

Preventing species invasion: a role for integrative taxonomy?

MAZZAMUTO MARIA VITTORIA¹, GALIMBERTI ANDREA², CREMONESI GIACOMO³, PISANU BENOÎT⁴,
CHAPUIS JEAN-LOUIS⁴, STUYCK JAN⁵, AMORI GIOVANNI⁶, SU HAI-JUN^{1,7}, ALOISE GAETANO⁸,
PREATONI DAMIANO G.¹, WAUTERS LUCAS A.¹, CASIRAGHI MAURIZIO², MARTINOLI ADRIANO¹

1-

Unità di Analisi e Gestione delle Risorse Ambientali - *Guido Tosi Research Group*

Dipartimento di Scienze Teoriche e Applicate

Università degli Studi dell'Insubria

Via J. H. Dunant, 3 - I-21100 Varese (Italy)

Maria Vittoria Mazzamuto: maria.mazzamuto@uninsubria.it +390332421538 (corresponding author)

Damiano Preatoni: damiano.preatoni@uninsubria.it

Lucas A. Wauters: lucas.wauters@uninsubria.it

Adriano Martinoli: adriano.martinoli@uninsunria.it

2-

ZooPlantLab

Dipartimento di Biotecnologie e Bioscienze

Università degli Studi di Milano-Bicocca

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Piazza della Scienza 2 - 20126 Milano (Italy)

Andrea Galimberti: andrea.galimberti@unimib.it

Maurizio Casiraghi: maurizio.casiraghi@unimib.it

3-

Department of Biology and Biotechnology "Charles Darwin"

Sapienza - Rome University

Viale dell'Università 32

00185 Roma (Italy)

Giacomo Cremonesi: cremonesi.giacomo@gmail.com

4-

Centre d'Ecologie et des Sciences de la Conservation

UMR 7204, Sorbonne Universités, MNHN, CNRS, UPMC,

Muséum National d'Histoire Naturelle

61 rue Buffon,

CP n°53, 75231 PARIS cedex 05 (France)

Benoît Pisanu: esnm2@mnhn.fr

Jean-Louis Chapuis: chapuis@mnhn.fr

5-

Instituut voor Natuur- en Bosonderzoek (INBO)

Research Institute for Nature and Forestry

Gaverstraat 4

B 9500 Geraardsbergen (Belgium)

This article is protected by copyright. All rights reserved.

Jan Stuyck: jan.stuyck@inbo.be

6-

CNR - Institute of Ecosystem Studies

c/o Department of Biology and Biotechnology "Charles Darwin"

Sapienza - Rome University

Viale dell'Università 32

00185 Roma (Italy)

giovanni.amori@uniroma1.it

7-

Forestry College

Guizhou University,

Huaxi District, Guiyang City,

Guizhou Province, China

haijun_su@163.com

8-

Università della Calabria,

Museo di Storia Naturale della Calabria e Orto Botanico

Via Savinio – Edificio Polifunzionale

87030 Arcavacata di Rende (CS), Italy

aloise@unical.it

RUNNING TITLE: Integrative taxonomy to manage invasive species.

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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. This study is the first on introduced populations of *Callosciurus*, Asiatic tree squirrels, known as potentially invasive species in Europe (Italy, Belgium and France). We combined molecular (mitochondrial DNA markers: *CoxI*, D-loop) and morphometric analysis on skulls, comparing them to the widest morphological and molecular datasets ever assembled for *Callosciurus*. Squirrels collected in Italy and Belgium shared the same haplotypes and skull characteristics, but are conspicuously different from the French population in Antibes. Genetic data revealed close similarity between French squirrels and Pallas's squirrels, *C. erythraeus*, from Taiwan. Italian and Belgian squirrels formed an independent taxonomic lineage in genetic analyses, whose taxonomic rank needs further investigation. The morphological and morphometric characteristics of these two populations are however similar to known specimens assigned to *C. erythraeus*. These results may indicate a common origin for the populations found in Belgium and Italy. In contrast, French specimens suggest an independent introduction event of squirrels originating from Asia.

ADDITIONAL KEYWORDS: Invasive species – *Callosciurus*– morphometric analysis – D-loop – *CoxI*

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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. As a matter of fact, species are not unequivocally defined and their designations based on a single category of taxonomic features (e.g., morphology) could be questionable also in mammals (Shoshani & McKenna, 1998; Wilson & Reeder, 2005). Such condition places the discipline of taxonomy at the forefront of invasive species research. Molecular techniques, and more recently the DNA barcoding, triggered a small revolution inside

taxonomy: the process of identifying biological entities opened the doors to a real integration of knowledge to improve practical or theoretical purposes (Galimberti et al. 2015).

Integrative taxonomy is a multi-disciplinary approach to traditional taxonomy where the morphological features are combined to other approaches and additional data (e.g., molecular, behavioural, developmental, ecological, etc.) (Dayrat, 2005; Flagella *et al.*, 2010; Padial *et al.*, 2010; Wu *et al.*, 2011). Nowadays, many studies in the field of bioinvasion are conducted with an integrative approach where molecular markers and morphological features are two complementary, independent, systems of identification, each using a separate set of criteria (Gotzek *et al.*, 2012; Pisanu *et al.*, 2013).

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

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

Although there are 15 species in the genus *Callosciurus*, some of which are morphologically very similar (Wilson & Reeder, 2005), the two species most commonly introduced worldwide are the Finlayson's squirrel *C. finlaysonii* (Horsfield, 1823), native to Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Vietnam and the Pallas's squirrel *C. erythraeus* (Pallas, 1778), native to south-east China, eastern India, Malaya, Indochina, Bhutan and Taiwan (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 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. *C. flavimanus*) (Ellerman & Morrison-Scott, 1951; Moore & Tate, 1965; Corbet & Hill,

1992), whereas recent molecular analysis suggested that the subspecies *C. e. griseimanus* is genetically distinct from other *C. erythraeus* subspecies and other *Callosciurus* species (Oshida *et al.*, 2013). In these cases, modern taxonomy standards call for several biological criteria being taken into account to pursue reliable species identification (Larsen, 2001; Malhotra & Thorpe, 2004; Galimberti *et al.*, 2012b).

In 2014, Gabrielli and co-workers, carried out the 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 three populations introduced in Europe to i) assess the taxonomic status of specimens assigned to *C. cfr. erythraeus*; ii) study and compare morphological and genetic characteristics among specimens; iii) assess the provenance of European *Callosciurus*. Morphometric analysis (skull measurements) and a molecular investigation (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 collected in Asia and kept at the “Andrea Doria” Natural History Museum and the Natural History Museum of Milan, 11 specimens of *C. finlaysonii* collected from an introduced population in Southern Italy (Potenza), and 54 skulls of *Callosciurus* cfr. *erythraeus* introduced in Italy, Belgium and France (Table 1) (Aloise & Bertolino, 2005; Bertolino & Lurz, 2013).

In Italy and Belgium, the introduced squirrels were captured using Tomahawk live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA) baited with apples and hazelnuts. Traps were set in the morning and checked at least twice a day to minimize stress during trapping. For each trapped squirrel sex and reproductive condition were recorded following Wauters & Dhondt (1989) with lactating females (large nipples, milk excretion if stimulated) being immediately released. All males and non-reproductive females were euthanized 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 N° 68-012) and following the European Union recommendations (Annex IV Directive 63 EU). Each individual was weighed to the nearest 5 g using a Pesola spring-balance.

MORPHOMETRIC ANALYSIS

We performed 10 measurements of the cranium (Fig. 1, 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 (Flury, 1997) using the stats R package (Venables & Ripley, 2002; R Development Core Team, 2014). Standardization and scaling were carried out automatically by the princomp R function.

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

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

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

MOLECULAR ANALYSIS

Sampling, DNA extraction, amplification and sequencing

A total of 17 tissue samples were collected from the three introduced *Callosciurus cfr. erythraeus* populations in Europe: five from Brezzo di Bedero, Varese province, Italy (45°58'09.2" N, 08°43'57.6" E), six from Dadizele, Moorsele province, Belgium (50°51'05.5" N, 03°05'40.3" E) and six from

Antibes, Alpes-Maritimes district, France (43°33'51.0" N, 07°07'27.0" E). The sampling dataset also includes two samples of *C. erythraeus* from Yanyuan and Muli counties, Sichuan province, People's Republic of China (hereafter PRC, 27°38'23.7" N, 101°48'43.8" E and 28°9'23.3" N, 100°48'43.8" E respectively) and three samples of *C. finlaysonii* from the two known Italian populations, introduced in recent years: Maratea, Potenza province and Acqui Terme, Alessandria province (39°59'35.3" N, 15°42'22.2" E and 44°40'25.0" N, 8°28'23.6" E , respectively; sampling details are provided in 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 manufacturer's instructions. Purified DNA concentration of each sample was estimated fluorometrically with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA) by measuring the absorbance at 260 nm.

Molecular characterization of European *C. cfr. erythraeus* populations was conducted by analyzing sequence differences at two 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 for example Galimberti *et al.*, 2012a;

Ermakov *et al.*, 2015) and because a huge number of D-loop sequences is available for the species group treated in this study (Gabrielli *et al.*, 2014). Moreover, the two markers typically show high mutation rates, that 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 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 μ L reaction (including 10 ng of DNA as template) by using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Freiburg, Germany), according to manufacturer's instructions. After checking for amplicons presence and quality on 1.5 % agarose gel, sequencing was carried out on both strands using an ABI 155 3730XL (Macrogen Inc., Seoul, Korea), with the same amplification primers.

Data analysis

Sequences were corrected by visual inspection of automated sequencer chromatograms in BIOEDIT 7.1 (Hall, 1999). Codons were then translated into amino acid sequences to check for the presence of nuclear pseudogenes using MEGA6 (Tamura *et al.*, 2013). To avoid the inclusion of nuclear sequence of mitochondrial origin (i.e. NUMTs, Nuclear Mitochondrial DNAs, Bensasson *et al.*, 2001) we also followed the guidelines proposed in Song *et al.* (2008) and Buhay (2009). Sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI) (see Table S1 for accession numbers). Bioinformatics analyses were conducted separately on the two datasets (i.e. *CoxI* and D-loop). Except for the samples analyzed in

this study, very few specimens among those reported in Genbank had sequences for both mitochondrial markers. As far as the *CoxI* is concerned, we considered the 17 samples of *C. cfr. erythraeus* obtained in the three European naturalized populations, two samples of *C. erythraeus* from its native range in People's Republic of China, three samples of Italian *C. finlaysonii*. We also used 33 public available sequences of the genus *Callosciurus*, including accessions of *C. erythraeus* from native (PRC) and introduced range (Argentina), *C. notatus*, *C. orestes* and *C. prevostii*. The D-loop dataset encompassed *Callosciurus* sequences obtained in this study and 99 Genbank sequences of *C. erythraeus* belonging to native (China and Taiwan) and introduced (Japan and Argentina) ranges, *C. finlaysonii* and *C. prevostii*.

Sequences of each marker were aligned using MUSCLE online (<http://www.ebi.ac.uk/Tools/msa/muscle/>; Edgar, 2004) with default options. For both datasets, the number of haplotypes and nucleotide diversity per site and per species were computed with DnaSP v. 5.10.1 software (Librado & 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 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 barcode gap occurs and groups sequences into lineages corresponding to putative species without any *a priori* hypothesis. Analysis results can be finally compared with other taxonomic approaches (e.g., morphology) within an integrative taxonomy

perspective. ABGD was run with default settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, X relative gap width = 1.5, Nb bins = 20) and Kimura distance model.

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

RESULTS

MORPHOMETRIC ANALYSIS

The fur color of all museum specimens was analyzed to confirm species identification (Amori & Doria; 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 & Tate (1965), Corbet & Hill (1992) and Thorington & Hoffmann (2005), all the specimens from Europe could be included in the species *C.*

erythraeus. According to pelage characteristics the French population could be included in three different subspecies: *C. e. erythrogaster* (Blyth, 1842: from India and Burma), *C. e. castaneoventrif* (Gray, 1842: from China) or *C. e. taiwanensis* (Bonhote, 1901: from Taiwan). The Italian and Belgian population could be assigned to the subspecies *C. e. styani* Thomas, 1894 (from China). For details on the pelage colors and skull measurements see Table S2, S3.

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

MOLECULAR CHARACTERIZATION

Alignments characteristics

DNA extracted from the ethanol preserved tissues was of high quality (ratios of absorbance, $A_{260/280}$ and $A_{260/230} \sim 1.80$ and >1.90 , respectively) and provided good yields (> 50 ng/ μ l). Amplification with the selected primer pairs was successful and resulting DNA concentration of purified amplicons was >50 ng/ μ l. High quality sequences showing a strong chromatogram signal along the entire read were

Accepted Article

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 $\pi_T = 30.9$ %. Concerning D-loop, the multiple alignment showed seven indel positions, which were concentrated in the variable 5'-end variable domain, and 184 variable sites of which 155 parsimony informative were detected. The *CoxI* alignment contained 172 variable positions, of which 158 were parsimony-informative and a mean transition/transversion ratio (over all sequence pairs) of 7.907. 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*.

Concerning *C. cfr. erythraeus* European populations, which have been investigated for the first time in this study, two different haplotypes were found at each marker. Interestingly, both the Italian and Belgian populations shared the same haplotypes for both markers, whereas French *Callosciurus* showed a different haplotype. Italian *C. finlaysonii* populations shared a single haplotype at *CoxI*, whereas at D-loop, they show two haplotypes. Moreover, the haplotypes found in European populations of *C. cfr. erythraeus* and *C. finlaysonii* had not been previously observed in other populations with the only exception of the *C. finlaysonii* individual from Acqui Terme (North-West Italy) that is identical at D-loop to a squirrel from Thailand (see 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 three groups with prior values of 0.036 and 0.060. The primary partition was stable on the whole range of prior values and the ten groups were coherent with the lineages identified by the NJ approach (see Fig. 3). Considering these lineages as putative different taxonomic units, the mean \pm standard error of K2P distance between groups (Table S5) was $12.6 \pm 5.9 \%$ (range: 3% – 19.9%). 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 PRC ($4.8 \pm 0.9 \%$ and $5.0 \pm 0.9 \%$). Interestingly, the lineage including the two *C. erythraeus* from Sichuan (PRC) sequenced in this study was closer to *C. finlaysonii* group ($3.5 \pm 0.7 \%$), whereas its K2P distance to the other two lineages belonging to Chinese *C. erythraeus* was two times higher (see Table S5 and Fig.3).

Genetic structure at D-loop

Differently from the case of *Cox1*, the higher number of D-loop sequences available in Genbank permitted to better define relationships among different species (and geographic populations) of *Callosciurus* squirrels.

Both NJ reconstruction (see Fig. 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 populations being their nearest neighbor (K2P distance = $12.0 \pm 1.34\%$), French *Callosciurus* resulted even closer to *C. erythraeus* from Taiwan (K2P distance = $3.6 \pm 0.6\%$). Once again, the lineage including the two Chinese samples of *C. erythraeus* from Sichuan analysed in this study (C_ery_PRC_II, see Table S6 and Fig. 4), resulted 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 three populations. Our molecular dataset is the widest ever assembled for the study of *Callosciurus* species with new haplotypes never described by previous studies and deposited in public databases. Moreover, museum collections confirmed their important role as a great source of knowledge (Wandeler *et al.*, 2007), allowing the comparison of the introduced specimens of unknown taxonomical position with reference specimens. The analyses conducted in this study provided three main findings concerning the investigated European *Callosciurus* populations.

The first important result emerging from molecular analyses is that the introduced *Callosciurus* of Italy and Belgium shared the same haplotypes for both markers, forming a supported MOTU (Molecular Operational Taxonomic Unit *sensu* Floyd *et al.*, 2002) that was conspicuously different from that of the French population. Morphometric data supported such molecular framework: skull

size measures of the Italian and Belgian groups were totally overlapped, while the French one was clearly separated from the Italian group and bordered little with the specimens from Belgium. Moreover, squirrels from Italy and Belgium had the same fur 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 two European MOTUs were included into the putative '*erythraeus*' group in our NJ reconstructions, thus partly confirming the field identification. Surprisingly, in the *CoxI* dataset, the two MOTUs formed two new independent lineages never described before, whereas the French one was included into the MOTUs of *C. erythraeus* from Taiwan in the D-loop dataset (Fig. 3, 4). K2P distance matrix and the comparison with the identification thresholds for 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 two morphologically recognized *C. erythraeus* collected in the Sichuan province (PRC). Both markers used in our study indicated that these samples constitute a new lineage closer to *C. finlaysonii* and to the Argentinean samples, even though their taxonomic status is still unknown. As recently reported by Ermakov *et al.* (2015), such a situation should also be evaluated considering possible introgressive hybridization events and/or incomplete lineage sorting of mtDNA haplotypes.

Both phenomena can lead to misidentification when comparing morphological and molecular data as frequently documented in bats (Nesi *et al.*, 2011; Galimberti *et al.*, 2012b), squirrels (Chang *et al.*, 2011; Ermakov *et al.*, 2015) and even *Callosciurus* species (Oshida *et al.*, 2007; Kuramoto *et al.*, 2012). In this context, the use of fast-evolving nuclear markers and a wider sampling coverage in native *C. erythraeus* distribution range 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 two 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, fur color patterns) from Belgian and Italian *Callosciurus* (Moore & Tate, 1965), thus confidently suggesting to classify the latter as *C. erythraeus*. In contrast, *Callosciurus* skulls from France are similar to the museum specimens of *C. phayrei* and *C. melanogaster*, but also in this case these species are different in fur 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 founder effect mostly referable to captive-bred stocks used for pet trade as also inferred from the low haplotype diversity at the analysed 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 such area is called “Villaggio Olandese” (Dutch Village) is known for the large number of vacation houses belonging to people from Belgium and the Netherlands. However, the origin of the Belgian population still remains unknown. On the contrary, as revealed by D-loop data and fur description, French *Callosciurus* probably originated from an independent introduction event of Taiwanese squirrels to France. Even though no document that ascertains the origin of *Callosciurus* in France, we know that a very small number of squirrels were imported directly from Asia by a single person in the 1960s (Chapuis and Pisanu pers. comm.).

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

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

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Table 1. Specimens whose cranial measurements were included in PCA analysis.

M= male; F= female; Un= sex unknown; n= number of specimens

Species	Origin of specimens	M	F	Un	n
<i>Callosciurus caniceps</i> (Gray, 1842)	“Andrea Doria” Natural History Museum	9	9	8	26
<i>Callosciurus erythraeus</i> (Pallas, 1778)	“Andrea Doria” Natural History Museum	2	2	8	12
<i>Callosciurus melanogaster</i> (Thomas, 1895)	“Andrea Doria” Natural History Museum	-	-	9	9
<i>Callosciurus nigrovittatus</i> (Horsfield, 1824)	“Andrea Doria” Natural History Museum	11	8	2	21
<i>Callosciurus notatus</i> (Boddaert, 1785)	“Andrea Doria” Natural History Museum	7	8	7	22
<i>Callosciurus phayrei</i> (Blyth, 1856)	“Andrea Doria” Natural History Museum	12	16	13	41
<i>Callosciurus prevostii</i> (Desmarest, 1822)	“Andrea Doria” Natural History Museum	-	1	1	2
<i>Callosciurus pygerythrus</i> (I.Geoffroy Saint Hilaire, 1833)	“Andrea Doria” Natural History Museum	-	-	2	2
<i>Callosciurus finlaysonii</i> (Horsfield, 1823)	“Andrea Doria” Natural History Museum	-	-	2	
	Potenza, Italy	3	8	-	14
	Natural History Musuem, 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

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
loc	Interorbital constriction
Lbc	Least breadth of caudal point of zygomatic process

APPENDIX

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 two mitochondrial markers) are reported.

Specimen Voucher	Species name	Provenance	Country	H coxI	H D- loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
MIB:ZPL:03830	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Brezzo di Bedero (VA)	Italy	CXH 10	DLH7	LN899 425	LN899 447	this study	this study
MIB:ZPL:07382	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Brezzo di Bedero (VA)	Italy	CXH 10	DLH7	LN899 429	LN899 451	this study	this study
MIB:ZPL:07383	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Brezzo di Bedero (VA)	Italy	CXH 10	DLH7	LN899 430	LN899 452	this study	this study
MIB:ZPL:07384	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Brezzo di Bedero (VA)	Italy	CXH 10	DLH7	LN899 431	LN899 453	this study	this study
MIB:ZPL:07385	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Brezzo di Bedero (VA)	Italy	CXH 10	DLH7	LN899 432	LN899 454	this study	this study
MIB:ZPL:07386	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Dadizele (Moorsele)	Belgium	CXH 10	DLH7	LN899 433	LN899 455	this study	this study
MIB:ZPL:07387	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Dadizele (Moorsele)	Belgium	CXH 10	DLH7	LN899 434	LN899 456	this study	this study
MIB:ZPL:07388	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Dadizele (Moorsele)	Belgium	CXH 10	DLH7	LN899 435	LN899 457	this study	this study
MIB:ZPL:07389	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Dadizele (Moorsele)	Belgium	CXH 10	DLH7	LN899 436	LN899 458	this study	this study
MIB:ZPL:07390	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Dadizele (Moorsele)	Belgium	CXH 10	DLH7	LN899 437	LN899 459	this study	this study
MIB:ZPL:07391	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Dadizele (Moorsele)	Belgium	CXH 10	DLH7	LN899 438	LN899 460	this study	this study
MIB:ZPL:07392	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Antibes (Alpes Maritimes)	France	CXH 11	DLH4 2	LN899 439	LN899 461	this study	this study
MIB:ZPL:07393	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Antibes (Alpes Maritimes)	France	CXH 11	DLH4 2	LN899 440	LN899 462	this study	this study
MIB:ZPL:07394	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Antibes (Alpes Maritimes)	France	CXH 11	DLH4 2	LN899 441	LN899 463	this study	this study
MIB:ZPL:07395	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Antibes (Alpes Maritimes)	France	CXH 11	DLH4 2	LN899 442	LN899 464	this study	this study
MIB:ZPL:07396	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Antibes (Alpes Maritimes)	France	CXH 11	DLH4 2	LN899 443	LN899 465	this study	this study
MIB:ZPL:07397	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Antibes (Alpes Maritimes)	France	CXH 11	DLH4 2	LN899 444	LN899 466	this study	this study
EMiB	<i>Callosciurus</i> cfr.	Luján, Buenos Aires	Argenti	CXH	DLH6	KF8562	KF7860	Gabrielli et	Gabrielli et

Specimen Voucher	Species name	Provenance	Country	H cox1	H D- loop	GenBank a.n.		Source	
						cox1	D-loop	cox1	D-loop
	<i>erythraeus</i>		na	14		33	12	<i>al.</i> , 2014	<i>al.</i> , 2014
EMiC	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 31	KF7860 13	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMiD	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 19	KF7860 14	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMiA	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 32	KF7860 15	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CG11	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Cañada de Gómez, Santa Fe	Argentina	CXH 14	DLH6	KF8562 12	KF7860 11	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CG13	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Cañada de Gómez, Santa Fe	Argentina	CXH 14	DLH6	KF8562 28	KF7860 10	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CG18	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Cañada de Gómez, Santa Fe	Argentina	CXH 14	DLH6	KF8562 27	KF7860 09	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CG19	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Cañada de Gómez, Santa Fe	Argentina	CXH 14	DLH6	KF8562 26	KF7860 08	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CG20	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Cañada de Gómez, Santa Fe	Argentina	CXH 14	DLH6	KF8562 25	KF7860 07	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CU24	<i>Callosciurus</i> cfr. <i>erythraeus</i>	La Cumbecita, Córdoba	Argentina	CXH 14	DLH6	KF8562 14	KF7860 06	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CU27	<i>Callosciurus</i> cfr. <i>erythraeus</i>	La Cumbecita, Córdoba	Argentina	CXH 14	DLH6	KF8562 29	KF7860 05	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
CU30	<i>Callosciurus</i> cfr. <i>erythraeus</i>	La Cumbecita, Córdoba	Argentina	CXH 14	DLH6	KF8562 15	KF7860 04	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi32	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 16	KF7860 16	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi33	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	–	DLH6	–	KF7860 17	–	Gabrielli <i>et al.</i> , 2014
EMi34	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 17	KF7860 18	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi35	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	–	DLH6	–	KF7860 19	–	Gabrielli <i>et al.</i> , 2014
EMi37	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 24	KF7860 20	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi38	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	CXH 14	DLH6	KF8562 23	KF7860 21	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
EMi74	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	–	DLH6	–	KF7860 22	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi75	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	–	DLH6	–	KF7860 23	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi78	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	–	DLH6	–	KF7860 24	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi79	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	–	DLH6	–	KF7860 25	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi80	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	–	DLH6	–	KF7860 26	Gabrielli <i>et al.</i> , 2014	Gabrielli <i>et al.</i> , 2014
EMi4	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	–	KF8562 11	–	Gabrielli <i>et al.</i> , 2014	–
CU23	<i>Callosciurus</i> cfr. <i>erythraeus</i>	La Cumbecita, Córdoba	Argentina	CXH 14	–	KF8562 13	–	Gabrielli <i>et al.</i> , 2014	–
CU29	<i>Callosciurus</i> cfr. <i>erythraeus</i>	La Cumbecita, Córdoba	Argentina	CXH 14	–	KF8562 30	–	Gabrielli <i>et al.</i> , 2014	–
EMi39	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Escobar, Buenos Aires	Argentina	CXH 14	–	KF8562 18	–	Gabrielli <i>et al.</i> , 2014	–
EMi55	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	–	KF8562 20	–	Gabrielli <i>et al.</i> , 2014	–
EMi63	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	–	KF8562 21	–	Gabrielli <i>et al.</i> , 2014	–
EMi71	<i>Callosciurus</i> cfr. <i>erythraeus</i>	Luján, Buenos Aires	Argentina	CXH 14	–	KF8562 22	–	Gabrielli <i>et al.</i> , 2014	–
MIB:ZPL:07398	<i>Callosciurus erythraeus</i>	Yanyuan county (Sichuan)	PRC	CXH 12	DLH1	LN899 445	LN899 467	this study	this study
Hongya County 06	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	–	DLH1 0	–	GU474 437	–	Guo <i>et al.</i> , 2011
Hongya County 03	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	–	DLH1 1	–	GU474 434	–	Guo <i>et al.</i> , 2011
Hongya County 18	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	–	DLH1 1	–	GU474 449	–	Guo <i>et al.</i> , 2011
Hongya County 04	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	–	DLH1 2	–	GU474 435	–	Guo <i>et al.</i> , 2011
Hongya	<i>Callosciurus</i>	Hongya county (Sichuan)	PRC	–	DLH1	–	GU474	–	Guo <i>et al.</i> ,

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
County 13	<i>erythraeus</i>				3		444		2011
Hongya County 16	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH1 4	-	GU474 447	-	Guo <i>et al.</i> , 2011
Hongya County 10	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH1 5	-	GU474 441	-	Guo <i>et al.</i> , 2011
Hongya County 01	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH1 6	-	GU474 432	-	Guo <i>et al.</i> , 2011
Hongya County 09	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH1 7	-	GU474 440	-	Guo <i>et al.</i> , 2011
Hongya County 07	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH1 8	-	GU474 438	-	Guo <i>et al.</i> , 2011
-	<i>Callosciurus erythraeus</i>	Mt. Hemei (Sichuan)	PRC	CXH 13	DLH1 8	KM502 568	KM502 568	Hu <i>et al.</i> , In press	Hu <i>et al.</i> , In press
Hongya County 12	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH1 9	-	GU474 443	-	Guo <i>et al.</i> , 2011
MIB:ZPL:07399	<i>Callosciurus erythraeus</i>	Muli county (Sichuan)	PRC	CXH 12	DLH2	LN899 446	LN899 468	this study	this study
Hongya County 14	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH2 0	-	GU474 445	-	Guo <i>et al.</i> , 2011
Hongya County 02	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH2 1	-	GU474 433	-	Guo <i>et al.</i> , 2011
Hongya County 08	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH2 2	-	GU474 439	-	Guo <i>et al.</i> , 2011
Hongya County 15	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH2 3	-	GU474 446	-	Guo <i>et al.</i> , 2011
Hongya County 17	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH2 4	-	GU474 448	-	Guo <i>et al.</i> , 2011
NMNS3748	<i>Callosciurus erythraeus</i>	Wufeng (Taichung)	Taiwan	-	DLH2 5	-	AB181 249	-	Oshida <i>et al.</i> , 2006
NMNS842, NMNS1345	<i>Callosciurus erythraeus</i>	Wufeng (Taichung)	Taiwan	-	DLH2 6	-	AB181 256	-	Oshida <i>et al.</i> , 2006
NMNS1191	<i>Callosciurus erythraeus</i>	Wufeng (Taichung)	Taiwan	-	DLH2 7	-	AB181 253	-	Oshida <i>et al.</i> , 2006
NMNS6408, NMNS1184,	<i>Callosciurus erythraeus</i>	Dadushan, Wufeng (Taichung)	Taiwan	-	DLH2 8	-	AB181 257	-	Oshida <i>et al.</i> , 2006

Specimen Voucher	Species name	Provenance	Country	H coxI	H D- loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
NMNS4366									
NMNS6616, NMNS1025, NMNS1178	<i>Callosciurus erythraeus</i>	Shinshe, Wufeng (Taichung)	Taiwan	-	DLH2 9	-	AB181 260	-	Oshida <i>et al.</i> , 2006
NMNS5439	<i>Callosciurus erythraeus</i>	Shuili (Nantou)	Taiwan	-	DLH3 0	-	AB181 254	-	Oshida <i>et al.</i> , 2006
NMNS6406	<i>Callosciurus erythraeus</i>	Longtan (Taoyuan)	Taiwan	-	DLH3 1	-	AB181 255	-	Oshida <i>et al.</i> , 2006
NMNS5280, NMNS5383	<i>Callosciurus erythraeus</i>	Tunglou (Taichung)	Taiwan	-	DLH3 2	-	AB181 259	-	Oshida <i>et al.</i> , 2006
NMNS5401	<i>Callosciurus erythraeus</i>	Tunglou (Taichung)	Taiwan	-	DLH3 3	-	AB181 252	-	Oshida <i>et al.</i> , 2006
NMNS6609, NMNS1905	<i>Callosciurus erythraeus</i>	Paoshan Dam (Hsinchu), Chilan (Yilan)	Taiwan	-	DLH3 4	-	AB181 258	-	Oshida <i>et al.</i> , 2006
NMNS6422	<i>Callosciurus erythraeus</i>	Shuanglianpi (Yilan)	Taiwan	-	DLH3 5	-	AB181 250	-	Oshida <i>et al.</i> , 2006
NMNS4244	<i>Callosciurus erythraeus</i>	Fushan (Yilan)	Taiwan	-	DLH3 6	-	AB181 251	-	Oshida <i>et al.</i> , 2006
NMNS5380	<i>Callosciurus erythraeus</i>	Lugu (Nantou)	Taiwan	-	DLH3 7	-	AB181 272	-	Oshida <i>et al.</i> , 2006
NMNS6606	<i>Callosciurus erythraeus</i>	Baolai (Kaohsiung)	Taiwan	-	DLH3 8	-	AB181 263	-	Oshida <i>et al.</i> , 2006
NMNS5440	<i>Callosciurus erythraeus</i>	Neimen (Kaohsiung)	Taiwan	-	DLH3 9	-	AB181 261	-	Oshida <i>et al.</i> , 2006
NMNS6413, NMNS6410, NMNS6597	<i>Callosciurus erythraeus</i>	Tsaolian, Alishan (Chiayi)	Taiwan	-	DLH4 0	-	AB181 262	-	Oshida <i>et al.</i> , 2006
NMNS278, NMNS6614	<i>Callosciurus erythraeus</i>	Baolai (Kaohsiung)	Taiwan	-	DLH4 1	-	AB181 264	-	Oshida <i>et al.</i> , 2006
NMNS5281, NMNS5402	<i>Callosciurus erythraeus</i>	Shitou (Nantou)	Taiwan	-	DLH4 3	-	AB181 265	-	Oshida <i>et al.</i> , 2006
NMNS5381	<i>Callosciurus erythraeus</i>	Lugu (Nantou)	Taiwan	-	DLH4 4	-	AB181 267	-	Oshida <i>et al.</i> , 2006
NMNS6610	<i>Callosciurus erythraeus</i>	Tsaolian (Chiayi)	Taiwan	-	DLH4 5	-	AB181 287	-	Oshida <i>et al.</i> , 2006

Specimen Voucher	Species name	Provenance	Country	H coxI	H D-loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
NMNS6805	<i>Callosciurus erythraeus</i>	Sandimen (Pingtung)	Taiwan	-	DLH4 6	-	AB181 290	-	Oshida <i>et al.</i> , 2006
NMNS6806, NMNS6834	<i>Callosciurus erythraeus</i>	Sandimen (Pingtung)	Taiwan	-	DLH4 7	-	AB181 291	-	Oshida <i>et al.</i> , 2006
NMNS6415	<i>Callosciurus erythraeus</i>	Shizi (Pingtung)	Taiwan	-	DLH4 8	-	AB181 273	-	Oshida <i>et al.</i> , 2006
184	<i>Callosciurus erythraeus</i>	Oshima Island (Kantō)	Japan	-	DLH4 9	-	AB259 594	-	Oshida <i>et al.</i> , 2007
-	<i>Callosciurus erythraeus</i>	Kumamoto (Kyūshū)	Japan	-	DLH5 0	-	AB576 365	-	Ikeda <i>et al.</i> , 2011
183,185,186,2 73,274	<i>Callosciurus erythraeus</i>	Oshima Island (Kantō), Fukue Island (Kyūshū)	Japan	-	DLH5 1	-	AB259 593	-	Oshida <i>et al.</i> , 2007
192	<i>Callosciurus erythraeus</i>	Izu peninsula (Chūbu)	Japan	-	DLH5 1	-	AB259 598	-	Oshida <i>et al.</i> , 2007
NMNS5417, NPUST14	<i>Callosciurus erythraeus</i>	Kenting, Jialeshuei (Pingtung)	Taiwan	-	DLH5 2	-	AB181 282	-	Oshida <i>et al.</i> , 2006
NMNS5420, NMNS5421	<i>Callosciurus erythraeus</i>	Kenting (Pingtung)	Taiwan	-	DLH5 3	-	AB181 270	-	Oshida <i>et al.</i> , 2006
NMNS5393	<i>Callosciurus erythraeus</i>	Fangliao (Pingtung)	Taiwan	-	DLH5 4	-	AB181 266	-	Oshida <i>et al.</i> , 2006
NMNS5399, NMNS5403, NMNS5407	<i>Callosciurus erythraeus</i>	Fangliao (Pingtung)	Taiwan	-	DLH5 5	-	AB181 285	-	Oshida <i>et al.</i> , 2006
NMNS5415, NMNS5416	<i>Callosciurus erythraeus</i>	Dunghe (Taitung)	Taiwan	-	DLH5 6	-	AB181 279	-	Oshida <i>et al.</i> , 2006
NMNS5283, NMNS5396, NMNS6412	<i>Callosciurus erythraeus</i>	Fangliao, Shizi (Pingtung)	Taiwan	-	DLH5 7	-	AB181 284	-	Oshida <i>et al.</i> , 2006
275	<i>Callosciurus erythraeus</i>	Miyazaki (Kyūshū)	Japan	-	DLH5 8	-	AB259 599	-	Oshida <i>et al.</i> , 2007
NMNS5414, NMNS6595	<i>Callosciurus erythraeus</i>	Wuling Farm (Taichung), Datong (Yilan)	Taiwan	-	DLH5 9	-	AB181 274	-	Oshida <i>et al.</i> , 2006
NMNS6615	<i>Callosciurus erythraeus</i>	Shinbaiyang (Hualien)	Taiwan	-	DLH6 0	-	AB181 288	-	Oshida <i>et al.</i> , 2006
NMNS6603, NMNS6613	<i>Callosciurus erythraeus</i>	Shinbaiyang (Hualien)	Taiwan	-	DLH6 1	-	AB181 289	-	Oshida <i>et al.</i> , 2006

Specimen Voucher	Species name	Provenance	Country	H coxI	H D- loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
NMNS5418, NMNS5425, NMNS5437, NMNS5438	<i>Callosciurus erythraeus</i>	Kuanfu (Hualien)	Taiwan	-	DLH6 2	-	AB181 275	-	Oshida <i>et al.</i> , 2006
NMNS6612	<i>Callosciurus erythraeus</i>	Dongshan (Yilan)	Taiwan	-	DLH6 3	-	AB181 277	-	Oshida <i>et al.</i> , 2006
NMNS6611	<i>Callosciurus erythraeus</i>	Dongshan (Yilan)	Taiwan	-	DLH6 4	-	AB181 286	-	Oshida <i>et al.</i> , 2006
NMNS5426	<i>Callosciurus erythraeus</i>	Rentze (Yilan)	Taiwan	-	DLH6 5	-	AB181 271	-	Oshida <i>et al.</i> , 2006
NMNS5423	<i>Callosciurus erythraeus</i>	Kuanfu (Hualien)	Taiwan	-	DLH6 6	-	AB181 280	-	Oshida <i>et al.</i> , 2006
NMNS5422	<i>Callosciurus erythraeus</i>	Kuanfu (Hualien)	Taiwan	-	DLH6 7	-	AB181 281	-	Oshida <i>et al.</i> , 2006
NMNS5436	<i>Callosciurus erythraeus</i>	Kuanfu (Hualien)	Taiwan	-	DLH6 8	-	AB181 268	-	Oshida <i>et al.</i> , 2006
NMNS5419	<i>Callosciurus erythraeus</i>	Kuanfu (Hualien)	Taiwan	-	DLH6 9	-	AB181 269	-	Oshida <i>et al.</i> , 2006
NMNS5424, NMNS5432, NMNS5434, NMNS5435	<i>Callosciurus erythraeus</i>	Kuanfu (Hualien)	Taiwan	-	DLH7 0	-	AB181 276	-	Oshida <i>et al.</i> , 2006
182	<i>Callosciurus erythraeus</i>	Oshima Island (Kantō)	Japan	-	DLH7 1	-	AB259 592	-	Oshida <i>et al.</i> , 2007
NMNS5406, NMNS5287	<i>Callosciurus erythraeus</i>	Fangliao (Pingtung)	Taiwan	-	DLH7 2	-	AB181 283	-	Oshida <i>et al.</i> , 2006
NPUST15	<i>Callosciurus erythraeus</i>	Neipu (Pingtung)	Taiwan	-	DLH7 3	-	AB181 278	-	Oshida <i>et al.</i> , 2006
190	<i>Callosciurus erythraeus</i>	Hamamatsu (Chūbu)	Japan	-	DLH7 4	-	AB259 596	-	Oshida <i>et al.</i> , 2007
Hongya County 05	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH8	-	GU474 436	-	Guo <i>et al.</i> , 2011
Hongya County 11	<i>Callosciurus erythraeus</i>	Hongya county (Sichuan)	PRC	-	DLH9	-	GU474 442	-	Guo <i>et al.</i> , 2011
HN117	<i>Callosciurus erythraeus</i>	Qiongzong (Hainan)	PRC	CXH 1	-	HM031 932	-	Lu <i>et al.</i> , 2012	-

Specimen Voucher	Species name	Provenance	Country	H coxI	H D- loop	GenBank a.n.		Source	
						coxI	D-loop	coxI	D-loop
HN118	<i>Callosciurus erythraeus</i>	Qiongzong (Hainan)	PRC	CXH 2	–	HM031 933	–	Lu <i>et al.</i> , 2012	–
HN119	<i>Callosciurus erythraeus</i>	Qiongzong (Hainan)	PRC	CXH 3	–	HM031 934	–	Lu <i>et al.</i> , 2012	–
HN120	<i>Callosciurus erythraeus</i>	Qiongzong (Hainan)	PRC	CXH 1	–	HM031 935	–	Lu <i>et al.</i> , 2012	–
MIB:ZPL:04253	<i>Callosciurus finlaysonii</i>	Maratea (PZ)	Italy	CXH 9	DLH7 7	LN899 426	LN899 448	this study	this study
MIB:ZPL:04254	<i>Callosciurus finlaysonii</i>	Maratea (PZ)	Italy	CXH 9	DLH7 7	LN899 427	LN899 449	this study	this study
MIB:ZPL:04255	<i>Callosciurus finlaysonii</i>	Acqui Terme (AL)	Italy	CXH 9	DLH7 8	LN899 428	LN899 450	this study	this study
187, 188, 189, 191, 507,508	<i>Callosciurus finlaysonii</i>	Hamamatsu (Chūbu)	Japan	–	DLH5	–	AB259 595	–	Oshida <i>et al.</i> , 2007
506	<i>Callosciurus finlaysonii</i>	Hamamatsu (Chūbu)	Japan	–	DLH5	–	AB259 597	–	Oshida <i>et al.</i> , 2007
M31312	<i>Callosciurus finlaysonii</i>	Vientianne	Laos	–	DLH3	–	AB259 600	–	Oshida <i>et al.</i> , 2007
M31313	<i>Callosciurus finlaysonii</i>	Vientianne	Laos	–	DLH4	–	AB259 601	–	Oshida <i>et al.</i> , 2007
–	<i>Callosciurus finlaysonii</i>	–	Thailand	–	DLH7 8	–	AB621 590	–	Kuramoto <i>et al.</i> , 2012
ATCC CRL- 1926	<i>Callosciurus notatus</i>	cell culture	–	CXH 4	–	HM102 291	–	Cooper <i>et al.</i> , 2007	–
BIOUG<CAN>: ROM 102085	<i>Callosciurus notatus</i>	Lalut Birai R.S. (Kalimantan Timur)	Indonesia	CXH 5	–	JF4442 86	–	unpublished	–
ROM:102139	<i>Callosciurus orestes</i>	Lalut Birai R.S. (Kalimantan Timur)	Indonesia	CXH 8	–	JF4442 87	–	unpublished	–
ROM:102178	<i>Callosciurus prevostii</i>	Long Sungan (Kalimantan Timur)	Indonesia	CXH 6	–	JF4442 88	–	unpublished	–
ROM:102221	<i>Callosciurus prevostii</i>	Lalut Birai R.S. (Kalimantan Timur)	Indonesia	CXH 7	–	JF4596 23	–	unpublished	–
311	<i>Callosciurus prevostii</i>	Sumatra Island	Indonesia	–	DLH7 5	–	AB259 602	–	Oshida <i>et al.</i> , 2007
312	<i>Callosciurus</i>	Sumatra Island	Indonesia	–	DLH7	–	AB259	–	Oshida <i>et al.</i> ,

Specimen Voucher	Species name	Provenance	Country	H coxl	H D-loop	GenBank a.n.		Source	
						coxl	D-loop	coxl	D-loop
	<i>prevostii</i>		ia		6		603		2007

*PRC= People's Republic of China

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

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

Fig. 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; PRC-People's Republic of China; IT-Italy; FR-France; BE-Belgium. European samples newly sequenced in this study are marked with red dots.

Fig. 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; PRC-People's Republic of China; IT-Italy; FR-France; BE-Belgium; TAI-Taiwan; THA-Thailand; LAO-Laos; JAP-Japan. European samples newly sequenced in this study are marked with red dots.

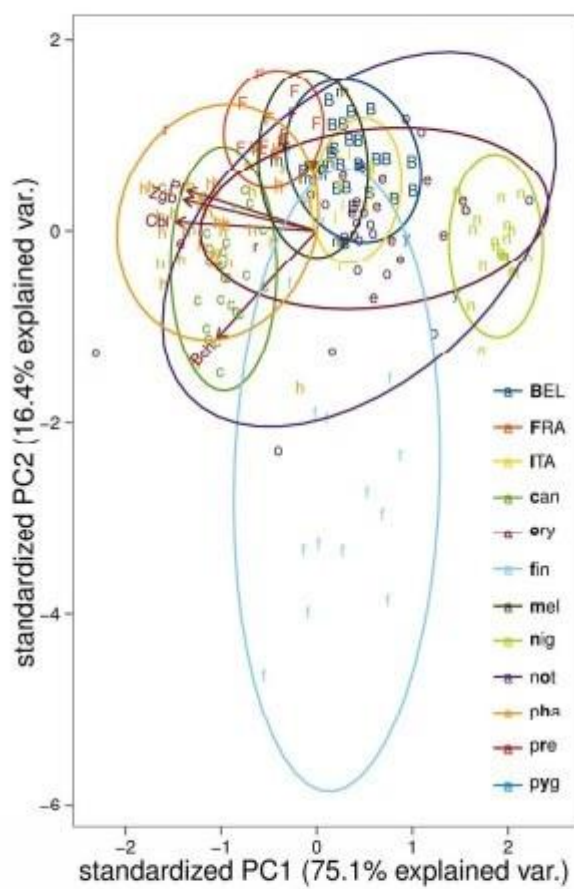
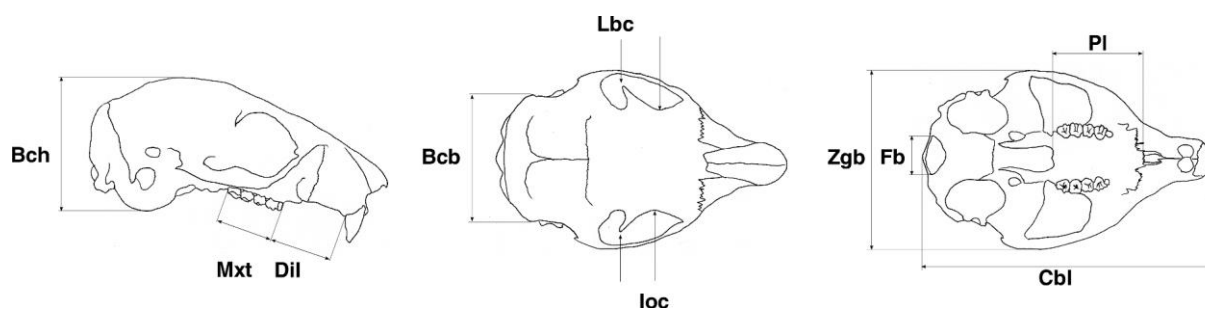


Fig. 2
177x177mm (300 x 300 DPI)

