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-occurring 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, 2005; 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 12512, 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 CO₂, 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 H2O. 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).

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185Phylogenetic 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. (Huelsenbeck 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
different models of molecular evolution HKY+I for 18S data set; GTR+I+G for ITS 1, ITS 2 and 5.8S; GTR+G for \textit{cox} I and HKY+G for concatenated data. Convergence of runs was checked in AWTY (Nylander et al. 2008). Maximum-likelihood (ML) analyses were generated using the PHYML 3.0 software (Guindon and Gascuel 2003) and were performed under search parameters suitable for individual data sets mentioned above. Reliability of branching patterns within trees was tested by the bootstrap method with 1000 resamplings. 

\textit{Eimeria exigua} was used as an outgroup for all four datasets to root phylogenetic trees.

### Statistical analysis

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

In each case, we first explored full models and then obtained minimal models through backward selection of non-significant variables. Interpretation of significant factors with more
218than two levels was based on pair-wise t-tests of Differences of Least Square Means (DLSM),
219applying sequential Bonferroni correction for multiple comparisons.
220All statistical analysis were performed using SAS/STAT 9.4 software (Copyright © 2013,
221SAS Institute Inc., Cary, NC, USA).

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223Results

224Oocyst morphology

225Morphological examination of oocysts across the three host species revealed 3 morphotypes
226(Fig.1, 2), which differ in oocysts/sporocysts size (Tab. 1), morphological characteristics, host
227species, pattern of co-occurrence and prevalence.

228Morphotype E1

229Morphotype E1 was detected only in *S. vulgaris*. Morphotype E1 oocysts were large (Fig. 1,
2302), with piriform bottleneck shape and brown scabrous thick wall (~3 µm) with micropyle.
231Neither oocyst residuum, nor polar granule developed. The sporocysts were elongated and
232had flat Stieda body, each inside with 2 sporozoites encircle a residuum consisting of several
233~1 µm granules. The oocysts of *Eimeria* sp. E1 morphologically correspond with *E. mira*
234characteristics (Levine and Ivens 1965; Pellérdy 1974).

235Morphotype E2

236Morphotype E2 was detected in all 3 species of examined squirrels and the oocysts/sporocysts
237dimensions slightly differed between host species (Fig. 1, 2). Morphotype E2 oocysts were
238ellipsoideal to cylindrical with smooth, bi-layered wall 1-2 µm thick without micropyle. No
239oocyst 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* ($\chi^2 = 490.9; p < 0.0001$). 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).
*Eimeria* sp. E2 was the most prevalent morphotype across host species (for details on prevalence and 95% CI, see Fig. 1), being the only one infecting *C. erythraeus* (4.1%) and also the dominant type in mixed infections in both *S. vulgaris* (with E1 and E3) and *S. carolinensis* (with E3). Overall, morphotype E2 prevalence was 95.6 % in *S. vulgaris* and 95.7 % in *S. carolinensis* and it was found in all sites where these two hosts occurred, independently from host cohabitation (RED, GREY or RED-GREY). Mean E2 OPG values in red and grey squirrels were 7282 (± 968) and 13552 (± 1680), respectively. In both red and grey squirrels, E2 intensity of infection was affected by age class (Tab. 2, Fig. 3): OPG in red squirrels were significantly higher in adults than in juveniles or subadults (both p<0.02), whereas in grey squirrels we observed an opposite pattern, with adults significantly less infected than either juveniles or subadults (both p<0.05). In addition, E2 OPG in grey squirrels varied seasonally (Fig. 4), showing significantly lower values in summer than in all the other seasons (all p<0.02), whereas in red squirrels no such temporal variation was detected.

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

**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 22JQ993657 *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. mira*).
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), 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. Coccidium 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 66-45379%, 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. lan-
casterensis oocysts in grey squirrels were significantly less abundant during dry summer sea-
son. Also E3 prevalence varied seasonally in both hosts, although with slightly different pat-
terns (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 demo-
graphic changes in the host populations than in E3 characteristics. On the other hand, we can-
not 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 un-
til identification of morphotype E3 is made certain. Similarly, Eimeria infection in C. eryth-
raeus 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 (Kvíčerová 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.
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