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ORIGINAL PAPER

Grey squirrels in central Italy: a new threat for endemic red squirrel subspecies

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Abstract A new population of the invasive American Eastern grey squirrel (*Sciurus carolinensis*) has recently settled in central Italy from an accidental release in Perugia, Umbria in the early 2000s. The grey squirrel is known to compete with and exclude native red squirrels (*S. vulgaris*) in the British Isles and Northern Italy, so it represents a potentially important new conservation threat to the red squirrel subspecies of south and central Italy, *S. vulgaris italicus* and *S. v. meridionalis,* which are endemic to peninsular Italy. The grey squirrel population range in Perugia is currently expanding at a rate of about 0.29 km/year (SD 0.19), slower than grey squirrel invasions elsewhere in Europe. Nuclear DNA analysed at 12 different microsatellite loci revealed that the grey squirrels in Perugia have extremely low genetic diversity, consistent with a small founder size. Genetic assignment tests indicate that the Perugia population was founded by translocations from an established population in Piedmont, Italy. No genetic substructure is evident yet in the Perugia population. These results together have serious consequences for the management of the grey squirrel invasion in Perugia and the conservation of the red squirrel subspecies: the Perugia grey squirrel population should be eradicated if politically feasible; otherwise new releases of grey squirrels, especially from sources other than the Piedmont population, should be prevented because such introductions could increase genetic diversity, thereby potentially increasing population range expansion rate to the much higher levels seen for more diverse grey squirrel populations elsewhere in Europe.

Keywords Invasive · Alien species · Microsatellites · *Sciurus carolinensis* · Translocations · Competition

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Introduction

Most invasions do not spread as a simple wave in a diffusion-like process from the initial introduction site. Instead, invaders usually radiate from multiple, separate foci arising either from multiple introductions from their native range or from long-distance jumps of dispersal, often human mediated, from the first introduced population (Kot et al. 1996). These foci can differ greatly in size and ability to spread as a consequence of the sizes of their founding events, their dates of establishment, local environments, and stochastic events which are more extreme in small populations (Zeisset and Beebee 2003; Walker et al. 2003; Thulin et al. 2006). The spreading of the American Eastern grey squirrel (*Sciurus carolinensis*) in Europe is an archetype of this pattern.

The grey squirrel invasion in Europe, and especially in Great Britain, is one of the best documented cases of an animal invasion anywhere, and has caused damage and great conservation concern: the species is considered one of the most invasive species worldwide (Lowe et al. 2000). The grey squirrel is highly adaptable, and this adaptability, together with multiple introductions and translocations in Great Britain in the 19th and 20th centuries (Middleton 1930, 1931), have contributed to the spread of the species in the United Kingdom. The grey squirrel's expansion throughout most of Britain (Lloyd 1983; Reynolds 1985; Tompkins et al. 2003) has allowed researchers to observe and begin to understand the mechanisms and consequences of invasive population spread (Rushton and Garson 1995; Rushton et al. 1997, 2002), including aspects of disease dynamics, competitive displacement of the native red squirrel, *Sciurus vulgaris* (Gurnell et al. 2004), and invasion population genetics (Signorile. et al. in review; Sainsbury et al. 2008). In Italy, grey squirrels were first introduced in 1948 in Turin, a population that is now spreading steadily and leading to red squirrel local extinctions (Wauters et al. 1997; Wauters and Gurnell 1999; Wauters et al. 2002). Other grey squirrel population foci have also appeared in Genoa (Venturini et al. 2005) and, more recently, in several areas in Lombardy (Martinoli et al. 2010). With the exception of a failed introduction in Rome (Bertolino et al. 2005), no mention of grey squirrels in the peninsular part of Italy has been reported except for the population in Perugia, Umbria, that we study here; that population sprang from an introduction that occurred at the beginning of the 2000s (Paoloni et al. 2010). Squirrels were accidentally released in a suburban wildlife park near Perugia and from there they have spread to the edge of the city and outward away from the city (Paoloni et al. 2012).

An endemic subspecies of Eurasian red squirrel, *S. vulgaris italicus* Bonaparte, 1838, lives in Central Italy around Perugia (Toschi 1965; Lurz et al. 2005), and another subspecies, *S. v. meridionalis* Lucifero, 1907, lives in southern Italy, in Calabria. Although it is known that the range of *S. v. italicus* includes the north-central part of the Apennine Mountains (Toschi 1965; Sidorowicz 1971; Amori et al. 2008), the distribution of this red squirrel subspecies has never been clearly mapped and no data on its abundance are available. Although we here refer to *S. v. italicus* and *S. v. meridionalis* as subspecies because they have been traditionally identified as such based on phenotypic measurements (Sidorowicz 1971; Lurz et al. 2005), a more recent study using mitochondrial DNA and microsatellites suggests that, of these two, only *S. v. meridionalis* is sufficiently differentiated from other *S. vulgaris* samples to be properly called a subspecies (Grill et al. 2009).

At the moment, red and grey squirrels both occur together in the Perugia area, but grey squirrels have eventually excluded red squirrels in essentially all previous areas the two species have come into contact. So the presence of grey squirrels in Perugia represents a serious conservation threat for two poorly known red squirrel subspecies—in the longer term, the grey squirrel could cause their extinction. Therefore understanding the spread of the new grey squirrel population and its mechanisms, the origin of the new population, its founding size, genetic structure, and the possible presence of ecological and genetic constraints that could affect grey squirrels' ability to spread in the new area is a priority for the conservation of *S. v. italicus* and *S. v. meridionalis*, and is the aim of this paper. We accomplish this aim by evaluating the empirical support for two hypotheses: (1) the population in Perugia grew from a small introduction from Piedmont, Italy, a population known to have low genetic diversity (Signorile et al. in review), and hence Perugia squirrels have very low genetic diversity; and (2) population range expansion in Perugia is very slow compared to other populations in Europe, possibly because of the low genetic diversity. Alternative hypotheses considered for origins of the introduction include the United Kingdom and the United States of America. We evaluate the implications of results for future expansion and management of the grey squirrel and for the nature of the threat the grey squirrel represents to the red squirrel subspecies.

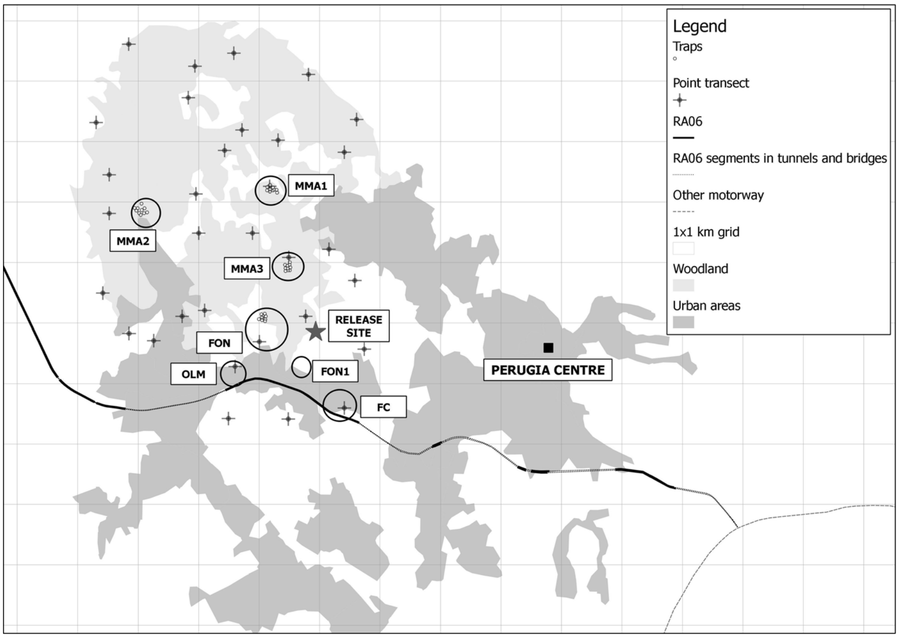

Fig. 1 Map of *grey* squirrel trapping and survey effort in Monte Malbe, Perugia. *Circled areas* represent the positions of genotyped squirrels

Methods

Study areas and sampling

Seven grey squirrels were reported to have been purchased in 1999 from a pet retailer by a private wildlife park near the city of Perugia. The squirrels were kept on display in wire cages outdoors, and these animals were reported to have escaped at the beginning of the 2000s. The wildlife park is located within the Site of Community Importance IT5210021, called "Monte Malbe" (Lat: 43.133 Long: 12.367), west of the city of Perugia, Umbria. It is a 945 ha forested area characterized by a mosaic of different vegetation patterns, dominated by holm oak (*Quercus ilex*) and other large-seeded deciduous trees such as chestnut (*Castanea sativa*).

Samples for genetic analysis were collected from road kills and from live-trapping sessions scattered at locations within the Monte Malbe site: Ferro di Cavallo (FC), Fontana (FON), Fontana 1 (FON1), Olmo 1 (OLM), MonteMalbe 1 (MMA1), MonteMalbe 2 (MMA2), and MonteMalbe 3 (MMA3) (Fig. 1). Roadkills were collected throughout 2010–2011. Live trapping was done from November 2011 to June 2012 using 10 ground-placed Tomahawk traps (model 104, Tomahawk Live Trap Co.). Tissue samples were taken from the ear pinna and placed in an Eppendorf vial containing 95 % ethanol and stored at 4 °C until DNA extraction. Squirrel densities were estimated at MMA1 and FON1 by the removal method (Zippin 1958).

DNA extraction and genotyping

DNA was collected from 25 animals. From each sample, about 5 mg of tissue were used to extract DNA according to the Wizard SV 96 Genomic DNA Purification System (Promega) protocol. Ten to twenty nmol/μL of DNA from each sample were amplified with 6 microsatellite markers developed for *S. vulgaris*, SCV1, SCV4, SCV6, SCV13, SCV18, SCV31 (Hale et al. 2001), and 6 developed for *S. niger*, FO11, FO36, FO46, FO54, FO63 (Fike and Rhodes 2009), DFS27 (Lance et al. 2003), for a total of 12 loci. Multiplex set ups and DNA amplification by PCR were carried out as described in (Signorile et al. in review). Diluted PCR products were run with an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) and alleles were scored with GeneMapper 4.0. To minimise genotyping errors, PCRs were repeated twice and positive and negative controls were added to the plate.

Data analysis

All genotypes were scored for deviations from Hardy–Weinberg equilibrium with a Fisher's exact test as implemented in GENEPOP 4.1 (Raymond and Rousset 1995) (default parameters were used). Fstat for Windows (2.9.3.2) (Goudet 1995) was used to test for pairwise linkage disequilibrium. This software runs the analysis using the Markov chain algorithm as implemented by Raymond and Rousset (1995) and automatically gives the adjusted *P* value for the 5 % nominal level after Bonferroni correction. MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) was used to assess if there was any evidence for allelic drop-outs, null alleles or stuttering. Mean number of alleles and inbreeding coefficient (F_{IS}) values were calculated and tested for significance with Genetix 4.05 (Belkhir et al. 2004).

STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to assess if there was any substructure in the Perugia population. This software assigns individuals to clusters using a Bayesian approach. A burn-in period discarding 100,000 Markov Chain Monte Carlo steps from each run was selected, followed by a chain of length 200,000 that was used. An admixture model with correlated allelic frequencies was chosen, with 20 iterations for each K.

Assignment tests were run with the software Oncor (Kalinowski et al. 2008). This software uses use a partial Bayesian method derived from the exclusion method of Rannala and Mountain (1997). A baseline composed of 338 individuals clustered in 5 populations from North and East England, Northern Ireland, Piedmont, Italy (Signorile et al., in review), and the USA (Table 1) was used for the exclusions. 95 % CI were estimated with 100,000 bootstrap resamplings. Fstat was also used to calculate the genetic distance (F_{ST}) of Perugia squirrels from the putative populations in the database, as implemented by Weir and Cockerham (1984).

Squirrel spread surveys

Data on squirrel presence and spread since the time of introduction was collected from 2010 through 2012 with point transects and hair tubes scattered across Monte Malbe. The position of each point transect (Fig. 1) was opportunistically chosen to maximize visibility in this very close and dense canopy, but also

Table 1 Population origins and numbers of individuals in the reference database used to investigate the source of the new grey squirrel population in Perugia

Location	Lat/Long	N
Piedmont		125
Stupinigi	54.80° 6.81°	19
Piobesi	54.64° 6.82°	6
Borgo Cornalese	54.40° 6.60°	54
Racconigi	54.31° 6.51°	32
Cavallermaggiore	54.27° 6.39°	14
Northern Ireland		122
Derrynoyd Forest	44.99° 7.60°	32
Drum Manor	44.94° 7.59°	20
Loughgall Forest	44.91° 7.73°	22
Gosford	44.79° 7.65°	29
Drumbanagher	44.72° 7.68°	19
Northumberland		38
Briar Wood	54.95° 2.32°	8
River Tyne Corridor	55.00° 2.11°	10
Morpeth	55.17° 1.70°	20
East Anglia	52.19° 0.83°	30
West Virginia	39.00° −80.23°	23

selecting spots were the composition of the arboreal vegetation was representative of the whole area. In each sampling location three different observations of 30 min each were carried out in different seasons during squirrels' peak activity times (early morning and late afternoon in springtime and summer, midday in autumn and winter). Further records were collected from citizens, park-owners, roadkills and occasional sightings and their positions georeferenced. All the point transect observations were carried out by one of the authors (DP) and a trained field worker. All the other observations were verified by DP. Since the study area has high-density tree growth, very poor visibility, and difficult terrain, distance sampling or other visual methods of assessing squirrel density could not be used.

Approximate squirrel spread rate since the release date was estimated, with uncertainties, by a simple method that is very similar to methods of Andow et al. (1990). The method measured maximum linear expansions from the release point in the eight main compass directions (Fig. 2). The eight distances were computed and two possible starting dates were used (2000 and 2003) to take into account uncertainties in

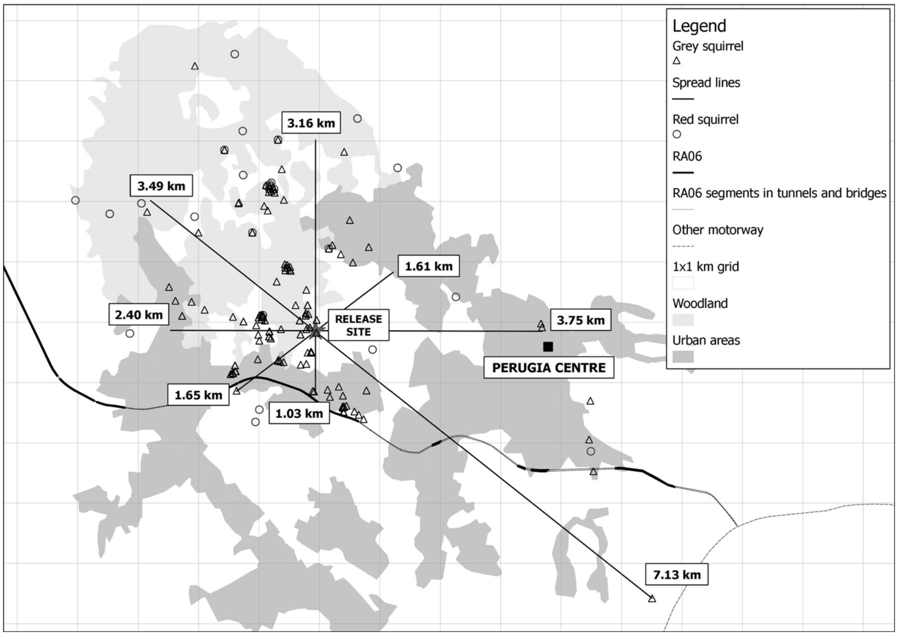

Fig. 2 Spatial distribution of red and grey squirrels in the urban and sub-urban area of Perugia and distances from the release point along the main directions of the compass. Squirrel records include trappings, sightings, and road kills from all years data were gathered (2010–2012)

the release time. Each distance was divided by the minimum and maximum Δt. The 16 individual estimates were averaged to get the point estimate of spread rate, but the individual measurements were retained as a measure of uncertainty. Andow et al. (1990) had species range data from several years, so they were able to evaluate whether spread was asymptotically linear, thereby computing the asymptotic rate; we had only 3 years data so this was infeasible and we computed only rates over the entire period from introduction to 2012 as an approximation of the asymptotic rate. We mention possible implications of this approximation in the Discussion.

Results

Genetic diversity and assignment tests

Across the 25 examined samples two loci (SCV4 and SCV13) were monomorphic, while DSF27 was monomorphic for all but one heterozygotic individual (PG22). The three markers were therefore kept to calculate genetic diversity and for the assignment tests but were excluded from the rest of the analysis. No evidence of deviation from Hardy–Weinberg equilibrium, linkage disequilibrium, allelic drop-outs, null alleles or stuttering was found for the Perugia population. The F_{IS} value was -0.023 (95 % CI -0.163 to 0.078) so the inbreeding coefficient was not significant. Mean number of alleles, averaged across all loci, was 2.17 ± 0.21, whereas the effective number of alleles (Ne) was 1.71 ± 0.16. Unbiased expected heterozygosity was 0.35 ± 0.067. The CI presented here and throughout the paper are 95 % ranges.

STRUCTURE analysis showed no clear evidence of substructure across the different sampling locations within the Perugia region. The assignment tests attributed all the individuals from Perugia to the population in Piedmont (Signorile et al. in review) and the population on the whole was attributed to Piedmont with a 100 % probability. The genetic distance (F_{ST}) from the Piedmont population was relatively low but still significant, with a value of 0.162 ($P < 0.001$) (Table 2). This was expected since allelic frequencies can vary greatly as a consequence of bottlenecks and genetic drift after a translocation event.

Grey squirrel expansion in Umbria

The mean spread rate was 0.29 km/year, with the 16 estimates ranging from 0.09 to 0.79 km/year. The SD of the 16 estimates was 0.19 km/year, and the standard error of the mean spread rate estimate was 0.05 km/year. Data showed that point transects for detecting

Table 2 Genetic distances between grey squirrels in Perugia and other populations. Stars indicate significance at a 95 % CI

Region	Genetic distance (F_{st})
Piedmont	0.1620***
Northern Ireland	0.3021***
Northumberland	0.3134***
East Anglia	0.2516***
West Virginia	0.2611***

squirrel presence were effective, because for every observation for which squirrels were detected, they were detected again on subsequent observations in the same location. Densities estimated at the two trapping locations MMA1 and FON1 were 21.7 and 32.2 individuals per hectare, respectively. During our capture sessions in the years 2011–2012 the capture ratio of reds to greys was 1:8 (10 captured reds versus 77 greys).

Discussion

Our data supported both of our initial hypotheses. They demonstrated that genetic diversity of grey squirrels around Perugia is very low following the severe bottleneck imposed when a few individuals were introduced from Piedmont, an area where genetic diversity was already low (Signorile et al. in review). It was not known prior to our work that the Perugia population had its origins in Piedmont; the double effect of low founder sizes, combined with founders from a region that had low diversity already, means diversity in Perugia is very low. Our results also showed that range expansion in Perugia has so far been very slow—comparisons to spread rates for other European populations are made below.

Genetic diversity and spread

There is limited consensus on the intrinsic physiological and ecological characteristics of good invaders, and the relationship between intrinsic characteristics of species and invasion may be complex and of limited predictive use (Kolar and Lodge 2001; Crawford and Whitney 2010). Instead, research often indicates that invasion success depends on the size of introduction events (Veltman et al. 1996; Kolar and Lodge 2001; Cassey et al. 2004; Lockwood et al. 2005; Sol et al. 2007; Dlugosch and Parker 2008; LeRoux and Wieczorek 2009; Hardesty et al. 2012). Genetic bottlenecks at the time of introduction can cause low genetic diversity, inbreeding depression and reduced ability to adapt to the new environment, limiting the probability of successful invasion (Frankham et al. 2002). When effective population size is low, stochastic loss of genetic variation is more likely and drift can overcome selection on evolutionary pathways. This can have a long term impact on fitness and population dynamics (Lammi et al. 1999; Reed and Frankham 2003; Reed et al. 2007; Roman and Darling 2007). Conversely, many introduced individuals or multiple introductions can lead to much genetic variation, which limits inbreeding depression, promotes adaptation, and facilitates invasion. These ideas and evidence supporting them are reviewed by Sakai et al. (2001), Lee (2002), and Dlugosch and Parker (2008). The invasiveness demonstrated by grey squirrels around Perugia and elsewhere in Italy therefore may be unexpected if expectations are based on the work cited above. The success of the invasion in spite of the genetic disadvantage of the population demonstrates the overall ability of the species as an invader in Europe. Further supporting this viewpoint, by compiling records on squirrel introductions worldwide, Bertolino (2009) estimated that *Sciurus* populations can be established from one introduced pair with 50 % success rate.

Although the population in Perugia has established and is spreading, the spread is slow in comparison with other populations in Europe. Low genetic diversity is the most probable explanation for the slowness—although squirrels can invade even when genetically poor, the rate of invasion may be decreased. Average spread rate in Piedmont was estimated to be about 0.6 km/year. For Northumberland, England it was 8.25 km/year. For East Anglia, England, it was 5.7 km/year, and for Northern Ireland it was 1.92 km/year (Signorile et al. in review). Earlier studies (Williamson and Brown 1986; Tangney and Montgomery 1995; O'Teangana et al. 2000) estimated 7.7 km/year in East Anglia and 1.94 km/year in Ireland, similar values. These are all markedly higher than our spread rate estimate for Perugia, 0.29 km/year. Bertolino and Genovesi (2003) also show results that indicate a higher expansion rate in Piedmont than in Perugia. The spread rates listed here are consistent with the hypothetical explanation that genetic diversity is negatively correlated with population spread rate (Signorile et al. in review). Although spread rate in Perugia is slow, ecological factors appear to be entirely in favor of rapid spread, in the sense that the largely unfragmented forests surrounding Perugia are dominated by large-seeded deciduous trees, and are an ideal habitat for fast population growth for an arboreal species native to the forests of the Eastern USA were oaks, walnuts, hickory and beeches are abundant (Koprowski 1994). Densities measured at MMA1 and FON1 were high—most densities previously measured in other European woodlands have been approximately in the range 1–5 individuals per hectare (Lurz et al. 2001).

There are at least two alternative hypothetical explanations for the slow spread in Perugia that can be compared in future work to the genetic explanation we suggest here. First, "lag phases" of slow expansion are often observed in the initial stages of an invasion (Crooks 2005). The Perugia expansion may accelerate after some years of initial slow expansion. Second, it was suggested by a referee of this study that fragmentation may speed population range expansion in some circumstances (With 2002). Hence slow expansion in Perugia may have been caused by the unfragmented nature of the Perugia forest instead of happening in spite of it, according to this hypothesis. One possible mechanism for such an effect would be if fragmentation forces individual squirrels to undergo larger dispersal events in order to bridge gaps between fragments. But the modelling and empirical results reviewed by With (2002) seem to suggest that greater habitat coverage is usually or always associated with faster range expansion. Another possible mechanism is that some degree of fragmentation is actually beneficial to grey squirrel ecology. Koprowski (2005) found a negative relationship between grey squirrel density and fragment size, and Fisher and Merriam (2000) found higher grey squirrel densities in fragmented landscapes than in continuous forest. However, as mentioned above, densities of grey squirrels in the Perugia region were quite high, casting doubt, in our view, on the hypothesis that lack of fragmentation is what has slowed expansion in the Perugia area. Nevertheless, landscape effects on spread rate involve a complex interaction of species demography, dispersal behaviour, ability to detect distant fragments, and precise landscape layout; further evaluation of landscape effects on spread rate and comparison to genetic effects will be an interesting topic for future research.

We detected no spatial genetic structure in the Perugia population using STRUCTURE; this contrasts with our earlier work in which we found structure at small spatial scales in populations in Piedmont and elsewhere (Signorile et al. in review). Lack of structure is probably due to the recency of the introduction or dispersal within the area or both. Structure would be expected to emerge as the population range expands, mirroring what was found, for instance, in Piedmont.

The method used here for calculating spread rate was quite similar to widely used methods described in Andow et al. (1990), but with a few differences because of the nature of our data. Andow et al. (1990) had several population range maps for their species of study, gathered at intervals over a long period of time. So they were able to compute spread rates over intervals and investigate whether the theoretical expectation held that spread was asymptotically linear. But Andow et al. (1990) selected species for study for which data were especially abundant, whereas we were interested for this paper in an important new population of grey squirrel, for which data were good but more limited than those of Andow et al. (1990). We calculated one spread rate per direction for each possible start date, using 2012 data, rather than extrapolating successive estimates over time to an asymptotic rate. This is an approximation of the asymptotic rate of Andow et al. (1990). We tried calculating spread rates for the successive years 2010–2012, and results were similar to those presented here, but because only 3 years of data were available this was not an improvement, and the simpler method was presented. Our estimated spread rate may be an underestimate if asymptotic rate has not been acheived (e.g., if the Perugia population is in a lag phase of expansion—see above), but because spread rate differences with other European populations are so large, spread rate will have to increase dramatically to approach levels seen elsewhere. Andow et al. (1990) also make a comparison between spread rates calculated from range map data and rates estimated using the formula $\sqrt{4\alpha D}$, where α is an intrinsic growth rate and D is the diffusion coefficient for the species. But this formula is not suitable for our study because estimates of α and D are not available for the Perugia population or for the other populations to which comparisons of spread rate were made.

Ecological issues

Umbria is covered by extensive and largely unfragmented secondary forests for over 60 % of its territory (Regione Umbria et al. 2009). Therefore, grey squirrels have the potential to spread dramatically. From Umbria, squirrels may well move freely northwards and southwards along the Apennine mountains to occupy the whole Italian peninsula. This is the first time that grey squirrels have occupied an extensive, continuous, forested area in continental Europe. Although grey squirrel spread is currently slow, ecological consequences could be serious in the long term, or in the shorter term if spread rate increases.

Although red and grey squirrels are sympatric at present, it is unlikely the peninsular woodlands will be able to indefinitely support both species. Competitive exclusion mechanisms (Wauters and Gurnell 1999) could already be acting on the red squirrel population; grey squirrels were much more commonly captured in the area than were red squirrels. Exclusion effects could become more evident in the next few years. If this proves to be the case, the presence of the grey squirrel will lead to local and regional extinctions of red squirrels.

Invasion of the grey squirrel may have consequences for the forest ecosystems of central and southern Italy more broadly than disappearance of the red squirrel. Squirrels have an important ecological role as seed dispersers because of their scatter-hoarding behaviour, and mutualistic relationships have evolved between tree species and squirrels (Wall 2001). Further studies would be needed to assess the capacity of the ecosystem to adapt to the replacement of the native red squirrel species with the grey squirrel, a species usually living at higher densities and with potentially subtly different seed hoarding and consumption behaviours. Red and grey squirrels overlap in their food preferences (Wauters et al. 2002), so replacement of red with grey squirrels may or may not lead to any substantive change in forest composition in the longer term. Although grey squirrels in the UK are known to debark and kill forest trees (Rowe and Gill 1985; Kenward and Parish 1986; Dagnall et al. 1998), this behaviour has not been observed in Italy (Signorile and Evans 2006). We have focussed here on the importance of the grey squirrel expansion in Perugia as derived from the threat the grey squirrel represents for *S. v. italicus* and *S. v. meridionalis* because that threat is very clear based on past observed interactions between grey and red squirrels elsewhere in Europe, whereas wider effects of the species replacement on southern Italian ecosystems may also be important but require further study.

The single squirrel seen at distance 7.13 km from the release site (Fig. 2) may have come from a human-mediated dispersal event from the main population centre West of the city, or it may have been part of a natural spread through a green belt north of the city. Future work should include sampling north of the city in the green belt. In addition, the distribution of grey squirrel sightings (Fig. 2) seems to suggest that the motorway RA06 has been a barrier to southward grey squirrel expansion. But because a few squirrels have recently been seen south of the road, the barrier appears to recently have been traversed. The section of the motorway closest to the release point is surface highway, but nearby sections are bridges or tunnels, which provide reduced or no barrier. Faster expansion south may be expected in the next several years now that the motorway barrier has been passed.

Conservation and research priorities

The grey squirrel population around Perugia should be eradicated. We have demonstrated that the population is expanding, albeit slowly, and it seems likely that new releases that increase genetic diversity will also increase spread rate: introgression of new genotypes, even of just a few individuals, could generate novel phenotypes, reduce the genetic load and have an effect on the evolutionary responses of invading populations (Lee 2011). The Perugia grey squirrel population is probably the only grey squirrel population that overlaps with the range of *S. v. italicus*, and is by far the closest population to the range of *S. v. meridionalis*. The expansion of the Perugia grey squirrel nucleus is probably the principle conservation threat to these subspecies (see below). New releases and increased spread rate are not unlikely given current enforcement of laws on invasive species. Grey squirrels were included in 2012 in Annex B of European Community Regulation 338/97, and this should prevent further imports from the species' natural range. However, the grey squirrels released in Perugia and purchased from the pet trade were taken from Piedmont, another Italian location. Grey squirrel live-captures for any purpose other than scientific studies are strictly regulated in Italy by law 157/92. Squirrels in the pet trade circuit can therefore come from other Italian locations only through an initial illegal poaching act, and it cannot be ruled out that this could happen again, especially given lax enforcement. Hence eradication is probably the only way to eliminate the threat posed to the peninsular Italian red squirrel subspecies. Eradication is very likely still possible and practical. For instance, eradication of the Piedmont population of grey squirrels was considered practical in 1997, when that population covered 380 km^2 (Bertolino and Genovesi 2003), far more than the <50 km^2 covered in the Perugia region. Eradication seems likely to be feasible for at least the next 10 years or longer, since the Piedmont population was established in 1948, and it was still considered practical to eradicate in 1997. But this is not guaranteed, especially if additional genetic diversity is imported to the Perugia population. Rapid action is considered crucial to successful eradication (Genovesi et al. 2010), as the task gets harder with population expansion. In addition, further translocations within the area or into the range of *S. v. meridionalis* are more likely with a nucleus at Perugia.

Past attempts to eradicate grey squirrel populations in Italy have been thwarted by animal rights activists (Bertolino and Genovesi 2003). If eradication is not possible because of this human dimension, the recent EU Regulation 757/2012 and the Legislative decree 24/12/2012 signed by the Italian government are steps in the right direction—these laws are aimed at forbidding the grey squirrel pet trade, releases in the wild, and import. Citizens should be educated about the ecological risks of buying, moving or releasing exotic species, and enforcement of these regulations should be vigilant.

Broader conservation of the Italian peninsular red squirrel subspecies, beyond the mitigation of the threat posed by grey squirrels, should focus on research because, to our knowledge, little has been published on the subspecies *S. v. italicus* and *S. v. meridionalis* regarding many aspects of their range, density, demography, genetics, behaviour, ecology or the threats they face, if any, other than the grey squirrel. Habitat loss and destruction have been found in extensive research to strongly affect *S. vulgaris* generally (Wauters et al. 1994; Verbeylen et al. 2003, 2009), with sensitivity to habitat fragmentation especially emphasized, for instance, by Koprowski (2005). Mortelliti et al. (2011) argued instead that what matters is habitat loss, not fragmentation *per se*. However, the vast majority of studies of red squirrel ecology use study locations outside the likely ranges of *S. v. italicus* and *S. v. meridionalis* (but see Mortelliti et al. (2011)), and we are aware of no studies that compare and contrast the ecology and demography of these subspecies with other red squirrel populations. Perhaps more importantly, there has been no research on whether the particular sensitivities of *S. vulgaris* to habitat layout and amount, combined with the past and projected changes of habitat in peninsular Italy, actually represent a real threat to *S. v. italicus* and *S. v. meridionalis* in the way that the grey squirrel represents a real and present danger to the subspecies.

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