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Research paper

Coccidian parasites of fish encompass profound phylogenetic diversity and gave rise to each of the major parasitic groups in terrestrial vertebrates

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ABSTRACT

Fish are the oldest and most diverse group of vertebrates; it therefore stands to reason that fish may have been the original hosts for many types of extant vertebrate parasites. Here, we sought to determine whether coccidian parasites of fish are especially diverse. We therefore sampled such parasites from thirty-nine species of fish and tested phylogenetic hypotheses concerning their relationships, using 18S rDNA. We found compelling phylogenetic support for distinctions among at least four lineages of piscine parasites presently ascribed to the genus *Goussia*. Some, but not all parasites attributed to *Eimeria* were confirmed as such. Major taxonomic revisions are likely justified for these parasites of fish, which appear to have given rise to each of the major lineages of coccidian parasites that subsequently proliferated in terrestrial vertebrates, including those such as *Toxoplasma gondii* that form tissue cysts in intermediate hosts.

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1. Introduction

Fish are the oldest and most diverse group of vertebrates; it therefore stands to reason that fish may have been the original hosts for many types of extant vertebrate parasites. Did the parasites of terrestrial mammals, birds, amphibians, and reptiles descend from parasites of fish? Here, we explore this question as it relates to a diverse and important group of Apicomplexan parasites (the coccidia), ubiquitous among tetrapods but less well characterized in fish.

Coccidia are intracellular parasitic eukaryotes encompassing (at least) two clearly differentiated lineages. Species of the first type complete their 'simple' lifecycle in the gastrointestinal tract of a single host that host ingests the infectious stage (oocysts), supports parasite asexual and sexual reproduction, and then excretes oocysts to begin the cycle anew.

Oocysts of other coccidians are excreted only by carnivores (which acquire infection by eating tissues in which the parasite has encysted). For such "tissue-cyst" forming coccidians, sexual development occurs in the carnivorous "definitive host." Species of *Sarcocystis*, and the agent of human toxoplasmosis exemplify this group. Phylogenetic data suggest that coccidians characterized by the "simple" lifecycle most likely arose in fish (Molnár et al., 2012). The origins of "tissue-cyst" coccidians have not, to our knowledge, been sufficiently explored. If tissue cyst-forming coccidians arose and persist in piscine hosts, they may pose risk to those who consume fish raw, such as in sashimi or in ceviche.

The physical structures of oocysts and sporocysts that have traditionally been used to diagnose the coccidia of terrestrial vertebrates are difficult to study in parasites of aquatic hosts; protection against desiccation is not required for oocysts or sporocysts of piscine coccidians, which are therefore enveloped by only a thin, single layered membrane (Lom and Dyková, 1992; Molnár, 2006). Most fish coccidia sporulate prior to being excreted, further limiting the ability to discern differences among their physical characteristics.

Diverse parasitic forms occur in fish, only some of which closely resemble better-studied, economically important parasites of birds, mammals, and reptiles. Some piscine coccidia, for instance, clearly belong to the genus *Eimeria* (established by Schneider in 1875). Oocysts of *Eimeria* harbour four pairs of sporozoites, each pair enveloped by a sporocyst wall. Egress from the sporocyst occurs through a Stieda body. The parasites distinguished by this apical plug (whether in fish or other types of vertebrates) descended from a unique, common ancestor (Molnár et al., 2012). Fish may have been the first vertebrates to be parasitized by *Eimeria*, given how evolutionarily diverse these parasites are in even the modest available sample of such hosts. "Fish origins" would be further substantiated if the *Eimeria* of fish could be shown to have descended from a broader, and even more diverse, assemblage of other piscine coccidia.

About half of the known piscine coccidians lack the Stieda body characteristic of Eimeria; instead, their sporocyst walls consist of two

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equally sized valves adhering to each other at a suture in the longitudinal plane. Their sporozoites are released when the two valves separate. Labbe (1896) regarded differences in the structure of the sporocysts as diagnostic for a distinct genus, *Goussia*. In addition to *Goussia*, Labbe (1896) created another genus, *Crystallospora*, for parasites characterized by bivalved sporocysts but which were also pyramidally hexagonal. Some taxonomists accepted Labbé's classification (Léger and Hesse, 1919; Stankovitch, 1920, Levine, 1983). So distinct did Levine (1983) deems *Goussia* that he recommended transferring the genus *Goussia* from Eimeriidae to Barouxidae. Others, however, regarded *Goussia* and *Crystallospora* as synonyms of *Eimeria* (Doflein, 1909; Grassé, 1953; Reichenow, 1953; Pellérdy, 1974. Pellérdy (1964) endorsed subdividing the genus *Eimeria*. Dyková and Lom (1981), focusing on aspects of parasite life cycles and pathogenicity, revitalized *Goussia* and *Crystallospora* and created a new genus, *Epieimeria* for species developing in an epicellular position of the intestinal epithelium. Though the revitalization of the genus *Goussia* has this historical basis, Upton (2012) deemed *Goussia* to be a synonym of *Eimeria* in his taxonomical review of the suborder Eimeriorina. Thus, parasites employing this strategy of sporozoite egress have alternatively been ascribed to *Eimeria* (Upton, 2012), *Goussia*, *Crystallospora*, *Choleoeimeria*, and *Acroeimeria* (Paperna and Landsberg, 1989) and the taxonomy of this enigmatic group has remained controversial for almost 140 years.

Although the vast majority of species ascribed to *Goussia* infect fish, certain others have been described from tadpoles (*Goussia neglecta* Nöller, 1920 and *Goussia noelleri* Jirku et al., 2009). These correspond morphologically to *Goussia carpelli*, a parasite of the common carp. Detailed histological characterization of these enigmatic parasites is warranted.

Only a few representatives of fish *Goussia* (*G. janae*, *G. metchnikovi*, *G. kuehae*, *G. ameliae*) and three species from tadpoles (*G. neglecta*, *G. noelleri* and an unnamed *Goussia* sp.) have yet been included in molecular phylogenies of the coccidia (Jirku et al., 2002, 2009; Gibson-Kueh et al., 2011a, 2011b; Székely et al., 2013; Lovy and Friend, 2015), in spite of the fact that such Apicomplexan protozoans are prevalent in both freshwater and marine fishes (recently reviewed by Molnár, 2005, 2006 and by Steinhagen and Davies, 2008). Although these sequences are dissimilar from those previously described from piscine *Eimeria*, sampling has not yet sufficed to determine whether other types of *Goussia* belong to this same lineage. Molecular data have also been acquired from three species of *Calyptospora* (*C. funduli*, *C. serrasalmi* and *C. spinosa*) (Whipps et al., 2012).

There are reasons to suspect that the myriad species ascribed to *Goussia* may not all share a close relationship. A wide diversity of developmental strategies has been described for such parasites. For example, although certain species complete their development within only weeks, others develop according to an annual cycle, disseminating only in the spring. Similarly, although certain ones infect the gut dispersedly, other species develop focally in nodules. Finally, although some species develop in the cytoplasm or nuclei of enterocytes, others develop in epicellular locations (i.e. in endocellular but ectocytoplasmal environments). Several other species develop in non-endothelial sites in the liver, spleen, kidney and the peritoneum. Genetic data provide a means to evaluate which, if any, of these developmental characteristics demarcate groups of parasites sharing specific evolutionary histories. A few studies have begun to evaluate phylogeny using 18S rDNA (Molnár et al., 2012; Lovy and Friend, 2015; Bartošová-Sojková et al., 2015) and one has used a transcriptomic approach to evaluate the position of an epicellular species of *Goussia* using several single-copy housekeeping genes (Dogga et al., 2015). The latter found that genes of *G. janae* typically were inferred as basal to the sarcocystids or as basal to the whole eimeriorinid clade; less frequently, a gene from this epicellular parasite was placed at the base of the Eimeriid subclade or as within the sarcocystid subclade (Dogga et al., 2015).

Here, we investigated the genetic diversity in a sample of parasites ascribed to *Goussia* which, though sharing common sporocyst morphology, nonetheless exhibit a diversity of developmental attributes. In general, we wished to understand their relationships to each other, and to the coccidia infecting other vertebrate groups. More specifically, we sought to determine whether or not the marginal suture, which serves as the principal diagnostic criterion for species of *Goussia*, demarcates only one, monophyletic assemblage of species. We also sought to understand whether shared ancestry was evident among parasites sharing a common developmental schedule, tissue localization, and/or morphology. We were particularly interested to determine whether any piscine parasites are specifically related to tissue-cyst forming coccidians. In total, we wanted to better understand how extensive the evolutionary diversity among these piscine parasites might be, and whether excessive diversity might substantiate the notion that they collectively comprise an especially old and diversified biological assemblage from which coccidian parasites of terrestrial vertebrates subsequently evolved.

2. Materials and methods

During the course of regular surveys of the parasite fauna of Hungarian waters, several hundred fish were examined; coccidian infections observed in 174 of those were preserved in 70% ethanol in hopes of deriving DNA that could be used for sequencing. Of these, suitable DNA was obtained from 86 specimens (as described below). We retained for this report all resulting sequences that produced clean, unambiguous reads. We suspect mixed infections as a source of ambiguity in other, excluded sequence reads. In the end, fish of thirty-nine species (Table 1) were collected by seine or by using an electric fishery device and transported live to the laboratory in oxygenated plastic bags and kept in aerated aquaria for 2 to 3 days before examination. These specimens derived from Hungarian rivers, lakes, and fish farms as denoted in Table 2.

After passing most of its faeces, each fish was anaesthetized by clove oil and sacrificed by decapitation, and its gut opened. Mucus covering the epithelium, and scrapings of the epithelium, were placed on a slide and examined under a cover slip at 100–200 times magnification with a Zeiss compound microscope. The occurrence of oocysts in inner organs was determined by compressing a piece of the organ between two glass plates or, when possible, under a coverslip. When sporulated oocysts were found, microscopic pictures were prepared by an Olympus microscope equipped with Nomarski interference contrast equipment. When unsporulated oocysts were encountered, their subsequent development (24, 48, and 72 h later) was observed by placing them in a small Petri dish with water containing a loop of penicillin and streptomycin, to prevent the excess growth of bacteria. In other cases, the mucus was placed in a small petri dish, covered by a fine-meshed sieve under tap water, and observed until the sporocysts matured. Photos and hand drawings were prepared of the sporulated oocysts and their physical dimensions recorded. Once identified morphologically (examples depicted in Fig. 1), the remaining oocysts were stored in 70% ethanol for subsequent genetic characterization. Of the coccidians found during the surveys and illustrated in Table 1, we attempted to genetically characterize only those where oocysts were not obviously mixed with those of other *Goussia* and *Eimeria* spp. and were relatively free from mucus and other cell elements. Dispersed developing species derived mostly from mucus or from the intensively infected part of the gut epithelium; by contrast, oocysts and sporogonic stages of the nodular species were selected from the center of nodules, while epicellular *Goussia* oocysts were obtained from fish specimens lacking a concurrent infection with the nodular species. In addition, an isolate of *C. funduli*, originating from the Mississippi coast of the Gulf of Mexico, was provided by Dr. Robin Overstreet, Table 2.

DNA was subsequently extracted from frozen or fixed specimens using DNeasy columns per manufacturer's recommendations (Qiagen Corp.). Portions of the small subunit of nuclear rDNA were amplified in 20 μl reactions using 2 μl of template (total DNA of varying concentrations) and 10 pmols of each primer and 0.35 U High Fidelity Platinum