Parasitology Research (2019) 118:2121–2127
https://doi.org/10.1007/s00436-019-06320-y

FISH PARASITOLOGY - ORIGINAL PAPER

First molecular data on *Eimeria truttae* from brown trout (*Salmo trutta*)

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Received: 21 February 2019 / Accepted: 9 April 2019 / Published online: 9 May 2019

Abstract

The genus *Eimeria* comprises obligate intracellular protozoan parasites belonging to the phylum Apicomplexa. Members of this genus cause enteric disease in a wide range of vertebrate hosts, including fish, reptiles, birds, and mammals. A total of 157 species of *Eimeria* that parasitize fish have been described; however, molecular information regarding these piscine parasites is scarce. In the present study, *Eimeria* oocysts were detected in 189 of 613 (30.8%) gastrointestinal tracts of brown trout (*Salmo trutta*) captured in several rivers in Galicia (NW Spain). Measurements of the sporulated oocysts, sporocysts, and other morphological characteristics enabled identification of the oocysts as *Eimeria truttae*. By molecular analysis of the small subunit ribosomal RNA (SSU-rRNA) gene, a single sequence of ~ 420 bp was obtained in 100 fish samples. After amplification of a ~ 1300-bp fragment of the same locus, two representative sequences that exhibited five nucleotide differences were obtained. Phylogenetic analysis grouped the samples within the piscine clade closest to *Eimeria nemethi* as they exhibited 96.7% similarity with this species. This study is the first to characterize *E. truttae* at the molecular level, thus helping to clarify the phylogenetic relationships between this and other *Eimeria* species isolated from fish and contributing further to the knowledge about this protozoan parasite.

Keywords *Eimeria truttae* · Wild trout · Morphometric analysis · Molecular characterization

Introduction

The genus *Eimeria* comprises obligate intracellular protozoan parasites belonging to the phylum Apicomplexa. Members of this genus cause enteric disease in a wide range of vertebrate hosts, including fish, reptiles, birds, and mammals. These parasites complete their development in a single host species and their sporocysts can be recognized by the presence of a Stieda body, an organelle through which the sporozoites excyst. Several species cause high levels of morbidity and/or mortality in some hosts, resulting in economic losses in various animal production industries (Daugschies and Najdrowski 2005; Aarthi et al. 2010; Sharma et al. 2018).

A total of 157 species of *Eimeria* that parasitize fish have been described on the basis of the morphology of sporulated oocysts, host specificity, pathology, and geographical distribution (Belova and Krylov 2000). Although these characteristics have been used traditionally in the identification of *Eimeria* species (Duszynski and Wilber 1997), they are often insufficient for reliable differentiation between species owing to overlapping morphometric and biological features (Long et al. 1977; Zhao and Duszynski 2001). A combination of morphological and molecular analyses is therefore required in order to delimit species and to determine the phylogenetic relationships between them.

Molecular information regarding the diversity of *Eimeria* species that infect fish is scarce. Thus, only a few species of *Eimeria* isolated from different marine, estuarine, and freshwater fish have been genetically characterized: *Eimeria percae* from perch (*Perca fluviatilis*); *Eimeria anguillae* from European eel (*Anguilla anguilla*); *Eimeria variabilis* from Longspined bullhead (*Taurulus bubalis*); *Eimeria daviesae* from gudgeon (*Gobius fluviatilis*); *Eimeria rutili* from roach

Section Editor: Berit Bangoura

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(*Rutilus rutilus*); and *Eimeria nemethi* from bleak (*Alburnus alburnus*) (Molnár et al. 2012). Moreover, in a recent study on apicomplexan parasites that infect commercially valuable fish species, Xavier et al. (2018) identify *Eimeria* sp. in the intestine of European sea bass (*Dicentrarchus labrax*) and pout (*Trisopterus luscus*), which sequences are closely related to *E. variabilis* and *E. nemethi*.

The brown trout (*Salmo trutta* Linnaeus, 1758) is the most widely distributed salmonid species in the world, and outside of its native geographical range (Europe, northern Africa, and western Asia), this species has been successfully introduced into waterways on every continent except Antarctica (MacCrimmon and Marshall 1968; Cobo et al. 2015). The wild trout is indigenous to Spain, where two types of populations are found: a migratory community, with a northern distribution (Galician and Cantabrian Mountain rivers), and a sedentary population that inhabits the remaining rivers, but it is absent from several rivers in the south and east of Spain (Doadrio 2002; Sánchez Hernández 2009). Although the brown trout is not cultured for commercial purposes in Spain, it is an important angling species and is consequently of high socioeconomic status (Almodóvar and Nicola 2004).

Eimeria truttae was described by Léger and Hesse (1919) in brown trout from France. The species was later found in trout in Canada, Spain, and Russia (Molnar and Fernando 1974; Álvarez-Pellitero and González-Lanza 1983; Pugachev et al. 2010); however, molecular analysis of this species has not been undertaken. The aim of the present study was to characterize molecularly, at the small subunit ribosomal RNA (SSU-rRNA) locus, *Eimeria* isolates obtained from brown trout (*S. trutta*), which resembled morphologically to *E. truttae*, and to determine the phylogenetic relationship between this and other species of *Eimeria*.

Material and methods

Collection and processing of the samples

A total of 613 brown trout (*S. trutta*) were captured by local anglers in several rivers in Galicia (NW Spain) and processed as previously described (Couso-Pérez et al. 2019). Briefly, the gastrointestinal tracts were differentiated into the pyloric caeca and intestine. The pyloric caeca were homogenized using an Ultra-Turrax® T10 homogenizer (Ika®-Werke GmbH and Co., KG, Staufen, Germany). The intestinal contents were removed by scraping with a scalpel blade and then ground in a mortar with phosphate-buffered saline (PBS) 0.04 M pH 7.2. The homogenates thus obtained were filtered through a set of two sieves (mesh size, 150 and 45 μm) before being subjected to diphasic concentration in PBS 0.04 M pH 7.2/diethyl ether (2:1) by centrifugation at 1250×*g*, 4 °C, for 15 min. The supernatants were carefully discarded, and the concentration step was repeated until lipid-free sediments were obtained. Finally, the pellets were resuspended in 500–1000 μL of PBS and stored at − 20 °C.

Aliquots of 10 μL of the sediments were examined under bright-field microscopy in order to detect *Eimeria* oocysts (× 400 magnifications), which were confirmed by a spherical appearance, presence of four sporocysts, and a thin wall. A total of 50 oocysts from several fish specimens were observed under differential interface contrast (DIC) microscopy (× 1000 magnifications) and measured in a light microscope (AX70 Olympus Optical Co., Ltd., Tokyo, Japan) by using an ocular micrometer and DP Controller software 2.1.1.183 (©2001-2004 Olympus Optical Co., Ltd.).

Molecular characterization

Nucleic acids were extracted from 200 μL of positive samples by using the Stool DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions, and the DNA thus extracted was stored at − 20 °C until use. A hemi-nested PCR technique was used to amplify a fragment of the SSU-rRNA gene. Primers targeting a hypervariable region of the SSU-rRNA locus were designed by aligning sequences of *Eimeria* spp. available in the GenBank® database. A PCR product of ~ 550 bp was amplified in the primary PCR using the forward primer Ei18sF (5′-CCCAATGAAAACAGYTTCGAGG-3′) and the reverse primer Ei18sR (5′-AAACCCCCTACTGTCGTTCTTG-3′). The secondary PCR was conducted using the primers Ei18sF and ER10 (5′-GCCCCCAACTGTCCCTATTA-3′), which amplify a product of ~ 420 bp. The reaction mixture contained 2.0 μL template DNA or 1 μL of diluted amplicons (1:10) of the primary amplification, 0.2 μM each primer, 1 mM $MgCl_2$, 2% dimethyl sulfoxide and EmeraldAmp® MAX PCR Master Mix 2× (Takara Biotechnology, Kusatsu, Japan), in a total volume of 25 μL. The PCR conditions consisted of initial denaturation of 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. Genomic DNA of *Eimeria tenella* from naturally infected chickens and molecular grade water were used as positive and negative controls, respectively, in all PCR runs. The amplicons were electrophoresed on 2% agarose gels and stained with Real Safe (Real Laboratory S.L., Paterna, Valencia, Spain). Positive samples were also amplified following Yang et al. (2012) in order to obtain a longer fragment of the *Eimeria* SSU-rRNA gene (~ 1300 bp).

Positive PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN®, Hilden, Germany) and sequenced in both directions. The sequencing reactions were assembled using the SeqMan™ 7.0 (DNASTAR®, Madison, WI, USA) and BioEdit 7.2.3 software programs (©1997-2013 Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). The resulting sequences were compared with *Eimeria* spp. sequences deposited in GenBank® (National Institute of Health, Bethesda, MD, USA), by using the public web interface of the BLAST® 2.2.31 program (https://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information). Phylogenetic and molecular evolutionary analyses were conducted with MEGA 7 (Kumar et al. 2016), on the basis of genetic distances calculated by the two-parameter model proposed by Kimura (1980).

Data analysis

Statistical analysis was performed using Statgraphics® Centurion XVI v.16.2.04 Statistical Software (©1982-2013 StatPoint Technologies, Inc., Warrenton, VA, USA). Differences in the prevalence rates were determined by the χ^2 test. Differences were considered statistically significant at $P < 0.05$.

Results

Prevalence and morphological features of *Eimeria* oocysts

Oocysts of *Eimeria* sp. were detected in 189 of 613 gastrointestinal samples of brown trout (30.8%), at a mean intensity of 2145.9 oocysts/trout. The oocysts were found in the pyloric caeca (164 trout, 86.8%), in the intestine (9 trout, 4.8%), or in both locations (16 trout, 8.4%), observing statistically significant differences between the two locations ($P < 0.01$).

The oocysts visualized were already sporulated and showed a spherical shape (diameter, 10.3–11.8 μm) and had a thin wall. Oocysts contained four sporocysts (length, 8.1–9.2 μm; width, 4.3–5.2 μm) with a disc-like Stieda body (diameter, 2.2–3.0 μm). Each sporocyst showed two flexed sporozoites (length, 7.1–8.1 μm; width, 1.1–1.9 μm) and a granular residual body (Fig. 1). Measurements of the sporulated oocysts, sporocysts, and other morphological characteristics enabled identification of the oocysts as *E. truttae* (Table 1).

Molecular characterization of *E. truttae* from brown trout

Partial sequences (~ 420 bp) of the SSU-rRNA gene were obtained from 100 fish samples by the hemi-nested PCR, and the same sequence was observed in all samples. This representative sequence showed a similarity of 96% relative to the sequence of *E. nemethi* deposited in the GenBank® database under the accession number GU479634.

By amplification of a larger fragment (~ 1300 bp) of the SSU-rRNA gene, two representative partial sequences that exhibited five nucleotide differences and 99.6% genetic similarity between them were obtained. The phylogenetic analysis revealed that these sequences were genetically distinct and grouped within the piscine clade. The sequences showed 96.7% similarity to *E. nemethi*, 91.1% similarity to *E. percae*, 90.8% similarity to *E. variabilis*, 88.5% similarity to *E. anguillae*, 87.5% similarity to *E. rutili*, and 83.5% similarity to *E. daviesae* (GenBank® database, accession numbers GU479634, GU479663, GU479674, GU479633, GU479667, and GU479675, respectively) (Fig. 2).

Nucleotide accession numbers

Representative sequences of the *E. truttae* isolates obtained in this study have been deposited in the GenBank® database under accession numbers MK425194–MK425196.

Discussion

This study reports the first sequences of the SSU-rRNA gene of *E. truttae* from brown trout, *S. trutta*, and analyzes the phylogenetic relationships with available sequences of *Eimeria* from fish and other representative hosts.

Morphological identification of *E. truttae* was first carried out by Léger and Hesse in 1919 in brown trout from the Furon stream (France). These authors described spherical oocysts of 12-μm diameter with a thin wall and four oval sporocysts of 8 × 4 μm with flattening on a pole and no oocyst residuum.

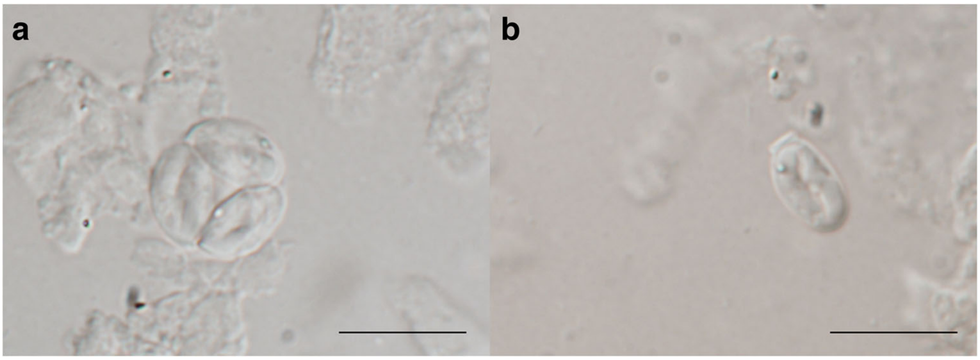

Fig. 1 Microphotographs of *E. truttae* oocysts observed in a homogenized sample of pyloric caeca from brown trout (*S. trutta*) under Nomarski differential interface contrast. **a** Sporulated oocyst. **b** Sporocyst with two sporozoites inside. Bar = 10 μm

Table 1 Comparison of morphometric (µm) data of *Eimeria* oocysts from brown trout captured in several rivers in Galicia (NW Spain) with those previously published for *E. truttae*

	Molnar and Hanek (1974)	Álvarez-Pellitero and González-Lanza (1983)	This study
Oocyst			
Mean (range)	12.8 (12.3–13.0)	11.5 (10.5–12.0)	11.2 (10.3–11.8)
SD	–	–	0.4
Sporocyst length			
Mean (range)	10.1 (9.3–11.0)	8.4 (8.0–9.0)	8.6 (8.1–9.2)
SD	–	–	0.3
Sporocyst width			
Mean (range)	6.0 (5.0–6.5)	4.6 (4.5–5.0)	4.8 (4.3–5.2)
SD	–	–	0.2
Stieda body			
Mean (range)	2.7 (2.6–2.8)	2.6 (2.4–2.8)	2.6 (2.2–3.0)
SD	–	–	0.3
Sporozoite length			
Mean (range)	8.5 (7.8–9.0)	8.1 (7.0–9.0)	7.6 (7.1–8.1)
SD	–	–	0.2
Sporozoite width			
Mean (range)	1.6 (1.3–2.0)	1.5 (1.0–1.8)	1.4 (1.1–1.9)
SD	–	–	0.2

SD standard deviation

The oocysts were located in the pyloric caeca, and sporulation was completed in the host gut (Léger and Hesse 1919). In 1974, Molnar and Hanek reported the presence of *E. truttae* oocysts in the anterior portion of the gut of brook trout (*Salvelinus fontinalis*) captured in the Matamek River (Canada) and described the morphological characteristics of a disc-like Stieda body (2.7 µm in diameter) and of the two flexed vermiform sporozoites (8.5 × 1.6 µm) contained in each sporocyst (Molnar and Hanek 1974). This coccidium was also detected by Álvarez-Pellitero and González-Lanza in specimens of wild trout from the Porma River (Spain). These authors observed spherical sporulate oocysts (diameter, 11.5 µm) with a thin wall, containing four oval sporocysts of 8.4 × 4.6 µm with a disc-like Stieda body of 2.6-µm diameter and two sporozoites of 8.1 × 1.5 µm (Álvarez-Pellitero and González-Lanza 1983).

The morphometric characteristics of the *E. truttae* oocysts identified in the present study are consistent with previously published data, although with slight variations in the measurements (see Table 1). The size of sporulated oocysts within individual species of *Eimeria* can vary, as morphometric polymorphism has been reported both within and between different hosts from several geographical locations (Duszynski 1971; Gardner and Duszynski 1990). Moreover, some members of the Eimeriidae family are known to display morphological polymorphisms (up to 40%) during the patent period, and the size of sporulated oocysts may increase during this period (Duszynski 1971). Thus, in recent studies on marsupial *Eimeria* species, in which molecular analysis was also conducted, several authors reported morphometric plasticity in *Eimeria trichosuri*, *Eimeria quokka*, and *Eimeria setonicis* (Power et al. 2009; Austen et al. 2014). Similarly, morphometric variations were observed in *Eimeria hessei* oocysts from bats (Afonso et al. 2014). Unfortunately, studies that identify morphologically and molecularly *Eimeria* from fish hosts are not available, being the present work, the first study that includes both characterizations.

Prevalence rates of 16.3% and 12.3% were reported for *E. truttae* in brown trout from Spain and Russia, respectively (Álvarez-Pellitero and González-Lanza 1983; Pugachev et al. 2010). A higher prevalence rate was observed in the present study (30.8%). Molecular analysis of the SSU-rRNA gene enabled identification of a single sequence of ~ 420 bp in 100 fish samples. After amplification of a ~ 1300-bp fragment of the same gene, two representative sequences that exhibited five nucleotide differences between them were obtained. Phylogenetic analysis grouped the samples within the piscine clade closest to *E. nemethi* and exhibiting 96.7% of similarity to this species.

The SSU-rRNA gene is a well-known marker for molecular characterization. It has been used extensively for classifying apicomplexan parasites and has become the standard target for elucidating phylogenetic relationships among this group of parasites (Morrison et al. 2004). Sequencing of the SSU-rRNA gene is currently the most extensively used molecular technique for characterizing *Eimeria* species and

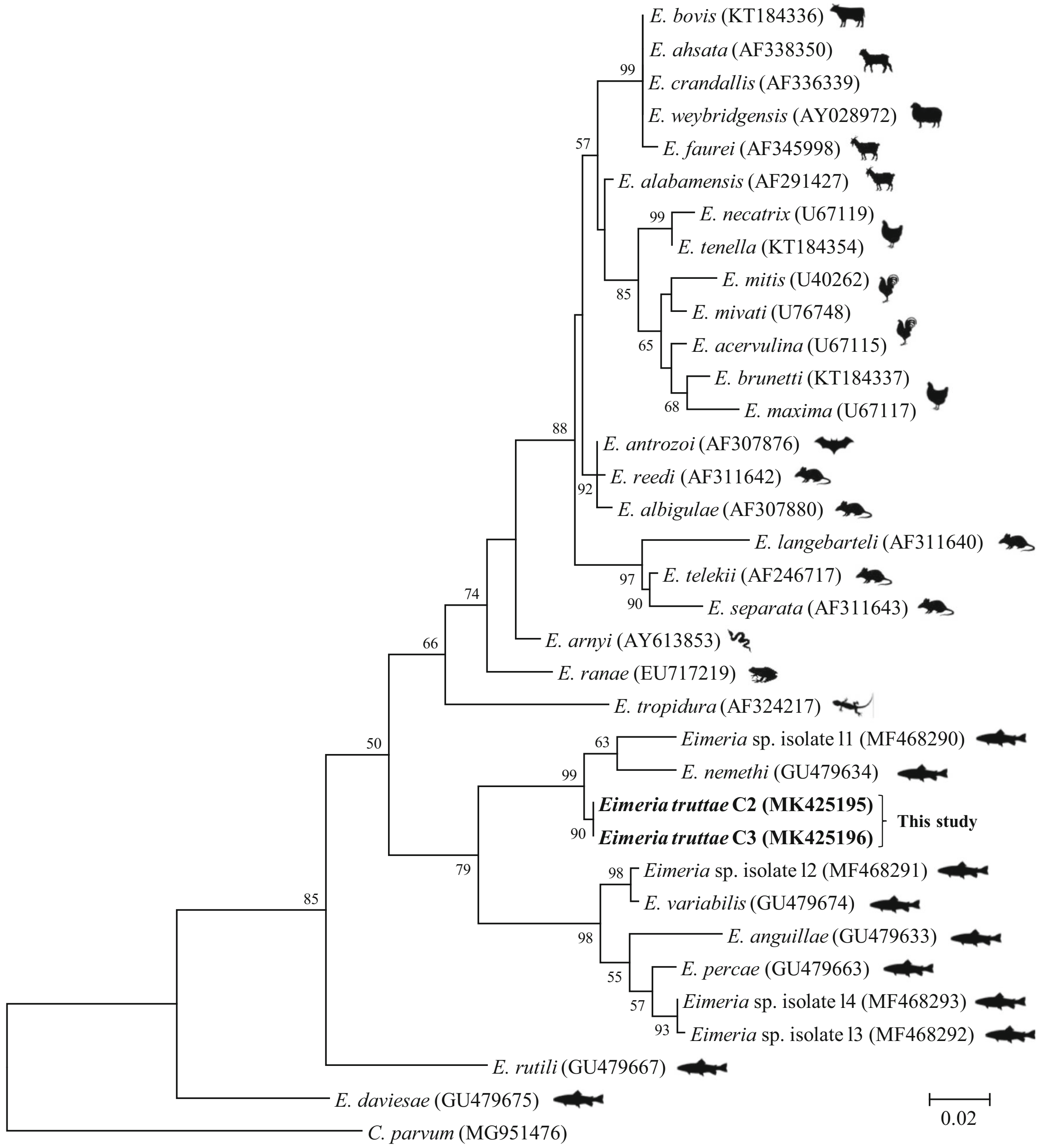

Fig. 2 Phylogenetic relationships among *E. truttae* isolates from brown trout (*S. trutta*) and other *Eimeria* species inferred by maximum likelihood analysis of the small subunit ribosomal RNA (SSU-rRNA) gene on the basis of genetic distances calculated by the Kimura 2-parameter model. The percentages of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes for distance (> 50% only). Isolates and accession numbers obtained in this study are highlighted in bold type

examining their phylogenetic relationships (Zhao et al. 2001; Power et al. 2009; Gibson-Kueh et al. 2011; Yang et al. 2012; Afonso et al. 2014; Austen et al. 2014; Sharma et al. 2018). Amplification of other less conserved loci may shed more light on the position of species of *Eimeria* that parasitize fish relative to other species of the genus. However, in the case of *Eimeria* species that infect piscine hosts, sequences for more variable genes are not available. Nonetheless, the results obtained in the present work are consistent with the phylogenetic findings reported in previous studies (Molnár et al. 2012; Rosenthal et al. 2016; Xavier et al. 2018).

Several authors have suggested that fish may have been the first vertebrates to be parasitized by *Eimeria*. Phylogenetic analysis of this genus has revealed that the parasites and their hosts co-evolved over time, indicating that the species of *Eimeria* that infect fish are ancestors of the *Eimeria* that parasitize terrestrial vertebrates (Power et al. 2009; Rosenthal et al. 2016). Previous analysis of piscine *Eimeria* species has revealed that they form a basal clade relative to the other Eimeriidae members, being clearly distinct from clades that include species of *Eimeria* that parasitize anurans, reptiles, rodents, birds, and mammals. Moreover, phylogenetic data indicate that *Eimeria* species inhabiting the posterior intestine of piscine hosts (i.e., *E. anguillae*, *E. percae*, *E. daviesae*, and *E. variabilis*) evolved separately from those occupying the pyloric caeca or extraintestinal tissues (i.e., *E. truttae*, *E. rutili*, and *E. nemethi*). Morphological differences between these two groups have also been observed. Thus, the Stieda body in intestinal *Eimeria* has a lid-like structure, whereas sporocysts of *E. truttae*, *E. rutili*, and *E. nemethi* show a disc-like Stieda body (Molnár et al. 2012). The results obtained in this study (detection of sporulated oocysts in the pyloric caeca, presence of a disc-like Stieda body, and phylogenetic position of *E. truttae*) are consistent with the previously mentioned observations on *Eimeria* species that parasitize piscine hosts.

In conclusion, the present study contributes further to the current knowledge about *Eimeria* piscine–derived species, providing the first DNA sequences of the SSU-rRNA gene for *E. truttae* from brown trout and confirming that it is closely related to *Eimeria* species that infect other fish hosts.

Acknowledgments The authors thank Dr. Emilio del Cacho from the Faculty of Veterinary Sciences of University of Zaragoza (Spain) for providing the *E. tenella* isolate.

Funding information This study was funded by the Autonomous Government of Galicia (grants GPC2014/069 and ED431C 2017/31).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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