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The invasive tree squirrel *Callosciurus erythraeus* in Italy:

Ecology, Management and Impact on the native squirrel *Sciurus vulgaris*

PhD Thesis

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Callosciurus erythraeus (Pallas, 1779)

… conservation is primarily not about biology

but about people and the choices they make.

Balmford and Cowling 2006

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ABSTRACT

The introduction of alien species is one of the most important causes of biodiversity loss and represents a long-term threat to ecosystem functioning. Invasive alien species (IAS) can alter the natural evolution of the native ones by competitive exclusion, hybridization, predation and niche displacement which can, ultimately, result in native species extinction. The outcome of the invasion process may be affected also by parasites, and infection transmission can become a threat to native species and human health. The best way to deal with the threat represented by IAS to biodiversity and society is through a combination of applied prevention measures, early detection and, when prevention fails, management and eradication of established invasive species.

In this study I investigated the competition mechanisms between the invasive Pallas's squirrel (*Callosciurus erythraeus*) and the native Eurasian red squirrels (*Sciurus vulgaris*) in Italy. The first necessary step, once confirmed the introduction and establishment of a new alien squirrel in the Northern Italy, was to identify the taxon (species) using an integrative approach performing morphometric analysis (skull measurements) and molecular investigations (variability at mtDNA markers). My analysis classified the species as *Callosciurus erythraeus*, the Pallas's squirrel, a tree squirrel native to Southeast Asia, known for its high invasiveness.

Pallas's squirrel in Italy co-occurs with the native Eurasian red squirrel, hence I explored different hypothesis of interspecific competition. To test competition for food resources, I compared the body mass and size of the native red squirrel in red-only areas with those of red squirrels in co-occurrence with the invasive squirrel. Results suggested that red squirrels in syntopy suffer from an interspecific competition that does not allow to reach the optimum body condition that they would have if the competitor was not present. This could be related to competition for food resources due to a change in the foraging efficiency of red squirrel when in syntopy, or to competition for cached food.

The next hypothesis was competition for space: squirrels were trapped and radio-tagged in different sites. Neither home range size, nor patterns of overlap of the alien and native species were affected by the presence of the other one. Patterns of interspecific home

range overlap suggested there was no spatial niche segregation between the two species. These results did not give support for inter-specific competition for space among adult red and Pallas's squirrels.

Competition between two species can also be mediated by parasites. I explored the parasite community of the Pallas's squirrel population and compared it to literature data from the native and introduced range, in order to detect any loss, acquisition or introduction of parasite species that may support enemy release hypothesis or potentially lead to parasite-mediated competition. Pallas's squirrel in Italy harbor a poor parasite fauna, characterized by two main ectoparasite species, the flea *Ceratophyllus* (*M.*) *sciurorum sciurorum* and the tick *Ixodes ricinus*, few helminths (*Rodentoxyuris sciuri*, *Trichuris muris*, *Strongyloides callosciureus*) and one *Eimeria* morphotype. The loss of native parasites might have contributed to their successful invasion. The acquisition of local parasites *C. sciurorum* and *T. sciuri*, and the introduction of the Asiatic nematode *S. callosciureus* may lead to spill-back and spill-over processes towards red squirrels, with potential impacts on this threatened native species.

In the following part of the study, I estimated the fecundity and reproductive output of the Pallas's squirrel as indicator of adaptation to the new environment and potential capacity to spread and recover after reduction of population size through control campaigns. I used a reliable staining technique of uterine scars that allows to determine individual fecundity in terms of both seasonal and total (annual) number of young born/female, and thus to define litter size at birth. Fecundity of the IAS in Italy was similar to what reported in the native range with 58% of females with a spring litter, 35% also with a summer litter and a few even with a third litter in autumn (10%). These results confirmed a good adaptation to the new environment, a higher fecundity than the native species and a potentially high capacity to spread and recover after reduction of population size.

Next, I tested the effect of the IAS on the red squirrel population dynamics and in particular I examined red squirrel population density, breeding rate and local survival in the red-Pallas area. I compared the red squirrel population parameters in the red-Pallas with a control red-only area. The reproductive success for red squirrels in co-occurrence was not affected, but the local survival rate was significantly lower (11%) than in the control area (50%). Consequently, comparison of the red squirrel density in the experimental and control area demonstrated a critically low number of red squirrels in co-occurrence (red-Pallas area: 0.05 ha^{-1}; red-only area: 0.39 ha^{-1}). Moreover, Pallas's squirrel density was 7.84 squirrels per hectare, almost 8 times higher than that of the native red squirrel.

Finally, after having removed more than 600 Pallas's squirrels, I identified the techniques to maximize trap efficiency, minimizing trapping effort, using live traps with the aim to improve the management practices of this species after a new introduction event or during ongoing control/eradication programs. Indications to improve trapping efficiency of this IAS were to (1) use single capture live traps with at least one week of prebaiting, (2) plan capture sessions 3 days long, (3) increase the capture effort in winter and (4) set traps where the forest access is easier because the vegetation did not affect capture success of Pallas's squirrel in deciduous forests.

Overall, the present study highlights the impact of invasive species on ecologically similar native species. In particular this research indicates that red squirrels are not yet locally extinct in the area of introduction of the invasive Pallas's squirrel, but they are strongly affected by interspecific competition. Pallas's squirrel is well adapted to the new environment and has a high reproductive and spread potential, probably in part due to parasite release. It successfully competes for food resources with the native squirrel which is gradually disappearing from the area. Red squirrels are probably forced to move to the borders of the distribution area to avoid Pallas's squirrels that occupy the high-quality habitat patches. Such border areas are generally of lower quality, in terms of degree of fragmentation, human disturbance and potentially forest composition, which seems to affect the persistence of the red squirrel population, in particular local survival of squirrels and consequently density.

Understanding the impacts of invasive tree squirrels is important for the conservation of native squirrels and the assessment of current distributions and knowledge of the invasive population's ecology is crucial in predicting the path of spread and planning ways to possibly reduce or eliminate the impacts on native populations. The eradication of the Pallas's squirrel in Italy is imperative to protect the long-term survival of the native squirrel population, but a long-term commitment and adequate resources, supported by the current ban of the species from pet trade, will be crucial to achieve this result.

INTRODUCTION

The introduction of alien species is one of the most important causes of biodiversity loss and represents a long-term threat to ecosystem functioning (Mack et al. 2000; Ehrenfeld 2010; Strayer 2012). The issue of biological invasions has long been recognized (Elton 2000), but the attention on alien species has increased over the last decade. The invasion of alien species is a process consisting of several stages, each with an independent probability of failure. To be defined as invasive, a species has to be able to establish population outside its distribution range, must have a fast demographic increase and should have an impact on native species and/or ecosystems (Kolar and Lodge 2002). In the past, human trade, transport, travel and tourism have dramatically helped the spread of invasive alien species. In recent decades the rate of new introductions and the number of invasive alien species (IAS) in Europe are increasing for all taxa (except mammals) raising extinction risk for many groups and affecting our health and economy (Clavero and García-Berthou 2005; European Environment Agency 2012). Introduced species can be advantaged in the new environment, increasing rapidly in numbers, due to a reduction or even absence of predators and/or parasites (so-called enemy-release hypothesis) (Wolfe 2002; Torchin et al. 2003; Romeo et al. 2014). Moreover, invasive species can alter the natural evolution of the native ones by competitive exclusion, hybridization, predation and niche displacement which can, ultimately, result in native species extinction (Kats and Ferrer 2003; Cox 2004; Largiadèr 2007; Ehrenfeld 2010). The outcome of the invasion process may be affected also by parasites, as has been reported for several introduced species, and infection transmission can become a threat to native species and human health (Torchin and Mitchell 2004; Dunn et al. 2012; Marsot et al. 2013; Mazza et al. 2013).

Invaders themselves evolve in response to their interactions with natives, as well as in response to the new abiotic environment (Elton 2000; Mooney and Cleland 2001; Ehrenfeld 2010). Interspecific competition, which occurs when two species co-evolve competing for the same resources, has negative effects on the individual, such as reduced survival and/or reproductive success, which will be reflected at the population level (distribution, population density, persistence). Among native species interspecific competition tends to shape some degree of niche differentiation, resulting in a stable and thus long-term coexistence (Emmons 1980; Riege 1991). However, If there is no (sufficient) niche differentiation and resources are limited, (Holway 1999; Byers 2000; Shuttleworth et al. 2015), it may ultimately cause the extinction of one of the two competing species (Ricciardi et al. 1998; Mooney and Cleland 2001). This problem is common when an alien species is introduced in a new environment where its niche will overlap with that of an ecologically similar native species (Broennimann et al. 2007). As opposed to naturally co-evolved competitor systems where co-evolution occurs, in the case of human-mediated introductions the two species (alien and native) come into contact abruptly, each one being in some way "trapped" by its evolutionary background. In most cases the only possible outcome, lacking the time for niche differentiation to evolve, is the establishment of a competitive exclusion mechanism with the alien species replacing the native one (Hardin 1960; Ricciardi et al. 1998; Strong and Pemberton 2000; Mooney and Cleland 2001; Bertolino 2008).

The best way to deal with the threat represented by IAS to biodiversity and society is through a combination of applied prevention measures (e.g., reducing or blocking pathways by which alien species can be introduced), early detection and prompt response to new invaders (European Environment Agency 2012). When prevention fails, eradication and management of established invasive species are the most concrete and economically advantageous responses to biological invasions to prevent or mitigate the negative impacts of those species on the environment and on native species (Wittenberg and Cock 2001; Veitch and Clout 2002; Genovesi et al. 2014; Adriaens et al. 2015). In Europe the economic costs of biological invasions are estimated to be at least EUR 12.5 billion per year (Kettunen et al. 2009). Eradication programs are now globally recognized as one of the keys to manage biological invasions and there is an increasing evidence of their success. In Europe, 37 successful eradication programs have been recorded, as the removal of the coypu and the muskrat from Great Britain, of rats, goats, rabbits and American minks from several small islands of the Macronesia, Mediterranean, Brittany, Great Britain and the Baltic sea (Genovesi 2005). Many European species belonging to different taxa have improved their conservation status as a result of eradications of invasive species (McGeoch et al. 2010). But it is important that countries establish stringent biosecurity policies and improve responsiveness after a detection of a new introduction in their territory (Harris and Timmins

2009; Simberloff et al. 2013; Genovesi et al. 2014) because, when the risk level for introduced species is underestimated, management actions become very difficult, expensive and often useless to prevent the loss of biodiversity as well as the economic losses that are already taking place (Bertolino and Genovesi 2003; Genovesi 2011).

That is, for example, the well known case of the Eastern grey squirrel (*Sciurus carolinensis*), native to North America and introduced in Great Britain, Ireland and Italy. The effects of the introduction were underestimated for several years causing the replacement of the native Eurasian red squirrel (*Sciurus vulgaris*) in most of its range, as well as serious impacts on trees due to bark stripping (Wauters et al. 2002b; Wauters et al. 2002a; Gurnell et al. 2004b; Mayle et al. 2009; Martinoli et al. 2010; Goldstein et al. 2014; Bertolino et al. 2014).

Among rodents, tree squirrels have been mainly introduced through the international pet trade for aesthetic reasons, or to increase hunting opportunities (Aprile and Chicco 1999; Long 2003) and their capability to establish viable populations from only a few founders has made them successful invaders (Palmer et al. 2007; Bertolino 2009; Martinoli et al. 2010).

In this study I have investigated the competition mechanisms between invasive alien species and ecologically similar native species; in particular between the alien Pallas's squirrel (*Callosciurus erythraeus*) and the native Eurasian red squirrels (*S. vulgaris*) in Italy.

The native range of this alien squirrel covers a large part of North-eastern South Asia, much of central and southern China, and mainland Southeast Asia (Wilson and Reeder 2005; Thorington et al. 2012; Bertolino and Lurz 2013). The story of the introduction as the number of squirrels released is still unknown, but the first sighting of the species in the province of Varese (Lombardy) was in 2007. The main pathway for the introduction of squirrels in Italy was the pet trade: in fact, grey and Pallas's squirrels could be traded and sold as pets until February 2013, which represented a big problem for any Italian eradication program for many years (Martinoli et al. 2010; Bertolino et al. 2014). Eventually, in 2013 a complete trade ban on three tree squirrel species (*S. carolinensis, S. niger, Callosciurus erythraeus*) was issued in Italy (Inter-Ministerial Decree n. 28 of February 2, 2013) following the EU wildlife trade regulation No. 338/97 (Annex B).

Pallas's squirrel has been introduced so far in seven different countries around the world (Argentina, France, Belgium, Italy, the Netherlands, China – Hong Kong and Japan), and of the 29 known introduction events 21 (including Italy) are documented as successful with a viable population established in the wild, whereas only two populations have been successfully eradicated (Bertolino and Lurz 2013; Lurz et al. 2013; Adriaens et al. 2015). This species causes damage by debarking commercial trees in forest plantations, eating fruits in orchards, and gnawing parts of buildings, cables and irrigation systems (Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). Moreover, in Japan the native squirrel species, *Sciurus lis*, is locally declining probably because of competition for food and nesting sites (Ministry of the Environment, Japan, 2002; Miyamoto et al. 2004) and in Argentina the risk of negative impacts on native squirrel species, if a translocation of the invasive squirrel occurs, is considered high (Cassini and Guichón 2009). In its current range in Italy, this Asian tree squirrel co-occurs with the native Eurasian red squirrel, in areas where the grey squirrel is not present. However, detailed hypotheses about the mechanisms of interspecific competition between Pallas's squirrel and native squirrels have not been investigated so far.

In this research, to be able to highlight any effect of the invasive squirrel on the native Eurasian red squirrel, the first necessary step was to identify the species of the new squirrel introduced in Northern Italy using an integrative approach performing morphometric analysis (skull measurements) and molecular investigations (variability at mtDNA markers) (**Chapter 1**). Next, I explored different hypothesis of interspecific competition when the two species co-occur: competition for food resources that can affect the red squirrel's body condition and have many effects not just on the life of the single animal but can also be reflected at the population level with long-term effects on its persistence, distribution and/or reproductive rate (**Chapter 2**); competition for space predicting red squirrel space use changes if competition occurs (**Chapter 3**). I also explored the parasite community of the Pallas's squirrel population and compared it to literature data from the native and introduced range, in order to detect any loss, acquisition or introduction of parasite species that may support enemy release hypothesis or potentially lead to parasite-mediated competition (**Chapter 4, 5**). Moreover, I estimated the fecundity and reproductive output of the Pallas's squirrel as indicator of adaptation to the new environment and potential capacity to spread and recover after reduction of population size through control campaigns (**Chapter 6**); then I presented the results of the Pallas's squirrel eradication program in Italy and its effect on the red squirrel population dynamic (**Chapter 7**). Finally I identified the techniques to maximize trap efficiency, minimizing trapping effort using live traps with the aim to improve the management practices of this species after a new introduction event or during ongoing control/eradication programs (**Chapter 8**).

CHAPTER 1
Preventing species invasion: a role for integrative taxonomy?

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Abstract

Integrative taxonomy, a multi-disciplinary approach adding modern techniques to traditional morphology-based methods (e.g., molecular variability and morphological criteria), can play an important role in bioinvasion research to identify introduced taxa, discover pathways of introduction and inform authorities to control and prevent future introductions. This study is the first on introduced populations of *Callosciurus*, Asiatic tree squirrels, known as potentially invasive species in Europe (Italy, Belgium and France). We combined molecular (variability at mitochondrial DNA markers CoxI, D-loop) and morphometric analysis on skulls, comparing them to the widest morphological and molecular datasets ever assembled for *Callosciurus*. Squirrels collected in Italy and Belgium shared the same haplotypes and skull characteristics but were conspicuously different from the French population in Antibes. Genetic data revealed close similarity between French squirrels and Pallas's squirrels, *C. erythraeus*, from Taiwan. Italian and Belgian squirrels formed an independent taxonomic lineage in genetic analyses, whose taxonomic rank needs further investigation. The morphological and morphometric characteristics of these two populations were however similar to known specimens assigned to *C. erythraeus*. These results may indicate a common origin for the populations found in Belgium and Italy. In contrast, French specimens suggest an independent introduction event of squirrels originating from Asia.

Introduction

The introduction of alien species is one of the most important causes of biodiversity loss and represents a long-term threat to ecosystem functioning (Mack et al. 2000; Ehrenfeld 2010; Strayer 2012). Modern management strategies to reduce the overall risks associated with the spread of invasive species are based on prevention (e.g., reducing or blocking pathways by which alien species can be introduced), early warning systems and a quick and efficient response to eradicate the species (Wittenberg and Cock 2001; Bertolino and Genovesi 2003; Bertolino and Lurz 2013).

To achieve this goal, it is necessary to obtain permits and decrees to manage the invasive species over the short term (e.g., obtaining blocking importation, permits for eradication and control) and to prevent or mitigate negative impacts (Mack et al. 2000; Grosholz 2005). Thus, critical first steps are the accurate identification of the invading taxon and its source area (e.g., Pisanu et al. 2013), and the detection of potential pathways to avoid delay in decisions for preventive control actions (Boykin et al. 2011).

Similar studies have focused on the well-known case of the invasive Eastern grey squirrel *Sciurus carolinensis* Gmelin, 1788, native to North America, where molecular markers were used to assess the origin and spread dynamics of the introduced populations (Hale et al. 2001; Signorile et al. 2014). Taxon identification is sometimes challenging. One reason is misidentification when sibling species occur (Galimberti et al. 2012a). A second reason is that many mammal taxa are still described using only morphological parameters which can be poor descriptors (Shoshani and McKenna 1998; Wilson and Reeder 2005). Moreover, there are also different views and debate on the species concept (Zachos and Lovari 2013; Gippoliti et al. 2013). These problems place the discipline of taxonomy at the forefront of invasive species research. Integrative taxonomy is a multi-disciplinary approach to traditional taxonomy where the morphological features are combined to other approaches and additional data (e.g., molecular, behavioral, developmental, ecological, etc.) (Dayrat 2005; Flagella et al. 2010; Padial et al. 2010; Wu et al. 2011). Nowadays, many studies in the field of bioinvasion are conducted with an integrative approach where molecular markers and morphological features are two complementary, independent, systems of identification, each using a separate set of criteria (Gotzek et al. 2012; Pisanu et al. 2013).

Among rodents, tree squirrels have been mainly introduced through the international pet trade for aesthetic reasons, or to increase hunting opportunities (Aprile and Chicco 1999; Long 2003) and their capability to establish viable populations from only a few founders has made them successful invaders (Palmer et al. 2007; Bertolino 2009; Martinoli et al. 2010). In the last decade a new alien invasive tree squirrel of the genus *Callosciurus* Gray, 1867, native to South-East Asia, has established a wild population in Northern Italy (Mazzamuto et al. 2015) in co-occurrence with the native Eurasian red squirrel *Sciurus vulgaris* Linnaeus, 1758. The history of *Callosciurus* invasions is recent and there is still little information on the impacts of these squirrels at the local scale despite their high invasiveness worldwide (Bertolino and Lurz 2013). A few *Callosciurus* species are well known to damage infrastructures and to debark forest trees and orchards (Noor 1992; Bertolino et al. 2004; Tamura and Ohara 2005; Hori et al. 2006). Moreover, experience with other invasive tree squirrels (e.g., *Sciurus carolinensis*) has shown that native species may be affected by competitive interactions arising from niche overlap such as food competition and parasite spill-over (Wauters et al. 2002a; Gurnell et al. 2004b; Romeo et al. 2013; Romeo et al. 2014).

Recent works have highlighted the growing importance of DNA barcoding in clarifying the taxonomic status and provenance of sciurid populations (see for example Gabrielli et al. 2014; Stevenson-Holt and Sinclair 2015; Ermakov et al. 2015). These studies also provided evidence for the importance of molecular surveys in management and conservation actions on sciurid species.

Although there are 15 species in the genus *Callosciurus*, some of which are morphologically very similar (Wilson and Reeder 2005), the two species most commonly introduced worldwide are the Finlayson's squirrel *C. finlaysonii* (Horsfield, 1823), native to Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Vietnam and the Pallas's squirrel *C. erythraeus* (Pallas, 1779), native to south-east China, eastern India, Malaya, Indochina, Bhutan and Taiwan (Corbet and Hill 1992; Oshida et al. 2001; Wilson and Reeder 2005; Thorington et al. 2012; Lurz et al. 2013). *C. finlaysonii* has established self-sustaining populations in Italy, Singapore and Japan, while *C. erythraeus* has been introduced to Argentina, France, Belgium, The Netherlands, Hong Kong and Japan (Bertolino and Lurz 2013). These species cause damage by debarking commercial trees in forest plantations, eating fruits in orchards, and gnawing parts of buildings, cables and irrigation systems (Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). In Argentina and Japan, the risk of negative impacts by introduced *C. erythraeus* on native squirrel species is also considered high (Miyamoto et al. 2004; Cassini and Guichón 2009; Tamura 2009).

A first phenotypic analysis of *Callosciurus* specimens from Belgium, France and Italy using body size, body mass and coat color as key characteristics, assigned them to *Callosciurus erythraeus*. However, this species is highly variable in fur color and body measurements and, so far, only morphological characters have been used to describe and differentiate the 25 subspecies (Wilson and Reeder 2005). According to this approach, 18 subspecies of *C. erythraeus* were previously assigned to a distinct species (i.e. *C. flavimanus*) (Ellerman and Morrison-Scott 1951; Moore and Tate 1965; Corbet and Hill 1992), whereas recent molecular analysis suggested that the subspecies *C. e. griseimanus* is genetically distinct from other *C. erythraeus* subspecies and other *Callosciurus* species (Oshida et al. 2013). In these cases, modern taxonomy standards call for several biological criteria being taken into account to pursue reliable species identification (Larsen 2001; Malhotra and Thorpe 2004; Galimberti et al. 2012b).

In 2014, Gabrielli and co-workers, carried out the genetic characterization of the

Table 1. Specimens whose cranial measurements were included in PCA analysis (M= male; F= female; Un= sex unknown; n= number of specimens).

Species	Origin of specimens	M	F	Un	n
Callosciurus caniceps (Gray, 1842)	"Andrea Doria" Natural History Museum	9	9	8	26
Callosciurus erythraeus (Pallas, 1779)	"Andrea Doria" Natural History Museum	2	2	8	12
Callosciurus melanogaster (Thomas, 1895)	"Andrea Doria" Natural History Museum	-	-	9	9
Callosciurus nigrovittatus (Horsfield, 1824)	"Andrea Doria" Natural History Museum	11	8	2	21
Callosciurus notatus (Boddaert, 1785)	"Andrea Doria" Natural History Museum	7	8	7	22
Callosciurus phayrei (Blyth, 1856)	"Andrea Doria" Natural History Museum	12	16	13	41
Callosciurus prevostii (Desmarest, 1822)	"Andrea Doria" Natural History Museum	-	1	1	2
Callosciurus pygerythrus (I.Geoffroy Saint Hilaire, 1833)	"Andrea Doria" Natural History Museum	-	-	2	2
Callosciurus finlaysonii (Horsfield, 1823)	"Andrea Doria" Natural History Museum	-	-	2	
	Potenza, Italy	3	8	-	14
	Natural History Musuem, Milan	1	-	-	
Callosciurus sp.	Dadizele, Belgium	11	14	-	25
Callosciurus sp.	Antibes, France	7	8	-	15
Callosciurus sp.	Varese, Italy	7	7	-	14
	Total	**70**	**81**	**52**	**203**

introduced Argentinean *Callosciurus* populations. These squirrels had been initially assigned to *C. erythraeus*; however, genetic analyses depicted a more complicated situation for both the studied population and the taxonomic status of the whole *Callosciurus* genus. One of their conclusive remarks was a plea for a multi-criteria taxonomic approach for this group of squirrels.

In the present study, we adopted an integrative approach using specimens of the three populations introduced in Europe to i) assess the taxonomic status of specimens assigned to C. cfr. *erythraeus*; ii) study and compare morphological and genetic characteristics among specimens; iii) assess the provenance of European *Callosciurus*. Morphometric analysis (skull measurements) and a molecular investigation (variability at mtDNA markers) were performed and compared to the widest morphological and molecular datasets ever assembled for *Callosciurus* with samples originally collected in their native range and in localities inhabited by introduced populations.

Materials and methods

Specimen collection

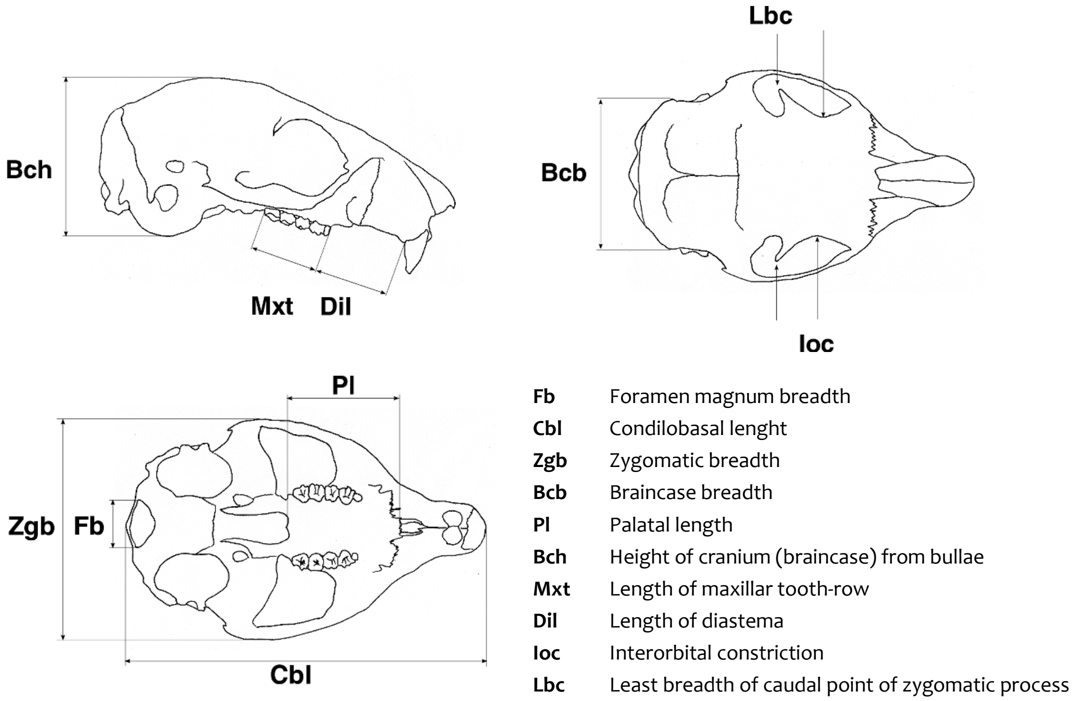
Natural history museum collections represent an important resource for bioinvasion research as they host good references for proper species identification (Wandeler et al. 2007). Morphological observations were based on a total of 203 skulls of adult *Callosciurus* specimens (70 males and 81 females, 52 of unknown sex). Specifically we used 138 *Callosciurus* specimens collected in Asia and at the "Andrea Doria" Natural History Museum and the Natural History Museum of Milan, 11 specimens of *C. finlaysonii* collected from an introduced population in Southern Italy (Potenza), and 54 skulls of *Callosciurus* cfr. *erythraeus* introduced in Italy, Belgium and France (Table 1) (Aloise and Bertolino 2005; Bertolino and Lurz 2013).

In Italy and Belgium, the introduced squirrels were captured using Tomahawk live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA) baited with apples and hazelnuts. Traps were set in the morning and checked at least twice a day to minimize stress during trapping. For each trapped squirrel sex and reproductive condition were recorded following (Wauters and Dhondt 1989) with lactating females (large nipples, milk excretion if stimulated) being immediately released. All males and non-reproductive females were euthanized by CO_2 inhalation following EC and AVMA guidelines (Close et al. 1996; Close et al. 1997; Leary et al. 2013). In France, all live-trapped individuals were immediately killed by cranial shock according to the current French ethic statements (Ethics Committee Cuvier: MNHN, Sorbonne Universities, agreement N° 68-012) and following the European Union recommendations (Annex IV Directive 63 EU). Each individual was weighed to the nearest 5 g using a Pesola spring-balance.

Morphometric analysis

We performed 10 measurements of the cranium (Fig. 1). Skull measurements, always taken by the same operator, were scaled at 0.02 mm by vernier calipers and they were analyzed by Principal Component Analysis (Flury 1997) using the stats R package (Venables and Ripley 2002; R Development Core Team 2014). Standardization and scaling were carried out automatically by the princomp R function.

A first Principal Component Analysis (PCA) took into account the whole data set, including incomplete biometric records (i.e. measurements derived from incomplete specimens). In accordance with standard practice in PCA data conditioning, missing values were imputed by substituting them with the mean value of the relative variable.



Fb	Foramen magnum breadth
Cbl	Condilobasal lenght
Zgb	Zygomatic breadth
Bcb	Braincase breadth
Pl	Palatal length
Bch	Height of cranium (braincase) from bullae
Mxt	Length of maxillar tooth-row
Dil	Length of diastema
Ioc	Interorbital constriction
Lbc	Least breadth of caudal point of zygomatic process

Figure 1. Skull measurements used in this study. Abbreviations explained in the table.

Since most of the measurements were highly autocorrelated, we performed a second PCA using a "reduced" dataset, i.e. considering only the variables with the highest scores and those that were most morphologically sensible. In this case the variables used were: condilobasal lenght (Cbl), zygomatic breadth (Zgb), palatal length (Pl), height of braincase from bullae (Bch).

For both PCAs, 95% confidence ellipses were calculated using the ggbiplot version 0.55 R package (Vu 2011) and the ggplot2 R package (Wickham 2009).

Molecular analysis

Sampling, DNA extraction, amplification and sequencing

A total of 17 tissue samples were collected from the three introduced *Callosciurus* cfr. *erythraeus* populations in Europe: five from Brezzo di Bedero, Varese province, Italy (45°58'09.2" N, 08°43'57.6" E), six from Dadizele, Moorsele province, Belgium (50°51'05.5" N, 03°05'40.3" E) and six from Antibes, Alpes-Maritimes district, France (43°33'51.0" N, 07°07'27.0" E). The sampling dataset also includes two samples of *C. erythraeus* from Yanyuan and Muli counties, Sichuan province, People's Republic of China (hereafter PRC, 27°38'23.7'' N, 101°48'43.8" E and 28°9'23.3" N, 100°48'43.8" E respectively) and three samples of *C. finlaysonii* from the two known Italian populations, introduced in recent years: Maratea, Potenza province and Acqui Terme, Alessandria province (39°59'35.3" N, 15°42'22.2" E and 44°40'25.0" N, 8°28'23.6" E , respectively; sampling details are provided in Supplemental material 1). The latter species is not yet characterized by DNA barcoding.

Animals were trapped and manipulated as described previously and all specimens were morphologically identified prior to sequencing by expert field operators. A 4-mm diameter sample of skin was taken from one ear for genetic analysis using a biopsy punch. Samples were vouchered and then stored in 99% ethanol at -20°C. Voucher codes are listed in Supplemental material 1.

DNA was extracted from a 3 mm (diameter) tissue punch by using the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) following manufacturer's instructions. Purified DNA concentration of each sample was estimated fluorometrically with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA) by measuring the absorbance at 260 nm.

Molecular characterization of European *C.* cfr. *erythraeus* populations was conducted by analyzing sequence variability at two mitochondrial DNA markers and comparing this data with sequences already available in GenBank (see Supplemental material 1). We analyzed the standard DNA barcoding region for metazoans (i.e. 648 bp at the 5' end of *CoxI* as described by (Hebert et al. 2003) and a fragment (1080 bp long) of the control region (D-loop). These markers were chosen because of their growing relevance in the field of modern integrative taxonomy (see for example Galimberti *et al.*, 2012a; Ermakov *et al.*, 2015) and because a huge number of D-loop sequences is available for the species group treated in this study (Gabrielli et al. 2014). Moreover, the two markers typically show high variability rates, an essential condition to better highlight similarities and differences among different species (*CoxI*) and even populations (D-loop).

CoxI fragment was amplified using universal primers LCO1490 and HCO2918 (Folmer et al. 1994) with the thermal profile described in Bellati *et al.* (2014). Amplification of D-loop region was performed with primer pair L15933 – H637 (Oshida et al. 2001) using the thermal conditions described in (Oshida et al. 2006). In both cases, PCRs were conducted in a 25 µL reaction (including 10 ng of DNA as template) by using puReTaq Ready-To-Go PCR beads (Amersham

Bioscience, Freiburg, Germany), according to manufacturer's instructions. After checking for amplicons presence and quality on 1.5 % agarose gel, sequencing was carried out on both strands using an ABI 155 3730XL (Macrogen Inc., Seoul, Korea), with the same amplification primers.

Data analysis

Sequences were corrected by visual inspection of automated sequencer chromatograms in BIOEDIT 7.1 (Hall 1999). Codons were then translated into amino acid sequences to check for the presence of nuclear pseudogenes using MEGA6 (Tamura et al. 2013). To avoid the inclusion of nuclear sequence of mitochondrial origin (i.e. NUMTs, Nuclear Mitochondrial DNAs, Bensasson *et al.*, 2001) we also followed the guidelines proposed in Song *et al.* (2008) and (Buhay 2009). Sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI) (see Supplemental material 1 for accession numbers – ACCESSION NUMBERS WILL BE PROVIDED UPON PAPER ACCEPTANCE). Bioinformatics analyses were conducted separately on the two datasets (i.e. *CoxI* and D-loop). Except for the samples analyzed in this study, very few specimens among those reported in GenBank had sequences for both mitochondrial markers. As far as the *CoxI* is concerned, we considered the 17 samples of *C.* cfr. *erythraeus* obtained in the three European naturalized populations, two samples of *C. erythraeus* from its native range in People's Republic of China, three samples of Italian *C. finlaysonii.* We also used 33 public available sequences of the genus *Callosciurus*, including accessions of *C. erythraeus* from native (PRC) and introduced range (Argentina), *C. notatus*, *C. orestes* and *C. prevostii*. The D-loop dataset encompassed *Callosciurus* sequences obtained in this study and 99 GenBank sequences of *C. erythraeus* belonging to native (China and Taiwan) and introduced (Japan and Argentina) ranges, *C. finlaysonii* and *C. prevostii*.

Sequences of each marker were aligned using MUSCLE online (http://www.ebi.ac.uk/Tools/msa/muscle/; (Edgar 2004) with default options. For both datasets, the number of haplotypes and nucleotide diversity per site and per species were computed with DnaSP v. 5.10.1 software (Librado and Rozas 2009).

The taxonomic status of *C.* cfr. *erythraeus* was also tested by comparing obtained *CoxI* data with private sequences stored in the Barcode of Life Database using the

Identification Engine tool (IDS) (http://www.boldsystems.org/index.php/IDS_OpenIdEngine; Species Level Barcode Records database), which returns unique species assignments based on 99% sequence similarity of the barcode sequence. Moreover, the DNA barcoding dataset (i.e., *CoxI*), was explored for species limits and barcoding gap occurrence with ABGD tool (Automatic Barcode Gap Discovery, available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html; Puillandre *et al.*, 2012). This software aims at finding the genetic distance at which barcode gap occurs and groups sequences into lineages corresponding to putative species without any *a priori* hypothesis. Analysis results can be finally compared with other taxonomic approaches (e.g., morphology) within an integrative taxonomy perspective. ABGD was run with default settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, X relative gap width = 1.5, Nb bins = 20) and Kimura distance model.

For both datasets, average genetic sequence divergences (and relative standard errors, SE) between and within distinct lineages were calculated and a NJ reconstruction was performed for both molecular regions using MEGA 6 with the same settings described in (Galimberti et al. 2012a). Although more sophisticated tree building methods are available for deep branch resolution, several studies confirmed that in a DNA barcoding context the NJ approach is was sufficient to resolve relationships at terminal branches (see for example Hebert et al. 2004; Kerr et al. 2009).

Results

Morphometric analysis

The fur color of all museum specimens was analyzed to confirm species identification (Moore and Tate 1965; Corbet and Hill 1992; Thorington and Hoffmann 2005; Lurz et al. 2013; Amori and Doria). *Callosciurus* squirrels from the introduced populations in Italy and Belgium showed a low variability, all individuals displaying yellow-creamy ventral fur and no black stripe on their backs. The French population, however, showed a red mahogany ventral fur with some individuals having an agouti ventro-central line and no black stripe on the back (Supplemental material 2). Based on Moore & Tate (1965), Corbet & Hill (1992) and Thorington & Hoffmann (2005), all the specimens from Europe could be included in the species *C. erythraeus.* According to pelage characteristics the French population could be included in three different subspecies: *C. e. erythrogaster* (Blyth, 1842: from India and Burma), *C. e. castaneoventris* (Gray, 1842: from China) or *C. e. thaiwanensis* (Bonhote, 1901: from Taiwan).

The Italian and Belgian population could be assigned to the subspecies *C. e. styani* Thomas, 1894 (from China). For details on the pelage colors and skull measurements see Supplemental material 2, 3.

In the PCA performed on skull measurements the first PCA axis (PC1) explained 75.1% of the variance. The first principal component was correlated with three measurements: it increases with decreasing Cbl (-0.56), Zgb (-0.53) and Pl (-0.52). The second principal component (explained variance 16.4%) is strongly correlated with Bch (-0.91) suggesting that it is primarily a measure of the Bch. Fig. 2 indicates that *Callosciurus* from Italy and Belgium have similar skulls and both of them mostly overlap with *C. notatus*, *C. melanogaster* and *C. erythraeus* reference samples. On the contrary, *Callosciurus* skulls from France seem to be separated from the Italian ones and exhibit a little overlap with the Belgian *Callosciurus.* In fact, they overlap much more with museum specimens of *C. notatus*, *C. melanogaster*, *C. phayrei* than with *C. erythraeus*.

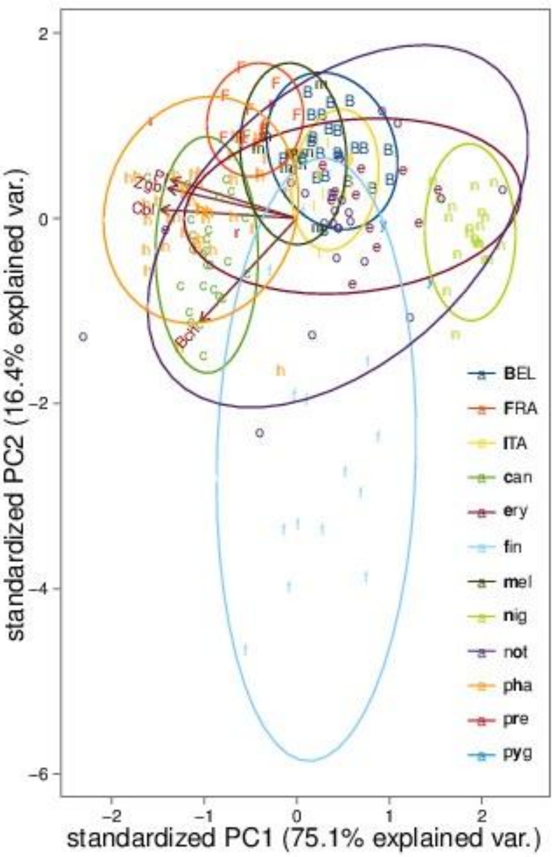


Figure 2. Principal Component Analysis biplot for the first two canonical axes (cumulative variance explained: 91.5%). Ellipses are 95% confidence intervals for each species or alien population. In the legend lowercase abbreviations indicates species names, uppercase abbreviations indicate the three introduced populations. B= Belgium, F= France; I= Italy; c= *C. caniceps*; e= *C. erythraeus*; f= *C. finlaysonii*; m= *C. melanogaster*; n= *C. nigrovittatus*; o= *C. notatus*; h= *C. phayrei*; r= *C. prevostii*; y= *C. pygerythrus*.

Molecular characterization

Alignments characteristics

DNA extracted from the ethanol preserved tissues was of high quality (ratios of absorbance, $A_{260/280}$ and $A_{260/230}$ ~ 1.80 and >1.90, respectively) and provided good yields (> 50 ng/µl). Amplification with the selected primer pairs was successful and resulting DNA concentration of purified amplicons was >50 ng/µl. High quality sequences were obtained for all the individuals sampled for the present study. Due to different lengths of GenBank sequences, we trimmed the alignments to the same final lengths of 622 bp and 511 bp for *CoxI* and D-loop, respectively. Regarding the *CoxI* DNA barcoding dataset, no sequence contained insertion/deletions (indels), stop codons or were biased by NUMT interference. Alignment analysis revealed average base composition as π_A: = 25.2, π_C = 27.1, π_G = 16.8 and p π_T = 30.9 %. Concerning D-loop, the multiple alignment showed seven indel positions, which were concentrated in the variable 5'-end variable domain, and 184 variable sites of which 155 parsimony informative were detected. The *CoxI* alignment contained 172 variable positions, of which 158 were parsimony-informative and a mean transition/transversion ratio (over all sequence pairs) of 7.907. Supplemental material 4 reports the number of haplotypes and values of nucleotide diversity (Π) of (Nei 1987) per population as well as the overall values for *C. erythraeus* and its sibling taxon *C. finlaysonii*.

Concerning *C.* cfr. *erythraeus* European populations, which have been investigated for the first time in this study, two different haplotypes were found at each marker. Interestingly, both the Italian and Belgian populations shared the same haplotypes for both markers, whereas French *Callosciurus* showed a different haplotype. Italian *C. finlaysonii* populations shared a single haplotype at *CoxI*, whereas at D-loop, they show two haplotypes. Moreover, the haplotypes found in European populations of *C.* cfr. *erythraeus* and *C. finlaysonii* had not been previously observed in other populations with the only exception of the *C. finlaysonii* individual from Acqui Terme (North-West Italy) that is identical at D-loop to a squirrel from Thailand (see Supplemental material 1). Overall, haplotype and nucleotide diversity of morphologically identified *C. erythraeus* and *C. finlaysonii* groups from native ranges were very high if compared to single population values and especially to European populations (Supplemental material 4).

DNA barcoding characterization

When using the BOLD-IDS tool on the European samples, no taxonomic assignment was possible relying on the identification threshold posed by the system. The ABGD approach on the DNA barcoding dataset resulted in 10 groups for the recursive partition with prior values ranging from 0.001 to 0.022 and three groups with prior values of 0.036 and 0.060. The primary partition was stable on the whole range of prior values and the ten groups were coherent with the lineages identified by the NJ approach (see Fig. 3). Considering these lineages as putative different taxonomic units, the mean ± standard error of K2P distance between groups (Supplemental material 5) was 12.6 ± 5.9 % (range: 3% – 19.9%). French *C.* cfr. *erythraeus* population was closer to the group encompassing Belgian and Italian squirrels (4.2 ± 0.8 %), and the nearest neighbour of both groups being represented by *C. erythraeus* from Hainan province of PRC (4.8 ± 0.9 % and 5.0 ± 0.9 %). Interestingly, the lineage including the two *C. erythraeus* from Sichuan (PRC) sequenced in this study was closer to *C. finlaysonii* group (3.5 ± 0.7 %), whereas its K2P distance to the other two lineages belonging to Chinese *C. erythraeus* was two times higher (see Supplemental material 5 and Fig.3).

D-loop variability

Differently from the case of *CoxI*, the higher number of D-loop sequences available in GenBank permitted to better define relationships among different species (and geographic populations) of *Callosciurus* squirrels.

Both NJ reconstruction (see Fig. 3) and the K2P genetic distance matrix (Supplemental material 6) confirmed the marked difference among European populations of *C.* cfr. *erythraeus* (11.4 ± 1.4 % Italy and Belgium *vs.* France). Although the mixed Italian and Belgian lineage remained still taxonomically uncharacterized with Taiwan populations being their nearest neighbor (12.0 ± 1.34 %), French *Callosciurus* resulted even closer to *C. erythraeus* from Taiwan (3.6 ± 0.6 %). Once again, the lineage including the two Chinese samples of *C. erythraeus* from Sichuan analyzed in this study (C_ery_PRC_II, see Supplemental material 6 and Fig. 3), resulted quite similar to *C. finlaysonii* (8.8 ± 1.0%) and even more to the invasive population of Argentinean squirrels (8.4 ± 1.2 %). In contrast, they are highly divergent from other *C. erythraeus* (C_ery_PRC_I, see Supplemental material 6 and Fig.4) sampled in the same province (14.0 % ± 1.9%).

Discussion

As far as we know, the present study is the first on the taxonomy of the European introduced populations of *Callosciurus*. The integrative approach employing molecular and morphometric data allowed a more complete view on the identification and provenance of the three populations. Our molecular dataset is the widest ever assembled for the study of *Callosciurus* species with new haplotypes never described by previous studies and deposited in public databases. Moreover, museum collections confirmed their important role as a great source of knowledge (Wandeler et al. 2007), allowing the comparison of the introduced specimens of unknown taxonomical position with reference specimens. The analyses conducted in this study provided three main findings concerning the investigated European *Callosciurus* populations.

The first important result emerging from molecular analyses is that the introduced *Callosciurus* of Italy and Belgium shared the same haplotypes for both markers, forming a supported MOTU (Molecular Operational Taxonomic Unit *sensu*(Floyd et al. 2002) that was conspicuously different from that of the French population. Morphometric data supported this molecular result: skull size measures of the Italian and Belgian groups were totally overlapped, while the French one was clearly separated from the Italian group and bordered little with the specimens from Belgium. Moreover, squirrels from Italy and Belgium had the same fur color unlike the French squirrels.

The second relevant finding of this study concerns the taxonomic implications of our results. Relying on external morphological characteristics all the investigated European populations were supposed to belong to the species *C. erythraeus*. At the molecular level, the two European MOTUs were included into the putative '*erythraeus*' group in our NJ reconstructions, thus partly confirming the field identification. Surprisingly, in the *CoxI* dataset, the two MOTUs formed two new independent lineages never described before, whereas the French one was included into the MOTUs of *C. erythraeus* from Taiwan in the D-loop dataset (Fig. 3, 4). K2P distance matrix and the comparison with the identification thresholds for sciurid species recently calculated by different authors (Gabrielli et al. 2014; Ermakov et al. 2015) suggest that the MOTU encompassing Italian and Belgian samples formed an independent taxonomic lineage at both markers, whose taxonomic rank (e.g., species or subspecies) needs to be further investigated.

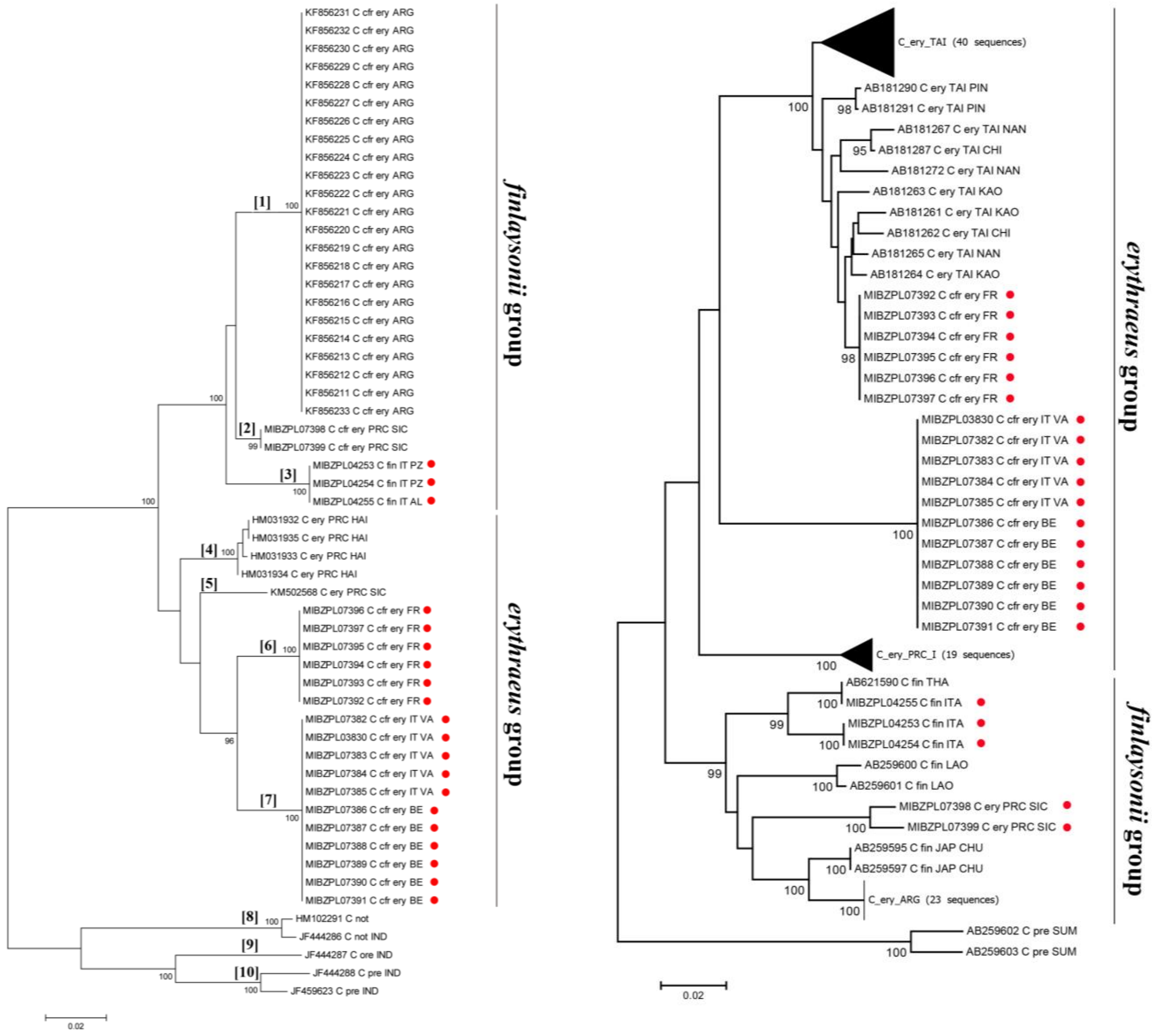


Figure 3. Neighbour joining tree based on CoxI sequences (on the left) and D-loop sequences (on the right) of *Callosciurus* generated with MEGA.

Numbers in square brackets indicate the different lineages resulting from the ABGD analysis. For each squirrel, voucher number and sampling locality are also provided (further details can be retrieved from Supplemental material 1). Bootstrap support (1000 replicates) values >90% are indicated above the nodes. *Callosciurus* species names are abbreviated as follows: *ery*-erythreaeus; *fin*-finlaysonii; *not*-notatus; *ore*-orestes; *pre*-prevostii. The countries where the different lineages and species occur are abbreviated as follows: ARG-Argentina; PRC-People's Republic of China; IT-Italy; FR-France; BE-Belgium; TAI-Taiwan; THA-Thailand; LAO-Laos; JAP-Japan. European samples newly sequenced in this study are marked with red dots.

A similar scenario was found for introduced *Callosciurus* in Argentina, where exhaustive phylogenetic and DNA barcoding analyses revealed the occurrence of a new lineage more closely related to *C. finlaysonii* than to *C. erythraeus*, in contrast to what was assumed during sampling (Gabrielli et al. 2014). Interestingly, the same unexpected misidentification occurred in our dataset for the two morphologically recognized *C. erythraeus* collected in the Sichuan province (PRC). Both markers used in our study indicated that these samples constitute a new lineage closer to *C. finlaysonii* and to the Argentinean samples, even though their taxonomic status is still unknown. As recently reported by Ermakov *et al.* (2015), such a situation should also be evaluated considering possible introgressive hybridization events and/or incomplete lineage sorting of mtDNA haplotypes. Both phenomena can lead to misidentification when comparing morphological and molecular data as frequently documented in bats (Nesi et al. 2011; Galimberti et al. 2012b), squirrels (Chang et al. 2011; Ermakov et al. 2015) and even *Callosciurus* species (Oshida et al. 2007; Kuramoto et al. 2012). In this context, the use of fast-evolving nuclear markers and a wider sampling coverage in native *C. erythraeus* distribution range would allow is likely to better characterize of the taxonomic status of Belgian and Italian populations. Finally, the analysis of D-loop variability also revealed a certain degree of variation between the two Italian populations of *C. finlaysonii* (K2P divergence of 3.5 ± 0.8%) that deserve to be further investigated after additional sampling.

The PCA performed on skull measurements indicates that *Callosciurus* from Italy and Belgium have similar skulls, both of them overlapping with *C. notatus*, *C. melanogaster* and *C. erythraeus* museum reference samples. However, *C. notatus* and *C. melanogaster* are very different in their body morphology (i.e. size, fur color patterns) from Belgian and Italian *Callosciurus* (Moore and Tate 1965), thus confidently suggesting to classify the latter as *C. erythraeus*. In contrast, *Callosciurus* skulls from France are similar to the museum specimens of *C. phayrei* and *C. melanogaster*, but also in this case these species are different in fur color and body size (Moore and Tate 1965). We also want to underline that the specimens of *C. finlaysonii* analyzed by PCA are mostly from the Italian introduced population confirmed as belonging to this species by molecular analyses. Skull measurements of this introduced population could be influenced by founder effects mostly referable to captive-bred stocks used for pet trade (see also Ashton & Zuckerman, 1950; Yom-Tov *et al.*, 1999; Dlugosch & Parker, 2008). Therefore, it is necessary to measure skulls of *C. finlaysonii* specimens from the native range to better compare molecular and morphological data.

The third main result of our investigation concerns the assessment of the provenance of European *Callosciurus*. Molecular data allowed to hypothesize a common origin for the populations found in Belgium and Italy. The single haplotype shared between these populations at both markers may suggest an introduction event in Belgium (Stuyck et al. 2009; Adriaens et al. 2015) followed by transport of captured animals and illegal release into the Italian site. In fact while the Belgian introduction occurred in the early 2000s, the first Italian sighting was recorded in a small area in Varese province in 2007. Interestingly such area is called "Villaggio Olandese" (Dutch Village) is known for the large number of vacation houses belonging to people from Belgium and the Netherlands. However, the origin of the Belgian population still remains unknown. On the contrary, as revealed by D-loop data and fur description, French *Callosciurus* probably originated from an independent introduction event of Taiwanese squirrels to France. Even though no document that ascertains the origin of *Callosciurus* in France, we know that a very small number of squirrels were imported directly from Asia by a single person in the 1960s (Chapuis and Pisanu pers. comm.).

In conclusion, combining morphological data and sequence variability at two mitochondrial markers, it was possible to clarify issues related to the taxonomy and provenance of the introduced European *Callosciurus*. Regarding the French specimens, both external morphology and molecular diagnosis confirm the Taiwanese origin of the population that led to the introduction event. Our results however support the complex taxonomy of *Callosciurus* species (e.g., Oshida *et al.*, 2007, 2013; Gabrielli *et al.*, 2014), underlining how only skull morphology or fur color cannot be used as exhaustive diagnostic characters for species assignment. More information on specimens from the original range is still necessary to allow a precise identification of introduced Pallas's squirrels in Belgium and Italy.

When new alien organisms are introduced government authorities require to have detailed information on its taxonomic status (i.e. genus, species and even subspecies if possible), provenance and the dynamics of introduction before taking actions to control or prohibit import and sale of potentially invasive species. However, in the case of invasive alien tree squirrels, that are able to establish viable populations from a few individuals (Bertolino 2009), should governments wait to know the exact identity of a taxon or better act preventively directing actions towards an entire genus, or even the entire Sciuridae family? Based on our result, we recommend that the legislation prohibiting the introduction of squirrels in European countries should provide taxonomic lists at a higher resolution than the species level. What is becoming increasingly clear is that the attention of governments and international bodies on pet trade should increase. In any case, the use of integrative approaches appears to be ever more urgent and will allow us to obtain more and faster information useful to the management of the new invasive alien species.

CHAPTER 2

The weight of invasive squirrels on the Eurasian red squirrel: effects of interspecific competition on body mass and size

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Abstract

Alien species can affect native species through several ecological processes such as competition. In this study we test the hypothesis that the native Eurasian red squirrel competes for food resources with the invasive North American Eastern grey squirrel and Pallas's squirrel in syntopy areas in Italy. In particular we compared the body mass and size of the native red squirrel in red-only areas with those of red squirrels in co-occurrence with invasive squirrels. We observed red squirrels heavier when they were the only squirrel species present than when in syntopy with competing squirrels. Moreover our results showed that red squirrels having the same skeletal size have a reduced body mass in IAS areas than in the red-only. However we did not confirmed the prediction of a reduction of size in syntopy areas as a consequence of reduced juvenile growth caused by food depletion by the IAS. Our results suggest that red squirrels occurring in syntopy with the invasive species suffer from an interspecific competition that does not allow to reach the optimum condition that they would have if the competitor was not present. This could be related to competition for food resources due to a change in the foraging efficiency of red squirrel when in syntopy, or to an indirect competition for cached food. Moreover this study represents an example of how in invasion biology the body condition can be used as an indicator of competition between native and invasive species.

Introduction

When two species co-evolve competing for the same resources, a stable and thus long-term coexistence is possible only when some degree of niche differentiation occurs (Emmons 1980; Riege 1991). If there is no (sufficient) niche differentiation and resources are limited, interspecific competition occurs and effects on the individual, such as reduced survival and/or reproductive success, will be reflected at the population level (distribution, population density, persistence) (Shuttleworth et al. 2015), ultimately leading one of the two competing species to extinction (Ricciardi et al. 1998; Mooney and Cleland 2001). This problem is common especially when an alien species is introduced in a new environment where its niche will overlap with that of an ecologically similar native species (Broennimann et al. 2007). As opposed to naturally co-evolved competitor systems where co-evolution occurs, in the case of human-mediated introductions the two species (alien and native) come into contact abruptly, each one being in some way "trapped" by its evolutionary background. In most cases the only possible outcome, lacking the time for niche differentiation to evolve, is the establishment of a competitive exclusion mechanism with the alien species replacing the native one (Hardin 1960; Mooney and Cleland 2001). In this process, food availability and the amount of food consumed can be a major factor in competition between native and alien species and food resources competition, in turn, can affect individuals' body condition. Food intake plays an important role not just on body growth, but also on the maintenance of body condition. In many vertebrates a relatively high body mass (in relation to body size) is an indication of good condition (Kuntz et al. 2006; Wilson and Nussey 2010). Body condition also affects the animal's survival: fat reserves allow individuals to survive during periods of lack of food or to cope better with cold temperatures (Gosler 1996; Monteith et al. 2013). Moreover, a good body condition seems to play a role in the prevention of diseases or in the ability to contrast parasite infections (Neuhaus 2003).

Particularly in mammals, high body masses are often related to a high reproductive success both for males and females. Females tend to prefer males in better condition for mating, and males in good condition generally have access to a higher number of females than competitors of lower body mass (Andersson 1994). Females in good condition are better capable of sustaining the high energy-costs of lactation, increasing their fertility and/or having larger litters (Wauters and Dhondt 1989; Wauters and Dhondt 1995; Oftedal 2000).

Also animal behavior can be influenced by body mass and size: individuals in good body condition tend to be better competitors then smaller or lighter conspecifics (Wauters and Dhondt 1989) and individuals in good condition have more chances to escape from predators (Veasey et al. 1998; van der Veen 1999; Wirsing et al. 2002). Thus, competition for food resources that affects the individual's body condition can have many effects not just on the life of the single animal but can also be reflected at the population level with long-term effects on its persistence, distribution and/or reproductive rate (Mooney and Cleland 2001).

In biological invasions, a well know case is the replacement of the Eurasian red squirrel (*Sciurus vulgaris*) by the North American Eastern grey squirrel (*Sciurus carolinensis*) in Great Britain and Ireland. The grey squirrel is among the most successful invasive alien species (IAS, Long 2003; Bertolino 2009). In Northern Italy the grey squirrel was first reported in Piedmont in 1948, with subsequent introductions in Liguria and Lombardy in 1966 and in the 1990s respectively (Martinoli et al. 2010). All predictive models of the species future spread in Northern Italy show that if no control or eradication actions are undertaken, grey squirrels will invade the neighboring countries within the next 15 to 70 years and invasion of the rest of Europe is only a matter of time (Bertolino et al. 2008). In Italy, as in the British Isles, the grey squirrel lives in syntopy with native red squirrel in part of its current range. Mechanisms of competitive replacement of red by grey squirrel have been examined in detail (reviewed by Gurnell et al. 2015). Similar food choices, a general lack of niche partitioning and the fact that grey squirrels can successfully pilfer in winter-spring the seeds cached by red squirrels the previous autumn, all support strong interspecific competition for food resources, at least in mixed deciduous forests (Wauters and Gurnell 1999; Wauters et al. 2001a; Wauters et al. 2002b; Wauters et al. 2002a; Wauters et al. 2005). The existence of this kind of competition is further supported by red squirrels smaller size and lower body mass recorded in co-occurrence with greys, if compared to similar habitats where greys are absent (Wauters et al. 2000; Wauters et al. 2001a). Lower body mass could negatively affect female reproductive success (Gurnell et al. 2015). Furthermore, many data support the view that interspecific competition acts strongly during on the growth phase of red squirrels when juvenile and subadults disperse and look for a place to settle (Gurnell et al. 2004a; Gurnell et al. 2015).

In the last decade, another invasive squirrel was introduced in Northern Italy, the Pallas's squirrel (*Callosciurus erythraeus*) (Mazzamuto et al. 2015). The native range of this species covers a large part of North-eastern South Asia, much of central and southern China, and mainland Southeast Asia (Thorington et al. 2012). In its current range in Italy (9800 ha), this Asian tree squirrel co-occurs with the native red squirrel, in areas where the grey squirrel is not present. Pallas's squirrel was introduced also in Argentina, France, Belgium, The Netherlands, Hong Kong and Japan (Bertolino and Lurz 2013; Guichón et al. 2015; Adriaens et al. 2015). This squirrel is known to have negative impacts on habitats and biodiversity (Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). In Japan, the native squirrel species, *Sciurus lis*, is locally declining probably because of competition for food and nesting sites (Ministry of the Environment, Japan, 2002; Miyamoto et al. 2004) and in Argentina the risk of negative impacts on native squirrel species, if a translocation of the invasive squirrel occurs, is considered high (Cassini and Guichón 2009). However, detailed hypotheses about the mechanisms of interspecific competition between Pallas's squirrel and native squirrels have not been investigated so far.

In this paper we examined three different kinds of areas in Northern Italy, that is areas where only native red squirrel is present or areas where an invasive squirrel species (either grey or Pallas's squirrel) co-occurs because of human-mediated release. In particular, we tested the hypothesis that the presence of an IAS negatively affects red squirrel body condition. We measured the hind foot length and the body mass of adult red squirrels in the different co-occurrence situations, and predict a) a reduction of size in syntopy areas as a consequence of reduced juvenile growth caused by food depletion by the IAS; b) that red squirrel body mass will be lower in syntopy areas because of a reduction of the energy intake caused by a lowering of the availability of high quality food resources, or the inability to exploit them; c)that red squirrels of a given size will weigh less in syntopy than in red-only area. Finally, we discuss the implications of our findings for red squirrel conservation.

Materials and methods

Study area

Red squirrels were trapped in broadleaf woodlands in Lombardy and Piedmont over a period of 5 years. Areas were divided in "red-only" areas (Lombardy), "red-grey" (Lombardy and Piedmont) and "red-Pallas" (Lombardy) areas where the red squirrel was in co-occurrence with the grey squirrel and the Pallas's squirrel respectively. These areas are mainly composed of continuous mixed deciduous forests with patches of grassland and small villages. Vegetation composition and structure is typical of woodlands of the Upper Po-plain areas and low hills and of the lower elevations submontane forest.

Data were collected from many different trapping sites for each of the three areas. Although we do not have detailed estimates of food availability, the 5 years longitudinal data would allow levelling out local effects. Furthermore, time has been taken into account in modelling, precisely to take into account temporal variation in body mass that might have been caused by variation in food availability between years in each of our study areas.

Trapping and handling squirrels

Trapping was carried out from 2010 to 2014 for red-only areas and from 2011 to February 2015 for red-grey and red-Pallas areas. Two types of traps were used, single capture traps (Tomahawk trap model 202, Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.) and multi capture traps (see Mayle et al. 2007). Both trap types were placed with baling wire on tree trunks at breast height or on the ground. Pre-baiting started one week before each trapping session: hazelnuts were placed inside the blocked traps and each trap was re-baited every two days. Traps were checked at least twice a day and they were baited and reset after each capture. Each trapped squirrel was weighed to the nearest 5 g with a Pesola spring balance, identified to species and sex (reproductive conditions identified following (Wauters et al. 2000), and the length of the right hind foot (nails excluded) was measured (0.5 mm precision) with a thin ruler (Wauters et al. 2007a). Red squirrels were individually marked with numbered metal ear tags (type 1003 S National Band and Tag Co, Newport, Kentucky, USA) and released. Since female tree squirrels increase their body mass during pregnancy and lactation (Humphries and Boutin 1996), pregnant or lactating females were excluded from analysis to avoid any bias due to presence of embryos (pregnancy) or adaptive weight changes (early and late phases of lactation).

Data analysis

Individual variation in body mass of red squirrels was analyzed using Generalised Linear Mixed Models (GLMMs, Bolker et al. 2009a), testing the effects of foot length as measure of body size, sex, year and the area type. We added individual squirrel ID as a repeated measure, and the field operator and season as random factors. The first was added since many animals were trapped and measured several times, and the second to control for potential measurement errors (observer bias) (Blackwell et al. 2006). Seasons were classified following the same criteria described by Wauters et al. (2007) (winter: December- February, spring: March- May, summer: June- August, autumn: September- November).

Model selection was carried out performing a backward stepwise analysis: first a backward elimination of the model random part is performed, followed by a backward elimination of the fixed-effects part. The p-values for the fixed effects are calculated from type III F-test based on Kenward-Roger approximation, p-values for the random effects are based on likelihood ratio test.

Statistical analysis were done using the R software (R Development Core Team 2014) in particular the lme4 package v. 1.1-7 and the lmerTest v. 2.0-25 package for fitting and analyzing mixed models (Bates et al. 2014; Kuznetsova et al. 2015). Graphs were created using the ggplot2 R package (Wickham 2009).

Results

Overall, we had 989 capture and recapture events (thus measurements), related to 279 different red squirrels. We analyzed 183 records of 79 individuals in the red-only area, 613 records of 146 different animals in the red-grey area, and 193 records of 54 individuals in the red-Pallas area (Supplemental material 7). The mean body mass and hind foot length for each area type are reported in Table1.

The hind foot length of adult red squirrels was significantly different in the 3 areas ($F_{[2,983]}$= 8.35, p< 0.001) but there was no sexual dimorphism in body size (as in body mass). However, the interaction between sex and area type was slightly significant ($F_{[2,983]}$= 3.24, p= 0.04). The post-hoc

Table 1. Red squirrel average body mass (g ± SD) and foot length (mm ± SD) for each area type.

Area type	Body mass			Hind foot length		
	Both sexes	*Males*	*Females*	*Both sexes*	*Males*	*Females*
Red-only	315.87 ± 32.14	314.20 ± 30.72	317.86 ± 33.82	58.47 ± 1.30	58.68 ± 1.03	58.22 ± 1.53
Red-grey	310.95 ± 32.72	308.81 ± 31.90	313.19 ± 33.47	58.97 ± 1.97	58.78 ± 1.80	59.17 ± 2.13
Red-Pallas	313.50 ± 37.91	309.32 ± 34.62	319.04 ± 41.44	58.32 ± 3.35	58.41 ± 3.32	58.20 ± 3.40

comparisons using the Tukey's HSD test indicated that the mean hind foot length of females in the red-grey area is greater than for females in the red-only area ($p< 0.01$), and that the mean foot length of red squirrels in the red-Pallas area is shorter than the one recorded for females in the red-grey area ($p< 0.001$) (Table1). However, differences in mean values are about only 1 mm.

The minimal model describing squirrel body mass retained as explanatory factors foot length, area type and their interaction, and as random factors the field operator. Fixed effects and their interaction were all strongly significant ($p< 0.001$) and in particular body mass was lower in syntopy areas than in the red-only and the slope of body mass on foot length regression line in the red-grey and red-Pallas areas is less steep then the one for red-only area (differences in slope ± SE = -8.13 ± 2.04 and -12.13 ± 1.93 respectively; Fig.1).

Discussion

Alien species can affect native species through several ecological processes such as competition, predation and hybridization (Kats and Ferrer 2003; Cox 2004; Largiadèr 2007; Ehrenfeld 2010). Here we test the hypothesis that a native species can be affected in its body condition by IAS because of food resources competition. In particular we looked at the body mass and size of a native species in syntopy with IAS as indicators of the competition, measurements that are often overlooked but that can give information about the native species response to the alien species introduction (Latini and Petrere 2004).

In Italy the native Eurasian red squirrel is in co-occurrence with two invasive squirrels, the North American Eastern grey squirrel and the Pallas's squirrel (Martinoli et al. 2010; Mazzamuto et al. 2015). The replacement of red by grey squirrels has been often related to competition for food resources due to a change in the foraging

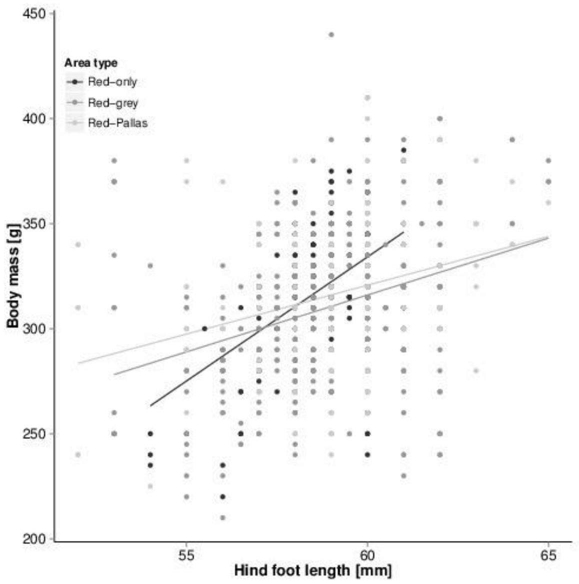

Figure 1. Body mass (g) on hind foot length (mm) of red squirrels in the three area types. Every dot represents a red squirrel measurement, dots can overlap.

efficiency of red squirrel when in syntopy, or to an indirect competition for cached food (Wauters and Gurnell 1999; Wauters et al. 2002a; Gurnell et al. 2015). Seemingly contrary to our predictions, we found female red squirrels with large body size in the red-grey area, that is with greater than expected hind foot lengths. Previous studies of red squirrel populations in syntopy with grey squirrels suggested that interspecific competition acts primarily on the juvenile and subadult stages, that is during the growth phase, ultimately causing smaller adult body sizes (Wauters et al. 2000; Gurnell et al. 2004b). A difference in size was also reported for red squirrels in large and fragmented woodlands: squirrels were larger in large woodlands than in small woodland fragments because of environmental stress such as decreased food availability (Wauters et al. 1996).

If foot length is just a skeletal measurement of squirrel size, body mass is also indicative of the squirrel's condition (Wauters and Dhondt 1995). The two predictions we made about the squirrels' body mass were confirmed. We actually observed an area type effect on body mass, with red squirrels heavier when they were the only squirrel species present (red-only area) than when in syntopy with other competing species. But, variation in body mass depends to an important extent on variation in skeletal size, so the interaction between foot length and area type on body mass gives information on variation in body condition of the squirrels, corrected for the existing differences in body size (foot length). Our results showed that red squirrels having the same skeletal size have a reduced body mass in IAS areas than in the red-only. This suggests that red squirrel individuals occurring in syntopy with the invasive species suffer from an interspecific competition that does not allow to reach the optimum condition that they would have if the competitor was not present. The effect of direct or indirect competition for food resources was detected in previous studies on red squirrels in red-grey areas. Red squirrel average body mass, in deciduous as well as in coniferous forests, was lower when red squirrels co-occurred with greys than in the red-only area, which is believed to be at least partly caused by reduced energy intake because of the pilfering of red squirrels' food caches by grey squirrels (Wauters et al. 2001a; Wauters et al. 2002b). Similarly, we demonstrated for the first time that the Pallas's squirrel can compete with the Eurasian red squirrel. Our results showed how the Pallas's squirrel has a similar effect on red squirrel body condition than the grey squirrel. Thus, further research on competition for resources may be a first direction to follow to know more about the interspecific competition between these two tree squirrels. Food availability has an effect on the mechanism of replacement of red squirrel by invasive squirrels and if food is scarce the competition will be more severe especially in autumn or winter (Gurnell 1987). Body mass in red squirrels is a good indicator of competitive ability and of dominance position (Wauters and Dhondt 1989; Wauters and Dhondt 1993), and heavier squirrels are more likely to reproduce successfully (females) or to mate (males) (Wauters and Dhondt 1989; Wauters et al. 1990; Wauters and Dhondt 1992). Thus a loss of body mass is likely to reduce reproductive output in female red squirrels and plays an important role in the replacement of red by grey (see also Wauters et al. 2002b) squirrels and our results suggest this may be the case also in competition of the native species with Pallas's squirrels.

CHAPTER 3

Space invaders: home range dynamics and interspecific competition between the invasive alien Pallas's squirrel (*Callosciurus erythraeus*) and the native Eurasian red squirrel (*Sciurus vulgaris*)

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Abstract

Alien species introduced outside their native range can become competitors of native species. Here we tested the hypothesis of interspecific competition for space between the native Eurasian red squirrel and the invasive Pallas’s squirrel introduced in Italy. Squirrels were trapped and radio-tagged in three different sites, two Pallas-only areas and one red-Pallas area. Home range size was estimated using both 95% kernel Density Estimator and 100% Minimum Convex Polygons. Male Pallas’s squirrels had larger home ranges than females, and neither home range size, nor patterns of overlap of the alien species were affected by the presence of the native one. Likewise red squirrel home ranges in the area of co-occurrence did not differ from values reported in red-only areas. Patterns of interspecific home range overlap suggested there was no spatial niche segregation between the two species. After the removal of 13 Pallas’s squirrels from the red-Pallas area, the same red squirrels were monitored again in a situation without the alien competitor but there were no significant changes in red squirrel home range size and overlap compared to the pre-removal situation. These results do not give support for inter-specific competition for space between adult red and Pallas’s squirrels. However, the failure to find other areas of co-occurrence of red and Pallas’s squirrels with

more than a single resident individual of the native species, suggests that the invasive squirrel may affect the native species' survival, dispersal and/or fertility rather than its space use.

Introduction

The invasion of alien species is a process consisting of several stages, each with an independent probability of failure. To be defined as invasive, a species has to be able to establish population outside its distribution range, must have a fast demographic increase and should have an impact on native species and/or ecosystems (Kolar and Lodge 2002). Introduced species can be advantaged in the new environment, increasing rapidly in numbers, due to a reduction or even absence of predators and/or parasites (so-called enemy-release hypothesis) (Wolfe 2002; Torchin et al. 2003; Romeo et al. 2014). Moreover, invasive species can alter the natural evolution of the native ones by competitive exclusion, hybridization, predation, spread of diseases, and niche displacement which can, ultimately, result in native species extinction. Invaders themselves evolve in response to their interactions with natives, as well as in response to the new abiotic environment (Elton 2000; Mooney and Cleland 2001; Ehrenfeld 2010). In natural communities, species that occupy similar ecological niches tend to compete for limited resources (Douglas et al. 1994; Gurnell et al. 2004b). When an alien species is introduced, it can become a competitor of a native one. This interspecific competition may adversely affect fertility, growth or survival of both native and invader species (Holway 1999; Wauters et al. 2000; Byers 2000). For the long-term persistence of both species, niche differentiation must happen (MacArthur and Levins 1964; Douglas et al. 1994; Carlton et al. 1999) and, in the absence of that differentiation, one of the two species will exclude the other, eventually causing its extinction (Hardin 1960; Ricciardi et al. 1998; Strong and Pemberton 2000; Bertolino 2008).

In the past, human trade, transport, travel and tourism have dramatically helped the spread of invasive alien species. Prevention of the introduction and early detection of a potential invasive species are the first and more cost-effective options (Wittenberg and Cock 2001; Finnoff et al. 2007). But when prevention has failed, eradication and management of established invasive species are the key to prevent or mitigate the negative impacts of those species on the environment and on native species (Veitch and Clout 2002; Genovesi et al. 2014; Adriaens et al. 2015). In Europe, many species belonging to different taxa have improved their conservation status as a result of eradications of invasive species (McGeoch et al. 2010). But when the risk level for introduced species is underestimated, management actions become very difficult, expensive and often useless to prevent the loss of biodiversity as well as the economic losses that are already taking place (Bertolino and Genovesi 2003; Genovesi 2011). That is, for example, the well known case of the North American Eastern grey squirrel (*Sciurus carolinensis*) in Great Britain, Ireland and Italy, whose introduction and subsequent spread has caused a dramatic decline of the native Eurasian red squirrel (*Sciurus vulgaris*), as well as serious impacts on trees due to bark stripping (Wauters et al. 2002b; Wauters et al. 2002a; Gurnell et al. 2004b; Mayle et al. 2009; Martinoli et al. 2010; Goldstein et al. 2014; Bertolino et al. 2014). Tree squirrels are successful invaders thanks to their capability to establish wild viable populations from a few founders (Palmer et al. 2007; Bertolino 2009; Martinoli et al. 2010).

In Italy, another invasive tree squirrel species, *Callosciurus erythraeus*, or Pallas's squirrel, native to Southeast Asia (Oshida et al. 2001), has been introduced (Mazzamuto et al. 2015). The story of the introduction and the number of squirrels released are still unknown, but the first sighting of the species in the province of Varese occurred in 2007. Pallas's squirrel has been introduced so far in seven different countries around the world, and of the 29 known introduction events 21 (including Italy) are documented as successful with a viable population established in the wild, whereas only two populations have been successfully eradicated (Bertolino and Lurz 2013; Lurz et al. 2013; Adriaens et al. 2015). This species debarks forest trees, consumes fruits in orchards and can also damage parts of buildings, electrical cables and irrigation systems (Tamura and Ohara 2005; Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). Moreover, in Argentina and Japan, the risk of negative impacts from the introduced *C. erythraeus* on native squirrel species is considered high (Miyamoto et al. 2004; Cassini and Guichón 2009). Today in Europe there are four introduced populations of Pallas's squirrel (Italy, two in France, The Netherlands) and in Italy the introduced squirrel has established in an area where it co-occurs with the native Eurasian red squirrel.

We conducted this study to test the hypothesis of interspecific competition for space between the native and the introduced squirrel. If competition occurs we can make different predictions of red squirrel space use changes. (1) Red squirrels might use larger home ranges than in non invaded sites with the same habitat (literature data from Wauters et al. 2001b) to reduce competition for food with Pallas's squirrel. (2) An alternative response could be that the native species reduces its home range size and uses smaller core areas that are exclusive with respect to the alien species. (3) Moreover, if competition for space occurs, we expect a change in the red squirrel home range size or home range (or core area) overlap among red squirrels, once the competing Pallas's squirrel is removed from invaded sites. In contrast, (4) if space competition between the two species is low, then home range size will be similar than in non invaded sites and home range or core area overlap among red squirrels (due to strong intraspecific competition, Wauters and Dhondt 1992) will be lower than between red and Pallas's squirrels.

Materials and methods

Trapping and handling squirrels

Pallas's squirrels were studied in the North of Varese province (Lombardy region, Italy), where the alien species co-occurs with native Eurasian red squirrels. The study area, bounded on the northeast by Lake Maggiore and on the southwest by a state road with intense traffic, is mainly composed of continuous mixed deciduous forests with patches of grassland and small villages (Mazzamuto et al. 2015).

Single-capture traps (model 202, Tomahawk Live Trap Co., Tomahawk, WI, USA) and multi-capture traps (Long Meadow Publishing, Shaftesbury, Wiltshire, UK; see Mayle et al. 2007) were arranged in grids and spaced ca. 80 m from each other. Both trap types were fixed on tree trunks at breast height using baling wire. Prebaiting started one week before each trapping session: hazelnuts and apple slices were placed inside the blocked traps, and each trap was checked and rebaited every two days. When activated, traps were checked two to three times per day to reduce the time squirrels were confined in traps. Each trapped squirrel was weighed to the nearest 5 g with a Pesola spring balance, identified to species and sex, and the length of the right hind foot (nails excluded) was measured (0.5 mm precision) with a thin ruler (Wauters et al. 2007a). All squirrels of both species that were radio-tagged, were individually marked with numbered metal ear tags (type 1003 S National Band and Tag Co, Newport, Kentucky, USA) and released.

Radio tracking

Squirrels were trapped in three different sites, selected because of signs of presence of the alien species (visual surveys, hair tubes) between 2013 and 2014. Two of them (A, B) after 6 weeks of capture-mark-recapture, were classified as sites with presence of the Pallas's squirrel only. The third selected site (C) was the only one we could find, in the whole area of introduction of the Pallas's squirrel with no ongoing removal program, where there was more than a single resident (more than 4 months or 4 trapping sessions in the area) Eurasian red squirrel in co-occurrence with the invasive squirrel (red-Pallas area) in 2014. A total of 23 Pallas's squirrels were tracked in the three sites. In site C, all resident red squirrels were radio-tracked (n = 5) while just 7 Pallas's squirrels of the entire (local) population were monitored with telemetry (Table 1). In this site, once sufficient locations from Pallas's squirrel and red squirrels were collected (Mar - Jun 2014), we removed 13 Pallas's squirrels (included all the radio-tagged ones) during July 2014 and subsequently monitored again the same red squirrels (one female disappeared) in a situation without the alien competitor. Hence we had monitored home ranges of about half on the individuals of the alien species that had been present in the area.

Two different types of radio tags were used, the PD-2C model by Holohil Systems Ltd. (Ontario, Canada) for Pallas's squirrels and Brass Collars by Biotrack Ltd. (Dorset, UK) for red squirrels. We monitored radio-tagged squirrels using a Sika Radio Tracking Receiver (Biotrack Ltd., Dorset, UK) and a foldable three-element Yagi antenna. Locations were determined by following the radio signal until the squirrel was seen or pinpointed by signal strength and direction (homing-in) and we collected from 28 to 83 locations for each animal (Wauters and Dhondt 1992). For red squirrels we collected another 25 to 30 fixes for each animal from August to October 2014 to compare the native species space use pre- and post-removal of the alien competitor.

Vegetation analysis

Forest composition was analyzed in detail in the three radio tracking sites (A, B, C). In each site a minimum convex polygon (MCP) was calculated from all the locations of both squirrel species to which a further 50 meters buffer zone was added. Within that area, a regular 150 x 150 m grid of points was created using a geographic information system (QGIS Development Team 2015). At each point of the regular grid that fell in the MCP area a 20 x 20 m sampling plot was set up in which we recorded species name of tree/shrub, number of trees/shrubs for every species, percentage cover of tree and shrub layer (see also Cagnin et al. 2000).

Data analysis

All analysis were performed using R software 3.0.3 (R Development Core Team 2014). Home ranges were calculated with the R package HRTools (Wauters et al. 2007b) using the 95% fixed kernel density estimator (KDE) and also the 100% MCP to allow comparison with literature data. To determine if the range of each squirrel contained an exclusive core-area, we visually examined the utilization distribution (UD, range size included in 5% incremental isopleths containing between 20% and 95% of fixes) of the KDE. We also used a one-way ANOVA with size of range included in a given isopleth as dependent variable and isopleth from the UD with a given percent of fixes (at 5% increments from 20% to 95% of all fixes) as a factor to define the core-area (Kerr and Bull 2006; Di Pierro et al. 2008; Bisi et al. 2010). However, visual inspection, for both species, did not show an inflection point in the slope of the utilization distribution curve, and the ANOVA nor any of its pairwise comparison between consecutive isopleths were significant. Thus we could not identify areas within the home range used most intensively. Consequently, home range size was used to estimate overlap expressed as percentage of overlap of a squirrel's range with the home range of all other squirrels (Wauters and Dhondt 1992). Analysis of variance (ANOVA) was used to test differences in mean home range sizes and overlaps between sexes and areas and where necessary a post-hoc Tukey's HSD test was made to identify significance in pairwise comparisons.

All measurements are expressed as mean values ± standard deviation (SD).

Results

Radio tracking

We collected 1166 locations of Pallas's squirrels and 327 locations of red squirrels. Number of fixes per individual varied between 28 and 83 (47 ± 21).

Using data from all three sites, mean home range size for Pallas's squirrels using 95% KDE was 7.95 ± 6.28 ha (100% MCP: 4.01 ± 3.14 ha). On average, males had larger home ranges than females ($F_{[1,16]}$= 8.95, $p< 0.01$) and there was a marginal effect of the area on home range size ($F_{[2,16]}$= 3.13, $p= 0.05$) while the interaction sex-area was not significant ($F_{[2,16]}$= 5.3, $p= 0.19$) (Table 1). For comparison with previous studies mean 100% MCP of males and females was 5.58 ± 3.94 ha and 2.80 ± 1.66 ha respectively.

Pallas's squirrel home range overlap differed between sites ($F_{[2,126]}$= 13.71, $p< 0.001$), being higher in site B than in the other two (Tukey HSD, B-C and B-A, $p< 0.001$). The mean home range overlap in the areas A, B and C are respectively 40% (± 27%), 67% (± 27%) and 44% (± 33%). Males and females had different patterns of home range overlap with other squirrels ($F_{[3,126]}$ = 4.57, $p< 0.01$), however pairwise comparisons showed that the only significant difference in overlap was that home ranges of males overlap less those of females than females' home ranges those of males (MM: 46 ± 33%; FF: 49 ± 27%; FM: 63 ± 33%; MF: 37 ± 28%, Tukey HSD MF-FM $p< 0.001$). In area C (red-Pallas site) red squirrels had a mean 95% KDE home range size of 17.87 ± 8.61 ha (100% MCP 6.53 ± 2.36 ha). Because of the low number of radio tagged animals we could not test statistically for a sex effect. The mean overlap among red squirrels (n=5) before removal of the alien species was 70 ± 29%.

We also explored the home range overlap between the two species, and Pallas's squirrels average overlap on red squirrels was estimated as 53 ±34%, while red on Pallas's overlap resulted 30 ±26%.

The home range size of red squirrels after the removal of 13 Pallas's squirrels in area C was 34.38 ± 16.92 ha but it was not significantly different from home range size prior to removal ($t(4)= -1.78$, $p= 0.15$). After the removal, red squirrels moved the centroids of their home ranges for an average of 187 m (± 125) but there was no difference on the red squirrels' intraspecific home range overlap before and after the removal (n = 4, 65 ± 34%; $t(6)= 0.23$, $p=0.83$) (Supplemental material 8).

Table 1. Pallas's and red squirrels mean home ranges (95% KDE ± SD in ha) in the three radio tracking areas.

Area	**Period**	***Callosciurus erythraeus***		***Sciurus vulgaris***	
		Male	*Female*	*Male*	*Female*
A	Apr - Oct 2013	14.14 ± 10.60 (3)	7.39± 3.66 (5)	-	-
B	Apr - Oct 2013	4.86 ± 4.03 (4)	3.14 ± 0.99 (4)	-	-
C	Mar - Jun 2014	16.66 ± 4.82 (3)	4.97 ± 1.25 (4)	17.55 ± 6.49(3)	18.36 ± 14.55 (2)
Total		11.34 ± 7.90 (10)	5.33 ± 2.92 (13)	17.55 ± 6.49(3)	18.36 ± 14.55 (2)
		7.95 ± 6.28 (23)		17.87 ± 8.61 (5)*	

* these red squirrels were followed also Aug- Oct 2014

Vegetation

The tree layer is dominated by *Robinia pseudoacacia* in site A (65%) and by *Castanea sativa* in site B (88%). Site C has a 32% coverage of *Castanea sativa*, 20% of *Pinus sylvestris* and 12% of *Prunus serotina*. The shrub layer is mainly composed of *Sambucus nigra* and *Corylus avellana* in site A (63 and 3% respectively), *Ilex aquifolium* (68%) and *Prunus lauroceraso* (25%) in B and *Corylus avellana* (80%) in site C (Supplemental material 9). Thus home range overlap among individuals of the alien species was highest in the site (B) dominated by sweet chestnut.

Discussion

In Europe the number of invasive alien species (IAS) and the rate of new introductions have increased in the last decades, raising the extinction risk of many species belonging to different taxa (Clavero and García-Berthou 2005; European Environment Agency 2012). Understanding the behavior of introduced aliens in the new invaded range and the risks associated to their introduction is one of the first steps to better manage IAS and to prevent and/or mitigate their ecological, economic or sanitary effects (Wittenberg and Cock 2001; Palmer et al. 2007). Therefore, we have studied the space use of the Pallas's squirrel, an invasive tree squirrel introduced in many different countries in the world (Bertolino and Lurz 2013), in temperate broadleaf forests in Italy, and tested the hypothesis of a possible competition with the native Eurasian red squirrel. We will first discuss space use of the IAS and then the patterns of space sharing with the native species.

Space use of Pallas's squirrels

The space use patterns of an alien species outside its natural distribution area may be different from the ones in its native range, because of different habitat and resources or different competitors and predators that mainly influence the species home range (Alterio 1998; Melero et al. 2008; Alexander and Edwards 2010).

In Italy we found that males used larger home ranges than females (MCP estimates: males 5.58 ha; females 2.80 ha), but both sexes had larger home ranges than reported in the native range and in Japan. In their native range in Taiwan, males of *C. erythraeus thaiwanensis* had a mean home range of 1.40 ha and females of 0.28 ha (MCP estimates; Tamura et al. 1989). In Japan, where it was introduced, the home range sizes were 1.25–3.83 ha for males and 0.48–0.72 ha for females (MCP estimates; Tam*ura et* al. 1987; Tam*ura et* al. 1988). In Japan, as in Taiwan, female Pallas's squirrels avoided other females reducing home range overlap, while male ranges overlap extensively with other males and females (Tamura et al. 1989). In Italy we did not find any difference in the amount of home range overlap of a male by other males and a female by other females, rejecting the hypothesis of intra-sexual territoriality among females (Wauters and Dhondt 1992; Wauters et al. 2001b for native red squirrels). Contrary to previous studies, males seemed to overlap less on females than females on males. Our data were gathered over a too short period to explore whether this pattern was due to temporal variation in food resources and/or mating behavior, or other factors linked to sex-related differences in habitat use. Interestingly, there were differences in the percentage home-range overlap among the three study sites, with higher overlap values in site B (only Pallas's squirrel present) than in the others. We investigated the vegetation composition in the three areas with the aim to find some marked habitat preferences that could help planning control measures. Higher overlap was probably related to forest composition: while sites A and C are mixed broadleaf woods, site B is an almost pure chestnut wood (88% chestnut trees). Chestnut seeds are an abundant trophic resource, heavily used by native red squirrels (Wauters et al. 2001b; Wauters et al. 2001a), and by a large number of small and large mammal species and some birds. In fact, chestnuts have a high energy-content and are not immediately perishable, allowing the possibility of being stored in caches as a resource for winter (Wauters et al. 2001a).

Greater food availability in site B may also explain the tendency (although not statistically significant) for smaller home ranges (Wauters and Dhondt 1992; Lurz et al. 2000). Hazelnuts, most abundant in site C, are also an energy-rich food for squirrels, but the productivity of the shrub layer may be not comparable to that of the tree layer. Hence, so far our data do not allow to distinguish clear selection patterns of the alien species for certain tree or shrub species, although occasional observations show they intensively use cherry trees and chestnuts. Further studies will be needed combining data on population density, space use, forest productivity and food availability to shed light on the factors affecting social organization in this IAS.

Core area

The total home range overlap is extensive in red squirrels (Wauters and Dhondt 1992). However they defend a portion of their home range (core area) that is intensively used and from which an individual excludes competitors from resources (food, suitable partners) through social interactions (Wauters and Dhondt 1992; Lurz et al. 2000; Di Pierro et al. 2008). In particular breeding females occupy core areas from which neighboring females are excluded, so called intrasexual territoriality (Lurz et al. 2000; Wauters et al. 2001b; Wauters et al. 2005). Therefore the core area overlap is a better indicator of intra and interspecific competition for space. In this study we did not find a core area both for the red and the Pallas's squirrel indicating a homogeneous space use in this forest. Previous researches on red squirrels showed that core area exclusiveness can change with local conditions of food abundance and distribution or squirrel density (Wauters and Dhondt 1992; Lurz et al. 2000; Di Pierro et al. 2008; Verbeylen et al. 2009), but our results may indicate a non-territorial behavior possibly related to low density and/or a homogeneous distribution of food resources. There are no previous studies about an animal's activity centre in the space use of the Pallas's squirrel, either in native or introduced range. It would be interesting to reanalyze existing data from studies on total home range size in Taiwan and Japan to investigate whether individuals use space homogeneously also in these countries or whether core areas can be revealed.

Interspecific competition for space with red squirrels

Our data suggested that neither red squirrel nor Pallas's squirrel home ranges differed in size in a single species situation compared to when the other species co-occurs. This is in contrast with the first two predictions of our hypothesis: a smaller or larger home range size due to interspecific competition for space. In fact, mean home range size for red squirrels in a similar habitat in Northern Italy (Lombardy) using the MCP estimate was 5.78 ha (Wauters et al. 2001b), a value not significantly different from the average home range size in our red-Pallas area (6.53 ha; t(11)= -0.62, p=0.54). Hence, these results supported our alternative, fourth prediction of limited competition for space between the two species.

We also predicted that, if competition for space does not occur, home range overlap among red squirrels would have been much lower (due to intraspecific competition) than between red and Pallas's squirrels. On the contrary, our results showed a larger overlap among red squirrels (70%) than between red and Pallas's squirrel (30%). Pallas's squirrels were radio-tagged both in Pallas-only (A and B) and red-Pallas (C) sites but there was no difference in mean home range sizes, or in overlap between the two types of areas. Home range overlap results can be considered an underestimate because while we radio-tagged all the red squirrels in site C, we followed only 7 Pallas's squirrels instead of the entire local population. Hence, we most certainly underestimated interspecific home range overlap, suggesting that there is no differentiation of the space-use niche dimension between the two species (e.g., Wauters and Gurnell 1999; Gurnell et al. 2015). In any case, these results on home range overlap are in contrast with our second prediction and, as our data on home range size, rather tend to give support for the fourth prediction of a limited effect of interspecific competition on space use.

We also tested whether removing introduced squirrels led to a change in red squirrel space use patterns. We found that red squirrel home range size and overlap did not change, at least in the two months immediately after the removal, which disagreed with our third prediction. Red squirrels showed marked individual differences (high SD) in home range sizes both before and after the removal, suggesting that other factors play a role in determining space use. For example, an individual squirrel's personality may play a role in the response to the competition with Pallas's squirrel (Boon et al. 2008). In addition, it might take more time (than two months) for red squirrels to adjust their space use to the absence of the potential competitor species, and/or short-term temporal variation in distribution of preferred food resources may be driving, at least partly, the animal's movements. Finally, we must admit that our sample size for red squirrels was small, for reasons explained below.

Conclusions

The data presented here do not give support for the three predictions made under the hypothesis of a strong inter-specific competition for space between adult red and Pallas's squirrels. A similar pattern was reported also for the red squirrel in co-occurrence with the North American grey squirrel in deciduous forests in Italy. Red squirrels did not completely avoid grey squirrels and spatial overlap of red with grey squirrels occurred in a similar manner as with conspecifics (Wauters and Gurnell 1999; Wauters et al. 2002b). Comparing two red-Pallas sites, and thus having a replicate for site C, could have clarified space use dynamics of the two species in syntopy. However, we could not find such an area of co-occurrence of the two species with a good number of resident red squirrels, suggesting that the IAS may affect the native species' survival, dispersal and/or fertility rather than its space use.

Red squirrels in the study area might be relegated to the borders of the distribution area where the forest may have a lower quality in terms of productivity, food availability and degree of fragmentation (Wauters et al. 1996; Wauters et al. 2010; Santicchia et al. 2015). The permanence in marginal areas in the long run could have an adverse effect on the red squirrel population affecting both survival and reproduction, with a consequent decrease in red squirrel density, as suggested for the replacement mechanism of red squirrels by introduced grey squirrels (Gurnell et al. 2015). Thus studying red squirrel population dynamics in the area of introduction of Pallas's squirrels could clarify if and how a competition between the two species is occurring. Finally, also other mechanisms should not be excluded, such as direct interference of Pallas's squirrels with the behavior of red squirrels (Wauters and Gurnell 1999; Gurnell et al. 2015) or parasite-mediated competition through the spillover of parasites carried by the alien species to the native one (Romeo et al. 2013; Romeo et al. 2014). The latter is currently being investigated through parasitological studies on both species (Chapter 4; Mazzamuto et al. in press).

CHAPTER 4

Poor parasite community of an invasive alien species: macroparasites of Pallas's squirrel introduced in Italy

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Abstract

The aim of the present study was to investigate the macroparasite fauna of the Pallas's squirrel, *Callosciurus erythraeus,* native to Southeast Asia, recently introduced to Italy in co-occurrence with the native Eurasian red squirrel *Sciurus vulgaris*. A total of 161 Pallas's squirrels harbored a poor macroparasite fauna, characterized by two main ectoparasite species, the flea *Ceratophyllus* (*M.*) *sciurorum sciurorum* (prevalence 50%, mean intensity 1.0±0.1) and the tick *Ixodes ricinus* (prevalence 47%, mean intensity 3.0±0.7), and few helminths (*Rodentoxyuris sciuri*, *Trichuris muris*, *Strongyloides callosciureus*) all with prevalence below 10%. All parasites, except *S. callosciureus*, are European species acquired in the invaded range. The loss of native parasites might have contributed to their successful invasion. The acquisition of local parasites *C. sciurorum* and *T. sciuri*, and the introduction of the Asiatic nematode *S. callosciureus* may lead to spill-back and spill-over processes towards red squirrels, with potential impacts on this threatened native species.

Introduction

The issue of biological invasions has long been recognized (Elton 2000), but the attention on alien species has increased over the last decade. Biological invasions can affect native species through several ecological processes such as competition, predation and hybridization (Kats and Ferrer 2003; Cox 2004; Largiadèr 2007; Ehrenfeld 2010).

The outcome of the invasion process may also be affected by parasites, as has been reported for several introduced species, and infection transmission can become a threat to native species and human health (Torchin and Mitchell 2004; Dunn et al. 2012; Marsot et al. 2013; Mazza et al. 2013). An invader can import parasites from its native range, transferring infectious agents to native host species that may be more susceptible to them (spill-over) (Asakawa 2005; Taraschewski 2006). Moreover, exotic hosts may acquire local parasites from native hosts (Tompkins and Poulin 2006; Pisanu et al. 2009; Romeo et al. 2014), thus altering their local dynamics and inducing higher transmission rates to native species because of the increased number of available hosts (spill-back) (Kelly et al. 2009). Finally, introduced species may also lose part of their parasites during the introduction process as a consequence of founder effect, absence of intermediate hosts or unsuitable environmental conditions (MacLeod et al. 2010). This loss may translate into higher individual fitness, increasing invasion success (parasite-release) (Torchin et al. 2003).

Table 1. Macroparasite species hosted by Pallas's squirrels *Callosciurus erythraeus* in Italy. *N*: total number of hosts examined; *P*: prevalence; *mI*: mean intensity (ectoparasite counts in italic when number of infected hosts<10).

Macroparasite species	P	mI±SE
Helminths	**N=73**	
Trypanoxyuris (R.) sciuri (Hugot, 1984)	5%	*1; 1; 1; 6*
Trichuris muris (Schrank, 1788)	4%	*1; 1; 1*
Strongyloides callosciureus Sato et al., 2007	1%	*1*
Strongyloides sp.	1%	*1*
Capillariinae [gen. sp.]	1%	*1*
Spiruroidea [gen. sp.]	1%	*1*
Arthropods	**N=135**	
Ceratophyllus (M.) s. sciurorum (Schrank, 1803)	50%	1±0.1
Ixodes (I.) ricinus (Linné, 1758)	47%	3±0.7
Trombiculidae	7%	-
Ctenophtalmus (C.) agyrtes sardiniensis (Ioff, 1929)	1%	*1*
Ctenophtalmus sp.	1%	*1*

Many introduced rodents have become invasive and cause problems to native wildlife and/or damage to urban infrastructure, agricultural crops, natural woods and tree plantations (Wauters et al. 2005; Tamura and Ohara 2005; Bertolino 2008; Bertolino 2009; Bertolino and Lurz 2013). Among rodents, tree squirrels have been introduced in several countries, mainly through the international pet trade or to increase hunting opportunities (Davis and Brown 1988; Aprile and Chicco 1999; Long 2003) and their capability to establish viable populations from only few founders has made them excellent invaders (Wood et al. 2007; Bertolino 2009; Martinoli et al. 2010; Bertolino et al. 2014).

The Pallas's squirrel, *Callosciurus erythraeus* (Pallas, 1979) is a tree squirrel native to Southeast Asia that has been introduced to Japan, Argentina, Belgium, France, the Netherlands and Italy (Bertolino and Lurz 2013). This species causes damage by gnawing tree bark, parts of buildings, cables and irrigation systems (Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). The origin of the Italian population, located in the province of Varese (Northern Italy), where it co-occurs with native Eurasian red squirrel *Sciurus vulgaris* L., is uncertain and no previous parasitological studies have been conducted on this exotic species in Italy. In countries where the species has been introduced, populations showed a low parasite species richness of imported macroparasite taxa, with most of the helminth or arthropod species detected only locally and accidentally acquired (Durden and Musser 1994; Shinozaki et al. 2004b; Asakawa 2005; Sato et al. 2007; Dozières et al. 2010; Gozzi et al. 2013; Gozzi et al. 2014).

Therefore, the main aim of the present study was to investigate the macroparasite fauna of the Pallas's squirrel established in Italy. In particular, we expected that introduced *C. erythraeus* harbors a macroparasite community poor in species, consisting of a few helminths and arthropods imported by the founding hosts, but composed mostly of i) local specialized parasites acquired from closely-related hosts (i.e. Eurasian red squirrels that co-inhabit with Pallas's squirrel) or ii) local generalist taxa able to develop in a wide range of hosts.

Materials and methods

Between May 2011 and June 2014 Pallas's squirrels (females=79, males=82) were captured from the only known Italian population located in the North of Varese province (45°58'09.2" N, 08°43'57.6" E, five kilometers away from Swiss borders).

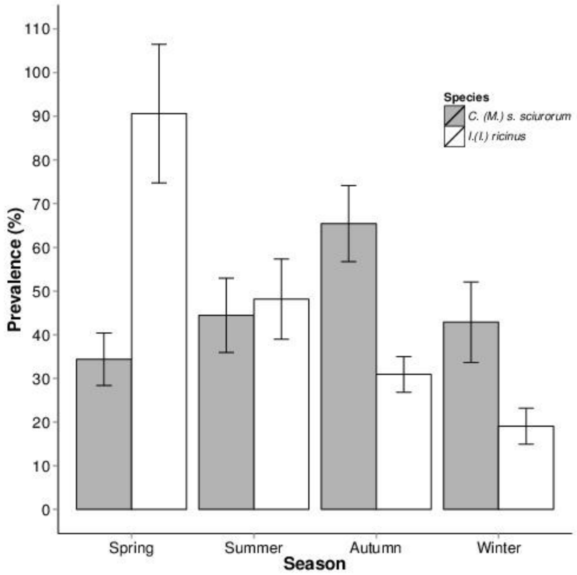

Figure 1. Prevalence (% of infected hosts ± SE) of C. (M.) sciurorum e I. (I.) ricinus during different seasons. Sample size above bars.

Trapping was carried out within a European Community LIFE Project (LIFE09 NAT/IT/00095 EC-SQUARE) aimed at eradicating the alien species in Italy. Traps (live-traps model 202, Tomahawk Live Trap Co., Wisconsin, USA) were set in the morning, baited with apple and hazelnuts and checked twice a day to minimize stress during trapping. For each trapped squirrel, sex and reproductive condition were recorded (Wauters and Dhondt 1989) and lactating females (nipples large, milk excretion if stimulated) were immediately released in order to comply with welfare of newborn animals. All males and non reproductive females were euthanized by CO_2 inhalation, following EC and AVMA guidelines (Close et al. 1996; Close et al. 1997; Leary et al. 2013). Each individual was weighed to the nearest 5 g using a Pesola spring-balance. Each carcass was immediately placed in a sealed plastic bag and stored at -20°C for further examinations.

Among all trapped squirrels only adult specimens were examined. The whole gastrointestinal tract was examined for endoparasites following standard parasitological procedures as described in (Romeo et al. 2013). Helminths were counted and stored in lactophenol for morphological identification using a microscope equipped with camera lucida (Romeo et al. 2013). In the laboratory, carcasses were also examined for ectoparasites by combing the whole body with a flea comb above a white surface. Recovered specimens were counted and stored in 70% ethanol for later identification. Identifications followed species descriptions and nomenclature provided by (Quentin 1971; Moravec 1982; Hugot 1984; Feliu et al. 2000; Sato et al. 2007) for nematodes, (Beaucournu and Launay 1990) for fleas, and (Pérez-Eid 2007) for hard ticks (Pisanu et al. 2007; Pisanu et al. 2008;

Pisanu et al. 2009; Dozières et al. 2010; Romeo et al. 2013; Romeo et al. 2014).

The probability of squirrels being infested by ectoparasites was analyzed through logistic regression to investigate the effects of hosts characteristics (sex and body mass) and season on the presence/absence of fleas and of ticks. In each model, the effect of the presence of the other species was also tested. We used Generalised Linear Mixed Models (GLMMs, Bolker et al. 2009b) adding year of sampling as random factor. Seasons were classified following the same criteria described by Wauters et al. (2007b) (winter: December- February; spring: March- May; summer: June- August; autumn: September- November). Model selection was carried out based on the minimum value of AIC. We checked model fit for overdispersion and through visual inspection of residuals (Zuur et al. 2009). Statistical analysis were done using R software (R Development Core Team 2014) and in particular the lme4-package v. 1.1-7 for fitting and analyzing mixed models (Bates et al. 2014). We decided to analyze presence/absence rather than abundance because the number of ectoparasites on an individual host showed a reduced variability with low values (see Table 1).

Results

Endoparasites

A total of 74 squirrels (females=34, males=40) were examined for gastrointestinal parasites. This sample size allows parasites detection with a confidence of 95%, with an expected prevalence greater than 4% from a population of 1000 individuals (Thrusfield 2013). Overall, 16 specimens belonging to 5 different nematode species were identified (Table 1). Four hosts harboured the Oxyurid nematode *Rodentoxyuris* (*Trypanoxyuris*) *sciuri* (Cameron 1932) with intensity of infection ranging from 1 to 6 helminths/squirrel (Table 1). Four other hosts had a single specimen of *Trichuris muris* (Schrank, 1788) each. One specimen of *Strongyloides callosciureus* (Sato et al. 2007) was found in the small intestine of a squirrel and a female belonging to the genus *Strongyloides* sp. was found in the small intestine of another individual. A single immature female of an unidentified Spirurid nematode was found in the stomach of a squirrel. Also, an immature female of a Capillariinae was found in the small intestine of another squirrel. There were no co-infections.

Ectoparasites

We identified three species of ectoparasites from the examination of 497 specimens collected in the fur of 135 Pallas's squirrels (females=61, males=74): two species of fleas and one hard tick species (Table 1). Of these specimens, 48 Ixodids could not be identified to species level (43 larvae and 5 nymphs). Both the common hard tick *Ixodes* (*Ixodes*) *ricinus* L. (131 larvae; 212 nymphs) and the flea *Ceratophyllys* (*Monopsyllus*) *sciurorum sciurorum* (Schrank, 1803) (51 females and 53 males) were the most prevalent parasitic arthropods with prevalence of 47% and 50%, respectively (Table 1). Other arthropods found were a single unidentified female of a flea genus *Ctenophtalmus* sensu-stricto, and a single male of *Ctenophtalmus* (*Ctenophtalmus*) *agyrtes sardiniensis* (Ioff, 1928). Acarid mites belonging to the family Trombiculidae were found on 10 squirrels and only their presence/absence on each squirrel was recorded. Species richness ranged from 0 to 3 (mean value=1.0±0.1 species per host). The minimal model describing ectoparasite prevalence retained as explanatory factors only trapping season for C. *(M.) s. sciurorum* and trapping season and sex for *I. (I.) ricinus* (Supplemental material 10), however in this last model sex was not statistically significant (p= 0.13). These results suggest that body mass, sex and presence of the other parasite did not affect the prevalence of the two main ectoparasites. Wald test performed for the pairwise comparisons among different seasons shows that prevalence of fleas was significantly higher in autumn than in spring (p< 0.01), whereas prevalence of ticks was significantly higher in spring than in all the other seasons (all p<0.05) and also higher in summer than in winter (p<0.01) (Fig.1).

Discussion

Pallas's squirrels introduced in Italy harbor a poor macroparasite fauna, characterised by 2 main ectoparasite species, and few helminth species all occurring with prevalence below 10%. Moreover, all the recorded parasite species, with the exception of the nematode S. *callosciureus*, are European, which were acquired in the invaded range (see Supplemental material 11 for the parasite species in its native range). This agrees with previous analyses on macroparasites of C. *erythraeus* s.l. introduced worldwide (Japan: Shinozaki et al. 2004a; France and Belgium: Dozières et al. 2010; Argentina: Gozzi et al. 2013; Gozzi et al. 2014) (Supplemental material 11).

In the present study, the gastro-intestinal helminth with the highest prevalence was *T. (R.) sciuri*, an Oxyurid species specific to the native Eurasian red squirrel that is transmitted mainly vertically (Hugot 1984; Romeo et al. 2013; Santicchia et al. 2015). In our study area, Pallas's squirrels co-occur with Eurasian red squirrels and no other squirrel species are present in the area. Hence, it is likely that the alien species acquired the local parasites through indirect contact, for example by using the same nests, where helminth eggs can be shed through grooming, on different nights. Moreover, in one host we recovered gravid *T. (R.) sciuri* females, indicating that the worm can fulfill its reproductive cycle in *C. erythraeus*. However, its prevalence remained very low, suggesting that this parasite is still not fully adapted to the Pallas's squirrel since it does not reach abundances observed in its natural host (Romeo et al. 2013). Moreover, the reported low prevalence may derive from both low environmental contamination and reduced direct contact between the native and alien species, as supported by radio-tracking data (Chapter 3).

In this study, *S. callosciureus* was found in only one host. This nematode primarily infects sciurids (Sato et al. 2007) and was originally found in Pallas's squirrels introduced to Japan, where it shows high prevalence of infection (i.e. 73%, n=100; Sato et al. 2007). Additionally, Sato et al. (2007) revealed a genetic variability of *S. callosciureus* in Japanese populations associated with strong differences in prevalence of infestation. Since our *C. erythraeus* genetic lineage still remains taxonomically uncharacterized (Chapter 1; Mazzamuto et al. in press), we believe that these differences in susceptibility according to host taxonomy found from Sato et al. (2007), could also explain the low prevalence of *S. callosciureus* in the Italian population.

The other species of helminths reported in this study were likely acquired through accidental infection. For example, *T. muris* was most likely acquired from terrestrial small rodents (Murid and Arvicolid; see Feliu et al. 2000) co-inhabiting with Pallas's squirrels (Dozières et al. 2010; Gozzi et al. 2014).

The ectoparasites detected in Italy have not previously been documented for Pallas's squirrel in its native (Gozzi et al. 2013; Lurz et al. 2013; Durden and Beaucournu 2014) or introduced range (e.g., Shinozaki et al. 2004a; Dozières et al. 2010; Gozzi et al. 2013; Lurz et al. 2013). The acquisition of *C. (M.) s. sciurorum* confirms that this species can adapt to various introduced squirrel species (i.e. *S. carolinensis*: Romeo et al. 2014; *T. sibiricus*: Pisanu et al. 2008). Indeed, *C. (M.) s. sciurorum* has a wide distribution in Europe where its primary host is the Eurasian red squirrel (Beaucournu and Launay 1990; Romeo et al. 2013). This flea occasionally infests mammals using arboreal nests, such as dormice (i.e. *Elyomis quercinus*, *Glis glis*, *Dryomis nitedula* and *Muscardinus avellanarius*) (Traub et al. 1983; Beaucournu and Launay 1990), providing that its primary host is present. Romeo et al. (2013) found an autumn peak of infestation by *C. (M.) s. sciurorum* in red squirrels and the same was detected in this study for infested alien hosts. Comparing prevalence of fleas in red squirrel (Romeo et al. 2013) and Pallas's squirrel in the same Italian biogeographic region, the alien species had a higher prevalence (red squirrel: 31%; Pallas's squirrel: 50%) probably due to its tendency to live at higher densities. These results suggest that parasite spill back towards red squirrels could occur, with the risk of a marked change in the native host-parasite dynamics.

The other most prevalent ectoparasite species found on Pallas's squirrels in Italy was the hard tick *I.* (*I.*) *ricinus*, which infests a wide variety of terrestrial vertebrate hosts (lizards, birds and mammals; e.g. Pérez-Eid 2007) in many Italian habitats (Dantas-Torres and Otranto 2013). It was found on both native and introduced squirrels in France (Vourc'h et al. 2007; Pisanu et al. 2010; Pisanu et al. 2014), but not on *S. vulgaris* and *S. carolinensis* in Italy (Romeo et al. 2013; Romeo et al. 2014). Such a discrepancy in infestations by *I.* (*I.*) *ricinus* between sciurids in France and Italy has been explained by the low densities or absence of ungulates, that are the key hosts species for *I.* (*I.*) *ricinus* (e.g., Ruiz-Fons et al. 2006; Pérez-Eid 2007; Tagliapietra et al. 2011), in the Italian sites where squirrels were collected (e.g., Romeo et al. 2013). In contrast, in our study area where Pallas's squirrels were sampled, roe deer is present. The seasonal pattern of tick prevalence observed in our Pallas's squirrel population peaked in spring as for other sciurids in France (i.e. Siberian chipmunks: Pisanu et al. 2010; Eurasian red squirrel: Pisanu et al. 2014). This peak coincides with the wettest season of temperate climates to which *I.* (*I.*) *ricinus* could be sensitive. Interestingly, the ratio of nymph to larvae on Pallas's squirrel was close to 2:1, which is similar to observations made on *S. vulgaris* in Switzerland and France (Humair and Gern 1998; Pisanu et al. 2014). This ratio is much higher than those usually observed for ground-foraging rodents with smaller body size (e.g., 1:80 for bank voles *Myodes glareolus*, and 1:10 for Siberian chipmunks *Tamias sibiricus*; Pisanu et al. 2010). This pattern of infestation could be related to the arboreal behavior and the wider home ranges of these larger body-sized sciurids, which may lead to a more frequent pick-up of nymphs from the undergrowth than larvae occurring on the ground.

We did not observe any gender-related differences in arthropod infestation for Pallas's squirrel, and this lack was also observed for *S. vulgaris* in France and Italy (Romeo et al. 2013; Pisanu et al. 2014) and *S. carolinensis* in Italy (Romeo et al. 2014). These observations deserve further attention, as sex-biased infestations by arthropods are critically related to behavioral processes associated with reproduction in small rodents (e.g., Krasnov et al. 2005; Ferrari et al. 2007; Krasnov et al. 2012). The complex interactions prevailing in this context can be difficult to assess (Le Coeur et al. 2014), especially for parasite species that do not have a long co-evolutionary history with a new host.

In contrast to what has been reported for *C. erythraeus* in its invading range in other European countries and in Japan (Shinozaki et al. 2004b; Dozières et al. 2010; Lurz et al. 2013), we did not find any sucking lice infesting the Italian population.

The low richness of macroparasite species found in our survey could be attributed to the history of this introduction. Despite the lack of precise information, the Italian population was likely founded by few individuals during just one or few introduction events. Such a small subset of founding individuals reduces the probability of parasites being introduced along with the founding hosts due to sampling effects (Torchin et al. 2003). Moreover, some parasites may have arrived in the new area, but they may have been lost during the first steps of establishment of the new population because of low host densities or adverse environmental conditions (Shinozaki et al. 2004b; MacLeod et al. 2010; Romeo et al. 2014). Finally, the founders likely originated from pet shops where antiparasitic treatments could had been applied. However, we must stress out that this limited parasite fauna could also be transitory because this population is still young and new host-parasite associations need time to establish (Torchin and Mitchell 2004; Gozzi et al. 2014).

Our findings suggest that, even though tree-squirrels are in general poorly parasitized because of their arboreal habits, which reduce the probability of acquiring infective free living stages in the environment (Aprile and Chicco 1999; Lurz et al. 2013), the loss of native parasites might indeed have contributed to their successful invasion (parasite release or enemy release hypothesis, Torchin et al. 2003).

We also showed that *C. erythraeus* acquired the flea *C. (M.) s. sciurorum* and the oxyurid nematode *T.(R.) sciuri* from red squirrels and introduced the nematode *S. callosciureus* with potential impacts on native red squirrels because of spill-back and spill-over processes (Kelly et al. 2009).The low prevalence of *T. (R.) sciuri* seems however to preclude, at least at present, any premises for parasite spill-back. On the other hand, further studies are needed to better understand the potential consequences of *C. (M.) s. sciurorum* spill-back and *S. callosciureus* spill-over to red squirrels (see also Romeo et al. 2014) and the role of the ongoing parasite colonization process on the invasion success of *C. erythraeus*. It must be stressed that, despite the parasite fauna of Pallas's squirrel in its native range has recently been reviewed (e.g., Gozzi et al. 2013; Lurz et al. 2013; Gozzi et al. 2014), effects of parasites at the host population level (e.g., Torchin et al. 2003) are unknown, which impedes any conclusions about enemy release *per se*. Finally, special attention should be paid to vector-borne diseases, especially pathogens transmitted by *I.* (*I.*) *ricinus* in Europe, since the high nymph to larvae ratio might indicate that Pallas's squirrels actually play a significant role in the circulation and transmission of such pathogens in the vertebrate community (see Pisanu et al. 2014), humans included.

CHAPTER 5

Do coccidia influence competition between native and invasive squirrels?

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Abstract

Introduction of alien species into new areas can have detrimental effect on native ecosystems and impact the native species. The present study aims to identification of *Eimeria* spp. infecting native and invasive squirrels in Italy, providing an gain insight into possible transmission patterns and, consequently, on the potential role of monoxenous coccidia in mediating the competition between native and invasive hosts. We collected fecal samples of native red squirrels *Sciurus vulgaris*, invasive grey squirrels S. *carolinensis* and invasive Pallas's squirrels

Callosciurus erythraeus. We examined 540 fecal samples from 466 animals. 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 of oocysts across the 3 host species revealed 3 *Eimeria* morphotypes. All the 3 morphotypes were found in *S. vulgaris*, 2 in *S. carolinensis* and only 1 morphotype in *C. erythraeus*. Partial sequences of nuclear and/or mitochondrial DNA were obtained from 40 *Eimeria* isolates. Phylogenetic analyses based on 18S, ITS, *CoxI* 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 low *Eimeria* species richness observed in 2 alien squirrel species suggests parasite-release which might facilitate the establishment of these species in the invaded range.

Introduction

Biological invasions are among the most prominent threats for biodiversity. Introduction of alien species into new geographic areas can have detrimental effect 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) (Zavaleta et al. 2001; Clavero and García-Berthou 2005; Hartigan et al. 2011; Pizzatto and Shine 2011). 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 (enemy-release hypothesis, 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 that may spill over to native hosts (Paterson and Gray 1997; Dubey and Shine 2008). Although majority of parasites are host-specific (Poulin 2011; Pizzatto and Shine 2011), a range of examples of successful invasion (i.e. 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 (Skerratt et al. 2007; Marzal et al. 2014; Atkinson et al. 2014). 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 (Tompkins et al. 2003; Collins et al. 2014). 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. 2004b; Wauters et al. 2005). However, in British Isles, the replacement process is accelerated by the SQPV (Rushton et al. 2006) 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; Romeo et al. 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 (1966) and, since 1999, in many sites in Lombardy (Martinoli et al. 2010; Bertolino et al. 2014). 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 (Bertolino and Lurz 2013; Mazzamuto et al. 2015). Since both species are a threat for local fauna, 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 (Levine and Ivens 1965; Hakkarainen et al. 2007; Winternitz et al. 2012). Despite *Eimeria* species being considered highly host-specific, cross-transmission of these species between different hosts has been demonstrated (Levine and Ivens 1988). The present study aims to identification of *Eimeria* spp. infecting native and invasive squirrels in Italy, providing an gain insight into possible transmission patterns and, consequently, on the potential role of monoxenous coccidia in mediating the competition between native and invasive hosts.

Material and methods

Trapping and sample collection

Fecal 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 fecal 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 habited 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 was placed at the bottom of each trap to collect the feces 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.

Feces 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 fecal 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 (Levine and Ivens 1965; Duszynski and Wilber 1997). 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 transcribed spacer 1 (ITS 1), 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS 2) and mitochondrial cytochrome c oxidase subunit I (*Cox I*) DNA, were amplified following PCR protocols and PCR primers published by Motriuk-Smith et al. (2009) and Kvičerová and Hypša (2013). The PCR reaction was performed in a 25 µl volume containing 2 µl (1-10 ng) of total DNA, 12.5 µl of commercial premix PPP master mix (Top-Bio s.r.o), 1 µl (400 µM) of each primer and 8.5 µl PCR H_2O. Each PCR reaction contained a negative control with PCR water instead DNA. Total DNA of *Eimeria*-positive fresh feces of *S. vulgaris* from rescue centres (CZ=Czech Republic), *E. exigua* oocysts from rabbit and *E. ferrisi* endogenous stages from mice (D7) were used as positive controls for all genes. The PCR products were separated by electrophoresis in 1.5% agarose gel stained with GoodView (ECOLI, Slovakia). Amplicons were purified using ExoSAP-IT® for PCR Product Cleanup (Affymetrix, USA). The selected amplicons were cloned into pGEM-T Easy Vector (Promega) and three plasmid clones of each were isolated using PureLink Quick Plasmid Miniprep Kit (Invitrogen). Sequencing of plasmids and PCR amplicons was carried out by the commercial company Macrogen (Amsterdam, The Netherlands).

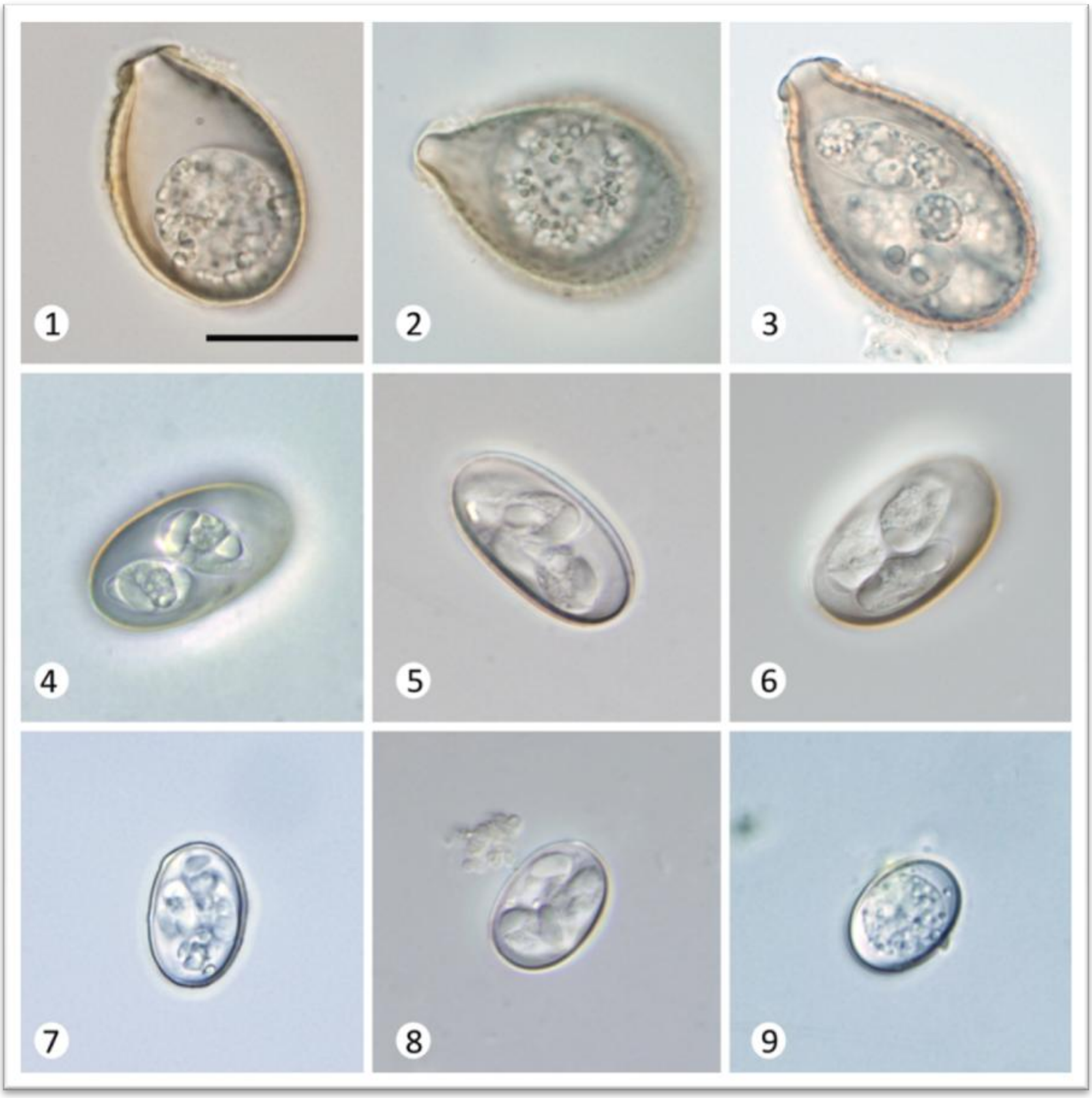
Phylogenetic analyses

Sequences were identified by BLAST analysis, edited and aligned using GENEIOUS Pro software package version 6.1 (Kearse et al. 2012) and deposited to the NCBI GenBank database under accession numbers KT360976 – KT361068, KT368144. Suitable model of molecular evolution was selected using jMODELTEST 0.1.1. (Posada 2008). The model with the best likelihood was chosen using AIC criteria and phylogenetic trees were reconstructed using Bayesian inference (BI) in the program MrBayes v. 3.2.2. (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). MrBAYES analyses were run for 2 million MCMC generations and with four chains. Runs for individual data sets were performed under the different models of

Table 1. ***Eimeria*** **spp. morphotypes, their counts of oocysts per gram of feces sediment (OPG) and prevalence with 95% CI in the examined host species.**

***Eimeria* morphotypes**	**OPG**	**Prevalence (%)**	**95%CI(%)**	**Species**
E1	102 – 79 394	7. 8	(4.1 – 11.4)	*S.vulgaris*
E2	116 – 66 550	95.6	(92.8 – 98.4)	*S.vulgaris*
	112 – 115 833	95.7	(92.6 – 98.9)	*S.carolinensis*
	$<10^2$ – 1 068	4.1	(1.1 – 7.1)	*C.erythraeus*
E3	$<10^2$	20.9	(15.3 – 26.5)	*S.vulgaris*
	$<10^2$	34.8	(27.4 – 42.1)	*S.carolinensis*

Figure 1. 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.



molecular evolution HKY+I for 18S data set; GTR+I+G for ITS 1, ITS 2 and 5.8S; GTR+G for *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. *Eimeria exigua* was used as an outgroup for all four datasets to root phylogenetic trees.

Statistical analysis

We analyzed variation in *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 modeled through generalized 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 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 (Table 1, Fig. 1), which differ in oocysts/sporocysts size (Supplemental material 12), 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 (Table 1, Fig. 1), 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 1965).

Morphotype E2

Morphotype E2 was detected in all 3 species of examined squirrels and the oocysts/sporocysts dimensions slightly differed between host species (Table 1, Fig. 1). 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; Pellérdy 1965; Joseph 1972).

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 (Table 1, Fig. 1). 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 was poorly visible, but present. Elongated sporozoites had bold refractile bodies. The *Eimeria* sp. E3 morphologically corresponds only with *E. silvana* oocysts characterization (Pellérdy 1965).

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

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

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).

Eimeria sp. E2 was the most prevalent morphotype across host species (for details on prevalence and 95% CI, see Table 1), being the only one infecting *C. erythraeus* (4.1%) and also the dominant finding 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 were these two hosts occurred, independently from host cohabitation (RED, GREY or RED-GREY). Mean E2 OPG values (±SE) 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 (Table 2, Fig. 2): OPG in red squirrels were significantly higher in adults than in juveniles or subadults (both p<0.019), whereas in grey squirrels we observed an opposite pattern, with adults significantly less infected than either juveniles or subadults (both p<0.028). In addition, E2 OPG in grey squirrels varied seasonally (Fig. 3), showing significantly lower values in summer than in all the other seasons (all p<0.018), 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 Table 1 for detailed prevalence). In both host species, infection by morphotype E3 varied across seasons (Table 2, Fig. 4): in red squirrels prevalence in winter was

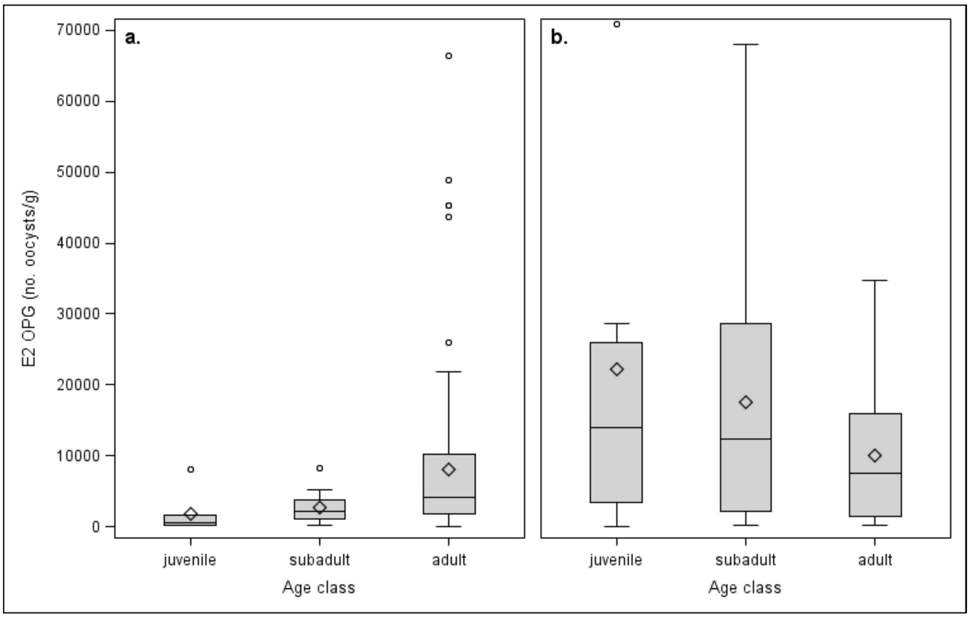


Figure 2. Morphotype E2 intensity of infection (OPG) by age class in red squirrels (a) and grey squirrels (b).

significantly lower than in all the other seasons (all p<0.039) and in grey squirrels E3 showed an infection peak during spring (all p<0.0097). Finally, presence of morphotype E1 in red squirrels, was affected by habitat type (Table 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 *S. vulgaris* and 17 *S. carolinensis*) originated from all three types of areas (RED = 19, GREY = 6, RED-GREY = 15) (Supplemental material 13). From the 40 sequenced samples, 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 analyzed in one dataset. All datasets (18S, ITS, *Cox I* and combined dataset) were analyzed by BI and ML based programs (see Materials 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 (Supplemental material 14, 15, 16, 17). 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 analyzed dataset was 1329 bp, sequences were yielded from 26 individuals and final data also contained referent sequences from NCBI (JQ993645, JQ993653, JQ993657, JQ993661 from Kvičerová and Hypša 2013). Samples of *Eimeria* sp. obtained from grey squirrels created separated clade from red squirrel samples with high branch support for both groups. The NCBI sequence JQ993653 *E. vilasi* created the sister group to the red squirrel samples. KT360995 *E. ferrisi* D7 clustered separately with JQ993657 *Eimeria* sp., sequences JQ993645 *E. cahirinensis* and JQ993661 *Eimeria* sp. clustered together and formed the most derived branch for the rest of the dataset.

The final length of ITS sequences in dataset was 1146 bp and alignment contained 25 sequences from our samples 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 samples created separate group from grey squirrel samples, which clustered together with EU302672, EU302673, EU302675, EU302676, EU302677, EU302678 and EU302681. All NCBI sequences were determined as *E. lancasterensis* (Motriuk-Smith et al., 2009). Two samples from red squirrels (KT361048 and KT361045) created sister branch with *E. ontarioensis* (EU302685, EU302686) (Motriuk-Smith et al. 2009).

Final length of *Cox I* alignment was 716 bp and sequences were obtained from 43 samples of *Eimeria* sp. in total. 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 control sample of laboratory mouse *E. ferrisi* D7 (KT361028) clustered with *E. burdai* (JQ993709) (Kvičerová and Hypša 2013) and formed separated branch with NCBI sequences JQ993707, JQ993704 *Eimeria* sp. and HM771682 *E. falciformis*

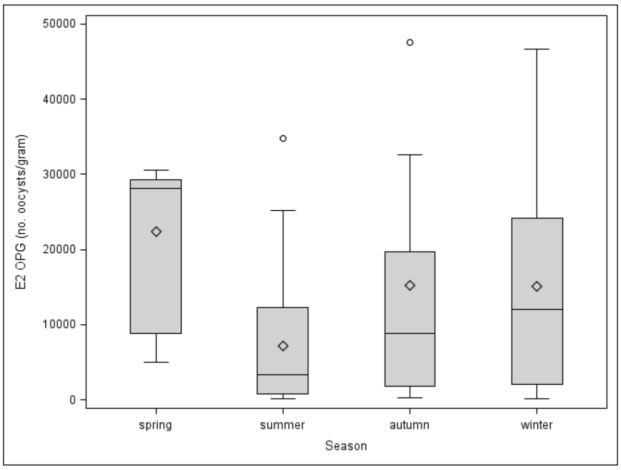


Figure 3. Seasonal variations in morphotype E2 intensity of infection (OPG) in grey squirrels.

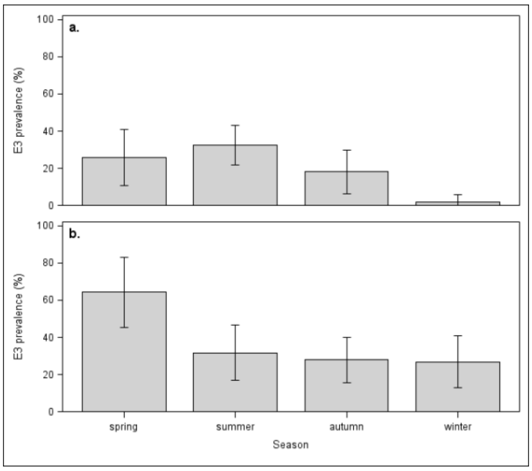


Figure 4. Prevalence of morphotype E3 by season in red squirrels (a) and grey squirrels (b). Error bars indicating 95% CI.

(Ogedengbe et al. 2011a). 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). Red squirrel samples clustered into monophyletic clade and formed well-supported branch likewise *Eimeria* sp. from grey squirrels. Concatenated tree corroborates with results of previous phylogenetic analyses of *Eimeria* sp. from red squirrels and grey squirrels and confirmed that each squirrel species is host for different *Eimeria* species.

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

Among the invasive mammalian species in European territory, the American and Asian squirrels are considered of enormous importance (Bertolino and Lurz 2013; Bertolino et al. 2014). Our study focused on comparison of coccidia of genus *Eimeria* infecting native (*S. vulgaris*) and alien (*S. carolinensis* and *C. erythraeus*) squirrels in Italy.

The traditional species concept and the identification of eimeriid coccidia rely 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 greatly complicated by the fact that several species can co-occur in a single host (Levine and Ivens 1965; Pellérdy 1974). The variability of oocysts of *Eimeria* in sciurids is lower that than e. g. in lagomorphs, where successful co-invasion by eimerian parasites with cottontail rabbit (*Sylvilagus floridanus*) was based on morphological identification of individual *Eimeria* spp. differences in oocysts (Bertolino et al. 2010).

Based on the feces examination, we identified 3 morphotypes of *Eimeria* oocysts in examined squirrels, in different prevalence and combinations of coinfections. The large thick wall oocysts of E1 morphotype apparently belong to *E. mira*. This species was found in *S. vulgaris* only, which suggests significant host specificity. The identity of morphotype E2 oocysts is not that straightforward, as it can include at least two nominal species, *E. sciurorum* (in *S. vulgaris*) and *E. lancasterensis* (in *S. carolinensis*). Oocysts of this morphotype were found in high prevalence both in red (%) and grey (%) squirrels, with occasional findings in Pallas squirrels. E3 was found in low prevalence in all three species. The prevalences of E2 and E3 morphotypes did not differ between single-species and mixed squirrel sub-populations. As the morphological examination was not efficient to distinguish the sciurid *Eimeria* species, we attempted to further analyse the *Eimeria* morphotypes using molecular identification and comparison.

Techniques of molecular taxonomy are helpful tool with better taxonomic resolution on species level in eimeriid coccidia (Motriuk-Smith et al. 2009; Motriuk-Smith et al. 2010; Ogedengbe et al. 2011b; Kvičerová and Hypša 2013), using various markers. 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; Motriuk-Smith et al. 2010; Ogedengbe et al. 2011b). So far, ITS1 and ITS2 are the only published sequences of coccidia from the Sciuridae, including the sequences of *E. lancasterensis* and *E. ontarioensis*.

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.

The results of phylogenetic analyses of E2 morphotype based on 18S, ITS, *cox I* markers displayed fairly distinct monophyletic clades from different host species isolates and proved indisputable distinction between E2 morphotype in red and grey squirrels, showing that E2 morphotype are two distinct species, with different hosts.

Coinfection with dominant morphotype E2 complicated analyses and DNA yield of other two morphotypes, 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. The results of phylogenetic analysis of ITS sequences showed the morphotype E1 (=*E. mira*) as a sister branch with *E. ontarioensis* (EU302685, EU302686) (Motriuk-Smith et al. 2009).

In conclusion, the morphological determination (*E. mira*) and molecular analyses (morphotype E2) do not support the hypothetical exchange of *Eimeria* spp., but revealed different *Eimeria* species in allopatric and sympatric population of native and introduced squirrels of genus *Sciurus*. Morphotype E3 was determined only morphologically, in accordance with valid species it morphologically corresponds with *E. silvana* oocysts, described in *S. vulgaris.* As we failed to obtain reliable molecular data, its possible transmission between red and grey squirrels remains unresolved. Similarly, the situation with E2 in Pallas´s squirrels deserves further attention.

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 1972; Spurgin and Hnida 2002; Motriuk-Smith et al. 2009). 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 nature host and native red squirrels.

The ecology of communities of *Eimeria* in sciurid hosts is intriguing phenomenon, underlying the prevalences of individual species in natural host populations. Motriuk-Smith et al. (2009) hypothesized two transmission strategies of *Eimeria* spp., as a trade-off between the oocyst production and their survival in environment. The highly prevalent species with thin-walled oocysts are less resistant in the external environment, but produce more oocysts with rapid sporulation and high transmission rates. In contrast, the species with thick-walled more resilient oocysts produce fewer oocysts, sporulate slowly and are considerably rare in host populations. We revealed higher prevalence of thin-walled morphotypes E2 (*E. sciurorum*/*E. lancasterensis,* >95 %) and E3 (*E.* cf. *silvana,* 20.9-34.8 %), compared to 7.8% prevalence of thick-walled morphotype E1 (*E. mira*). These findings correspond well with previously reported results, when *E. lancasterensis* with 65-91 % and *E. sciurorum* with 66-79 % showed higher prevalence than thick-walled *E. ontarioensis* 3-29%, *E. mira* 2.6 % or *E. confusa* 23 % (Joseph 1972; McAllister and Upton 1989; Spurgin and Hnida 2002; Bertolino et al. 2003; Motriuk-Smith et al. 2009; Ball et al. 2014).

The introduction and resulting 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 (Romeo et al. 2014; Collins et al. 2014; Romeo et al. 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.

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 that prevented specific identification of the single detected morphotype. Therefore, in both cases, additional investigation is 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 needed to ascertain definitely whether been indeed already demonstrated for the macroparasite fauna of both alien species in Italy (Romeo et al. 2014; Chapter 4). 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.

CHAPTER 6

The use of uterine scars to explore fecundity levels in invasive alien tree squirrels.

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Abstract

In invasion ecology, reliable measures of female fecundity are necessary to infer population growth rate and develop control programs to determine the proportion of animals that should be culled to reduce population size. Here, we present a reliable staining technique of uterine scars to determine individual fecundity in terms of both seasonal and total (annual) number of young born per female. We applied this method to two alien squirrels species (grey squirrel, *Sciurus carolinensis* and Pallas's squirrel, *Callosciurus erythraeus*) introduced in Northern Italy, obtaining carcasses from control campaigns from 2011 to 2013. We also investigated environmental and phenotypic variables that might affect individual variation in fecundity and compared annual reproductive output between the two species. For grey squirrels (n=44), 25% of examined females produced a single litter and 61% two litters. Females which reproduced in both seasons tended to have larger summer than spring litters (on average 2.61 and 1.94 offspring, respectively) and mean annual fecundity was 3.4 scars/female ranging from 1 to 8 births. There was no effect of year, eye lens weight, body size or body mass on total fecundity. For Pallas's squirrel (n=31), 58% of females had a spring litter, some of these also produced a summer litter (35%) and a few even a third litter in autumn (10%). Heavier and older females (higher eye lens weight) had more uterine scars than younger animals with lower body mass.

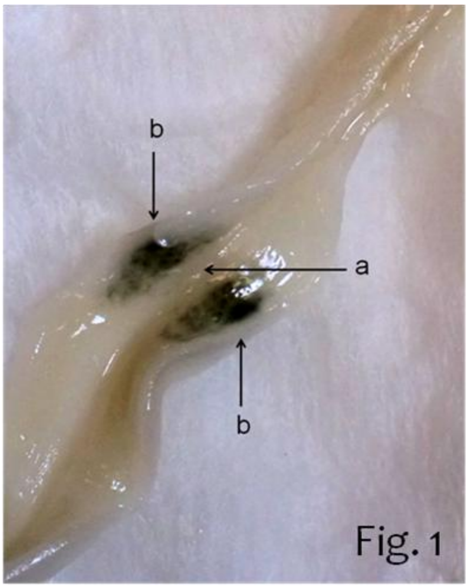
Finally, fecundity of the two IAS in Italy was similar or even higher than in the native range and/or in other countries of introduction, suggesting they are well adapted to their new environment and potentially have a high capacity to spread and recover after reduction of population size.

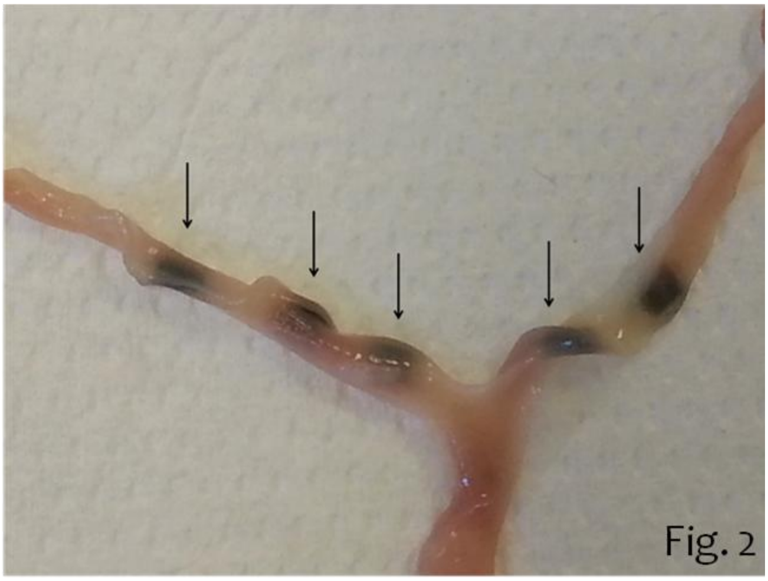
Introduction

Invasive alien species (IAS) are a major threat to long-term survival of local native species (Clavero and García-Berthou 2005; European Environment Agency 2012). Among mammals, many IAS have arrived in the new environment through pet trade (e.g. many tree squirrels) or by accidental or deliberate releases from fur farms (e.g. muskrat, coypu, mink) (Kolar and Lodge 2002; Bonesi and Palazon 2007; Martinoli et al. 2010; Bertolino and Lurz 2013; Simberloff et al. 2013).

Most of these species need to be controlled to avoid or reduce direct or indirect damage to native species, agriculture and forestry, buildings and infrastructure or human health (Keller et al. 2011; Simberloff et al. 2013; Genovesi et al. 2014). Control campaigns generally consist in removing a proportion of all animals present in a given population. In order for control to be effective, the number of individuals removed must be scaled to the net population growth rate to define adequate harvesting quotas (Baker 2006). Moreover, in addition to supplying practical information to directly implement control strategies, reliable estimates of population growth rates of IAS can support further investigations aimed at disclosing impacts of such species on native species and/or human health and activities.

One of the primary parameters affecting population growth rate is the annual reproductive output of females, which is the product of the number of young weaned per litter and the number of litters produced in a year (Gurnell et al. 2004b). This value is generally referred to as fecundity. In small mammals fecundity can be estimated by intensive capture-mark-recapture studies which, however, require a large investment in terms of time, manpower and funding (e.g. Wauters and Lens 1995; A. Wauters et al. 2008). Therefore, in species that are harvested for hunting and/or control, alternative methods have been developed. When carcasses of





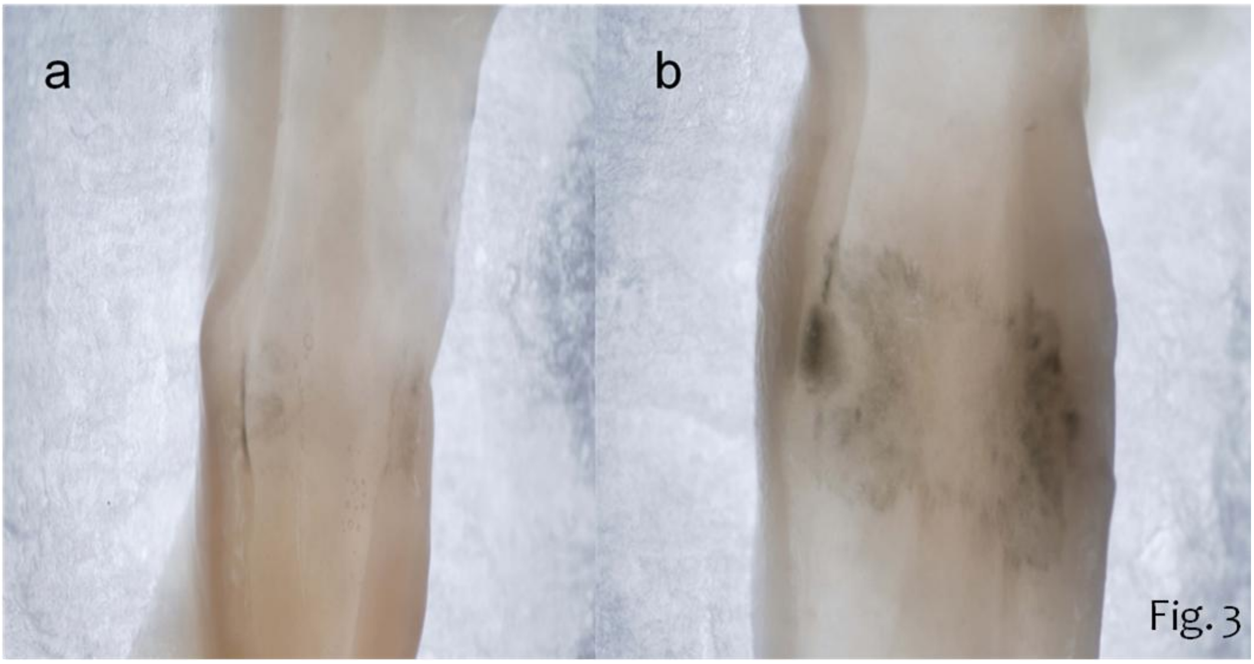


Figure 1. Detail of a stained placental scar in a longitudinally dissected uterus of grey squirrel. A single scar is constituted by a pale, central crater (a) surrounded by two dark bands (b). Each of the bands shows a circular area with darker pigmentation due to blood and macrophages accumulation (antimesometrial depression).

Figure 2. Uterus of grey squirrel (*Sciurus carolinensis*) stained to highlight placental scars (indicated by arrows). Uniformity of staining indicates that all the five scars are referable to the same gestation/parturition.

Figure 3. Light-stained/paler scar referable to an older gestation (a) and dark-stained/darker scar referable to a more recent parturition (b).

pregnant females are available, the number of embryos can be directly counted (Wauters et al. 1995). Otherwise, when carcasses are obtained after parturition or for species producing more than one litter per year, counting uterine scars can be an effective method (Bengtson and Siniff 1981; Lindström 1981; Rolley 1985; Bray et al. 2003). Such scars are left by the detachment of the placenta from uterine walls, hence each scar corresponds with the parturition of a single young, thus providing information on litter size at birth. Fecundity has been estimated through placental-scar counts in rodents (Nixon and McClain 1975; Martin et al. 1976), lagomorphs (Hackländer et al. 2011) and terrestrial carnivores (Allen 1983; Lindström 1994; Mowat et al. 1996; Ruette and Albaret 2010; Melero et al. 2015) and seals (Kauhala et al. 2014).

One of the issues involved in counting uterine scars resides in the difficulties to count them a long time after parturition (e.g. Martin et al. 1976; Elmeros and Hammershøj 2005; Ruette and Albaret 2010). Here we adapted a staining technique which allows to distinguish the number of uterine scars of different litters to two alien squirrels present in Italy (Eastern grey squirrel, *Sciurus carolinensis* and Pallas's squirrel, *Callosciurus erythraeus*). This method allows to estimate fecundity of female squirrels as a demographic parameter that may subsequently be used i) in studies on population dynamics of the alien species aimed at predicting necessary levels of control (i.e. number of animals to be culled) to successfully reduce population size; and ii) in spatially explicit population dynamics models to predict future range expansion and demographic trends (e.g. Lurz et al. 2001; Tattoni et al. 2006; Bertolino et al. 2008).

Materials and methods

Trapping and handling squirrels

Over three years (2011-2013), from September to December, grey squirrels (*Sciurus carolinensis*) and Pallas's squirrels (*Callosciurus erythraeus*) were trapped in Lombardy as part of a European Community LIFE Project (LIFE09 NAT/IT/00095 EC-SQUARE) aimed at eradicating the alien species in Italy. Traps (live-traps model 202, Tomahawk Live Trap Co., Wisconsin, USA) were set in the morning, baited with hazelnuts and checked twice to three times a day to minimize stress to captured animals during trapping. For each trapped squirrel sex and reproductive condition were recorded (Wauters and Dhondt,

Table 1. Fecundity data of female grey squirrels trapped in Northern Italy. The mean number of uterine scars (± SD), lowest and highest number of scars (range) and mean litter size (± SD) of females which produced at least a litter (scars ≥ 1). Data for spring and summer parturition separately, and over the entire year (total).

Litters	Uterine scars			Litter size	
	n	*mean ± SD*	*range (min- max)*	*n*	*mean ± SD*
Spring	44	1.41 ± 1.33	0 – 6	32	1.94 ± 1.19
Summer	44	1.95 ± 1.49	0 – 5	33	2.61 ± 1.12
Total	44	3.36 ± 1.71	0 – 8	38	3.89 ± 1.13

1989). Animals were euthanized by CO_2 inhalation, following EC and AVMA guidelines (Close et al. 1996; Close et al. 1997; Leary et al. 2013). Each individual was weighed to the nearest 5 g using a Pesola spring-balance and hind foot length was measured with a thin ruler (± 0.5 mm). Each carcass was immediately placed in a sealed plastic bag and stored at -20°C for further examinations.

Laboratory methods

We analyzed uterine scars of 49 female grey squirrels trapped in three different sites in Lombardy, Northern Italy and of 40 Pallas's squirrels (from the only population in Varese province, Lombardy, N. Italy). All the analyzed animals were culled at the end of the last breeding season (mid September – early December). This sampling strategy is justified since after the winter diapause the process of endometrium repair is completed and uterine scars can no longer be revealed by staining techniques. The collection of animals in autumn-early winter allow thus to reveal all the uterine scars of the previous breeding season. During *post mortem* examination, the uterus was collected, placed in clean tap water and sealed in plastic bags which were stored at -20°C. From the same individuals, we also took both eyes and stored them in vials with a 10% formalin solution. After two weeks, eye lenses were removed and dried for 48 h at 80°C following the procedure described in Beale (1962). Dried eye lens weight was then used to distinguish immature subadults from adults in *C. erythraeus* (body mass < 240g and dry eye-lens weight ≤ 13 mg), and as a proxy for age in both species (e.g. Beale 1962). Since, in populations at low density, grey squirrels are capable of breeding at 7-8 months-old (Gurnell et al. 2001), we did not use eye lens weight, nor body mass, to exclude animals from our analyses.

Fecundity was determined by counting the number of uterine-placental scars which are formed by the detachment of each embryo's placenta at parturition, thus allowing to estimate the total number of young born from a single female during the entire reproductive season (from February-

March to July-September in these alien tree squirrels) (Koprowski 1994; Gurnell 1996; our unpubl. data). Uterine scars were identified after staining which reveals dark pigments of macrophages involved in processes of repairing the endometrium after detachment of the hemochorial placenta (Figs. 1, 2, 3; Wild 1971).

After removing the connective tissue, the uterus was opened over its entire length and stained using the Turnbull reaction developed by Salewski (1964) on rats (*Rattus rattus*) and later adapted by Bray et al. (2003) on lagomorphs. Uteri were first immersed for 10 minutes in a fresh 10% solution of ammonium sulphide (H_8N_2S), rinsed thoroughly with tap water and then soaked for 10 minutes in a solution made of equal parts of 1% chlorhydric acid (HCl) and of a 20% solution of potassium hexacyanoferrate ($K_4[Fe(CN)_6]$, $3H_2O$). Then the staining process was ended by flushing the uterus with cold tap water to eliminate all traces of reagents. To prevent changes in uterine scars color over time, they were counted within the next hour using a microscope equipped with a digital camera. Since the macrophages are rich in hemosiderin, they are colored from blue to beige based on the age of the scar. A scar is recognized as having a paler central crater surrounded by two darker bands. In each band there are circular depressions with darker pigmentation due to concentration of blood and macrophages, indicated as antimesometrial depressions (Figs. 1, 2, 3).

Table 2. GLM (dependent variables: Number of litters or total annual fecundity measured by uterine scars of all litters) exploring the effects of study area (only grey squirrel), sampling year and of phenotypic parameters on variation in fecundity among (a) female grey squirrels; (b) female Pallas's squirrels.

Explanatory variables	Number of litters/year	Total annual fecundity
(a) Grey squirrel		
Body mass	χ2 = 0.00; df = 1; p = 0.95	χ2 = 2.06; df = 1; p = 0.15
Foot length	χ2 = 0.01; df = 1; p = 0.91	χ2 = 0.32; df = 1; p = 0.57
Eye lens weight	χ2 = 0.43; df = 1; p = 0.51	χ2 = 0.32; df = 1; p = 0.64
Year	χ2 = 0.73; df = 2; p = 0.70	χ2 = 3.33; df = 2; p = 0.19
Study area	χ2 = 0.45; df = 2; p = 0.80	χ2 = 2.26; df = 2; p = 0.32
(b) Pallas's squirrel		
Body mass	χ2 = 2.45; df = 1; p = 0.12	χ2 = 7.51; df = 1; p = 0.006
Foot length	χ2 = 1.42; df = 1; p = 0.23	χ2 = 4.39; df = 1; p = 0.036
Eye lens weight	χ2 = 6.24; df = 1; p = 0.013	χ2 = 15.3; df = 1; p < 0.0001
Year	χ2 = 0.01; df = 1; p = 0.92	χ2 = 1.46; df = 1; p = 0.23

We based the classification on the colors of crater, surrounding bands and antimesometrial depression. In particular, older scars, appear much paler than the more recent scars which show a darker staining. Since the animals have been collected in the same period and squirrels have synchronized seasonal parturitions, we assumed that the paler scars refer to the first parturition, while the darker ones to the second litter. In some Pallas's squirrels, three different shades of staining occurred, indicating that these females produced a total of three litters/year.

Statistical analyses

For both species, we explored the effects of phenotypic and environmental factors on individual variation in number of litters/year and on total fecundity (total number of uterine scars over the entire breeding season). Since our dependent variables were counts following a Poisson distribution, we used generalized linear models (GLM) with Poisson error structure to test effects of site of collection (study area, only for grey squirrels), sampling year, body mass, foot length and eye lens weight. We also explored effects of species, period (spring vs. summer litter; spring parturition from February to April; summer parturition from June to August) and the species * period interaction on litter size (number of births per litter) thus considering only females that had at least one uterine scar for a given reproductive period, using a generalized linear model with Poisson error structure. All GLM were done using the Proc Genmod procedure (SAS/STAT 9.4 software, Copyright © 2011, SAS Institute Inc., Cary, NC, USA).

Results

Grey squirrel

Of 49 female grey squirrels, 5 had a very small uterus, typical of immature nulliparous individuals and were not analyzed. Of the remaining 44 specimens, 6 did not have any uterine scars (0 litters, 14% of females), 11 (25%) had scars of only one litter and the remaining 27 (61%) had scars from two litters. Out of the 11 females that had a single litter/year, five reproduced only in spring and six only in summer. Overall, females produced between 0 to 8 young/year, with an average of 3.4 scars/female (Table 1). The highest litter size at birth (maximum number of scars for a single litter) was six (Table 1). There was no difference in average number of scars between spring and summer reproduction

Table 3. Fecundity data of female Pallas's squirrels trapped in Northern Italy. The mean number of uterine scars (± SD), lowest and highest number of

scars (range) and mean litter size (± SD) of females which produced a litter (scars ≥ 1). Data for spring, summer and autumn parturition separately, and over the entire year (total).

Litters	Uterine scars			Litter size	
	n	*mean ± SD*	*range*	*n*	*mean ± SD*
Spring	31	1.90 ± 1.97	0 – 6	18	3.28 ± 1.45
Summer	31	0.90 ± 1.49	0 – 6	11	2.55 ± 1.44
Autumn	31	0.26 ± 0.81	0 – 3	3	2.67 ± 0.58
Total	31	3.06 ± 3.00	0 – 9	18	5.28 ± 1.87

(Mann-Whitney U-test, p = 0.087). However, if we only considered the 27 females that had scars for both periods, they produced on average a larger summer than spring litter (difference 0.74 ± 0.25 young/litter, Wilcoxon matched pairs signed ranks test, p = 0.01).

Body mass of female grey squirrels varied between 380 and 660 g (mean ± SD = 533 ± 51 g) and right hind foot length between 56.0 and 67.0 mm (mean ± SD = 62.1 ± 2.5 mm). Neither body mass (Fig. 4a) nor foot length significantly affected variation in total number of uterine scars, or in number of litters produced. Also we found no effects of eye lens weight, study site or year (Table 2a).

Pallas's squirrel

Of 40 female Pallas's squirrels, 9 had a small uterus, typical of immature nulliparous individuals and were not used in further analyses. Of the remaining 31 specimens, 13 did not have any uterine scar (or litters, 42% of females), 7 (22%) had scars of only one litter, 8 (26%) of two litters, and 3 females (10%) had scars from three litters. All females that produced a summer or summer and autumn litter (thus with more recent uterine scars) had also produced an early spring litter (oldest uterine scars). Hence, out of the 18 reproductive females, all of them had a spring litter, 11 reproduced in spring and summer and three in spring, summer and autumn. Overall, females produced between 0 and 9 young/year, with an average of 3.1 scars/female (Table 3). The highest litter size at birth (maximum number of scars for a single litter) was 6 (Table 3). Mean number of uterine scars of early spring litters was higher than of summer (second) litters (Mann-Whitney U-test, p = 0.015), but this was mainly due to more females having a spring than a summer litter resulting in more specimens with 0 scars for summer litters. In fact, considering only the 11 females with both spring and summer parturition, there was no difference in average litter size (Wilcoxon test, p = 0.60).

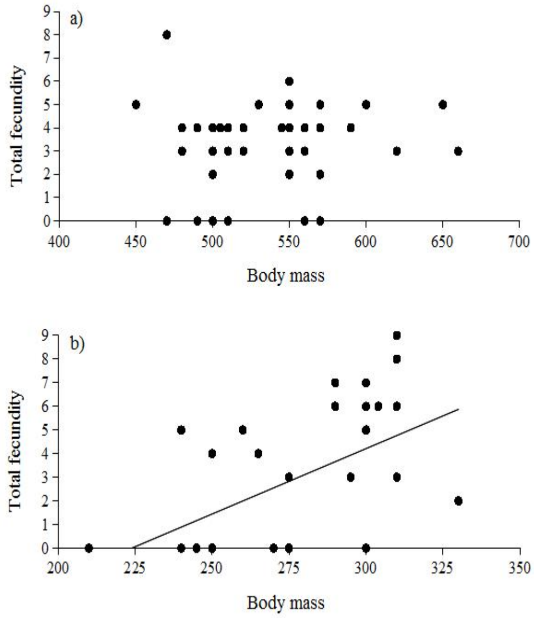


Figure 4. Relationship between female body mass (g) and total fecundity (total number of uterine scars over the entire breeding season) in: a) grey squirrels; b) Pallas's squirrels.

Body mass of adult female Pallas's squirrels varied between 210 and 330 g (mean ± SD = 279 ± 28 g) and right hind foot length between 45.0 and 51.0 mm (mean ± SD = 49.1 ± 1.5 mm). Number of litters/year increased with eye lens weight, indicating that older female produced more litters (Table 2b). There was no effect of sampling year on number of uterine scars, but the total number of uterine scars increased with a female’s foot length (estimate ± SE = 0.19 ± 0.09), body mass (Fig. 4b, estimate ± SE = 0.012 ± 0.004) and age (eye lens weight 0.064 ± 0.016; Table 2b). Body mass and eye lens weight were correlated (n = 31; r = 0.39; p = 0.029) suggesting an increase of body mass with age.

Grey squirrel vs Pallas's squirrel

There was a nearly significant species * period interaction (χ2 = 3.75; df = 1; p = 0.053) on litter size. Therefore, we compared litter size between the two species for spring and summer litters separately (for values see Tables 1 and 3). In spring, Pallas’s squirrels produced on average larger litters than grey squirrels (Kruskal-Wallis χ2 = 12.0; df = 1; p = 0.0005), but in summer there was no difference between the two species in mean litter size (Kruskal-Wallis χ2 = 0.29; df = 1; p = 0.59). As a consequence, total litter size was higher in Pallas’s squirrel than in grey squirrel (Kruskal-Wallis χ2 = 8.10; df = 1; p = 0.004) with breeding females of the former producing on average 1.4 young more per year than grey squirrels.

Discussion

The uterine staining technique described in this paper and applied to invasive tree squirrels allows to distinguish individual uterine scars and to discriminate scars produced earlier (spring litters) or later (summer – autumn litters) in the breeding season. Although the small dimensions of squirrel's uteri makes the measure of the scars as discriminant tool unpractical, we found that the use of variability in macrophage coloration is a reliable method to recognize different parturitions. The technique was successfully applied to both grey squirrels and Pallas's squirrels and allowed us to distinguish from two (grey squirrel) to even three (Pallas's squirrel) parturition events (litters) in a year and to determine number of births (litter size) for each litter separately as well as a female's total fecundity over the entire year (e.g., Nixon and McClain 1975; Martin et al. 1976; Hackländer et al. 2011).

Earlier works made use of uterine scars to estimate fecundity in these same squirrel species, however details on the staining method used and on the number of litters/year are often lacking. More in detail, Tang and Alexander (1979), T'sui et al. (1982) and Tamura (1999) analysed fecundity of *C. erythraeus* in its native range without describing the staining method and with no indication of how older scars (from earlier litters) could be distinguished from more recent ones. Also, the number of scars from single and/or multiple litters was not reported and authors only analyze data of number of embryos/litter in pregnant females (see Table 5). Humason (1962) on grey squirrels in their native range, refers to the staining method used as "Prussian blue reaction" without giving further details. Similarly, Shorten and Elton (1951) on grey squirrels introduced in the UK did not describe how the uterus was stained or how uterine scars were counted and apparently no attempt was made to distinguish between older and more recent scars. Moreover, this paper only presented average number of uterine scars per female for a given litter (spring or summer/autumn litter) without range of scars or a measure of variance (SD or SE, see Table 4). Only Nixon and McClain (1975) distinguish between older (spring litter) and more recent scars (summer litter), using darker staining as the discriminating factor. Distinguishing placental scars from more recent litters from those of, previous, older litters based on a darker unstained appearance and/or on darker staining has been used successfully also in other species (e.g., voles, foxes, lynx, mink; Martin et al. 1976; Allen 1983; Mowat et al. 1996; Elmeros and Hammershøj 2006; Melero et al. 2015).

Grey squirrel

In our sample of grey squirrels from N. Italy, collected over a period of 3 years, 61% of females produced two litters/year. This was higher than the average reported in a multi-year study in the native range (Ohio, 27%, Nixon and McClain 1975). These authors also documented that in their study site the percentage of females producing two litters/year varied between 0% and 36% in relation to tree seed abundance; consequently total number of uterine scars/female varied over years and was highest in years with good seed crops (Nixon and McClain 1975). In contrast, we did not find a significant year effect on total number of uterine scars/female in this study. Medium to long-term studies have documented that grey squirrel reproductive rate varies markedly among years in both the native range and in areas in Great Britain where the species has been introduced (e.g. Koprowski 1994; Gurnell 1996; Kenward et al. 1998). Reproductive rate is density dependent (with adult female density) and increases when food abundance is high. In our case, the lack of significant variation in total reproductive output between years might be due to small sample size and/or less annual variation in food availability in the Italian sites (deciduous woodland and park habitats) than in the woods and forests in Great Britain and North America.

In this study, females that had reproduced in both seasons tended to have a larger summer than spring litter (2.61 and 1.94 offspring respectively, see Table 4). Larger summer litters were also reported for grey squirrels introduced to England Shorten and Elton (1951) and in the native range, particularly for adult (> 1-year old) females (Table 4). In the study in Ohio, adult females tended to produce larger litters than yearlings and, consequently, their total annual reproductive output was higher (Table 4 and Nixon and McClain 1975). We did not divide our sample in these two age-classes, but used eye lens weight as a continuous variable and proxy for age. However, we did not find any effect of eye lens weight on number of litters or on total fecundity. Among the grey squirrels analyzed in this study, variation in number of litters and in total number of uterine scars was not correlated with body size or body mass. Body mass and age are important factors in determining female reproductive success in other tree squirrels, for example Eurasian red squirrels (*Sciurus vulgaris*), through both indirect effects (on dominance status, home range quality) and direct effects (entering oestrus, successful weaning of offspring, number of litters in lifetime) (Wauters and Dhondt 1989; Wauters and Dhondt 1995; Lurz et al. 2000; Wauters et al. 2001b; Lurz et al. 2005; A. Wauters et al. 2008). There can be several reasons why we did not find any effect of

Table 4. Fecundity data of grey squirrels: Comparison between populations from the native range and introduced populations (this study).

	Native range				Introduced populations							
	Nixon and McClain 1975				*Shorten 1951*				*This study*			
Breeding season	age	placental scar count *n*	placental scar count *mean±SD*	range	age	placental scar count *n*	placental scar count *mean±SD*	range	age	placental scar count *n*	placental scar count *mean±SD*	range
Spring	Yearling	23	2.43±0.58	1-3	All	66	2.70	1-4*	All	32	1.94±1.19	1-6
	Adult	40	2.70±0.89	1-4								
Summer	Yearling	35	2.51±0.77	1-4	All	36	3.04	1-7*	All	33	2.61±1.12	1-5
	Adult	63	3.49±0.87	2-6								
Annual	Yearling	46	2.54±0.75	1-4	-	-	-	-	All	38	3.89±1.13	1-8

n = number of squirrels examined; mean number of scars/female with SD; range = minimum and maximum number of uterine scars per litter or over the entire year (annual); *= embryos/female, no data on placental scars range.

eye lens or body mass on number of uterine scars in this study. First, our data are few and larger datasets are necessary to investigate how individual variation in phenotypic characteristics might affect variation in reproductive rate. Second, age effects might be important only between primiparous yearlings and multiparous adults, as suggested by Nixon and McClain (1975): in this case using a continuous variable (i.e. eye lens weight) might not reveal a significant pattern. Third, body mass, which is known to fluctuate seasonally in this species (Kenward and Tonkin 1986; Koprowski 1994), could be important for successful reproduction at the beginning of the breeding season, but not relevant when measured at the end of it (as in this study). Fourth, the lack of effects of body mass and age might indicate an adaptation of the IAS to the new environment: grey squirrel females invest in producing offspring independent of their age, size or body condition and their relative success (variation in number of young) is more determined by other factors not measured here.

Finally, it is interesting to note that mean litter size estimated with number of uterine scars was slightly higher for both spring and summer litters in Ohio than in this study (Table 4). In contrast, in our case more females reproduced twice a year and consequently mean number of uterine scars/female over the entire year was higher. Also, maximum number of scars found in a female was higher in N. Italy than in the population in the native range (Table 4). This higher reproductive rate may be an adaptation of the introduced grey squirrel

Table 5. Comparison between studies on fecundity (mean ± SD and range) in Pallas's squirrel native range and in introduced populations.

	Native range		**Introduced populations**	
	Tang and Alexander 1979	*T'sui et al. 1982*	*Tamura et al. 1988* *Tamura 1999*	*This study*
Litters/year	1-2	1-2	1-3	1-3
Embryos/female	1.68	Spring 1.83 Summer 1.75	2.4±0.7 (1-4)	-
Scars/ breeding female	-	-	-	Spring 3.28±1.45 (1-6) Summer 2.55±1.44 (1-6) Autumn 2.67±0.58 (1-3)

population to the spreading phase in act. We found only one paper with partly comparable data on uterine scars of other introduced populations (grey squirrels in the UK, Shorten, 1951). However, as above mentioned, this paper only presents average number of uterine scars per female for a given litter (spring or summer/autumn litter) without any range of scars or a measure of variance. Overall, mean number of scars for both spring and summer litters, as well as the maximum number of young/litter in summer/autumn (based on embryo counts) tended to be higher in the UK populations than in Italy (Table 4). It has been shown that grey squirrels in the UK spread more rapidly than in Italy (Bertolino et al. 2014), but both studies presented preliminary results of fecundity and it would be speculative trying to explain differences in fecundity between invasive populations in the two countries based on these data.

Pallas’s squirrel

Although our dataset was small, relationships between phenotypical characteristics of females and reproductive output revealed some interesting patterns. In the introduced population in N. Italy, females invested heavily in an early spring litter. In fact, 58% of animals examined had a spring litter, and only among the females which had already produced a spring litter, some also produced a summer litter and a few even a third litter in autumn. In contrast with grey squirrels examined in this study, individual variation in total number of uterine scars in Pallas’s squirrels depended on eye lens weight (age) and body mass: older females and those of higher body mass produced more offspring in a year than younger ones and/or than females of lower body mass. The preponderance of early (spring) breeding in our study site might be related to seasonal variation in food availability or quality. We often observed Pallas's squirrels feeding first on flowers and later on fruits of wild cherry (*Prunus avium*), a common tree species in the mixed deciduous forest where the population occurs. These food items are abundant and rich in carbohydrates and may constitute an energy-rich food supply for breeding females in spring. Furthermore, although we do not yet know if and how frequent Pallas's squirrels hoard tree seeds in our study site, in similar habitats cached tree seeds are an important food source for spring breeding in native red squirrels (Wauters and Casale 1996; Zong et al. 2010; Zong et al. 2014).

We found few data in the literature reporting measures of fecundity in this species, either in its native or introduction range (Table 5). Data from the native range, based on counts of embryos in animals shot throughout the year, reveal two litters per year (concentrated in spring and summer) with only 1-2 embryos/litter (Tang and Alexander 1979; T'sui et al. 1982). These authors suggest that small litter size was related to high population density in the areas where animals were collected (commercial conifer forests, orchards and woods). In any case, all data from populations introduced to other countries show higher fecundity than in the native range (Table 5), suggesting that this species might be extremely adaptive to new environments and habitats, as supported by its high invasiveness (Bertolino and Lurz 2013). High fecundity in colonizing populations in Italy and Japan could also be due to low squirrel density. However, in our case, large number of animals removed for control and high estimated population density (ca. 8 individuals/ha, Chapter 7) seem to contradict this. Comparing fecundity between Japan and Italy, although based on different methods, showed that in both countries females could produce up to 3 litters per year, with average and maximum litter size slightly higher in Italy than in Japan (Table 5) (Tamura et al. 1988; Tamura 1999). In contrast, data from a population introduced at Antibes (France), suggest lower fecundity in this country (n = 84, mean ± SE = 1.8 ± 0.1 embryos/female, range = 1-3, Chapuis et al. unpubl. data) maybe due to poor habitat quality at the collection site.

Conclusions

Our first data of uterine scar counts in female grey squirrels culled in N. Italy suggest than fecundity over an entire year was slightly larger than in populations in the native range. Hence, this IAS seems well adapted to the new environment in Italy, where habitats most commonly occupied are mixed lowland woods with presence of oaks (*Quercus* sp.) and urban, suburban or private parks where densities tend to be high(Wauters et al. 1997; Venturini et al. 2005; Bertolino et al. 2008; Martinoli et al. 2010). Furthermore, it has been reported that the grey squirrels in Italy have lost many of the macroparasite species they normally host in their native range, setting the premises for positive effects of parasite release on host fitness (Romeo et al. 2014), which could also include a higher fecundity in animals with low helminth abundance in the Italian populations.

A similar pattern was found in the Pallas's squirrel where uterine scar counts definitively indicate a high fecundity of individual females when compared with the (scarce) data available for the native range. Also for this species, the extremely poor parasite fauna and absence of gastrointestinal helminths in most animals, suggest that parasite release might be involved in the high fecundity of the IAS in this area (Chapter 4) and in other countries where it has been introduced (Dozières et al. 2010; Gozzi et al. 2014). Although there are no directly comparable fecundity data for native red squirrels, which can have large litters in some circumstances (Mari et al. 2008), estimates of reproductive rate from percentage females breeding and litter size at weaning in lowland mixed woodlands (e.g., Wauters and Lens 1995; Wauters et al. 2001b) suggest that the two IAS have similar or even higher fecundity than the native species. Thus, non only grey but also Pallas's squirrels might become a risk for the long-term persistence of populations of the native red squirrel. Preliminary data on trapping success and removal of the IAS linked with occurrence/density of red squirrels (Chapter 7) suggest that this is actually the case.

Our data also have implications for Spatially Explicit Population Dynamics Models (SEPMs) used to predict future range expansion of invasive squirrels. In fact, in these models, parameter input of fecundity values have a high impact on model results of future population size and distribution (Lurz et al. 2001; Guichón and Doncaster 2008). Based on fecundity values found in our study, the range of litter sizes and % females breeding used in SEPMs predicting future expansion of grey squirrels in Italy are realistic (litter size range: 2-4 in Lurz et al. 2001; 1.5 – 3.5 in Tattoni et al. 2006; 2 – 3.5 in Bertolino et al., 2008). However, a SEPM developed to predict range expansion of Pallas's squirrel in Argentina used a mean litter size of 2 young/female per year (Guichón and Doncaster 2008), which, based on our data, seems an underestimation of true fecundity.

In conclusion, the results presented in this paper indicate that uterine scar counts are a good method to estimate reproductive output in the two introduced squirrel species and that both IAS have a high reproductive output in their new range in Italy. Therefore, and since both species negatively affect native red squirrels (Gurnell et al. 2004; Bertolino et al. 2014; Chapter 2), management plans must aim to eradicate both species in Italy, or, when in the case of the grey squirrel this is considered impossible, control populations to avoid further expansion.

CHAPTER 7

Interspecific competition between alien Pallas's squirrels and Eurasian red squirrels reduces density of the native species

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Abstract

Pallas's squirrel has been introduced in Northern Italy in its current range in it co-occurs with the native Eurasian red squirrel that could suffer from interspecific competition. In this paper we present the results of the control campaign and the effects of Pallas's squirrel presence on red squirrel population dynamics. We tested the hypothesis that the alien Pallas's squirrel negatively affects red squirrel population density, fecundity and local survival comparing an the experimental area where Pallas's and red squirrel share the same space and food resources, and a control area without the invasive squirrel. According to our predictions, we found a significant lower density of red squirrels in the red-Pallas than in the red-only area with a heterogeneous distribution in the experimental area. A low local survival of red squirrel when in co-occurrence was confirmed, but its reproductive success did not seem to be affected by the Pallas's squirrel presence. Moreover the removal of the invasive squirrel throughout the study period had only local effects in some trapping sites allowing the return of the native species suggesting a low trapping effort to increase the demographic parameters of the red squirrel population.

Introduction

It is generally in press that stable, long-term coexistence between two species that compete for the same resources is possible only when some degree of niche differentiation occurs (Emmons 1980; Riege 1991). If there is no (sufficient) niche differentiation and resources are limited, interspecific competition can be severe and affect individual's fitness (e.g., reduced survival and/or reproductive success), which will be reflected at the population level (distribution, population density, persistence; Holway 1999; Byers 2000; Shuttleworth et al. 2015). When there is no sufficient time for co-evolution to shape niche differentiation, competition can ultimately lead to extinction of one of the two species (Ricciardi et al. 1998; Mooney and Cleland 2001).

This problem of exclusion competition can occur when an alien species is introduced in a new environment where its niche overlaps strongly with that of an ecologically similar native species (Broennimann et al. 2007). As opposed to naturally co-evolved competitor systems, in the case of human-mediated introductions the two species (alien and native) come into contact abruptly, each one being in some way "trapped" by its evolutionary background. In many cases the outcome, lacking the time for niche differentiation to evolve, is the establishment of mechanisms of competitive exclusion with the alien species replacing the native one (Hardin 1960; Ricciardi et al. 1998; Strong and Pemberton 2000; Mooney and Cleland 2001; Bertolino 2008).

The introduction of alien species is one of the most important causes of biodiversity loss. Many invasive alien species (IAS), once established outside their native range, have a fast demographic increase and severe impacts on native species and/or ecosystems (Mack et al. 2000; Kolar and Lodge 2002; Ehrenfeld 2010; Strayer 2012). Invasive species can alter the natural evolution of the native ones not just by competitive exclusion, but also by hybridization, predation and parasite-mediated competition (Kats and Ferrer 2003; Cox 2004; Largiadèr 2007; Romeo et al. 2014), raising extinction risk for many taxa and affecting our health and economy (Clavero and García-Berthou 2005; European Environment Agency 2012).

Among mammals, tree squirrels are good examples of IAS. They have been introduced worldwide mainly through the international pet trade (Aprile and Chicco 1999; Long 2003) and their capability to establish viable populations from only a few founders, the high vagility and diverse food habits have made them a successful invasive taxon (Palmer et al. 2007; Bertolino 2009; Martinoli et al. 2010). Alien – native squirrel species interactions are good model systems to explore patterns and mechanisms involved in interspecific (resource) competition and disease-mediated competition (Gurnell et al. 2015; Shuttleworth et al. 2015). The replacement of the native Eurasian red squirrel (*Sciurus vulgaris*) by the alien Eastern grey squirrel (*Sciurus carolinensis*) in the Brithish Isles and Italy is a well-known example of invasion leading to local extinction of the native species. The presence of the invasive grey squirrel negatively affects the food exploitation and, in some forests, habitat use, of red squirrels resulting in smaller body size and reduced female reproductive success and juvenile recruitment, ultimately causing population size to decrease until local extinction (Gurnell et al. 2004b; Gurnell et al. 2015). Among squirrels, another case is the competition between the American red squirrel (*Tamiasciurus hudsonicus*) and the Abert's squirrel (*Sciurus abertii*). Abert's squirrel is naturally sympatric with red squirrels over much of its range in the United States (Thorington et al. 2012). However in the Pinaleño Mountains (Arizona, USA) the Abert's squirrel was recently introduced in an area where the endemic Mt. Graham red squirrel (*T. h. grahamensis*) has been isolated for approximately 10,000 years from other tree squirrel species (Lomolino et al. 1989; Edelman and Koprowski 2005). An on-going research is trying to determine the impact of the Abert's squirrel on the Mt. Graham red squirrel population (Edelman and Koprowski 2005; Derbridge pers. comm.). Preliminary results suggest that, even if red squirrels are territorial (Steel 1998) and appear to be dominant over Abert's squirrels in inter-specific encounters (Steel 1998; Hutton et al. 2003), the non-native species may have an effect on the space use of the red squirrel because of extensive home range overlap and use of common food resources, which may force the native species to forage more widely and consume sub-optimal food items (Ferner 1974; Edelman and Koprowski 2005; Edelman and Koprowski 2006; Derbridge pers. comm.).

In the last decade, a tree squirrel native to South-East Asia, the Pallas's squirrel (*Callosciurus erythraeus*), has been introduced in Northern Italy, but the story of the introduction as the number of squirrels released are still unknown. So far, Pallas's squirrel has been introduced in seven countries around the world (Argentina, France, Belgium, Italy, the Netherlands and Japan), and of the 29 known introduction events 21 (including Italy) are documented as successful with a viable population established in the wild (Bertolino and Lurz 2013). The species is known to be invasive and to damage commercial and forest trees, electric wires and parts of buildings (Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). Moreover, in Japan the native squirrel species, *Sciurus lis*, is locally declining probably because of competition for food and nesting sites (Ministry of the Environment, Japan, 2002; Miyamoto et al. 2004).

In its current range in Italy, Pallas's squirrel co-occurs with the native Eurasian red squirrel that suffers from interspecific competition for food resources, due to ecological niche overlap. For this reason in 2011 a removal program of the invasive squirrel started (European Life Project LIFE09 NAT/IT/00095 EC-SQUARE; http://www.rossoscoiattolo.eu/en).

In this paper we present the results of the control campaign and the effects of Pallas's squirrel presence on red squirrel population dynamics. In particular we examined red squirrel population density, fecundity and local survival in the experimental area where Pallas's and red squirrel share the same space and food resources, and in a control area without the invasive squirrel. Based on earlier work on interspecific competition between native red and alien grey squirrels (Wauters et al. 2000; Gurnell et al. 2004b; Gurnell et al. 2015), we tested the hypothesis that the alien Pallas's squirrel negatively affects the demography of the native red squirrel. Hence, we predict that: (i) distribution and/or density of red squirrels will be lower in the mixed-species than in the red-only area; (ii) local survival and/or reproductive success of the native species will be lower in the mixed-species than in the red-only area. Finally, we predict that removal of Pallas's squirrels throughout the study period should result in an increase in the demographic parameters of the red squirrel population over time in the experimental area.

Materials and methods

Study design

We used an unbalanced study design, using data from different studies on red squirrel ecology (control area) carried out both

Table 1. Trapping sites in the red-Pallas area.

Trap site	N traps	Sessions	*C. erythraeus*	*S. vulgaris*
01	20	6	16	-
02	20	5	22	-
03	3	6	30	1
04	33	32	225	19
05	44	38	123	22
06	20	5	6	2
07	28	10	74	2
08	13	19	46	5
09	25	13	50	8
10	16	13	33	5
11	8	4	8	-
12	15	2	2	-
13	16	9	10	5
14	9	3	7	-
15	3	1	2	-
16	14	1	2	-
17	5	3	8	-
18	6	2	5	-
19	14	5	15	-

before and during the period of our monitoring of red and Pallas's squirrels in several trapping grids in the only area in Italy where they co-occur (experimental area). We are aware that this implies that the different red-only sites are not true replicates, but we will show that both previously recorded data (years 1996-1998, see Wauters et al., 2001a; Gurnell et al., 2004) and those recorded by us in 2011-2012 in mixed forests of Parco Pineta produce comparable results for population parameters and can be used as replicates of red-only area.

Squirrels were studied in two areas, one experimental red-Pallas area with both red and Pallas's squirrels present, and one control red-only area where only red squirrels occurred. The first study area contains 19 trapping sites (see Table 1) and extends over 326 ha. It is part of 9000 ha of continuous mixed deciduous forests with patches of grassland and small villages where the native and alien squirrel co-occur (North of Varese province, Lombardy region, Italy 45°58'09.2" N, 08°43'57.6" E). The red-only area contains two trapping sites of 31 and 37 ha, monitored in 1996-1998 and 2011-2012, respectively. They are part of an extensive mixed forest of 3000 ha on the northern edge of the upper Po plain in Lombardy (Parco Pineta regional park, 45°46'43.7" N, 8°54'35.1"E). Shortest distance between the two study areas is 23 km and both fall within the Upper Po plain and lower hills of the Insubria region (Tosi and Zilio 2002). For comparison, tree species composition in our two study areas is given in Table 2. Although there are slight differences in tree-species composition, both red-only and red-Pallas's squirrel areas have a diverse forest structure with a variety of seed-bearing species used by both squirrel species for feeding (e.g., Wauters et al. 2001b). All study areas have between 20-30% of hazel *Corylus avellana* in the understorey.

Table 2. Tree species composition in the red-Pallas and red-only areas.

	Cover %		
		red- only area	
Species	*red-Pallas area*	*2011-12*	*1996-98*
Tree			
Carpinus betulus	1	3	5
Castanea sativa	43	62	20
Fraxinus excelsior	9	-	-
Juglans regia	1	-	-
Picea abies	1	-	17
Pinus strobus	1	-	
Pinus sylvestris	-	8	19
Prunus sp.	9	2	2
Quercus sp.	2	11	9
Robinia pseudoacacia	26	12	25
Other	7	2	3

Trapping and handling squirrels

In the red-only area red squirrels were trapped in 2 trapping sites from July 1996 to December 1998 and from December 2011 to June 2012. During 1996-1998, trapping was carried out for at least 5 days every season (4 trapping sessions/year), while during 2011-2012, three 5-day trapping sessions were held over a period of 8 months. In the red-Pallas area squirrels were trapped in 19 different trapping sites and trapping was carried out for at least 5 days every month from May 2011 to February 2015.

Single-capture traps (model 202, Tomahawk Live Trap Co., Tomahawk, WI, USA) and, in the experimental area, both single-capture and multi-capture traps (Long Meadow Publishing, Shaftesbury, Wiltshire, UK; see Mayle et al. 2007) were arranged in grids and spaced 70 - 100 m from each other. Both trap types were fixed on tree trunks at breast height using baling wire. In the red-only area, traps were also set on the ground (Wauters et al. 2001a). Prebaiting started one week before each trapping session: hazelnuts and apple slices were placed inside the blocked traps, and each trap was checked and rebaited every two days. When activated, traps were checked two times per day to reduce the time squirrels were confined in traps. Each trapped squirrel was weighed to the nearest 5 g with a Pesola spring balance, identified to species and sex, and the length of the right hind foot (nails excluded) was measured (0.5 mm precision) with a thin ruler (Wauters et al. 2007a). Red squirrels were individually marked with numbered metal ear tags (type 1003 S National Band and Tag Co, Newport, Kentucky, USA) and immediately released, while all males and non reproductive females (in order to comply with welfare of newborn animals) of the invasive species were euthanized by CO_2 inhalation, following EC and AVMA guidelines (Close et al. 1996, Close et al. 1997, Leary et al. 2013). For each trapped squirrel, trap ID, date, the capture session number and the occasion number were recorded. As 'capture session' we defined a period of 5 consecutive days of trapping (from Monday to Friday); each session generally comprehending 8 trap checks or ‘occasions’, one on Monday and Friday, two on the other days (one in the morning and the other in the afternoon).

Population parameters and data analyses

Pallas’ squirrel population size and densities

Population size estimates of the alien species were based on removal sampling. Applying the R package fishmethods v. 1.8-0 (Nelson 2015), the maximum likelihood estimation from catch-effort data was used to estimate the maximum number of Pallas’s squirrels in the study area (Gould and Pollock 1997). This model improves standard linear regression methods to estimate the number of individuals present at the start of a series of trapping sessions (Y-variable), based on the number of animals trapped and removed (X-variable) in subsequent sessions, assuming a closed population during the entire trapping period (Leslie and Davis 1939).

Next, we compared the occurrence of the native and the invasive squirrel in the trapping grids with the Fisher’s exact test and used the Wilcoxon matched-pairs signed-ranks test to compare the catch per unit effort per grid per year (number of squirrels trapped/ number of occasions) between the two species.

Red squirrel population parameters

Red squirrel population size (N) was estimated using Capture-Mark-Recapture (CMR) models, precisely loglinear models for open population (Baillargeon and Rivest 2007; Rivest and Baillargeon 2014). Population size was first estimated for every capture session and, a later stage, averaged per year and trapping grid. To allow comparisons with previous papers on squirrel demography, we also used the minimum number of animals known to be alive (MNA, Krebs 1966), from trapping and radio-tracking, in each trapping session as a second estimator of population size.

Next, to express squirrel numbers as density ha^{-1}, we calculated the total trapping area including a buffer around each grid of 160 m for the Pallas’s squirrel, 238 m for the red squirrel in the red-Pallas area and 126 m for red squirrels in the red-only (considering the mean home range size of each species in the two areas, Wauters et al. 2001b; Chapter 3). All the areas with the buffer were reshaped eliminating habitats not suitable for squirrels (e.g., big meadows, major village centers, commercial and industrial areas, etc.) using QGIS (QGIS v. 2.8.2 Development Team, 2013).

Local survival was explored using Kaplan-Meier survival curve (Kaplan and Meier 1958). Differences in survival between the sexes and between experimental and control areas were tested with Proportional Hazard Regression (Cox 1972). To allow comparisons with previous papers on squirrel demography, we estimated local survival also with the survival index (number of animals present in year t and still alive in year t+1/number of animals present in year t) used in earlier studies on squirrel demography (Wauters et al. 2001a; Wauters et al. 2004; Gurnell et al. 2004b). The local survival rate was calculated at 6 and 12 months.

Because of low capture-recapture rate in the experimental area, the proportion of adult females that were at least one time in the year in post-oestrus (hence pregnant) or lactating was used as a measure of reproductive rate.

Results

Red-Pallas area

A total of 684 Pallas's squirrels (370 males, 314 females; sex ratio (M/F) 1.18) were removed from our study area in 45 months (117 sessions). During the same period only 69 different Eurasian red squirrels (34 males, 35 females; sex ratio 0.97) were captured in the same areas (Table 1). Many of these were captured only once (25, 36%) and only 32 (46%) of them were considered residents (more than 4 months or 4 trapping sessions in the trap site). While the Pallas's squirrel was trapped in all 19 trapping sites, red squirrel was captured in only 9 of them (Fisher Exact test p= 0.0004) and the first red squirrel capture was recorded after the removal of on average 25 ± 20 (SD) Pallas's squirrels (in 36 ± 28 occasions) (Supplemental material 18). The catch per unit effort in all trapping grids was significantly higher for the invasive squirrel than for the native one (n=36 sites, Wilcoxon matched-pairs signed-ranks test p<0.001); also when using only the 15 sites where red squirrel catch per unit effort was > 0 (Wilcoxon matched-pairs signed-ranks test p<0.001) (Supplemental material 19).

The estimated mean density of Pallas's squirrel was of 7.84 animals ha^{-1} while average red squirrel density throughout the study period was 0.05 individual ha^{-1} using the CMR method (MNA: 0.04 ha^{-1}; Table 3). The two methods used to estimate mean density in the area gave overlapping results (Supplemental material 20). Differences between grids ($F_{4,5}$=1.017, p=0.48) and years ($F_{4,5}$=0.62, p=0.67) were not significant and density was always lower than 0.2 ha^{-1} (Supplemental material 20).

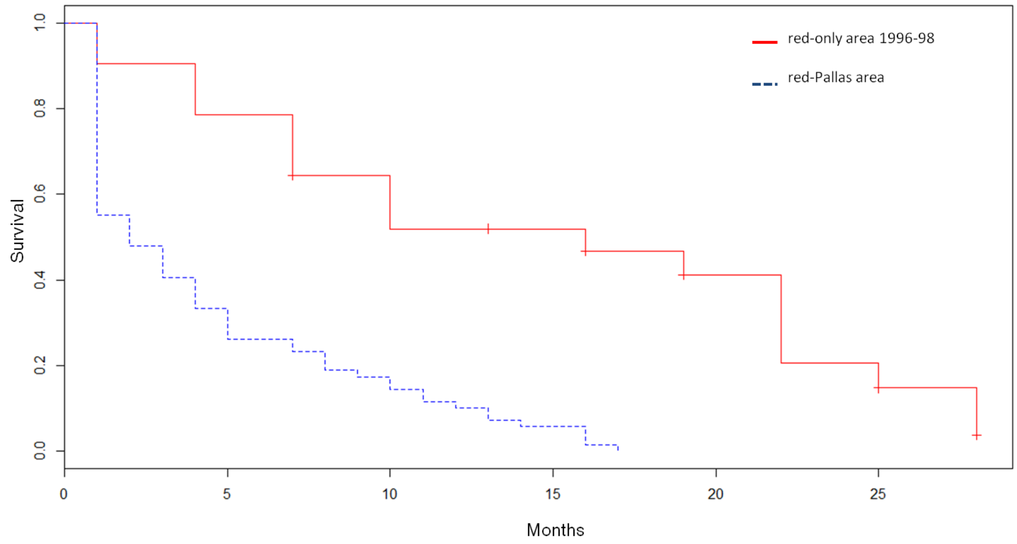

Figure 1. Kaplan Meier survival curve of red squirrel population in red-Pallas and red-only area.

Proportion of female red squirrels breeding ranged from 82 to 100% for the years 2012 (n=3), 2013 (n=9), 2014 (n=11). Kaplan-Meier survival rate for red squirrels (Fig. 1) was 0.25 at 6 months (95% CI = 0.18-0.4) and 0.11 (95% CI = 0.05-0.22) at 12 months. The local survival index reported comparable rates of 0.36 and 0.20 at 6 and 12 months, respectively (Table 3). Local survival did not differ between sexes (Likelihood ratio test=0.76, p=0.38).

Red-only area

Totally 16 red squirrels (7 males, 9 females; sex ratio: 0.78) were trapped in 8 months (3 capture sessions) in Parco Pineta in 2012. The estimated density with CMR was of 0.39 ha^{-1} and MNA reported a similar density of 0.35 squirrels ha^{-1} (Table 3). All adult females in the area reproduced in 2012. Because of the small number of capture sessions in 2011-12 we could not calculate the Kaplan Meier survival curve but the survival index at 6 months was 0.78. Regarding data from the late 90s, the Kaplan Meier estimate of local survival in the control area was significantly higher than in the red-Pallas area (Table 3; Likelihood ratio test=43.06, p< 0.001).

Discussion

In this study we tested the hypothesis that the alien Pallas's squirrel negatively affects the demography of the native red squirrel. According to our first prediction, we found a significant lower density of red squirrels in the red-Pallas than in the red-only area with a heterogeneous distribution in the experimental area. The second prediction was confirmed in terms of a low local survival of red squirrel when in co-occurrence, but its reproductive success did not seem to be affected by the Pallas's squirrel presence. Moreover the removal of the invasive squirrel throughout the study period had only local effects in some trapping sites allowing the return of the native species suggesting a low trapping effort to increase the demographic parameters of the red squirrel population.

Distribution of native and invasive species in the experimental area

We found a low occurrence of Eurasian red squirrels in trapping grids in the area occupied by the invasive Pallas's squirrel. Although forest structure is heterogeneous, the entire study area is covered by mixed deciduous forests dominated by sweet chestnut *Castanea sativa* mixed with black locust *Robinia pseudoacacia* and a good understorey of hazelnut *Corylus avellana,* suitable habitat for red squirrels (Wauters et al. 2001b). However, both occurrence in the trapping grids and catch per unit effort were lower for red than for alien Pallas's squirrels, demonstrating a higher presence and wider distribution of the invasive species than of the native one. Historically, red squirrels seemed to occur throughout the study area and were regularly observed, suggesting they occurred at relatively high densities (Prigioni et al. 2001). We exclude that low catch per unit effort of native red squirrels was affected by lack of traps not occupied by captured Pallas's squirrels, since the high trap-density in the trapping sites assured that during each trapping occasion many traps remained open, thus available for red squirrels to enter. The Pallas's squirrel density in Italy was 7.8 animals ha^{-1}, higher than reported for the invasive species in fragmented landscape in Belgium (5.6 ha^{-1}, (Adriaens et al. 2015). Our estimates were closer to those reported in Taiwan (native range) and Japan (introduced range) with 4.3 to 6.8 individuals ha^{-1} (Tamura et al. 1988; Tamura et al. 1989). However, they were still slightly higher, confirming the high invasiveness of the species. In contrast, density of native red squirrels was much lower than of alien Pallas's squirrels, with about 150 Pallas's squirrels for every single red squirrel. Red squirrel population densities in mixed deciduous woodland in other countries varied from 0.2 to 1.6 individuals per ha (Gurnell 1987; Lurz et al. 2000; Wauters et al. 2004). Hence, our estimates reported a low number of red squirrels in the experimental area.

Table 3. Population parameters reported for red squirrels in red-Pallas and red-only area.

Areas		Breeding rate	Density		Survival			
			CMR	MNA	Kaplan Meier		Survival Index	
					6months	*12months*	*6months*	*12months*
red-Pallas		90%	0.05	0.04	0.25	0.11	0.36	0.20
red-only	**2011-2012**	100%	0.39	0.35	-	-	0.78	-
	1996-1998	67.50%	0.72	0.55	0.69	0.50	0.84	0.53

Red squirrel: Red-Pallas vs. red-only

Comparison of red squirrel density between experimental and control areas demonstrated a critically low density, and population size, of red squirrels in the red-Pallas area. Independent of the method used (CMR or MNA), density in the control area (0.39 – 0.72 individuals ha^{-1}) was about 8 to 14 times higher than in the experimental area (on average 0.05 individuals ha^{-1}). Within the red-only area, densities had changed little over time, fluctuating between 0.4 and 0.7 squirrels ha^{-1} with CMR estimates slightly higher than MNA (Table 3). Because the observed differences in red squirrel density between the experimental and control area were so large, we feel confident that, although data were gathered in habitats with small differences in forest composition and, partly, in different years, they reveal a negative effect of interspecific competition with alien Pallas's squirrel on red squirrel population dynamics.

Breeding success of female red squirrels did not seem to differ between the two areas. Values reported in this study were high both in the red-only area (100%) and in the red-Pallas area (90%). Estimates of red squirrel breeding in broadleaf forests was 51% in England and ranged from 39 to 67 % in Belgium (Wauters and Dhondt 1990; Wauters et al. 2004). In the control area for the period 1996-1998, annual breeding rate ranged from 61 to 69% (Wauters et al. 2001a). Thus, so far, our results seem to exclude a negative effect of the invasive squirrel on the reproductive success of the native red squirrel. This is in contrast with the competition mechanism between red and alien grey squirrels in England and Italy. Studies have shown that the presence of grey squirrels reduces the breeding rate of red squirrels with a significantly smaller percentage of females succeeding in weaning a summer litter, and consequently, very low number of females raising two litters/year (Wauters et al. 2000; Gurnell et al. 2004b; Gurnell et al. 2015). However, because of the low capture-recapture rate in the red-Pallas site, we could not separate spring from summer reproduction as was done in earlier studies. Therefore, our estimates of breeding success are not fully comparable with those of previous studies. Nevertheless, most red squirrel females in our experimental area were in good condition and produced at least one litter per year.

A study on the fecundity of the Pallas's squirrel in our experimental area showed that 58% of females had a spring litter, some of these also produced a summer litter (35%) and a few even a third litter in autumn (10%) (Santicchia et al. in press; Chapter 6). Although there are no directly comparable fecundity data for native red squirrels, which can have large litters in some circumstances (Mari et al. 2008), estimates of reproductive rate from percentage females breeding and litter size at weaning in mixed woodlands (Wauters and Dhondt 1995; Wauters and Lens 1995; Wauters et al. 2001a) suggest that the IAS has a higher fecundity than the native species. Hence, in addition to a good adaptation to the new environment, the Pallas' squirrel, potentially, has a high capacity to spread and recover after reduction of population size, more and faster than the red squirrel.

Where reproductive success did not seem to be affected by the Pallas's squirrel presence, local survival rate of red squirrels in co-occurrence with Pallas's squirrels was lower than in red-only sites. Both methods used, the Kaplan Mayer curve and the survival index, reported similar results. In Parco Pineta, late in the 90s, the annual local survival rate was 67.5%, with females survival significantly higher than that of males (Wauters et al. 2001). In the red-Pallas area there was no difference between sexes, as reported for stable habitats in England and Belgium (Wauters and Dhondt 1990; Kenward et al. 1998). Comparative studies of demographic parameters in red squirrel populations between control and experimental sites, showed that the adult survival of reds was not reduced when grey squirrels where in co-occurrence with red squirrels, suggesting that the main effects were on the reduced breeding rate and recruitment decreased with the increasing density of grey squirrels (Gurnell et al. 2004b; Gurnell et al. 2015). In the red-Pallas area the low survival rate may be related to a competition for food resources appearing to red squirrels not reaching the optimum condition that they would have if the competitor was not present (Chapter 2). This could be related to a change in the foraging efficiency of red squirrel when in syntopy or to an indirect competition for cached food which is expressed in a higher mortality.

Pallas's squirrel management and red squirrel conservation

In some trapping grids the removal of the invasive squirrel allowed red squirrels to gradually recolonize the sites in a relative short period. This confirms the importance of the removal program and indicates that red squirrels are not locally extinct but probably relegated to the borders of the distribution area of Pallas's squirrel. Red squirrels may avoid good quality patches occupied by Pallas's squirrel and be forced to move to poorer-quality habitats or even emigrate and disperse to new areas. These areas may be of lower quality in terms of productivity of forests, food availability and degree of fragmentation (Wauters et al. 1996; Wauters et al. 2010; Santicchia et al. 2015). The permanence of red squirrels in marginal areas, in the long run, could have an adverse effect on the red squirrel population, negatively affecting the survival and the reproduction with a consequent decrease in red squirrel density as suggested for the replacement mechanism of red squirrels by introduced grey squirrels (Gurnell et al. 2015). On the other side, and in contrast with our third prediction, the overall results of the removal program did not result in an increase in red squirrel density. After an initial return of the red squirrel in several sites in the experimental area, the density in those trapping sites monitored for 3-4 years (e.g. grids 4, 5) remained the same, as demonstrated by the absence of a significant year-effect on red squirrel population size. This could be related to a trapping effort not sufficient to control and drastically reduce the numbers of the invasive species, possibly related to its high reproductive rate (Santicchia et al. in press; Chapter 6).

The question is: how can we improve the control of this alien species? Lessons can be learned from studies and projects carried out in the British Isles. Modeling the effects of grey squirrel population control methods on red squirrel population viability in UK, (Rushton et al. 2002) indicated that an integrated control strategy, incorporating both trapping and immunocontraception, may be the best option for controlling the alien species. In Scotland, the Saving Scotland's Red Squirrel project, set up a robust and co-ordinated network of grey squirrel control with three components: Project staff providing intensive but adaptable control effort; landowners funded under the Scottish Rural Development Programme trapping simultaneously over a wide area; and a trap-loan scheme providing assistance in areas beyond the capacity of Project staff control (Tonkin et al. 2011). These could be some options to consider for the next years in the management plan for the invasive Pallas's squirrel. Worldwide, only one Pallas's squirrel population was eradicated of the 22 established outside its native range (Bertolino and Lurz 2013; Adriaens et al. 2015). The invasive Pallas's squirrel is still not eradicated in Italy, despite the four year trapping activity. Hence, in the next management plan a higher trapping effort and a better trapping efficiency (Mazzamuto et al. 2015), supported by alternative control methods, such as planned shooting (Chapuis et al. 2011) should improve the efficiency of the management of this alien species and help the conservation of the native red squirrel.

CHAPTER 8

Knowledge, management and optimization: the use of live traps in control of non-native squirrels

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Abstract

This study identifies the techniques to maximize trap efficiency and minimizing trapping effort, using live traps to capture the invasive alien Pallas's squirrel (*Callosciurus erythraeus*) introduced in Italy near Swiss borders. We explored the effects of time of the day, season, number of the check in the capture session and type of the live trap (single or multi-capture). Moreover the vegetation around traps (characteristics of the tree supporting the trap, vegetation growth, vegetation cover, vegetation richness and similarity index) was tested. Squirrels were caught more frequently in the morning but trapping success was not affected by the trap type used. Squirrel trap response varied significantly in relation to season and a higher trapping success in the first days of the trapping session suggests the importance of prebaiting. We rejected the hypothesis that vegetation around traps affected capture success of Pallas's squirrel in deciduous forests. Thus, indications to improve trapping efficiency of this species are to (1) use single capture live traps with at least one week of prebaiting, (2) increase the capture effort in winter and (3) set traps where access is easier and where there are signs of high activity of individuals of the alien species.

Introduction

In recent decades the rate of new introductions and the number of invasive alien species (IAS) in Europe are increasing for all taxa (except mammals) raising extinction risk for many groups and affecting our health and economy (Clavero and García-Berthou 2005; European Environment Agency 2012). In Europe the economic costs of biological invasions are estimated to be at least EUR 12.5 billion per year (Kettunen et al. 2009) and the best way to deal with the threat represented by IAS to biodiversity and society is through a combination of applied prevention measures, early detection and prompt response to new invaders (European Environment Agency 2012). When prevention fails, eradication and management of established invasive species are the most concrete and economically advantageous responses to biological invasions (Veitch and Clout 2002; Genovesi et al. 2014). Eradication programs are now globally recognized as one of the keys to manage biological invasions and there is an increasing evidence of their success. In Europe, 37 successful eradication programs have been recorded, as the removal of the coypu and the muskrat from Great Britain; of rats, goats, rabbits and American minks from several small islands of the Macronesia, Mediterranean, Brittany, Great Britain and the Baltic sea (Genovesi 2005).

But even if there are many good examples of successful eradication programs, it is important that countries establish stringent biosecurity policies and improve responsiveness after a detection of a new introduction in their territory (Harris and Timmins 2009; Simberloff et al. 2013; Genovesi et al. 2014). A well known problematic case is the introduction of the eastern grey squirrel (*Sciurus carolinensis*) in Great Britain, Ireland and Italy. The effects of the introduction were underestimated for several years causing the replacement of the native Eurasian red squirrel (*Sciurus vulgaris*) in most of its range and huge damages to forestry (Wauters et al. 2002a; Gurnell et al. 2004b; Bertolino 2008; Bertolino et al. 2014).

The main pathway for this species in Italy was the pet trade: in fact, grey squirrels could be traded and sold as pets until February 2013, which represented a big problem for any Italian eradication program for many years (Martinoli et al. 2010; Bertolino et al. 2014). Eventually, in 2013 a complete ban on grey squirrel trade was issued (Inter-Ministerial Decree n. 28 of February 2, 2013).

In Italy, another invasive tree squirrel species, *Callosciurus erythraeus* or Pallas's squirrel, native to Southeast Asia (Oshida et al. 2001), has been introduced too. The story of the introduction as the number of squirrels released is still unknown, but the first sighting of the species in the province of Varese was in 2007. This species, is known to cause damage to forest plantation and commercial trees by gnawing tree bark, consumes fruits, and can also damage parts of buildings, cables and irrigation systems (Hori et al. 2006; Guichón and Doncaster 2008; Stuyck et al. 2009). Pallas's squirrel was introduced also in Argentina, France, Belgium, Netherlands, Hong Kong and Japan (Bertolino and Lurz 2013). In Argentina and Japan, the risk of negative impacts by introduced *C. erythraeus* on native squirrel species is considered high (Miyamoto et al. 2004; Cassini and Guichón 2009). Eradication programs are ongoing in Argentina, France, Belgium and from 2010 and 2013 in Italy within two EU LIFE Projects (LIFE09 NAT/IT/00095 EC-SQUARE and LIFE13 BIO/IT/000204 U-SAVEREDS).

Especially for squirrels that are able to establish viable populations from few individuals (Bertolino 2009), early management of new introduction events is essential. Therefore, the aim of this study is to identify the techniques to maximize trap efficiency, minimizing trapping effort using live traps. In particular, we investigate which type of trap (single vs. multi-capture) is best, and how time of day, season and local vegetation structure can influence trapping rate. Moreover, having reached the third year of the eradication program, here we present intermediate results obtained so far.

Materials and methods

Study area

Pallas's squirrels were studied in the North of Varese province (Lombardy region, Italy), where the alien species co-occurs with native Eurasian red squirrels *Sciurus vulgaris.* The study area, bounded on the northeast by Lake Maggiore and covering 9836 ha, is mainly composed of continuous mixed deciduous forests with patches of grassland and small villages. Vegetation composition and structure is typical of submontane forest: it is dominated by oaks (*Quercus robur, Q. petrae*), chestnut (*Castanea sativa*), ash (*Fraxinus excelsior*), with some maples (*Acer campestris, A. pseudoplantatus*), alder (*Alnus glutinosa*) and bird cherry (*Prunus avium*). The understory is dominated by hazel (*Corylus avellana*), blackberry (*Sambucus nigra*) and holly (*Ilex aquifolium*). In addition, there are exotic species as black locust (*Robinia pseudoacacia*), black cherry (*Prunus serotina*), walnut (*Juglans nigra*) and red oak (*Quercus rubra*) (Tosi and Zilio 2002).

Table 1. Data collected for each live trap in relation to trap type and to the surroundings vegetation.

Type	Support			Vegetation plot	
single capture	species			growth	young
	size	<10 cm			middle-age
multi capture		10-20 cm			mature
		20-30 cm		cover	high: >70%
		>30 cm			medium: 30-70%
	state	alive			low: <30%
		dead	standing	vegetation richness	
			felled		
	age	young		similarity index	
		adult			

Trapping and handling squirrels

Traps were spaced 100 m each other and set in grids with 12 up to 35 traps in 13 different sites selected because of signs of presence of the alien species (visual survey, hair tubes). Trapping was carried out for at least 5 days every month from May 2011 to September 2014 in 13 different sites. Two types of traps were used, single capture traps (Tomahawk trap model 202, Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.) and multi capture traps (see Mayle et al. 2007) arranged in grids. Both trap types were placed with iron wires on tree trunks at breast height and were covered with clear plastic panels to protect animals from the weather. Prebaiting started one week before each trapping session: hazelnuts and apple were placed inside the blocked traps and each trap was re-baited every two days. Traps were set on Monday morning and checked twice a day until Friday morning, and they were rebaited and reset after each capture. Each trapped squirrel was weighed to the nearest 5 g with a Pesola spring balance, identified to species and sex, and the length of the right hind foot (nails excluded) was measured (0.5 mm precision) with a thin ruler (Wauters et al. 2007a). 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 released. 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 were euthanized using CO_2, following EC and

AVMA guidelines (Close et al. 1996; Close et al. 1997; Leary et al. 2013). Lactating females (large nipples, milk excretion if stimulated) (Wauters and Dhondt 1989) were immediately released.

Data collection and statistical analyses

Data recorded for each trap, which had a unique Trap ID, are reported in Table 1. Around each trap a 10 x 10 m vegetation plot was delineated with the trap as centre. We did not consider for analysis plots where the support tree species occurred less than 10 times: therefore support species considered were *Castanea sativa*, *Fraxinus excelsior*, *Ilex aquifolium* and *Robinia pseudoacacia*. Tree size was measured as the diameter at breast height (dbh) and the age (young or adult) of the four support species was assigned on the base of leaf shape and structure, shoots, thorns, dbh and bark aspect (Trippi 1963a; Trippi 1963b; Kozlowski 2012). The vegetation cover level was measured by estimating the amount of shady parts on the ground and the age of the forest around the plot was assigned on a qualitative base. We classified as 'young', forests where vegetation was mainly composed by shrub species with low densities of young trees; 'mature' forests where large, high adult trees dominated the plot with a good structured shrub level; and 'middle-aged' forests if in the plot there were some old trees but disturbance from land management occurred in part of the area. We determined vegetation richness as number of tree and shrub species in the plot and calculated a qualitative similarity index, relating the occurrence of the support species to other species present in the vegetation plot.

Similarity Index = a/vegetation richness

where *a* took as a value 1 if the species used as support for the trap was elsewhere present in the plot, otherwise *a* took value of 0 (i.e. the support tree was the only individual of that species in the vegetation plot).

For each trapped squirrel, trap ID, date, time of day (morning or afternoon), the capture session number and the occasion number were recorded. As 'capture session' we defined a period of 5 consecutive days of trapping (from Monday to Friday); each session comprehending 8 trap checks or 'occasions', one on Monday and Friday, two on the other days (one in the morning and the other in the afternoon). Seasons were classified following Wauters et al. (2007a) (winter: December-February; spring: March-May; summer: June-August; autumn: September-November).

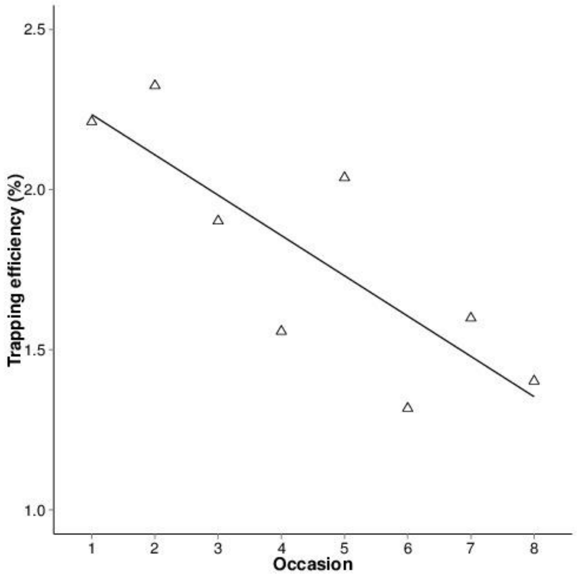

Figure 1. Trapping efficiency by occasion (trap check) during the capture session for Pallas's squirrel in Italy.

Statistical analysis

A trap capture success for Pallas's squirrels was analyzed using logistic regression where each occasion constituted an observation, and as dependent variable was defined a value of 1 when at least one squirrel was captured, or 0 if empty (binary dependent variable). In a first model we analyzed data from all 299 traps, exploring the effects of season, time of day and occasion to test for temporal trends in trapping success. In a second model (N=214 traps) we explored the effects of trap type, support species, support size, support state, support age, vegetation growth, vegetation cover, vegetation richness and similarity index (Table 1). For both regressions, model selection was carried out based on the differences between models in the value of AIC where lower values indicate better fit (stepwise analysis) (Burnham and Anderson 2004). Trapping efficiency was calculated as the number of captured squirrels on the number of occasions. All statistical analysis were done using R (R Development Core Team 2014).

Results

A total of 667 Pallas's squirrels were captured in 299 traps (115 multi-capture and 184 single-capture). Multi-capture traps caught more than a single animal just 2 times. The first logistic model, after stepwise variable selection still included all factors (season, time of day and occasion; Supplemental material 21). The number of squirrels captured was significantly higher in the morning than in the afternoon (z=0.52 p<0.001) with 426 (18931 occasions) and 241 (17510 occasions) captures respectively. The occasion number influenced significantly the capture success (p<0.001) (Supplemental material 22) and this temporal trend is shown in Fig. 1.

Traps efficiency in the four seasons (winter=2.15%, spring=1.93%, summer=1.78%, autumns=1.52%; Supplemental material 23) was higher in winter than autumn (pairwise comparison: z=0.32 p<0.01) (Fig. 2). In the second logistic model, the model obtained after stepwise variable selection included as independent variables support species and vegetation cover only (delta AIC with full model Δ*i*=12; Supplemental material 24) but no significant difference in affecting trapping outcome was found (all partial p>0.05). Trapping efficiency by trap type (single-capture: 3.43%, multi-capture: 3.86%) was not significantly different and was also the first factor eliminated in the stepwise analysis (p>0.05).

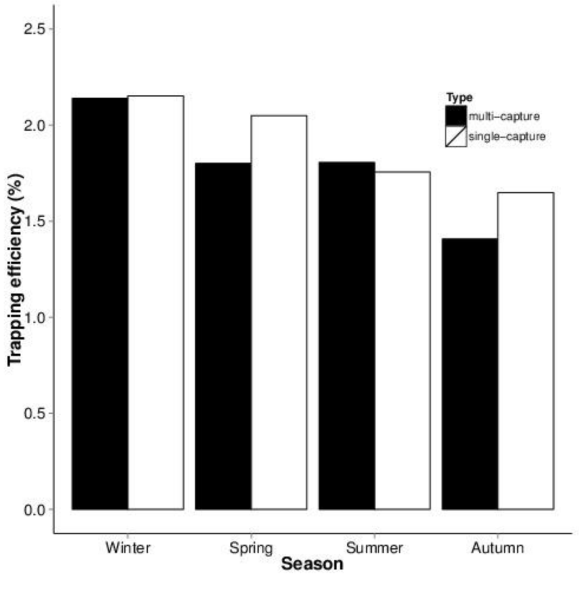

Figure 2. Trapping efficiency of traps by season and trap type for Pallas's squirrel in Italy.

Discussion

The 'catchability' of invasive small mammals is an important issue when control or eradication programs are being carried out. In case of a new introduction event, promptness of action is needed to prevent the establishment of the alien species population. In this study we have tested which factors can affect the capture success of Pallas's squirrel introduced in Italy: time (season, time of the day, day of the capture session), trap types and the vegetation around traps.

Overall, 667 Pallas's squirrel were removed using live traps with trapping success higher in the morning than in the afternoon. This agrees with previous studies on *S. vulgaris* (Wauters and Dhondt 1987) confirming that activity levels are higher in the morning also for this sciurid (Tamura et al. 1987; Tamura et al. 1988). But analyzing the capture trend during a 5-day trapping session, there is a strong evidence of a trapping efficiency decrease since first session occasions, with a progressive reduction towards the end of the session. All the traps were prebaited before each capture session, so prebaiting could have affected the trapping success. It is likely that in the first occasions any animals which accustomed to enter the traps in search of food in the previous week were captured. Many studies have tested the importance of prebaiting in many rodent species (Chitty and Kempson 1949; Babińska and Bock 1969; Gurnell 1980; Edalgo and Anderson 2007) and we underline the importance of prebaiting also on squirrels live trapping. Further studies are needed to understand which type of bait is the most successful in attracting squirrels (Andelt and Woolley 1996) and to test effects of initial captures on subsequent capture success, because of residual odors in traps (Gurnell and Little 1992; Tobin et al. 1994; Banks 1998; Vander Wall et al. 2008). Another possible explanation of the temporal effect throughout occasions is that the removal of the squirrels led to an emptying of the trapping grid area and that subsequent re-colonization of the area from neighborhood occurred in the weeks following the end of the trapping session. So, to improve trapping effectiveness, we suggest to prebait traps on several trapping areas but activating them alternatively and for a maximum of 4-5 controls (occasions) each. In this way the operator will be able to cover more trapping areas in one week. Another ‘time dependent’ factor is a seasonal effect: capture sessions were differently effective in different seasons: autumn was the worst season for captures because of the large amounts of natural high-energy food resources available in the forest (Zong et al. 2014). In contrast, squirrels entered traps more readily in winter, probably because in deciduous woods there was a gradual decrease in the availability of high-energy tree seeds and squirrels were also attracted by hazelnuts for hoarding (Grodziński et al. 1966; Wauters and Dhondt 1987; Wauters and Casale 1996; Gurnell 1996; Vernes 2004). Our results agreed with (Perry et al. 1977) and (Gurnell 1996) for gray squirrels: capture probabilities of squirrels in winter were inversely related to tree seed availability. Moreover, food availability is the most important factor limiting grey squirrel densities, but this factor both positively and negatively interacts with the severity of winter weather in affecting grey squirrel population dynamics.

Two types of traps were used for live trapping squirrels (Mayle et al. 2007). This study showed that both types, single and multi-capture traps, were successful, but multi-capture traps did not fulfill the opportunity of catching more than one animal at a time. Pallas’s squirrels vocalize often to communicate (Tamura et al. 1987; Tamura et al. 1988; Tamura 1989); hence a trapped animal might emit distress calls thereby repelling other squirrels coming close to that trap. A final consideration on trap types is that multi-capture traps are less comfortable for the operator to flush the trapped squirrel into the handling bag for manipulation, due to their dimensions and trap-flap position. Moreover, studies on squirrels (*Tamias ruficauda, T. amoenus, Spermophilus lateralis, Tamiasciurus hudsonicus*) and other rodents species, such as Norway rat and mice, showed that also trap distribution might affect trapping success (Stickel 1948; Weihong et al. 1999; Pearson and Ruggiero 2003). In this study we used the same grid distribution in all trapping areas, thus our current data do not allow to evaluate this aspect.

Many vegetation factors which theoretically could affect capture efficiency were explored. Forest productivity (in terms of food availability), and therefore forest age and composition, could influence distribution and density of squirrels. Therefore, we expected that some tree or shrub species should have been selected for food, or that plots with middle-aged trees (not too old or too young to produce seeds) should have had the most successful trapping (Perry et al. 1977). In spite of this none of the factors investigated by us affected capture success. *C. erythraeus* seemed to use the habitat in a continuous fashion, without any detectable selection at microhabitat level. (Perry et al. 1977) demonstrated that trees producing mast and dbh of trees were generally associated with grey squirrel's capture success as moderate tree densities in mature mixed habitat, but these patterns were not found in our study site. Detailed studies in more different forest types may shed light on the subject in the future.

C. erythraeus is an invasive species and our results suggest that it is well adapted to forest types in the Italian study site, even though they are very different from their native forests. Significant factors affecting trappability in our study were mostly related to squirrels ecology, as the time of the day and season. In conclusion, our indications to improve trapping efficiency of this species in the countries where it has been introduced are to use single-capture live traps with at least one week of prebaiting, increase the capture effort in winter and set traps where the operator believes operator believes the access is easier and where there are signs of high activity of individuals of the alien species. The LIFE project results obtained until now support the importance of eradication programs: many Pallas's squirrels have been removed in North Italy and, even though the alien species is not completely eradicated yet, the competition pressure on native red squirrel has been reduced showing a progressive return of the native species in many areas (Chapter 7).

CONCLUSIONS

The present study highlights the impact of invasive species on ecologically similar native species. The continued growth in global interest in invasive species and the rapid anthropogenic spread of tree squirrels make these species excellent model systems for the study of impacts (Palmer et al. 2007; Bertolino 2009; Bertolino and Lurz 2013). Understanding the impacts of invasive tree squirrels is important for the conservation of native squirrels and the assessment of current distributions and knowledge of the invasive population's ecology is crucial in predicting the path of spread and planning ways to possibly reduce or eliminate the impacts on native populations.

In particular this research was focused on the competitive interactions between the native Eurasian red squirrel and the Pallas's squirrel, an invasive alien species introduced in Northern Italy. In my project I went through the necessary steps to identify a new alien species, its distribution and effects on native species, and the best management actions to control it. The Pallas's squirrel is an invasive squirrel native to Southeast Asia introduced in the North of Varese province (Lombardy) in the last decade. It is well adapted to the new environment and has a high reproductive rate and potential to spread. This is related to its ecology but also to the new environment that provides a good availability of food resources as demonstrated by the high density of invasive squirrels in the area. Moreover parasite release may result in increased reproduction and/or survival. Co-occurring red squirrels are not yet locally extinct but are strongly affected by interspecific competition. Pallas's squirrel successfully competes for food resources with the native red squirrel that is gradually disappearing from the area. Adult red squirrels are not affected by space use competition; nevertheless the native species mainly occurs at the borders of the distribution area of Pallas's squirrels which occupy high-quality habitat patches. These border areas are generally of lower quality in terms of forest composition, human disturbance and degree of fragmentation, which negatively affects persistence of the red squirrel population, in particular local survival and consequently population density. The removal program did not reach the complete eradication of the Pallas's squirrel in 4 years, mainly because of insufficient trapping effort in relation to the habitat type (mainly continuous forests) and the high reproductive rate of the species which apparently compensates for the reduction of adult density. However, the removal in some trapping areas allowed red squirrels to recolonize these areas. Thus, considering the interspecific competition, the eradication of the Pallas's squirrel in Italy is imperative to protect the long-term survival of the native squirrel population. A long-term commitment and adequate resources, supported by the current ban of the species from pet trade, will be crucial to achieve this result. Hence, the next management plan with higher trapping effort and better trapping efficiency supported by alternative control methods, such as planned shooting, should improve the efficiency of the management of this alien species and help the conservation of the native red squirrel.

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SUPPLEMENTAL MATERIALS

Supplemental material 1. List of *Callosciurus* samples analyzed in this study. This molecular dataset includes both the samples newly sequenced in this study and the reference sequences retrieved from GenBank. For each sample, the Specimen voucher (if available), the species name, the provenance of samples and the molecular details (i.e. accession number and haplotype (H) for the two mitochondrial markers) are reported.

Specimen Voucher	**Species name**	**Provenance**	**Country**	**H coxI**	**H D-loop**	**GenBank a.n.**		**Source**	
						coxI	**D-loop**	**coxI**	**D-loop**
MIB:ZPL:03830	*Callosciurus* cfr. *erythraeus*	Brezzo di Bedero (VA)	Italy	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07382	*Callosciurus* cfr. *erythraeus*	Brezzo di Bedero (VA)	Italy	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07383	*Callosciurus* cfr. *erythraeus*	Brezzo di Bedero (VA)	Italy	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07384	*Callosciurus* cfr. *erythraeus*	Brezzo di Bedero (VA)	Italy	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07385	*Callosciurus* cfr. *erythraeus*	Brezzo di Bedero (VA)	Italy	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07386	*Callosciurus* cfr. *erythraeus*	Dadizele (Moorsele)	Belgium	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07387	*Callosciurus* cfr. *erythraeus*	Dadizele (Moorsele)	Belgium	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07388	*Callosciurus* cfr. *erythraeus*	Dadizele (Moorsele)	Belgium	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07389	*Callosciurus* cfr. *erythraeus*	Dadizele (Moorsele)	Belgium	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07390	*Callosciurus* cfr. *erythraeus*	Dadizele (Moorsele)	Belgium	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07391	*Callosciurus* cfr. *erythraeus*	Dadizele (Moorsele)	Belgium	CXH10	DLH7	submitted	submitted	this study	this study
MIB:ZPL:07392	*Callosciurus* cfr. *erythraeus*	Antibes (Alpes Maritimes)	France	CXH11	DLH42	submitted	submitted	this study	this study
MIB:ZPL:07393	*Callosciurus* cfr. *erythraeus*	Antibes (Alpes Maritimes)	France	CXH11	DLH42	submitted	submitted	this study	this study
MIB:ZPL:07394	*Callosciurus* cfr. *erythraeus*	Antibes (Alpes Maritimes)	France	CXH11	DLH42	submitted	submitted	this study	this study
MIB:ZPL:07395	*Callosciurus* cfr. *erythraeus*	Antibes (Alpes Maritimes)	France	CXH11	DLH42	submitted	submitted	this study	this study
MIB:ZPL:07396	*Callosciurus* cfr. *erythraeus*	Antibes (Alpes Maritimes)	France	CXH11	DLH42	submitted	submitted	this study	this study
MIB:ZPL:07397	*Callosciurus* cfr. *erythraeus*	Antibes (Alpes Maritimes)	France	CXH11	DLH42	submitted	submitted	this study	this study
EMiB	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	DLH6	KF856233	KF786012	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMiC	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	DLH6	KF856231	KF786013	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMiD	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	DLH6	KF856219	KF786014	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMiA	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	DLH6	KF856232	KF786015	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CG11	*Callosciurus* cfr. *erythraeus*	Cañada de Gómez, Santa Fe	Argentina	CXH14	DLH6	KF856212	KF786011	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CG13	*Callosciurus* cfr. *erythraeus*	Cañada de Gómez, Santa Fe	Argentina	CXH14	DLH6	KF856228	KF786010	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CG18	*Callosciurus* cfr. *erythraeus*	Cañada de Gómez, Santa Fe	Argentina	CXH14	DLH6	KF856227	KF786009	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
CG19	*Callosciurus* cfr. *erythraeus*	Cañada de Gómez, Santa Fe	Argentina	CXH14	DLH6	KF856226	KF786008	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CG20	*Callosciurus* cfr. *erythraeus*	Cañada de Gómez, Santa Fe	Argentina	CXH14	DLH6	KF856225	KF786007	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CU24	*Callosciurus* cfr. *erythraeus*	La Cumbecita, Córdoba	Argentina	CXH14	DLH6	KF856214	KF786006	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CU27	*Callosciurus* cfr. *erythraeus*	La Cumbecita, Córdoba	Argentina	CXH14	DLH6	KF856229	KF786005	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
CU30	*Callosciurus* cfr. *erythraeus*	La Cumbecita, Córdoba	Argentina	CXH14	DLH6	KF856215	KF786004	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi32	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	CXH14	DLH6	KF856216	KF786016	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi33	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	_	DLH6	_	KF786017	_	Gabrielli *et al.*, 2014
EMi34	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	CXH14	DLH6	KF856217	KF786018	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi35	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	_	DLH6	_	KF786019	_	Gabrielli *et al.*, 2014
EMi37	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	CXH14	DLH6	KF856224	KF786020	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi38	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	CXH14	DLH6	KF856223	KF786021	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi74	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	_	DLH6	_	KF786022	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi75	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	_	DLH6	_	KF786023	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi78	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	_	DLH6	_	KF786024	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi79	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	_	DLH6	_	KF786025	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi80	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	_	DLH6	_	KF786026	Gabrielli *et al.*, 2014	Gabrielli *et al.*, 2014
EMi4	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	_	KF856211	_	Gabrielli *et al.*, 2014	_
CU23	*Callosciurus* cfr. *erythraeus*	La Cumbecita, Córdoba	Argentina	CXH14	_	KF856213	_	Gabrielli *et al.*, 2014	_
CU29	*Callosciurus* cfr. *erythraeus*	La Cumbecita, Córdoba	Argentina	CXH14	_	KF856230	_	Gabrielli *et al.*, 2014	_
EMi39	*Callosciurus* cfr. *erythraeus*	Escobar, Buenos Aires	Argentina	CXH14	_	KF856218	_	Gabrielli *et al.*, 2014	_
EMi55	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	_	KF856220	_	Gabrielli *et al.*, 2014	_
EMi63	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	_	KF856221	_	Gabrielli *et al.*, 2014	_
EMi71	*Callosciurus* cfr. *erythraeus*	Luján, Buenos Aires	Argentina	CXH14	_	KF856222	_	Gabrielli *et al.*, 2014	_
MIB:ZPL:07398	*Callosciurus erythraeus*	Yanyuan county (Sichuan)	PRC*	CXH12	DLH1	submitted	submitted	this study	this study
Hongya County 06	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH10	_	GU474437	_	Guo *et al.*, 2011
Hongya County 03	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH11	_	GU474434	_	Guo *et al.*, 2011
Hongya County 18	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH11	_	GU474449	_	Guo *et al.*, 2011
Hongya County 04	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH12	_	GU474435	_	Guo *et al.*, 2011
Hongya County 13	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH13	_	GU474444	_	Guo *et al.*, 2011
Hongya County 16	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH14	_	GU474447	_	Guo *et al.*, 2011
Hongya County 10	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH15	_	GU474441	_	Guo *et al.*, 2011
Hongya County 01	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH16	_	GU474432	_	Guo *et al.*, 2011
Hongya County 09	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH17	_	GU474440	_	Guo *et al.*, 2011
Hongya County 07	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH18	_	GU474438	_	Guo *et al.*, 2011
_	*Callosciurus erythraeus*	Mt. Hemei (Sichuan)	PRC*	CXH13	DLH18	KM502568	KM502568	Hu *et al.*, In press	Hu *et al.*, In press

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	**D-loop**	**coxI**	**D-loop**
Hongya County 12	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH19	_	GU474443	_	Guo *et al.*, 2011
MIB:ZPL:07399	*Callosciurus erythraeus*	Muli county (Sichuan)	PRC*	CXH12	DLH2	submitted	submitted	this study	this study
Hongya County 14	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH20	_	GU474445	_	Guo *et al.*, 2011
Hongya County 02	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH21	_	GU474433	_	Guo *et al.*, 2011
Hongya County 08	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH22	_	GU474439	_	Guo *et al.*, 2011
Hongya County 15	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH23	_	GU474446	_	Guo *et al.*, 2011
Hongya County 17	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH24	_	GU474448	_	Guo *et al.*, 2011
NMNS3748	*Callosciurus erythraeus*	Wufeng (Taichung)	Taiwan	_	DLH25	_	AB181249	_	Oshida *et al.*, 2006
NMNS842, NMNS1345	*Callosciurus erythraeus*	Wufeng (Taichung)	Taiwan	_	DLH26	_	AB181256	_	Oshida *et al.*, 2006
NMNS1191	*Callosciurus erythraeus*	Wufeng (Taichung)	Taiwan	_	DLH27	_	AB181253	_	Oshida *et al.*, 2006
NMNS6408, NMNS1184, NMNS4366	*Callosciurus erythraeus*	Dadushan, Wufeng (Taichung)	Taiwan	_	DLH28	_	AB181257	_	Oshida *et al.*, 2006
NMNS6616, NMNS1025, NMNS1178	*Callosciurus erythraeus*	Shinshe, Wufeng (Taichung)	Taiwan	_	DLH29	_	AB181260	_	Oshida *et al.*, 2006
NMNS5439	*Callosciurus erythraeus*	Shuili (Nantou)	Taiwan	_	DLH30	_	AB181254	_	Oshida *et al.*, 2006
NMNS6406	*Callosciurus erythraeus*	Longtan (Taoyuan)	Taiwan	_	DLH31	_	AB181255	_	Oshida *et al.*, 2006
NMNS5280, NMNS5383	*Callosciurus erythraeus*	Tunglou (Taichung)	Taiwan	_	DLH32	_	AB181259	_	Oshida *et al.*, 2006
NMNS5401	*Callosciurus erythraeus*	Tunglou (Taichung)	Taiwan	_	DLH33	_	AB181252	_	Oshida *et al.*, 2006
NMNS6609, NMNS1905	*Callosciurus erythraeus*	Paoshan Dam (Hsinchu), Chilan (Yilan)	Taiwan	_	DLH34	_	AB181258	_	Oshida *et al.*, 2006
NMNS6422	*Callosciurus erythraeus*	Shuanglianpi (Yilan)	Taiwan	_	DLH35	_	AB181250	_	Oshida *et al.*, 2006
NMNS4244	*Callosciurus erythraeus*	Fushan (Yilan)	Taiwan	_	DLH36	_	AB181251	_	Oshida *et al.*, 2006
NMNS5380	*Callosciurus erythraeus*	Lugu (Nantou)	Taiwan	_	DLH37	_	AB181272	_	Oshida *et al.*, 2006
NMNS6606	*Callosciurus erythraeus*	Baolai (Kaohsiung)	Taiwan	_	DLH38	_	AB181263	_	Oshida *et al.*, 2006
NMNS5440	*Callosciurus erythraeus*	Neimen (Kaohsiung)	Taiwan	_	DLH39	_	AB181261	_	Oshida *et al.*, 2006
NMNS6413, NMNS6410, NMNS6597	*Callosciurus erythraeus*	Tsaolian, Alishan (Chiayi)	Taiwan	_	DLH40	_	AB181262	_	Oshida *et al.*, 2006
NMNS278, NMNS6614	*Callosciurus erythraeus*	Baolai (Kaohsiung)	Taiwan	_	DLH41	_	AB181264	_	Oshida *et al.*, 2006
NMNS5281, NMNS5402	*Callosciurus erythraeus*	Shitou (Nantou)	Taiwan	_	DLH43	_	AB181265	_	Oshida *et al.*, 2006
NMNS5381	*Callosciurus erythraeus*	Lugu (Nantou)	Taiwan	_	DLH44	_	AB181267	_	Oshida *et al.*, 2006
NMNS6610	*Callosciurus erythraeus*	Tsaolian (Chiayi)	Taiwan	_	DLH45	_	AB181287	_	Oshida *et al.*, 2006
NMNS6805	*Callosciurus erythraeus*	Sandimen (Pingtung)	Taiwan	_	DLH46	_	AB181290	_	Oshida *et al.*, 2006
NMNS6806, NMNS6834	*Callosciurus erythraeus*	Sandimen (Pingtung)	Taiwan	_	DLH47	_	AB181291	_	Oshida *et al.*, 2006
NMNS6415	*Callosciurus erythraeus*	Shizi (Pingtung)	Taiwan	_	DLH48	_	AB181273	_	Oshida *et al.*, 2006
184	*Callosciurus erythraeus*	Oshima Island (Kantō)	Japan	_	DLH49	_	AB259594	_	Oshida *et al.*, 2007
_	*Callosciurus erythraeus*	Kumamoto (Kyūshū)	Japan	_	DLH50	_	AB576365	_	Ikeda *et al.*, 2011
183,185,186,273,274	*Callosciurus erythraeus*	Oshima Island (Kantō), Fukue Island (Kyūshū)	Japan	_	DLH51	_	AB259593	_	Oshida *et al.*, 2007

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	**D-loop**	**coxI**	**D-loop**
192	*Callosciurus erythraeus*	Izu peninsula (Chūbu)	Japan	_	DLH51	_	AB259598	_	Oshida *et al.*, 2007
NMNS5417, NPUST14	*Callosciurus erythraeus*	Kenting, Jialeshuei (Pingtung)	Taiwan	_	DLH52	_	AB181282	_	Oshida *et al.*, 2006
NMNS5420, NMNS5421	*Callosciurus erythraeus*	Kenting (Pingtung)	Taiwan	_	DLH53	_	AB181270	_	Oshida *et al.*, 2006
NMNS5393	*Callosciurus erythraeus*	Fangliao (Pingtung)	Taiwan	_	DLH54	_	AB181266	_	Oshida *et al.*, 2006
NMNS5399, NMNS5403, NMNS5407	*Callosciurus erythraeus*	Fangliao (Pingtung)	Taiwan	_	DLH55	_	AB181285	_	Oshida *et al.*, 2006
NMNS5415, NMNS5416	*Callosciurus erythraeus*	Dunghe (Taitung)	Taiwan	_	DLH56	_	AB181279	_	Oshida *et al.*, 2006
NMNS5283, NMNS5396, NMNS6412	*Callosciurus erythraeus*	Fangliao, Shizi (Pingtung)	Taiwan	_	DLH57	_	AB181284	_	Oshida *et al.*, 2006
275	*Callosciurus erythraeus*	Miyazaki (Kyūshū)	Japan	_	DLH58	_	AB259599	_	Oshida *et al.*, 2007
NMNS5414, NMNS6595	*Callosciurus erythraeus*	Wuling Farm (Taichung), Datong (Yilan)	Taiwan	_	DLH59	_	AB181274	_	Oshida *et al.*, 2006
NMNS6615	*Callosciurus erythraeus*	Shinbaiyang (Hualien)	Taiwan	_	DLH60	_	AB181288	_	Oshida *et al.*, 2006
NMNS6603, NMNS6613	*Callosciurus erythraeus*	Shinbaiyang (Hualien)	Taiwan	_	DLH61	_	AB181289	_	Oshida *et al.*, 2006
NMNS5418, NMNS5425, NMNS5437, NMNS5438	*Callosciurus erythraeus*	Kuanfu (Hualien)	Taiwan	_	DLH62	_	AB181275	_	Oshida *et al.*, 2006
NMNS6612	*Callosciurus erythraeus*	Dongshan (Yilan)	Taiwan	_	DLH63	_	AB181277	_	Oshida *et al.*, 2006
NMNS6611	*Callosciurus erythraeus*	Dongshan (Yilan)	Taiwan	_	DLH64	_	AB181286	_	Oshida *et al.*, 2006
NMNS5426	*Callosciurus erythraeus*	Rentze (Yilan)	Taiwan	_	DLH65	_	AB181271	_	Oshida *et al.*, 2006
NMNS5423	*Callosciurus erythraeus*	Kuanfu (Hualien)	Taiwan	_	DLH66	_	AB181280	_	Oshida *et al.*, 2006
NMNS5422	*Callosciurus erythraeus*	Kuanfu (Hualien)	Taiwan	_	DLH67	_	AB181281	_	Oshida *et al.*, 2006
NMNS5436	*Callosciurus erythraeus*	Kuanfu (Hualien)	Taiwan	_	DLH68	_	AB181268	_	Oshida *et al.*, 2006
NMNS5419	*Callosciurus erythraeus*	Kuanfu (Hualien)	Taiwan	_	DLH69	_	AB181269	_	Oshida *et al.*, 2006
NMNS5424, NMNS5432, NMNS5434, NMNS5435	*Callosciurus erythraeus*	Kuanfu (Hualien)	Taiwan	_	DLH70	_	AB181276	_	Oshida *et al.*, 2006
182	*Callosciurus erythraeus*	Oshima Island (Kantō)	Japan	_	DLH71	_	AB259592	_	Oshida *et al.*, 2007
NMS5406, NMNS5287	*Callosciurus erythraeus*	Fangliao (Pingtung)	Taiwan	_	DLH72	_	AB181283	_	Oshida *et al.*, 2006
NPUST15	*Callosciurus erythraeus*	Neipu (Pingtung)	Taiwan	_	DLH73	_	AB181278	_	Oshida *et al.*, 2006
190	*Callosciurus erythraeus*	Hamamatsu (Chūbu)	Japan	_	DLH74	_	AB259596	_	Oshida *et al.*, 2007
Hongya County 05	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH8	_	GU474436	_	Guo *et al.*, 2011
Hongya County 11	*Callosciurus erythraeus*	Hongya county (Sichuan)	PRC*	_	DLH9	_	GU474442	_	Guo *et al.*, 2011
HN117	*Callosciurus erythraeus*	Qiongzhong (Hainan)	PRC*	CXH1	_	HM031932	_	Lu *et al.*, 2012	_
HN118	*Callosciurus erythraeus*	Qiongzhong (Hainan)	PRC*	CXH2	_	HM031933	_	Lu *et al.*, 2012	_
HN119	*Callosciurus erythraeus*	Qiongzhong (Hainan)	PRC*	CXH3	_	HM031934	_	Lu *et al.*, 2012	_
HN120	*Callosciurus erythraeus*	Qiongzhong (Hainan)	PRC*	CXH1	_	HM031935	_	Lu *et al.*, 2012	_
MIB:ZPL:04253	*Callosciurus finlaysonii*	Maratea (PZ)	Italy	CXH9	DLH77	submitted	submitted	this study	this study
MIB:ZPL:04254	*Callosciurus finlaysonii*	Maratea (PZ)	Italy	CXH9	DLH77	submitted	submitted	this study	this study

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
MIB:ZPL:04255	*Callosciurus finlaysonii*	Acqui Terme (AL)	Italy	CXH9	DLH78	submitted	submitted	this study	this study
187, 188, 189, 191, 507,508	*Callosciurus finlaysonii*	Hamamatsu (Chūbu)	Japan	_	DLH5	_	AB259595	_	Oshida *et al.,* 2007
506	*Callosciurus finlaysonii*	Hamamatsu (Chūbu)	Japan	_	DLH5	_	AB259597	_	Oshida *et al.,* 2007
M31312	*Callosciurus finlaysonii*	Vientianne	Laos	_	DLH3	_	AB259600	_	Oshida *et al.,* 2007
M31313	*Callosciurus finlaysonii*	Vientianne	Laos	_	DLH4	_	AB259601	_	Oshida *et al.,* 2007
_	*Callosciurus finlaysonii*	_	Thailand	_	DLH78	_	AB621590	_	Kuramoto *et al.,* 2012
ATCC CRL-1926	*Callosciurus notatus*	cell culture	_	CXH4	_	HM102291	_	Cooper *et al.,* 2007	_
BIOUG<CAN>:ROM 102085	*Callosciurus notatus*	Lalut Birai R.S. (Kalimantan Timur)	Indonesia	CXH5	_	JF444286	_	unpublished	_
ROM:102139	*Callosciurus orestes*	Lalut Birai R.S. (Kalimantan Timur)	Indonesia	CXH8	_	JF444287	_	unpublished	_
ROM:102178	*Callosciurus prevostii*	Long Sungan (Kalimantan Timur)	Indonesia	CXH6	_	JF444288	_	unpublished	_
ROM:102221	*Callosciurus prevostii*	Lalut Birai R.S. (Kalimantan Timur)	Indonesia	CXH7	_	JF459623	_	unpublished	_
311	*Callosciurus prevostii*	Sumatra Island	Indonesia	_	DLH75	_	AB259602	_	Oshida *et al.,* 2007
312	*Callosciurus prevostii*	Sumatra Island	Indonesia	_	DLH76	_	AB259603	_	Oshida *et al.,* 2007

*PRC= People's Republic of China

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Supplemental material 2. Description of the main fur characteristics of the three European populations of *Callosciurus*. Colors classified following Ridgway, 1912*.

Body regions	France (Antibes)	Italy	Belgium
Venter color	Mahogany Red	Antimony yellow (XV)	Antimony yellow (XV)
	Light Brownish Olive (XXX) agouti stripe	Warm Buff (XV)	Warm Buff (XV)
		Pinkish Buff (XXIX)	Pinkish Buff (XXIX)
Dorsum color	Brownish Olive (XXX) agouti	Light Brownish Olive (XXX) agouti	Light Brownish Olive (XXX) agouti
	No black stripe	No black stripe	No black stripe
Tail	-	0 or from 5 to 9 blackish stripes	0 or from 5 to 9 blackish stripes
Paws	Dark Brownish Olive (XXX) agouti	Smoke Grey (XLVI)	Smoke Grey (XLVI)
Head	Brownish Olive (XXX) agouti	“Light Brownish Olive” (XXX) agouti	“Light Brownish Olive” (XXX) agouti
Throat	Brownish Olive (XXX) agouti	Smoke Grey (XLVI)	Smoke Grey (XLVI)

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Supplemental material 3. Skull measurements for each species and for the three European populations of *Callosciurus* sp. For measurements and abbreviations see Fig. 1 and Table 2.

	Fb		**Cbl**		**Zgb**		**Bcb**		**Pl**		**Bch**		**Mxt**		**Dil**		**Ioc**		**Lbc**	
Species	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**	**mean**	**± SD**
Callosciurus caniceps	12.10	0.38	55.09	0.83	32.03	0.75	22.53	0.62	16.88	0.53	17.58	0.52	10.83	0.41	13.17	0.51	18.99	0.75	27.91	0.81
Callosciurus erythraeus	10.96	0.81	49.75	2.46	28.98	1.64	20.86	1.14	15.19	1.08	15.77	0.78	9.67	0.58	11.64	0.68	17.05	1.43	25.34	1.76
Callosciurus finlaysonii	7.77	1.40	49.28	2.32	29.51	1.26	20.90	0.93	14.48	0.78	18.49	1.41	9.70	0.70	11.21	0.62	17.78	0.95	26.26	1.58
Callosciurus melanogaster	12.24	0.34	51.43	1.26	29.93	0.52	21.36	0.69	16.84	0.44	15.89	0.36	10.51	0.34	12.55	0.61	17.50	1.02	25.74	1.05
Callosciurus nigrovittatus	9.94	0.29	43.83	0.46	27.06	0.51	19.15	0.75	13.45	0.63	15.02	0.39	8.55	0.46	10.62	0.31	16.79	0.73	24.15	0.71
Callosciurus notatus	11.13	0.58	49.06	2.78	29.38	1.90	20.48	0.99	15.58	1.21	16.19	1.27	9.32	0.65	11.94	1.08	17.23	1.39	25.12	1.93
Callosciurus phayrei	12.16	0.45	55.33	1.41	32.43	0.93	22.86	0.83	17.14	1.01	17.20	0.60	10.88	0.46	13.50	0.99	19.58	1.10	27.72	0.63
Callosciurus prevostii	12.54	0.00	52.76	0.00	33.88	1.47	23.44	1.24	17.56	2.18	17.12	0.00	10.74	0.82	13.67	0.75	22.42	0.48	30.08	2.38
Callosciurus pygerythrus	10.02	0.25	46.07	1.57	28.28	0.00	20.34	0.00	14.26	1.19	15.79	0.04	8.66	0.31	11.41	0.16	15.35	0.78	21.77	0.16
European populations																				
BELGIUM	11.21	0.36	49.87	1.14	30.79	0.55	21.19	0.55	15.20	0.70	15.43	0.34	10.53	0.48	11.48	0.42	17.44	0.80	24.53	1.08
FRANCE	11.58	0.46	52.23	0.93	32.48	0.66	22.17	0.30	16.66	0.39	15.90	0.30	11.24	0.31	12.09	0.52	19.71	1.06	28.21	1.03
ITALY	11.18	0.35	49.18	0.83	30.17	0.75	21.03	0.46	15.49	0.40	15.75	0.37	10.21	0.42	11.55	0.49	16.93	0.51	24.89	0.80

Supplemental material 4. Haplotype characteristics. Number of haplotypes and nucleotide diversity values in the *Callosciurus* European and Asiatic populations and in the two main species analyzed in this study. *Π* = Nei (1987) nucleotide diversity; sd (*Π*) = standard deviation of the Nei's nucleotide diversity. Values are given separately for the two molecular markers.

Group	**Status**	***CoxI***				**D-loop**			
		n° of sequences	**n° haplotypes**	**πd**	**sd(πd)**	**n° of sequences**	**n° haplotypes**	**πd**	**sd(πd)**
Italy	introduced	5	1	0	0	5	1	0	0
Belgium	introduced	6	1	0	0	6	1	0	0
France	introduced	6	1	0	0	6	1	0	0
Europe	introduced	17	2	0.01951	0.00316	17	2	0.10433	0.05217
Argentina	introduced	23	1	0	0	23	1	0	0
People's Republic of China	native	7	5	0.04303	0.00975	86*	19	0.03376	0.01204
Taiwan	native	_	_	_	_	71*	43	0.03605	0.00123
Japan	introduced	_	_	_	_	71*	6	0.02887	0.00585
C. *erythraeus*	-	7	5	0.04303	0.00975	228*	68	0.06636	0.00401
C. *finlaysonii*	-	3	1	0	0	13*	5	0.06023	0.00750

* number of sequences retrieved from original literature (when only haplotypes have been deposited in GenBank)

Supplemental material 5. Within and between species genetic divergence values (%) of *cox1* sequences, based on the K2P distance calculation (bottom left), for the taxa belonging to the genus *Callosciurus* considered in this study. Standard errors are reported in upper right portion of the matrix. Lineages and species, showed by numbers in square brackets, belong to the ABGD analysis conducted on the *CoxI* dataset (see also the NJ tree in Fig. 3). *Callosciurus* species names are abbreviated as follows: ery-*erythreaeus*; fin-*finlaysonii*; not-*notatus*; ore-*orestes*; pre-*prevostii*. The countries where the different lineages occur are abbreviated as follows: ARG-Argentina; PRC-People's Republic of China; IT-Italy; FR-France; BE-Belgium.

	C_cfr_ery_ARG_[1]	C_ery_PRC_[2]	C_fin_IT_[3]	C_ery_PRC_[4]	C_ery_PRC_[5]	C_cfr_ery_FR_[6]	C_cfr_ery_IT_BE_[7]	C_not_[8]	C_ore_[9]	C_pre_[10]
C_cfr_ery_ARG_[1]		0.007	0.009	0.011	0.012	0.013	0.013	0.020	0.021	0.020
C_ery_PRC_[2]	0.030		0.007	0.010	0.011	0.011	0.012	0.020	0.020	0.019
C_fin_IT_[3]	0.052	0.035		0.012	0.012	0.013	0.013	0.020	0.021	0.021
C_ery_PRC_[4]	0.075	0.069	0.083		0.010	0.009	0.009	0.020	0.021	0.021
C_ery_PRC_[5]	0.083	0.070	0.085	0.062		0.010	0.010	0.019	0.020	0.018
C_cfr_ery_FR_[6]	0.094	0.079	0.098	0.050	0.054		0.008	0.020	0.021	0.020
C_cfr_ery_IT_BE_[7]	0.096	0.079	0.094	0.048	0.056	0.042		0.019	0.021	0.020
C_not_[8]	0.190	0.183	0.189	0.177	0.174	0.189	0.180		0.017	0.018
C_ore_[9]	0.194	0.183	0.190	0.186	0.171	0.181	0.181	0.142		0.012
C_pre_[10]	0.191	0.181	0.199	0.184	0.167	0.185	0.180	0.141	0.082	

Supplemental material 6. Within and between species genetic divergence values (%) of D-loop sequences based on the K2P distance calculation (bottom left) for the taxa belonging to the genus *Callosciurus* considered in this study. Standard errors are reported in the upper right portion of the matrix. *Callosciurus* species names are abbreviated as follows: ery-*erythreaeus*; fin-*finlaysonii*; pre-*prevostii*. The countries where the different lineages occur are abbreviated as follows: ARG-Argentina; PRC-People's Republic of China; IT-Italy; FR-France; BE-Belgium; TAI-Taiwan.

	C_ery_TAI	C_ery_Jap	C_ery_PRC_I	C_cfr_ery_ARG	C_cfr_ery_IT_BE	C_cfr_ery_FR	C_ery_PRC_II	C_fin	C_pre
C_ery_TAI		0.005	0.015	0.016	0.013	0.006	0.018	0.015	0.018
C_ery_Jap	0.039		0.016	0.016	0.013	0.008	0.018	0.015	0.020
C_ery_PRC_I	0.119	0.129		0.018	0.017	0.015	0.019	0.016	0.021
C_cfr_ery_ARG	0.142	0.146	0.137		0.014	0.017	0.012	0.009	0.019
C_cfr_ery_IT_BE	0.120	0.128	0.143	0.125		0.014	0.018	0.014	0.019
C_cfr_ery_FR	0.036	0.045	0.112	0.135	0.114		0.018	0.014	0.019
C_ery_PRC_II	0.155	0.156	0.140	0.084	0.168	0.153		0.010	0.018
C_fin	0.135	0.140	0.130	0.067	0.136	0.124	0.088		0.016
C_pre	0.213	0.220	0.214	0.212	0.215	0.222	0.207	0.205	

Supplemental material 7. Number of measurements (obs) and of individual red squirrels (squ) analyzed by area and year.

	2010		2011		2012		2013		2014		2015		Total	
	obs	**squ**	**obs**	**squ**	**Obs**	**squ**	**obs**	**squ**	**obs**	**squ**	**obs**	**squ**	**obs**	**squ**
Red only	17	13	17	14	92	42	55	23	2	2	0	0	183	79
Red-grey	0	0	10	4	90	36	148	54	277	66	88	39	613	146
Red-Pallas	0	0	17	6	16	7	63	23	96	29	1	1	193	54
													989	279

Supplemetal material 8. Red squirrels' home ranges (95% KDE in ha) before and after the removal of 13 Pallas's squirrels in area C and movement (m) of the home range centroids after the removal.

ID squirrel	Sex	Home range before removal	Home range after removal	Movement of home range centroid
4081	M	15.54	14.62	104
4180	M	24.80	55.96	373
4201	M	12.30	32.90	128
4221	F	28.65	34.05	144
4036	F	8.07	-	-

Supplemental material 9. Percentage of tree (a) and shrub (b) species in areas A, B and C.

Area	A	B	C
a) Tree			
Acer campestre	0	0	2.7
Acer pseudoplatanus	4.3	0	0
Betula sp.	0	0.6	0
Castanea sativa	3.6	88	32.3
Fraxinus sp.	14.5	0	13
Ilex aquifolium	0	1.1	2.1
Juglans nigra	5.1	0	0
Picea sp.	2.2	0	0
Pinus sylvestris	0	0	19.9
Prunus avium	2.2	0.6	1.1
Prunus serotina	0	0	11.8
Quercus sp.	2.2	0.6	2.7
Robinia pseudoacacia	65.2	9.1	11.3
Taxus baccata	0	0	2.1
Tilia sp.	0.7	0	1.1
b) Shrub			
Corylus avellana	36.5	4.5	79.6
Ilex aquifolium	0	68.2	14.8
Prunus laurocerasus	0	25	1.9
Prunus serotina	0	2.3	0
Sambucus nigra	63.5	0	3.7

Supplemental material 10. Model selection in the logistic regression to investigate the influence of Pallas's squirrels' sex and body mass, season of sampling and the presence/absence of *Ixodes (I.) ricinus* on the presence/absence of *Ceratophyllus (M.) s. sciurorum*, and of Pallas's squirrels' sex and body mass, season of sampling and the presence/absence of *Ceratophyllus (M.) s. sciurorum* on the presence/absence of *Ixodes (I.) ricinus*. Random term for all models:"year of sampling".

Presence of fleas ~					
Body mass	**Presence of ticks**	**Season**	**Sex**	**AIC**	**Δ AIC**
		+		186.6	-
	+	+		188.5	1.88
		+	+	188.5	1.90
+		+		188.6	1.99
	+	+	+	190.4	3.73
+	+	+		190.5	3.87
+		+	+	190.5	3.89
				101.1	4.50
	+			192.3	5.61
+	+	+	+	192.4	5.72
+				192.9	6.28
			+	193.1	6.49
+	+			194.0	7.39
	+		+	194.2	7.60
+			+	194.9	8.27
+	+		+	196.0	9.38

Presence of ticks ~					
Body mass	**Presence of fleas**	**Season**	**Sex**	**AIC**	**Δ AIC**
		+	+	137.7	-
		+		138.0	0.31
	+	+	+	139.3	1.52
+		+	+	139.4	1.64
+		+		139.4	1.69
	+	+		139.6	1.83
+	+	+	+	140.9	3.16
+	+	+		140.9	3.21
	+			164.2	26.49
	+		+	164.3	26.58
				165.2	27.43
			+	165.2	27.51
+	+			165.8	28.12
+	+		+	166.1	28.38
+				167.0	29.27
+			+	167.2	29.45

Supplemental material 11. Macro-parasite species list recorded on sub-species of *Callosciurus erythraeus* (Pallas's squirrel) on the native range (re-arranged from Durden & Beaucournu 2014; Gozzi et al. 2013, 2014; Lurz et al. 2013).

Species [1]	Type hosts		Other hosts' records		Reference
	Species[2]	Locality	Species[2]	Locality	
HELMINTH					
NEMATODA					
Heligmosomidae					
Brevistriata skrjabini (Schulz & Lubimov, 1932)	*Sciurus vulagris mantshuricus*	Eastern Russia	*C. e. thaiwanensis*	Taiwan	[1,2]
Brevistriata sinensis Li, 1941	*C. e. castaneoventris*	China	*C. e. gordoni*	Yunnan, China	[3]
Calypsostrongylus ogdeni Schmidt, Myers & Kuntz, 1967	*C. e. thaiwanensis*	Nan-Tou, Tai-Pei ; Taiwan	-	-	[4]
Calypsostrongylus titasuthi Kliks & Durette-Desset, 1976	*C. e. flavimanus*	Chang Mai; Thailand	-	-	[5]
Brevistriata callosciuri Wang, 1981	*C. e. ningpoensis*	Fujian; China	-	-	[6]
Trichostrongylidae					
Trichostrongylus colubriformis (Giles, 1852)	Ruminants	Cosmopolite	*C. e. castaneoventris*	Yunnan, China	[3]
Spiruridae					
Protospirura sp.	?	-	*C. e. thaiwanensis*	Taiwan	[1]
Oxyuridae					
Syphacia obvelata (Rudolphi, 1802) Seurat, 1916	*M. musculus* (L.)	Cosmopolite	*C. erythareus s.l.*	Taiwan	[1]
Enterobius sp.	?	-	*C. e. thaiwanensis*	Taiwan	[1]
Rictulariidae					
Pterygodermatites (*Mesopectines*) *tani* (Hoeppli, 1929)	Murids	Southern Vietnam	*C. e. gordoni*	Yunnan, China	[3]
TREMATODA					
Dicrocoellidae					
Zonorchis taiwanensis Fischthal & Kuntz, 1981	*C. e. thaiwanensis*	Taiwan	-	-	[7]
Zonorchis callosciuri Nguyen Thi Le, 1968	*C. erythraeus s.l.*	Vietnam	-	-	[7]
ARTHROPODS					
IXODIDA					
Ixodidae					
Ixodes kuntzi Hoogstraal & Kohls, 1965	*Petaurista* spp.	Taiwan	*C. e. thaiwanensis*	Taiwan	[8]
SIPHONAPTERA					
Ceratophyllidae					
Macrostylophora bispiniforma Li, Hsieh & Yang, 1976	*C. e. gloveri*	Yunnan, China	*Tamiops s. swinhoei*	Yunnan, China	[9,10]
Macrostylophora cuiae Liu, Wu & Yu, 1964	*Hylopetes alboniger*	Zheijiang, China	*C. erythraeus s.l.* and other Sciurids	Zheijiang, China	[11]
Macrostylophora euteles (Jordan & Rothschild, 1911)	*Sciurotamias davidianus consobrinus*	Sichuan, China	Sciuridae	Yunnan, China; Thailand	[12,10]
Macrostylophora hastata malayensis Traub, 1950	*C. erythraeus s.l.*	West Malaysia	-	-	[10]
Macrostylophora liae Wang, 1957	*C. erythraeus s.l.*	Fukien; China	Sciurids	Taiwan; Vietnam	[10]
Macrostylophora lupata lupata (Jordan & Rothschild, 1921)	*C. erythraeus s.l.*	India; Nepal; Sikkim	Sciurids; Murids	-	[10]
Macrostylophora lupata bamana Jordan, 1939	*C. e. griseimanus*	Myanmar	-	-	[10]
Megathoracipsylla pentagonia Liu, Liu & Zang, 1980	*Dremomys pernyi*	Shaanxi, China	*C. erythraeus s.l.*	Yunnan, China	[13]
Pygiopsyllidae					
Medwayella robinsoni (Rothschild, 1905)	*C. erythraeus s.l.*	Malaysia	Other sciurids		
ANOPLURA					
Enderleinellidae					
Enderleinellus corrugatus Johnson, 1959	*Tamiops maclellandi*	Thailand	*C. erythraeus s.l.*	South-east Asia	[14-16]
Hoplopleuridae					
Hoplopleura erismata Ferris, 1921	*C. finlaysonni*[x]	Thailand	*C. e. hendeei*	Yunnan, China	[14-18]
Polyplacidae					
Neohaematopinus callosciuri Johnson, 1959	*C. finlaysonni*	Thailand	*C. erythraeus s.l*	South-east Asia	[14-16]

[1] Nomenclature for Nematoda follows Durette-Desset 1983, Hugot (1988), and Quentin (1969); for Ixodida: Guglielmone et al. 2010; Siphonaptera: Durden and Beaucournu (2014); Anoplura: Durden and Musser (1994a, b)

[2] Nomenclature for *C. eryhtraeus* (Pallas) subspecies follows Lurz et al. 2013: *C. e. castaneoventris* (Gray) [=*C. e. michianus* (Robinson & Wroughton)]; *C. e. thaiwanensis* (Bonhote, 1901) [= *C. e. centralis* (Bonhote, 1901); = *C. e. roberti* (Bonhote, 1901)]; For *Sciurus vulgaris* (L.) see Lurz et al. 2005; For *Tamias sibiricus* (Laxmann, 1759) see Obolenskaya et al. 2009.

[x] Ferris (1921) report *H. erismata* from *Sciurus ferrugineus cinnamomeus* but do not mention any name authority: we admit here *S. f. cinnamoeus* = *C. finlaysonni cinnamomeus* (Temminck) (see also Durden and Musser 1994b)

[1] Myers and Kuntz (1964); [2] Durette-Desset (1976); [3] Yen (1973); [4] Schmidt et al. (1967); [5] Kliks and Durette-Desset (1976); [6] Wang (1981); [7] Fischthal and Kuntz (1981); [8] Hoogstraal and Kohls (1965); [9] Li et al. (1976); [10] Durden and Beaucournu (2014); [11] Liu et al. (1964); [12] Jordan and Rothschild (1911); [13] Liu et al. (1982); [14] Durden and Musser (1994a); [15] Durden and Musser (1994b); [16] Johnson (1959); [17] Ferris (1921); [18] Blagoveschensky (1972)

Supplemental material 12. 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.

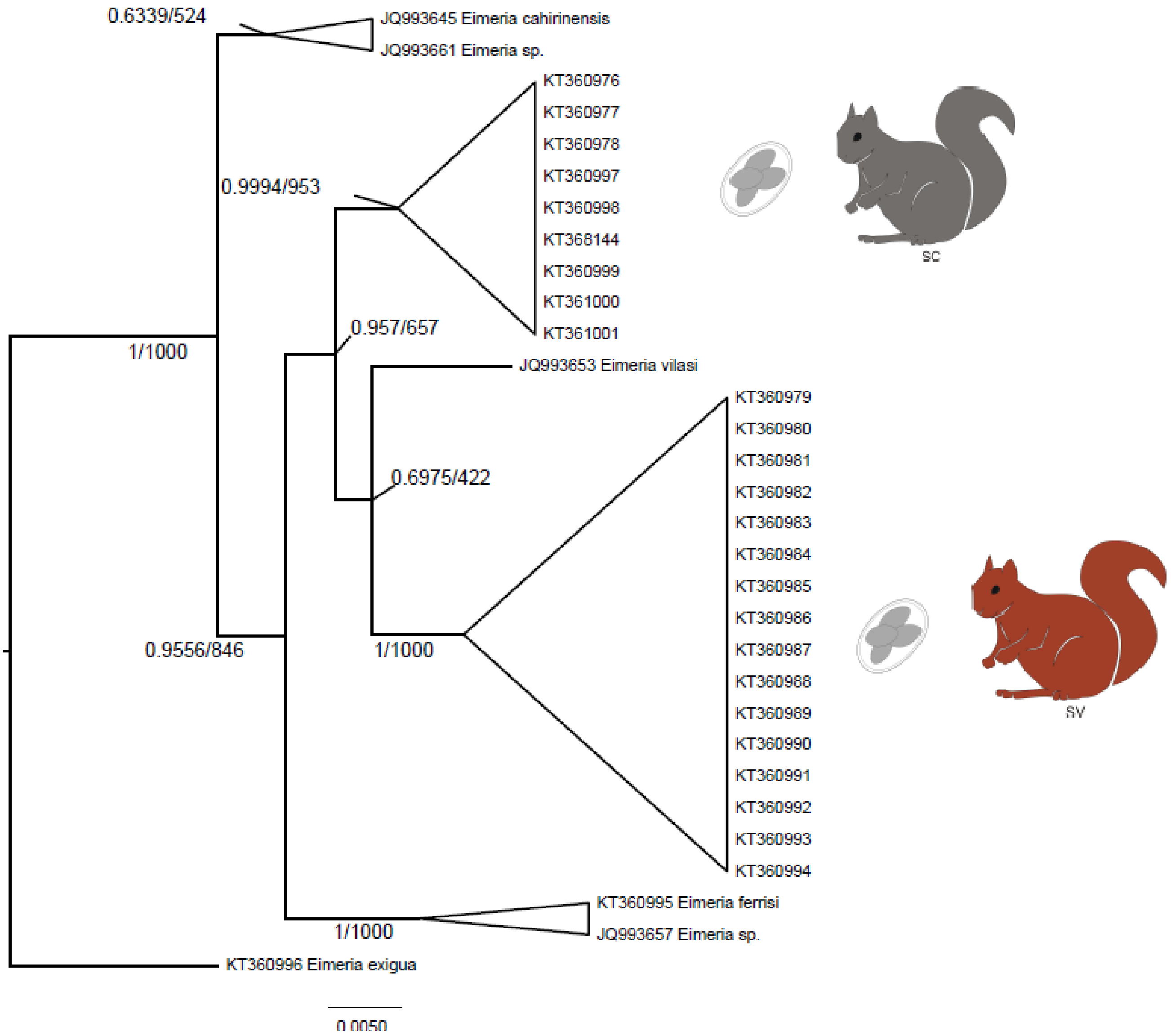
	Host	Oocysts	SI	Sporocysts	SI
E1	SV	39.5 (36-43) x 26.5 (25-30)	1.5	19.3 (16-22) x 8.2 (7-10)	2.4
E2	SV	27.2 (20-36) x 16.3 (11-20)	1.7	11.5 (7-15) x 6.8 (5-9)	1.7
	SC	28.4 (21-35) x 16.8 (13-23)	1.7	12.3 (9-17) x 7.3 (5-9)	1.7
	CE	30.9 (28-35) x 17.4 (15-19)	1.8	12.3 (10-15) x 7.5 (6-8)	1.7
E3	SV	18.5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

Supplemental material 13. Sequenced isolates of *Eimeria* spp. of 40 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.

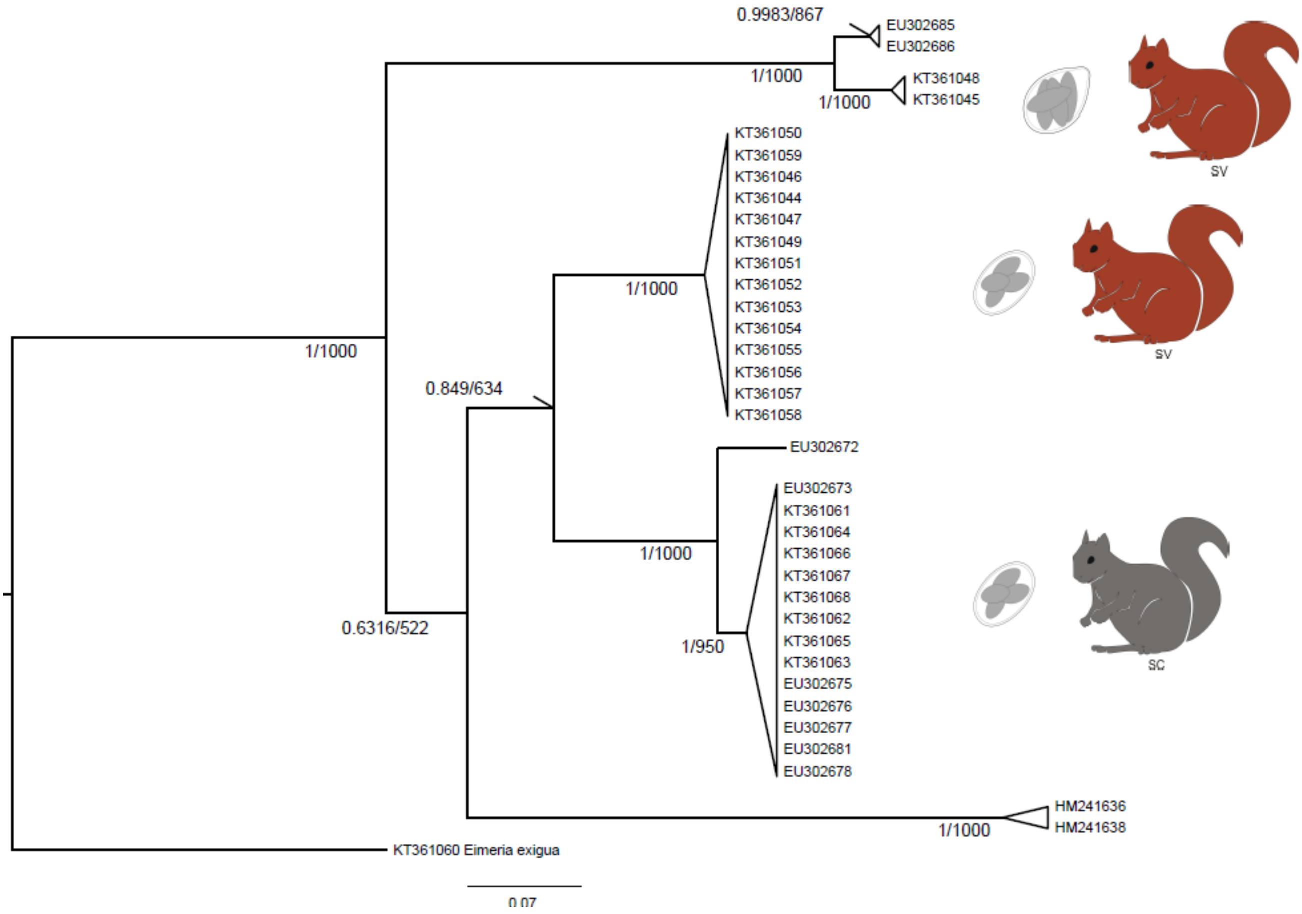
No.	Host	Site	*Eimeria* morphotype/OPG	18S	ITS	Cox I
L05	SC	GREY	E2/68000		KT361061	KT361031
L28	SC	GREY	E2/115833	KT360997		KT361032
L348	SC	GREY	E2/1667			KT361035
L356	SC	GREY	E2/6154		KT361067	KT361041
L357	SC	GREY	E2/46667; E3/<100	KT360998	KT361064	KT361038
GF01	SC	RED-GREY	E2/<100			KT361030
L345	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	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

No.	**Host**	**Site**	***Eimeria* morphotype/OPG**	**18S**	**ITS**	**Cox I**
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

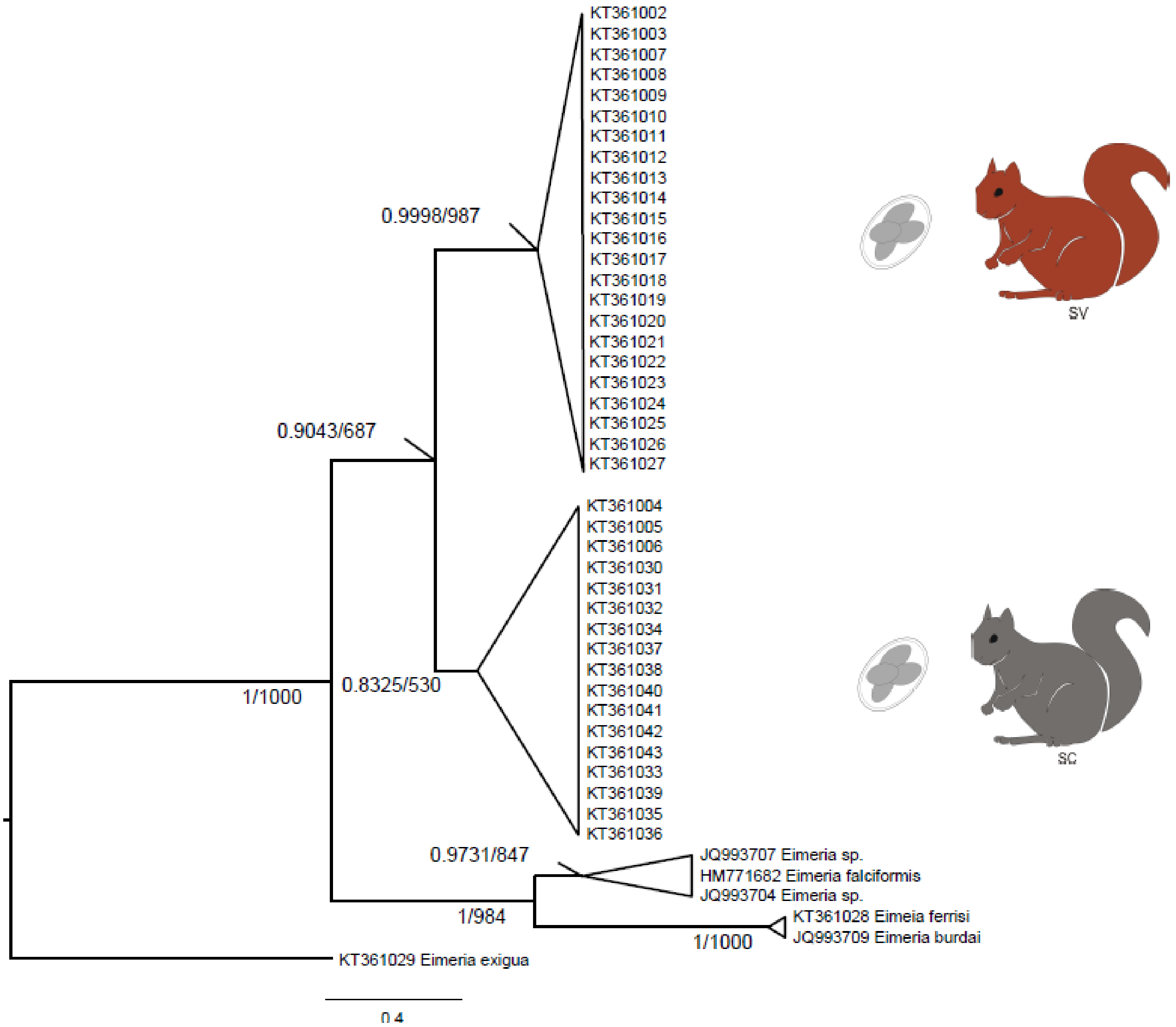
Supplemental material 14. Phylogenetic tree based on 18S sequences of *Eimeria* spp. constructed by MrBayes software run under HKY+I model of molecular evolution with combined branch supports of PP for BI/ML bootstrap.



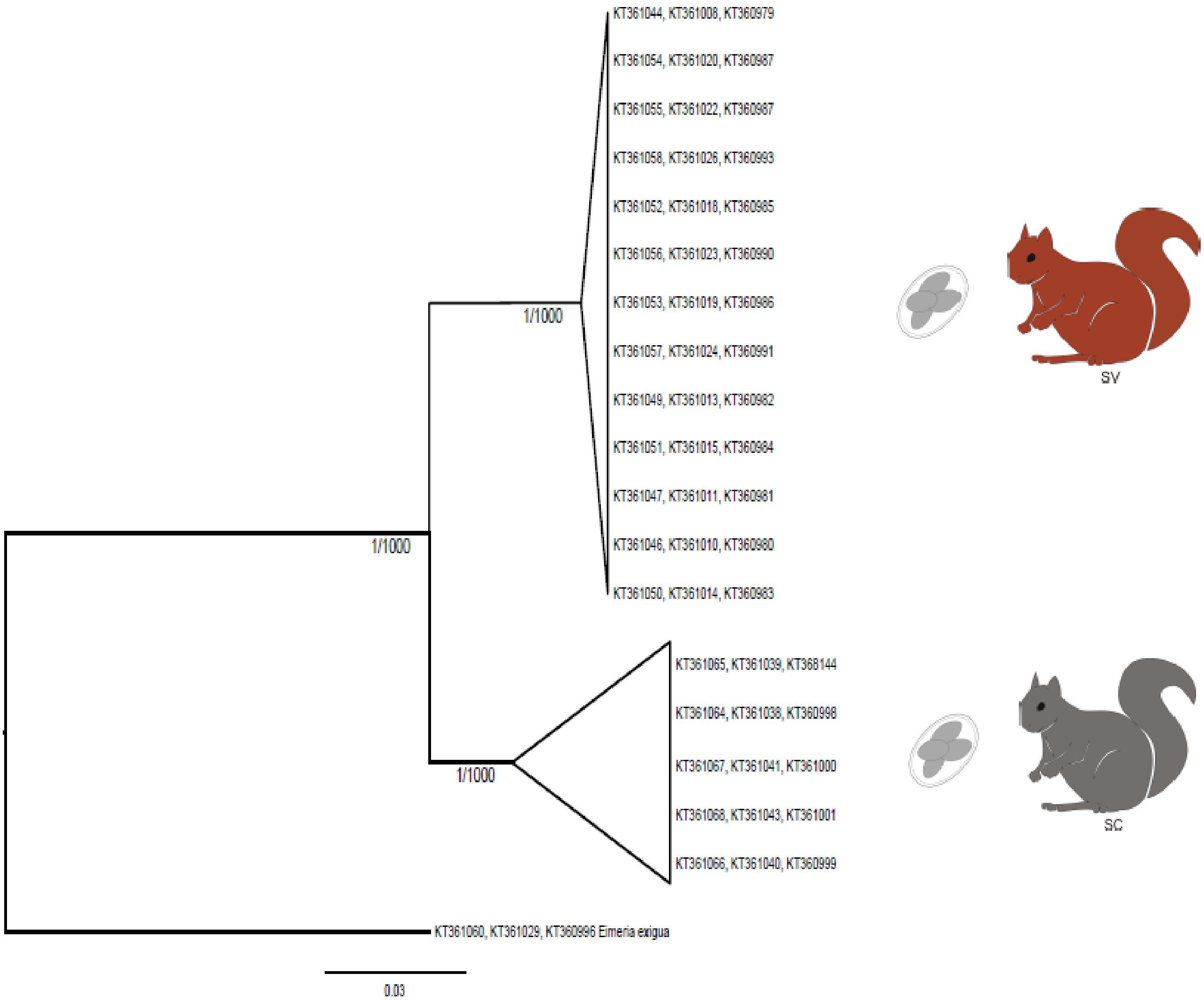
Supplemental material 15. Phylogenetic tree based on ITS1, 5.8S and ITS2 sequences of *Eimeria* spp. constructed by MrBayes software run under GTR+I+G model of molecular evolution with combined branch supports of PP for BI/ML bootstrap.



Supplemental material 16. Phylogenetic tree based on *Cox I* sequences of *Eimeria* spp. constructed by MrBayes software run under GTR+G model of molecular evolution with combined branch supports of PP for BI/ML bootstrap.



Supplemental material 17. Concatenated phylogenetic tree based on 18S, ITS 1, ITS 2, 5.8S and *cox I* sequences of *Eimeria* spp. constructed by MrBayes software run under HKY+G model of molecular evolution with combined branch supports of PP for BI/ML bootstrap.



Supplemental material 18. Number of session, occasions and Pallas's squirrels removed at the first red squirrel capture.

Trap grid	Session	Occasions	*C.erythraeus*
3	12	91	29
4	7	55	67
5	1	3	6
6	4	25	4
7	2	15	28
8	3	18	8
9	3	20	25
10	8	58	20
13	6	42	41

Supplementary material 19. Squirrels removed/captured for each trapping site (cpue= catch per unit effort).

Trap grid	Year	Sessions	Occasions	*Callosciurus erythraeus*		*Sciurus vulgaris*		
				removed	cpue	residents	tot	cpue
1	2011	6	49	16	0.33	0	0	0
2	2011	2	11	17	1.55	0	0	0
	2012	3	27	5	0.19	0	0	0
3	2012	2	16	3	0.19	0	0	0
	2013	6	35	8	0.23	0	0	0
	2014	6	58	13	0.22	0	1	0.02
	2015	2	20	8	0.40	0	0	0
4	2012	9	81	71	0.88	2	6	0.07
	2013	12	76	111	1.46	7	13	0.17
	2014	7	65	36	0.55	4	6	0.09
	2015	4	38	7	0.18	0	0	0
5	2011	11	79	39	0.49	6	10	0.13
	2012	4	33	9	0.27	2	5	0.15
	2013	11	66	32	0.48	5	6	0.09
	2014	12	85	27	0.32	5	9	0.11
6	2011	5	39	6	0.15	0	0	0
7	2012	5	39	58	1.49	2	2	0.05
	2013	5	22	16	0.73	0	0	0
8	2012	1	6	7	1.17	0	0	0
	2013	10	66	20	0.30	1	2	0.03
	2014	6	47	17	0.36	0	2	0.04
	2015	2	14	4	0.29	0	1	0.07
9	2013	3	21	18	0.86	2	2	0.10
	2014	8	57	29	0.51	4	7	0.12
	2015	2	20	3	0.15	0	0	0
10	2013	5	17	11	0.65	0	0	0
	2014	8	65	22	0.34	2	5	0.08
11	2013	2	16	3	0.19	0	0	0
	2014	2	16	5	0.31	0	0	0
12	2013	2	16	2	0.13	0	0	0
13	2014	9	63	10	0.16	2	5	0.08
14	2015	3	30	7	0.23	0	0	0
15	2015	1	8	2	0.25	0	0	0
16	2014	1	8	2	0.25	0	0	0
17	2015	3	30	8	0.27	0	0	0
18	2015	2	10	5	0.50	0	0	0
19	2015	5	42	15	0.36	0	0	0

Supplemental material 20. Eurasian red squirrel population estimates using Capture Mark Recapture and Minimum Number Alive to estimate the abundance (d= density ha^{-1}; dmax= maximum density ha^{-1}).

		CMR		MNA	
Trap grid	**Year**	**d**	**dmax**	**d**	**dmax**
4	2012	0.03	0.04	0.01	0.02
	2013	0.05	0.30	0.03	0.04
	2014	0.03	0.05	0.04	0.04
	2015	<0.01	0.01	0	0
5	2011	0.09	0.13	0.07	0.09
	2012	0.04	0.06	0.03	0.03
	2013	0.04	0.07	0.05	0.07
	2014	0.06	0.07	0.05	0.07
8	2013	0.03	0.03	0.02	0.04
	2014	0.01	0.01	0	0
	2015	0.09	0.09	0	0
9	2013	0.04	0.05	0.03	0.03
	2014	0.09	0.18	0.10	0.13
10	2014	0.11	0.14	0.09	0.10

Supplemental material 21. Model selection in the logistic regression to investigate the influence of occasion (trap check), period (time of the day) and season on the trap success.

Occasion	Period	Season	AIC	Δ AIC
+	+	+	6602,70	–
+	+		6605,30	2,60
	+	+	6619,60	16,90
	+		6623,30	20,60
+		+	6642,40	39,70
+			6645,80	43,10
		+	6656,50	53,80

Supplemental material 22. Trapping efficiency in the occasions (trap checks) during the capture sessions.

Occasion	Individuals	n	Trapp. efficiency (%)
1	118	5335	2,21
2	118	5076	2,32
3	96	5047	1,90
4	77	4944	1,56
5	95	4663	2,04
6	53	4024	1,32
7	53	3315	1,60
8	42	2995	1,40

Individuals: squirrels captured; n: number of controls made in the occasion; Trapping efficiency: individuals/n %

Supplemental material 23. Trapping efficiency in the four seasons.

Season	Individuals	Occasions	Trapp. efficiency (%)
Winter	166	7736	2,15
Spring	183	9461	1,93
Summer	174	9771	1,78
Autumn	144	9473	1,52

Individuals: squirrels captured; Occasions: number of controls made in the season; Trapping efficiency: individuals/Occasions %

Supplemental material 24. Model selection in the logistic regression to investigate the influence of trap type and vegetation on the trap success.

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
+						+			2949,70	-
		+				+			2949,99	0,29
+		+				+			2950,03	0,32
						+			2950,33	0,63
+						+		+	2950,55	0,85
		+				+		+	2950,63	0,93
		+			+	+			2950,66	0,96
					+	+			2950,70	0,99
			+			+			2950,71	1,00
+			+			+			2950,75	1,05
		+	+			+			2950,78	1,07
+					+	+			2950,84	1,14
						+		+	2950,88	1,18
+		+				+		+	2950,89	1,19
					+	+		+	2951,10	1,40
		+			+	+		+	2951,13	1,43
+		+	+			+			2951,28	1,58
+		+			+	+			2951,31	1,61
+					+	+		+	2951,59	1,89
+						+	+		2951,69	1,99
			+		+	+			2951,92	2,21
+									2951,98	2,28
		+				+	+		2951,99	2,29
+		+				+	+		2952,02	2,32
+		+			+	+		+	2952,06	2,36
			+			+		+	2952,08	2,38
		+	+			+		+	2952,10	2,40
+			+			+		+	2952,14	2,44

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
		+	+		+	+			2952,15	2,45
				+		+		+	2952,23	2,53
+				+		+			2952,26	2,55
						+	+		2952,27	2,57
			+	+		+			2952,30	2,60
+				+		+		+	2952,37	2,67
		+			+	+	+		2952,38	2,67
+						+	+	+	2952,43	2,73
				+		+			2952,45	2,75
				+	+	+		+	2952,52	2,82
+			+		+	+			2952,53	2,82
		+				+	+	+	2952,54	2,84
					+	+	+		2952,56	2,86
+		+	+			+		+	2952,62	2,92
						+	+	+	2952,63	2,93
			+			+	+		2952,64	2,94
+					+	+	+		2952,65	2,95
		+		+		+		+	2952,72	3,02
+			+			+	+		2952,73	3,03
		+	+			+	+		2952,77	3,07
				+	+	+			2952,84	3,14
		+		+		+			2952,84	3,14
+		+				+	+	+	2952,86	3,16
+			+	+		+			2952,89	3,19
	+	+				+			2952,93	3,22
			+		+	+		+	2952,93	3,23
+					+				2952,95	3,25
+		+		+		+			2952,98	3,27
+		+			+	+	+		2952,99	3,28
		+			+	+	+	+	2953,05	3,35
		+	+		+	+		+	2953,07	3,37
					+	+	+	+	2953,09	3,39
+		+	+		+	+			2953,11	3,41
+		+		+		+		+	2953,15	3,45
+	+	+				+			2953,19	3,49
	+	+			+	+			2953,22	3,52
			+	+		+		+	2953,26	3,55
+							+		2953,27	3,57
		+	+	+		+			2953,28	3,58
+		+	+			+	+		2953,28	3,58
		+		+	+	+		+	2953,30	3,60
+	+					+			2953,38	3,68
+				+	+	+			2953,44	3,74
+				+	+	+		+	2953,50	3,79
+		+							2953,52	3,81

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
		+		+	+	+			2953,53	3,82
+					+	+	+	+	2953,55	3,85
+			+		+	+		+	2953,57	3,86
			+	+	+	+			2953,61	3,91
+								+	2953,65	3,95
	+	+	+			+			2953,71	4,01
+			+						2953,82	4,12
+			+	+		+		+	2953,84	4,13
			+		+	+	+		2953,85	4,14
									2953,87	4,17
			+			+	+	+	2953,89	4,18
	+					+			2953,92	4,22
+		+			+	+	+	+	2953,94	4,23
		+	+		+	+	+		2953,95	4,25
	+				+	+			2953,96	4,26
+		+	+	+		+			2954,00	4,30
		+	+			+	+	+	2954,03	4,33
+			+			+	+	+	2954,05	4,35
+		+	+		+	+		+	2954,06	4,36
	+	+				+		+	2954,06	4,36
+	+	+			+	+			2954,08	4,37
				+	+	+	+		2954,12	4,42
		+	+	+		+		+	2954,13	4,43
+				+					2954,17	4,47
+	+				+	+			2954,20	4,50
			+	+	+	+		+	2954,20	4,50
				+		+	+	+	2954,21	4,51
+				+		+	+		2954,22	4,51
				+	+	+	+	+	2954,23	4,52
			+	+		+	+		2954,25	4,55
	+		+			+			2954,25	4,55
+		+		+	+	+			2954,26	4,55
+				+		+	+	+	2954,37	4,67
+		+		+	+	+		+	2954,37	4,67
+	+		+			+			2954,38	4,68
+	+					+		+	2954,39	4,69
+			+		+	+	+		2954,40	4,69
	+	+			+	+		+	2954,41	4,71
+	+	+				+		+	2954,41	4,71
+	+	+	+			+			2954,42	4,71
				+		+	+		2954,42	4,72
+							+	+	2954,55	4,85
+		+			+				2954,58	4,88
+		+	+			+	+	+	2954,60	4,90
+					+			+	2954,62	4,91

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
		+		+		+	+	+	2954,67	4,97
							+		2954,70	4,99
	+	+				+	+		2954,70	5,00
					+				2954,75	5,04
	+					+		+	2954,75	5,05
		+	+	+	+	+			2954,77	5,07
+					+		+		2954,81	5,11
+			+	+	+	+			2954,82	5,12
+			+	+		+	+		2954,82	5,12
	+	+	+		+	+			2954,82	5,12
+		+					+		2954,84	5,13
		+		+		+	+		2954,84	5,13
+		+	+		+	+	+		2954,84	5,14
+				+	+	+	+		2954,85	5,15
+		+	+	+		+		+	2954,85	5,15
	+				+	+		+	2954,88	5,18
			+		+	+	+	+	2954,92	5,22
+			+		+				2954,94	5,23
			+	+	+	+	+		2954,95	5,25
+		+		+		+	+		2954,96	5,26
		+	+		+	+	+	+	2955,00	5,30
		+		+	+	+	+		2955,01	5,31
		+							2955,07	5,37
+			+				+		2955,10	5,39
+	+	+				+	+		2955,11	5,41
		+		+	+	+	+	+	2955,12	5,42
+		+		+		+	+	+	2955,13	5,43
				+					2955,20	5,50
		+	+	+	+	+		+	2955,21	5,50
	+	+			+	+	+		2955,22	5,51
+				+	+	+	+	+	2955,24	5,53
+	+								2955,24	5,54
+		+						+	2955,25	5,55
	+		+		+	+			2955,25	5,55
			+	+		+	+	+	2955,26	5,55
		+	+	+		+	+		2955,26	5,56
+	+					+	+		2955,26	5,56
+	+				+	+		+	2955,36	5,66
+				+	+				2955,37	5,67
								+	2955,39	5,69
+	+	+			+	+		+	2955,40	5,70
+		+	+						2955,40	5,70
+			+	+	+	+		+	2955,40	5,70
	+	+	+			+		+	2955,42	5,72
			+						2955,43	5,73

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
	+	+	+			+	+		2955,46	5,76
+				+				+	2955,47	5,77
	+		+	+		+			2955,48	5,78
+			+		+	+	+	+	2955,53	5,83
+			+					+	2955,59	5,88
	+					+	+		2955,59	5,89
							+	+	2955,64	5,93
	+	+		+		+			2955,66	5,96
+			+	+					2955,73	6,03
	+			+	+	+			2955,73	6,03
+	+			+		+			2955,74	6,03
	+			+		+			2955,76	6,06
	+	+				+	+	+	2955,80	6,10
+		+		+	+	+	+		2955,82	6,12
+			+	+		+	+	+	2955,83	6,13
+				+			+		2955,84	6,14
	+	+		+	+	+			2955,86	6,16
			+	+	+	+	+	+	2955,87	6,17
	+		+			+		+	2955,87	6,17
	+		+			+	+		2955,88	6,18
+		+		+					2955,89	6,18
+	+		+		+	+			2955,91	6,21
+	+	+	+		+	+			2955,91	6,21
+		+	+		+	+	+	+	2955,94	6,23
+		+	+	+	+	+			2955,94	6,24
+	+		+			+		+	2955,95	6,25
	+				+	+	+		2955,96	6,26
		+					+		2955,96	6,26
+		+	+	+		+	+		2955,97	6,27
+	+	+		+		+			2956,03	6,33
	+			+		+		+	2956,05	6,34
+	+	+			+	+	+		2956,05	6,35
	+	+	+	+		+			2956,06	6,36
				+	+				2956,07	6,37
+	+	+	+			+		+	2956,07	6,37
		+			+				2956,09	6,39
		+	+	+		+	+	+	2956,11	6,41
	+			+	+	+		+	2956,17	6,46
+	+				+	+	+		2956,19	6,49
					+			+	2956,20	6,50
+	+			+		+		+	2956,20	6,50
+		+		+	+	+	+	+	2956,21	6,51
+	+		+			+	+		2956,22	6,51
+		+					+	+	2956,22	6,52
	+	+		+		+		+	2956,23	6,52

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
+			+	+	+	+	+		2956,23	6,52
+	+					+	+	+	2956,24	6,53
+	+		+	+		+			2956,24	6,54
			+				+		2956,24	6,54
		+	+	+	+	+	+		2956,26	6,56
				+				+	2956,28	6,58
			+	+					2956,28	6,58
+					+		+	+	2956,30	6,59
+		+			+			+	2956,30	6,60
+	+	+	+			+	+		2956,30	6,60
+	+	+				+	+	+	2956,31	6,61
+	+				+				2956,32	6,62
	+	+	+		+	+		+	2956,32	6,62
	+					+	+	+	2956,36	6,66
					+		+		2956,36	6,66
+		+	+	+	+	+		+	2956,37	6,67
	+	+			+	+	+	+	2956,41	6,71
+		+			+		+		2956,45	6,75
+	+			+	+	+			2956,49	6,79
+			+				+	+	2956,51	6,81
+			+		+			+	2956,54	6,84
+	+	+							2956,55	6,85
+		+	+		+				2956,56	6,85
+	+						+		2956,56	6,85
	+		+	+	+	+			2956,56	6,86
	+	+		+	+	+		+	2956,57	6,87
	+		+		+	+		+	2956,67	6,97
			+		+				2956,68	6,98
+				+	+			+	2956,70	7,00
		+						+	2956,70	7,00
+		+	+				+		2956,70	7,00
+	+	+		+		+		+	2956,71	7,01
+	+							+	2956,74	7,04
		+	+						2956,74	7,04
				+			+		2956,77	7,07
+				+			+	+	2956,78	7,07
		+		+					2956,79	7,09
+	+	+		+	+	+			2956,79	7,09
+			+		+		+		2956,81	7,10
	+	+	+		+	+	+		2956,82	7,12
+		+	+	+		+	+	+	2956,85	7,14
	+				+	+	+	+	2956,88	7,18
	+		+	+		+		+	2956,91	7,20
+	+	+	+	+		+			2956,99	7,29
		+	+	+	+	+	+	+	2957,00	7,30

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
+	+		+						2957,06	7,36
		+					+	+	2957,08	7,37
				+	+			+	2957,08	7,38
	+								2957,11	7,41
+			+	+	+	+	+	+	2957,12	7,42
+		+		+	+				2957,13	7,43
			+					+	2957,15	7,45
	+	+	+			+	+	+	2957,15	7,45
+	+			+	+	+		+	2957,16	7,46
+		+	+					+	2957,20	7,50
	+		+		+	+	+		2957,24	7,54
	+	+	+	+	+	+			2957,25	7,55
+			+	+				+	2957,25	7,55
+		+		+				+	2957,26	7,56
+	+		+		+	+		+	2957,32	7,62
+			+	+	+				2957,34	7,64
+	+				+	+	+	+	2957,36	7,66
+				+	+		+		2957,36	7,66
				+			+	+	2957,38	7,68
+	+	+			+	+	+	+	2957,39	7,69
+	+	+	+		+	+		+	2957,39	7,69
	+	+		+		+	+		2957,42	7,71
+			+	+			+		2957,43	7,73
+		+		+			+		2957,44	7,73
	+		+	+		+	+		2957,44	7,74
					+		+	+	2957,45	7,75
			+				+	+	2957,48	7,77
	+		+			+	+	+	2957,48	7,78
	+	+	+	+		+		+	2957,49	7,79
+		+	+	+	+	+	+		2957,49	7,79
+		+	+	+					2957,54	7,84
+	+		+	+		+		+	2957,57	7,87
	+			+	+	+	+		2957,58	7,87
		+	+				+		2957,61	7,91
		+			+			+	2957,64	7,93
+	+	+		+	+	+		+	2957,68	7,98
	+			+		+	+		2957,70	8,00
			+	+	+				2957,71	8,01
+	+			+					2957,72	8,02
		+			+		+		2957,72	8,02
+	+			+		+	+		2957,72	8,02
+	+	+			+				2957,73	8,02
			+	+				+	2957,76	8,06
	+		+	+	+	+		+	2957,77	8,07
+	+		+			+	+	+	2957,77	8,07

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
		+		+	+				2957,80	8,10
	+						+		2957,81	8,11
+	+	+					+		2957,83	8,13
	+	+		+	+	+	+		2957,85	8,15
+	+		+	+	+	+			2957,86	8,16
	+	+	+	+		+	+		2957,89	8,19
+	+	+		+		+	+		2957,90	8,20
+	+	+	+		+	+	+		2957,90	8,20
+	+		+		+	+	+		2957,91	8,21
			+	+			+		2957,91	8,21
+	+						+	+	2957,92	8,22
	+	+		+		+	+	+	2957,93	8,23
	+			+		+	+	+	2957,94	8,24
+	+	+	+			+	+	+	2957,95	8,25
+	+				+			+	2957,96	8,26
		+		+				+	2957,98	8,27
	+	+							2957,98	8,28
+		+			+		+	+	2958,01	8,30
		+	+	+					2958,04	8,34
		+	+		+				2958,06	8,35
				+	+		+		2958,06	8,36
	+			+	+	+	+	+	2958,06	8,36
	+				+				2958,07	8,37
+	+				+		+		2958,10	8,39
+	+			+		+	+	+	2958,16	8,46
+	+	+						+	2958,19	8,48
+		+	+				+	+	2958,19	8,49
			+		+			+	2958,20	8,49
+		+	+		+			+	2958,20	8,50
+		+	+	+	+	+	+	+	2958,20	8,50
		+		+			+		2958,22	8,52
+	+		+	+		+	+		2958,23	8,53
+			+		+		+	+	2958,23	8,53
			+		+		+		2958,26	8,55
+	+		+		+				2958,31	8,61
	+	+	+		+	+	+	+	2958,32	8,62
+	+	+	+	+		+		+	2958,33	8,63
+	+		+				+		2958,33	8,63
+	+			+	+	+	+		2958,36	8,66
	+		+	+	+	+	+		2958,42	8,71
+	+	+	+						2958,42	8,72
+		+	+		+		+		2958,44	8,73
	+	+	+	+	+	+		+	2958,44	8,74
+		+		+			+	+	2958,44	8,74
	+							+	2958,45	8,74

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
		+	+					+	2958,52	8,81
+		+		+	+			+	2958,52	8,82
+	+	+	+	+	+	+			2958,53	8,83
+	+	+		+		+	+	+	2958,55	8,85
	+	+		+	+	+	+	+	2958,56	8,86
+				+	+		+	+	2958,57	8,86
+			+	+			+	+	2958,64	8,94
	+		+						2958,64	8,94
	+		+		+	+	+	+	2958,66	8,95
	+	+					+		2958,66	8,96
+	+		+					+	2958,69	8,99
+			+	+	+			+	2958,70	9,00
+	+	+		+	+	+	+		2958,79	9,09
	+			+					2958,81	9,11
	+		+	+		+	+	+	2958,84	9,14
+	+			+	+				2958,86	9,16
+	+	+	+	+		+	+		2958,90	9,19
		+		+	+			+	2958,90	9,20
				+	+		+	+	2958,92	9,21
		+		+			+	+	2958,92	9,22
		+			+		+	+	2958,95	9,25
		+	+				+	+	2958,95	9,25
	+						+	+	2958,96	9,26
+	+			+				+	2958,96	9,26
			+	+	+			+	2958,97	9,27
			+	+			+	+	2959,01	9,30
+	+		+	+	+	+		+	2959,02	9,32
+	+			+	+	+	+	+	2959,06	9,36
	+	+			+				2959,08	9,38
+		+		+	+		+		2959,08	9,38
+		+	+	+				+	2959,10	9,39
+		+	+	+	+				2959,12	9,42
+		+	+	+			+		2959,14	9,44
+	+			+			+		2959,19	9,49
	+	+	+	+	+	+	+		2959,23	9,53
+	+		+	+					2959,26	9,56
	+	+	+	+		+	+	+	2959,27	9,57
	+		+				+		2959,29	9,59
+	+	+		+					2959,31	9,60
+	+		+		+	+	+	+	2959,32	9,62
+			+	+	+		+		2959,32	9,62
+	+	+					+	+	2959,36	9,66
+	+	+	+		+	+	+	+	2959,38	9,68
	+				+			+	2959,44	9,74
			+		+		+	+	2959,44	9,74

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
+	+	+			+			+	2959,47	9,77
+	+	+			+		+		2959,48	9,78
	+	+						+	2959,53	9,82
	+				+		+		2959,54	9,83
		+	+	+	+				2959,54	9,84
+	+		+	+		+	+	+	2959,55	9,84
		+	+	+				+	2959,55	9,85
		+	+	+			+		2959,55	9,85
	+			+	+				2959,60	9,90
	+	+	+						2959,63	9,93
		+	+		+			+	2959,64	9,93
	+		+	+	+	+	+	+	2959,66	9,96
		+	+		+		+		2959,66	9,96
+	+	+	+	+	+	+		+	2959,66	9,96
+	+	+	+				+		2959,67	9,97
+	+	+		+	+	+	+	+	2959,67	9,97
+	+				+		+	+	2959,68	9,97
			+	+	+		+		2959,69	9,99
+	+	+	+		+				2959,71	10,00
+	+		+	+	+	+	+		2959,73	10,03
		+		+	+		+		2959,76	10,06
	+		+	+					2959,85	10,15
+	+		+				+	+	2959,86	10,16
+	+		+		+			+	2959,90	10,20
	+			+				+	2959,90	10,20
+		+	+		+		+	+	2959,92	10,22
	+		+		+				2959,98	10,28
	+			+			+		2960,02	10,32
	+	+					+	+	2960,06	10,36
+	+		+		+		+		2960,10	10,39
+	+	+	+					+	2960,15	10,45
+	+	+	+	+		+	+	+	2960,20	10,50
	+		+					+	2960,23	10,53
	+	+		+					2960,26	10,56
+	+			+			+	+	2960,27	10,57
	+	+	+				+		2960,27	10,57
+	+			+	+			+	2960,29	10,59
+		+		+	+		+	+	2960,33	10,63
+		+	+	+			+	+	2960,36	10,66
	+	+	+	+	+	+	+	+	2960,43	10,73
+	+	+		+	+				2960,49	10,79
+	+	+		+			+		2960,50	10,79
+		+	+	+	+			+	2960,52	10,81
	+	+			+		+		2960,52	10,82
+	+	+	+	+	+	+	+		2960,52	10,82

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
+			+	+	+		+	+	2960,56	10,86
	+	+			+			+	2960,64	10,94
		+	+	+			+	+	2960,66	10,96
		+		+	+		+	+	2960,67	10,97
+	+	+		+				+	2960,67	10,97
+	+			+	+		+		2960,73	11,03
+	+		+	+			+		2960,74	11,03
	+		+				+	+	2960,74	11,04
	+			+	+			+	2960,76	11,06
+	+		+	+				+	2960,77	11,07
	+				+		+	+	2960,79	11,09
			+	+	+		+	+	2960,82	11,12
+	+		+	+	+				2960,82	11,12
		+	+	+	+			+	2960,83	11,13
	+			+			+	+	2960,88	11,18
+	+		+	+	+	+	+	+	2960,92	11,22
		+	+		+		+	+	2960,95	11,25
+	+	+	+	+					2960,96	11,26
	+	+	+		+				2961,04	11,34
+		+	+	+	+		+		2961,08	11,37
	+		+	+			+		2961,08	11,38
	+	+		+			+		2961,09	11,39
+	+	+			+		+	+	2961,17	11,47
	+	+		+	+				2961,20	11,50
	+		+	+	+				2961,23	11,53
+	+	+	+				+	+	2961,31	11,61
	+	+	+					+	2961,36	11,65
+	+	+	+		+			+	2961,39	11,69
	+		+	+				+	2961,39	11,69
	+		+		+		+		2961,41	11,70
	+		+		+			+	2961,44	11,73
	+			+	+		+		2961,46	11,76
+	+	+	+		+		+		2961,47	11,77
	+	+	+	+					2961,50	11,79
		+	+	+	+		+		2961,50	11,80
	+	+		+				+	2961,50	11,80
+	+		+		+		+	+	2961,63	11,93
+	+	+	+	+	+	+	+	+	2961,66	11,96
+	+	+		+			+	+	2961,73	12,03
	+	+	+				+	+	2961,89	12,19
	+	+			+		+	+	2961,99	12,29
+	+	+		+	+			+	2962,02	12,32
+	+			+	+		+	+	2962,09	12,38
+	+		+	+			+	+	2962,11	12,41
	+	+		+			+	+	2962,15	12,45

Cover	Cover growth	Supp. age	Similarity Index	Supp. size	Supp. state	Supp. species	Trap type	Vegetation richness	AIC	ΔAIC
+	+	+		+	+		+		2962,19	12,49
+	+	+	+	+			+		2962,19	12,49
+	+		+	+	+			+	2962,29	12,59
+		+	+	+	+		+	+	2962,32	12,61
	+	+	+	+			+		2962,41	12,71
	+		+	+			+	+	2962,44	12,74
	+	+	+		+		+		2962,45	12,75
+	+	+	+	+	+				2962,48	12,78
	+	+		+	+			+	2962,49	12,79
	+			+	+		+	+	2962,51	12,81
+	+	+	+	+				+	2962,54	12,83
		+	+	+	+		+	+	2962,62	12,92
	+		+	+	+			+	2962,64	12,93
	+	+	+		+			+	2962,64	12,94
+	+		+	+	+		+		2962,69	12,98
	+		+		+		+	+	2962,78	13,08
	+	+		+	+		+		2962,87	13,17
	+	+	+	+	+				2962,95	13,25
	+		+	+	+		+		2963,07	13,37
	+	+	+	+				+	2963,09	13,39
+	+	+	+		+		+	+	2963,11	13,41
+	+	+	+	+			+	+	2963,63	13,93
+	+	+		+	+		+	+	2963,66	13,96
	+	+	+	+			+	+	2963,84	14,14
	+	+	+		+		+	+	2963,99	14,28
+	+	+	+	+	+			+	2964,01	14,31
	+	+		+	+		+	+	2964,07	14,37
+	+		+	+	+		+	+	2964,09	14,38
+	+	+	+	+	+		+		2964,18	14,48
	+		+	+	+		+	+	2964,39	14,69
	+	+	+	+	+			+	2964,42	14,72
	+	+	+	+	+		+		2964,63	14,93
+	+	+	+	+	+		+	+	2965,64	15,94
	+	+	+	+	+		+	+	2966,01	16,31

PEER-REVIEWED PAPERS

- Preventing species invasion: a role for integrative taxonomy? 2015. *Integrative Zoology* in press.
  Mazzamuto M.V., Galimberti A., Cremonesi G., Pisanu B., Chapuis J-L., Stuyck J., Amori G., Su H-J., Aloise G., Preatoni D., Wauters L., Casiraghi M., Martinoli A.

- The weight of invasive squirrels on the Eurasian red squirrel: effects of interspecific competition on body mass and size. *Ethology Ecology and Evolution* submitted.
  Mazzamuto M.V., Panzeri M., Wauters L.A., Preatoni D.G., Martinoli A.

- Space invaders: home range dynamics and interspecific competition between the invasive alien Pallas's squirrel (*Callosciurus erythraeus*) and the native Eurasian red squirrel (*Sciurus vulgaris*). *Mammal Research* submitted.
  Mazzamuto M.V., Morandini M., Wauters L., Preatoni D., Martinoli A.

- Poor parasite community in an invasive alien species: macroparasites of Pallas's squirrels in Italy. 2015. *Annales Zoologici Fennici* in press.
  Mazzamuto M.V., Pisanu B., Chapuis J-L., Romeo C., Ferrari N., Wauters L., Preatoni D., Martinoli A.

- Do coccidia influence competition between native and invasive squirrels? *Oecologia* submitted.
  Hofmannová L., Štohanzlová L., **Mazzamuto M.V.**, Jirsová D., Romeo C., Wauters L.A., Ferrari N., Modrý D.

- Uterine scars analysis reveals high fecundity in invasive alien tree squirrels. 2015. *Hystrix The Italian Journal of Mammalogy* in press.
  Santicchia F., Romeo C., Grilli G., Vezzoso L., Wauters L.A., **Mazzamuto M.V.**, Martinoli A., Ferrari N.

- Interspecific competition between alien Pallas's squirrels and Eurasian red squirrels reduces density of the native species. In prep.
  Mazzamuto M.V., Bisi F., Preatoni D., Martinoli A., Wauters L.

- Knowledge, management and optimization: the use of live traps in control of non-native squirrels. 2015. *Mammalia* on line first.
  Mazzamuto M.V., Panzeri M., Wauters L., Preatoni D., Martinoli A.

- Shape variation and modularity of mandible of *Callosciurus erythraeus* introduced in Europe. In prep.
  Su H.J., **Mazzamuto M.V.**, Wauters L.A., Martinoli A., Preatoni D.