Using DNA profiling to investigate human-mediated translocations of an invasive species

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ABSTRACT

The reconstruction of invasion history is the goal or foundation of many investigations of biological invasions. This study applies DNA profiling to investigate the sources and vectors of new propagules, to detect illegal human-mediated translocations and to improve the management of invasions by identifying invasion pathways that can be targeted for more stringent control. We use the European invasion of the American Eastern grey squirrel as a focal example. Many human-mediated translocations of this species have occurred in Europe since the 19th century. We built a genotype database of 1421 individuals from 59 locations across Europe and one in the USA, with each individual genotyped at 12 highly polymorphic microsatellites. DNA from newly detected populations and individuals of unknown sources were compared with this database. Our results indicated illegal human-mediated translocations in Aberdeen, the Isle of Skye and Northumberland in the UK, and revealed precise details of illegal squirrel trade in Italy. We recommend that multi-locus genotype databases be set up for all major invasive species considered attractive or ornamental, as these are often subject to illegal translocations through human actions such as the pet trade.

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1. Introduction

Molecular diagnostic techniques provide a potentially useful but underused tool for biosecurity. Biosecurity was defined by Pyšek and Richardson (2010) as "the management of risks posed by organisms to the economy, environment, and human health through exclusion, mitigation, adaptation, control, and eradication". Molecular methods are potentially very valuable in reconstructing the history of invasion processes, a critical step in analysing biological invasions (Cristescu, 2015; Estoup and Guillemaud, 2010). The geographic pathways followed by the founders of the invading populations, the type of invasion vector, the number of introduced individuals and the pathways of spread are all components of the invasion history, and provide key information about the origin and genetic composition of the invading populations (Dlugosch and Parker, 2008a; Estoup and Guillemaud, 2010). This information can be useful for management of an invasion, and may be substantially illuminated through molecular diagnostic techniques.

Over the last few decades, the reconstruction of invasion history has become the foundation of many long-term investigations of biological invasions (Dlugosch and Parker, 2008b; Estoup and Guillemaud, 2010; Ficetola et al., 2008; Walker et al., 2003). Humans are the main force facilitating the global spread of invasive species: geographic barriers to dispersal and gene flow are continually being removed by human trade and travel (Cristescu, 2015; Mooney and Cleland, 2001). The relatively recent history of many human-mediated invasions suggests that approaches that adopt DNA profiling can be very useful in serving the goal of reconstructing the most likely invasion scenarios (Cristescu, 2015).

DNA profiling is already an important tool for identifying illegal trades in protected species. Its application to detect crimes against protected species is becoming widespread, with examples including detection of the sources of ivory, tiger bone, mouflon carcasses, and other products (Lorenzini et al., 2011; Ogden et al., 2009; Wasser et al., 2008, 2015; Wetton et al., 2002). DNA profiling has also been successfully applied to the detection of illegal introductions and translocations of invasive or potentially invasive species that could threaten biodiversity (Richardson and Pyšek, 2006) or have huge economic impacts (Kettunen et al., 2008), though such applications have been less common than applications to protected species. It is a goal of this study to advocate expanded application of DNA profiling to the study and management of invasions.

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Applications of DNA profiling to invasions involve special difficulties not encountered in typical applications to protected species; the example applications of this study provide an illustration of how some difficulties can be overcome or circumvented. Human mediated invasions often involve complex routes with multiple sources of introduction and repeated introductions (Wilson et al., 2009). New propagules of invasive species are often noticed only once populations establish and densities become high, sometimes many years after the introductory event (Crooks and Soulé, 2001; Geller et al., 1997). Allelic frequencies of the new population may have started to differentiate from those of the source by that time, making it more challenging to find the source of the new invasive population and to reconstruct its history. Many difficulties associated with the reconstruction of the dispersal history of widespread invasive organisms are primarily related to the availability of an extensive reference database, to minimize errors in results caused by 'ghost populations' which have not been sampled but that acted as sources of new populations (Estoup and Guillemaud, 2010).

The example species used for this study is the American Eastern grey squirrel (*Sciurus carolinensis*), a highly invasive rodent that has been introduced and translocated multiple times in Europe and elsewhere (Bertolino, 2009; Martinoli et al., 2010; Middleton, 1931). In the UK and Ireland, grey squirrels were introduced for the first time in 1876 and 1911 respectively and have since expanded quickly. Through a combination of competition and a disease they carry but do not suffer mortality from, their presence in the UK has resulted in the almost complete replacement of red squirrels (*Sciurus vulgaris*) in much of the red squirrels' original distribution (Gurnell et al. 2004). Restrictions of grey squirrel movements are now the subject of legislation in Europe. The trade of this species among countries within Europe is now regulated by the inclusion of the species in Annex B of European Community Regulation no. 338/97, the European Union Wildlife Trade Regulation that enforces the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES); and by the Regulation 1143/2014 on invasive alien species, which came into effect on 1 January 2015. The species is further regulated by national laws such as the Italian Legislative decree 24/12/2012, aimed at forbidding the importation and trade of grey squirrels and their release into the wild. In the UK, the import, keeping and release of grey squirrels has been prohibited by several national laws since the 1930s (Bosch and Lurz, 2012): the 1932 Destructive Imported Animals Act; the 1937 grey squirrel Prohibition of Importation and Keeping Order; and Section 14 of the Wildlife and Countryside Act of 1981.

We use four case studies to illustrate the potential of DNA profiling for reconstructing invasion histories and to examine how methods might be adapted from conservation applications to the peculiarities of this different context. We defined our tasks as building an extensive genetic database that can reduce the risks of errors from ghost populations, and answering the following questions: 1) What is the accuracy of the available assignment methods and software in the identification of sources of invasions, and what are the best available tools for determining source populations in the invasion context? 2) Can we detect the origins of the specific individuals and populations of interest in the aforementioned case studies, and do the results point to human-mediated releases or to natural expansion? And 3) Can we detect the origin of a population even if the population is sampled for the first time some years after its introduction? By addressing these questions, we assess the potential effectiveness of DNA profiling as a biosecurity tool for understanding and managing invasions.

2. Material and methods

2.1. Case studies

We present four case studies. Case study 1 involves the Isle of Skye (Scotland, UK; henceforth SKYE, all location abbreviations are given on Fig. 1 and Table A.1 in Supplementary material). SKYE is connected to the mainland only by the A87 road bridge. There were no known squirrels, either the Scottish-native red or invasive grey, on the island prior to 2010. In October 2010 a grey squirrel was captured in the town of Breakish, on SKYE. The nearest known grey squirrel populations to Breakish are on the Scottish mainland at Fort William, and on the east side of Loch Ness, both approximately 130 km away. An anonymous resident informed authorities that a grey squirrel stowed away under the bonnet of a car from Glasgow and was released at Breakish. One of the authors (LS) was asked to assess the reliability of this report.

Case study 2 refers to a grey squirrel caught in 2010 in Northumberland (UK), near Wooler, in the Happy Valley (henceforth HAPPY) (Fig. 1), an area where grey squirrels were not previously known. Grey squirrels are now (2015) well established in Northumberland, having colonized the area from adjacent counties in Scotland to the north and from southern Northumberland and Country Durham to the south (Signorile et al., 2014b, 2015). For conservation purposes it would have been important to know the origin of the isolated squirrel to control and minimize grey squirrel spread and to assess the relative likelihoods that the captured squirrel represented natural expansion or a human-mediated translocation.

Case Study 3 illustrates some of the potential differences between applications of DNA profiling methods to new populations, as opposed to isolated individuals. There is an established population of grey squirrels in Aberdeen, Scotland (henceforth AB) (Fig. 1), and the origin, time and mechanism of arrival of the founders of this population are poorly understood (Harris et al., 2006). Bryce (1997), examining Forestry Commission questionnaires assessing red/grey squirrel presence for the period 1980–1994, indicated that the animals arrived in that time range. However, records registered in the National Biodiversity Network's (NBN) Gateway database suggests that the squirrels arrived in the late 1960s. Furthermore, it is not known whether the population in AB was the result of a human-mediated introduction event or, as suggested by Staines (1986), a natural expansion from populations in Tayside, approximately 60 km away. Our aim was to determine the source of this population. In addition to providing a useful simple illustration of differences between conservation and invasion biosecurity, this case study also provides information about invasion history.

Case study 4 is the most complex and interesting of our case studies. In Italy the most important introduction occurred in 1948 when four grey squirrels were introduced near Turin and spread throughout the river Po plain, south of the city. In the last 10 years, 22 new grey squirrel populations have appeared in Lombardy, northern Italy, and one new population has appeared in the central part of the peninsula, in Umbria (Martinoli et al., 2010; Signorile et al., 2014a). The origin of the populations in Lombardy is unclear. The first nucleus was found in the Ticino Valley, in a regional park (Fig. A.2). The Valley is not far from the town of Trecate (Novara, Piedmont) where in 1994 six squirrels purchased from a known pet retailer were released for ornamental purposes. The pet retailer claimed he had bought the squirrels from a Dutch importer (Bertolino et al., 2000). The animals were thought to have been removed from Trecate, but some individuals could already have dispersed to the Ticino Valley before the removal (Bertolino et al., 2000). Other grey squirrel populations in Lombardy can therefore have one or more of the following origins: a natural expansion from the populations in the Ticino Valley; human-mediated introductions from that source; human-mediated introductions from Piedmont, the nearby region where the grey squirrel was first introduced into Italy; or introductions from the native range (North America) or from elsewhere outside Italy. The origin of the population in Umbria, in the peninsular part of Italy, is better known. In 1999 seven squirrels were purchased from an Italian pet trader by a private wildlife park and kept on display outdoors in Perugia (henceforth PG). The following year all the squirrels were declared to have escaped and the resulting population is now slowly spreading (Signorile et al., 2014a). In addition to the Lombardy and Umbria (PG) populations, a single grey squirrel was observed in 2009
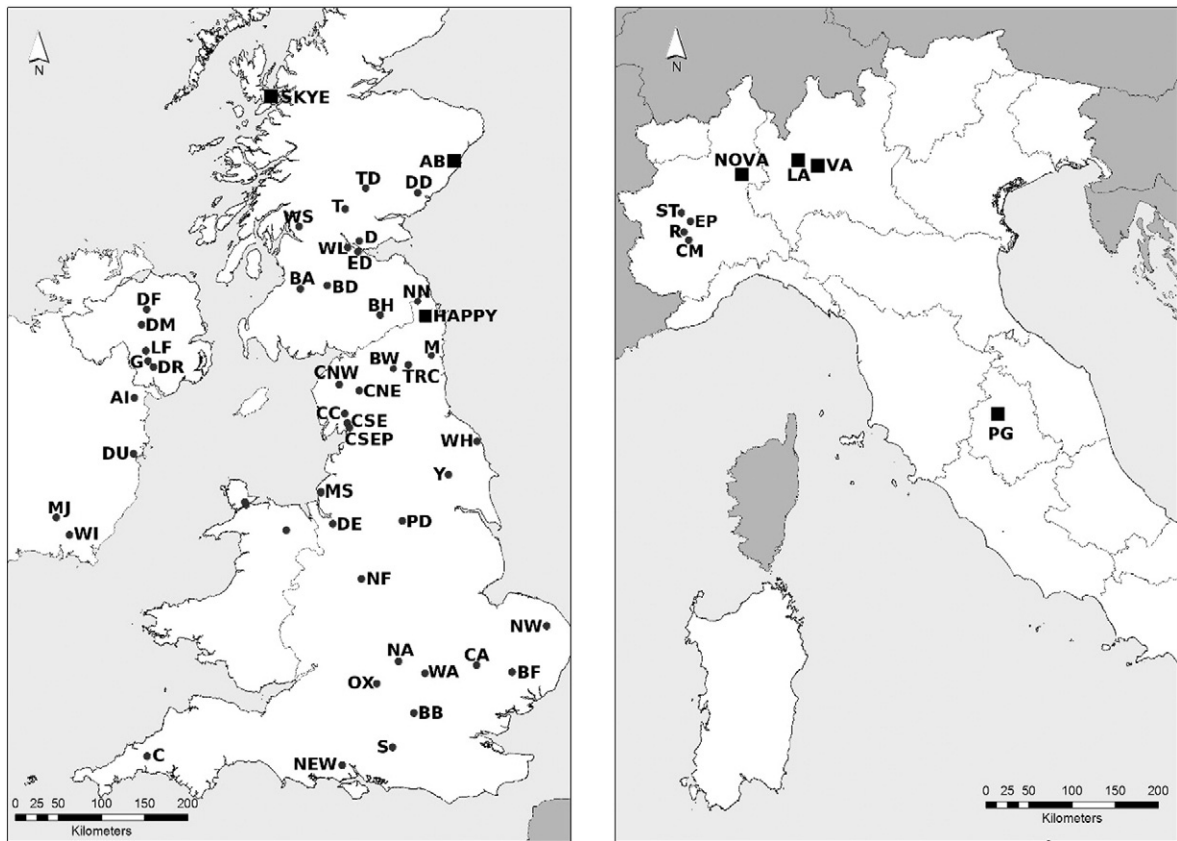
Fig. 1. UK (left) and Italy (right) maps indicating the geographic position of all the sampled eastern grey squirrels forming the database. The position of the single individuals or populations examined for the case studies are indicated by black squares. The position of the population in West Virginia (USA) is not shown, but the latitude and longitude of all populations are given in Table A.1 in Appendices. Abbreviations shown on the map and used in the text correspond to place names provided in Table A.1.

in San Nazzaro Sesia, Novara, Piedmont (Martinoli et al., 2010; henceforth NOVA), a location previously not known to have grey squirrels, 24 km from Trecate and about 70 km from the large population around Turin (Fig. A.2). The origin of this squirrel is unknown.

2.2. Sampling

A total of 1421 individuals were sampled in 60 locations: 13 in Scotland, 28 in England and Wales, nine in the Island of Ireland, nine in Italy, and one in the Eastern US (West Virginia) (Fig. 1, Table A.1). Each location was defined as the same isolated forest or group of forests interlinked with evident corridors. Samples ranged in size from 1 (the isolated squirrels found at SKYE, HAPPY, NOVA) to 56 individuals per location, with a mean of 24.9 ($\pm$10.5) excluding the isolated squirrels. Tissue samples from carcasses were obtained through official control schemes in the UK and Lombardy (Italy) and opportunistic control in Ireland and Umbria (Italy), and were obtained from animals killed as game by a squirrel hunting association in West Virginia. In Piedmont, where there was no official control, samples were collected by live-trapping squirrels and taking a tissue sample from the ear (Signorile et al., 2014b). The two individual squirrels from SKYE and HAPPY and the AB population were used for the UK case studies; and two populations from Lombardy (Parco del Lambro, henceforth LA; and Vaprio d'Adda, henceforth VA), one population from Umbria (PG), and the NOVA individual were used for the Italian case study (Fig. 1).

2.3. Laboratory procedures

DNA was extracted using a QIAGEN DNeasy Blood and Tissue Kit (Quiagen Ltd) or Wizard SV 96 Genomic DNA Purification System (Promega) according to manufacturers' protocols. Microsatellite

primers for *S. carolinensis* are unavailable, but those developed for the phylogenetically related species *S. vulgaris* and *Sciurus niger* proved to be effective for *S. carolinensis*. Twelve independent loci were selected based on their polymorphism and absence of detected genotyping errors such as null alleles or stuttering. The primers used were SCV1, SCV4, SCV6, SCV13, SCV18, SCV31 (Hale et al., 2001), FO11, FO36, FO46, FO54, FO63 (Fike and Rhodes, 2009), and DFS27 (Lance et al., 2003). Squirrel DNA was genotyped with the methods described in Signorile et al. (2014b). To minimize genotyping errors, PCRs were repeated twice and positive and negative controls were added to each plate. The allele scoring was automated and data were cross read and visually double-checked to mitigate human and machine errors.

2.4. Genotyping errors

Deviations from Hardy-Weinberg equilibrium, pairwise linkage disequilibrium, and F-statistics were assessed with FSTAT for Windows (2.9.3.2) (Goudet, 1995) and GENEPOP 4.1 (Raymond and Rousset, 1995). Significance levels for multiple comparisons were adjusted for type I errors with Bonferroni corrections with nominal significance 5%. Observed and expected heterozygosity was assessed with the Excel Microsatellite Toolkit (Park, 2001). Evidence for large allelic drop-out, null alleles or stuttering was assessed with MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

2.5. Assignment tests

The aim of assignment methods is to assign or exclude reference populations as the origin of tested individuals, given the allele frequencies of the reference populations and the genotypes of the tested individuals at a number of loci (Piry et al., 2004). Available methods were

reviewed by several authors (Cornuet et al., 1999; Manel et al., 2005; Latch et al., 2006; Hauser et al., 2006; Alacs et al., 2010; Linacre and Tobe, 2013). Two partially Bayesian statistical methods were selected for the assignment tests in this study. They are implemented in the software GENECLASS (Piry et al., 2004), developed for genetic assignments and detection of first-generation migrants, and Oncor, developed to estimate mixture proportions and to make population assignments (Kalinowski et al., 2007). Both methods derive from the method originally described by Rannala and Mountain (1997). According to Cornuet et al. (1999), Bayesian methods are more powerful than frequency-based methods, which are also implemented in GENECLASS2 but were not used here for assignments.

Manel et al. (2002) argued that for wildlife assignment tests the fully Bayesian clustering method implemented in STRUCTURE (Pritchard et al., 2000) performs better than partial Bayesian assignment methods, though requiring the assumption that the true population of origin was sampled (Bonizzoni et al., 2001; Estoup and Guillemaud, 2010; Frantz et al., 2006). It was not known if the true population of origin had been sampled, so we used both STRUCTURE and partial Bayesian (GENECLASS2, Oncor) methods, as recommended by Manel et al. (2002) in such cases. Hauser et al. (2006) found small differences between STRUCTURE and the clustering method implemented in BAPS (Corander et al., 2004). We use STRUCTURE rather than BAPS because it has been used in conservation applications of DNA forensics (Lorenzini et al., 2011) and in litigation (Glover, 2010).

Both Oncor and GENECLASS2 were evaluated for their ability to correctly assign a multi-locus genotype to its population of origin. Both programmes can be made to automatically test their own performance on a dataset by sequentially removing each multi-locus genotype from the data and then estimating its origin using the remaining individuals as reference (this is a "leave-one-out" analysis). However, in this evaluation, Oncor ignores those multi-locus genotypes with missing data, and GENECLASS2 assigns them to a population. For a fair test, therefore, individuals with missing data were removed from the database prior to the leave-one-out tests with both software packages. A further test was performed by assigning each individual to a population without first removing its genotype from the database (called a "self-assignment test"). Both software packages can be made to automatically assign all individuals to populations in this latter test, regardless of missing data. For both tests, accuracy was in the form of the number of correctly assigned individuals on a per-population basis and overall. The method with the higher percentage of correct assignments is considered better for application to our dataset.

Cluster analysis with STRUCTURE 2.3.4 was conducted with a burn-in period of 50,000 followed by a chain of length 100,000. An admixture model with correlated allelic frequencies was chosen, with 5 replicates for each value of the number of clusters, K. The method of Evanno et al. (2005) was used to infer the most likely K value in a dataset. STRUCTURE HARVESTER (Earl and VonHoldt, 2011) was used to estimate the optimal K and to visualize STRUCTURE's output.

With GENECLASS2 we used the exclusion technique of Paetkau et al. (2004) based on Monte Carlo resampling of 10,000 simulated individuals for each candidate population. A threshold was set to 0.001 to decide if each individual genotype originated from a population in the database. Prior to assignment of a population the corresponding genotypes were removed from the database.

3. Results

3.1. Data quality control and assignment methods tests

All the assessed populations were tested for Hardy–Weinberg and linkage disequilibrium which could bias estimates of the probability of origin. Although some linkage and Hardy–Weinberg disequilibria were detected for some loci in some populations of the database, possibly due to sampling errors (See Supplementary material, Appendix A.1

for details), analysis with MICRO-CHECKER did not reveal any significant genotyping errors, so all markers were kept. Self-assignment tests showed that both Oncor and GENECLASS2 performed better when all loci were used than they did when some were omitted. Therefore, we ignored these small deviations and choose to use all loci in our case studies.

In tests of assigning single genotypes to populations of origin, Oncor performed better than GeneClass2 for our data. In the leave-one-out test done after removing incomplete genotypes, 79.6% of individuals were correctly assigned by Oncor, whereas only 55.6% were correctly assigned by GeneClass2 (Table A.2). Oncor also performed better for self-assignment tests. Oncor correctly assigned 91.31% of individuals versus 81.31% for GENECLASS2 (Table A.2). Given these results, GENECLASS2 was not further used in case studies.

Cluster analysis was performed on 57 populations of the database, excluding the three individuals from SKYE, HAPPY and NOVA. The results show a complex genetic structure. The optimal K value was estimated to be 3 by STRUCTURE HARVESTER. STRUCTURE partitions the database in three main clusters corresponding to Scotland, the rest of the British Islands (England, Wales and Ireland), and Italy (Fig. 2). However, the absolute maximal posterior probability (mean LnP(K) across the five repeats) indicates a complex nested structure of at least 15 clusters (Fig. A.1). This nested structure was confirmed by conducting a STRUCTURE analysis of the populations in each of the 3 main clusters identified above.

3.2. Case study 1: the Isle of Skye

Case study 1 results support the anonymous report that the squirrel came from Glasgow. Oncor results show that the multi-locus genotype of the examined individual matches a population sampled 40 km south-east of Glasgow (BD) with a 98.5% probability (Table 1). The second best probability (1.5%) was awarded to a phylogenetically related population in West Lothian (WL), also close to Glasgow. Our database includes populations from areas neighbouring Glasgow, but does not include squirrels sampled directly from the city. A finer-scale STRUCTURE analysis of 10 neighbouring populations from Scotland and North England confirms the Oncor results (Fig. 3): the SKYE individual clusters in the same group with populations BD, WL, and BA, the latter being sampled about 40 km south of the city. The membership proportion in that cluster was 68.5% for the SKYE individual, and was on average 70.4%, 35.1%, and 74% for BD, WL and BA individuals respectively. These results support an origin of the SKYE individual in the Glasgow area but not an origin in the squirrel population in our database nearest to SKYE (WS, 2.1% membership proportion).

3.3. Case study 2: Northumberland

Results for Case Study 2 strongly support the conclusion that the HAPPY squirrel was translocated, and is not a migrant from a nearby population. Oncor attributes the HAPPY squirrel to population WL with 96% probability. The population with the second best probability is BD, at 4% (Table 1). These populations are the same ones implicated in the SKYE squirrel, are over 100 km distant from HAPPY, and are not among the closest populations to HAPPY in our database (Fig. 1). The results are confirmed by a cluster analysis performed using 12 neighbouring populations from Scotland and North England: the maximal posterior probability (mean LnP(K) across the five repeats) indicates a partition into five clusters. The genotype of the HAPPY squirrel has the highest membership proportion in the same cluster as squirrels from populations BD, BA and WL (Fig. 3; see also Fig. A.1), and the Isle of Skye squirrel. In this cluster, the HAPPY squirrel has a membership proportion 71.7%, and WL, BD and BA have average membership proportions 83.5%, 87.5%, and 71.7% respectively. In contrast, the northern population nearest to HAPPY, NN, has a membership proportion of only 1.3%, and M, the nearest population to the south of HAPPY, has a
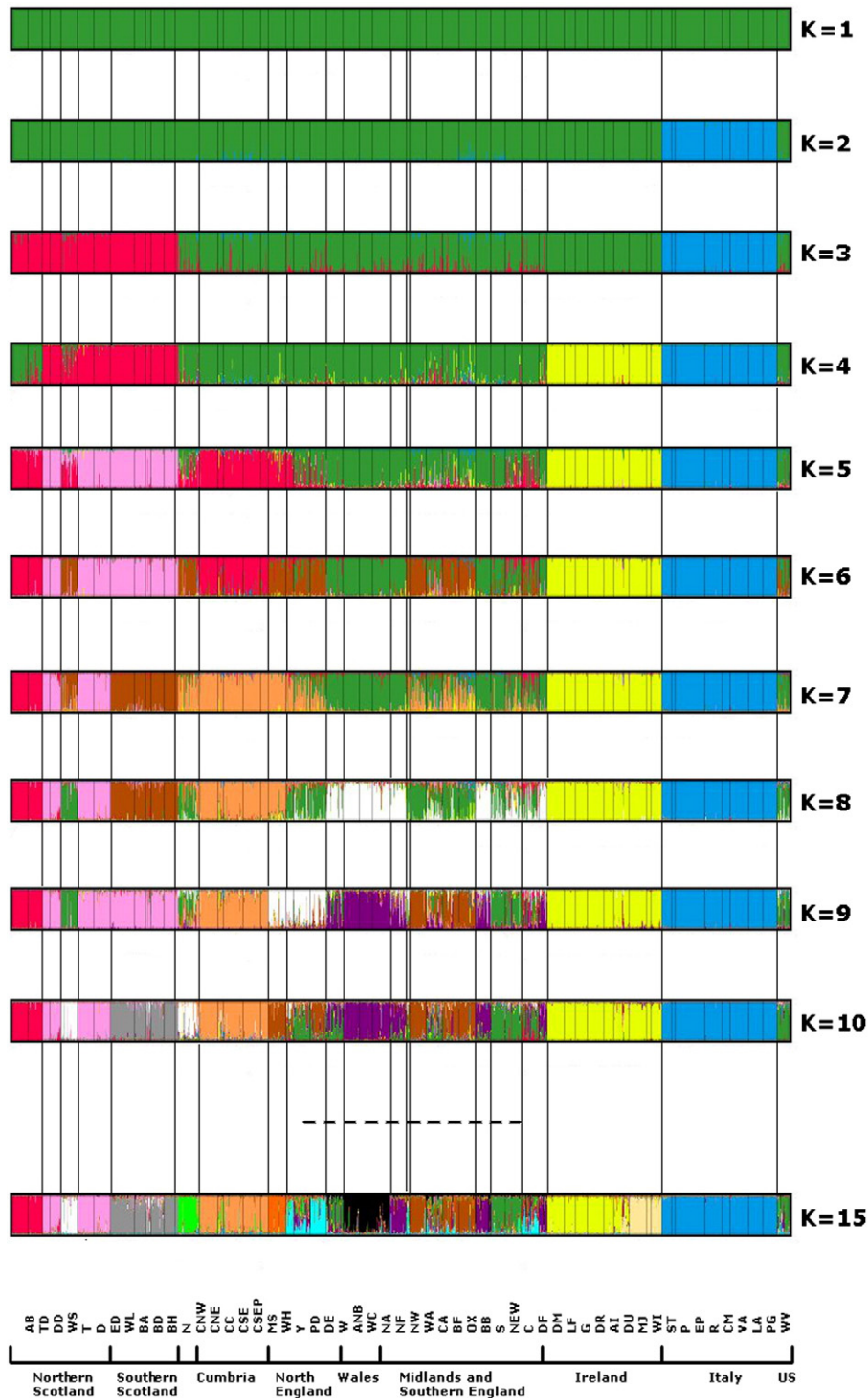
Fig. 2. Cluster analysis via STRUCTURE of the grey squirrel database.

membership proportion of 1.1%. The HAPPY squirrel was therefore very unlikely to have arrived in Northumberland from any of the closest populations to the north or south.

3.4. Case study 3: Aberdeen

Results for Case Study 3 strongly support the conclusion that the AB population was a result of a human-mediated translocation. This case study differs from the previous two and from classical applications of DNA profiling in conservation, since a large sample of 56 squirrels from a population, rather than one individual, is examined for source

of origin, and since the AB population was founded decades ago. Oncor assigned 64.3% of the sampled individuals (36 individuals) to a population sampled in the New Forest, Hampshire (NEW), in southern England (Table 1), with an average assignment probability of 93.7 ± 12.4%. Only three individuals assigned to NEW had a probability lower than 80% and in all three cases the second highest probability was with the nearby population of S in Surrey, also in southern England. Seven squirrels (12.5% of the samples) were attributed to S with an average probability of 79.2 ± 16.0%. NEW was the second most likely source population in six of these cases. Ten squirrels (17.9%) were attributed to a population from the north-western coast of Wales

Table 1
Assignment results with Oncor. For SKYE, HAPPY and NOVA assignment probabilities for individual samples are reported. For AB, VA, LA, and PG the number of squirrels assigned to each database population are reported. Database populations for which assignment results were always zero are not shown.

Location	Pop	SKYE	HAPPY	NOVA	AB	VA	LA	PG
Edinburgh	ED				1			
West Lothian	WL	0.0147	0.9602		1			
Auchinleck	BA							
Douglas	BD	0.9853	0.0398					
Wales Coast	WC				10			
Northampton	NA				1			
Alice Holt	S				7			
New Forest	NEW				36			
Stupinigi	ST			0.0002		16		
East River Po	EP			0.9998		17	23	20
Vaprio d'Adda	VA						3	5
Lambro	LA					1		

(WC) with an average probability of 80.9 ± 18.1% (Table A.3). Of the remaining three individuals two were assigned to the southern coast of the river Forth in Scotland (populations WL and ED) and one to population NA in the Midlands. However, the assignment probabilities are low in all of these three cases, so the sources of the three squirrels are uncertain (Table A.3).

Cluster analysis supports the conclusion that AB squirrels were originally introduced from a source near NEW. Accepting the finer nested structure containing K = 15 clusters (Fig. A.1), the AB population is partitioned into a single cluster (94.9% membership proportion). The only other population partially sharing the same cluster is NEW (Fig. 3), which shares a 13.7% membership proportion with the AB cluster. After the passage of a few decades since introduction, the allelic frequencies at AB may have changed considerably due to genetic drift and founder effects, producing this partial sharing of a cluster with NEW. Alternatively multiple source populations or a recent undetected introduction from a source population not included in our database, such as a US population, could be an explanation for the origin of AB, though the latter would then make it difficult to explain clustering of AB with NEW in our STRUCTURE results.

3.5. Case study 4: Italy

In Case Study 4, the NOVA individual and the LA, PG and VA populations were all clearly introduced, from the Piedmont region, and probably came from close to the EP population. Oncor attributed the NOVA individual to EP with 99.98% probability (Table 1). Out of 26 individuals from LA, 23 were attributed to EP with an average probability of 98.3 ± 6.3%. The remaining three squirrels were attributed to VA with probability 95.7 ± 6.6% (Table A.3). These three animals could also have come from EP, because VA itself mainly comes from EP (see below). Most of the individuals from PG were attributed to EP: 80% of the squirrels were assigned to EP with an average probability of 98.0 ± 6.0%. The five remaining squirrels were assigned to VA with average probability 91.1 ± 5.1% (Table A.3). These 5 individuals may again also have come from EP, since this population is one of the possible sources of VA. The evaluation of the origin of VA is more complex. Oncor attributed 17 individuals to EP, 16 to ST and one to LA (Table 1) with average probabilities of 88.5 ± 14.2, 79.1 ± 15.2, and 87.6 respectively (Table A.3). The individual attributed to LA may have been a migrant, since LA is close to VA. The difficulty in clearly assigning the VA population to ST or EP might be due to an admixed population or to not having sampled the true population of origin, which may have been geographically and genetically placed between ST and EP. However, ST and EP are close, and regardless of the exact location, the origin of the VA population somewhere in the Turin plane is highly likely: when populations from Piedmont are merged in the dataset, all the examined individuals from VA were assigned to Piedmont with 99.0 ± 3.1% probability.

STRUCTURE (Fig. A.3) and F_{ST} (Table 2) results support the conclusion that the VA, LA, and PG populations came from introductions of squirrels captured from Piedmont in three independent capture events, and also further support the conclusion that these populations share the same or similar origin and are not new introductions from outside Italy. From their level of differentiation (Table 2) the three examined populations do not appear to be closely related, so it can be hypothesised that they came from three independent captures from the Piedmont region. STRUCTURE separates the populations from each other (Fig. A.3) when just the Italian populations are examined, further supporting independent captures. However, when all populations are examined (Fig. 2), all Italian populations cluster together and separate from all other populations for all values of K greater than 1. On balance, all three events establishing the VA, LA and PG populations were probably translocations, which are unlawful in Italy, from independent Piedmont captures; drift and bottleneck effects changed allelic frequencies so that the populations cluster separately in a finer-scaled STRUCTURE analysis.

4. Discussion

The results of this study highlight how methods based on genetic markers can have useful practical applications to the integrated management of invasive species. Our results show that translocations that are often undetected or considered to be natural expansion processes can indeed be identified as human-mediated releases. Our results indicate a previously under-estimated anthropogenic role in the observed invasion success of grey squirrels which may apply to other species. Furthermore, testing the source of new populations can help detect illegal translocations and trades. The ecological and economic costs of invasive species are substantial (Kettunen et al., 2008; Lizarralde et al., 2007; Lockwood et al., 2005; Manchester and Bullock, 2000; Pimentel, 2011; Pimentel et al., 2005). Translocations can contribute greatly to invasive species spread (Dlugosch and Parker, 2008a; Kolbe et al., 2004); and if populations are structured, as for grey squirrels in Europe (Signorile et al., 2015) translocations can also contribute to the genetic merging of populations, and hence to increased local genetic diversity, reduced inbreeding, and increased population fitness (Signorile et al., 2014b). However, control measures are usually oriented toward reducing numbers of individuals rather than preventing translocations. Efforts to detect and prosecute illegal translocations have been limited or non-existent so far. Our results suggest that individual or population attributions using standardized genetic databases can be a useful tool to detect long-jump dispersal events. It remains to be seen, however, whether such information can lead to prosecutions and ultimately be a deterrent to illegal translocations in future.

Our results therefore have potential implications for biosecurity and the management of invasive species, generally, as well as specific implications for grey squirrels in Europe. Genetic methods to determine the sources of invasive species can be applied to any invasive species and there is an extensive literature on this subject (Cristescu, 2015; Dlugosch and Parker, 2008a; Estoup and Guillemaud, 2010; Guillemaud et al., 2010; Lombaert et al., 2014; Rius et al., 2014), though such application of the methods has been insufficiently utilized in practical management of invasions. Limited attention has been paid to the underlying mechanisms involved in the invasion process and to the prevention of human-mediated dispersal of ornamental species. For invaders subject to management that are also at risk of human translocation, including mammals, birds and reptiles regarded as attractive or ornamental, culling programmes should be linked and resources should be allocated to genotyping efforts to establish databases for DNA profiling, an essential pre-requisite to analyse the genetics of invasion processes. Populations and individuals in new areas could then be periodically tested to assess their origin, possibly revealing unexpected release pathways which could then be mitigated. Similar methods have been successfully applied in assessing the origin of an invading alga in the Mediterranean (Papini et al., 2013) or on the invasive brown tree
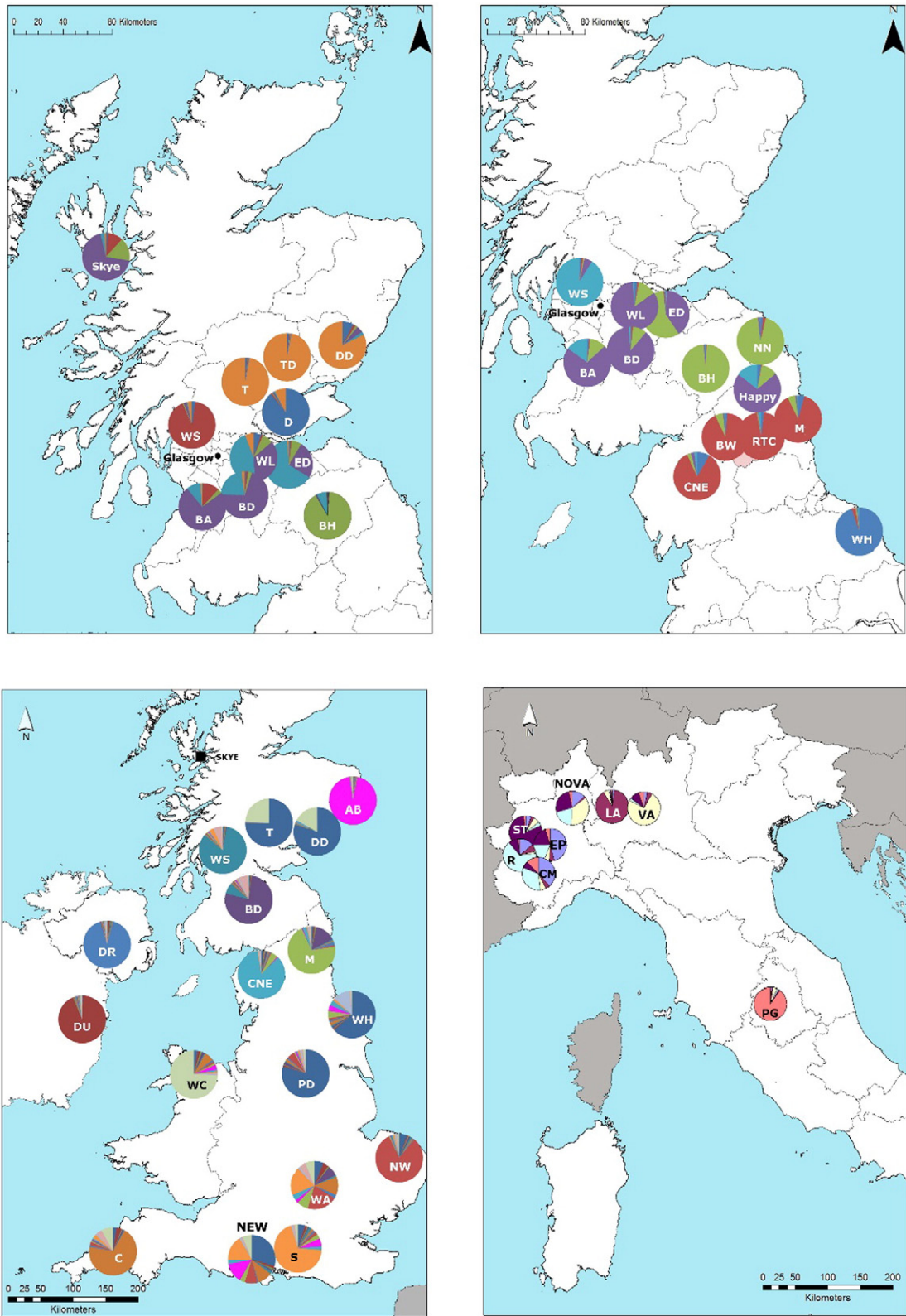
Fig. 3. Cluster distributions showing the proportion of membership of each pre-defined population for the Skye squirrel (K = 6, top left), the Happy Valley squirrel (K = 5, top right), and the Aberdeen (K = 15, bottom left) and Italy (K = 6, bottom right) populations.

snake (Richmond et al., 2014). Gaining an understanding of the origins of translocations may also allow us to target public education programmes to problem areas thereby increasing their effectiveness in helping to reduce translocations. Our SKYE and HAPPY results indicate

potential opportunities for mitigating translocations mediated by trucks from Glasgow to surrounding areas.

Several types of genetic marker for individual or population identification are available, e.g. mtDNA sequences, nuclear single nucleotide

Table 2

F_{ST} values indicating the genetic differentiation between the Italian populations. Stars indicate F_{ST} values different from zero at the p = 5% significance level. ST = Stupinigi.

Location	Pop	ST	EP	R	CM	VA	LA	PG
East River Po	EP	0.0719*						
Racconigi	R	0.1187*	0.0575*					
Cavallermaggiore	CM	0.1483*	0.038 ns	0.0517*				
Vaprio d'Adda	VA	0.1161*	0.1205*	0.1648*	0.1999*			
Lambro	LA	0.1887*	0.1304*	0.1874*	0.2432*	0.1686*		
Perugia	PG	0.2144*	0.1655*	0.2377*	0.2270*	0.2548*	0.3241*	
West Virginia	WV	0.1670*	0.1593*	0.2020*	0.1844*	0.2135*	0.2829*	0.2574*

polymorphisms (SNPs), and microsatellites (Alacs et al., 2010; Jobling and Gill, 2004; LeRoux and Wieczorek, 2009). Although microsatellite methods are an ageing technology, they are still the most frequently used genetic tool in wildlife trade enforcement applications, provided there are available primers and databases (Alacs et al., 2010). Microsatellites are probably also still the most appropriate tool for applications to invasions. Microsatellites are still more cost effective than genomic tools, having a lower overall cost and a vast choice of user-friendly applications for their analysis (Estoup and Guillemaud, 2010; Ogden et al., 2009). This is an important advantage in the invasion management field as managers may have limited expertise in genetic analyses. As costs of genomic methods decline, other techniques may supersede microsatellites, particularly in light of their greater capacity for comparison of measurements taken by different researchers, but current factors favour microsatellites for invasion applications.

Although our results highlight the promise of DNA profiling in helping manage invasions, there are both logistical and methodological challenges that complicate this application; our work has helped address some of these challenges, but they will need to be further investigated in future work. Logistical difficulties should be straightforward to overcome. Target species, especially recent invaders, might be little studied, so genetic markers may be unavailable at first. Also, mangers may initially view genetic studies and the building of a genetic database as expensive and as a lower priority compared to culling, a well-accepted method. But these challenges should be more easily addressed for invasion applications than they were in the original conservation applications, because culling programmes make it relatively easy to obtain genetic material. And logistical difficulties have been overcome in conservation applications.

Methodological challenges unique to invasion applications of DNA profiling arise when one seeks to identify the origin of a new population instead of a single individual, because this often involves multiple sources of introduction or repeated introductions from the same source. These challenges, and the degree of certainty that will be required for litigation and translocation-prevention applications, suggest there is opportunity for further methods development. In addition to sampling errors and genotyping errors which also affect conservation applications, possibly admixed new populations and genetic drift complicate identification of population founder origins. Genetic drift can alter allele frequencies compared to the origin population (Hauser et al., 2006; Manel et al., 2002), as probably occurred in the Aberdeen case study. This applies in particular when the number of founders is small, the effective population size is low, and the introduction event was far in the past. The problem can be mitigated by genotyping new populations and doing comparisons with a baseline database immediately when new populations are noticed. The simultaneous use of clustering methods (e.g., STRUCTURE) and partial Bayesian methods (e.g., Oncor) can increase the reliability of the assignment, or at least help exclude some locations as possible origins. But optimizations of methods to the invasion application could likely also help, and standard use of genetic methodologies in biosecurity, especially when concerning illegal translocations, will require standardization of methods specific to the invasion application and the establishment of specific protocols, tasks remaining for future work.

Appendix A. Supplementary data

Microsatellite genotypes: Dryad DOI: http://dx.doi.org/10.5061/dryad.md6m0/1. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.biocon.2015.12.026.

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