Detection and quantification of house mouse *Eimeria* at the species level – challenges and solutions for the assessment of Coccidia in wildlife

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Abstract

Detection and quantification of coccidia in studies of wildlife can be challenging. Therefore, prevalence of coccidia is often not assessed at the parasite species level in non-livestock animals. Parasite species – specific prevalences are especially important when studying evolutionary questions in wild populations. We might expect, for example, highly prevalent parasite species to have a lower virulence than lowly prevalent species.

We studied free-living commensal populations of the house mouse (*Mus musculus*) in Germany, and established a strategy to detect and quantify *Eimeria* infections. We show that a novel diagnostic primer targeting the apicoplast genome (Ap5) and coprological assessment after flotation provide complementary detection results increasing sensitivity. Genotyping PCRs confirm detection in a subset of samples and cross-validation of different PCR markers does not indicate bias towards a particular parasite species in genotyping. We were able to detect double infections and to determine the preferred parasite occurrence along the distal-proximal axis of the intestine. Parasite genotyping from tissue samples provides additional indication for the absence of species bias in genotyping amplifications. Three *Eimeria* species were found infecting house mice at different prevalences: *Eimeria ferrisi* (16.1%; 95% CI 12.7 – 20.2), *E. falciformis* (4.2%; 95% CI 2.6 – 6.8) and *E. vermiformis* (1.1%; 95% CI 0.4 – 2.7).

We provide methods for the assessment of prevalences of coccidia at the species level in rodent systems. We discuss the need for broader species level assessment in Coccidia. Prevalence negatively correlates with virulence for *Eimeria* species of house mice, as the more prevalent *E. ferrisi* has been shown to be less virulent than *E. falciformis*. It is an open question whether house mouse *Eimeria* are host specialist and whether prevalence in our system correlates with host range.

Key words

Eimeria, Coccidia, house mice, diagnostic PCR, species specific prevalence, qPCR

Highlights

- Flotation and PCR provide complementary results for *Eimeria* detection in house mice.
- Genotyping PCRs confirm detections.
- *E. ferrisi*, *E. falciformis,* and *E. vermiformis* infect natural populations of *M. musculus*.

- Double infections and preferentially infected tissues could be identified using qPCR.
- Potential virulence prevalence trade-off for *Eimeria* of house mice.

1. Introduction

House mice (*Mus musculus*) are the most commonly used mammalian model for biomedical research worldwide (Houdebine, 2004; Vandenbergh, 2013). Laboratory mouse strains are derived mainly from the subspecies *M. m. domesticus* with genetic contributions from other subspecies (*M.m musculus* and *M. m. castaneus*) (Nishioka, 2011; Yang et al., 2007). Establishment of suitable mouse models to better understand infections with coccidia is an ongoing process. Wild rodents and especially wild house mice are an attractive system for first steps in this direction (Ehret et al., 2017).

Eimeria (Schneider, 1875) is with around 1700 species the most specious genus in the phylum Apicomplexa (Duszynski, 2011; Perkins et al., 2000). For economical reasons, the most studied parasites in this group are those infecting livestock (Shirley et al., 2005; Su et al., 2003). However, at least one third of the total number of described species infects rodents (Levine and Ivens, 1965; Zhao and Duszynski, 2001).

The most used detection and identification method for coccidia is the flotation and microscopical observation of oocysts shed in faeces during the patent period of infection (Ryley et al., 1976). However, unsporulated oocyst are difficult to differentiate into different species (Levine and Ivens, 1988, 1965) and a sporulation process under specific conditions and high degree of expertise in the observation are required (Duszynski and Wilber, 1997; Long and Joyner 1984). For that reason, tools based on DNA amplification and sequencing have been included as routine strategy not only for detection, but also for taxonomic assessment (Fernandez et al., 2003; Hnida and Duszynski, 1999; Kawahara et al., 2010; Morris et al., 2007; Schnitzler et al., 1999; Su et al., 2003; Vrba et al., 2010).

Up to 16 species of *Eimeria* have been described from house mice (Levine and Ivens, 1965) and some of them use different niches in the intestine. The reasons for this diversity are still elusive (Zhao and Duszynski, 2001) and artificial splitting of morphologically plastic forms of the same species (in the same of different hosts) might contribute to this.

Eimeria species described from house mice include *E. falciformis,* the first coccidia described in

house mice (Eimer, 1870), which has sometimes been regarded as the most prevalent species in mice (Becker, 1934; Owen, 1976). This species (and especially the BayerHaberkorn1970 isolate) are the most commonly studied coccidia model in laboratory mice. Life cycle progression (Haberkorn, 1970) and host response (Mesfin et al., 1978; Schelzke et al., 2009; Schmid et al., 2012) are relatively well studied and the whole genome of this species has been sequenced and annotated in detail (Heitlinger et al., 2014).

E. vermiformis was first described in 1971 (Ernst et al., 1971) but since then, to our knowledge, not reported in wild house mice. Similar to *E. falciformis*, most of the information on this species comes from laboratory infection experiments (Figueiredo-Campos et al., 2018; Todd and Lepp, 1971; Rose et al., 1990; Rose and Millard, 1985), making the timing of life cycle progression and its effect on the host relatively well studied.

E. ferrisi was originally described from *M. m. domesticus* from North America (Ankrom et al., 1975a; Levine and Ivens, 1965) but since then to our knowledge investigated only in a single recent laboratory infection showing its virulence to be lower and life cycle to be shorter than that of *E. falciformis* (Al-khlifeh et al., 2019).

To the best of our knowledge, no investigation of prevalences and intensities of coccidia has been conducted in free-living populations of *M. musculus* and its sub-species. In the present work we studied the prevalence of *Eimeria* in house mice from a transect of the well-studied European house mice hybrid zone (HMHZ) (Boursot et al., 2003; Ďureje et al., 2012; Phifer-Rixey and Nachman, 2015). We established methods for detection, species identification and quantification of *Eimeria* in these wild commensal populations of house mice.

2. Material and methods

2.1. Collection of samples

Between 2015 and 2017, 378 house mice (*Mus musculus*) were captured in 96 farms and private properties in a transect 152.27 km long and 114.48 km wide, within the German federal state of Brandenburg (capture permit No. 2347/35/2014) (Figure 1A, Supplementary data 1). On average 20 traps were set overnight per locality. Faeces for microscopical diagnosis of *Eimeria* spp. were preserved in potassium dichromate ($K_2Cr_2O_7$) 2.5% (w/v) and stored at 4°C until further processing,

colon content was preserved in liquid nitrogen and stored at -80°C. For a subset of 163 mice (from Brandenburg in 2016) tissue samples from cecum and ileum were collected for DNA extraction and molecular identification of *Eimeria* spp. All samples were kept in liquid nitrogen during transportation and maintained at -80°C until processing.

2.2. Flotation and microscopical analysis of oocyst

Fecal samples were washed with tap water to eliminate potassium dichromate and homogenized. After oocyst were flotated using a saturated salt solution (specific gravity = 1.18—1.20), they were collected by centrifugation (3,234 × *g*/room temperature/ 10 minutes), washed with distilled water (1,800 × *g*/room temperature/10 minutes). The flotations were screened for the presence of oocyst using a Leica® DM750 M light microscope under the 10X objective. To estimate the intensity of infection, flotated oocysts were counted using a Neubauer chamber and the results were expressed as oocyst per gram (OPG) of faeces. Samples were then preserved in a fresh solution of potassium dichromate 2.5% (w/v) and sporulated in a water bath at 30°C for 10-12 days for further characterisation.

Eimeria isolates, corresponding to different phylogenetic groups (see below), were selected to take photomicrographs of sporulated oocysts using a Carl-Zeiss microscope at 100x magnification. Measurements were made on ~30 oocysts and ~30 sporocysts, using Adobe Photoshop CC v14.2.1 (3778 pixels = 100, 0000 µm). Length and width were measured and reported in micrometers. The Length/Width (L/W) ratio was calculated for both oocysts and sporocysts including means, standard deviation and variation coefficients. Additionally, main morphological traits (oocyst wall, oocyst residuum, micropyle, polar granule, sporocyst residuum, refractile bodies and Stieda body) were described, according to the protocol of Duszynski and Wilber (1997).

2.3. DNA extraction

DNA from colon content was extracted using the NucleoSpin® Soil kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendations, adding a mechanical lysis process in a Mill Benchtop Mixer MM 2000 (Retsch GmbH, Haan, Germany).

DNA from cecum and ileum tissues was isolated using the innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany) following the instructions of the manufacturer after disruption of the tissue with liquid nitrogen in a mortar. Quality and quantity of isolated DNA was measured spectrophotometrically in a NanoDrop 2000c (Thermo Scientific, Waltham, USA).

2.4. PCR amplification for detection (ap tRNA) and identification (nu 18S rDNA and mt COI)

For detection of *Eimeria*, amplification of a conserved tRNA region of the apicoplast genome (Ap5) was used. Primers Ap5_Fwd (YAAAGGAATTTGAATCCTCGTTT) and Ap5_Rev (YAGAATTGATGCCTGAGYGGTC) were designed based on the complete apicoplast genomes sequences available in the GenBank from *Eimeria tenella* (NC_004823.1), *E. falciformis* (CM008276.1) and *E. nieschulzi* (JRZD00000000.1).

For all samples with oocysts detected during flotation or successful amplification of Ap5, genotyping PCRs were performed to confirmation of detection and further identification of parasite species. A fragment of nuclear small subunit ribosomal RNA (~1,500 bp) and mitochondrial cytochrome C oxidase subunit I (~ 800 bp) were amplified using primers 18S_EF and 18S_ER (Kvičerová et al., 2008) and Cocci_COI_For/Rev (Ogedengbe et al., 2011), respectively. An alternative pair of primers was used in case of failure to amplify COI: Eim_COI_M_F (ATGTCACTNTCTCCAACCTCAGT) and Eim_COI_M_R (GAGCAACATCAANAGCAGTGT). These primers were designed based on the mitochondrial genome of *E. falciformis* (CM008276.1) (Heitlinger et al., 2014) and amplify a ~700 bp fragment of COI gene.

PCR reactions were carried out in a Labcycler (SensoQuest GmbH, Göttingen, Germany) using 0.025 U/µL of DreamTaq™ DNA Polymerase (Thermo Scientific, Waltham, USA), 1X DreamTaq Buffer, 0.5 mM dNTP Mix, 0.25 µM from each primer and 1—20 ng/µL of DNA template in 25 µL reaction. A concentration of 0.25 mM dNTP Mix and a supplementation with 2 mM $MgCl_2$ was used for the amplification of Ap5. The thermocycling protocol consist of 95 °C initial denaturation (4 min) followed by 35 cycles of 92 °C denaturation (45 s), annealing at 52°C (30 s/Eim_COI); 53 °C (45 s/18S_E); 55 °C (30 s/Cocci_COI); 56 °C (30 s/Ap5); 72 °C extension 90 s (18S_E), 20 s (Cocci_COI/Eim_COI) or 45s (Ap5) and a final extension at 72 °C (10 min). DNA from oocyst of *E. falciformis* BayerHaberkorn1970 strain and DNA from colon content of a non-infected NMRI mouse

were used as positive and negative controls, respectively.

All PCR products with the expected size were purified using the SAP-Exo Kit (Jena Bioscience GmbH, Jena, Germany) and Sanger sequenced from both directions by LGC Genomics (Berlin, Germany). Quality assessment and assembly of forward and reverse sequence was performed in Geneious v6.1.8. All sequences were submitted to NCBI GenBank (Accession numbers: nu 18S rDNA [MH751925—MH752036] and mt COI [MH777467—MH777593 and MH755302—MH755324] (Supplementary data S2).

2.5. Eimeria detection in tissue by qPCR

For mice collected in 2016 (*n*= 163) cecum and ileum tissue was screened using qPCR. Primers targeting a short fragment of mt COI were used to amplify DNA from from intracellular stages of *Eimeria* (Eim_COI_qX-F, TGTCTATTCACTTGGGCTATTGT; Eim_COI_qX-R GGATCACCGTTAAATGAGGCA). Amplification reactions with a final volume of 20 µL contained 1X iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany), 400 nM of each primer and 50 ng of DNA template. Cycling in a Mastercycler® RealPlex 2 (Eppendorf, Hamburg, Germany) was performed with the following program: 95 °C initial denaturation for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and extension 68 °C for 20 s. Melting curves were analysed to detect eventual primer dimer formation or non-specific amplification. As internal reference for relative quantification the CDC42 gene from the nuclear genome of the house mouse was amplified (Ms_gDNA_CDC42_F CTCTCCTCCCCTCTGTCTTG; Ms_gDNA_CDC42_R TCCTTTTGGGTTGAGTTTCC). Infection intensity was estimated as the ΔCt between mouse and *Eimeria* amplification (CtMouse-Ct*Eimeria*). To correct for background noise a detection threshold was estimated at ΔCt = -5 and only results above this value were considered infected. ΔCtIleum and ΔCtCecum were compared for samples above the threshold in both tissues to assess primary tissue occurrence (Ahmed et al., 2019). In samples positive for qPCR, *Eimeria* genotyping was performed based on DNA extracted from tissue, as described above (see *2.4*).

2.6. Molecular identification of Eimeria spp. isolates: 18S and COI phylogenetic analysis.

As strategy for molecular identification, datasets of nu 18S and mt COI sequences were compiled. Sequences generated for the present work were compared to databases sequences using NCBI BLAST and most similar sequences were selected. Based on this, sequences for *E. falciformis, E. vermiformis* and *E. ferrisi* were downloaded from GenBank as a reference. COI sequences were aligned by translation using the Multiple Align algorithm and translation frame 1 with the genetic code for “mold protozoan mitochondrial”, 18S sequences were aligned using MUSCLE (Edgar, 2004), both through Geneious v6.1.8.

Phylogenetic trees for all datasets were constructed using Maximum Likelihood (ML) and Bayesian inference (BI) methods, implemented in PhyML v3.0 (Guindon et al., 2010) and MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012), respectively. The most appropriate evolutive models for each datasets were determined in JModelTest v2.1.10 (Posada, 2008). For ML trees, a bootstrap analysis with 1000 replicates was performed, whereas MCMC for BI was run with two cold and two hot chains for 1,000,000 generations or until the average split frequency was below 0.05. The concatenated dataset was analysed using partitions and locus-specific models. Visualization of the trees was done with FigTree v1.4.2 (Rambaut, 2012).

2.7. Statistical analysis

All statistical analyses were performed in R (R Development Core Team, 2008). Prevalence of *Eimeria* was calculated as the proportion of positive samples in the total number of analysed samples. The 95% confidence interval [CI 95%] was calculated using the Sterne's exact method (Sterne, 1954) implemented in the package “epiR” v0.9-99 (Nunes et al., 2018).

To assess the significance in primer bias, logistic regression models were used to estimate the probability to successfully amplify and sequence a specific genetic marker for each *Eimeria* species. The response variable in these models was the amplification and sequencing success with a particular primer pair (COCCI_COI_F/R, Eim_COI_M_F/R or 18S_E F/R), and the predictors were the species identity (as determined with the other markers only, to make response and predictors independent) and additionally the detection of an infection with Ap5 and Flotation. These models were fitted first for COCCI_COI_F/R as response, then for the combined probability of successful COI genotyping and finally for 18S genotyping as response using the package “stats”

(R Development Core Team). Tables were produced for the summary of models using the package "jtools" v2.0.0 (Long, 2019).

Differences on oocyst and sporocyst L/W ratios between *Eimeria* species were tested for significance with an analysis of variance fitting a linear model using the species assignment as predictor with a Tukey HSD post hoc test adjusting for multiple comparisons, with the package "stats" (R Development Core Team).

3. Results

3.1. Sampling and Eimeria spp*. detection*

We used flotation of oocyst from faeces and PCR amplification of a novel diagnostic maker (Ap5) from colon content DNA to detect *Eimeria* parasites in a total of 378 house. Overall prevalence was 25.9% [95% CI = 21.7 – 30.7] (98/378) for PCR and 27.0% [95% CI = 22.7 – 31.7%] (102/378) for flotation. These estimates are not significantly different. Nevertheless, both techniques considered together estimate a higher prevalence of 37.6% [95% CI = 32.8 – 42.6] (142/378), meaning that some positive results were detected by only one of the techniques (Fig. 1B). We further aimed to provide species specific specific identification and to consolidate results from the two different detection methods.

3.2. Molecular identification of Eimeria isolates - (phylogenetic analysis nu 18S and mt COI)

Eimeria species were identified by phylogenetic analysis of nu 18S and mt COI sequences, the most commonly used molecular markers of apicomplexan parasites. To identify our isolates, sequences were compared with references from the NCBI database. Sequences from three previously described *Eimeria* species infecting *M. musculus* showed highest BLAST similarities and phylogenetic clustering. This approach ignores the problem whether isolates from different hosts would be assigned to the same phylogenetic clusters while they are regarded different species by taxonomists.

The nu 18S phylogenetic tree was inferred based on 80 sequences (540—1,795 bp), 73 of them from wild house mice generated in our study (3 from ileum tissue, 16 from cecum tissue and 54 from colon content, see below). *Eimeria* species previously described in house mouse were

represented by *E. falciformis* (AF080614), *E. vermiformis* (KT184355) and *E. ferrisi* (KT360995). In addition, one newly generated sequence from *E. falciformis* strain BayerHaberkorn1970 (MH751998) was also included. Sequence identity of our isolates to this references sequences was above 98% and even 100% in most of the cases for this marker. *Isospora* sp. sequences identified in *Talpa europaea* moles were used as outgroup. Both ML and BI rooted trees shared a topology placing our sequences at the same positions in relation to reference sequences with high support (bootstrap values and posterior probabilities are shown in Fig. 2A). The sequences clustered in three well supported monophyletic groups (Fig. 2A).

The phylogenetic tree for mt COI was based on 103 sequences (519—804 bp), 97 of which were obtained from *Eimeria* infecting wild house mice (3 from ileum, 16 from cecum tissue and 78 from colon content). Reference sequences from house mouse *Eimeria* (*E. ferrisi*, KT361028; *E. falciformis,* KX495129 and MH777539; *E. vermiformis* JN205071) identified by BLAST searches showed an identity of above 98% to respective groups of our isolates. We defined *Isospora* sp. from *Talpa europaea* as outgroup for rooting. ML and BI rooted trees based on alignments of these COI sequences shared a general topology with respect to the placement of our isolates in relation to reference sequences. Bootstrap values and posterior probabilities for support of these placements are shown in Fig. 2B. The sequences derived from house mice cluster in three monophyletic groups including reference sequences for *E. falciformis* (*n*= 17, sequences from our study), *E. ferrisi* (*n*= 72) and *E. vermiformis* (*n*= 8) (Fig 2B). Phylogenies based on concatenated supermatrices for the two markers show the same topology concerning placement of isolates from the present study (Supplementary data S3 and S4).

3.3. Morphometrical and morphological comparison of oocysts

For further support assignment of the three phylogenetic groups of *Eimeria* from house mouse, we characterized sporulated oocysts morphologically (Table 1). *E. falciformis, E. ferrisi* and *E. vermiformis* oocyst shared most of the traits we evaluated and showed overlapping morphometry (Fig. 3A). The length/width ratio of *E. vermiformis* oocysts, however, was significantly higher (1.29; 95%CI = 1.26—1.33; *n*= 35) than that of *E. falciformis* (1.17; 95%CI = 1.14—1.20; *n*= 31) and *E. ferrisi* oocysts (1.23; 95%CI = 1.21—1.25; *n*= 127) (Tukey HSD, $p < 0.05$) (Fig. 3B; Supplementary

data S5). This means that *E. vermiformis* has more ellipsoidal oocysts than the other two species. Other morphological characteristics of oocysts (smooth wall, absence of micropyle, presence of polar granule and absence of oocyst residuum) are very similar or identical between the three species (Table 1).

Morphological measurements of sporocysts are not significantly different between the three species found in house mice (Fig. 3C). We also observed the presence of a sporocyst residuum and refractile bodies uniformly in all species. Nevertheless, Stieda bodies were absent in *E. falciformis* only, in disagreement with previous descriptions (Eimer, 1870; Haberkorn, 1970).

3.5. Proximal-distal occurrence of infection and double infections

We detected DNA from endogenous stages by qPCR in 27 of 163 samples analysed (Supplementary data S6). We differentiate detection between small and large intestine, analysing ileum as the most distal tissue of the small intestine and cecum as the most proximal tissue of the large intestine. Detection was either limited just to cecum (n = 19), to ileum (n = 2) or possible in both tissues (n = 6). Infections in cecum were identified as *E. falciformis* (n = 4), *E. ferrisi* (n = 17) and *E. vermiformis* (n = 1) Detections in ileum (n = 2) were identified as *E. vermiformis*. In two mice positive in both tissues, it was possible to identify *E. ferrisi* in cecum and *E. vermiformis* in ileum, providing evidence that these animals presented a double infection (that is, simultaneous infections with different isolates; Fig. 4).

3.6. Amplification efficiency of different markers

As *Eimeria* detection by PCR and the determination of species identity could be biased especially in cases of double infections, we analysed differences in amplification efficiency of the three primer pairs used for the molecular identification of *Eimeria* species (Table 2). In a cross-validation approach we compare the likelihood to amplify a maker given the species identification with the other marker. The amplification and sequencing efficiency of Cocci_COI primer pair was significantly higher for *E. ferrisi* isolates (logistic regression, $p < 0.001$) than for *E. falciformis* isolates (the letter determined by the 18S marker). Using the novel primer pair Eim_COI_M_F/R the sequencing results were complemented and we detected no significant differences in the

combined amplification efficiency for both primers (logistic regression; $p = 0.62$).

Similarly, we did not detect significant differences in the probability to obtain an 18S sequence (using the 18S_EF/R primer pair) between *Eimeria* species as determined by (combined) COI assessment (logistic regression; $p = 0.25$). Differences in PCR efficiency for Cocci_COI make it likely that markers amplify different species in case of double infections in a single isolate. Both detection by flotation and diagnostic (Ap5) PCR significantly increase the likelihood amplification for all primers (COI or 18S). The statistical models for biased amplification hence also confirm that both detection methods provide complementary results, even while controlling for detection with the other method (Table 2).

3.7. Prevalence of the different Eimeria species

Genotyping amplification and sequencing with either 18S or COI primers (or both) was possible for samples in which infections had been detected (n=81) (Fig. 1B). Both COI and 18S genotyping PCRs hence support detection by flotation and diagnostic PCRs in this subset of samples. Furthermore amplification of both or either one maker was fully sufficient to assign the isolate to an *Eimeria* species (Fig. 2), allowing us to resolve prevalence on the species level.

These corrections and controls allow us to determine prevalence at the species level: *E. ferrisi* was identified at a higher prevalence of 16.1% (61/378, 95%CI = 12.7 — 20.2) in comparison to *E. falciformis* (16/378, 4.2% [95%CI = 2.6—6.8]) and *E. vermiformis* (4/378, 1.1% [0.4—2.7]).

4. Discussion

In this study we identify *Eimeria* species in wild commensal populations of house mice (*Mus musculus*). We show that detection and identification of this group of rodent coccidia can be challenging and propose to complement classical coprological assessment with molecular tools: a highly sensitive detection PCR, genotyping PCRs for species identification and qPCR for localization and detection of double infections. Based on this we identified three different *Eimeria* species in the house mouse: *E. ferrisi*, *E. falciformis* and *E. vermiformis.* Morphological characteristics and preferential occurrence were congruent with the assignment of isolates to the above species. We give examples for the need for *Eimeria* species identification in predictions for

two evolutionary relevant hypotheses: virulence-prevalence and host range-prevalence trade-off.

Only few studies report prevalences of *Eimeria* in wild populations of rodents. Prevalences ranging between 20 and 40% for isolates unclassified as either as *E. falciformis*, *E. ferrisi* and/or *E. vermiformis* were estimated before for wild populations of *Mus musculus* in UK, USA and Australia (Ball and Lewis, 1984; Moro et al., 2003; Tattersall et al., 1994). A recent study in rodents (other then *Mus musculus*) reported an *Eimeria* spp. prevalence of 32.7% based on coprological observations (Mácová et al., 2018). In comparison, overall prevalence for all *Eimeria* species in our study is only slightly higher (37.6%) in the house mouse. While flotation is the most commonly used for detection and quantification of coccidia (Hobbs et al., 1999; Hu et al., 2016; Rinaldi et al., 2011), we here used a complementary approach of flotations and diagnostic PCRs.

Traditional identification of *Eimeria*, depends on the expertise to recognise the morphology of sporulated oocyst (Levine and Ivens, 1965). We show that interpretation of morphometrical data is complex due overlap between species while measurement means agree with literature (Table 1). Considering the challenges of identification and characterisation of *Eimeria* isolates from field samples, we conclude that characterisation of *Eimeria* species requires molecular markers and phylogenetic analysis.

Sequence identity of our isolates to reference sequences from *Eimeria* species previously described in *M. musculus* was above 98% for COI, which is sometimes assumed sufficient correspond differences within species of *Eimeria* (Yang et al., 2015). We confirm taxonomic assignment based on highly supported maximum likelihood and Bayesian phylogenetic clustering of 18S and COI sequences. Moreover, the three identity of the three *Eimeria* species was supported by phenotypic characteristics: morphometry of oocysts and tissue occurrence of the infection.

For some *Eimeria* species precise tissue localization is described based on histology or electron microscopy (Ankrom et al., 1975; Šlapeta et al., 2001). Both methods provide detailed information on developmental stages, but are also time consuming and require a high level of expertise. As an alternative to determine (only) the rough occurrence of the infection along the proximal-distal axis of the intestine, a DNA based qPCR method allowed us not only to detect the presence of *Eimeria*, but also to estimate tissue specific intensity of infection. While infection with rodent *Eimeria* in general can be limited to the duodenum and jejunum (Kvičerová et al., 2007), house mouse *Eimeria* have been described to be mostly found in either the small or the large intestine (Ankrom et al., 1975; Ernst et al., 1971; Haberkorn, 1970; Levine and Ivens, 1965). The ileum as the most distal part of the small intestine and the cecum as the most proximal part of the large intestine thus provide an easy differentiation in these large small intestinal categories of infection. Genotyping DNA derived from tissue allowed to detect double infections with *E. vermiformis* in the small intestine and *E. ferrisi* in the large intestine. Localization generally agrees with previous descriptions for the isolates we identified as *E. ferrisi*, *E. falciformis* and *E. vermiformis* by phylogenetic clustering. To our knowledge we provide the first report of double infections in wild rodents.

Double infections can be problematic for identification of species by genotyping. Simultaneous infection of the caecum with *E. ferrisi* and *E. falciformis* would not be recognized with our qPCR method. We shown that amplification of COI with the commonly used primer pair Cocci_COI (Ogedengbe et al., 2011) is differentially efficient for different *Eimeria* isolates. This primer preference can lead to a misidentification in double infections due to the generation of “chimeric” isolates that present different and contradictory information for different markers. Attention to such discrepancies is needed when collating database sequences and when developing multi-marker approaches in general. For rodent *Eimeria* systems, we develop an alternative COI primer pair and find no evidence for differential amplification bias in our cross-validation of the different primer pairs.

E. ferrisi is by far the most prevalent species in our study area infecting *M. musculus*. Concerning the house mouse hybrid zone we find infections in *M. m. domesticus, M. m. musculus* and hybrids, suggesting that there are no strict geographical or host subspecies constraints for this species.

Population structure for *E. ferrisi* (which could in turn correspond to host subspecies (Goüy de Bellocq et al., 2018; Kváč et al., 2013) can not be found at the resolution the analysed markers provide. In addition to the currently used *E. falciformis* and *E. vermiformis* (Al-khlifeh et al., 2019)

Differences in prevalence between parasites found in wildlife systems can be integrated in an evolutionary perspective, allowing predictions on virulence and host range:

A lower virulence (reduction of host fitness caused by a parasite (Frank, 1996) should be associated with higher prevalences, because of virulence-transmission trade-offs (Anderson and May, 1982). Especially in relatively virulent parasites (such as *Eimeria* in general) infected individuals might suffer from moribundity and mortality reducing the rate of interaction between infected and uninfected hosts and the length of transmission periods (Leggett et al., 2013). While both the highly prevalent *E. ferrisi* and the lowly prevalent *E. falciformis* and *E. vermiformis* has been described pathogenic and potentially lethal in mice in laboratory infections (Blagburn and Todd, 1984; Klesius and Hinds, 1979; Mesfin et al., 1978), a direct comparison found virulence of *E. ferrisi* to be lower than that of *E. falciformis* (Al-khlifeh et al., 2019). In this regard the species specific prevalence in our system matches a prediction from one of the most central hypotheses in evolutionary epidemiology of parasites.

Two different hypotheses predict different prevalence of parasites with different host ranges. Under the trade-off hypothesis (Poulin and Combes, 1999) specialism is associated with higher prevalence as generalist parasites are less well adapted to infect many individuals in single species. Under the resource breadth hypothesis (Krasnov et al., 2005), however, species with a broad host range would be generally better at exploiting all host species and have higher prevalence. Predictions from both hypotheses might be integrated when taking into account the structure of differences between hosts (Pinheiro et al., 2016). We emphasize that we did not test host range of rodent *Eimeria* in the present work but used the assumption of biologically meaningful taxonomy and traditional “morphospecies” within house mice. This means that open questions on host specificity of rodent *Eimeria* (Mácová et al., 2018) could in future be investigated on the background of prevalences resolved on the level of “morphospecies” in the house mouse host.

In conclusion we argue that *Eimeria* in wildlife populations should be identified more frequently at

the level of species previously described by taxonomist. We propose to integrate a set of simple methods into a reproducible procedure to achieve this aim. For Coccidians, as important parasites of vertebrates, only species specific assessment will allow to test hypotheses under the framework of evolutionary epidemiology.

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Tables

Table 1. Morphological and morphometrical characteristics from *Eimeria* wild-derived isolates and reference morphotypes[§].

Species	Oocysts							
	Shape	Wall	Micropyle	Polar granule	Residuum	Length (µm)	Width (µm)	L/W ratio
E. falciformis	Spherical/Ellipsoidal	Smooth	Absent	Present	Absent	18.62 (15.29 – 20.76)	15.92 (13.89 – 17.6)	1.17 (1.00 – 1.36)
E. falciformis	Ovoid/Spherical	Smooth	Absent	Present	Absent/Present	21 (15 – 26)	18 (13 – 24)	1.17 (1.1 – 1.2)
E. ferrisi	Spherical/Ellipsoidal	Smooth	Absent	Present*	Absent	17.32 (13.59 – 21.47)	14 (10.97 – 17.44)	1.23 (1.02 – 1.56)
E. ferrisi	Ellipsoidal/Subspherical	Smooth	Absent	Present	Absent	17 (12 – 22)	14 (11 – 18)	1.22 (1.0 – 1.6)
E. vermiformis	Spherical/Ellipsoidal	Smooth	Absent	Present*	Absent	20.02 (16.22 – 22.8)	15.57 (12.37 – 18.44)	1.29 (0.99 – 1.45)
E. vermiformis	Spherical/Ellipsoidal	NA	Absent	Present	Absent	23.1 (18 – 26)	18.4 (15 – 21)	1.25 (1.1 – 1.4)

Species	Sporocysts					Tissue localization	Reference
	Residuum	Refractile body	Stieda body	Length (µm)	Width (µm)		
E. falciformis	Present	Present	Absent	8.88 (7.03 – 10.30)	5.52 (4.16 – 6.93)	Cecum	This work
E. falciformis	Present	Present	Present	11 (10 – 12)	7 (6 – 8)	Ileum,**cecum**and colon	Eimer, 1870; Haberkorn, 1970
E. ferrisi	Present	Present	Present	7.75 (5.17 – 11.47)	5.03 (2.88 – 7.73)	Cecum	This work
E. ferrisi	Absent/Present	Present	Present	10.5 (10 – 11)	5.5 (5 – 6)	**Cecum**and colon	Levine & Ivens, 1965; Ankrom et al., 1975
E. vermiformis	Present	Present	Present	8.35 (6.18 – 11.29)	5.43 (4.19 – 7.00)	Cecum and ileum	This work
E. vermiformis	Present	Present	Present	12.8 (11 – 14)	7.9 (6 – 10)	Jejunum,**ileum**and cecum	Ernst et al., 1971

§ Measurements are means in micrometers with ranges in parenthesis

*Observed in more than 80% of the oocysts

Table 2. Statistical models used to analyse primer preference to different *Eimeria* species.

Predictors	Model 1 (Cocci_COI)	Model 2 (Cocci_COI + Eim_COI)	Model 3 (18S_EF/R)
Intercept	-5.80 ***	-1.55	-1.40
	(1.11)	(1.27)	(0.87)
Ap5	3.50 ***	3.99 ***	0.78
	(0.59)	(0.60)	(0.62)
Flotation	1.64 **	2.15 ***	1.59 **
	(0.52)	(0.54)	(0.51)
Identification *E. ferrisi* (other marker)	5.10 ***	0.28	0.32
	(1.14)	(1.35)	(0.63)
Identification *E. vermiformis* (other marker)	17.23	11.98	1.71
	(1385.38)	(1385.38)	(1.33)
No identification (other marker)	0.97	-3.57 **	-3.44 ***
	(0.92)	(1.23)	(0.87)
Identification *Eimeria* spp. (COI)			-14.80
			(1455.40)
N	378	378	378
AIC	128.09	119.18	143.39
BIC	151.70	142.79	170.93
Pseudo R2	0.77	0.82	0.70

The upper number represents the estimate and numbers in brackets represent standard error for each predictor.
Intercept is *Eimeria falciformis* identification with other marker
Other marker refers to 18S based identification for COI models or vice versa
N (Total number of samples), AIC (Akaike information criterion), BIC (Bayesian information criterion).
*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure captions

Figure 1. Geographical localization of house mice (*Mus musculus*) collected for this study and comparison of diagnostic methods for *Eimeria*. A) Localization from the 378 mice included in the present study, colors indicate the *Eimeria* species identified for each. B) Venn diagram showing the overlap between detection methods and successful genotyping identification of the isolates.

Figure 2. Phylogenetic trees based on 18S rRNA and COI sequences. Sequences of 18S A) and COI B) were used to infer the molecular identification of wild-derived isolates of *Eimeria*. In both phylogenies, our isolates clustered in three groups one close to *E. falciformis* (red), other close to *E. ferrisi* (green) and finally one to *E. vermiformis* (yellow). Numbers in the branches represent the Bayesian posterior probability and the non-parametric bootstrap value. In bold are indicate the reference sequences for each species. CE and IL make reference to sequence derived from cecum or ileum tissue DNA, respectively.

Figure 3. Morphological and morphometrical characteristics of *Eimeria* oocyst isolated from *Mus musculus*. a) Photomicrographs at 1000x amplification of *Eimeria* oocyst from the three species isolated from *Mus musculus* (red = *E. falciformis*; green = *E. ferrisi* and yellow = *E. vermiformis*). Length/Width ratio from b) oocyst and c) sporocysts corresponding to each species (*E. falciformis n*= 31; *E. ferrisi n*= 127 and *E. vermiformis* n= 35). Mean +/- 95% Confidence Interval is plotted. * Represent significant difference (Tukey HSD, $p<0.05$).

Figure 4. qPCR detection of intracellular stages of *Eimeria* in cecum and ileum from *Mus musculus*. -Delta Ct value ($Ct_{Eimeria}$ - Ct_{Mouse}) from each tissue for 164 mice are plotted on the graph. The dotted line indicate the threshold of -6, values above the line are considered positive for the corresponding tissue. Circles represent negative samples, triangles indicate samples with *Eimeria* species identification and colors correspond to the *Eimeria* species identified in those samples.

Supplementary data S1. Geographical coordinates for collected mice in Brandenburg, Germany.

Supplementary data S2. Accession numbers for 18S and COI sequences from *Eimeria* spp. wild isolates.

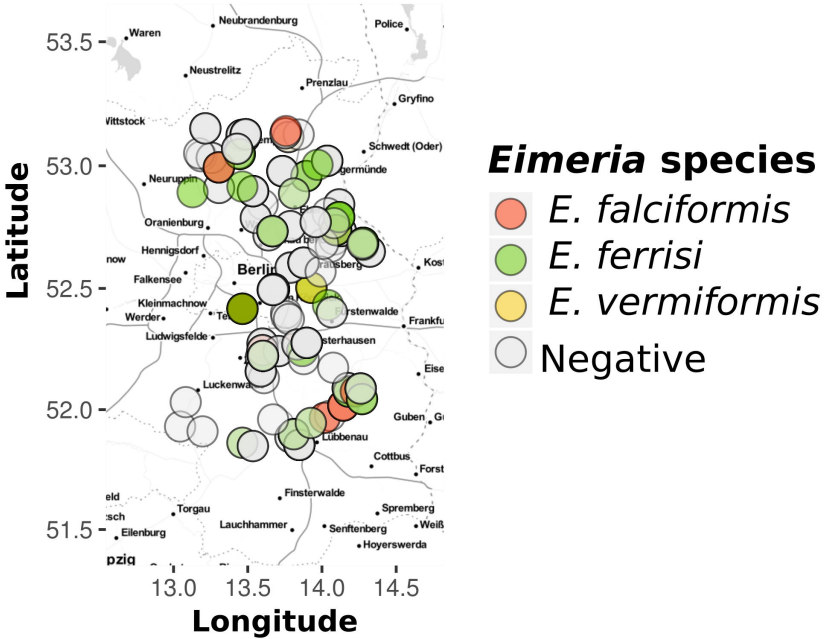
Supplementary data S3. Maximum-Likelihood phylogenetic tree inferred for 18S/COI sequences supermatrix.

Supplementary data S4. Bayesian phylogenetic tree inferred for 18S/COI sequences supermatrix.

Supplementary data S5. Oocysts measurements from wild-derive isolates of *Eimeria falciformis*, *E. ferrisi* and *E. vermiformis*

Supplementary data S6. Ct values from the qPCR detection of *Eimeria* in tissue.

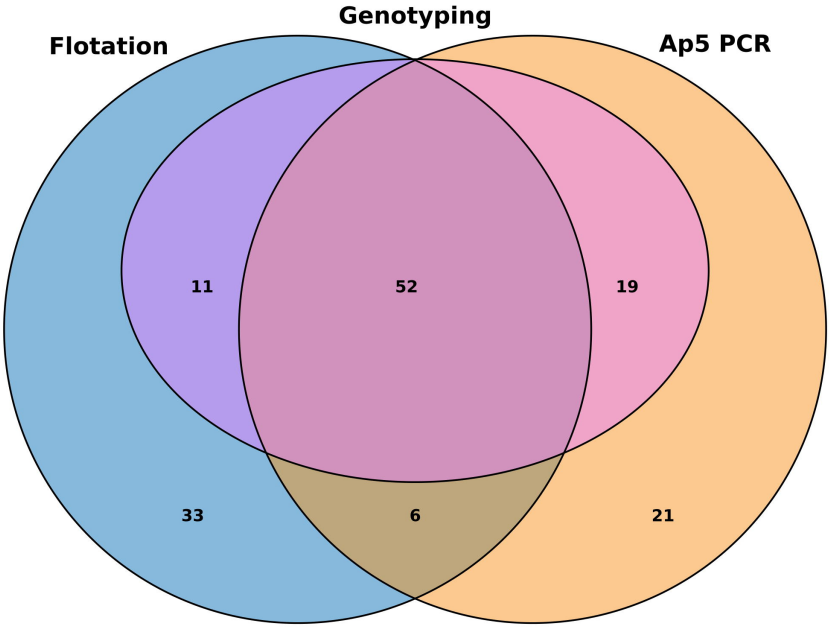
A)



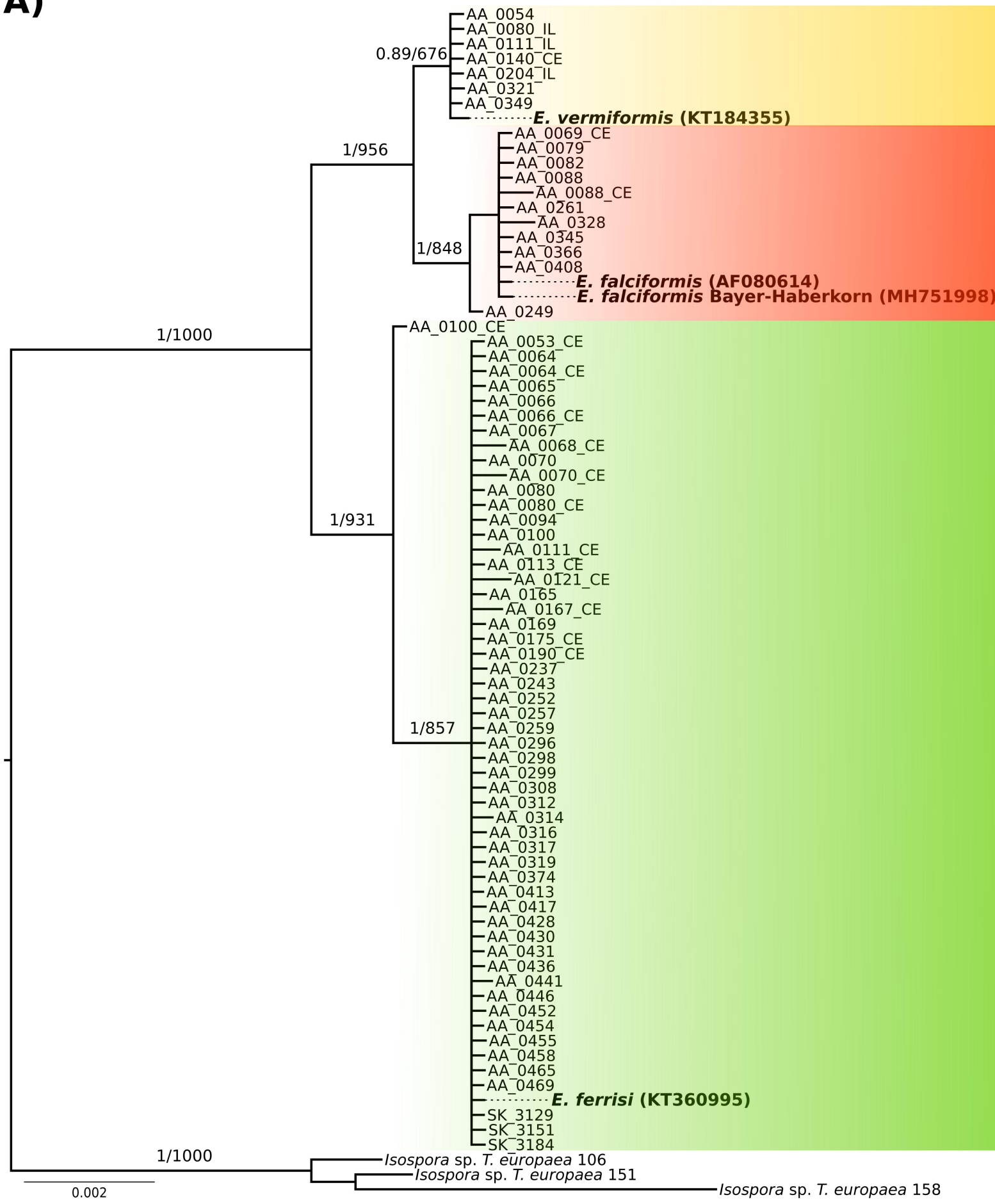
***Eimeria* species**

- *E. falciformis*
- *E. ferrisi*
- *E. vermiformis*
- Negative

B)



A)



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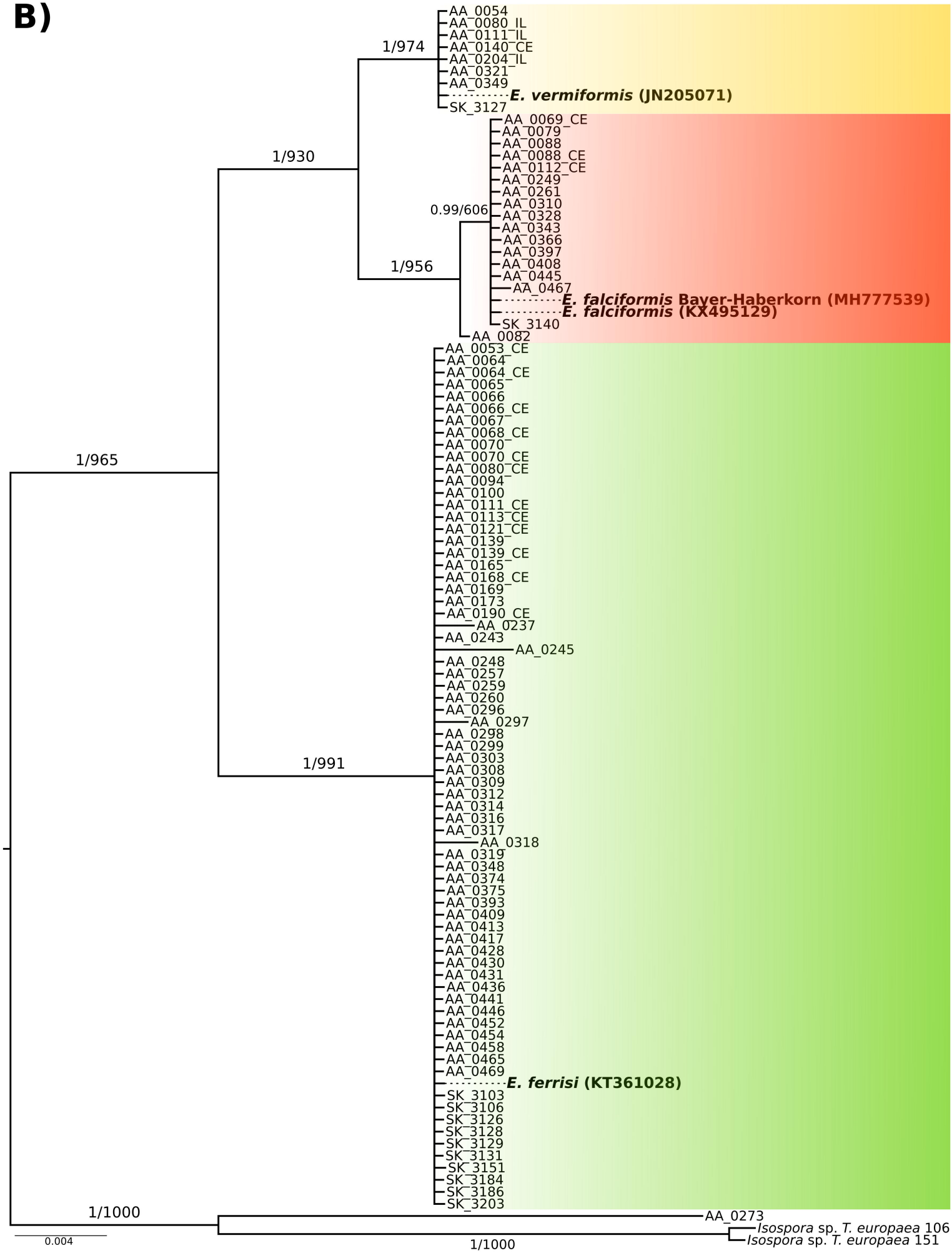
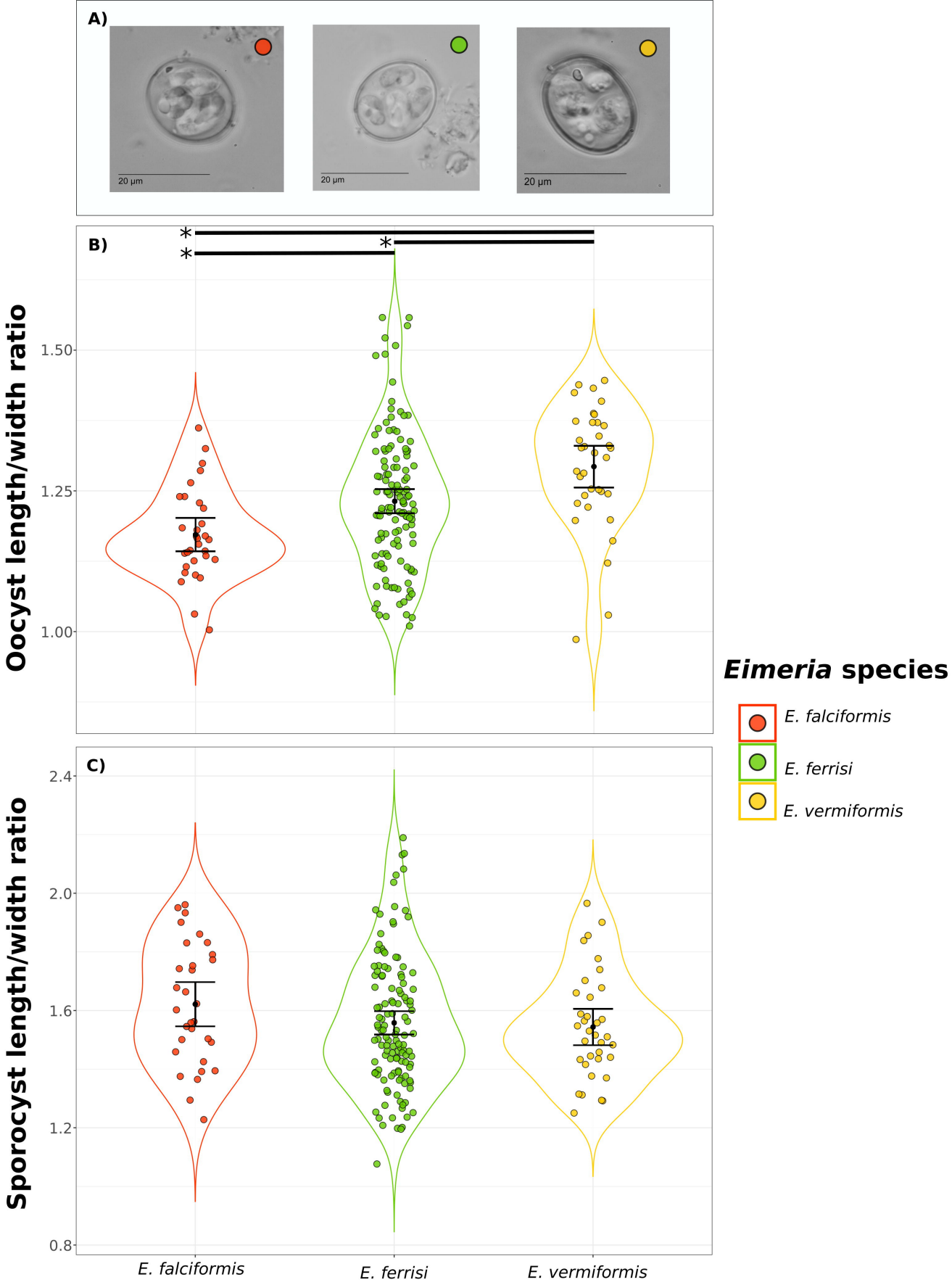
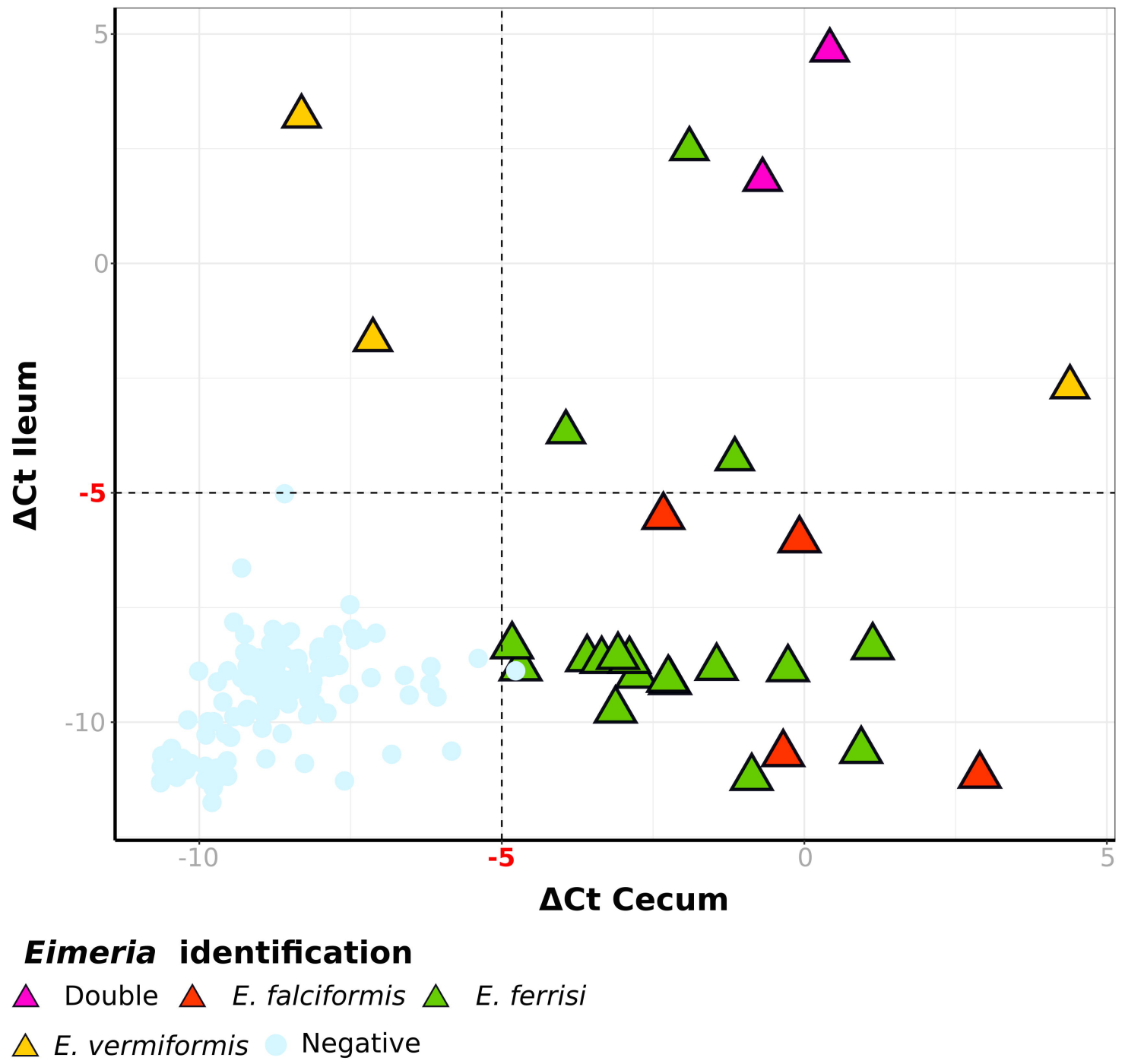

B)
1/974
1/930
0.99/606
1/956
1/965
1/991
1/1000
1/1000
0.004
AA_0054
AA_0080_IL
AA_0111_IL
AA_0140_CE
AA_0204_IL
AA_0321
AA_0349
E. vermiformis (JN205071)
SK_3127
AA_0069_CE
AA_0079
AA_0088
AA_0088_CE
AA_0112_CE
AA_0249
AA_0261
AA_0310
AA_0328
AA_0343
AA_0366
AA_0397
AA_0408
AA_0445
AA_0467
E. falciformis Bayer-Haberkorn (MH777539)
E. falciformis (KX495129)
SK_3140
AA_0082
AA_0053_CE
AA_0064
AA_0064_CE
AA_0065
AA_0066
AA_0066_CE
AA_0067
AA_0068_CE
AA_0070
AA_0070_CE
AA_0080_CE
AA_0094
AA_0100
AA_0111_CE
AA_0113_CE
AA_0121_CE
AA_0139
AA_0139_CE
AA_0165
AA_0168_CE
AA_0169
AA_0173
AA_0190_CE
AA_0237
AA_0243
AA_0245
AA_0248
AA_0257
AA_0259
AA_0260
AA_0296
AA_0297
AA_0298
AA_0299
AA_0303
AA_0308
AA_0309
AA_0312
AA_0314
AA_0316
AA_0317
AA_0318
AA_0319
AA_0348
AA_0374
AA_0375
AA_0393
AA_0409
AA_0413
AA_0417
AA_0428
AA_0430
AA_0431
AA_0436
AA_0441
AA_0446
AA_0452
AA_0454
AA_0458
AA_0465
AA_0469
E. ferrisi (KT361028)
SK_3103
SK_3106
SK_3126
SK_3128
SK_3129
SK_3131
SK_3151
SK_3184
SK_3186
SK_3203
AA_0273
Isospora sp. T. europaea 106
Isospora sp. T. europaea 151


A)
20 µm
20 µm
20 µm
B)
Oocyst length/width ratio
1.50
1.25
1.00
C)
Sporocyst length/width ratio
2.4
2.0
1.6
1.2
0.8
E. falciformis
E. ferrisi
E. vermiformis
Eimeria species
E. falciformis
E. ferrisi
E. vermiformis


5
0
-5
-10
ΔCt Ileum
-10
-5
0
5
ΔCt Cecum
Eimeria identification
Double
E. falciformis
E. ferrisi
E. vermiformis
Negative