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ORIGINAL PAPER

The Phylogeny of *Goussia* and *Choleoeimeria* (Apicomplexa; Eimeriorina) and the Evolution of Excystation Structures in Coccidia

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The phylogenetic relationships of *Goussia janae* and *Choleoeimeria* sp. were analyzed using the small subunit ribosomal RNA gene (SSU rDNA). This is a first attempt to study the molecular phylogeny of coccidian genera parasitizing strictly poikilotherm hosts. The biliary *Eimeria*-like coccidia of reptiles classified into the genus *Choleoeimeria* form a sister clade to the family Eimeriidae, which confirms the separate generic status of the genus *Choleoeimeria*. The position of *Goussia* is less robustly resolved, since it forms a trichotomy with the Eimeriidae and Sarcocystidae, or alternatively constitutes the earliest branch of the coccidian lineage. Morphological similarities, namely the extracytoplasmic location of the endogenous stages, and the presence of sutures in the sporocyst wall are discussed in the context of the traditional classification of eimeriids. In contrast to the morphology-based systematics, the monophyly of *Goussia* and *Choleoeimeria* is not supported by the SSU rDNA data.

Introduction

Classification of the coccidian parasites (Apicomplexa: Eimeriorina) on the generic and family levels is traditionally based on the mode of the life cycle and the number of sporocysts and sporozoites in the exogenous stages (= oocysts) (Levine 1982). In 1983, Levine wrote about the genus *Goussia* and closely related coccidia: "…. It is easy to believe that the latter two [*Goussia* and *Defretinella*] arose from the first [*Barroussia*] by stabilization of the number of sporocysts in each oocyst and change in the number of sporozoites per sporocyst. Whether this is true, however, cannot be proved because of the absence of fossils…." (Levine 1983). Although coccidian fossils have yet to be found, within the last decade, molecular phylogenetic techniques have provided a wealth of data that may help us to elucidate the evolution of coccidia.

Because of their importance to human and animal health, members of the families Eimeriidae and Sarcocystidae that are mostly parasites of homeiotherm vertebrates, have attracted substantially more attention than other coccidian groups. Species belonging to the genera *Goussia*, *Choleoeimeria* and *Acroeimeria* parasitize primarily poikiloterm hosts.

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Features like extracytoplasmic localization, endodyogony, absence of true wall-forming bodies, presence of membranaceous veils in oocysts and thin oocyst walls, unify these coccidians and may be regarded as ancestral traits (Paperna 1995). Therefore, the inclusion of representatives of these genera into phylogenetic studies is important for our understanding of coccidian evolution.

The genus *Goussia* Labbé, 1896 has a complex taxonomic history. Being created to accommodate the tetrasporocystic fish coccidia with a sporocyst wall composed of two joined plates, it was later treated as a subgenus or even as a synonym of *Eimeria* Schneider 1875 (Upton et al. 1984; Upton 2001). The genus *Goussia* was revived by Dyková and Lom (1981), who accommodated into it all fish coccidia with bivalved sporocysts lacking the Stieda body. Moreover, Levine (1983) also listed species from centipedes, beetles and reptiles as members of this genus. The genus *Goussia* is traditionally included into the family Eimeriidae Minchin, 1903 (Azevedo 2001; Dyková and Lom 1981; Upton et al. 1984), but some authors preferred its transfer into either the family Barroussiidae Léger, 1911 (Davies and Ball 1993; Landsberg and Paperna 1985; Levine 1983), or Calyptosporidae Overstreet, Hawkins and Fournie, 1984 (Diouf and Toguebaye 1993; Overstreet et al. 1984).

An assemblage of species with endogenous development localized in the gall bladder of reptiles represents another distinct group within the eimeriids (Paperna 1995). All species with biliary localization are characterized by the presence of elongated, usually cylindroidal oocysts that sporulate endogenously. The sporocyst wall of these coccidia is composed of two valves joined by a distinct suture. Infected biliary epithelial cells are hypertrophied and displaced to the surface of the epithelium (Paperna and Landsberg 1989a). Based on these unique features, Paperna and Landsberg (1989b) separated the biliary coccidians of reptiles into a new genus *Choleoeimeria*. Many authors, however, rejected this classification, awaiting more supportive data (Daszak and Ball 2001; Modrý et al. 2000, 2001a; Telford 1992; Upton et al. 1990, 1993). In the most recent review, all coccidians from reptiles with oocysts that have two sporocysts each with four sporozoites are treated as members of the genus *Eimeria* (Perkins et al. 2001).

Hence, the taxonomy of eimeriid coccidians of fish and reptiles is controversial. Herein, we include representatives of the genera *Goussia* and *Choleoeimeria* into a phylogenetic tree for the first time. Taxonomic criteria applied so far for *Goussia* spp. and *Choleoeimeria* spp. use only few informative characters. It is hoped that the relationships inferred from the molecular data presented here justify the resurrection of both enigmatic genera and thereby, will finally bring their synonymization with *Eimeria* to an end. Moreover, we address the (in)congruence of obtained relationships with the distribution of morphological characters involved in the process of excystation.

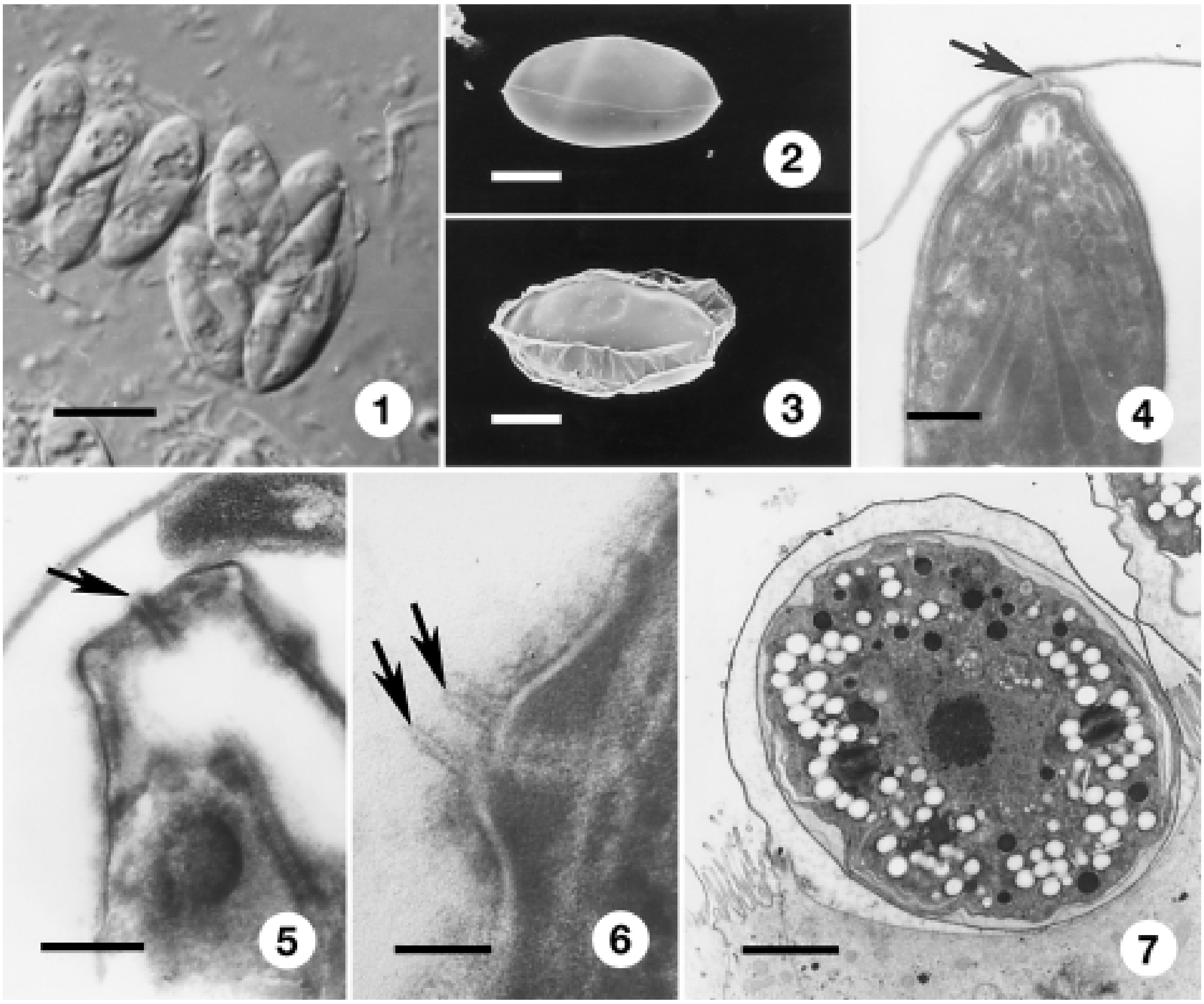
Results

Morphology of Oocysts and Sporocysts

Members of the genus *Goussia* have oocysts with the "*Eimeria*-type" features, typically having a thin and fragile oocyst wall, which in light microscopy appears to be single-layered (Fig. 1). Unsporulated oocysts recovered from the excrements of the dace were allowed to sporulate exogenously for several days in water. The dimensions of sporulated oocysts and sporocysts matched exactly those reported from the same host and locality for *G. janae* (Lukeš and Dyková 1990). Oocysts of *Choleoeimeria* sp. 1 and sp. 2 are also of the *Eimeria* type and are typically cylindroidal with a shape index (length/width ratio) of usually between 1.5–1.8, but always exceeding 1.4; a feature characteristic for the genus (Paperna and Landsberg 1989b). Endogenously sporulated oocysts (Fig. 8) were recovered from the lumen of heavily infected gall bladder of a colubrid snake and an agamid lizard (see Methods). The morphology of oocysts and other taxonomic criteria do not match any known species (DM, unpubl. data) and both new species will be described elsewhere.

The sporocyst wall of *G. janae* is composed of two identical valves joined by a longitudinal suture, which are widened at the margins where they appose each other. The suture is only barely visible in fresh material examined by light microscopy (Fig 1), but is prominent in micrographs of liberated sporocysts (Fig. 2). In *G. janae*, the suture is accompanied by a fine veil formed by two loose membranes, that is preserved only in a fraction of sporocysts processed for scanning electron microscopy (Fig. 3). On the ultrastructural level, the sporocyst wall of *G. janae* consists of a thick inner and thin outer layers that differ in electron density. Within the suture, a thin electron-lucent strip is interposed between the two valves (Figs 4, 5 and 6).

The sporocyst wall of *Choleoeimeria* sp. 1 has a similar structure. It consists of two layers; an outer electron-lucent layer and an inner electron-dense one. The longitudinal suture is formed by dense plates stretching into the sporocyst lumen that are angled at ca. 90° to the sporocyst wall and are inter-

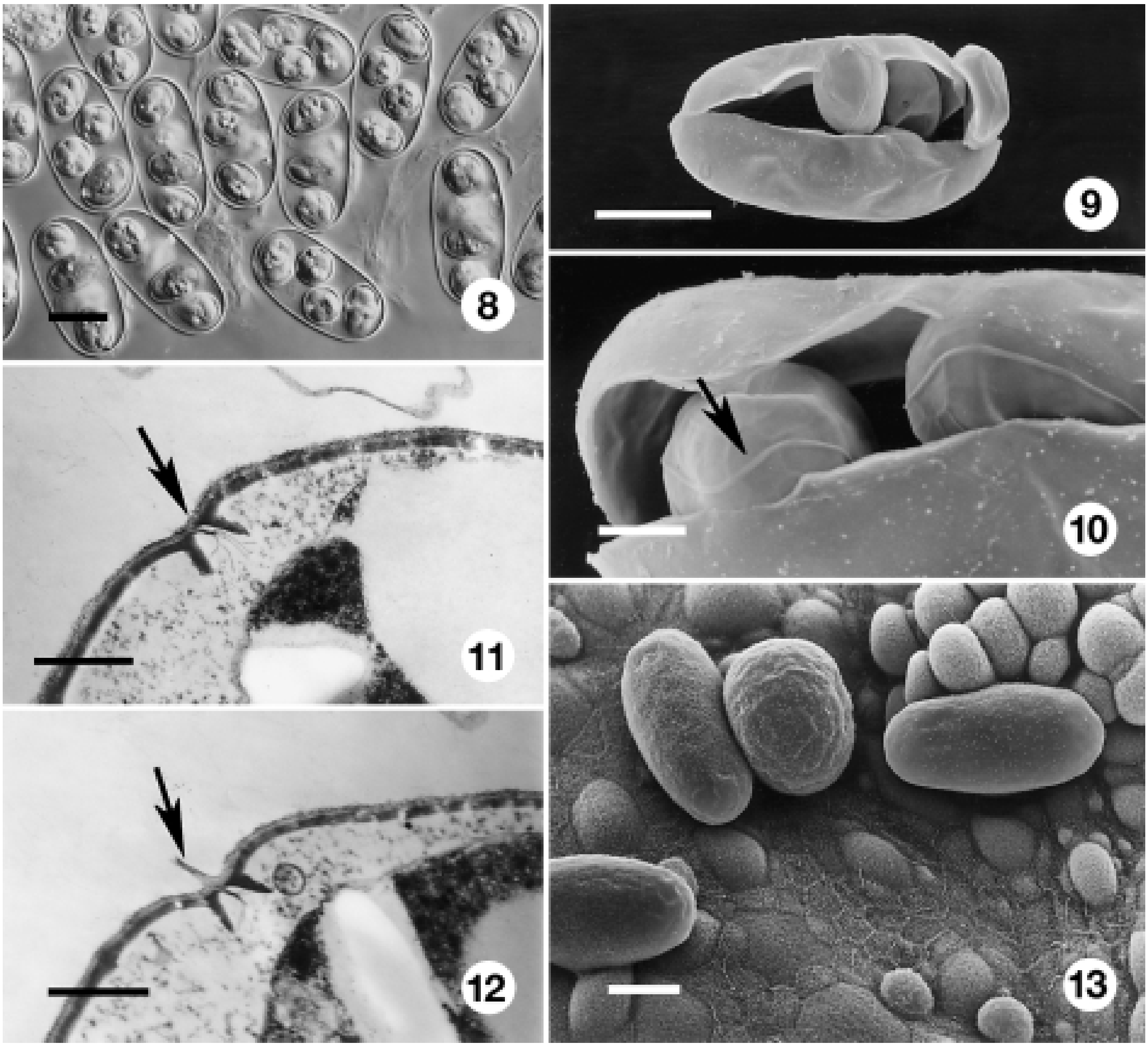


Figures 1–7. Morphology of *Goussia janae.* **1.** Nomarski interference contrast optics; **2–3.** Scanning electron microscopy; bar = 1 µm; **4–7.** Transmission electron microscopy). **1.** Sporulated oocysts in fresh preparation with four dizoic sporocysts without the Stieda body being released from an oocyst, after its fragile wall ruptured. Bar = 5 µm. **2.** Sporocyst lacking the pellicle; note the distinct longitudinal suture. **3.** Sporocyst with the pellicle obscuring the suture. **4.** Mature sporozoite within a sporocyst. The arrow points to the suture in the sporocyst wall. Bar = 1 µm. **5**. Section through the sporocyst wall. Note the meridional suture indicated by an arrow. Bar = 0.5 µm. **6**. Transverse section through the sporocyst wall extended at the suture. Note the tiny membranaceous veil indicated by arrows. Bar = 0.1 µm. **7.** Sporont in a monopodial extracytoplasmic location in the intestinal epithelium. Note the numerous amylopectin granules. Bar = 5 µm.

posed by a fine filament (Fig. 11). In some cases, a protruding plate from the suture, resembling the veil of *Goussia*, was observed in transmission electron micrographs (Fig. 12), but not in scanning electron micrographs (Figs 9 and 10). In contrast to the membranaceous oocyst wall of *G. janae* (Figs 1, 4 and 5), the oocyst wall of *Choleoeimeria* sp. 1 is thick, preserving the shape of the oocyst as observed by scanning electron microscopy (Fig. 9).

Morphology of Endogenous Stages

The endogenous development of *G. janae* occurs in the epithelial cells of the fish intestine. All merogonial, gamogonial and early sporogonial stages are localized in the enterocytes between the cell membrane and the cytoplasm in an intracellular and extracytoplasmic position (Fig. 7). Most merogonial stages are attached to the host cell cytoplasm at a



Figures 8–13. Morphology of *Choleoeimeria* sp. 1 (**8–10**) and *Choleoeimeria* sp. 2 (**11–13**). (**8.** Nomarski interference contrast optics; **9, 10, 13.** Scanning electron microscopy; **11, 12.** Transmission electron microscopy). **8.** Sporulated oocysts in fresh preparation showing typically elongated tetrasporocystic oocysts and dizoic sporocysts without the Stieda body. Bar = 10 µm. **9.** Artificially ruptured oocyst with extruding sporocysts. Note the suture in the sporocyst wall. Bar = 10 µm. **10.** Detail of two sporocysts protruding from a ruptured oocyst. The arrow points to the suture in the sporocyst wall. Bar = 2 µm. **11.** Transverse section through the suture (arrow) of the sporocyst wall; note the presence of fine filaments interposed between the suture plates. Bar = 0.5 µm. **12.** Transverse section through sporocyst of the same species showing the suture with a protruding plate (arrow). Bar = 0.5 µm. **13.** Part of the infected biliary epithelium. Note the extracytoplasmic localization of the coccidium. Bar = 10 µm.

single large area in a so-called “monopodial” position; however, stages in a “spider-like” formation (Lukeš 1992), connected with the host cell cytoplasm by two or more projections, were also observed (data not shown).

The endogenous development of both studied *Choleoeimeria* spp. was confined solely to the gall bladder epithelium. The infected host cells were hypertrophied and displaced from the epithelial layer to above the surface (Fig. 13). The attachment area between the parasitized cell and surrounding non-infected cells of the epithelial layer became reduced to a narrow peduncule. The general morphology of observed stages resembled that described for

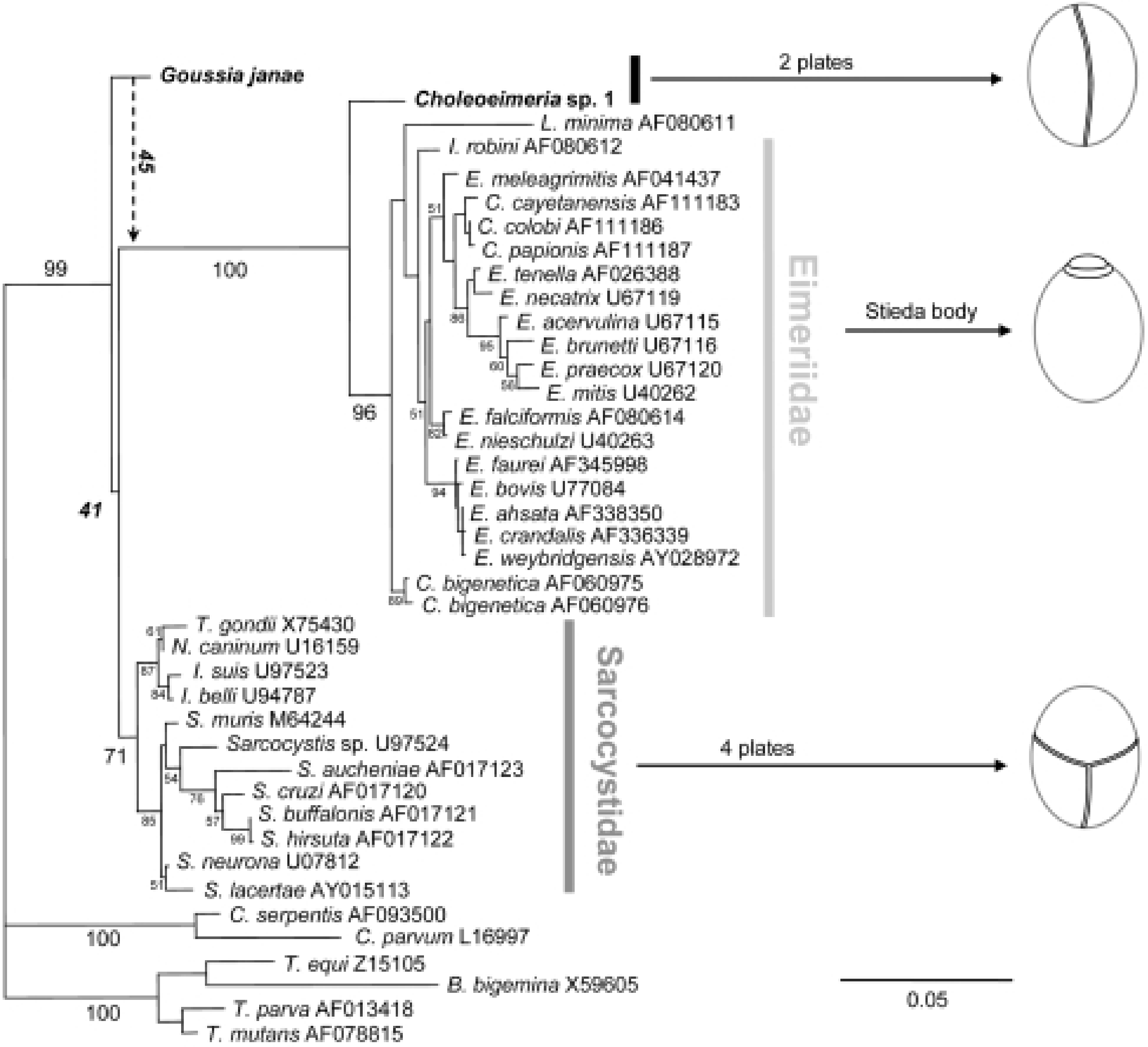


Figure 14. Maximum likelihood tree that includes the partial SSU rDNAs of *Choleoeimeria* sp. 1 and *Goussia janae*. The model of TrN+I+Γ was considered and analyzed using a heuristic search with NNI branch swapping (–*ln* = 4452.9). Values indicated on the branches represent bootstrap support (*n* = 300). The scale for substitution/site is given under the tree. Mutual position of *G. janae* and its bootstrap support is indicated by the broken arrow. Schematic drawings of the excystation structures of sporocysts and taxonomic classification are shown on the right.

Choleoeimeria rochalimai (Paperna and Lainson 2000).

Phylogenetic Analysis

We have obtained the complete SSU rDNA sequence of *G. janae* (1756 bp) and the partial SSU rDNA sequence of *Choleoeimeria* sp. 1 (1215 bp), which was lacking the 5′ end.

We began the analysis with a subset containing *Goussia*, *Choleoeimeria*, 21 representative eimeriids and 12 representative sarcosporidians. *Cryptosporidium* spp., three *Theileria* spp. and *Babesia bigemina* were used as outgroups. This alignment

Table 1. Likelihood ratio test for constrained trees using apicomplexan alignment of partial SSU rDNA with *Goussia janae* and *Choleoeimeria* sp.

	Tree	–*ln L*	Difference –*ln L*	KH test	SH test
				P	
1	((E), G, C,(S))	5466.15300	135.23883	0.000*	0.000*
2	((E), (G, C), (S))	5466.15300	135.23883	0.000*	0.000*
3	**(((E), C), G, (S))**	5332.67078	1.75662	0.485	0.871
4	**(((E), C), (G, (S)))**	5332.67079	1.75662	0.485	0.871
5	(((E), G, C),(S))	5465.65887	134.74470	0.000*	0.000*
6	((E, C, G), (S))	5514.13981	183.22564	0.000*	0.000*
7	**(((E), C), S, G)**	5344.91682	14.00265	0.062	0.417
8	**((((E), C), S), G)**	5335.41366	4.49949	0.186	0.751
9	(((E, C), S), G)	5343.63101	12.71684	0.033*	0.425
10	**(((E, C), (S)), G)**	5340.28070	9.36653	0.074	0.520
11	**((((E), C), (S)), G)**	5330.91417	Best		

Kishino-Hasegawa (SH) and Shimodaira-Hasegawa (SH) tests performed in PAUP*: KH test using RELL bootstrap, two-tailed test, SH test using RELL bootstrap (one-tailed test). * $P < 0.05$, suggests that the constrains are significantly different. Number of bootstrap replicates = 1000. Abbreviations: E, Eimeriidae; S, Sarcocystidae; C, *Choleoeimeria*; G, *Goussia janae*

Table 2. Likelihood ratio test for constrained trees using apicomplexan alignment of complete SSU rDNA with *Goussia janae*.

	Tree	–*ln L*	Difference –*ln L*	KH test	SH test
				P	
1–3	**((E), G, (S))**	7067.00712	0.49137	0.652	0.849
4	**((E), (G, (S)))**	7067.00712	0.49137	0.652	0.849
5	**(((E), G),(S))**	7066.93012	0.41438	0.741	0.868
6	**((E, G), (S))**	7289.50691	222.99116	0.000*	0.000*
7	((E), S, G)	7130.91114	64.39539	0.002*	0.022*
8–9	(((E), S), G)	7102.16356	35.64782	0.003*	0.098
10–11	**(((E), (S)), G)**	7066.51574	Best		

See Table 1 for further details.

(alignment I) was trimmed according to the *Choleoeimeria* SSU rDNA sequence. ModelTest supported the $TrN+I+\Gamma$ model of nucleotide evolution. The best tree returned by ML (–*ln* = 4452.9) analysis supported *G. janae* as a sister taxon to the families Eimeriidae and Sarcocystidae. In the same tree, *Choleoeimeria* sp. 1 constituted a sister species to the Eimeriidae, while the Sarcocystidae and Eimeriidae formed well-supported monophyletic groups (Fig. 14). The ML bootstrap strongly favours (99%) the inclusion of both studied species into the coccidia clade (*n* = 300). Within this group, however, the relationship of *G. janae* with each family was not completely resolved. Its affiliation with the Eimeriidae was as poorly supported (45–%) as its position as a sister taxon to both the Sarcocystidae and Eimeriidae (41%) (Fig. 14). Setting the transversion/transition ratio in MP to 3:1, *Goussia* appeared as a sister taxon either to Eimeriidae and Sarcocystidae, or to Eimeriidae only, but the bootstrap support for both alternatives was very low (36% and 37%, respectively) (*n* = 1000). In the best ML tree, a close relationship between *Choleoeimeria* sp. 1 and eimeriids was unequivocal (100% bootstrap) and, at the same time, monophyly of both families remained strongly supported (Eimeriidae, 96%; Sarcocystidae, 71%). Trees returned by ME using GTR + Γ ($\alpha = 0.5$) and MP analysis were highly congruent with the ML tree, minor differences being confined to the internal branches within the Eimeriidae and Sarcocystidae. The rooting position of *Choleoeimeria* sp. 1 as a sister species to eimeriids was consistently supported, while the position of *G. janae* remained unresolved. The LTR, Kishino-

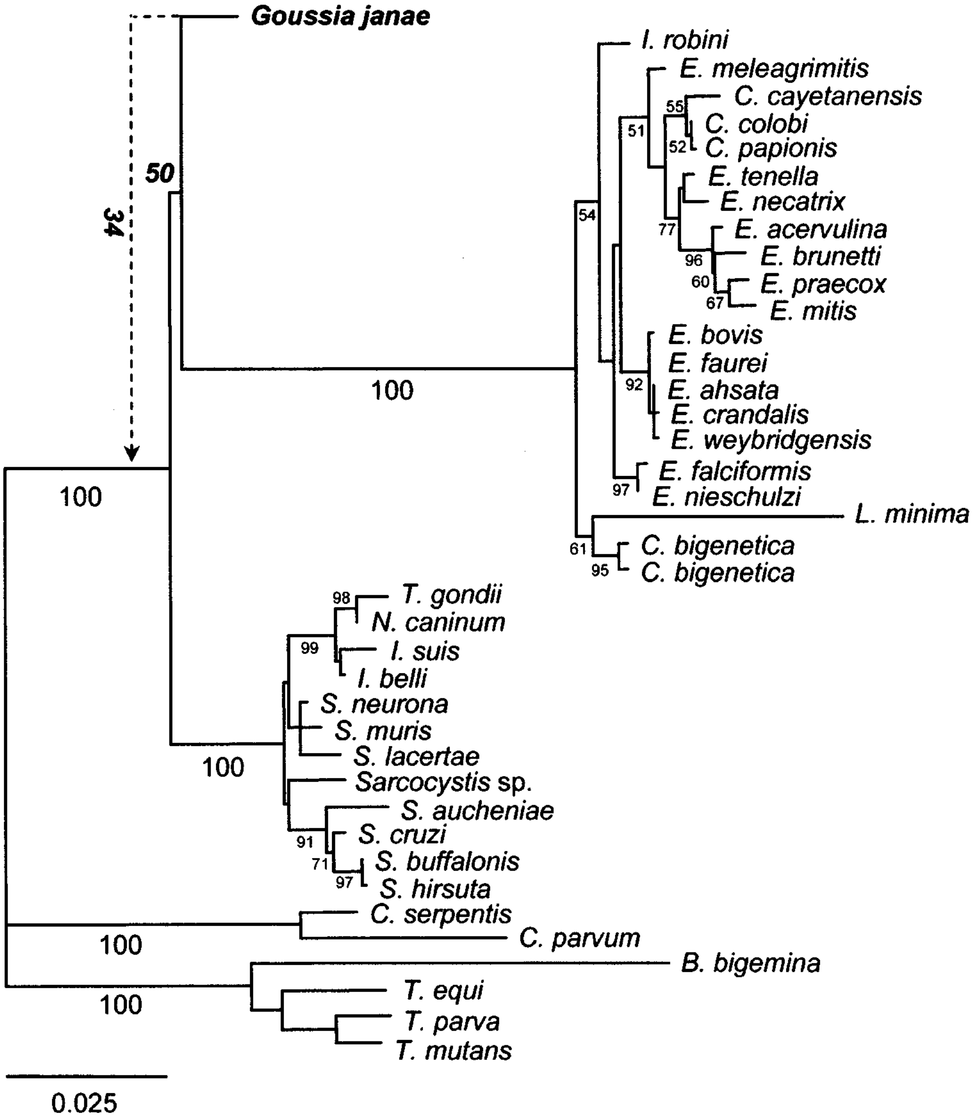


Figure 15. Maximum likelihood tree that includes the complete SSU rDNA of *Goussia janae*. The model of TrN+I+Γ was considered and analyzed using heuristic search with the NNI branch swapping (–*ln* = 7853.2). Values indicated on the branches represent bootstrap support (*n* = 300). The scale for substitution/site is given under the tree. Mutual position of *G. janae* and its bootstrap support is indicated by the broken arrow. In comparison with Fig. 14, note the increased support for the monophyly of Sarcocystidae, but low values for alternative branching of *G. janae*.

Hasegawa and Shimodaira-Hasegawa tests, designed to resolve the positions of taxa, were performed using 11 different tree topologies (Table 1). Monophyly of both families withstood the deletion of *Choleoeimeria* and/or *Goussia*, and the use of different outgroups (*Cryptosporidium* spp. and/or haemosporidia) did not affect the branching order and nodal support (data not shown). The results also show that the genera *Goussia* and *Choleoeimeria* are not monophyletic with each other.

In a second analysis, based on the full-length SSU rDNA sequences (alignment II) and using the same outgroups, *Choleoeimeria* sp. 1 could not have been included due to the lack of the 5' region. ModelTest supported the same model (TrN+I+Γ) as for the partial alignment. The best tree provided by ML analysis (–*ln* = 7853.2) identified *G. janae* as a sister taxon to the Eimeriidae, their monophyly being supported by 50% bootstrap (*n* = 300). Alternative rooting of *Goussia* on the base of the entire coccidian clade, garnered weak support (34%) (Fig. 15). In this analysis, the Sarcocystidae and Eimeriidae again formed strongly supported (99% and 100% bootstraps, respectively) monophyletic clades (Fig. 15). As with the previous data set, ME using GTR+Γ (α = 0.5) and MP showed trees almost identical with those yielded by the ML analysis, the branching order slightly altered only within the Eimeriidae and Sarcocystidae clades. ME supported early branching of *Goussia*, although MP (even when transversions were treated 3 times transitions) preferred its monophyly with eimeriids. As an outcome of the LTR, Kishino-Hasegawa and Shimodaira-Hasegawa tests, performed on the same tree topologies as with alignment I, any unequivocal affinities of *G. janae* with major clades were rejected (Table 2).

Conservative evaluation of the obtained results, supported by all performed analyses, holds that *Choleoeimeria* is a sister taxon to Eimeriidae, while *Goussia* branched off at the root of the coccidian clade. Moreover, due to the lack of resolution and limiting sampling, we conservatively regard *G. janae* to form a branch in the basal trichotomy, together with clearly monophyletic Eimeriidae and Sarcocystidae.

Discussion

In a first attempt to shed light on the molecular phylogeny of coccidians of cold blooded vertebrates, we have selected *Goussia janae* and *Choleoeimeria* sp. 1, parasites of fish and reptiles, respectively. In the case of *G. janae*, we have obtained infected type hosts from the type locality (Lukeš and Dyková 1990), and by light and electron microscopy determined features characteristic for the genus and species (Figs 1–7). Because *G. janae* is an extracytoplasmic coccidium, its developmental stages are found in the microvillous region of the epithelial cells. Similar localization in the epithelium of poikilotherms has been described for all members of the genera *Epieimeria*, *Choleoeimeria, Acroeimeria*, and some *Goussia* spp. and *Eimeria* spp. (Dyková and Lom 1981; Lukeš 1992; Molnár and Baska 1986; Paperna 1995; Paperna and Lainson 1999a; 2000). While *G. janae* is typically extracytoplasmic, communicating with the host cell cytoplasm by single or multiple stems, the displacement of stages of *Choleoeimeria* spp. involves the entire host cell (including its nucleus), which remains fixed to the epithelium by a narrow peduncule. This important difference leads us to assume that the displacement of the endogenous stages of several *Goussia* and all *Choleoeimeria* species to the surface of the infected epithelium, a feature rather common among coccidians of poikilotherms, may represent a homoplasy rather than plesiomorphy.

While the complete SSU rDNA gene of *G. janae* was easily obtained, the situation was less straightforward with *Choleoeimeria* sp. 1. We were able to clone only a partial SSU rDNA sequence, which fortunately contains enough information. Importantly, general structure of trees based either on the partial or the complete SSU rDNA sequences was almost identical. Presented molecular phylogenetic analysis placed *Choleoeimeria* sp. 1 as a sister taxon to the Eimeriidae, and *G. janae* as a branch that forms a trichotomy with the Eimeriidae and Sarcocystidae. In some tests, however, this aquatic parasite constitutes the earliest branch of the coccidian clade (excluding *Cryptosporidium*). The uncertainty with the placement of *Goussia* was not removed by testing different outgroups. The alignment of both SSU rDNA sequences was not easy due to their significant divergence from available coccidian homologues. Therefore, we have based our analysis on rather conservative alignments, from which all gaps and poorly alignable regions were omitted (Hickson et al. 2000; Morrison and Ellis 1997; Swofford et al. 1996). Although only partial SSU rDNA of *Choleoeimeria* sp. 1 is available, its position on the root of the Eimeriidae is highly supported. Regrettably, the phylogenetic relationships of *G. janae* remain less clear, since neither of the two alignments used unambiguously resolved the position of *Goussia* (Figs. 14 and 15; Tables 1 and 2).

The generic definitions of *Goussia* and *Choleoeimeria* are based on the presence of the tetrasporocystic oocysts containing dizoic sporocysts. However, the informativeness of the number of sporocysts/sporozoites as a key taxonomic character has its limits, as exemplified recently for the genus *Isospora* (Barta et al. 2001). The oocyst morphology is a variable feature for goussiids, while the oocysts of members of the genus *Choleoeimeria* are strikingly uniform, with a typical cylindroidal or ellipsoidal shape (Paperna and Landsberg 1989b). The thick wall, here shown in *Choleoeimeria* sp. 2, minimizes desiccation and is considered to be a relatively recent adaptation (Overstreet et al. 1984). Members of the genera *Goussia* and *Choleoeimeria* bear identical excystation structures in their sporocysts, represented by a meridional suture that divides the sporocyst wall into two identical shelves. Outside of these genera, similar excystation structures were described for the extracytoplasmic *Acroeimeria pintoi* (Paperna and Landsberg 1989b; Paperna and Lainson 1999a), and the intracytoplasmic *Eimeria boveroi* and *E. gastrosauris* (Paperna 1994; Paperna and Lainson 1999b), all parasites of geckoes. Additionally, bivalved sporocysts were also mentioned in early descriptions of some coccidians of invertebrates, e.g. *Eimeria lacazei*, *Cyclospora glomericola* and *Barroussia* spp. (Minchin 1903). However, no information on phylogenetic affinities of these coccidians is available.

The separation of biliary coccidia of reptiles from the genus *Goussia* into a separate genus *Choleoeimeria*, first proposed by Paperna and Landsberg (1989b), is justified by our data, since both species do not form a monophyletic taxon. We assume that all bivalved *Eimeria*-like coccidians from poikilotherm hosts should be reclassified into the genera *Goussia, Choleoeimeria* and/or *Acroeimeria*. Since it is impossible to properly classify species originally described without notes on the site of infection and the presence or absence of sutures and Stieda bodies, these species should be treated as *species inquirendae* or *incertae saedis* until more information is available.

The excystation structures were considered an important feature for the classification of coccidia (Box et al. 1980). Molecular data obtained so far support this notion (Fig. 14; and Carreno and Barta 1999). According to the most plausible scenario, the sporocyst suture has arisen early in coccidian evolution, substantially predating the origin of more complex excystation structures. Under naive parsimony, a simple longitudinal suture joining two identical valves in the sporocyst wall of *Goussia* and *Choleoeimeria* seem to be more primitive than the sporocyst wall composed of four plates. The latter arrangement represents the main diagnostic feature of the Sarcocystidae (Modrý et al. 2001b). This assumption is congruent with the results of our phylogenetic analysis, as well as with the presence of bivalved coccidians in invertebrates and poikilotherm vertebrates. The eimeriids solved the excystation problem by replacing the sutures with a specialized complex of the Stieda and substieda bodies.

Taken together, the phylogenetic analysis presented here supports an early branching of *Goussia* and a somewhat later emergence of *Choleoeimeria*, and the apparent paraphyly of the abandoned family Barrouxiidae *sensu* Levine (1983) (Fig. 14). The data are robust enough to support the conclusion that these parasites are distantly related to eimeriids (family Eimeriidae) and sarcosporidians (family Sarcocystidae), and may contain ancestral features. However, their affiliation remains unsettled, since more sequence data are needed for classification of both genera on the family level. *G. janae*, a causative agent of heavy infections in fish that can be obtained in large quantities, may qualify as a key model organism for understanding the evolution of coccidia. It was proposed that the frequent extraintestinal localization documented for many *Goussia* species is a primitive trait (Desser 1981).

It should be mentioned that the tree obtained with our data-set may be prone to artifacts due to insufficient sampling. Braking the long branch of *G. janae* by the inclusion of other members of the widely occurring and economically significant parasites of the genus *Goussia* would likely improve the resolution. Although the SSU rDNA seems to be a good molecular marker, the addition of other markers, preferably protein-coding sequences (Mugridge et al. 2000; Zhu et al. 2000) would help to resolve some uncertainties. Despite large improvement of taxon sampling over the last few years, the quest for proper classification of the bivalved coccidians is still in its infancy, and should be resumed by the inclusion of representatives of the so far neglected genera *Epieimeria*, *Acroeimeria, Aggregata, Calyptospora* and *Barroussia*.

The available data clearly placed *Goussia* and *Choleoeimeria* out of the families Eimeriidae and Sarcocystidae. Since at present, both families are not properly defined, we propose the following emendations:

Eimeriidae Minchin, 1903

Number of sporocysts and sporozoites variable, sporocysts excyst by dissolving the complex of Stieda and substieda bodies; monoxenous or with more complex development.

Sarcocystidae Poche, 1913

Bisporocystic oocysts and tetrazoic sporocysts; sporocyst wall composed of four plates joined by sutures; monoxenous or with more complex development.

Methods

Organisms: *Goussia janae* was isolated from twelve adult specimens of the dace, *Leuciscus leuciscus* (Linnaeus 1758) (Osteichthyes: Cyprinidae), caught in April 2000 in the Černovický brook, Soběslav, Czech Republic. *Choleoeimeria* sp. 1 was isolated from the gall bladder of the diadem snake, *Spalerosophis diadema* (Schlegel 1837) (Serpentes: Colubridae), imported from Jordan in 2000. *Choleoeimeria* sp. 2 parasitized the gall bladder of a horned tree lizard, *Acanthosaura crucigera* Boulenger, 1885 (Sauria: Agamidae) originating from Thailand. The only *Choleoeimeria* from which DNA was available for molecular analysis is *Choleoeimeria* sp. 1 from *S. diadema*.

Light and electron microscopy: Oocysts of *G. janae* were isolated from the feces of *L. leuciscus* and temporarily kept in an aquarium until they completed sporulation. Oocysts of both isolates of *Choleoeimeria* spp. were obtained directly from the gall bladder after dissection of euthanised hosts. Oocysts were examined, measured and photographed after flotation in Sheather's sugar solution at specific gravity 1.3, using an Olympus BX 60 microscope equipped with the Nomarski interference contrast optics. For transmission and scanning electron microscopy, small pieces of infected tissue and oocyst suspension were processed according to the protocols described elsewhere (Lukeš 1992; Šlapeta et al. 2001). The samples were examined with JEOL 1010 and JEOL JSM 6300 transmission and scanning electron microscopes, respectively.

DNA extraction, PCR amplification and sequencing: Total cellular DNA was isolated from tissues infected with merogonial and gamogonial stages by 2% sarcosyl (Sigma) and 0.5 mg/ml pronase E (Sigma), phenol/chloroform extraction and ethanol precipitation as described elsewhere (Maslov et al. 1996). PCR amplification, cloning and sequencing of the SSU rDNA gene of *G. janae* and *Choleoeimeria* sp. 1 were performed as described previously (Modrý et al. 2001b). The complete SSU rDNA of *G. janae* was amplified using the universal eukaryotic primers ERIB1 and ERIB10 (Barta et al. 1997), while these primers did not give any amplicon of expected size with the DNA of *Choleoeimeria* sp. 1. However, partial sequence of its SSU rDNA was amplified with the primers GJ-574 (5′- GCA AGT CTG GTG CCA GC -3′) and ERIB10. GJ-574 anneals to a region located approximately 500 bp from the 5′ end of the coccidian SSU rDNA gene. Three clones were completely sequenced on both strands using ABI or Beckman automatic sequencers. Nucleotide sequences obtained in this study were deposited in the GenBank under the accession numbers AY043206 (*Goussia janae*) and AY043207 (*Choleoeimeria* sp. 1).

Phylogenetic analysis: Forty-one almost complete SSU rDNA sequences were aligned using the multiple aligning program Clustal X version 1.81 (Thompson et al. 1997). The alignment was performed by profile alignment to sequences already aligned by secondary structure from the SSU rRNA database (Van de Peer et al. 2000), using a range of multiple alignment gap open- and gap-extension penalties for the optimization of gap introduction.

Two individual alignments were produced; (i) partial SSU rDNA spanning the region available for *Choleoeimeria* sp. 1 (alignment I; alignment residue summary: total 1351, excluded 358, included 993 out of which 282 are variable); and (ii) complete SSU rDNA from which *Choleoeimeria* sp. 1 was excluded (alignment II; alignment residue summary: total 2083, excluded 645, included 1438 out of which 396 are variable). Alignments have been analyzed by the program PAUP*4.0b8 (Swofford 2001) using heuristic search with NNI branch swapping. To control for alignment artifacts, we analyzed a conservative alignment where all gapped positions and positions in highly variable regions were excluded according to the user-set residue ranges in PAUP*. Maximum parsimony (MP), maximum likelihood (ML) and minimum evolution (ME) were performed. To further explore the effect of the outgroup on the ingroup taxa, a large data set of *Cryptosporidium* spp. and/or haemosporidians was used (data not shown). Because no effect on the tree was detected, two *Cryptosporidium* spp. and four haemosporidians were selected as the outgroup. The $GTR+\Gamma$ ($\alpha = 0.5$) was assumed for ME. For ML, the hierarchial likelihood test of ModelTest version 3.06 (Posada and Crandall 1998) to optimize the likelihood search was applied. The likelihood ratio tests (LRT), Kishino-Hasegawa and Shimodaira-Hasegawa tests were performed using different user-defined topologies. Alignments in the Nexus format are available on request or at ftp.vfu.cz/slapeta/alignments/poikil.

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