

ORIGINAL ARTICLE

Preventing species invasion: A role for integrative taxonomy?

Maria V. MAZZAMUTO,¹ Andrea GALIMBERTI,² Giacomo CREMONESI,³ Benoît PISANU,⁴ Jean-Louis CHAPUIS,⁴ Jan STUYCK,⁵ Giovanni AMORI,⁶ Haijun SU,^{1,7} Gaetano ALOISE,⁸ Damiano G. PREATONI,¹ Lucas A. WAUTERS,¹ Maurizio CASIRAGHI² and Adriano MARTINOLI¹

¹Environment Analysis and Management Unit -Guido Tosi Research Group, Department of Theoretical and Applied Sciences, University of Insubria, Varese, Italy, ²ZooPlantLab, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy, ³Department of Biology and Biotechnology “Charles Darwin,” Sapienza, Rome University, Rome, Italy, ⁴Centre of Ecology and Conservation Sciences, Sorbonne University, MNHN, CNRS, UPMC, National Museum of Natural History, Paris, France, ⁵Research Institute for Nature and Forestry, Geraardsbergen, Belgium, ⁶CNR - Institute of Ecosystem Studies, Department of Biology and Biotechnology “Charles Darwin,” Sapienza, Rome University, Rome, Italy, ⁷Forestry College, Guizhou University, Guiyang, Guizhou, China and ⁸University of Calabria, Museum of Natural History of Calabria and Botanical Garden, Arcavacata di Rende (CS), Italy

Abstract

Integrative taxonomy, a multi-disciplinary approach adding modern techniques to traditional morphology-based methods (e.g. molecular 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. The present 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 (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 share the same haplotypes and skull characteristics, but are conspicuously different from the French population in Antibes. Genetic data revealed close similarity between French squirrels and Pallas’s squirrels, *Callosciurus erythraeus*, from Taiwan, China. 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 2 populations are, however, similar to known specimens assigned to *Callosciurus 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.

Key words: *Callosciurus*, *CoxI*, D-loop, invasive species, morphometric analysis

Correspondence: Maria Vittoria Mazzamuto, Environment Analysis and Management Unit, Guido Tosi Research Group, Department of Theoretical and Applied Sciences, University of Insubria, Via J. H. Dunant, 3 - I-21100 Varese, Italy.
Email: maria.mazzamuto@uninsubria.it

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 & Cock 2001; Bertolino & Genovesi 2003; Bertolino & 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. Species are not unequivocally defined and their designations based on a single category of taxonomic features (e.g. morphology) can be questionable, even in mammals (Shoshani & McKenna 1998; Wilson & Reeder 2005). This places the discipline of taxonomy at the forefront of invasive species research. Molecular techniques, and more recently DNA barcoding, have triggered a small revolution inside taxonomy: the process of identifying biological entities has opened the doors to a real integration of knowledge to improve practical or theoretical methods (Galimberti *et al.* 2015).

Integrative taxonomy is a multi-disciplinary approach to traditional taxonomy where the morphological features are combined with other approaches and additional data (e.g. molecular, behavioural, developmental and ecological) (Dayrat 2005; Flagella *et al.* 2010; Padial *et al.* 2010; Wu *et al.* 2011). Nowadays, many studies in the field of bioinvasion are conducted following an integrative approach where molecular markers and morphological features are 2 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, or to increase hunting opportunities (Aprile & Chicco 1999;

Long 2003). 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). Over the past decade a new alien invasive tree squirrel of the genus *Callosciurus*, native to Southeast 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 & Lurz 2013). A few *Callosciurus* species are well known to damage infrastructure and to debark forest trees and orchards (Noor 1992; Bertolino *et al.* 2004; Tamura & 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 spillover (Wauters *et al.* 2002; Gurnell *et al.* 2004; Romeo *et al.* 2013, 2014).

Recent works have highlighted the growing importance of DNA barcoding in clarifying the taxonomic status and provenance of sciurid populations (see e.g. Gabrielli *et al.* 2014; Ermakov *et al.* 2015; Stevenson-Holt & Sinclair 2015). These studies also provide 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 & Reeder 2005), the 2 species most commonly introduced worldwide are Finlayson's squirrel, *Callosciurus finlaysonii* (Horsfield, 1823), native to Cambodia, Lao, Myanmar, Thailand and Vietnam and Pallas's squirrel, *Callosciurus erythraeus* (Pallas, 1778), native to southeast China, eastern India, Malaya, Indochina, Bhutan and Taiwan, China (Corbet & Hill 1992; Oshida *et al.* 2001; Wilson & 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, China and Japan (Bertolino & 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 & 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 & 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 & Reeder 2005). According to this approach, 18 subspecies of *C. erythraeus* were previously assigned to a distinct species (i.e. *Callosciurus flavimanus*) (Ellerman & Morrison-Scott 1951; Moore & Tate 1965; Corbet & Hill 1992), whereas recent molecular analysis suggested that the subspecies *Callosciurus erythraeus 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 & Thorpe 2004; Galimberti *et al.* 2012b).

In 2014, Gabrielli and co-workers carried out a genetic characterization of the 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 3 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; and (iii) assess the provenance of European *Callosciurus*. Morphometric analysis (skull measurements) and a molecular investigation (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.

MATERIAL 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

Table 1 Specimens whose cranial measurements were included in principal component analysis

Species	Origin of specimens	M	F	Un	<i>n</i>
<i>Callosciurus caniceps</i> (Gray, 1842)	“Giacomo Doria” Natural History Museum	9	9	8	26
<i>Callosciurus erythraeus</i> (Pallas, 1778)	“Giacomo Doria” Natural History Museum	2	2	8	12
<i>Callosciurus melanogaster</i> (Thomas, 1895)	“Giacomo Doria” Natural History Museum	—	—	9	9
<i>Callosciurus nigrovittatus</i> (Horsfield, 1824)	“Giacomo Doria” Natural History Museum	11	8	2	21
<i>Callosciurus notatus</i> (Boddaert, 1785)	“Giacomo Doria” Natural History Museum	7	8	7	22
<i>Callosciurus phayrei</i> (Blyth, 1856)	“Giacomo Doria” Natural History Museum	12	16	13	41
<i>Callosciurus prevostii</i> (Desmarest, 1822)	“Giacomo Doria” Natural History Museum	—	1	1	2
<i>Callosciurus pygerythrus</i> (I. Geoffroy Saint Hilaire, 1833)	“Giacomo Doria” Natural History Museum	—	—	2	2
<i>Callosciurus finlaysonii</i> (Horsfield, 1823)	“Giacomo Doria” Natural History Museum	—	—	2	
	Potenza, Italy	3	8	—	14
	Natural History Museum, Milan	1	—	—	
<i>Callosciurus</i> sp.	Dadizele, Belgium	11	14	—	25
<i>Callosciurus</i> sp.	Antibes, France	7	8	—	15
<i>Callosciurus</i> sp.	Varese, Italy	7	7	—	14
Total		70	81	52	203

F, female; M, male; *n*, number of specimens; Un, sex unknown; —, N/A.

collected in Asia and kept at the “Giacomo 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 & Bertolino 2005; Bertolino & Lurz 2013).

In Italy and Belgium, the introduced squirrels were captured using Tomahawk live-traps (model 202, Tomahawk Live Trap, 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 and milk excretion if stimulated) being immediately released. All males and non-reproductive females were killed by CO₂ inhalation following EC and AVMA guidelines (Close *et al.* 1996, 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 No. 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; see Table 2 for abbreviations). 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 (PCA [Flury 1997]) using the stats R package (Venables & Ripley 2002; R Devel-

opment Core Team 2014). Standardization and scaling were carried out automatically by the princomp R function.

A first PCA took into account the whole dataset, 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.

Because most of the measurements were highly autocorrelated, we performed a second PCA using a “reduced” dataset; that is, considering only the variables with the highest scores and those that were most morphologically sensible. In this case, the variables used were: condilobasal length (Cbl), zygomatic breadth (Zgb), palatal length (Pl) and 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).

Table 2 List of measurements and their abbreviations

Fb	Foramen magnum breadth
Cbl	Condilobasal length
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

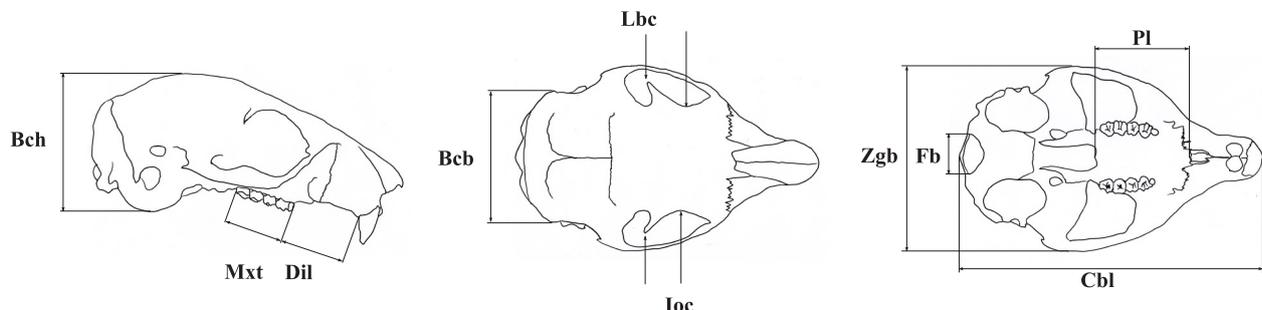


Figure 1 Skull measurements used in this study. Abbreviations are also explained in Table 2

Molecular analysis

Sampling, DNA extraction, amplification and sequencing

A total of 17 tissue samples were collected from the 3 introduced *Callosciurus* *cf.* *erythraeus* populations in Europe: 5 from Brezzo di Bedero, Varese Province, Italy (45°58'09.2"N, 08°43'57.6"E), 6 from Dاذezele, Moorsele Province, Belgium (50°51'05.5"N, 03°05'40.3"E) and 6 from Antibes, Alpes-Maritimes District, France (43°33'51.0"N, 07°07'27.0"E). The sampling dataset also includes 2 samples of *C. erythraeus* from Yanyuan and Muli Counties, Sichuan Province, China (27°38'23.7"N, 101°48'43.8"E and 28°9'23.3"N, 100°48'43.8"E, respectively) and 3 samples of *C. finlaysonii* from the 2 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 Table S1). To date, no DNA barcoding sequences are available for *C. finlaysonii*; thus, we decided to include this *Callosciurus* species in our molecular dataset.

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 Table S1.

DNA was extracted from a 3-mm (diameter) tissue punch by using the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) following the 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. cf.* *erythraeus* populations was conducted by analyzing sequence differences at 2 mitochondrial DNA markers and comparing this data with sequences already available in GenBank (see Table S1). 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 e.g. Galimberti *et al.* 2012a; Ermakov *et al.* 2015) and because a huge number of D-loop sequences are available for the species group treated in this study

(Gabrielli *et al.* 2014). Moreover, the 2 markers typically show high mutation rates, which is essential to better identify 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 the 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 the 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, 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 Table S1 for accession numbers). Bioinformatics analyses were conducted separately on the 2 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. cf.* *erythraeus* obtained in the 3 European naturalized populations, 2 samples of *C. erythraeus* from its native range in China, and 3 samples of Italian *C. finlaysonii*. We also used 33 publicly available sequences of the genus *Callosciurus*, including accessions of *C. erythraeus* from native (PRC) and introduced range (Argentina), *Callosciurus notatus*, *Callosciurus orestes* and *Callosciurus prevostii*. The D-loop dataset encompassed *Callosciurus* sequences obtained in this study and 99 Genbank sequences of *C. erythraeus* belonging to native (Mainland and Taiwan, China) 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 & Rozas 2009).

The taxonomic status of *C. cfr. erythraeus* was also tested by comparing obtained *CoxI* data with 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 the ABGD tool (Automatic Barcode Gap Discovery, available at <http://www.wabi.snv.jussieu.fr/public/abgd/abgdweb.html>; Puillandre *et al.* 2012). This software aims at finding the genetic distance at which the barcode gap occurs and groups sequence 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 the Kimura distance model.

For both datasets, average genetic sequence divergences (and relative standard errors, SE) between and within distinct lineages were calculated and an 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 sufficient to resolve relationships at terminal branches (see e.g. Hebert *et al.* 2004; Kerr *et al.* 2009).

RESULTS

Morphometric analysis

The fur color of all museum specimens was analyzed to confirm species identification (Amori & Doria, unpubl. data; Moore & Tate 1965; Corbet & Hill 1992; Thorington & Hoffmann 2005; Lurz *et al.* 2013). *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 (Table S2). Based on Moore and Tate (1965), Corbet and Hill (1992) and Thorington and Hoffmann (2005), all the specimens from Europe could

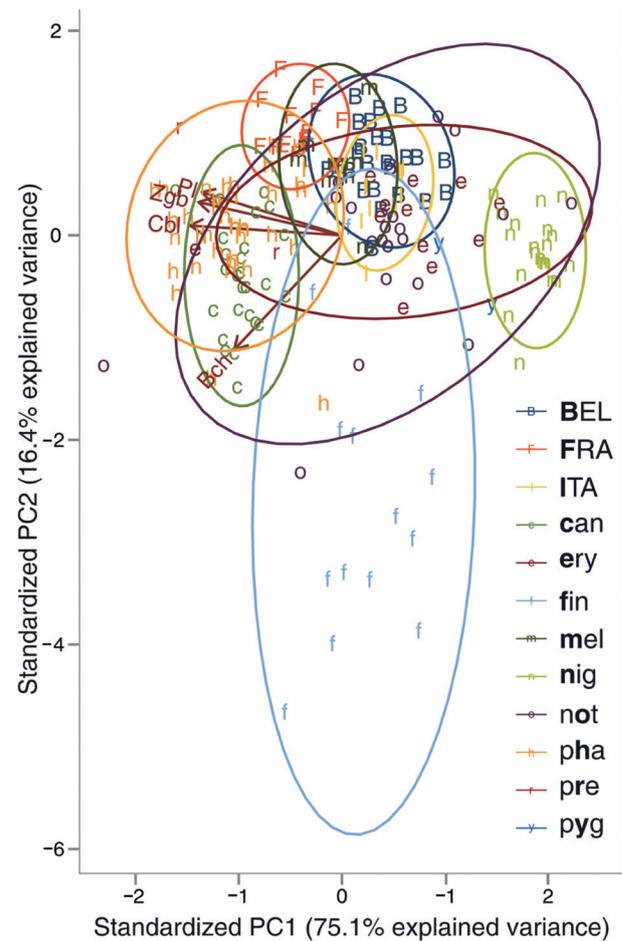


Figure 2 Principal component analysis biplot for the first 2 canonical axes (cumulative variance explained: 91.5%). Ellipses are 95% confidence intervals for each species or alien population. In the legend lowercase abbreviations indicate species names; uppercase abbreviations indicate the 3 introduced populations. BEL, Belgium; FRA, France; ITA, Italy; can, *Callosciurus caniceps*; ery, *Callosciurus erythraeus*; fin, *Callosciurus finlaysonii*; mel, *Callosciurus melanogaster*; nig, *Callosciurus nigrovittatus*; not, *Callosciurus notatus*; pha, *Callosciurus phayrei*; pre, *Callosciurus prevostii*; pyg, *Callosciurus pygerythrus*.

be included in the species *C. erythraeus*. According to pelage characteristics, the French population could be included in 3 different subspecies: *C. e. erythrogaster* (Blyth, 1842: from India and Burma), *C. e. castaneiventris* (Gray, 1842: from China) or *C. e. thaiwanensis* (Bonhote, 1901: from Taiwan, China). 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 Tables S2 and S3.

In the PCA performed on skull measurements the first PCA axis (PC1) explained 75.1% of the variance. The first principal component is correlated with 3 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. Figure 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. In contrast, *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* and *C. phayrei* than with *C. erythraeus*.

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 showing a strong chromatogram signal along the entire read 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 $\pi_A = 25.2$, $\pi_C = 27.1$, $\pi_G = 16.8$ and $\pi_T = 30.9$ %. For D-loop, the multiple alignment showed 7 indel positions, which were concentrated in the variable 5'-end variable domain, and 184 variable sites, of which 155 were parsimony-informative. 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. Table S4 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*.

For *C. cfr. erythraeus* European populations, which have been investigated for the first time in this study, 2 different haplotypes were found at each marker. Interestingly, 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 showed 2 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 being the *C. finlaysonii* individual from Acqui Terme (Northwest Italy), which is identical at D-loop to a squirrel from Thailand (see Table S1). 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 (Table S4).

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 3 groups with prior values of 0.036 and 0.060. The primary partition was stable on the whole range of prior values and the 10 groups were coherent with the lineages identified by the NJ approach (see Fig. 3). Considering these lineages as putative different taxonomic units, the mean \pm SE of K2P distance between groups (Table S5) was 12.6% \pm 5.9 % (range: 3%–19.9%). The French *C. cfr. erythraeus* population was closer to the group encompassing Belgian and Italian squirrels (4.2% \pm 0.8 %), and the nearest neighbour of both groups being represented by *C. erythraeus* from Hainan Province of China (4.8% \pm 0.9 % and 5.0% \pm 0.9 %). Interestingly, the lineage including the 2 *C. erythraeus* from Sichuan (China) sequenced in this study was closer to the *C. finlaysonii* group (3.5% \pm 0.7 %), whereas its K2P distance to the other 2 lineages belonging to Chinese *C. erythraeus* was 2 times higher (see Table S5 and Fig. 3).

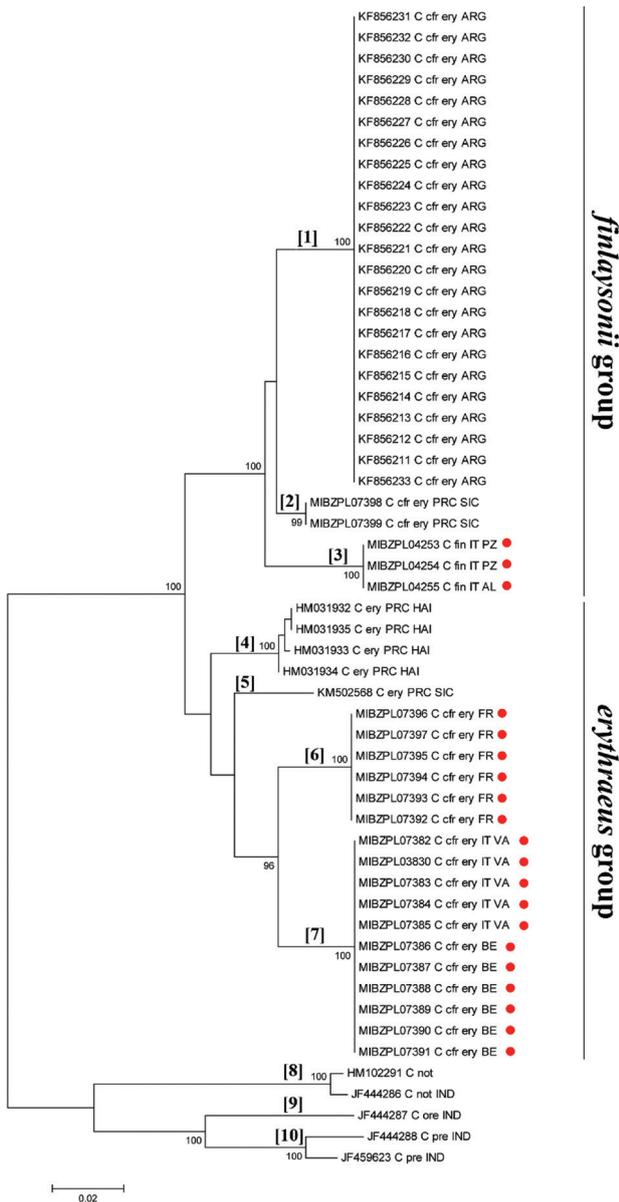


Figure 3 Neighbour joining tree based on *CoxI* sequences of *Callosciurus* generated with MEGA. Numbers in square brackets indicate the different lineages resulting from the ABGD analysis. For each squirrel, voucher number and sampling locality are also provided (further details can be retrieved from Table S1). Bootstrap support (1000 replicates) values >90% are indicated above the nodes. *Callosciurus* species names are abbreviated as follows: ery, *erythraeus*; fin, *finlaysonii*; not, *notatus*; ore, *orestes*; pre, *prevostii*. The countries where the different lineages occur are abbreviated as follows: ARG, Argentina; BE, Belgium; FR, France; IT, Italy; PRC, People's Republic of China. European samples newly sequenced in this study are marked with red dots.

Genetic structure at D-loop

Different from the case of *CoxI*, the higher number of D-loop sequences available in Genbank permitted a better definition of the relationships among different species (and geographic populations) of *Callosciurus* squirrels.

Both NJ reconstruction (see Fig. 4) and the K2P genetic distance matrix (Table S6) confirmed the marked difference among European populations of *C. cfr. erythraeus* (K2P distance = $11.4\% \pm 1.4\%$ Italy and Belgium vs France). Although the mixed Italian and Belgian lineage remained still taxonomically uncharacterized with Taiwan, China populations being their nearest neighbor (K2P distance = $12.0\% \pm 1.34\%$), French *Callosciurus* resulted even closer to *C. erythraeus* from Taiwan, China (K2P distance = $3.6\% \pm 0.6\%$). Once again, the lineage including the 2 Chinese samples of *C. erythraeus* from Sichuan analyzed in this study (*C_ery_PRC_II*, see Table S6 and Fig. 4), was quite similar to *C. finlaysonii* (K2P distance = $8.8\% \pm 1.0\%$) and even more to the invasive population of Argentinean squirrels (K2P distance = $8.4\% \pm 1.2\%$). In contrast, they are highly divergent from other *C. erythraeus* (*C_ery_PRC_I*, see Table S6 and Fig.4) sampled in the same province (K2P distance = $14.0\% \pm 1.9\%$).

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 3 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 3 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.

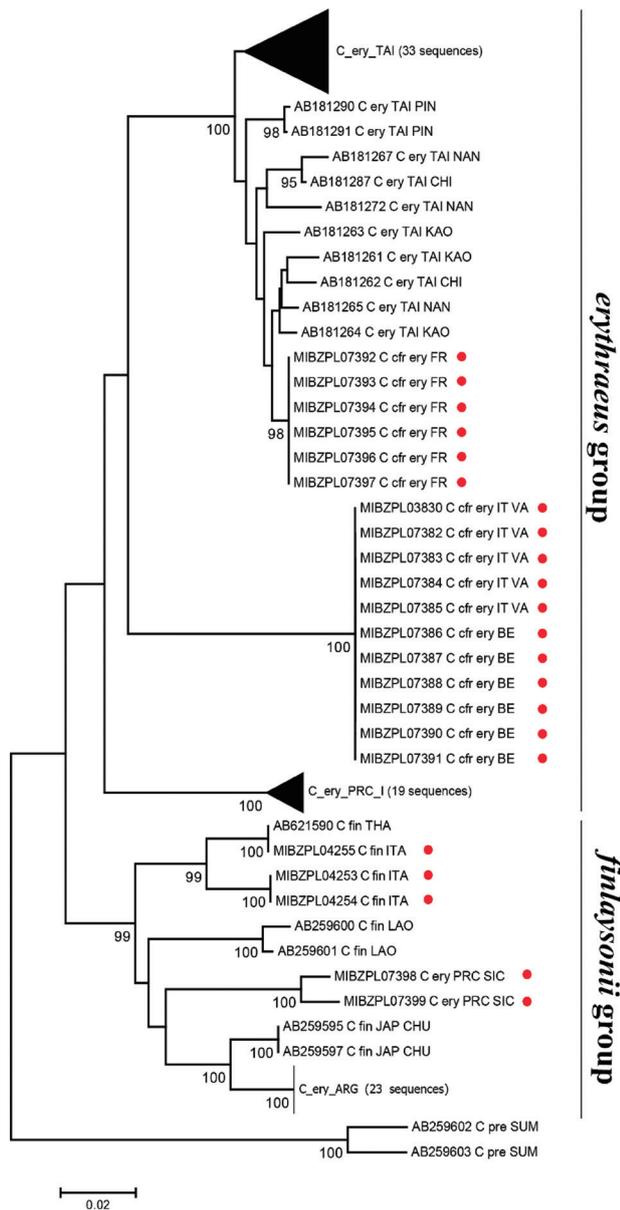


Figure 4 Neighbour joining tree based on D-loop sequences of *Callosciurus* generated with MEGA. For each squirrel, voucher number and sampling locality are also provided (further details can be retrieved from Table S1). Bootstrap support (1000 replicates) values >90% are indicated above the nodes. *Callosciurus* species names are abbreviated as follows: ery, *erythraeus*; fin, *finlaysonii*; pre, *prevostii*. The countries where the different lineages and species occur are abbreviated as follows: ARG, Argentina; BE, Belgium; FR, France; IT, Italy; JAP, Japan; LAO, Laos; PRC, Mainland of China; TAI, Taiwan, China; THA, Thailand. European samples newly sequenced in this study are marked with red dots.

Morphometric data supported such a molecular framework: skull size measures of the Italian and Belgian groups were totally overlapped, while the French group was clearly separated from the Italian group and overlapped little with the specimens from Belgium. Moreover, squirrels from Italy and Belgium had the same fur colour, 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 2 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 2 MOTUs formed 2 new independent lineages never described before, whereas the French one was included into the MOTUs of *C. erythraeus* from Taiwan, China in the D-loop dataset (Figs 3,4). The K2P distance matrix and the comparison with the identification thresholds for sciurids 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.

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 2 morphologically recognized *C. erythraeus* collected in Sichuan Province (China). 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 the native *C. erythraeus* distribution range

is likely to better characterize the taxonomic status of Belgian and Italian populations. Finally, the analysis of D-loop sequences also revealed a certain degree of variation between the 2 Italian populations of *C. finlaysonii* (K2P divergence of $3.5\% \pm 0.8\%$) 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 and fur color patterns) from Belgian and Italian *Callosciurus* (Moore & Tate 1965), thus confidently suggesting the latter be classified 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 colour and body size (Moore & 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 the founder effect mostly referable to captive-bred stocks used for pet trade as also inferred from the low haplotype diversity at the analyzed DNA markers (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 the hypothesis of 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, this area, 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. In contrast, as revealed by D-loop data and fur description, French *Callosciurus* probably originated from an independent introduction event of squirrels from Taiwan, China to

France. Even though no document 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 & Pisanu, pers. comm.).

In conclusion, combining morphological data and sequence analysis at 2 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 Taiwan, China 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 detailed information on their taxonomic status (i.e. genus, species and even subspecies if possible), provenance and the dynamics of introduction before taking action 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 should they take preventive direct actions towards an entire genus, or even the entire Sciuridae family? Based on our results, we recommend that the legislation prohibiting the introduction of squirrels in European countries should provide taxonomic lists at a higher level than the species level. It is clear that governments and international bodies should increase the attention they pay to pet trade. In any case, the application of integrative approaches appears to be ever more urgent and will allow us to obtain more and faster information that is useful for the management of the new invasive alien species.

ACKNOWLEDGMENTS

We thank the Province of Varese for cooperation, the Director Dr. Giuliano Doria of the “Giacomo Doria” Natural History Museum of Genoa and Dr. Michela Podestà of the Natural History Museum of Milan. Special thanks to Mattia Panzeri, Marina Morandini, Giovanni Zardoni, Olivier Gerret and all the students involved in the survey. This work was achieved under the LIFE09

NAT/IT/000095 EC-SQUARE Project. This is Paper 9 of the ECSQUARE project.

REFERENCES

- Adriaens T, Baert K, Breyne P *et al.* (2015). Successful eradication of a suburban Pallas's squirrel *Callosciurus erythraeus* (Pallas 1779) (Rodentia, Sciuridae) population in Flanders (northern Belgium). *Biological Invasions* **17**, 2517–26.
- Aloise G, Bertolino S (2005). Free-ranging population of the Finlayson's squirrel *Callosciurus finlaysonii* (Horsfield, 1824) (Rodentia, Sciuridae) in South Italy. *Hystrix, the Italian Journal of Mammalogy* **16**, 70–4.
- Amori G, Doria G. *Catalogue of Sciuridae (Mammalia, Rodentia) in the Museum of Genoa*, Annali Museo Civico Storia Naturale Genova eds, Genoa, unpublished.
- Aprile G, Chicco D (1999). Nueva especie exotica de mamifero en la Argentina: la ardilla de vientre rojo. *Mastozoología Neotropical* **6**, 7–14.
- Ashton EH, Zuckerman S (1950). The influence of geographic isolation on the skull of the green monkey (*Cercopithecus aethiops sabaeus*). I. A comparison between the teeth of the St Kitts and the African green monkey. *Proceedings of the Royal Society of London B: Biological Sciences* **137**, 212–38.
- Bellati A, Tiberti R, Cocca W *et al.* (2014). A dark shell hiding great variability: A molecular insight into the evolution and conservation of melanic *Daphnia* populations in the Alps. *Zoological Journal of the Linnean Society* **171**, 697–715.
- Bensasson D, Zhang D-X, Hartl DL, Hewitt GM (2001). Mitochondrial pseudogenes: Evolution's misplaced witnesses. *Trends in Ecology & Evolution* **16**, 314–21.
- Bertolino S (2009). Animal trade and non-indigenous species introduction: The world-wide spread of squirrels. *Diversity and Distributions* **15**, 701–8.
- Bertolino S, Genovesi P (2003). Spread and attempted eradication of the grey squirrel (*Sciurus carolinensis*) in Italy, and consequences for the red squirrel (*Sciurus vulgaris*) in Eurasia. *Biological Conservation* **109**, 351–8.
- Bertolino S, Lurz PWW (2013). *Callosciurus* squirrels: Worldwide introductions, ecological impacts and recommendations to prevent the establishment of new invasive populations. *Mammal Review* **43**, 22–33.
- Bertolino S, Mazzoglio PJ, Vaiana M, Currado I (2004). Activity budget and foraging behavior of introduced *Callosciurus finlaysonii* (Rodentia, Sciuridae) in Italy. *Journal of Mammalogy* **85**, 254–9.
- Boykin LM, Armstrong KF, Kubatko L, De Barro P (2011). Species delimitation and global biosecurity. *Evolutionary Bioinformatics Online* **8**, 1–37.
- Buhay JE (2009). “COI-like” sequences are becoming problematic in molecular systematic and DNA barcoding studies. *Journal of Crustacean Biology* **29**, 96–110.
- Cassini GH, Guichón ML (2009). Variaciones morfológicas y diagnosis de la ardilla de vientre rojo, *Callosciurus erythraeus* (Pallas, 1779), en Argentina. *Mastozoología Neotropical* **16**, 39–47.
- Chang S-W, Oshida T, Endo H *et al.* (2011). Ancient hybridization and underestimated species diversity in Asian striped squirrels (genus *Tamiops*): inference from paternal, maternal and biparental markers. *Journal of Zoology* **285**, 128–38.
- Close B, Banister K, Baumans V *et al.* (1996). Recommendations for euthanasia of experimental animals: Part 1. *Laboratory Animals* **30**, 293–316.
- Close B, Banister K, Baumans V *et al.* (1997). Recommendations for euthanasia of experimental animals: Part 2. *Laboratory Animals* **31**, 1–32.
- Corbet GB, Hill JE (1992). *The Mammals of the Indomalayan Region: A Systematic Review*. Oxford University Press, Oxford.
- Dayrat B (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society* **85**, 407–15.
- Dlugosch KM, Parker IM (2008). Founding events in species invasions: Genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology* **17**, 431–49.
- Edgar RC (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–7.
- Ehrensfield JG (2010). Ecosystem consequences of biological invasions. *Annual Review of Ecology, Evolution, and Systematics* **41**, 59–80.
- Ellerman JR, Morrison-Scott TCS (1951). *Checklist of Palaearctic and Indian mammals, 1758 to 1946*. Trustees of the British Museum (Natural History), London, United Kingdom.
- Ermakov OA, Simonov E, Surin VL *et al.* (2015). Implications of hybridization, NUMTs, and overlooked diversity for DNA barcoding of Eurasian ground squirrels. *PLoS ONE* **10**, e0117201.

- Flagella MM, Andreakis N, Hiraoka M *et al.* (2010). Identification of cryptic *Ulva* species (Chlorophyta, Ulvales) transported by ballast water. *Journal of Biological Research* **13**, 47–57.
- Floyd R, Abebe E, Papert A, Blaxter M (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology* **11**, 839–50.
- Flury B (1997). *A First Course in Multivariate Statistics*. Springer Science & Business Media, New York, USA.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**, 294–9.
- Gabrielli M, Cardoso YP, Benitez V, Gozzi AC, Guichón ML, Lizarralde MS (2014). Genetic characterization of *Callosciurus* (Rodentia: Sciuridae) Asiatic squirrels introduced in Argentina. *Italian Journal of Zoology* **81**, 328–43.
- Galimberti A, Romano DF, Genchi M *et al.* (2012a). Integrative taxonomy at work: DNA barcoding of taenids harboured by wild and domestic cats. *Molecular Ecology Resources* **12**, 403–13.
- Galimberti A, Spada M, Russo D *et al.* (2012b). Integrated operational taxonomic units (IOTUs) in echolocating bats: A bridge between molecular and traditional taxonomy. *PLoS ONE* **7**, e40122.
- Galimberti A, Sandionigi A, Bruno A, Bellati A, Casiraghi M (2015). DNA barcoding in mammals: What's new and where next? *Hystrix, The Italian Journal of Mammalogy* **26**, 13–24.
- Gippoliti S, Cotterill FPD, Groves CP (2013). Mammal taxonomy without taxonomists: A reply to Zachos and Lovari. *Hystrix, the Italian Journal of Mammalogy* **24**, 145–7.
- Gotzek D, Brady SG, Kallal RJ, LaPolla JS (2012). The importance of using multiple approaches for identifying emerging invasive species: The case of the raspberry crazy ant in the United States. *PLoS ONE* **7**, e45314.
- Grosholz ED (2005). Recent biological invasion may hasten invasional meltdown by accelerating historical introductions. *PNAS* **102**, 1088–91.
- Guichón ML, Doncaster PC (2008). Invasion dynamics of an introduced squirrel in Argentina. *Ecography* **31**, 211–20.
- Gurnell J, Wauters LA, Lurz PWW, Tosi G (2004). Alien species and interspecific competition: effects of introduced eastern grey squirrels on red squirrel population dynamics. *Journal of Animal Ecology* **73**, 26–35.
- Hale ML, Bevan R, Wolff K (2001). New polymorphic microsatellite markers for the red squirrel (*Sciurus vulgaris*) and their applicability to the grey squirrel (*S. carolinensis*). *Molecular Ecology Notes* **1**, 47–9.
- Hall TA (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: *Nucleic Acids Symposium Series*, Vol. 41. Oxford University Press, Oxford, UK, pp. 95–8.
- Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B* **270**, 313–21.
- Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *PNAS* **101**, 14812–7.
- Hori M, Yamada M, Tsunoda N (2006). Line census and gnawing damage of introduced Formosan squirrels (*Callosciurus erythraeus taiwanensis*) in urban forests of Kamakura, Kanagawa, Japan. In: Koike F, Clout MN, Kawamichi M, De Poorter M, Iwatsuki K, eds. *Assessment and Control of Biological Invasion Risks*. Shoukadoh Book Sellers, Kyoto, Japan and IUCN, Gland, Switzerland, pp. 204–9.
- Hu L, Peng R, Zou F (2016). Complete mitochondrial genome of the Pallas's squirrel *Callosciurus erythraeus* (Rodentia: Sciuridae). *Mitochondrial DNA* **27**, 1850–1.
- Kerr KC, Birks SM, Kalyakin MV, Red'kin YA, Koblik EA, Hebert PD (2009). Filling the gap - COI barcode resolution in eastern Palearctic birds. *Frontiers in Zoology* **6**, 29.
- Kuramoto T, Torii H, Ikeda H, Endo H, Rerkamnuaychoke W, Oshida T (2012). Mitochondria DNA Sequences of Finlayson's Squirrel Found in Hamamatsu, Shizuoka Prefecture, Japan. *Mammal Study* **37**, 63–7.
- Larsen K (2001). Morphological and molecular investigation of polymorphism and cryptic species in tanaid crustaceans: Implications for tanaid systematics and biodiversity estimates. *Zoological Journal of the Linnean Society* **131**, 353–79.

- Leary S, Underwood W, Anthony R *et al.* (2013). *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition*, American Veterinary Medical Association, Schaumburg, USA.
- Librado P, Rozas J (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–2.
- Long JL (2003). *Introduced Mammals of the World. Their History, Distribution and Influence*. CSIRO Publishing, Wallingford, UK.
- Lurz PWW, Hayssen V, Geissler K, Bertolino S (2013). *Callosciurus erythraeus* (Rodentia: Sciuridae). *Mammalian Species* **902**, 60–74.
- Mack RN, Simberloff D, Mark Lonsdale W, Evans H, Clout M, Bazzaz FA (2000). Biotic invasions: Causes, epidemiology, global consequences, and control. *Ecological Applications* **10**, 689–710.
- Malhotra A, Thorpe RS (2004). Maximizing information in systematic revisions: A combined molecular and morphological analysis of a cryptic green pitviper complex (*Trimeresurus stejnegeri*). *Biological Journal of the Linnean Society* **82**, 219–35.
- Martinoli A, Bertolino S, Preatoni DG *et al.* (2010). Headcount 2010: The multiplication of the grey squirrel populations introduced to Italy. *Hystrix, the Italian Journal of Mammalogy* **21**, 127–36.
- Mazzamuto MV, Panzeri M, Wauters AL, Preatoni DG, Martinoli A (2015). Knowledge, management and optimization: The use of live traps in control of non-native squirrels. *Mammalia* on-line first.
- Miyamoto A, Tamura N, Sugimura K, Yamada F (2004). Predicting habitat distribution of the alien Formosan squirrel using logistic regression model. *Global environmental research* **8**, 13–22.
- Moore JC, Tate GHH (1965). A study of the diurnal squirrels: Sciurinae, of the Indian and Indochinese subregions. *Fieldiana Zoology* **48**, 1–351.
- Nei M (1987). *Molecular Evolutionary Genetics*. Columbia University Press, New York.
- Nesi N, Nakouné E, Cruaud C, Hassanin A (2011). DNA barcoding of African fruit bats (Mammalia, Pteropodidae). The mitochondrial genome does not provide a reliable discrimination between *Epomophorus gambianus* and *Micropteropus pusillus*. *Comptes Rendus Biologies* **334**, 544–54.
- Noor HM (1992). Some notes on bark stripping behaviour of *Callosciurus netatus*. *Planter*. Available from URL: <http://agris.fao.org/agris-search/search.do?recordID=MY19930025163>
- Oshida T, Yasuda M, Endo H, Hussein NA, Masuda R (2001). Molecular phylogeny of five squirrel species of the genus *Callosciurus* (Mammalia, Rodentia) inferred from cytochrome b gene sequences. *Mammalia* **65**, 473–82.
- Oshida T, Lee J-K, Lin L-K, Chen Y-J (2006). Phylogeography of Pallas's squirrel in Taiwan: geographical isolation in an arboreal small mammal. *Journal of Mammalogy*, **87** 247–54.
- Oshida T, Torii H, Lin L-K *et al.* (2007). A preliminary study on origin of *Callosciurus* squirrels introduced into Japan. *Mammal Study* **32**, 75–82.
- Oshida T, Dang CN, Nguyen ST *et al.* (2013). Phylogenetic position of *Callosciurus erythraeus griseimanus* from Vietnam in the Genus *Callosciurus*. *Mammal Study* **38**, 41–7.
- Padial JM, Miralles A, De la Riva I, Vences M (2010). Review: The integrative future of taxonomy. *Frontiers in Zoology* **7**, 1–14.
- Palmer GH, Koprowski J, Pernas T (2007). Tree squirrels as invasive species: Conservation and management implications. *Managing Vertebrate Invasive Species*, paper 36.
- Pisanu B, Obolenskaya EV, Baudry E, Lissovsky AA, Chapuis J-L (2013). Narrow phylogeographic origin of five introduced populations of the Siberian chipmunk *Tamias (Eutamias) sibiricus* (Laxmann, 1769) (Rodentia: Sciuridae) established in France. *Biological Invasions* **15**, 1201–7.
- Puillandre N, Lambert A, Brouillet S, Achaz G (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21**, 1864–77.
- R Development Core Team (2014). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Romeo C, Pisanu B, Ferrari N *et al.* (2013). Macroparasite community of the Eurasian red squirrel (*Sciurus vulgaris*): poor species richness and diversity. *Parasitology Research* **112**, 3527–36.
- Romeo C, Wauters LA, Ferrari N *et al.* (2014). Macroparasite fauna of alien grey squirrels (*Sciurus carolinensis*): Composition, variability and implications for native species (ed. Russo D). *PLoS ONE* **9**, e88002.
- Shoshani J, McKenna MC (1998). Higher taxonomic relationships among extant mammals based on morphology, with selected comparisons of results from

- molecular data. *Molecular Phylogenetics and Evolution* **9**, 572–84.
- Signorile AL, Wang J, Lurz PWW, Bertolino S, Carbone C, Reuman DC (2014). Do founder size, genetic diversity and structure influence rates of expansion of North American grey squirrels in Europe? *Diversity and Distributions* **20**, 918–30.
- Song H, Buhay JE, Whiting MF, Crandall KA (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences* **105**, 13486–91.
- Stevenson-Holt CD, Sinclair W (2015). Assessing the geographic origin of the invasive grey squirrel using DNA sequencing: Implications for management strategies. *Global Ecology and Conservation* **3**, 20–7.
- Strayer DL (2012). Eight questions about invasions and ecosystem functioning. *Ecology Letters* **15**, 1199–210.
- Stuyck J, Baert K, Breyne P, Adriaens T (2009). Invasion history and control of a Pallas squirrel *Callosciurus erythraeus* population in Dadizele, Belgium. In: *Proceedings of the Science Facing Aliens Conference Brussels*. Belgian Biodiversity Platform, Brussels, Belgium, p. 46.
- Tamura N (2009). The *Callosciurus erythraeus* (Pallas's squirrel) datasheet. *Invasive Species Compendium*. [<http://www.cabi.org/isc/datasheet/91200>]
- Tamura N, Ohara S (2005). Chemical components of hardwood barks stripped by the alien squirrel *Callosciurus erythraeus* in Japan. *Journal of Forest Research* **10**, 429–33.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–9.
- Thorington R Jr, Hoffmann RS (2005). Family Sciuridae. In: *Mammal Species of the World: A Taxonomic and Geographic Reference*, 3rd edn, Johns Hopkins University Press, Baltimore, Maryland, pp. 754–818
- Thorington RWJ, Koprowski JL, Steele MA, Wharton JF (2012). *Squirrels of the World*. Johns Hopkins University Press, Baltimore, Maryland 472 pp.
- Venables WN, Ripley BD (2002). *Modern Applied Statistics with S*. Springer, New York.
- Vu V (2011). *ggbiplot: A ggplot2 based biplot*. R package version 0.55. Available from URL: <http://www.vince.vu/software/>
- Wandeler P, Hoeck PEA, Keller LF (2007). Back to the future: Museum specimens in population genetics. *Trends in Ecology & Evolution* **22**, 634–42.
- Wauters L, Dhondt AA (1989). Body Weight, longevity and reproductive success in red squirrels (*Sciurus vulgaris*). *The Journal of Animal Ecology* **58**, 637.
- Wauters L, Gurnell J, Martinoli A, Tosi G (2002). Interspecific competition between native Eurasian red squirrels and alien grey squirrels: Does resource partitioning occur? *Behavioral Ecology and Sociobiology* **52**, 332–41.
- Wickham H (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer, New York.
- Wilson DE, Reeder DM (2005). *Mammal Species of the World: A Taxonomic and Geographic Reference*. Johns Hopkins University Press, Baltimore, USA.
- Wittenberg R, Cock MJW (2001). *Invasive Alien Species: A Toolkit of Best Prevention and Management Practices*. CABI, 242 pp.
- Wu L-W, Liu C-C, Lin S-M (2011). Identification of exotic sailfin catfish species (*Pterygoplichthys*, Loricariidae) in Taiwan based on morphology and mtDNA sequences. *Zoological Studies* **50**, 235–46.
- Yom-Tov Y, Yom-Tov S, Moller H (1999). Competition, coexistence, and adaptation amongst rodent invaders to Pacific and New Zealand islands. *Journal of Biogeography* **26**, 947–58.
- Zachos FE, Lovari S (2013). Taxonomic inflation and the poverty of the phylogenetic species concept – A reply to Gippoliti and Groves. *Hystrix, the Italian Journal of Mammalogy* **24**, 142–4.

SUPPLEMENTARY MATERIALS

Table S1 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 information (i.e. accession number and haplotype (H) for the 2 mitochondrial markers) are reported

Table S2 Description of the main fur characteristics of the 3 European populations of *Callosciurus*

Table S3 Skull measurements for each species and for the 3 European populations of *Callosciurus* sp. For measurements and abbreviations see Fig. 1 and Table 2

Table S4 Haplotype characteristics: Number of haplotypes and nucleotide diversity values in the *Callosciurus* European and Asiatic populations and in the 2 main species analyzed in this study

Table S5 Within and between species genetic divergence values (%) of *CoxI* sequences, based on the K2P

distance calculation (bottom left), for the taxa belonging to the genus *Callosciurus* considered in this study

Table S6 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

Cite this article as:

Mazzamuto M, Galimberti A, Cremonesi G *et al.* (2016). Preventing species invasion: A role for integrative taxonomy? *Integrative Zoology* **11**, 214–28.