0992, Institute of Parasitology (projects Z602 20518 and LC 522), and by a grant MSM 0021622416. Authors are greatly indebted to the members of the Laboratory of Electron Microscopy (Institute of Parasitology), Věra Kučerová, Blanka Cíkáňová and Martina Tesařová for generous help and technical assistance. JS would like to acknowledge support from the Faculty of Veterinary Science, University of Sydney, towards the preparation of this manuscript. We also thank Jakub Bednár for provision of amphibians involved in this study. Fig. 31 was taken, with permission of the Australian Society for Parasitology, from Fig. 6A in Valigurová et al. (doi:10.1016/j.ijpara.2007.11.003), International Journal for Parasitology, published by Elsevier.

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Received 2 November 2007

Accepted 14 January 2008