Diversity and host specificity of coccidia (Apicomplexa: Eimeriidae) in native and introduced squirrel species

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Abstract

Introduction of alien species into new areas can have detrimental effects on native ecosystems and impact the native species. The present study aims to identify coccidia infecting native and introduced squirrels in Italy, to gain insight into possible transmission patterns and role of monoxenous coccidia in mediating the competition between alien and native hosts. We collected 540 faecal samples of native red squirrels *Sciurus vulgaris*, invasive alien grey squirrels *S. carolinensis* and introduced Pallas's squirrels *Callosciurus erythraeus*. Total prevalence of *Eimeria* spp. was 95.6% in *S. vulgaris*, 95.7% in *S. carolinensis* and only 4.1% in *C. erythraeus*. Morphological examination revealed 3 *Eimeria* morphotypes. Phylogenetic analyses of *Eimeria* DNA based on 18S, ITS, *cox I* markers displayed fairly distinct monophyletic clades in microscopically indistinguishable E2 morphotype, proving indisputable distinction between the isolates from red and grey squirrels. Grey squirrels successfully introduced *E. lancasterensis* from their native range, but this species does not spillover to native red squirrels. Similarly, there is no evidence for the transmission of *E. sciurorum* from red to grey squirrels. The possible transmission and the potential role of monoxenous coccidia in mediating the competition between native and invasive squirrels in Italy were not confirmed.

Keywords

Eimeria; Sciurus vulgaris; Sciurus carolinensis; squirrels; competition

Introduction

Biological invasions are among the most prominent threats for biodiversity. Introduction of alien species into new geographic areas can have detrimental effects on native ecosystems and impact the native species both directly (e.g. through predation or introduction of lethal pathogens) or indirectly (e.g. through competition, including the parasite-mediated competition) (Clavero and Garcia-Berthou 2005; Hartigan et al. 2011; Pizzatto and Shine 2011; Zavaleta et al. 2001). Moreover, invasive species and the pathogens they spread represent a threat for human health (Hulme 2014).

Parasites may play a role in biological invasions via three main mechanisms: (i) invaders may lose some of their parasites during translocation, leading to a competitive advantage (Torchin et al. 2003); (ii) invaders may serve as complementary hosts for local parasites, leading to spillback process or dilution effect, depending on their competence as hosts (Kelly et al. 2009); (iii) invaders may introduce with them new parasites, which may spill over to native hosts (Dubey and Shine 2008; Paterson and Gray 1997). Although majority of parasites are host-specific (Pizzatto and Shine 2011; Poulin 2007), a range of examples of successful invasion (i. a. avian malaria to Hawaii, *Fascioloides magna* to Europe, spreading of chytridiomycosis in amphibian populations) suggests that the interspecific transmission of parasites can be more frequent than expected (Atkinson et al. 2014; Marzal et al. 2015; Skerratt et al. 2007). In some of these cases, the introduced pathogens seriously impacted the naive host populations leading to their decline or extinction.

Parasite-mediated competition is likely common in natural populations although being difficult to observe (Price et al. 1988). Introduction of novel pathogens and parasites along with their hosts can play an important indirect role in invasion outcome by mediating competitive interactions with susceptible native hosts (Prenter et al. 2004). The phylogenetic relatedness between invaders and native hosts might facilitate the host-switch and spill over of

parasites (Torchin and Mitchell 2004). Parasites have the evolutionary advantage of having shorter generation times, which leads to fast adaption to new hosts (Kaltz and Shykoff 1998). Among the others, the squirrelpoxvirus (SQPV) accelerates replacement of susceptible native Eurasian red squirrel (*Sciurus vulgaris*) by alien Eastern grey squirrels (*Sciurus carolinensis*), which serve as unaffected reservoir (Collins et al. 2014; Tompkins et al. 2003). The North American Eastern grey squirrels have been repeatedly introduced to Europe (mainly Great Britain, Ireland and Italy) since the end of 19th century and cause local extinction of native Eurasian red squirrel mainly through competition for food resources (Gurnell et al. 2004; Wauters et al. 2005). However, in British Isles, the replacement process is accelerated by the SQPV (Rushton et al. 2005) and recent findings suggest that in Italy, where SQPV does not seem to occur, competition between these two squirrel species might be mediated by a North American nematode, introduced by the alien host (Romeo et al. 2014, 2015). The grey squirrels were introduced in Italy later than in Great Britain: they were first reported in Piedmont in 1948, but subsequent introductions were reported in Genova-Nervi in 1966 and, since the 1990, in many sites in Lombardy (Bertolino et al. 2014; Martinoli et al. 2010). During the last decade, Pallas's squirrel (*Callosciurus erythraeus*) has been introduced in Lombardy from South-East Asia and established a viable population in the North of Varese province, co-occuring with native red squirrels (Mazzamuto et al. 2015). Since both species are a threat for the local fauna, and in particular for the native red squirrel (Gurnell et al. 2004, 2015; Wauters et al. 2005; Bertlino et al. 2014; Mazzamuto et al. 2016) long-term conservation strategies aimed at preserving native biodiversity should not only include intensive control of populations of the alien species, but also surveys of parasites and infectious diseases and disease spread risk assessment (Guberti et al. 2014). As stated above, disease risk for native hosts may be greatly exacerbated by the introduction of alien species, especially when the two are phylogenetically related. Hence, our study focused on these three

squirrel species present in Italy (native *S. vulgaris* and alien *S. carolinensis* and *C. erythraeus*) and on coccidia of genus *Eimeria* infecting them. In general, these intestinal protozoan parasites affect individuals with reduced immunocompetence, such as young animals, and may represent an added threat to already endangered populations (Hakkarainen et al. 2007; Levine and Ivens 1965; Winternitz et al. 2012). Although *Eimeria* species are considered highly host-specific, cross-transmission of these species between different hosts has been demonstrated (Levine and Ivens 1988). The present study aims to identify *Eimeria* spp. infecting native and invasive squirrels in Italy to gain insight into possible transmission patterns and, consequently, on the potential role of monoxenous coccidia in mediating the competition between native and invasive hosts.

Tens of *Eimeria* spp. have been described in squirrels (Levine and Ivens 1965) and microscopic examination of oocysts is often insufficient for exact species determination, as different *Eimeria* spp. may have morphologically indistinguishable oocysts. This is especially true when, as in our case, data about endogenous stages or experimental infection are unavailable. Hence, when possible, we will make use of molecular tools for specific identification of oocysts.

Finally, we will also explore factors affecting variation of coccidia infections in our host species, to highlight possible differences in host-parasite relationships among the three squirrel species.

Material and methods

Trapping and sample collection

Faecal samples of native red squirrels (*S. vulgaris*) and invasive grey squirrels (*S. carolinensis*) were collected periodically between 2010 and 2014; sampling of Pallas's squirrels (*C. erythraeus*) was incorporated during the last 2 years of this study. We examined

a total of 540 faecal samples from 466 animals (*S. vulgaris* 206 samples/143 individuals, *S. carolinensis* 164/164, *C. erythraeus* 170/159), some individuals were screened repeatedly. The animals originated from 43 localities in regions Valle d'Aosta (1), Lombardia (35) and Piemonte (7). Some sites were inhabited by a single squirrel species (only *S. vulgaris* = RED 12, *S. carolinensis* = GREY 13), whereas in other sites more than one species was present (*S. vulgaris* with *S. carolinensis* = RED-GREY 9, *S. vulgaris* with *C. erythraeus* = RED-CALLO 9). There were no localities co-inhabited by all 3 squirrel species, no sites with *S. carolinensis* and *C. erythraeus* together and no *C. erythraeus*-only population.

In each site, the trapping was carried out for at least 5 continuous days every month, using single capture (Tomahawk trap model 202, Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.) or multi capture live-traps (Mayle et al. 2007) arranged in grids. Traps were placed on tree trunks and baited with hazelnuts. A plastic panel or mosquito-mesh was placed at the bottom of each trap to collect the faeces left by the trapped animal. Traps were set on Monday morning and checked twice a day until Friday morning, and they were rebaited and reset after each capture. For each squirrel we recorded species, sex and reproductive conditions, each individual was then weighed to the nearest 5 g with a Pesola spring balance and the length of its right hind foot (nails excluded) was measured (0.5 mm precision) with a thin ruler (Wauters et al. 2007). Red squirrels were individually marked with numbered ear tags (10 x 2 mm, type 1003 S National Band and Tag Co, Newport, Kentucky, U.S.A.) and immediately released. For alien squirrels, trapping was carried out within a European Community LIFE Project (LIFE09 NAT/IT/00095 EC-SQUARE) with the goal of eradicating the alien species in Italy, thus Pallas's squirrels and grey squirrels were euthanized using CO_2, following EC and AVMA guidelines (Close et al. 1996; Close et al. 1997; Leary et al. 2013).

Faeces found in the trap were collected, placed in tubes with 2.5% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$) solution, aerated for sporulation and stored at 4-8°C for later examination.

Samples examination

The faecal samples were examined for the presence of parasites microscopically, after centrifugation-flotation concentrations using modified Sheather's sugar solution (specific gravity 1.3). Coccidia were quantified as number of oocysts per gram of sediment (OPG) by counting in Bürker chamber in 100x magnification (Gunetti et al. 2012).

Coccidia were identified according to generally valid criteria for species separation by morphological characteristics of the oocysts. The morphological examination includes measurement (min. 30 oocysts and 30 sporocysts) and shape identification (shape index = SI) of oocysts and sporocysts, appearance of an oocyst wall, absence or presence (appearance) of a micropyle cap, a micropyle, polar granules, an oocyst residuum, Stieda bodies, sporocyst residua and appearance of sporozoites (Duszynski and Wilber 1997; Levine and Ivens 1965). The oocysts were measured and photographed using an Olympus Provis AX 70 microscope, equipped with a Nomarski interference-contrast (NIC) microscopy, a camera Olympus DP 70 and Olympus DP Controller Ver.03.01 PC software.

Molecular analyses

Identification of morphologically similar oocysts in different host species was followed by molecular identification and phylogenetic analyses. DNA was extracted from ~ 200 mg of sediment of representative samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA USA) according to the manufacturer's instructions. Three different markers, namely part of nuclear 18S rRNA, ITS region of nuclear rRNA = internal

169transcribed spacer 1 (ITS 1), 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS
1702) and mitochondrial cytochrome c oxidase subunit I (*cox I*) DNA, were amplified following
171PCR protocols and PCR primers published by Motriuk-Smith et al. (2011), Kvičerová and
172Hypša (2013). The PCR reaction was performed in a 25 µl volume containing 2 µl (1-10 ng)
173of total DNA, 12.5 µl of commercial premix PPP master mix (Top-Bio s.r.o), 1 µl (400 µM)
174of each primer and 8.5 µl PCR H_2O. Each PCR reaction contained a negative control with
175PCR water instead DNA. Total DNA of *Eimeria*-positive fresh faeces of *S. vulgaris* from
176rescue centres of the Czech Republic (CZ), *E. exigua* oocysts from rabbit and *E. ferrisi*
177endogenous stages from laboratory mouse (D7) were used as positive controls for all genes.
178The PCR products were separated by electrophoresis in 1.5% agarose gel stained with
179GoodView (ECOLI, Slovakia). Amplicons were purified using ExoSAP-IT® for PCR Product
180Cleanup (Affymetrix, USA). The selected amplicons were cloned into pGEM-T Easy Vector
181(Promega) and three plasmid clones of each were isolated using PureLink Quick Plasmid
182Miniprep Kit (Invitrogen). Sequencing of plasmids and PCR amplicons was carried out by the
183commercial company Macrogen (Amsterdam, The Netherlands).
184
185*Phylogenetic analyses*
186Sequences were identified by BLAST analysis, edited and aligned using GENEIOUS Pro
187software package version 6.1 (Kearse et al. 2012) and deposited to the NCBI GenBank
188database under accession numbers KT360976 – KT361068, KT368144. Suitable model of
189molecular evolution was selected using jMODELTEST 0.1.1. (Posada 2008). The model with
190the best likelihood was chosen using AIC criteria and phylogenetic trees were reconstructed
191using Bayesian inference (BI) in the program MrBayes v. 3.2.2. (Huelsenbe*ck et* al. 2001;
192Ronquist and Huelsenbeck 2003). MrBAYES analyses were run for 2 million MCMC
193generations and with four chains. Runs for individual data sets were performed under the

194different models of molecular evolution HKY+I for 18S data set; GTR+I+G for ITS 1, ITS 2
195and 5.8S; GTR+G for *cox I* and HKY+G for concatenated data. Convergence of runs was
196checked in AWTY (Nylander et al. 2008). Maximum-likelihood (ML) analyses were
197generated using the PHYML 3.0 software (Guindon and Gascuel 2003) and were performed
198under search parameters suitable for individual data sets mentioned above. Reliability of
199branching patterns within trees was tested by the bootstrap method with 1000 resamplings.
200*Eimeria exigua* was used as an outgroup for all four datasets to root phylogenetic trees.
201
202*Statistical analysis*

203We analysed variation in *Eimeria* infection only in red and grey squirrels since only a few
204Pallas's squirrels showed presence of oocysts. For both host species we examined i) variation
205in intensity of infection (i.e. OPG) for the most prevalent oocyst morphotype and ii) variation
206in infection status (i.e. presence/absence) for the less represented morphotypes. OPG values
207were modelled through generalised linear models with negative binomial error structure,
208whereas for presence/absence we run logistic regressions with binary response. On each
209dependent variable we explored the effect of sex, age class (i.e. juvenile, subadult or adult),
210season, year and area type (i.e. red-only, grey-only or red-grey). In addition, for red squirrels
211we run mixed models, with individual code as random factor, to account for repeated
212measures and we also added habitat type (i.e. mixed-deciduous or conifer forest) as a factor.
213We did not take into account habitat variability in the models about grey squirrels since all our
214sampling sites for this host had similar habitat conditions (i.e. lowland mixed-deciduous
215woods).

216In each case, we first explored full models and then obtained minimal models through
217backward selection of non-significant variables. Interpretation of significant factors with more

than two levels was based on pair-wise t-tests of Differences of Least Square Means (DLSM), applying sequential Bonferroni correction for multiple comparisons.

All statistical analysis were performed using SAS/STAT 9.4 software (Copyright © 2013, SAS Institute Inc., Cary, NC, USA).

Results

Oocyst morphology

Morphological examination of oocysts across the three host species revealed 3 morphotypes (Fig.1, 2), which differ in oocysts/sporocysts size (Tab. 1), morphological characteristics, host species, pattern of co-occurrence and prevalence.

Morphotype E1

Morphotype E1 was detected only in *S. vulgaris*. Morphotype E1 oocysts were large (Fig. 1, 2), with piriform bottleneck shape and brown scabrous thick wall (~3 µm) with micropyle. Neither oocyst residuum, nor polar granule developed. The sporocysts were elongated and had flat Stieda body, each inside with 2 sporozoites encircle a residuum consisting of several ~1 µm granules. The oocysts of *Eimeria* sp. E1 morphologically correspond with *E. mira* characteristics (Levine and Ivens 1965; Pellérdy 1974).

Morphotype E2

Morphotype E2 was detected in all 3 species of examined squirrels and the oocysts/sporocysts dimensions slightly differed between host species (Fig. 1, 2). Morphotype E2 oocysts were ellipsoidal to cylindrical with smooth, bi-layered wall 1-2 µm thick without micropyle. No oocyst residuum was formed, but polar granules (1-3) were distinct. Sporocysts were ovoidal,

with distinct nipple-like Stieda body; sporocyst residuum consisted of mass of small granules. Sporozoites were elongated, with tiny dotting and with large refractile bodies.

The characteristic features of the oocysts of *Eimeria* sp. E2 morphologically correspond with those of *E. sciurorum*, and *E. lancasterensis* (Levine and Ivens 1965; Joseph 1972; Pellérdy 1974).

Morphotype E3

Oocysts of morphotype E3 were detected in *S. vulgaris* and *S. carolinensis*, and only minor dimension differences between the two hosts were recorded. Morphotype E3 oocysts were small (maximum length 20 µm) and had cylindrical or subspherical shape (Fig. 1, 2). The oocyst wall was colourless, smooth, without micropyle and less than 1 µm thin. Small polar granules were visible inside the oocysts. Residua were present in ovoid sporocysts, but absent in oocysts. Stieda body were poorly visible, but present. Elongated sporozoites had bold refractile bodies. The *Eimeria* sp. E3 morphologically corresponds only with *E. silvana* oocysts characterization (Pellérdy 1974).

Variation in Eimeria *infection*

Overall, total prevalence of *Eimeria* spp. was 95.6% (197/206) in examined samples of *S. vulgaris*, 95.7% (157/164) in *S. carolinensis* and only 4.1% (7/170) in *C. erythraeus*; being significantly higher in the two *Sciurus* species than in *C. erythraeus* ($\chi^2_2 = 490.9$; $p < 0.0001$)

All the three identified *Eimeria* morphotypes were found in red squirrels (mean richness/host: 1.2±0.6 SE), with 24.7% of samples showing infection by more than 1 morphotype. In grey squirrels we observed only E2 and E3 (mean richness/host: 1.3±0.5 SE, with 34.8% of individuals showing mixed infection) and Pallas' squirrel were infected only by E2 (mean richness/host: 0.04±0.2 SE).

264*Eimeria* sp. E2 was the most prevalent morphotype across host species (for details on 265prevalence and 95% CI, see Fig. 1), being the only one infecting *C. erythraeus* (4.1%) and 266also the dominant type in mixed infections in both *S. vulgaris* (with E1 and E3) and *S.* 267*carolinensis* (with E3). Overall, morphotype E2 prevalence was 95.6 % in *S. vulgaris* and 26895.7 % in *S. carolinensis* and it was found in all sites were these two hosts occurred, 269independently from host cohabitation (RED, GREY or RED-GREY). Mean E2 OPG values 270(±SE) in red and grey squirrels were 7282 (± 968) and 13552 (± 1680), respectively. In both 271red and grey squirrels, E2 intensity of infection was affected by age class (Tab. 2, Fig. 3): 272OPG in red squirrels were significantly higher in adults than in juveniles or subadults (both 273$p<0.02$), whereas in grey squirrels we observed an opposite pattern, with adults significantly 274less infected than either juveniles or subadults (both $p<0.05$). In addition, E2 OPG in grey 275squirrels varied seasonally (Fig. 4), showing significantly lower values in summer than in all 276the other seasons (all $p<0.02$), whereas in red squirrels no such temporal variation was 277detected.

278As regards the other two morphotypes, E1 was found only in *S. vulgaris*, whereas E3 in both 279*S. vulgaris* and *S. carolinensis* (see Fig. 1 for detailed prevalence). In both host species, 280infection by morphotype E3 varied across seasons (Tab. 2, Fig. 5): in red squirrels prevalence 281in winter was significantly lower than in all the other seasons (all $p<0.05$) and in grey 282squirrels E3 showed an infection peak during spring (all $p<0.01$). Finally, presence of 283morphotype E1 in red squirrels, was affected by habitat type (Tab. 2) with a significantly 284higher prevalence of infection in mountain conifer forests (prevalence: 16.7%; 95% CI: 7.8% 285- 25.5%) than in lowland deciduous woods (prevalence: 3.0%; 95% CI: 0.1% - 5.9%).

286

287*Molecular taxonomy*

Partial sequences of nuclear and/or mitochondrial markers were obtained from 40 *Eimeria* samples (23 of *S. vulgaris* and 17 of *S. carolinensis*) originated from all three types of areas (RED = 19, GREY = 6, RED-GREY = 15) (Tab. 3). Oocysts morphologically classified as morphotype E2 were in all 40 samples, whereas 6 of these samples contained also oocysts of morphotype E1 (found only in *S. vulgaris*) and morphotype E3 (5 in *S. vulgaris* and 7 in *S. carolinensis*). With regard to low number of oocysts of morphotype E3 in *S. vulgaris* and *S. carolinensis* and morphotype E2 in *C. erythraeus* isolation was insufficient. Coinfection with dominant morphotype E2 complicated analyses and DNA yield of other 2 morphotypes and our effort of single oocysts isolations (Dolnik et al. 2009) failed in sciurid eimerias. Thus, we obtained only ITS sequences of morphotype E1 (from red squirrels 3564 and 2970) and no sequences from E3. From the 40 sequenced samples with morphotype E2, we obtained 40 amplicons of *cox I* DNA (~810bp), 25 of 18S rRNA (~1500bp) and 26 of ITS (~1000bp). All three genetic markers were obtained for 19 samples (concatenate tree) and analysed in one dataset. All datasets (18S, ITS, *cox I* and combined dataset) were analysed by BI and ML based programs (see Material and methods). Inasmuch as both analyses provide same tree topology, final graphic trees were generated by program MrBayes and will be presented with both branch supports i. e. posterior probabilities (PP) and bootstrap supports. The genetic analyses of *cox I* DNA in the dominant morphotype E2 showed different *Eimeria* haplotypes/species in *S. vulgaris* and *S. carolinensis*. A comparison of ITS sequences obtained from the oocysts classified as morphotype E2 obtained from *S. vulgaris* and *S. carolinensis* produced sequences having respectively 80-81% and 95-96% identity to the indexed sequences of the ITS region of *E. lancasterensis* isolated from a fox squirrel (*S. niger*) (GenBank accession numbers EU302675, EU302672, EU302681). The morphotype E1 (from *S. vulgaris*) showed 93-97% identity to *E. ontarioensis* from *S. niger* (EU302685) (Motriuk-Smith et al. 2009).

The total length of 18S analysed dataset was 1329 bp with sequences from 25 squirrel's samples, a control sample of laboratory mouse tissue with *E. ferrisi* (KT360995), a sequence originated of *E. exigua* oocysts of rabbit (KT360996), and final data also contained reference sequences from NCBI (JQ993645, JQ993653, JQ993657, JQ993661 from Kvičerová and Hypša 2013). Nine samples of *Eimeria* morphotype E2 obtained from grey squirrels created separated clade from 16 red squirrel's samples with high branch support for both groups. The NCBI sequence JQ993653 *E. vilasi* (originated of sciurid rodent *Spermophilus elegans*, from USA) created the sister group to the red squirrel samples. Sequences from murid coccidia clustered differently. KT360995 *E. ferrisi ex Mus musculus* clustered separately with JQ993657 *Eimeria* sp. *ex Apodemus agrarius*, while sequences JQ993645 *E. cahirinensis ex Acomys dimidiatus* and JQ993661 *Eimeria* sp. *ex Ap. sylvaticus* clustered together and formed the most derived branch for the rest of the dataset (Fig. 6a).

The final length of ITS sequences in dataset was 1146 bp and alignment contained 25 sequences from our samples (2 sequences of red squirrel's samples with dominant amount of morphotype E1 oocysts, 14 sequences of red squirrel's samples mainly with morphotype E2 oocysts, 8 sequences of grey squirrel's samples with dominant E2 oocysts, a sequence of *E. exigua* oocysts of rabbit KT361060) and 11 from NCBI database. Sequences in dataset showed high level of variability (100%-52.1%). Results of phylogenetic analysis showed same patterns as outcome of 18S phylogenetic runs. Majority of red squirrel's samples created separate group from grey squirrel's samples, which clustered together with EU302672, EU302673, EU302675, EU302676, EU302677, EU302678 and EU302681, which were determined as *E. lancasterensis* (Motriuk-Smith et al. 2009). Two our samples from red squirrels (KT361048 and KT361045) containing dominant amount of morphotype E1 oocysts (*E. mira*) created sister branch with *E. ontarioensis* (EU302685, EU302686) (Motriuk-Smith et al. 2009). NCBI sequences HM241638 and HM241636 (both sequences of *E.*

callospermophili ex Cynomys leucurus) formed separated branch (Motriuk-Smith et al. 2009, 2011) (Fig. 6b).

Final length of *cox I* alignment was 716 bp and sequences were obtained from 43 samples of *Eimeria* sp. in total: 17 sequences of samples of grey squirrels, 22 sequences of samples of red squirrels from Italy, 2 sequences of red squirrel's samples from CZ, a sequence of control sample of laboratory mouse tissue with *E. ferrisi* (KT361028) and a sequence of *E. exigua* oocysts of rabbit (KT361029). According the previously described results the phylogenetic analysis of *cox I* showed the same outcome as 18S and ITS i. e. *Eimeria* samples obtained from red squirrels formed strongly supported group segregated from monophyletic group of grey squirrel samples. Only our control sample of laboratory mouse *E. ferrisi* (KT361028) clustered with *E. burdai ex Heliophobius argenteocinereus* (JQ993709) (Kvičerová and Hypša 2013) and formed separated branch with NCBI sequences JQ993707 *Eimeria* sp. *ex A. sylvaticus*, JQ993704 *Eimeria* sp. *ex A. flavicollis* (Kvičerová and Hypša 2013) and HM771682 *E. falciformis* (mouse) (Ogedengbe et al., 2011), all from rodent hosts (Fig. 6c).

Dataset for concatenated tree consisted of *Eimeria* sp. samples, which sequences of all three genes (18S, ITS and *cox I*) were obtained (19 samples in total). Sequences of 13 red squirrel's samples with *Eimeria* morphotype E2 clustered into monophyletic clade and formed well-supported branch likewise *Eimeria* morphotype E2 from grey squirrels (5 samples). Concatenated tree corroborates with results of previous phylogenetic analyses of *Eimeria* morphotype E2 from red squirrels and grey squirrels and confirmed that each squirrel species is host for different *Eimeria* species (Fig. 6d).

The results of phylogenetic analyses of E2 morphotype based on 3 different markers (18S, ITS, *cox I*) displayed fairly distinct monophyletic clades from different host species isolates

with pairwise distance values for 18S (97.2-100%), ITS (57.3-100%) and *cox I* (94.1-100%) datasets and proved indisputable distinction between E2 morphotype in red and grey squirrels.

Discussion

The traditional species concept and the identification of eimeriid coccidia relies on morphological features of the oocysts (size, shape, wall, internal structures), combined with data about sporulation time and endogenous development and host specificity. Moreover, the species identification in *Eimeria* is further complicated by the fact that several species can co-occur in a single host (Levine and Ivens 1965; Pellérdy 1974). To reach desired resolution in distinguishing of possible cryptic species of *Eimeria* in our study, we combine the traditional morphology-based identification with molecular taxonomy.

Our study focused on identification and comparison of coccidia of genus *Eimeria* infecting native *S. vulgaris* and alien *S. carolinensis* and *C. erythraeus* in Italy. Microscopic determination allowed us to detect three different oocyst morphotypes, with differences in prevalence and infection patterns among the three squirrel species. Furthermore, molecular analysis revealed that morphologically similar oocysts in red and grey squirrels are actually two distinct *Eimeria* species, each one specific to its host, suggesting that no transmission of *Eimeria* spp. between native and introduced squirrels of genus *Sciurus* occurs.

Based on morphological examination, we initially identified 3 distinct oocyst morphotypes, one of which (E1) was present only in red squirrels, whereas the other two (E2 and E3) were shared by more than one squirrel species. As already mentioned, morphological identification of oocysts is often unreliable and, despite *Eimeria* spp. having usually a high host-specificity, a few studies gave evidence for the sharing of some species between different squirrel species (Levine and Ivens 1965; Motriuk-Smith et al. 2009). For example, among coccidia infecting squirrels of the genus *Sciurus*, *E. confusa*, *E. lancasterensis* and *E. ontarioensis* were origin-

ally described in *S. carolinensis*, but are able to infect other North American squirrel species such as *S. niger* or *S. aberti* (Joseph 1975; Motriuk-Smith et al. 2009). Hence, when feasible, we relied on subsequent molecular analysis to confirm morphological identification. Morphotype E1 morphological features corresponded with both *E. mira* and *E ontarioensis*. The results of phylogenetic analysis of ITS sequences showed that morphotype E1 is indeed a sister branch to *E. ontarioensis* (EU302685, EU302686) (Motriuk-Smith et al. 2009), what providing support for the recognition of this morphotype as a distinct species. Thus, based on morphology of oocysts, host specificity and the phylogenetic analysis, E1 infecting *S. vulgaris* can be identified as *E. mira*.

Phylogenetic analyses of morphotype E2 (detected in both hosts) were based on 3 different markers (18S, ITS, *cox I*) as suggested by recent studies, which have described the use of multiple genetic markers in *Eimeria* species as an helpful tool to identify species boundaries or cryptic species (Kvičerová and Hypša 2013; Motriuk -Smith et al. 2009, 2011; Ogedengbe et al. 2011). These analysis displayed fairly distinct monophyletic clades from each different host species isolates, with pairwise distance values for 18S (97.2-100%), ITS (57.3-100%) and *cox I* (94.1-100%) datasets, which proved indisputable distinction between E2 morphotypes in red and grey squirrels. Hence, by combination of host specificity, morphological and genetic analyses, we identified morphotype E2 as *E. sciurorum* in *S. vulgaris*, and as *E. lancasterensis* in *S. carolinensis*. The identification of oocysts E2 as two distinct species may also explain the different age-infection profiles observed for this morphotype in the two host species. Different *Eimeria* species are indeed known to elicit different immune responses and in this regard, red squirrels seem to mount a lower immune response towards *E. sciurorum* than grey squirrels towards *E. lancasterensis*, since the former show higher OPG values in adults.

The identification of *E lancasterensis* also means that grey squirrels carried successfully with them at least this one species from their native range. Coccidia parasites have been so far mostly overlooked in studies addressing diseases of alien species, however co-invasion of Eimerian parasites along with their hosts seems likely to be a common pattern in biological invasions, at least when morphological features of oocysts make identification obvious (e.g. seven *Eimeria* species introduced by the cotton-tail rabbit in Italy, Bertolino et al. 2010).

Also morphotype E3 was detected both in red and grey squirrels, but, unfortunately, low intensity of infection in both species (resulting in low availability of genetic material) coupled with the fact that it always co-occurred with E2, making its isolation difficult, prevented molecular determination of this *Eimeria* sp. Morphological features of E3 correspond only with *E. silvana* (described in the red squirrel), whereas to our knowledge no comparable species was ever described in grey squirrels (Joseph 1972; Levine and Ivens 1965; McAllister and Upton 1989; Pellérdy 1974).

Overall, the findings about E1 and E2 do not support an hypothetical transmission of *Eimeria* spp. between native red squirrels and alien grey squirrels, however uncertain identification of morphotype E3 in both hosts did not allow us to ascertain whether this third morphotype represents a single species (i.e. *E. silvana*) shared by the two hosts or whether E3 oocysts belongs to two morphologically indistinguishable species as was the case for E2. The same is true for Pallas's squirrels, which were infected only by morphotype E2 with low prevalence and abundance of oocysts, which did not allow for molecular analysis to disclose whether this *Eimeria* was a third species or whether it was *E. sciurorum* transmitted by the native red squirrel to the alien host. Actually, this same infection pattern (i.e. few infected individuals shedding a low number of oocysts), may suggest that morphotype E2 in Pallas's squirrel is a recently acquired species, however we cannot presently draw any conclusion on the matter.

In any case, both alien squirrels seem to show at least some measure of parasite-release since their coccidia communities in Italy resulted quite poor in terms of species richness. A minimum of 6 different species is indeed reported in studies dealing with *Eimeria* spp. infecting grey squirrels in North America (Joseph 1972; Levine and Ivens 1965; McAllister and Upton 1989; Pellérdy 1974). Hence, considering the successful co-introduction of *E. lancasterensis* and even assuming for morphotype E3 to be an unidentified Nearctic species, this still means that grey squirrels lost many eimerian parasites during the introduction process. As regards Pallas's squirrels, information about parasites infecting this tree squirrel in its native range is sorely lacking, however coccidia communities of Sciurids are usually quite rich in species with high-prevalence infections (Ball et al. 2014; Bertolino et al. 2003). Hence, since we found only one morphotype of oocysts, similar to that of the red squirrel, that had a very low prevalence in the examined population, we believe that this alien squirrel has lost all its original eimerian parasites and is acquiring a new species through parasite spillover.

Finally, overall the three detected morphotypes showed different patterns of infection depending on the morphology of their oocysts' walls: thin-walled morphotypes E2 (*E. sciurorum* and *E. lancasterensis*, ˃95 %) and E3 (*E. silvana* 20.9-34.8 %) showed a higher prevalence than thick-walled oocysts E1 (*E. mira*, prevalence 7.8%) found only in red squirrels. These findings correspond with results previously reported in other studies, when *E. lancasterensis* in grey squirrels and *E. sciurorum* in red squirrels showed higher prevalence (65-91% and 66-79%, respectively) than thick-walled *E. ontarioensis* (3-29%), *E. mira* (2.6%) or *E. confusa* (23%) (Ball et al. 2014; Bertolino et al. 2003; Joseph 1972; McAllister and Upton 1989; Motriuk-Smith et al. 2009; Spurgin and Hnida 2002). Authors Motriuk-Smith et al. (2009) suggested 2 contrasting infection strategies in *Eimeria* spp. of sciurids, with thin-walled species producing many oocysts, having rapid sporulation, high transmission rates, inducing little immune response, but being less resistant in the external environment. On the contrary, thick-

walled species produce fewer oocysts, sporulate slowly, stimulate more immune response and have more resilient exogenous stages. In our case, these alternative strategies may explain the higher prevalence and intensities observed in thin-walled species and the different effects of season and habitat conditions on the three morphotypes. In particular, infection by *E. mira* in red squirrels was significantly more common in mountain habitats, likely because its thick-walled oocysts are well adapted to harsh weather conditions. Conversely, thin-walled *E. lancasterensis* oocysts in grey squirrels were significantly less abundant during dry summer season. Also E3 prevalence varied seasonally in both hosts, although with slightly different patterns (i.e. highest peak during spring in grey squirrels and lowest peak during winter in red squirrels), but the reasons for this seasonal variation are less clear and may lie more in demographic changes in the host populations than in E3 characteristics. On the other hand, we cannot rule out the possibility of E3 in the two hosts actually representing two distinct species with different infection strategies.

Conclusions

Results from microscopic determination, molecular analyses and infection patterns point out that the dominant coccidian parasites of red and grey squirrels in Italy are two different and host-specific *Eimeria* species. Grey squirrels successfully introduced *E. lancasterensis* from their native range, but this species does not spillover to native red squirrels. Similarly, there is no evidence for the transmission of *E. sciurorum* from red to grey squirrels. However, cross-transmission of eimerian parasites between these two hosts cannot be completely ruled out until identification of morphotype E3 is made certain. Similarly, *Eimeria* infection in *C. erythraeus* occurred with a low shedding and prevalence, which prevented specific identification of the single detected morphotype. Therefore, in both cases, additional investigation is needed to

ascertain definitely whether cross-transmission between these three squirrel hosts occurs to some measure.

Finally, the low *Eimeria* species richness observed in two alien squirrel species suggests parasite-release which might facilitate the establishment of these species in the invaded range. A loss of species has been indeed already demonstrated for the macroparasite fauna of both alien species in Italy (Mazzamuto et al. 2016; Romeo et al. 2014). In order to verify this mechanism the next step should be to assess the pathological effect of these *Eimeria* species and quantify their impact on squirrel's fitness.

Techniques of molecular taxonomy are helpful tool with better taxonomic resolution on species level in eimeriid coccidia using various markers (Kvičerová and Hypša 2013; Motriuk-Smith et al. 2009, 2011; Ogedengbe et al. 2011). The 18S rDNA is broadly used for phylogenetic analyses within the genus *Eimeria*, facilitated by growing number of available sequences. The variability of this marker is insufficient for distinguishing of closely related taxa. In contrast, mitochondrial *cox I* sequences and ITS rDNA variable region are more reliable as species-specific markers (Motriuk-Smith et al. 2009, 2011; Ogedengbe et al. 2011). So far, ITS1 and ITS2 are the only published sequences of coccidia from the Sciuridae, including the sequences of *E. lancasterensis* and *E. ontarioensis*.

Host specificity is a key aspect of the parasite diversity. Similarly to other rodents, the squirrels host tens of described *Eimeria* species and waste majority of species is probably undescribed (Levine and Ivens 1965). Although the host specificity of *Eimeria* spp. is presumably high, few studies provided evidence for sharing species of *Eimeria* between different squirrel species. *Eimeria confusa*, *E. lancasterensis* and *E. ontarioensis*, all originally described in *S. carolinensis*, are able to infect also other North American squirrel species such as *S. niger* or *S. aberti* (Joseph 1975; Motriuk-Smith et al. 2009; Spurgin and Hnida 2002). Our study brings the evidence that *E. lancasterensis* successfully invaded the

European territory with its host; however, it was unable to cross the species barriers between its natural host and native red squirrels.

The introduction and subsequent invasion of exotic squirrel species into European continent represent not only an imminent threat for the native fauna, but also interesting experiment enabling to assess the host specificity of eimeriid parasites. In contrast to SQPV or nematodes introduced by the alien grey squirrels (Collins et al. 2014; Romeo et al. 2014, 2015), the possible transmission and the potential role of monoxenous coccidia in mediating the competition between native and invasive squirrels in Italy were not confirmed.

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Appendices

Table 1. Dimensions of oocysts and sporocysts of *Eimeria* morphotypes E1, E2, E3 in the examined host species - *S. vulgaris* (SV), *S. carolinensis* (SC) and *C. erythraeus* (CE). SI = shape index.

Table 2. Factors explaining variation in *Eimeria* spp. infection in red (SV) and grey squirrels (SC), df = degrees of freedom.

Table 3. Sequenced isolates of *Eimeria* spp. of 40 squirrel samples with identification number of the sample (No.), hosts - *S. vulgaris* (SV), *S. carolinensis* (SC), *C. erythraeus* (CE), type of trapping sites and Genbank accession numbers.

Figure 1. *Eimeria* spp. morphotypes (E1, E2, E3), their counts of oocysts per gram of faeces sediment (OPG) and prevalence with 95% CI in the examined host species - *S. vulgaris* (SV), *S. carolinensis* (SC) and *C. erythraeus* (CE).

Figure 2. Photos of sporulated and unsporulated oocysts of *Eimeria* spp. morphotypes. 1 – 3: the oocysts of morphotype E1 of *S. vulgaris* (1 - the unsporulated oocyst, 2 - the unsporulated oocyst with detailed oocyst wall, 3 - the sporulated oocyst). 4 – 5: the oocysts of morphotype E2 (4 - the sporulated oocyst of *S. vulgaris*, 5 - the sporulated oocyst of *S. carolinensis*, 6 - the sporulated oocyst of *C. erythraeus.* 7 – 9: the oocysts of morphotype E3 (7 - the sporulated oocyst of *S. vulgaris*, 8 - the sporulated oocyst of *S. carolinensis*, 9 - the unsporulated oocyst of *S. carolinensis.* The bar is 20 um and all oocysts are in the same scale.

Figure 3. Morphotype E2 intensity of infection (OPG) by age class in red squirrels (a) and grey squirrels (b).

Figure 4. Seasonal variations in morphotype E2 intensity of infection (OPG) in grey squirrels.

Figure 5. Prevalence of morphotype E3 by season in red squirrels (a) and grey squirrels (b). Error bars indicating 95% CI.

Figure 6. Results of phylogenetic analyzes constructed by MrBayes software presented with combined branch supports of PP for BI/ML bootstrap, genetic distances between individual sequences in clades are displayed in percentage. Phylogenetic trees of *Eimeria* spp. are based on: (a) 18S sequences and run under HKY+I model of molecular evolution; (b) ITS1, 5.8S and ITS2 data and performed under GTR+I+G model; (c) *cox I* sequences and run under GTR+G model; (d) the concatenated phylogenetic tree is based on 18S, ITS 1, ITS 2, 5.8S and *cox I* sequences and run under HKY+G model.

Table 1

	Host	Oocysts	SI	Sporocysts	SI
E1	SV	39.5 (36-43) x 26.5 (25-30)	1.5	19.3 (16-22) x 8.2 (7-10)	2.4
E2	SV	27.2 (20-36) x 16.3 (11-20)	1.7	11.5 (7-15) x 6.8 (5-9)	1.7
	SC	28.4 (21-35) x 16.8 (13-23)	1.7	12.3 (9-17) x 7.3 (5-9)	1.7
	CE	30.9 (28-35) x 17.4 (15-19)	1.8	12.3 (10-15) x 7.5 (6-8)	1.7
E3	SV	18.5 (17-20) x 14.5 (12-17.5)	1.3	9.0 (8-10) x 5.3 (5-6)	1.7
	SC	19.0 (17-20) x 12.7 (9-14)	1.3	8.7 (7-11) x 5.3 (4-6)	1.7

Table 2

Host species	Dependent variable	Factor	df	χ^2	p value
SV	E2 OPG	Age class	2	9.50	0.0087
	E3 presence/absence	Season	2	10.75	0.0132
	E1 presence/absence	Habitat type	1	9.70	0.0018
SC	E2 OPG	Age class	2	10.68	0.0048
		Season	3	11.52	0.0092
	E3 presence/absence	Season	2	11.97	0.0075

Table 3

No.	Host	Site	*Eimeria* morphotype/OPG	18S	ITS	*Cox I*
L05	SC	GREY	E2/68000		KT361061	KT361031
L28	SC	GREY	E2/115833	KT360997		KT361032
L348	SC	GREY	E2/1667			KT361035
L356	SC	GREY	E2/6154		KT361067	KT361041
L357	SC	GREY	E2/46667; E3/˂100	KT360998	KT361064	KT361038
GF01	SC	RED-GREY	E2/<100			KT361030
L345	SC	RED-GREY	E2/32500; E3/ <100		KT361062	KT361033
L347	SC	RED-GREY	E2/24118; E3/ <100			KT361034
L353	SC	RED-GREY	E2/27879			KT361036
L369	SC	RED-GREY	E2/6957	KT368144	KT361065	KT361039
LS47	SC	RED-GREY	E2/6857; E3/<100	KT360999	KT361066	KT361040
LS56	SC	RED-GREY	E2/1212; E3/<100	KT361000	KT361063	KT361037
LS61	SC	RED-GREY	E2/9167; E3/<100			KT361042
LS63	SC	RED-GREY	E2/7368; E3/<100	KT361001	KT361068	KT361043
64	SC	GREY	E2/16364	KT360976		KT361004
159	SC	RED-GREY	E2/952	KT360977		KT361005
160	SC	RED-GREY	E2/5294	KT360978		KT361006
2490	SV	RED	E2/4500			KT361007
2965	SV	RED-GREY	E2/20000	KT360979	KT361044	KT361008
2970	SV	RED	E1/13889; E2/694		KT361045	KT361009
3379	SV	RED-GREY	E2/11034	KT360980	KT361046	KT361010
3518	SV	RED	E2/7000	KT360981	KT361047	KT361011
3564	SV	RED	E1/79394; E2/43636		KT361048	KT361012
4017	SV	RED	E2/5000; E3/<100	KT360982	KT361049	KT361013
4036	SV	RED	E1/1042; E2/2083	KT360983	KT361050	KT361014
4081	SV	RED	E2/3333; E3/<100	KT360984	KT361051	KT361015
4136	SV	RED-GREY	E1/9000;E2/66500;E3/<100			KT361016
4167	SV	RED-GREY	E2/48889			KT361017

4180	SV	RED	E2/4762	KT360985	KT361052	KT361018
4201	SV	RED	E2/1290	KT360986	KT361053	KT361019
4208	SV	RED	E2/2222	KT360987	KT361054	KT361020
4210	SV	RED	E2/2667	KT360988		KT361021
4220	SV	RED	E2/6684	KT360989	KT361055	KT361022
4223	SV	RED	E2/4516	KT360990	KT361056	KT361023
4425	SV	RED	E2/3500	KT360991	KT361057	KT361024
4438	SV	RED	E2/9231; E3/<100	KT360992		KT361025
4448	SV	RED	E2/12000	KT360993	KT361058	KT361026
4451	SV	RED	E2/3448	KT360994	KT361059	KT361027
CZ2	SV	RED	E1/6429; E2/85000			KT361002
CZ6	SV	RED	E1/<100;E2/15102;E3/<100			KT361003